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Preparation of dihydroxy polycyclic aromatic hydrocarbons and activities of two dioxygenases in the phenanthrene degradative pathway

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ABSTRACT

Dihydroxy phenanthrene, fluoranthene, and pyrene derivatives are intermediates in the bacterial catabolism of the corresponding parent polycyclic aromatic hydrocarbon (PAH). Ring-opening of the dihydroxy species followed by a series of enzyme-catalyzed reactions generates metabolites that funnel into the Krebs Cycle with the eventual production of carbon dioxide and water. One complication in delineating these pathways and harnessing them for useful purposes is that the initial enzymatic processing produces multiple dihydroxy PAHs with multiple ring opening possibilities and products. As part of a systematic effort to address this issue, eight dihydroxy species were synthesized and characterized as the dimethoxy or diacetate derivatives. Several dihydroxy compounds were examined with two dioxygenases in the phenanthrene degradative pathway in *Mycobacterium vanbaalenii* PYR-1. One, 3,4-dihydroxyphenanthrene, was processed by PhdF with a k_{cat}/K_m of $6.0 \times 10^6\ M^{-1}s^{-1}$, a value that is consistent with the annotated function of PhdF in the pathway. PhdI processed 1-hydroxy-2-naphthoate with a k_{cat}/K_m of $3.1 \times 10^5\ M^{-1}s^{-1}$, which is also consistent with the proposed role in the pathway. The observations provide the first biochemical evidence for these two reactions in *M. vanbaalenii* PYR-1 and, to the best of our knowledge, the first biochemical evidence for the reaction of PhdF with 3,4-dihydroxyphenanthrene. Although PhdF is upregulated in the presence of pyrene, it did not process two dihydroxypyrenes. Methodology was developed for product analysis of the extradiol dioxygenases.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) and their derivatives are pervasive environmental contaminants that adversely affect air, soil, and water quality [1]. PAHs persist in the environment due to their limited aqueous solubility and low volatility. They also accumulate in the food chain. PAHs are toxic to humans (either directly or indirectly by reactive metabolites) as well as marine and aquatic organisms [2–4]. Hence, there is interest in the development of technologies to remove PAHs from the environment. One particularly attractive technology is bioremediation, which exploits the nutritional versatility of microorganisms to convert the PAHs into carbon dioxide and water [5]. This technology requires a comprehensive understanding of the degradative pathways.

PAHs are categorized according to their molecular weights when referring to their degradation by bacterial pathways. The low-molecular-weight (LMW) PAHs consist of three or less aromatic rings and are exemplified by naphthalene (**1**, Scheme 1) [6] and phenanthrene (**2**) [7,8]. The high-molecular-weight (HMW) PAHs have four or more aromatic rings and include fluoranthene (**3**) and pyrene (**4**). The LMW PAHs are more tractable systems, but the degradative pathway for only one of these, naphthalene, is understood in any significant detail. As the number of rings increases, the systems become less tractable, making our understanding of the degradative pathways for phenanthrene and the HMW PAHs limited, at best [9–11].

Several factors contribute to this lack of knowledge, but one troubling issue is that as the number of rings increases, the number of potential hydroxylation sites by the ring-hydroxylating enzymes increases [7–11]. This produces multiple dihydroxy species with multiple ring-opening possibilities and products. These observations (along with other factors) hinder a systematic examination of the PAH catabolic pathways. As part of an effort to address this problem, we developed methodology for the chemical synthesis of four dihydroxyphenanthrenes (**5a-d**, Scheme 2), two dihydroxyfluoranthenes (**6a,b**), and two dihydroxypyrenes (**7a,b**).[1] The compounds were characterized as the dimethoxy or diacetate derivatives. In

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[1] The literature for PAH degradation pathways generally refers to these compounds as dihydroxy PAHs. More precisely, they are named 1,2-, 2,3-, 3,4-, and 9,10-phenanthrenediol (**5a-d**), 2,3- and 7,8-fluoranthenediol (**6a,b**), and 1,2- and 4,5-pyrenediol (**7a,b**). In order to be consistent with the field, we retain the dihydroxy PAHs nomenclature.

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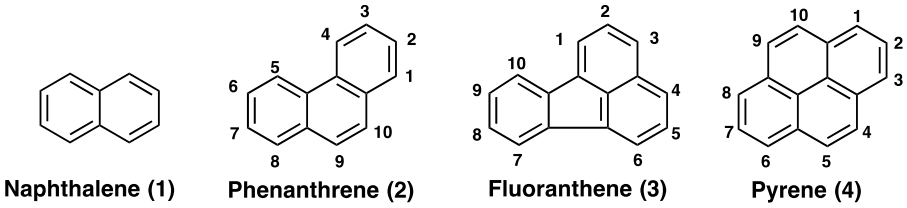
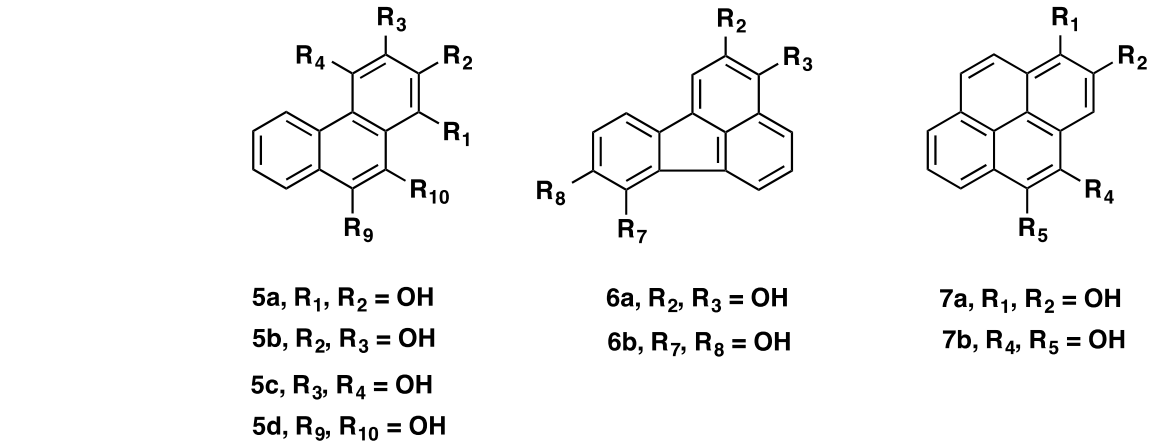


Scheme 1. Representative LMW and HMW polycyclic aromatic hydrocarbons (PAHs). The LMW PAHs include naphthalene (**1**) and phenanthrene (**2**) and the HMW PAHs include fluoranthene (**3**) and pyrene (**4**).



Scheme 2. Dihydroxy PAHs synthesized in this work and characterized as the dimethoxy or diacetate derivatives. The R group is a hydrogen unless otherwise indicated.

addition, the dihydroxypyrenes were examined with two ring-opening dioxygenases (PhdI and PhdF) from *M. vanbaalenii* PYR-1, the first bacterium identified to completely degrade pyrene [7,8,11]. Although PhdI and PhdF did not process the dihydroxypyrenes, ring-opening activities were observed for these enzymes using four other compounds. Most notably, PhdF processes **5c**, which is the first biochemical evidence for this reaction. The results, reported herein, along with the availability of the dihydroxy compounds, set the stage for future investigations of the enzymes in PAH catabolic pathways.

2. Results and discussion

Synthesis of 5a-d. The 1,2- and 2,3-dihydroxyphenanthrenes (**5a**, **5b**, respectively) were synthesized in 3 steps from 2,3- or 3,4-dimethoxybenzaldehyde (**8a** and **8b**, respectively, Scheme 3A). The starting material (**8a** or **8b**) was combined with potassium *t*-butoxide and benzyltriphenylphosphonium bromide in toluene to produce an *E,Z* mixture of the 1,2- or 3,4-dimethoxystilbene (**9a** and **9b**, respectively) [12]. Oxidative cyclization was carried out in the presence of O_2, I_2, and UV irradiation to yield the 1,2- and 2,3-dimethoxyphenanthrenes (**10a** and **10b**, respectively) [12]. The dimethoxy derivatives were deprotected, as needed, using BBr_3 [13], and the products were stored under argon at −20 °C.

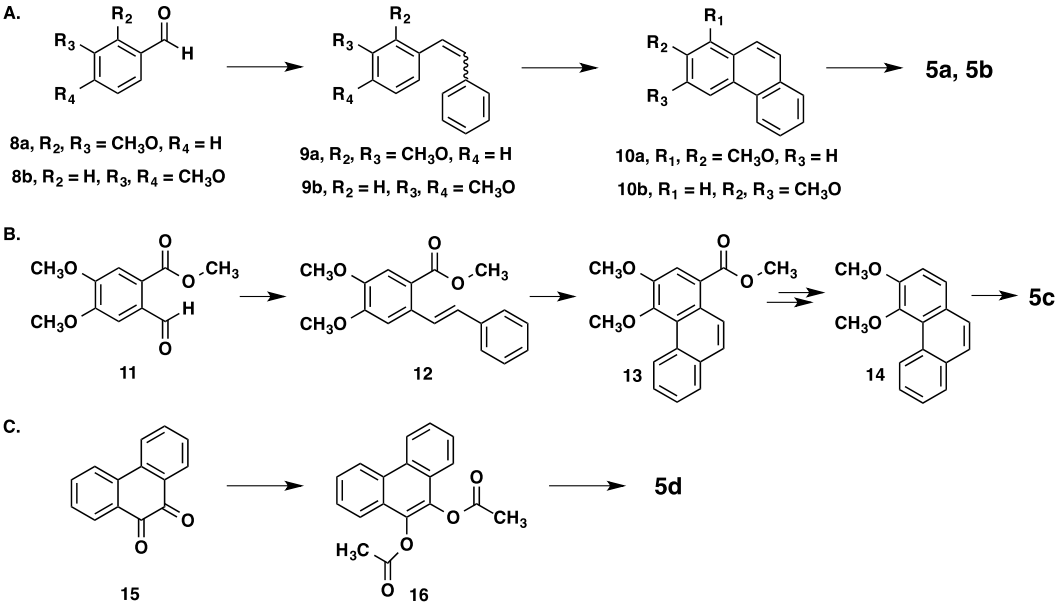
3,4-Dihydroxyphenanthrene (**5c**) was synthesized from methyl 2-formyl-4,5-dimethoxybenzoate (**11**, Scheme 3B), which was generated by a published protocol [13]. Subsequently, a Wittig reaction (using NaH and benzyltriphenylphosphonium bromide) was carried out on the aldehyde to produce the *E*-isomer of the stilbene (**12**) [12]. Oxidative cyclization was carried out as above, and the resulting methyl ester (**13**) was hydrolyzed and decarboxylated using copper in quinolone [14]. The product, 3,4-dimethoxyphenanthrene (**14**), was deprotected, as needed, by treatment with BBr_3 to afford **5c** [13].

9,10-Dihydroxyphenanthrene (**5d**) was generated from the commercially available 9,10-phenanthrenequinone (**15**, Scheme 3C), which was converted into phenanthrene-9,10-diyl diacetate (**16**) by a variation on published procedures [15]. When needed, small amounts were treated with acetyl chloride and ethanol (under argon) to afford **5d**.

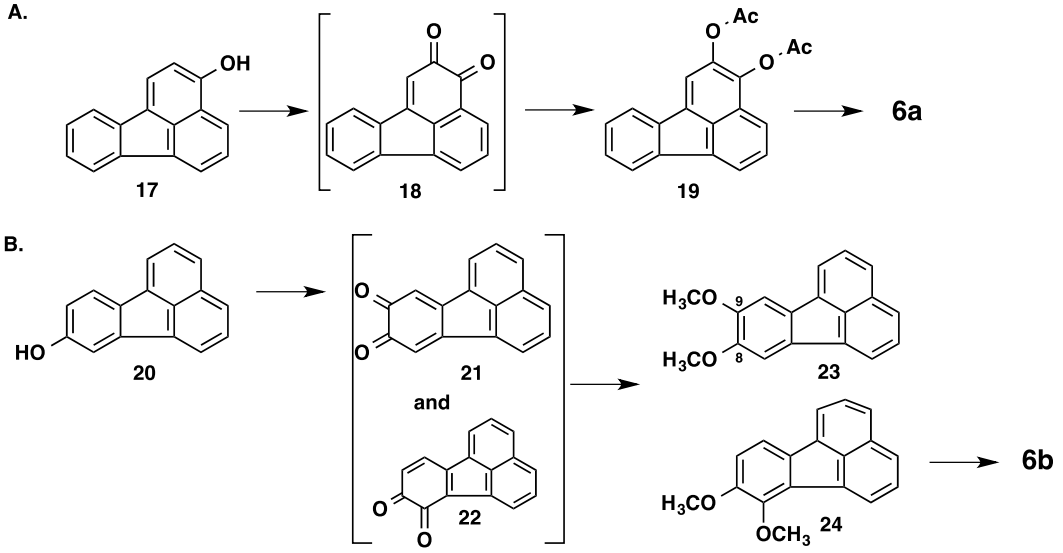
Synthesis of 6a,b. The 2,3- and 7,8-dihydroxyfluoranthenes (**6a,b**, respectively) were synthesized in 2 steps from 3- or 8-hydroxyfluoranthene (**17** and **20**, respectively, Scheme 4). The appropriately substituted hydroxyl fluoranthenes were synthesized by published procedures [16]. The individual hydroxyfluoranthenes were treated with *o*-iodoxybenzoic acid (IBX) to generate the corresponding *ortho*-quinones [17]. Treatment of 3-hydroxyfluoranthene with IBX yielded **18** and treatment of 8-hydroxyfluoranthene (**20**) yielded a mixture of **21** and **22**. The *ortho*-quinones were not isolated and the isomers were not separated. Acetylation of **18** generated the diacetyl derivative, **19**. As needed, small amounts were treated with acetyl chloride and ethanol (under argon) to afford **6a**. Treatment of **21**/**22** with $NaBH_4$ followed by dimethyl sulfate produced a mixture of the corresponding dimethoxy compounds (**23** and **24**). The isomers were separated by silica gel chromatography. From 42.6 mg of **20**, 19.2 mg of 8,9-dimethoxyfluoranthene (**23**) and 2.2 mg of **24** were obtained. As needed, small amounts of the dimethoxy derivative was treated with BBr_3 [13], and the product (**6b**) was stored under argon at −20 °C.

Synthesis of 7a,b. Oxidation of the commercially available 1-hydroxypyrene (**25**, Scheme 5) to pyrene-1,2-dione (**26**) was performed by treatment with *o*-iodoxybenzoic acid (IBX) [17]. Acetylation of **26** and pyrene-4,5-dione (**28**), prepared as described elsewhere [18], to the respective diacetates, pyrene-1,2-diyl diacetate (**27**) and pyrene-4,5-diyl diacetate (**29**), was performed by following published procedures [15]. The compounds were stored at −20 °C. When needed, small amounts of the diacetate esters were treated as described above (for **5d**) to produce 1,2-dihydroxypyrene (**7a**) or 4,5-dihydroxypyrene (**7b**).

Kinetic Characterization of PhdF and PhdI. There is an extensive body of literature on PAH degradation including the identification of several gene clusters and individual enzymes, the detection of metabolites by mass spectrometry or gas chromatography, and the demonstration of the conversion of specific PAHs to cellular intermediates [6–11]. However, there is limited knowledge about the actual substrates and products for many of the individual enzymes, and little biochemical confirmation of several proposed activities because many substrates are not readily available [7–11]. Assignments of functions are mostly based by analogy with the corresponding steps in the well-characterized



Scheme 3. Synthetic routes resulting in **5a-d**. The details for the individual steps are described in the text.



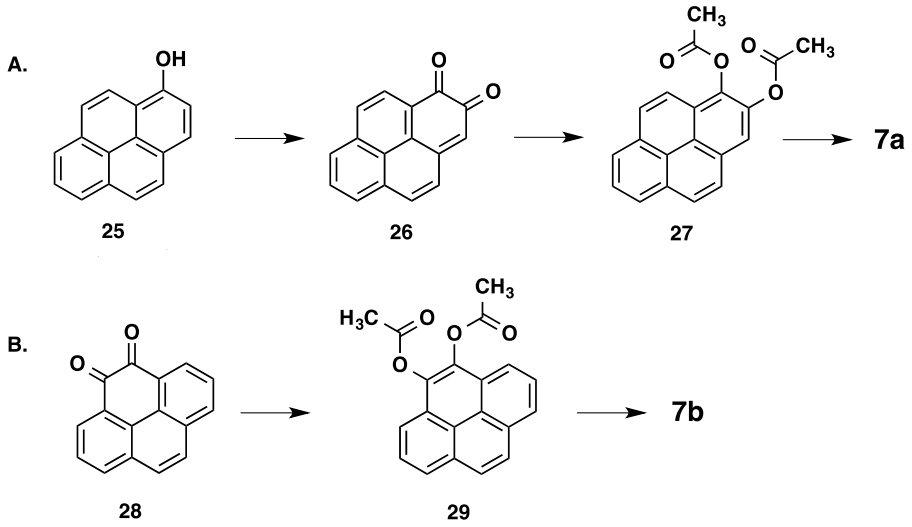
Scheme 4. Synthetic routes resulting in **6a,b**. The details for the individual steps are described in the text.

pathway for the degradation of naphthalene.

There are 9 annotated extradiol dioxygenases (including PhdF) and 3 annotated intradiol dioxygenases (including PhdI) in the PAH gene cluster in *M. vanbaalenii* PYR-1 [8]. PhdF and PhdI are proposed to carry out two steps in the degradation of phenanthrene [8]. PhdF is a Type I extradiol dioxygenase and a member of the vicinal oxygen chelate superfamily [19], that is proposed to convert 3,4-dihydroxyphenanthrene (**5c**, Scheme 6A) into *trans-o*-hydroxybenzylidenepyruvate (**31**) presumably via 2-hydroxybenzo[*h*]chromene-2-carboxylate (**30**) (Scheme 6A) [8]. PhdI is an intradiol dioxygenase and a purported member of the cupin superfamily [20], that is proposed to convert 1-hydroxy-2-naphthoic acid (**32**, Scheme 6B) into *trans-o*-carboxybenzylidenepyruvate (**33**) [8]. In addition to these proposed reactions, PhdF is upregulated in the bacterial degradation of pyrene [11]. This observation suggests that it might play a role in the ring-opening process of **7a** and **7b**.

The genes for both enzymes were cloned from *M. vanbaalenii* PYR-1 genomic DNA, the enzymes were expressed, and purified in good yield. PhdF has to be activated after purification, whereas PhdI does not [19,20]. Both enzymes were examined with 1,2-dihydroxynaphthalene (in ethanol), 1-hydroxy-2-naphthoate (**32**, in ethanol), **5a**,**c**,**d**, **6a**,**b**, **7a**,**b** (Scheme 2), 3-methylcatechol (**34**, Scheme 7A), and 2,3-dihydroxybiphenyl (**35**, Scheme 7B) following changes in the UV/Vis spectra. The only substrates processed were **5c**, **32** (by PhdI), **34**, and **35** (see supporting information for representative UV/Vis traces). Notably, PhdF did not process the dihydroxypyrenes (**7a**,**b**), suggesting that the observed upregulation of PhdF in the presence of pyrenes might be due to steps downstream of the dihydroxypyrene ring-opening step. However, PhdF processed **5c** to **31** (Scheme 6A), the expected extradiol product, and PhdI processed **32** to the intradiol product (i.e., **33**, Scheme 6B). Both activities are in accord with the proposed functions and the presence of the genes for other enzymes in the phenanthrene catabolic pathway in. *M. vanbaalenii* PYR-1 [8,11]. In addition, PhdF processed 3-methylcatechol (**34**, Scheme 7A) and 2,3-dihydroxybiphenyl (**35**, Scheme 7B), to the extradiol products (**36** and **38** in Scheme 7).

The kinetic parameters for PhdI and PhdF with these substrates are summarized in Table 1. The high value of the k_{cat}/K_m for **32** and PhdI indicates that **32** is a reasonably good substrate for the enzyme and



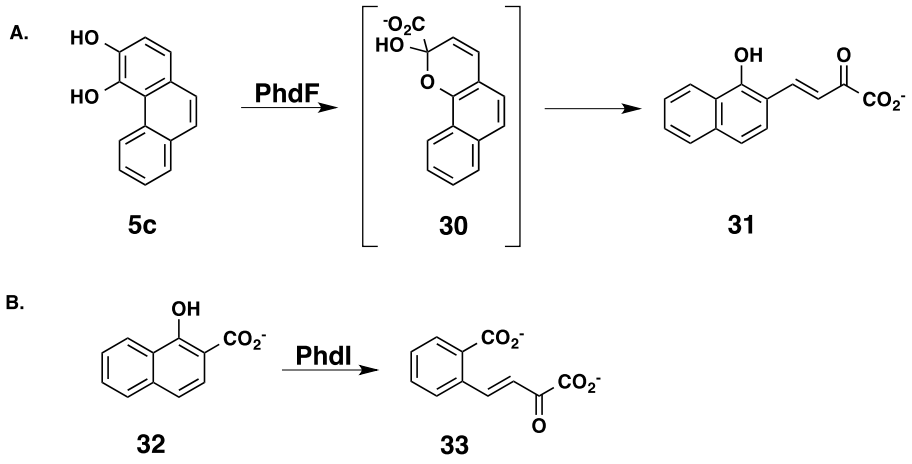
Scheme 5. Synthetic routes resulting in **7a,b**. The details for the individual steps are described in the text.

likely the biological one. Likewise, the high value of the k_{cat}/K_m for **5c** and PhdF indicates that **5c** is likely the biological substrate. A comparison of the k_{cat}/K_m values for **5c**, **34**, and **35** with PhdF (**5c** > **35** > **34**) shows that PhdF prefers larger substrates.

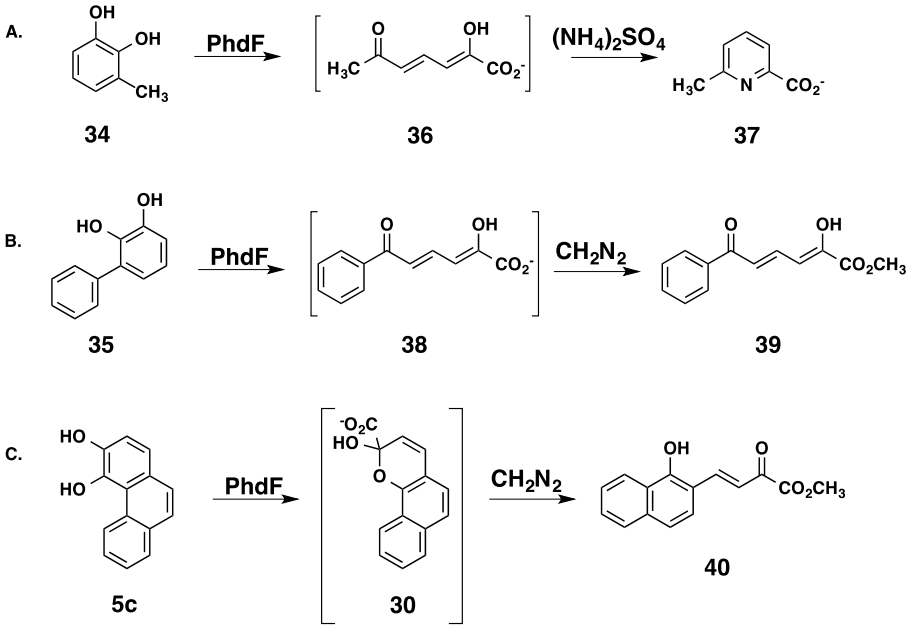
Product Analysis for PhdF and PhdI. In addition to the kinetic parameters, the products of the four reactions (Scheme 6B, Scheme 7A-C) were isolated and identified. The product analysis for the PhdF-catalyzed reactions with **34** and **35** was done in part to develop the methodology for future studies of these pathways where more complex products are generated. The product for the reaction of PhdI and **32** is *trans-o*-carboxybenzylidenepyruvate (**33**), which was identified by ^{1}H NMR analysis (and comparison to an authentic sample) [21]. The predicted extradiol product for the reaction of **34** and PhdF is 2-hydroxymuconaldehyde (**36**, Scheme 7A), which was trapped as the picolonate derivative (**37**). The identity was confirmed by ^{1}H, ^{13}C NMR, and mass spectral analysis. The predicted extradiol product for the reaction of **35** and PhdF is 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (**38**, Scheme 7B), which was isolated as the methyl ester, **39**. The predicted product (but not confirmed) for the reaction of **5c** and PhdF is **30** (Scheme 7C), which was isolated as its methyl ester, **40**. The identities of **39** and **40** were established by ^{1}H, ^{13}C NMR, and mass spectral analysis.

3. Experimental procedures

Materials. Chemicals, biochemicals, buffers, and solvents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Fluka Chemical Corp. (Milwaukee, WI), or EMD Millipore, Inc. (Billerica, MA). 2,3-Dimethoxybenzaldehyde (**8a**), 3,4-dimethoxybenzaldehyde (**8b**), 3,4-dimethoxybenzoic acid, 9,10-phenanthrenequinone (**15**), 1,2-dihydroxynaphthalene, 1-hydroxy-2-naphthoic acid (**32**), and 3-methylcatechol (**34**) were purchased from Sigma-Aldrich Chemical Co. 1-Hydroxypyrene (**25**) was acquired from Tokyo Chemical Industry Co., Ltd. (TCI) America (Portland, OR). 2,3-Dihydroxybiphenyl (99%) (**35**) was purchased from Wako Chemicals Inc. (Richmond, VA). Oligonucleotide primers were synthesized by Sigma-Aldrich Co. The EconoColumn® chromatography columns were obtained from BioRad (Hercules, CA). The DEAE-Sepharose resin, the HiLoad 16/60 Superdex resin, and the Sephadex G-25 resin were obtained from GE Healthcare (Piscataway, NJ). The GenElute gel extraction kit, the HisPur Ni-NTA resin, and *Escherichia coli* strain C41(DE3) were purchased from Sigma-Aldrich Co. Enzymes and reagents used for molecular techniques and the Gibson Assembly Cloning Kit were purchased from New England Biolabs, Inc. The QIAprep spin miniprep kit was purchased from Qiagen (Hilden,

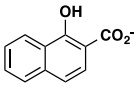
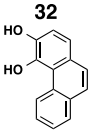
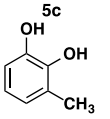
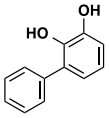


Scheme 6. Proposed PhdF- and PhdI-catalyzed reactions for **5c** and **32**. PhdF is proposed to convert **5c** into **31** via the presumed intermediate **30** and PhdI is proposed to convert **32** into **33**.



Scheme 7. Product analysis for the reactions catalyzed by PhdF. PhdF catalyzes a ring-opening reaction using **34**, **35**, or **5c**, where the products are trapped as their picolinate (**37**) or methyl ester derivatives (**39** or **40**).

Table 1
Kinetic parameters for PhdI and PhdI using the indicated substrates[a].

Substrate	Enzyme	k_{cat}	K_m	k_{cat}/K_m
		(s^{-1})	(μM)	(M^{-1} s^{-1})
 **32**	PhdI	7.0 ± 0.2	23 ± 2	3.1 (± 0.3) × 10^5
 **5c**	PhdF	9.3 ± 0.9	1.7 ± 0.5	6.0 (± 2.0) × 10^6
 **34**	PhdF	0.32 ± 0.01	50 ± 6	6.5 (± 0.9) × 10^3
 **35**	PhdF[b]	0.17 ± 0.01	< 11	> 1.5 × 10^4

[a] The steady-state kinetic parameters were determined under the conditions described in the text.

[b] The steady-state kinetic parameters can only be estimated for the reaction of PhdF and **35** because the enzyme was saturated at all substrate concentrations, as detailed in the experimental.

Germany). ArcticExpress cells were obtained from Agilent Technologies (Santa Clara, CA). Genomic DNA from *Mycobacterium vanbaalenii* PYR-1 was a gift from the laboratory of Dr. Carl Cerniglia (National Center for Toxicology Research, Food and Drug Administration, Jefferson, AR). The Amicon stirred cell concentrators and ultrafiltration membranes (10000 Da cutoff) were purchased from EMD Millipore Inc.

General Methods. The PCR amplification of DNA was conducted in a GeneAmp 2700 thermocycler (Applied Biosystems, Carlsbad, CA). DNA sequencing was performed in the DNA Core Facility in the Institute for Cellular and Molecular Biology (ICMB) at the University of Texas at Austin. Sonication was performed using a W385 sonicator of Heat systems-ultrasonics, Inc. (Farmingdale, NY). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Bio-Rad Mini-Protean II gel electrophoresis apparatus [22]. Protein concentrations were determined using the Bradford method [23]. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian UNITY + 300 MHz (Palo Alto, CA), Varian DirectDrive 400 MHz (Palo Alto, CA),or a Bruker AVANCE III 500 MHz spectrometer (Billerica, MA). NMR signals were analyzed using the software program SpinWorks 3.1.6 (Copyright 2009 Kirk Marat, University of Manitoba).

High-resolution mass spectrometry (HRMS) analysis was carried out on an Agilent 6530 Accurate Mass Q-TOF LC/MS housed in the Mass Spectrometry Facility, Department of Chemistry, University of Texas. Steady-state kinetic assays were performed on an Agilent 8453 diode-array spectrophotometer. Nonlinear regression data analysis was performed using the program Grafit (Erithacus Software Ltd., Staines, U.K.) [24]. Compounds **5a-c** and **6b** were characterized as the dimethoxy derivatives (**10a,b**, **14**, **and 21**, respectively) and compounds **5d**, **6a, 7a**,**b** were characterized as the diacetate derivatives (**16**, **19**, **27**, **29**, respectively) due to the rapid oxidation (and decomposition) of the dihydroxy species.

*Synthesis of 1,2-Dimethoxyyphenanthrene (**10a**) and 2,3-Dimethoxyphenanthrene (**10b**)*. An (*E*, *Z*)-mixture of 3,4-Dimethoxystilbene (**9b**) was generated by the addition of potassium *t*-butoxide (1.1 eq) to a stirred and chilled solution (on ice) of 3,4-dimethoxybenzaldehyde (**8b**, 1.0 g) and benzyltriphenylphosphonium bromide (1.1 eq) dissolved in toluene (25 mL) [12]. The reaction was monitored by TLC and determined to be complete after 3 h. The solvent was filtered and removed under reduced pressure and the residue was purified by silica gel chromatography (4:1 hexanes/ethyl acetate). The procedure generates 0.4 g of the (*Z*)-isomer as a clear colorless liquid along with 0.6 g of the (*E*)-isomer as a white solid. The same procedure was used to generate **9a**, using 2,3-dimethoxybenzaldehyde (**8a**) as the starting material. (*E*)-**9b**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.89 (s, 3H), 3.94 (s, 3H), 6.85 (d, 1H, J = 8.2 Hz), 6.96 (d, 1H, J = 16.3 Hz), 7.05 (m, 3H), 7.23 (m, 1H), 7.34 (m, 2H), 7.48 (m, 2H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 55.8, 55.9, 108.7, 111.2, 119.9, 126.2 (2C), 126.8, 127.3, 128.4 128.6 (2C), 130.4, 137.5, 148.9, 149.1 ppm. (*Z*)-**9b**: ^{1}H

NMR ($CDCl_3$, 400 MHz) δ 3.58 (s, 3H), 3.84 (s, 3H), 6.52 (m, 2H), 6.73 (m, 2H), 6.81 (ddd, 1H, J = 0.4 Hz, J = 2.0 Hz, J = 8.2 Hz), 7.17 (m, 1H), 7.26 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 56.1, 56.5, 111.5, 112.4, 122.6, 127.7, 129.0 (2C), 129.6 (3C), 130.5, 130.7 138.4, 148.9, 149.0 ppm. ^{13}C NMR (CD_3OD, 125 MHz) δ 56.2, 56.6, 112.9, 113.7, 123.6, 128.4, 129.6 (2C), 130.18, 130.23 (2C), 131.4, 131.7 139.5, 150.1 (2C) ppm.

Oxygen is bubbled into a solution of **9a** or **9b** (1 g) dissolved in ether (50 mL) and contained in a quartz glass reaction vessel [12]. After 10 min, the oxygen flow was stopped and I_2 (50 mg) was added. The flask was gently stoppered and irradiated by UV (UV transilluminator set on high). Oxygen and I_2 (10 mg) were added after 24 h until reaction is complete. The reaction was monitored by TLC and determined to be complete after 48 h. The ether layer was washed with 0.5 M sodium metabisulphite (50 mL) and the organic layer separated and removed. The aqueous layer was extracted with ethyl acetate (2 × 50 mL). The organic layers were combined, dried over anhydrous sodium sulfate, and the solvent was removed. The residue was purified by silica gel chromatography (4:1 hexanes/ethyl acetate) to afford 0.5 g of 2,3-dimethoxyphenanthrene (**10b**, 0.5 g). 1,2-Dimethoxyphenanthrene (**10a**): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.02 (s, 3H), 4.04 (s, 3H), 7.37 (d, 1H, J = 9.1 Hz), 7.54 (m, 1H), 7.62 (m, 1H), 7.74 (d, 1H, J = 9.1 Hz), 7.86 (dd, 1H, J = 1.4 Hz, 7.8 Hz), 8.09 (dd, 1H, J = 0.5 Hz, 9.1 Hz), 8.42 (d, 1H, J = 9.0 Hz), 8.59 (dd, 1H, J = 0.5 Hz, 8.2 Hz) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 56.4, 61.3, 113.4, 118.9, 120.2, 122.3, 125.4, 125.9, 126.7, 127.36, 127.41, 128.6, 130.3, 131.0, 143.8, 149.8 ppm. 2,3-Dimethoxyphenanthrene (**10b**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.08 (s, 3H), 4.16 (s, 3H), 7.28 (s, 1H), 7.57 (m, 1H), 7.65 (m, 1H), 7.69 (m, 1H), 7.91 (d, 1H, J = 7.9 Hz), 8.05 (s, 1H), 8.57 (d, 1H, J = 8.3 Hz) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 55.9, 56.0, 103.3, 108.3, 122.1, 124.9, 125.3, 125.6, 126.0, 126.2, 127.2, 128.7, 129.8, 131.4, 149.33, 149.35 ppm.

*Synthesis of 3,4-Dimethoxyphenanthrene (**14**).* (*E*)-2-Carboxymethyl-3,4-dimethoxystilbene (**12**) was generated from methyl 2-formyl-4,5-dimethoxybenzoate (**11**) [13], as follows. To a chilled mixture (on ice) of **11** (1.0 g) and NaH (1.1 eq of a 60% mineral oil dispersion) in anhydrous THF (25 mL) is added benzyltriphenylphosphonium bromide (1.1 eq) [12]. After 1.5 h, the reaction mixture was poured into chilled water (200 mL). The resulting solution was extracted with ethyl acetate (3 × 200 mL). The organic layers were combined, dried (over anhydrous Na_2SO_4), and the solvent removed. The residue was purified by silica gel chromatography (4:1 hexanes/ethyl acetate) to afford **12** (1 g) as a white solid. The product (**12**) was treated as described above for **9a** and **9b** to yield **13** [12]. (*E*)-2-Carboxymethyl-3,4-Dimethoxystilbene (**12**): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.89 (s, 3H), 3.92 (s, 3H), 6.90 (d, 1H, J = 16.2 Hz), 7.14 (s, 1H) 7.24 (m, 1H), 7.34 (m, 2H), 7.46 (s, 1H), 7.54 (m, 2H), 8.06 (d, 1H J = 16.2) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 52.0, 55.99, 56.03, 109.0, 113.0, 120.5, 126.7 (2C), 127.6, 127.7, 128.6 (2C), 130.0, 134.0, 137.5, 147.9, 152.0, 167.3 ppm.

1-Carboxymethyl-3,4-dimethoxyphenanthrene (**13**): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.96 (s, 3H), 4.01 (s, 3H), 4.05 (s, 3H), 7.62 (m, 2H), 7.68 (d, 1H, J = 9.3 Hz), 7.85 (m, 1H), 7.92 (s, 1H), 8.65 (d, 1H J = 9.3 Hz), 9.64 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 52.4, 56.5, 59.9, 116.0, 123.4, 123.9, 125.3, 126.8, 127.0, 127.5, 127.7, 128.0, 128.2, 129.3, 132.6, 150.1, 150.7, 168.1 ppm.

Subsequently, the methyl ester (**13**, 0.4 g) is suspended in 1 M NaOH (10 mL) and refluxed for 2 h. The reaction is allowed to cool and the pH is adjusted to 1.8 with phosphoric acid. The resulting solution was extracted with ethyl acetate (3 × 20 mL). The organic layers were combined, dried (over anhydrous Na_2SO_4), and the solvent removed to give 0.3 g of 1-carboxy-3,4-dimethoxyphenanthrene acid as a white solid. 1-Carboxy-3,4-dimethoxyphenanthrene acid: ^{1}H NMR (0.6 mL $CDCl_3$ with 30 μL DMSO-d_6, 300 MHz) δ 3.88 (s, 3H), 3.98 (s, 3H), 7.53 (m, 2H), 7.58 (d, 1H, J = 9.4 Hz), 7.77 (m, 1H), 7.95 (s, 1H), 8.73 (d, 1H, J = 9.4 Hz), 9.55 (m, 1H) ppm; ^{13}C NMR (0.6 mL $CDCl_3$ with 30 μL DMSO-d_6, 75 MHz) δ 56.3, 59.7, 116.2, 123.7, 124.5, 125.0, 126.5, 126.7, 127.0, 127.6, 127.8, 128.0, 129.2, 132.4, 149.9, 150.2, 169.6 ppm.

3,4-Dimethoxyphenanthrene (**14**) was generated from the carboxylic acid (derived from the hydrolysis of **13**) as follows. Copper powder (25 mg) was added to freshly distilled quinoline (0.5 mL), and the mixture was allowed to stir for 1 h under argon [14]. The carboxylic acid (200 mg) was added and the reaction was gently refluxed for 18 h. After cooling, the mixture was diluted with ether and slowly poured into 6 M HCl (10 mL). The resulting mixture was extracted with ether (3 × 20 mL). The organic layers were combined, dried (over anhydrous Na_2SO_4), and the solvent removed. The residue was purified by silica gel chromatography (4:1 hexanes/ethyl acetate) to afford **14** (110 mg) as a white solid. 3,4-Dimethoxyphenanthrene (**14**): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.0 (s, 1H), 4.1 (s, 1H), 7.4 (d, 1H, J = 8.7 Hz), 7.6 (m, 5H), 7.9 (dd, 1H, J = 1.8 Hz, 7.7 Hz), 9.7 (dd, 1H, J = 0.9 Hz, 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 56.5, 59.7, 113.0, 124.6, 124.8, 125.5, 126.48, 120.51, 126.9, 127.9, 128.26, 128.33, 129.6, 133.0, 147.1, 151.5 ppm.

Removal of the Dimethoxy Groups. In a typical procedure, the dimethoxy derivative (**10a,b** or **14**, ~20 mg) was dissolved in CH_2Cl_2 (3 mL) and cooled to −78 °C. A solution of BBr_3 (3 eq of a 1 M solution in CH_2Cl_2) was added [13]. After 1 h, the reaction mixture was allowed to warm to ambient temp and was then stirred for an additional 1 h. Subsequently, the mixture was poured into cold water (5 mL) and extracted with CH_2Cl_2 (3 × 10 mL). The CH_2Cl_2 layers were collected, purged with argon, dried over anhydrous Na_2SO_4, and the solvent is removed to yield 18 mg of the dihydroxy compound as a white solid. The material is kept under argon and stored at least at −20 °C (or below). The purity was assessed by TLC.

*Synthesis of Phenanthrene-9,10-diyl diacetate (**16**).* The commercially available 9,10-phenanthrenequinone was treated as described below for the synthesis of pyrene-4,5-diyl diacetate (**29**) to generate diacetate **16** [15]. As needed, small quantities of **16** were dissolved in ethanol (3 mL) and purged with argon. Three drops of acetyl chloride are added and the reaction heated to 70 °C. The reaction is allowed to cool and the solvent removed under reduced pressure. The purity of the resulting **5d** was assessed by TLC. The compound was stored under argon at least −20 °C, to minimize oxidation. Phenanthrene-9,10-diyl diacetate (**16**): ^{1}H NMR ($CDCl_3$, 300 M Hz) δ 2.5 (s, 6H), 7.7 (m, 4H), 7.9 (m, 2H), 8.7 (d, 2H, J = 8.5 Hz) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 20.5, 122.0, 123.0, 126.5, 127.1, 127.3, 129.7, 135.8, 168.2 ppm.

*Synthesis of 7,8-Dimethoxyfluoranthene (**24**).* In a typical procedure, **20** (42.6 mg) is added to a mixture of IBX (1.2 eq in 3 mL of a solution of 1% methanol/CH_2Cl_2) and reacted for 6–8 h (until starting material is consumed). Most of the solvent is removed, ethyl acetate is added, and most of the solvent is removed again (to remove CH_2Cl_2) and then ethyl acetate (~5–10 mL) is added. A solution of $NaBH_4$ (200 mg in 20 mL H_2O) is added to the round bottom flask and the mixture is stirred at room temperature. The mixture is then extracted with ethyl acetate, the ethyl acetate layers are collected, dried over anhydrous Na_2SO_4, while purging with argon, and the solvent is removed. Subsequently, $(CH_3)_2SO_4$ (5 eq) followed by K_2CO_3 (10 eq) are added to the residue, and the reaction is refluxed (under argon) in acetone (2 mL) for 2 days. The acetone is removed under reduced pressure to near dryness, the remaining liquid is diluted with ethyl acetate, filtered, and then the solvent is removed under reduced pressure. The residue is subjected to silica gel chromatography (8:1 hexanes/ethyl acetate). The appropriate fractions (for **21** and **22**) are collected, and the solvent is removed under reduced pressure to yield **23** (19.2 mg) and **24** (2.2 mg). 7,8-Dimethoxyfluoranthene (**24**): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.0 (s, 3H), 4.1 (s, 3H), 7.0 (d, 1H, J = 8.1 Hz), 7.6 (m, 2H), 7.7 (m, 1H), 7.8 (d, 2H, J = 8.2 Hz), 7.9 (m, 2H), 8.19 (d, 1H, J = 6.9 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 56.3, 60.3, 111.4, 117.3, 119.1, 123.3, 125.6, 126.4, 127.8, 128.2, 129.9, 132.4, 132.8. 133.4, 135.4, 136.9, 146.0, 153.0. The dimethoxy groups were removed as described above for **5a-c**.

*Synthesis of Fluoranthene-2,3-diyl (**19**) and Pyrene-1,2-diyl diacetate*

(27). In a typical procedure IBX (3 eq), prepared as described [17], is added to a solution of 1-hydroxypyrene (**25**, 60.0 mg) in a 2:1 mixture of CH_2Cl_2/CH_3OH (3 mL). The reaction is monitored by TLC until **25** is consumed (~4 h). The solvent is filtered and removed under reduced pressure. Ethyl acetate (5 mL) is added to the residue followed by the slow addition of a solution of $NaBH_4$ (150 mg in 1 mL of H_2O). The mixture is stirred until the gas evolution subsides (1 h). The organic layer is separated and the aqueous layer is extracted with ethyl acetate (3 × 2 mL). The organic layers are combined and dried over anhydrous Na_2SO_4. The solvent is removed under reduced pressure and the residue is dissolved in anhydrous pyridine. Acetic anhydride (3 eq) is added to the solution, and the reaction is allowed to proceed under argon for 24 h [18]. The solvent is then removed and the residue is purified by silica gel chromatography (8:1 hexanes/ethyl acetate). This gives **19** or **27** (14.7 mg) as off-white solids. Fluoranthene-2,3-diyl diacetate (**19**): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.4 (s, 3H), 2.5 (s, 3H), 7.4 (m, 2H), 7.65 (m, 1H), 7.8 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 20.5, 20.8, 116.5, 120.3, 121.4, 121.71, 121.73, 124.4, 127.9, 127.93, 129.0, 131.0, 135.7, 137.1, 137.6, 138.4, 139.7, 141.5, 168.4, 168.7. Pyrene-1,2-diyl diacetate (**27**): ^{1}H NMR (acetone-d_6, 500 MHz) δ 1.6 (s, 3H), 2.6 (s, 3H), 7.4 (d, 1H, J = 9.2 Hz), 8.0 (d, 1H, J = 9.3 Hz), 8.1 (m, 1H), 8.3 (d, 2H, J = 8.9 Hz), 8.37 (s, 1H), 8.41 (m, 2H); ^{13}C NMR (acetone-d_6, 125 MHz) δ 19.9, 20.5, 121.3, 124.0, 124.7, 124.9, 126.1, 126.2, 127.2, 127.3, 127.7, 129.1, 130.18, 130.21, 131.6, 131.8, 138.0, 141.1, 168.4, 139.5 ppm. As needed, the diacetate groups were removed as described above for **5d**.

Synthesis of Pyrene-4,5-diyl diacetate (***29***). Quinone **28** (0.75 g), prepared as described elsewhere [18], was dissolved in anhydrous THF (50 mL), and Pd/C (100 mg), K_2CO_3 (15 eq), and acetic anhydride (15 eq) were added [15]. The reaction is shaken on a Parr Hydrogenator (5 psi of H_2) for 48 h. Subsequently, the mixture was diluted with ethyl acetate (100 mL), purged with argon and filtered. The solvent is removed under reduced pressure and the residue is purified by silica gel chromatography (2:1 hexanes/ethyl acetate). The silica gel chromatography step is repeated using toluene to remove traces of a colored impurity. This gives **29** (0.44 g) as an off-white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.6 (s, 6H), 8.0 (t, 2H), 8.1 (s, 2H), 8.17 (dd, 2H, J = 1.1 Hz, 7.9 Hz), 8.23 (dd, 2H, J = 1.1 Hz, 7.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.6, 119.4, 123.6, 125.8, 125.9, 126.4, 127.6, 131.2, 136.7, 168.3 ppm. As needed, the diacetate groups were removed as described above for **5d**.

Construction of the Expression Vectors for PhdI and PhdF. Amplification of the desired genes was achieved through two rounds of PCR. In the first round, two overhang PCR primers that annealed within 100 bp around the desired gene were used (For PhdI: overhang forward primer, CCCAAGAGGTCAGCTCAGTG, and overhang reverse primer, GGAGGGCGTAGGGATAATGC; for PhdF: overhang forward primer, TGGGCCGCACTCCAGCAGCGCTAT, and overhang reverse primer, TTAGGAACCGGATCGCCATTTCAC). In the second round, the desired gene flanked with appropriate restriction sites was isolated (PhdI: *Bam*HI and *Eco*RI and PhdF: *Eco*RI and *Hind*III). For PhdI, the forward and reverse primers were TAGCCAGGATCCTCCACCGCC and GAATC GGAATTCTCAGCGGGC, respectively, where the restriction sites are underlined. For PhdF, the forward and reverse primers were GGACAG CACGAATTCTTGGTGAA and GCCGTCGAGAAGCTTTCATGTTG, respectively, where the restriction sites are underlined. The amplification reaction (100 μL) contained template DNA (15 ng), dNTPs (0.4 mM), MgCl (2 mM), primers (0.4 μM), 1 × buffer, and Vent Polymerase (1 unit). The PCR amplification protocol consisted of a 5 min denaturation step at 94 °C followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min and ended with a 10 min elongation step at 72 °C. The PCR reaction product size was confirmed on a 1% agarose gel and isolated using the GenElute gel extraction kit and elution into water (60 μL). The PCR products were inserted into pET 32 between appropriate restriction sites using digestion and ligation procedures reported elsewhere [23]. An aliquot of the individual ligation mixtures (30 μL) of PhdI or PhdF was transformed into *E. coli* C41(DE3) by the calcium heat shock method [25]. Transformed cells were grown on agar plates containing ampicillin (100 μg/mL) at 37 °C overnight. A single colony was used for the generation of additional plasmid for sequencing and protein expression.

For expression, LB media cultures (25 mL) containing ampicillin (100 μg/mL) were inoculated using a single colony and grown overnight at 37 °C. LB media cultures (1.6 L) were inoculated using an appropriate amount of the overnight culture to make OD_{600} = 0.05 and grown at 37 °C for 2.5 h. Protein expression was induced overnight (18 h) at 21 °C using a final isopropyl-β-D-thiogalactoside concentration of 0.1 mM. Cells were harvested by centrifugation (10,000 × g) with yields ranging from 3 to 5 g and stored at −80 °C.

Purification of PhdI and PhdF. Cells were suspended to a final concentration of 10 mg/mL in in Buffer A (20 mM HEPES, 300 mM NaCl, pH 7) made 15 mM in imidazole. Cells were disrupted by sonication and the resulting solution was centrifuged for 30 min at 17,700 × g and 4 °C. The supernatant was loaded onto a Ni-NTA column (1 × 10 cm, ~6 mL resin) pre-equilibrated with Buffer A. The column was washed with Buffer A (made 70 mM in imidazole) and the desired protein was eluted with Buffer A (made 90 mM imidazole). Protein purity was assessed by the observation of a single band on an SDS-PAGE gel. The eluant was concentrated to ~1 mg/mL and exchanged into Buffer B (20 mM HEPES, 300 mM NaCl, pH 7.0, and 5% glycerol) using an Amicon stirred cell equipped with an ultrafiltration membrane (10,000 Da cutoff). The typical yields were 2–4 mg of protein per gram of cells.

Activation of PhdF. PhdF is active when the cells are lysed, but loses activity when left overnight or passed through a column. Hence, kinetic analysis required activation (because the enzyme was purified), but product isolation did not (because the enzyme was used as crude lysate). To activate PhdF, ascorbate (4 mM final), $FeCl_2$ (2 mM final) and acetone (5% final) were added to aliquots (~500 μL) of protein (1 mg/mL final concentration) purified as described above [19,20]. The resulting mixture was incubated on ice for 30 min. The sample was centrifuged for 5 min at 20,800 × g and exchanged into Buffer B using a PD-10 column. Activity decreases within 8 h of activation so protein is activated and assayed on the same day. PhdI did not require activation.

Incubation of PhdI and PhdF with Various Substrates. Aliquots of PhdI or PhdF were incubated with 1,2-dihydroxynaphthalene (in ethanol), 1-hydroxy-2-naphthoate (**32**, in ethanol), **5a**,**c**,**d**, **6a**,**b**, **7a**,**b** (Scheme 2), 3-methylcatechol (**34**, Scheme 7A), and 2,3-dihydroxybiphenyl (**35**, Scheme 7B) in 1 mL of 20 mM HEPES buffer, pH 7.0. Reactions were generally followed up to 30 min, but the rapid non-enzymatic oxidation of some substrates limited the incubation times. With the exception of the incubation mixture of PhdI and **32**, PhdF and **5c**, (see supporting information for representative UV/Vis traces), **34**, and **35**, no change in absorbance other than that caused by non-enzymatic oxidation was observed. These reactions were subjected to kinetic and product analysis.

*Reaction of PhdI with 1-Hydroxy-2-naphthoate (**32**)*. Aliquots of PhdI (1–10 μL, 6–60 nM final concentration) and **32** (2–80 μM final concentration in ethanol) were added to 1 mL of 20 mM HEPES buffer (pH 7.0) and the reaction was monitored by UV/Vis spectroscopy for 30 s. The trace showed a clear decrease in absorbance at 250 nm with a concomitant increase in absorbance at 300 nm. A sample trace is found in the supporting information (Fig. S1A).

*Reaction of PhdF with 3,4-Dihydroxyphenanthrene (**5c**)*. Aliquots of PhdF (21–500 nM final concentration) and **5c** (1–20 μM final concentration in ethanol) were added to 1 mL of 20 mM HEPES buffer (pH 7.0) and the reaction was monitored by UV/Vis spectroscopy for 30 s. The trace showed a clear decrease in absorbance at 245 nm. Product absorbance overlaps with substrate absorbance so there is not an obvious increase in absorbance concomitant with the decrease at 245 nm. A sample trace is found in the supporting information (Fig. S2A).

*Kinetic Analysis of PhdI and **32***. An aliquot of PhdI (2 μL of a

0.55 mg/mL solution) was added to 20 mM HEPES buffer (1 mL, pH 7.0) to give a final concentration of 19 nM. Assays were initiated by the addition of aliquots of a stock solution of **32** in ethanol (34 mM). The final substrate concentration ranged from 3 to 270 μM. The reaction was monitored by following the increase in absorbance at 300 nm (ε = 8637 $M^{-1}cm^{-1}$). Initial rates were determined from the first 30 s of the reaction, plotted versus substrate concentration, and fit to the equation to calculate the steady-state parameters k_{cat} and K_M using Grafit [24]. The Michaelis-Menten plot is shown in the Supporting Information (Fig. S1B).

The extinction coefficient for **33** was determined as follows. A stock solution of **32** (34 mM) was made up and aliquots were removed and added to 1 mL of HEPES buffer (pH 7.0) containing a large amount of PhdI. The final concentrations of **32** ranged from 2.6 to 270 μM. The final concentration of product was assumed to be the same as substrate. Absorbance readings were taken at each concentration and plotted vs the concentration. The slope is the reported extinction coefficient.

*Product Analysis for the Reaction of PhdI and **32**.* PhdI was expressed as described above in *E.coli* C41(DE3) cells, and the cells (~1.2 g) were suspended in 20 mM HEPES buffer (40 mL, pH 7.0), lysed by sonication, and the resulting solution was centrifuged for 30 min at 17,700 × g and 4 °C. The supernatant was diluted to 150 mL with 20 mM HEPES buffer (pH 7.0) and 1-hydroxy-2-naphthoate (25 mg in 1.5 mL ethanol) was added dropwise to the supernatant, which was stirring on ice. Aliquots (1 mL) of the solution were removed every 5 min, centrifuged at 20,800 × g for 2 min, and the clarified supernatant was monitored by UV spectroscopy until the reaction was complete (30 min) as determined by no further increase in absorbance at 300 nm. The reaction was centrifuged at 10,000 × g and 4 °C for 20 min. The pH of the clarified supernatant was adjusted to 8.0 using drops of 10 M KOH. Protein was removed using an Amicon stirred cell concentrator equipped with an ultrafiltration membrane (10,000 Da cutoff). The protein-free flow-through was concentrated in vacuo to 1.5 mL and loaded onto a Sephadex G-25 column. The column was washed with water and the eluate monitored using UV spectroscopy (294 nm). The product-containing fractions (~10 mL) were combined and dried in vacuo. The product was resuspended in ~0.6 mL of NaD_2PO_4, pH 7.3, placed in an NMR tube, and a ^{1}H NMR spectrum was obtained. The ^{1}H NMR spectrum matched that of an authentic sample of *trans-o*-carboxybenzylidenepyruvate (**33**, Scheme 6B) [21].

*Kinetic Analysis of PhdF using 3,4-dihydroxyphenanthrene (**5c**), 3-methylcatechol (**34**), or 2,3-dihydroxybiphenyl (**35**).* Aliquots of PhdF (10–510 nM final concentration) were added to 20 mM HEPES buffer (1 mL, pH 7.0). The reaction was initiated by the addition of substrate (1–20 μL) in ethanol from stock solutions of 3,4-dihydroxyphenanthrene (**5c**, final concentration 1–9 μM), 3-methylcatechol (**34**, final concentration 6–580 μM) or 2,3-dihydroxybiphenyl (**35**, final concentration 11–570 μM). For **5c**, the decrease in absorbance at 245 nm (ε = 25,800 $M^{-1}cm^{-1}$) was monitored; for **34**, the increase in absorbance at 388 nm (ε = 13,800 $M^{-1}cm^{-1}$) was monitored; and for **35**, the increase in absorbance at 434 nm (ε = 21,700 $M^{-1}cm^{-1}$) was monitored. The extinction coefficients were determined as follows. For **5c**, **34**, and **35**, stock solutions were made up in ethanol (10.3, 59.6, and 2.15 mM, respectively), aliquots removed (0.5–32, 0.5–2.5, and 2–20 μL, respectively) and added to 1 mL of 20 mM HEPES buffer (pH 7.0) to give final concentrations of 5.2–330.9 μM for **5c**, 29.8–149 μM for **34**, and 4.3–43 μM for **35.** For **5c**, absorbance readings were taken at each concentration and plotted vs the concentration. The slope is the reported extinction coefficient. The extinction coefficients for the products of the reaction with **34** and **35** (**36** and **38**, respectively) were determined as described for **33** above.

Initial rates were determined from the first 15 s of the reaction, plotted versus substrate concentration, and fit to the Michaelis-Menten equation to calculate the steady-state parameters k_{cat}, K_M, and K_i using Grafit [24]. The Michaelis-Menten plot is shown in the Supporting Information (Fig. S2B). For the reaction of PhdF and **35**, the kinetic parameters could only be estimated because the enzyme was saturated at all concentrations of substrate (11–570 μM) that could be measured within the limits of the spectrophotometer. Hence, the observed rate is assumed to be V_{max} and the K_m is estimated to be less than 11 μM (as reported in Table 1).

*Product Analysis for the Reaction of PhdF and **34**.* PhdF was used to generate the ring-opened product for 3-methylcatechol (**34**). PhdF was expressed in *E.coli* C41(DE3) cells as described above, and the cells were resuspended in Buffer A at a concentration of 1 g/30 mL. The cells were disrupted via sonication and the resulting solution was centrifuged for 30 min at 17,700 × g and 4 °C. The clarified supernatant was mixed with **34** and the presumed product (**36**, Scheme 7A) was converted into the picolonic acid derivative (**37**) for isolation and purification. Briefly, 80 tubes containing 2 mL Buffer A, 100 μL of clarified supernatant, and **34** (40 μL of a 22 mg/mL solution in ethanol) were incubated at room temperature for ~20 min. Ammonium sulfate (2 mL of 4 mM stock solution) was added to each tube and the tubes were combined and extracted with methanol. The extract was mixed with silica, concentrated in vacuo, washed with 10% methanol in chloroform, and the product was recovered using chloroform:methanol:acetic acid (9:3:1). The solution was evaporated to dryness and the product resuspended in ~0.6 mL of deuteriated buffer and placed in an NMR tube. **37**: ^{1}H NMR (,0.6 mL of NaD_2PO_4, pH 7.3 with 30 μL of DMSO-d_6, 300 MHz) δ 2.4 (3H, s), 7.2 (1H, d, J = 8.1 Hz), 7.5 (1H, d, J = 7.8 Hz), 7.7 (1H, t); ^{13}C NMR (0.6 mL of NaD_2PO_4, pH 7.3 with 30 μL of DMSO-$d)_6$, 125 MHz) δ 24.58, 122.60, 127.40, 140.35, 154.39, 159.60, 174.69 ppm; HRMS (ESI-TOF) *m/z* 138.0550 $(M + H)^+$ calcd for $C_7H_8NO_2$, found 138.0552.

*Product Analysis for the Reaction of PhdF with 2,3-Dihydroxybiphenyl (**35**) or 3,4-dihydroxyphenanthrene (**5c**).* The *E.coli* C41(DE3) cells containing the PhdF expression vector were grown as indicated above. Subsequently, the cells were centrifuged at 17,700 × g for 30 min.. The cell pellet was washed 3 times by resuspension in Buffer A (OD_{600} = 0.5 for 10 μL suspension in 1 mL buffer) and then centrifuged (17,700 × g for 30 min). The pellet was then resuspended in Buffer A to an OD_{600} of 0.5 (10 μL in 1 mL of buffer). The final volume was 100 mL (for **35**) or 300 mL (for **5c**). Stock solutions (50 mg/mL) were prepared in ethanol immediately before use. An aliquot of the stock substrate (2 mL) was added to the cell suspension, which was stirring on ice, over the course of 5 min to a final concentration of 1 mg/mL (for **35**) or 0.33 mg/mL (for **5c**). Every 5 min, aliquots (500 μL) were removed, centrifuged at 20,800 × g for 2 min, and the cell free supernatant monitored by UV spectroscopy until the reaction was complete (~30 min) as determined by no further change in absorbance. The reactions were monitored by following the increase in absorbance at 434 nm (**35**) or decrease in absorbance at 245 nm (**5c**). The reaction was centrifuged at 17,700 × g for 20 min and the cell-free supernatant acidified to a pH of 2.25 using drops of 10 M HCl. The acidic solutions were extracted using 2.5 vol of ether and the ether layers were collected and dried over anhydrous $Mg(SO_4)_2$. The unstable reaction product was methylated using CH_2N_2 generated from a Diazald kit, following the manufacturer's instructions. The methyl esters were purified by silica gel chromatography using hexane:ethyl acetate (2:1) for **39** or (4:1) for **40**. The product-containing fractions, as determined by TLC, were combined and evaporated to dryness. The products were dissolved in $CDCl_3$ (~0.6 mL) and placed in NMR tubes. **39**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.9 (3H, s), 6.4 (1H, dd, J = 0.6 Hz, 11.7 Hz), 6.5 (1H, s), 7.1 (1H, dd, J = 0.6 Hz, 15.3 Hz), 7.5 (3H, m), 7.9 (1H, dd, J = 11.7 Hz, 15.3 Hz), 7.9 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 53.5, 109.4, 127.2, 128.45, 128.6, 132.8, 137.0, 138.0, 144.7, 165.1, 190.5 ppm; HRMS (ESI-TOF) *m/z* 255.0633 $(M + Na)^+$ calcd for $C_{13}H_{12}O_4Na$, found 255.0628. **40**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.9 (3H, s), 6.4 (1H, dd, J = 0.7 Hz, 11.7 Hz), 7.1 (1H, dd, J = 0.6 Hz, 15.3 Hz), 7.5 (2H, m), 7.58 (1H, m), 7.87 (1H, dd, J + 11.7 Hz, 15.3 Hz), 7.97 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.8, 145.8, 134.9, 127.8, 127.6, 127.0, 126.0, 124.7, 124.5, 122.0, 121.8, 117.7, 113.6, 94.0, 54.1 ppm; HRMS (ESI-TOF) *m/z* 279.0633 $(M + Na)^+$ calcd for $C_{15}H_{12}O_4Na$, found 279.0636.

4. Conclusions

Eight dihydroxy PAH derivatives were successfully synthesized and characterized (4 dihydroxyphenanthrenes, 2 dihydroxyfluoranthenes, and 2 dihydroxypyrenes) as the dimethoxy or diacetate derivatives. Two putative dioxygenases (PhdI and PhdF) in the phenanthrene degradative pathway in *M. vanbaalenii* PYR-1 were purified and examined for ring opening activity with 3,4-dihydroxyphenanthrene and two dihydroxypyrenes. The dihydroxypyrenes were not processed, but 3,4-dihydroxyphenanthrene was a substrate for PhdF, thereby confirming its role in the pathway. The product was identified by ^{1}H NMR analysis. The reaction of PhdI and 1-hydroxy-2-naphthoate was confirmed by kinetic and product analysis. This is the first biochemical evidence for these reactions in *M. vanbaalenii* PYR-1 and the first evidence for the reaction of PhdF with 3,4-dihydroxyphenanthrene. Both results are consistent with the presence of the genes for the other enzymes in the proposed phenanthrene degradative pathway in *M. vanbaalenii* PYR-1 [8,11]. Two additional substrates (3-methylcatechol and 2,3-dihydroxybiphenyl) were examined with PhdF and the products characterized in order to develop methodology for future work.

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ABBREVIATIONS

DMSO	dimethyl sulfoxide
DEAE	diethyl-aminoethyl
ESI-TOF	electrospray ionization time-of-flight
HMW	high-molecular-weight
HRMS	high resolution mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
IBX	*o*-iodoxybenzoic acid
LMW	low-molecular-weight
LB	Luria – Bertani
Ni-NTA	nickel-nitrolotriacetic acid
NMR	nuclear magnetic resonance
dNTP	deoxyribose nucleotide triphosphates
PAH	polycyclic aromatic hydrocarbons
PhdF	3,4-dihydroxyphenanthrene 2,3-dioxygenase
PhdI	1-hydroxy-2-naphtolate 1,2-dioxygenase
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	thin-layer chromatography

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2019.108081.

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