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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,[†] Satoshi Omura,[‡] Haruo Ikeda,[§] and David E. Cane*,[†]

*Department of Chemistry, Brown University, Box H, Providence, Rhode Island 02912-9108, The Kitasato Institute, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108-8642, Japan, and Kitasato Institute for Life Sciences, Kitasato University, 1-151-1, Kitasato, Sagamihara, Kanagawa 228-8555, Japan*

Received March 2, 2006; E-mail: david_cane@brown.edu

*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 7575 open reading frames (ORFs), including some 30 gene clusters thought to be related to secondary metabolism, corresponding to 7% of the genome.[1] 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).[2] 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).[2,3] The *gap1* (SAV2990) gene, which encodes a pentalenolactone-insensitive glyceraldehyde-3-phosphate dehydrogenase, corresponds to the pentalenolactone resistance gene.[2,4] 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*),[5] a dehydrogenase (*ptlF*), 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.[6] Pentalenolactone F (**4**) has also recently been isolated from *S. avermitilis*,[2] 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, α-ketoglutarate-dependent hydroxylase that catalyzes the conversion of 1-deoxypentalenic acid (**5**) to a new biosynthetic intermediate, 11$\beta$-hydroxy-1-deoxypentalenic acid (**8**).

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 α-hydroxylation of phytanoyl-CoA, belongs to the sub-family of Fe(II)/α-ketoglutarate-dependent hydroxylases.[7] 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).

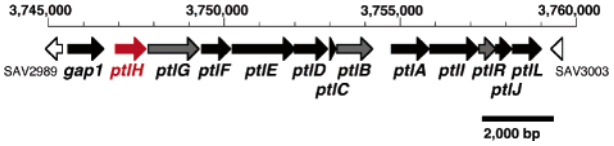
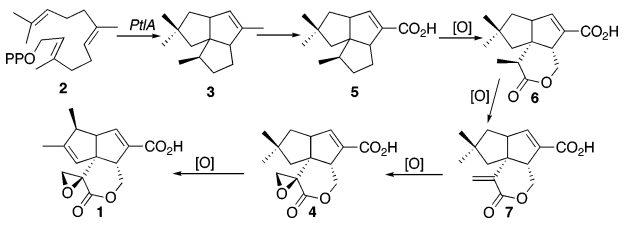
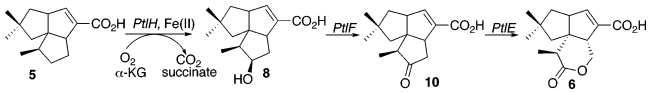



***Figure 1.*** Pentalenolactone biosynthetic gene cluster from *S. avermitilis*. See the website of the *S. avermitilis* Genome Project http://avermitilis.ls.kitasato-u.ac.jp/for annotations and detailed sequence alignments.

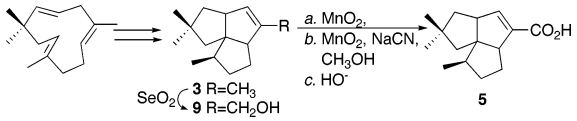
***Scheme 1***




***Scheme 2***




***Scheme 3***




*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 $His_6$-tag, was purified to homogeneity by Ni−NTA chromatography. The purified protein had a subunit $M_D$ by MALDI-TOF MS of *m*/*z* 37 139 ± 19 (calcd 37121). The presence of a second peak at *m*/*z* 73965 suggested that PtlH may be a homodimer.

The requisite substrate (±)-1-deoxypentalenic acid (**5**) was synthesized from α-humulene by way of (±)-pentalenene (**3**) and (±)-pentalen-13-ol (**9**), as previously described (Scheme 3).[8] A mixture (1.5 mL) of recombinant PtlH (1.66 $\mu$M), α-ketoglutarate (2 mM), L-ascorbic acid (2 mM), $FeSO_4$ (1 mM), DTT (1.5 mM),

[†] Brown University.
[‡] The Kitasato Institute.
[§] Kitasato University.



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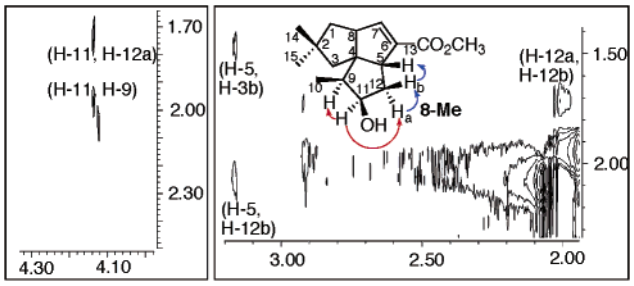


***Figure 2.*** Details of NOESY spectrum of methyl (−)-11$\beta$-hydroxy-1-deoxypentalenate (**8-Me**).

and bovine catalase (1 mg/mL) in 100 mM Tris buffer (pH 7.3) was incubated with (±)-(**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−$CHN_2$ 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 (±)-deoxypentalenic acid were well resolved, confirmed that the enzymatic reaction product was a single enantiomer. Control incubations that omitted α-ketoglutarate or Fe(II) showed no turnover of **5**. Neither (±)-pentalenene (**3**) (0.5 mM) nor (±)-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).[10]

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.[11] Fitting of the initial velocities to the Michaelis−Menten equation gave $k_{cat}$ = 4.2 ± 0.6 s$^{-1}$ and a $K_m$ (**5**) of 0.57 ± 0.19 mM.

These results firmly establish the biochemical function of the *ptlH* gene product, which is shown to catalyze the Fe(II)- and α-ketoglutarate-dependent hydroxylation of **5** to 11$\beta$-hydroxy-1-deoxypentalenic acid (**8**) (Scheme 2). Further conversion of **5** to pentalenolactone D (**6**) may involve oxidation of **5** to the ketone **10** by PtlF, an apparent NAD(P)$^+$-dependent dehydrogenase, followed by Baeyer−Villiger-like oxidation of **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).

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**Supporting Information Available:** Expression of recombinant PtlH, spectroscopic data for **8-Me**, and kinetic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) **8-Me:** $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.66 (1H, H-7, m), 4.10 (1H, H-11, m), 3.74 (3H, $OCH_3$, 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). $^{13}$C NMR (75.47 MHz, $CDCl_3$): $\delta$ 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 ($OCH_3$), 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 (C-10). $\alpha_D^{22}$ = −10.5° ($CH_2Cl_2$, $c$ = 0.1 g/100 mL). HRMS 264.1718; calcd $C_{16}H_{22}O_3$: 264.1725.
(10) The 11α-hydroxyl epimer of **8-Me** has a signal for H-11 at $\delta$ 370 (ref 8b).
(11) Kinetic assays were carried out at 23 °C with PtlH (0.093 $\mu$M) in MES (95 mM, pH 6.0) containing α-ketoglutarate (2.67 mM), L-ascorbate (2.67 mM), $FeSO_4$ (1.33 mM), catalase (0.95 mg/mL), DTT (1 mM) in a total volume of 200 $\mu$L. Reactions were initiated by adding a solution of (±)-deoxypentalenic acid (**5**) in DMSO to a concentration of 0.097−0.97 mM (final DMSO concentration 4%). The reactions were quenched with HCl at 30 min, and the mixtures were extracted with ether, treated with TMS−$CHN_2$, and analyzed by GC−MS.

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