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Inactivation of 4-Oxalocrotonate Tautomerase by 5-Halo-2-hydroxy-2,4-pentadienoates

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Abstract

5-Halo-2-hydroxy-2,4-pentadienoates (5-halo-HPDs) are reportedly generated in the bacterial catabolism of halogenated aromatic hydrocarbons by the meta-fission pathway. The 5-halo-HPDs, where the halogen can be bromide, chloride, or fluoride, result in the irreversible inactivation of 4-oxalocrotonate tautomerase (4-OT), which precedes the enzyme that generates them. The loss of activity is due to the covalent modification of the nucleophilic amino-terminal proline. Mass spectral and crystallographic analysis of the modified enzymes indicate that inactivation of 4-OT by 5-chloro- and 5-bromo-2-hydroxy-2,4-pentadienoate follows a different mechanism than that for the inactivation of 4-OT by 5-fluoro-2-hydroxy-2,4-pentadienoate. The 5-chloro- and 5-bromo derivatives undergo 4-OT-catalyzed tautomerization to their respective α -unsaturated ketones followed by attack at C-5 (by the prolyl nitrogen) with concomitant loss of the halide. For the 5-fluoro species, the presence of a small amount of the α,β -unsaturated ketone could result in a Michael addition of the prolyl nitrogen to C-4 followed by protonation at C-3. The fluoride is not eliminated. These observations suggest that the inactivation of 4-OT by a downstream metabolite could hamper the efficacy of the pathway, which is the first time that such a bottleneck has been reported for the meta-fission pathway.

Graphical Abstract

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The Supporting Information is available free of charge on the ACS Publications website at DOI.

The Supplementary Information consists of ESI-MS analysis of the *Lc* 4-OT enzymes as purified; both the $2F_o-F_c$ map and F_o-F_c map of the covalent adducts generated during the incubation of *Pp* 4-OT with **9** and **10**; ESI-MS analysis of the *Pp* 4-OT enzyme as purified, and of the *Pp* and *Lc* 4-OT enzymes after incubation with **8**, **9**, or **10**; and bromide release during incubation of **9** with *Pp* 4-OT.

Accession Codes

The atomic coordinates and structure factors have been deposited in the PDB (PDB entry 5TIG for the *Pp* 4-OT inactivated by 5-bromo-2-hydroxy-2,4Z-pentadienoate (**9**) and PDB entry 6BGN for the *Pp* 4-OT inactivated by 5-fluoro-2-hydroxy-2,4Z-pentadienoate (**10**).

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INTRODUCTION

Halogenated aromatic compounds have great commercial, industrial, and pharmaceutical significance (1–3). Past and present uses include solvents, lubricants, dielectric fluids, insecticides, herbicides, flame retardants, drugs, and synthetic intermediates. Due to their toxicity and persistence in the environment, many of these compounds have been banned from further use, most notably polychlorinated biphenyls (PCBs) (3). Many of these compounds are completely resistant to microbial degradation while others undergo partial degradation (2–4). Much effort has been devoted to understanding the microbial pathways that remove halogenated aromatic compounds from the environment along with the factors responsible for the incomplete degradation so that these pathways can be optimized for bioremediation purposes (1–7).

A major route for the bacterial degradation of aromatic compounds is the meta-fission pathway, which is typified by the one elucidated in *Pseudomonas putida* mt-2 (8–9). Monocyclic aromatic compounds (e.g., benzene, toluene, and alkyl-substituted derivatives) are first converted to catechol (**1**, Scheme 1) or a catechol derivative. Subsequently, the resulting species undergoes meta-fission, where this term refers to the position of the ring fission, and further processing to yield 2-hydroxymuconate (**2**). Ketoneization of **2** by 4-oxalocrotonate tautomerase (4-OT) produces 2-oxo-3-hexenedioate (**3**) (10). Decarboxylation of **3** by 4-oxalocrotonate decarboxylase (4-OD) generates 2-hydroxy-2,4-pentadienoate (**4**) (11–13). This compound is further degraded to pyruvate and acetyl-CoA, which are funneled into the Krebs cycle (14).

Variations of this pathway that process halogenated catechols have also been reported, such as one found in *Comamonas* sp. strain CNB-1 that grows on 4-chloronitrobenzene and nitrobenzene as sole carbon and energy sources (15,16). Although the reported pathway shows the chloro substituent at C5 of **2**, **3**, and **4** (R = Cl, Scheme 1), there is limited evidence for these intermediates. Moreover, the presence of the halogen on these intermediates raises questions about whether the bulk and/or electronegativity affect catalysis of the corresponding enzymes or whether the enzymes of this pathway have evolved to accommodate the 5-halo substituent (3).

As part of an ongoing effort to address these and other questions, we synthesized the 5-halo-2-hydroxymuconates (the 4*Z* isomers of **5**, **6**, and **7**, Scheme 2) and the 5-halo-2-hydroxy-2,4-pentadienoates (the 4*Z* isomers of **8**, **9**, and **10**) and examined their behavior with the corresponding enzymes from *Pseudomonas putida* mt-2 (representing the canonical meta-fission pathway and designated *Pp*) and *Leptothrix cholodnii* SP-6 (representing a haloaromatic degradative pathway and designated *Lc*) (13,17). In the course of this work, we found that incubation of 4-OT (from either species) with **8**, **9**, or **10** resulted in an irreversible loss of activity. For **8** and **9**, mass spectral analysis showed that the enzyme formed a covalent bond with a species having a mass consistent with the structure of **4** (or a tautomer where R = H). Inactivation of the enzyme was coincident with the loss of the halo substituent. Crystallographic analysis of the modified enzyme resulting from incubation with **9** showed that Pro1 was attached to C5. One mechanism for inactivation involves the 4-OT-catalyzed conversion of **8** or **9** to the α,β -unsaturated ketone (**11** and **12**, respectively in Scheme 2) followed by attack at C5 with concomitant loss of the chloride or bromide. A different outcome was observed for **10**. In this case, mass spectral and crystallographic analysis showed covalent attachment of Pro1 to C4 of a species derived from **10** where the fluoro substituent is still present. The inactivation mechanism relies on the presence of a small amount of the α,β -unsaturated ketone (**13** in Scheme 2). Michael addition to C4 followed by protonation at C3 results in the stable adduct on the prolyl nitrogen of Pro1. These observations suggest that the enzyme-catalyzed decarboxylation of the 5-halo-2-hydroxymuconates in a haloaromatic degradative pathway could generate products that become irreversible inhibitors of 4-OT. Inhibition of this upstream enzyme could limit the efficiency of the pathway if these intermediates accumulate. To the best of our knowledge, this type of metabolic block has not been previously reported for the meta-fission pathway.

EXPERIMENTAL PROCEDURES

Materials.

Chemicals, biochemicals, buffers, and solvents were obtained from sources reported elsewhere (13). The syntheses of halogenated compounds (**5**–**10**, Scheme 2) are reported elsewhere (13,17). The *Pp* 4-OT was purified as described elsewhere (18). The *Lc* 4-OT was purified as described elsewhere (17), and characterized as described below. The 4-oxalocrotonate decarboxylase/vinylpyruvate hydratase (4-OD/VPH) and 4-OD/E106QVPH mutant complexes (both from *P. putida* mt-2) were purified as described (11–13).

General Methods.

Mass spectral data were obtained on an LCQ electrospray ion-trap mass spectrometer (Thermo, San Jose, CA) in the ICMB Protein and Metabolite core facility. The samples were prepared as described previously (19). Kinetic data were obtained at 24 °C on an Agilent 8453 diode-array spectrophotometer. 4-OT was assayed using 2-hydroxymuconate (**2**), as previously reported (18). Nonlinear regression data analysis was performed using Mathematica (Wolfram Research, Inc., Mathematica, Version 8.0, Champaign, IL 2010). Protein concentrations were determined by the Waddell method (20).

Mass Spectral Analysis of 4-OT from *L. cholodnii* SP-6.

This procedure typically yields ~ 8 mg of 4-OT estimated to be ~95% pure (by SDS-PAGE). A sample was analyzed by electrospray ionization mass spectrometry (ESI-MS) to verify the molecular mass (6916 Da). In addition to this species (corresponding to the intact enzyme without the N-formylmethionine), there are five additional signals typically at 6475 Da, 6606 Da, 6634 Da, 7047 Da, and 7075 Da (Supplementary Figure 1). These signals correspond to enzyme without the four C-terminal amino acids, the same enzyme with an N-terminal methionine, the same enzyme with an N-formylmethionine, the intact enzyme with an N-terminal methionine, and the intact enzyme with an N-formylmethionine.

Inactivation of Pp 4-OT by **7** and **10**.

A quantity of **7** (5 mg) or **10** (1.8 mg) was dissolved in 20 mM Na₃PO₄ buffer (1.0 and 0.5 mL, respectively) to give a final concentration of 28 mM. The pH for the solution of **7** was adjusted to 8.6 with 1 M NaOH. The pH for the solution of **10** was 7.3 (and was not adjusted). Pp 4-OT (1.4 mM, 1.0 mL) in 20 mM NaH₂PO₄ buffer, pH 7.3 was added to 4 individual eppendorf tubes (1.5 mL). Subsequently, 0.5-mL aliquots of **7**, **10**, or **7** along with an aliquot of partially pure 4-OD/E106QVPH (~2–10 µL of ~2 mg/mL), were added to three of the eppendorf tubes. A fourth control eppendorf tube contained enzyme and a 0.5 mL-aliquot of 20 mM NaH₂PO₄ buffer, pH 7.3. The final pH in each reaction mixture was ~7.3. An aliquot of the 4-OT was assayed before the addition of compound and after an overnight incubation period (~18 h). Upon complete inactivation of the 4-OT in the three reaction mixtures (for **7** alone, inactivation took an additional 3 days), the three samples and the control sample were prepared as described elsewhere and analyzed by ESI mass spectrometry (19).

Inactivation of Lc and Pp 4-OT by **8**, **9**, and **10**.

Both Pp 4-OT and Lc 4-OT (~1 mg/mL) were diluted in 20 mM NaH₂PO₄ buffer, pH 7.3, and incubated with **8**, **9**, or **10** for a 16–18 h period in individual eppendorf tubes. The final concentration of enzyme and compound is 145 µM (per monomer) and 500 µM, respectively. The enzyme was inactivated in all mixtures except for the one containing the Lc 4-OT and **10**, which retained 30% activity. In this mixture, the enzyme was inactivated by adding another aliquot of **10** (250 µM) and allowing the resulting mixture to incubate for 2 h. The inactivated enzymes were isolated and prepared as described elsewhere and analyzed by ESI mass spectrometry (19).

Bromide Elimination from **9** in the Presence of Pp 4-OT.

The release of bromide ion was monitored using an Accumet AP63 pH/millivolt/ion meter equipped with an Accumet bromide combination ion selective electrode (Fisher Scientific Inc.). The amount of bromide in solution was determined from a standard curve following the manufacturer's directions. The reaction mixture (total volume of 3 mL) contained **9** (100 µM), Pp 4-OT (6 µL from 6.3 mM or a 43 mg/mL stock solution resulting in a final concentration of 126 µM), and 10 mM NaNO₃ (6 µL of a 5 M solution). The reaction was initiated by the addition of **9**. The presence of NaNO₃ maintained a constant ionic strength. The ION reading was measured, converted to bromide concentration, and plotted as a

function of time. The data were fit to an exponential curve. The reaction was followed until the readings appeared to be leveling off (135–165 min). A final value was obtained after 19 h.

Kinetics of Inactivation.

Stock solutions of **8** (30 mM and 100 mM for *Pp* and *Lc* 4-OT, respectively), **9** (30 mM and 50 mM for *Pp* and *Lc* 4-OT, respectively), and **10** (50 mM and 250 mM for *Pp* and *Lc* 4-OT, respectively) were made up in ethanol. A quantity of *Pp* 4-OT (43 mg/mL) or *Lc* 4-OT (1 mg/mL) was diluted into 20 mM Na₂HPO₄ buffer, pH 7.3 to give final concentrations of 0.63 μM or 2.1 μM, respectively (based on monomer molecular mass). The diluted enzyme was allowed to equilibrate for 1 h before use. The diluted enzymes were divided into 1-mL portions and placed in 1.5 mL eppendorf tubes. A 1 μL- or 1.25 μL-aliquot (*Pp* and *Lc* 4-OT, respectively) was removed prior to the addition of inhibitor and assayed for activity in triplicate. This activity represents 100% activity at time zero. Varying amounts of the stock solutions of **8**, **9**, or **10** (2–20 μL) were added to the incubation mixtures. The change in volume was negligible. The final pH was 7.2. Aliquots (1 or 1.25 μL) were removed at ~60 s intervals and assayed for 4-OT activity. The inactivation data for **9** were fit to a single exponential function as described in the results. The inactivation data for **8** and **10** could not be fit, as described in the results.

Crystallization and Determination of the Structures of the Inactivated 4-OTs.

The *Pp* 4-OT was inactivated by incubation with **9** or **10**, as follows. For **9**, the enzyme (0.5 mL of a 43 mg/mL solution in 20 mM NaH₂PO₄ buffer, pH 7.3) was inactivated by the addition of a 0.2 mL aliquot from a 150 mM solution of **9** made up in 100 mM Na₃PO₄ (in water). The enzyme had no activity after 2 days (9.5 eq of inhibitor were added per monomer). For **10**, the enzyme (1.6 mL of a 43 mg/mL solution in 20 mM NaH₂PO₄ buffer, pH 7.3) was inactivated by the addition of a solution of **10** (5.2 mg in 2.6 mL of 20 mM Na₃PO₄) (in water). After 3.6 eq and a 4-day incubation period, the enzyme had no activity. Both samples were concentrated to ~70 mg/mL in 20 mM HEPES buffer (pH 7.6) containing 1 mM dithiothreitol (DTT). Several crystallization conditions were identified by the sitting drop method during sparse-matrix screening using a Crystal Phoenix Liquid Handling System (Art Robbins Instruments, Sunnyvale, CA). The conditions resulting in the best diffraction-quality crystals for 4-OT inactivated by **9** and **10** were obtained by mixing 1 μL of inactivated 4-OT (~70 mg/mL) respectively with 1 μL of crystallization solution [100 mM TRIS buffer, pH 8.0, in 17.5% PEG 4600 (w/v) and 0.1 M potassium acetate] or [100 mM MES buffer, pH 6.5, in 20% PEG 4600 and 0.1 M sodium nitrate], followed by incubation at 25 °C. Within 1 day, crystals were formed through vapor diffusion in sitting drops. For both, a crystal from the crystallization drop was transferred to mother liquor with 30% (v/v) glycerol as the cryoprotectant. Following a brief equilibration period, the crystal was captured in a nylon loop and vitrified in liquid nitrogen for data collection. The X-ray diffraction data for 4-OT inactivated by **9** or **10** were collected at a wavelength of 1 Å at ~100 K respectively on beamline 5.0.3 of the Advanced Light Source (ALS, Berkeley, CA) or beamline 23-ID-B of the Advance Photon Source (Argonne, IL). The data were processed and scaled using HKL2000 (21). The data collection statistics are summarized in Table 1.

The inactivated *Pp* 4-OT structures were determined by molecular replacement (MR) using Phaser-MR from the Phenix suite with anisotropy correction (22). The *Pp* 4-OT model (Protein Data Bank entry 1BJP) was used as the search model. Structures were refined with Refmac5 (23) in the CCP4 Program Suite (24) along with iterative model building in COOT (25). In refinement, 5% test set (reflections) was excluded for R_{free} cross-validation (26). The covalent adduct was not modeled until the final stages of refinement, where the adduct was only added to monomers that displayed pockets of positive electron density. The final models were evaluated by PROCHECK (27) and MolProbity (28), and displayed adequate electron density around Pro1 and the covalent adduct (Supplementary Figures 2 and 3). The Figures were prepared with PyMol (29). The refinement statistics for both inactivated structures are summarized in Table 1.

RESULTS

Inactivation of *Pp* 4-OT by 5-Fluoro-2-hydroxymuconate (**7**) or 5-Fluoro-2-hydroxy-2,4-pentadienoate (**10**) and ESI-MS Analysis.

An initial analysis showed that incubation of *Pp* 4-OT with **7** or **10** results in a loss of enzymatic activity, but the loss of activity is more rapid with **10**. After 18 h, the *Pp* 4-OT retained ~83% activity when incubated with **7**. After an additional 72 h, the enzyme had no activity. Incubation of the enzyme with an equal amount of **10** (final concentration of 9.3 mM) for 18 h resulted in a complete loss of activity. (The untreated enzyme had nearly 100% activity over the same time intervals.) The slower inactivation of 4-OT by **7** could be due to the presence of **10**, which results from the non-enzymatic decarboxylation of **7**. Accordingly, 4-OT was incubated with **7** and 4-OD/E106QVPH (11–13). (4-OD is in a complex with VPH. In its absence, 4-OD is insoluble.) The E106Q mutant of VPH shows little hydratase activity, but the complex retains full decarboxylase activity (12). Under these conditions, there was a complete loss of 4-OT activity after 18 h. This result is consistent with the proposed mechanism for the loss of 4-OT activity in the presence of **7**. In all cases, gel filtration chromatography (using a PD-10 Sephadex G-25 column) did not result in the reactivation of the enzyme suggesting that a covalent bond had formed between the enzyme and the compound (or a derivative).

In order to identify the covalent species resulting in the covalent modification of the *Pp* 4-OT, the inactivated enzyme samples and the control sample of enzyme were isolated and analyzed by ESI-MS (19). The untreated sample of *Pp* 4-OT has a mass of 6812 Da (calculated mass of 6811 Da) (Supplementary Figure 4) (30,31). Mass spectral analysis of the samples that were incubated with **7** (in the presence or absence of 4-OD/E106QVPH) or **10**, showed two equal sized signals corresponding to masses of 6812 Da and 6943 Da (Supplementary Figure 5A). The first signal corresponds to the unmodified enzyme and the second signal is consistent with the enzyme modified by 2-oxo-5-fluoropentanoate (**14**, Scheme 3A) (132 Da), or the enol tautomer (**15**). (The indicated hydrogen bond is suggested by the crystal structure, as discussed later.) Notably, the fluoride is still present. These results indicate that the species responsible for the inactivation of the *Pp* 4-OT is an adduct derived from **10**. In addition, the equivalent amounts of labeled and unlabeled species

suggest that the hexameric enzyme is inactivated by 3 equivalents of inhibitor. This half-of-the-sites stoichiometry has been observed previously (31).

Inactivation of *Pp* 4-OT by 5-Chloro-2-hydroxy-2,4-pentadienoate (**8**) or 5-Bromo-2-hydroxy-2,4-pentadienoate (**9**).

The irreversible loss of 4-OT activity in the presence of **10** suggested that **8** and **9** might also cause irreversible inhibition of the enzyme. Accordingly, the *Pp* 4-OT was incubated with **8** or **9** for 18 h and 1 h respectively, after which there was no enzymatic activity. Again, gel filtration chromatography did not result in the reactivation of the enzyme suggesting that a covalent bond had formed between the enzyme and the compound. The inactivated enzymes in both samples were isolated and analyzed by ESI-MS in order to identify the covalent species responsible for covalent modification (19). Both samples show a small signal at 6812 Da (corresponding to the unmodified *Pp* 4-OT), and a major signal at 6923 Da (Supplementary Figures 5B and 5C). The mass difference is 112 Da, which is consistent with the covalent modification of the enzyme by **16** (Scheme 3B) or the dienol (i.e., **17**). For both species, the mechanism responsible for the inactivation of the enzyme has displaced the halide substituent. Hence, the inactivation mechanism for **8** and **9** is different from that for **10**.

Inactivation of *Lc* 4-OT by **8**, **9**, or **10**.

The *Lc* 4-OT was incubated with **8**, **9**, and **10** for 16 h, and analyzed for remaining activity. (The same reactions were carried out with the *Pp* 4-OT under identical conditions to compare enzyme activities.) After 16 h, all enzymes were inactivated except for the *Lc* 4-OT incubated with **10**, which retained 30% activity. For the *Lc* enzyme, the mass spectral analysis of the inactivated enzymes is more complicated due to a mixture of enzyme species. Mass spectral analysis of the untreated *Lc* 4-OT showed six signals with masses at 6475 Da, 6606 Da, 6634 Da, 6916 Da, 7047 Da, and 7075 Da (Supplementary Figure 1) (The underlined masses correspond to the enzyme species with an unblocked Pro1.) These masses correspond to the enzyme without the four C-terminal amino acids, the same enzyme with an N-terminal methionine, the same enzyme with an N-formylmethionine, the intact enzyme, the intact enzyme with an N-terminal methionine, and the intact enzyme with an N-formylmethionine. The masses are all within experimental error of the calculated masses (6474 Da, 6606 Da, 6633 Da, 6915 Da, 7047 Da, and 7074 Da). The removal of the initiating methionine (and the N-formyl group) in *E. coli* has been correlated with the identity of the second residue (in this case, proline) (32). It is not known why some of the *Lc* enzyme is processed to remove the N-formyl group and the initiating methionine and some is not. It is also not clear why the *Lc* 4-OT enzyme loses 4 amino acids at the C-terminus, but it might be related to the boiling step in the purification procedure. However, these observations are reproducible, and the mass spectral analysis of the inactivated enzyme is interpretable.

Mass spectral analysis of the inactivated *Lc* 4-OT shows covalent modification in the presence of **8**, **9**, and **10**. Treatment with **8** or **9** shows two new signals with masses of 7029 Da and 6587 Da. These masses represent increases of 113 Da and 112 Da, respectively, and indicate that the *Lc* 4-OT is modified by **16** or tautomer, **17** (Scheme 3B), like that observed

for the *Pp* 4-OT (with the loss of the halide) (Supplementary Figures 6A and 6B). Moreover, the observation that modification only occurs on the two enzyme species with unblocked amino-terminal prolines is consistent with Pro1 being the site of modification (31). Mass spectral analysis of the *Lc* 4-OT incubated with **10** shows a signal with a mass of 7046 (Supplementary Figure 6C). This mass is consistent with the enzyme modified by **14** or **15** (an increase of 132 Da) with the fluoride present (Scheme 3A). Unfortunately, this signal overlaps with the signal corresponding to the intact enzyme with the N-terminal methionine. However, the signal is higher in the modified sample than it is in the control sample. Hence, both the *Lc* and *Pp* 4-OT are inactivated by the same adduct, derived from **10** (Scheme 3A). (A signal is anticipated at 6607 Da, but this signal overlaps with the signal for the enzyme having an N-terminal methionine, but without the four C-terminal amino acids.)

Incubation of the *Lc* 4-OT with **8**, **9**, or **10** also results in a signal with a mass of 7001 Da. This mass represents an 84 Da increase in mass over that observed for the intact enzyme. The same signal is seen in the mass spectra for the incubation mixtures containing the *Pp* 4-OT with **8**, **9**, or **10**, but it is much smaller. The increase in mass may be the result of halide loss and an oxidative decarboxylation of **8**, **9**, or **10**.

Bromide Elimination from **9** in the Presence of *Pp* 4-OT.

A bromide electrode was used to determine if bromide ions were released during the inactivation of 4-OT (126 μ M) by **9** (100 μ M). A slight lag can be seen followed by an exponential increase in bromide ions until leveling off (after 150 min) (Supplementary Figure 7). The lag could be due to the conversion of the dienol (**9**) into the conjugated ketone (**12**), the species that likely inactivates the enzyme. The final concentration of bromide ions was determined to be 75 μ M, suggesting that 59% of the 4-OT monomers had been labeled, with 41% remaining activity. The remaining activity of the enzyme was determined spectrophotometrically to be ~44%, which is in good agreement with the number calculated by bromide ion release.

Kinetics of Inactivation for *Pp* and *Lc* 4-OT by **9**.

The inactivation of *Pp* 4-OT by **9** in each of the nine experiments (15–600 μ M) followed a single exponential decay (Figure 1A). The observed rate constant for inhibition, k_{obs} , at each inhibitor concentration, $[I]$, could be fit to the hyperbolic equation, $k_{\text{obs}} = k_2[I]/(K_i + [I])$, where K_i and k_2 (or k_{inact}) represent the affinity for noncovalent binding and the rate of the bond forming reaction, respectively (Figure 1B) (33,34). The calculated values from this plot are $K_i = 60 (\pm 10) \mu\text{M}$ and $k_2 = 0.19 (\pm 0.01) \text{ min}^{-1}$. The k_{inact}/K_i is $53 (\pm 9) \text{ M}^{-1} \text{ s}^{-1}$ (34). The observation of saturation kinetics (i.e., Figure 1B) reflects the formation of a dissociable complex of the enzyme and the inactivating species at the active site prior to covalent bond formation and inactivation.

The inactivation of the *Lc* 4-OT by **9** follows a similar pattern although at higher concentrations of inhibitor (Figures 2A and 2B). Accordingly, in each of ten experiments (75–1500 μ M), the inactivation of enzyme followed a single exponential decay (Figure 2A). A plot of the observed rate constants for inhibition, k_{obs} , vs inhibitor concentration results in a hyperbolic curve (Figure 2B) and yields a $K_i = 260 (\pm 90) \mu\text{M}$ and $k_2 = 0.43 (\pm 0.01) \text{ min}^{-1}$.

The k_{inact}/K_i is $30 (\pm 10) \text{ M}^{-1} \text{ s}^{-1}$. A comparison of these parameters shows that **9** (or **12**) binds better to *Pp* 4-OT, but that the inactivation rate constant is smaller than that for *Lc* 4-OT. The efficiency constants are nearly comparable within the margin of error.

Kinetics of Inactivation for *Pp* and *Lc* 4-OT by **8** and **10**.

Incubation of the *Pp* and *Lc* 4-OT with **8** and **10** also resulted in the irreversible loss of enzymatic activity (Figures 3 and 4). However, all attempts to fit the data were not successful. There is not an obvious explanation for the inability to fit the data. For **8**, it might be related to the lower amounts of **11** (39%, Scheme 4) along the higher amounts of **18** (39%) present in an equilibrium mixture. The data resulting from the inactivation of 4-OT by **9** (which can be fit) has significantly higher quantities of **12** (61%), the presumed inactivating species, than **19** (17%). The ESI-MS analysis of the inactivation of the *Pp* and *Lc* 4-OT with **10** displayed half-of-sites reactivity. This may result in different binding affinities and inactivation rates of neighboring active sites in the 4-OT hexamer. Nonetheless, some general trends are apparent for the inactivation data for **8** and **10**.

For both enzymes and both inhibitors (**8** and **10**), there is a clear loss of enzymatic activity. For the *Pp* 4-OT and **8**, the enzyme appears to lose about ~10–15% activity in the first minute (200 and 300 μM) and then drifts steadily downward (Figure 3A). It is only possible to capture the 100% activity reading at 100 μM of **8**. As the concentration of **8** increases, the enzyme loses significant activity in the first 60 s (25–55% for 500–1000 μM). The *Lc* 4-OT follows the same trend except the loss of activity is greater (due to the higher concentrations of **8**) (Figure 3B). The enzyme loses ~20% in the first 60 s at lower concentrations of **8** and upwards to 55% activity at the higher concentrations. The general trend for both enzymes is a rapid drop in activity followed by a slower loss of activity.

For the *Pp* and the *Lc* 4-OT with **10**, the same general trends are observed, but they are more pronounced (Figure 4). For example, at the lowest concentration of inhibitor, the *Pp* 4-OT loses 25% of activity within 60 s (Figure 4A), whereas the *Lc* 4-OT loses 15% of activity within 60 s (Figure 4B). At the higher concentrations (*Pp* 300 μM and above and *Lc* 500 μM and above), both enzymes lose ~40% of activity within 60 s. After the initial loss of activity, there is a slower downward trend.

Crystallographic Analysis of the Inactivated *Pp* 4-OTs.

The *Pp* 4-OT was inactivated by incubating the enzyme with **9** or **10** and the resulting inactivated enzyme was crystallized. The crystal structures were obtained to 2.7 Å resolution (4-OT-“**9**”) and 1.5 Å resolution (4-OT-“**10**”), respectively (Figures 5 and 6). Examination of the active site regions in both structures clearly shows (Supplementary Figures 2 and 3) a covalent bond between the prolyl nitrogen of Pro1 and C-5 of the adduct resulting from **9** (Scheme 3B) or C-4 of the adduct resulting from **10** (Scheme 3A). The latter structure shows the presence of the fluoride (Figure 6). In contrast, the complex resulting from the incubation of **9** and 4-OT shows that the bromide has been displaced (Figure 5). This observation is consistent with the results of the bromide release experiments and mass spectral analysis.

In Figure 5, the structure shows two interactions between the enzyme and the inactivating moiety (**16** or **17**). These interactions are the covalent modification of Pro1 by the adduct and the interaction of C1 carboxylate group with the ω -nitrogen of the side chain of Arg39". It is not possible to distinguish between adducts **16** and **17**, i.e. an α,β -unsaturated ketone or a dienol (**16** was modeled in the active site).

In Figure 6, several interactions are observed between the adduct (**14** or **15**) and the enzyme. The C1 carboxylate group interacts with the δ - and ω -nitrogen of the side chain of Arg39" (2.7 and 3.2 Å, respectively). The δ -nitrogen also interacts with the C2 oxygen. The C2 oxygen interacts with an ordered water molecule (2.5 Å), which interacts with the backbone carbonyl group of Ser37 (2.6 Å) and the ω -nitrogen of the Arg39 side chain (2.9 Å). The rigidity of the structure indicates a hydrogen bond between the fluoride and the protonated nitrogen (3.1 Å). There is also an interaction between the ω -nitrogen of Arg11' and the fluoride of the adduct mediated by a water molecule.

DISCUSSION

Aromatic hydrocarbons and their halogenated counterparts are frequently transformed by soil microorganisms into less toxic products (1–4). Incomplete degradation often frustrates efforts to use microbes in the cleanup of contaminated environments (1–7). This limitation has prompted research to identify vulnerable steps in a degradative pathway and determine the molecular basis for the bottleneck (2–4). Ultimately, this knowledge could be used to guide protein engineering efforts to circumvent the vulnerable steps.

There are two well-characterized examples of "roadblocks" in meta-fission pathways (3,4). The first involves the mechanism-based inhibition of extradiol-type dioxygenases (e.g., catechol 2,3-dioxygenase) by halogenated catechols (2,4). It was initially thought that ring opening generated an acyl chloride species, which results in the covalent modification of the enzyme. A more careful analysis of the reaction catalyzed by 2,3-dihydroxybiphenyl 1,2-dioxygenase (in the polychlorinated biphenyl degradation pathway) with 3-chlorocatechol showed that an acyl species is formed, but it does not lead to enzyme modification (4). Instead, dissociation of a bound superoxide species from a ternary complex of enzyme, substrate, and oxygen results in the oxidation of the bound Fe(II) into a catalytically inactive Fe(III) (4). A second example involves the inhibition of a carbon-carbon bond hydrolase (designated BphD) in the degradation of PCBs (3). The reaction requires a tautomerization step that sets up the molecule for C-C bond cleavage. The halogenated substrate binds in a non-productive mode that is stabilized by interactions between the halogen and non-polar side chains. In this mode, the hydroxyl group of the substrate is positioned too far away from His-265, which initiates tautomerization. In addition, access to this histidine is blocked by the halogen substituent (3).

We report herein a third example where a downstream product (i.e., **8**, **9**, or **10**) results in the irreversible inhibition of an upstream enzyme (4-OT). This phenomenon was uncovered in our studies of a bacterial strain that utilizes *p*-chloronitrobenzene as the sole source of carbon, nitrogen, and energy (15,16). The proposed degradative pathway parallels the conventional meta-fission route with an appended chloro group at C-5 (Scheme 5). Such a

pathway would include intermediates **20**, **21**, and **22**. The presence of the halogen raises some interesting questions including the effects of chloroacetaldehyde (**22**), in the event that it is generated. This compound would almost certainly cause problems for the cell unless there were some adaptations (35). The answer to this and other questions required the chemical and enzymatic synthesis of the 5-halo-2-hydroxymuconates and 5-halo-2-hydroxy-2,4-pentadienoates, and the characterization of their properties and fates in the proposed pathway.

In the course of the enzymatic synthesis of **10** from **7**, it was noted that incubation of 4-OT with **7** resulted in the slow inactivation of 4-OT (17). The slow rate of inactivation suggested that 4-OT converts **7** to **23**, which undergoes non-enzymatic decarboxylation to form **10** (Scheme 6), along with the β,γ - and α,β -unsaturated ketones (**24** and **13**, respectively in Scheme 6). In this scenario, **13** is responsible for the covalent modification and inactivation of 4-OT. Support for this proposal came from the observation that inclusion of 4-OD/E106QVPH in the reaction mixture accelerates inactivation. 4-OD/E106QVPH catalyzes the decarboxylation of **23**, thereby generating **10** (in equilibrium with **24** and **13**). In addition, incubation of 4-OT with **10** results in faster loss of enzymatic activity. Mass spectral analysis showed that the enzyme was modified by **14** or **15** (Scheme 3A), which results from nucleophilic attack at C4 of **13** (or **10**), respectively. A crystal structure of the inactivated enzyme showed that Pro1 is the site of modification and that the fluoride ion is present in the covalent adduct (Scheme 3A).

Based on these observations, inactivation of 4-OT can occur by the mechanism shown in Scheme 7A. The nucleophilic prolyl nitrogen of Pro1 ($pK_a \sim 6.4$) (36) attacks C-4 of **13** with protonation at C-3 to result in the final covalent adduct, **14** or **15**. This mechanism relies on the presence of **13**, for which we have no spectroscopic evidence (UV or ^1H NMR). After 24 h, analysis of the mixture shows a ~3:1 ratio of **24** to **10** (17). Nonetheless, there could be a very small amount of **13** present in the mixture. The covalent reaction with Pro1 of 4-OT would shift the equilibrium (to produce more **13**) and eventually drive everything present in the mixture to form a covalent adduct with the enzyme.

These observations prompted an examination of 4-OT by the two other halogenated species, **8** and **9**. Both lead to the irreversible inactivation of the enzyme. In these cases, the halogen is not present in the final covalent adduct. The loss of the halogen was confirmed by mass spectral analysis, and the position of attachment to the enzyme (via Pro1) was shown by the crystallographic analysis of 4-OT inactivated by **9**. Hence, the nucleophilic proline attacks the C5 position of **11** or **12**, which would be generated by 4-OT (Scheme 7B). The mechanism is favored by the combination of the highly electrophilic allylic position (i.e., C5) and the greater leaving group ability of bromide and chloride (vs. fluoride). Displacement of the halide results in the formation of **16**, which is presumably in equilibrium with **17**.

The *Lc* meta-fission pathway is representative of a haloaromatic degradative pathway (13). Previous work indicated that *Lc* 4-OT is less capable of performing the 1,5-keto-enol and 1,3-keto-enol tautomerization than *Pp* 4-OT (17). The results of this work show that a higher concentration of inhibitor (i.e., **8**, **9**, or **10**) is required for the inactivation of *Lc* 4-OT

(compared with *Pp* 4-OT). At first glance, this could suggest that *Lc* 4-OT has evolved a mechanism to protect itself from inactivation such as binding the compound less tightly (for example). However, there is no evidence to suggest this and the actual reason(s) are likely much more complicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DTT	dithiothreitol
ESI-MS	electrospray ionization mass spectrometry
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate
4-OD	4-oxalocrotonate decarboxylase
4-OT	4-oxalocrotonate tautomerase
<i>Pp</i> and <i>Lc</i> 4-OT	4-OT from <i>Pseudomonas putida</i> mt-2 and <i>Leptothrix cholodnii</i> SP-6, respectively
PCB	polychlorinated biphenyl
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
VPH	vinylpyruvate hydratase

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Figure 1.

Kinetics of *Pp* 4-OT inactivation (0.63 μM) upon incubation with 5-bromo-2-hydroxy-2,4Z-pentadienoate (**9**). (A) The percent of 4-OT activity remaining as a function of incubation time with varying amounts of **9**. (B) Plot of k_{obs} for inactivation vs the concentration of **9**. The values of K_i and k_{inact} are presented in the text.

Figure 2.

Kinetics of *Lc* 4-OT inactivation (2.1 μM) upon incubation with 5-bromo-2-hydroxy-2,4Z-pentadienoate (**9**). (A) The percent of 4-OT activity remaining as a function of incubation time with varying amounts of **9**. (B) Plot of k_{obs} for inactivation vs the concentration of **9**. The values of K_i and k_{inact} are presented in the text.

Figure 3.

Kinetics of *Pp* and *Lc* 4-OT inactivation (0.63 and 2.1 μM, respectively) upon incubation with 5-chloro-2-hydroxy-2,4Z-pentadienoate (**8**). (A) The percent of *Pp* 4-OT activity remaining as a function of incubation time with varying amounts of **8**. (B) The percent of *Lc* 4-OT activity remaining as a function of incubation time with varying amounts of **8**. For both, there is a sharp decrease in activity (in the first 60 s) followed by a slower loss of enzymatic activity, as detailed in the text.

Figure 4.

Kinetics of *Pp* and *Lc* 4-OT inactivation (0.63 and 2.1 μM , respectively) upon incubation with 5-fluoro-2-hydroxy-2,4 Z -pentadienoate (**10**). (A) The percent of *Pp* 4-OT activity remaining as a function of incubation time with varying amounts of **10**. (B) The percent of *Lc* 4-OT activity remaining as a function of incubation time with varying amounts of **10**. For both, the initial decrease in activity is more pronounced (in the first 60 s) than that observed in Figure 3, followed by a slower loss of enzymatic activity, as detailed in the text.

Figure 5.
The active site of the *Pp* 4-OT showing the covalently modified Pro1 after incubation with 5-bromo-2-hydroxy-2,4Z-pentadienoate (**9**).

Figure 6.

The active site of the *Pp* 4-OT showing the covalently modified Pro1 after incubation with 5-fluoro-2-hydroxy-2,4Z-pentadienoate (**10**). A single and double prime after a residue label designates that the residue comes from a second and third monomer, respectively.

Scheme 1.
Partial Meta-fission Pathway for Microbial Degradation of Aromatic Compounds.

Scheme 2.
Inactivators of 4-OT.

Scheme 3.
Structures of the Covalently Modified 4-OT at Pro1.

Scheme 4.

Isomers Mixtures for 8 and 9 with Percentages at Equilibrium.

Scheme 5.
Proposed Degradation Pathway for 5-Chloro Substituted Species via the Meta-fission Pathway.

Scheme 6.

Generation of 10 from the Ketonization of 7 Followed by Decarboxylation of 23.

Scheme 7.
Possible Inactivation Mechanisms.

Table 1.**X-ray Data Collection and Refinement Statistics**

Data Collection	4-OT-compound 9	4-OT- compound 10
space group	$P2_1$	$C2$
cell dimensions		
a, b, c (Å)	62.7, 90.1, 171.4	87.4, 88.0, 112.7
α , β , γ (°)	90.0, 96.8, 90.0	90, 98.3, 90
resolution (Å)	50–2.7 (2.75–2.7) ¹	50–1.51 (1.54–1.51) ¹
Total no. of reflections	918590	895292
Data cutoff	$F > 0$	$F > 0$
Asymmetric unit (asu)	30 monomers	15 monomers
Number of unique reflections	52302	131636
Redundancy	3.8 (3.8)	3.7 (3.4)
R_{sym} (%) (linear R factor)	11.0 (7.9) ¹	5.5 (27.6) ¹
$I/\sigma I$	13.7 (1.6) ¹	32.2 (4.38) ¹
completeness (%)	100.0 (100.0) ¹	99.6 (98.4) ¹
Refinement		
resolution (Å)	48.06–2.70	43.29–1.51
no. of reflections	49580	124537
$R_{\text{work}}/R_{\text{free}}$ (%)	21.7/25.5	11.8/16.9
no. of atoms	13647	7904
Protein	13532	7155
Water	115	641
solvent		120
Average B factor (Å ²)	58.0	23.7
Protein	58.2	22.8
Adduct alone	81.5	49.3
Water	35.0	33.5
Solvent		28.5
R.m.s. deviation		
bond lengths (Å)	0.008	0.01
bond angles (deg)	1.102	1.27
Ramachandran plot (%)		
residues in most favored regions	97.50	96.10
residues in additional allowed regions	2.50	3.90
residues in disallowed regions	0.00	0.00

¹Data for the highest resolution shell are shown in parentheses.

² R_{free} is calculated with 5% of data randomly omitted from refinement.