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# Pentalenolactone Biosynthesis. Molecular Cloning and Assignment of Biochemical Function to PtlH, a Non-Heme Iron Dioxygenase of *Streptomyces avermitilis*

Zheng You<sup>a</sup>, Satoshi Omura<sup>b</sup>, Haruo Ikeda<sup>c</sup>, and David E. Cane<sup>a,\*</sup>

aDepartment of Chemistry, Brown University, Box H, Providence, RI 02912-9108, USA

bThe Kitasato Institute, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108-8642, Japan

cKitasato Institute for Life Sciences, Kitasato University, 1-151-1, Kitasato, Sagamihara, Kanagawa 228-8555, Japan

## **Abstract**

The hydroxylase encoded by the ptlH (SAV2991) gene from the pentalenolactone gene cluster of Streptomyces avermitilis was cloned by PCR and expressed in Escherichia colias an N-terminal-His<sub>6</sub>-tag protein. Incubation of recombinant PtlH with ( $\pm$ )-1- deoxypentalenic acid ( $\bf 5$ ) in the presence of Fe(II),  $\alpha$ -ketoglutarate, and O<sub>2</sub> gave (-)-11 $\beta$ -hydroxy-1-deoxypentalenic acid ( $\bf 8$ ), whose structure and stereochemistry were determined by a combination of H, H, COSY, HMQC, HMBC, and NOESY NMR. The steady state kinetic parameters were  $k_{cat}$  4.2 $\pm$ 0.6 s<sup>-1</sup> and  $k_{m}$  ( $\bf 5$ ) 0.57 $\pm$ 0.19 mM.  $\bf 8$  is a new intermediate in the conversion of the sesquiterpene pentalenene ( $\bf 3$ ) to pentalenolactone ( $\bf 1$ ).

Streptomyces avermitilis is a Gram-positive soil organism that is responsible for the production of the widely used anthelminthic macrolide avermectins. (*S. avermectinius* is a junior homotypic synonym of *S. avermitilis*.) The 9.03 Mb linear chromosome harbors 7,575 open reading frames (ORFs), including some 30 gene clusters thought to be related to secondary metabolism, corresponding to 7% of the genome. We have recently reported the molecular genetic and biochemical identification of the gene cluster for the biosynthesis of the sesquiterpene antibiotic pentalenolactone (1), in a 13.4-kb segment centered at 3.75 Mb in the *S. avermitilis* genome that contains 13 unidirectionally-transcribed ORFs (Figure 1). Among these ORFs, the 1011-bp *ptlA* (SAV2998), encodes pentalenene synthase, a protein of 336 amino acids that catalyzes the cyclization of farnesyl diphosphate (2) to pentalenene (3), the established parent hydrocarbon of the pentalenolactone family of antibiotics (Scheme 1). A typical farnesyl diphosphate gene. A typical farnesyl diphosphate synthase is apparently encoded by *ptlB*, while *ptlR* and *ptlG* have been

assigned as a putative transcriptional regulator and a transmembrane efflux protein, respectively. All of the remaining 8 ORFs correspond to redox enzymes, among which is a cytochrome p450 (CYP183A1, ptlI), and six monooxygenases and dioxygenases.

Several presumptive intermediates as well as a number of shunt metabolites in the conversion of pentalenene to pentalenolactone have been isolated from cultures of a wide variety of pentalenolactone-producing *Streptomyces* species. Pentalenolactone F (4), has also recently been isolated from *S. avermitilis*, confirming that the pentalenolactone pathway is functional in this organism. Along with 1-deoxypentalenic acid (5),  $^{6a}$  pentalenolactone D (6),  $^{6g}$  and pentalenolactone E (7),  $^{6e}$  these metabolites can be organized into a plausible biosynthetic pathway (Scheme 1). Beyond the conversion of labeled pentalenene (3) to pentalenolactone (1),  $^{3b}$  these proposed biosynthetic relationships have yet to be demonstrated experimentally, and none of the enzymes linking pentalenene (3) to pentalenolactone (1) have been identified. We report below the biochemical characterization of PtlH (SAV2991), a non-heme iron,  $\alpha$ -ketoglutate dependent hydroxylase that catalyzes the conversion of 1-deoxypentalenic acid (5) to a new biosynthetic intermediate,  $\alpha$ -11 $\alpha$ 

BLAST searching indicates that ptlH has 26% amino acid sequence identity and 44% similarity over 244 residues to phytanoyl-CoA dioxygenase of Agrobacterium tumefaciens (PhyH, Genbank Accession No. YP\_086787). PhyH, which catalyzes the  $\alpha$ -hydroxylation of phytanoyl-CoA, belongs to the sub-family of Fe(II)/ $\alpha$ -ketoglutarate-dependent hydroxylases. We therefore hypothesized that PtlH might be responsible for hydroxylation of the methylcyclopentane ring of 1-deoxypentalenic acid (5) in the oxidative conversion of 5 to pentalenolactone D (6) (Scheme 2).

*PtlH* was amplified by polymerase chain reaction (PCR) from DNA of *S. avermitilis* cosmid CL\_216\_D07 and cloned into the vector pET28e. The resulting construct pET28e-*PtlH* was transformed into *Escherichia coli* BL21(DE3). After induction with IPTG, the expressed PtlH protein, carrying an N-terminal His<sub>6</sub>-tag, was purified to homogeneity by Ni-NTA chromatography. The purified protein had a subunit  $M_D$  by MALDI-TOF MS of m/z 37139 ±19 (calc. 37121). The presence of a second peak at m/z 73965 suggested that PtlH may be a homodimer.

The requisite substrate  $(\pm)$ -1-deoxypentalenic acid (5) was synthesized from  $\alpha$ -humulene by way of  $(\pm)$ -pentalenene (3) and  $(\pm)$ -pentalen-13-ol (9), as previously described (Scheme 3). A mixture (1.5 mL) of recombinant PtlH (1.66  $\mu$ M),  $\alpha$ -ketoglutarate (2 mM), L-ascorbic acid (2 mM), FeSO<sub>4</sub> (1 mM), DTT (1.5 mM), and bovine catalase (1 mg/mL) in 100 mM Tris buffer (pH 7.3) was incubated with  $(\pm)$ -(5) (0.64 mM) overnight at room temperature. After acidification with HCl, the mixture was extracted with diethyl ether and the organic extract was treated with TMS-CHN<sub>2</sub> to generate the methyl ester. GC-MS analysis (HP5ms, 30 m × 0.25 mm) revealed a single new peak with m/z 264, indicating the formation of the hydroxylated product. Chiral GC-MS analysis (Hydrodex- $\beta$ -6-TBDM, 25 m × 0.25 mm), under conditions in which the methyl esters of ( $\pm$ )-deoxypentalenic acid were well resolved. confirmed that the enzymatic reaction product was a single enantiomer. Control incubations that omitted  $\alpha$ -ketoglutarate or Fe(II) showed no turnover of 5. Neither ( $\pm$ )-pentalenene (3) (0.5 mM) nor ( $\pm$ )-pentalen-13-ol (9) (0.1 mM) underwent PtlH-catalyzed hydroxylation.

A preparative-scale incubation was carried out with PtlH and 1-deoxypentalenic acid (**5**). The isolated product was converted to the methyl ester **8-Me**, which was purified by preparative TLC and analyzed by  $^{1}$ H,  $^{13}$ C, COSY, HMQC, HMBC, and NOESY NMR as well as EI-MS.  $^{9}$  In the  $^{13}$ C NMR spectrum of **8-Me** the C-11 methylene of **5** was replaced by a new methine signal at 76.0 ppm that was correlated with the H-11 carbinyl proton signal at  $\delta$  4.10 (m),

consistent with the introduction of the hydroxyl group at C-11. This assignment was corroborated by the HMBC spectrum, which exhibited the expected cross peak between C-11 and the H-10 methyl protons at  $\delta$  0.99 (d, J=7 Hz, 3H). The configuration of the 11 $\beta$ -hydroxyl group of **8-Me** was readily established by the NOESY spectrum, which showed correlations between H-11 and both H-9 ( $\delta$  1.92) and H-12a ( $\delta$  1.72) as well as between H-12b ( $\delta$  2.05) and H-12a and H-5 ( $\delta$  3.19) (Figure 2).

PtlH showed a pH optimum of 6.0. The steady-state kinetic parameters were determined by carrying out a series of 30-min incubations with 0.097-0.97 mM (±)-1-deoxypentalenic acid (**5**) and quantitation of the product **8-Me** by calibrated GC-MS. Fitting of the initial velocities to the Michaelis-Menten equation gave  $k_{\text{cat}}$  4.2±0.6 s<sup>-1</sup> and a  $K_{\text{m}}$  (**5**) of  $0.57\pm0.19$  mM.

These results firmly establish the biochemical function of the ptlH gene product, which is shown to catalyze the Fe(II)- and  $\alpha$ -ketoglutarate-dependent hydroxylation of  $\bf 5$  to  $11\beta$ -hydroxy-1-deoxypentalenic acid ( $\bf 8$ ) (Scheme 2). Further conversion of  $\bf 5$  to pentalenolactone D ( $\bf 6$ ) may involve oxidation of  $\bf 5$  to the ketone  $\bf 10$  by PtlF, an apparent NAD<sup>+</sup>-dependent dehydrogenase, followed by Baeyer-Villiger-like oxidaton of  $\bf 10$  mediated by PtlE, which has 49% identity and 62% similarity over 591 aa to the cyclodecanone-lauryl lactone dioxygenase of *Rhodococcus ruber* (Genbank AY052630.1).

# Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

# Acknowledgements

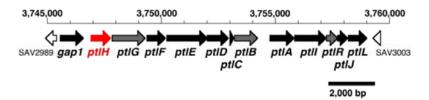
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- 9. 8-Me:  $^1\text{H}$  NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.66 (1H, H-7, m); 4.10 (1H, H-11, m); 3.74 (3H, OCH3, s); 3.19 (1H, H-5, m); 2.93 (1H, H-8, m); 2.05 (1H, H-12, m); 1.92 (2H, H-3, d, J=13.5 Hz, H-9, m);1.72 (2H, H-1, H-12, m); 1.45 (1H, H-3, d, J=13.5 Hz); 1.31(1H, H-1, dd, J=5.26, 12.78 Hz); 1.02 (3H, H-14 or H-15).  $^{13}\text{C}$  NMR 75.47 MHz, CDCl<sub>3</sub>) 166.0 (C-13); 147.9 (C-7); 137.7 (C-6); 76.0 (C-11); 62.8 (C-4); 59.5 (C-8); 55.9 (C-5); 51.8 (OCH3); 50.2 (C-3); 48.5 (C-9); 45.7 (C-1), 41.26 (C-2); 38.4 (C-12), 30.2 (C-14 or C-15); 29.5 (C-14 or C-15); 10.6 ppm (C-10).  $\delta_{\text{D}}^{22}$  =  $-10.5^{\circ}$  (CH2Cl<sub>2</sub>, c=0.1 g/100 mL). HRMS 264.1718; calc.  $C_{16}\text{H}_{22}\text{O}_{3}$ : 264.1725.
- 10. The  $11\alpha$ -hydroxyl epimer of 8-Me has a signal for H-11 at  $\delta$  370 (ref 8b).
- 11. Kinetic assays were carried at 23 °C with PtlH (0.093  $\mu$ M) in MES (95 mM, pH 6.0) containing  $\alpha$ -ketoglutarate (2.67 mM), L-ascorbate (2.67 mM), FeSO<sub>4</sub> (1.33 mM), catalase (0.95 mg/mL), DTT (1 mM) in a total vol. of 200  $\mu$ L. Reactions were initiated by adding a solution of ( $\pm$ )-deoxypentalenic acid (5) in DMSO to a concentration of 0.097 0.97 mM (final DMSO conc. 4%). The reactions were quenched with HCl at 30 min, and the mixtures were extracted with ether, treated with TMS-CHN2 and analyzed by GC-MS.



**Figure 1.** Pentalenolactone biosynthetic gene cluster from *S. avermitilis*. See the website of the *S. avermitilis* Genome Project http://avermitilis.ls.kitasato-u.ac.jp/ for annotations and detailed sequence alignments.

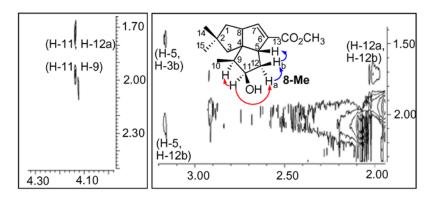


Figure 2. Details of NOESY spectrum of methyl (–)-11 $\beta$ -hydroxy-1-deoxypentalenate (8-Me).

Scheme 1.

Scheme 2.

Scheme 3.