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Pentalenolactone Biosynthesis. Molecular Cloning and Assignment of Biochemical Function to PtlI, a Cytochrome P450 of *Streptomyces avermitilis*

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Streptomyces are a rich source of bioactive secondary metabolites. The 9.03 Mb linear chromosome of *S. avermitilis*, the producer of the widely used antiparasitic avermectins, harbors 7575 open reading frames (ORFs)[1] of which 33 encode cytochrome P450 enzymes.[2] One of these CYP genes, *ptlI* (SAV2999, CYP183A1), is found within the gene cluster for the biosynthesis of the sesquiterpene antibiotic pentalenolactone (**1**). This cluster lies in a 13.4 kb segment, centered at 3.75 Mb in the *S. avermitilis* genome, that contains 13 unidirectionally transcribed ORFs (Figure 1).[3] Among these ORFs, the 1011 bp *ptlA* encodes pentalenene synthase (PtlA), which catalyzes the cyclization of farnesyl diphosphate (FPP) (**2**) to pentalenene (**3**), the established parent hydrocarbon of the pentalenolactone family of antibiotics (Scheme 1).[3,4] Besides the heme-dependent monooxygenase CYP183A1 (*ptlI*),[2] seven of the remaining ORFs correspond to redox enzymes, including the non-heme iron dioxygenase encoded by *ptlH*,[5] and six additional monooxygenases and dioxygenases.

We recently showed that PtlH, an Fe^{2+}/α-ketoglutarate-dependent hydroxylase, catalyzes the conversion of 1-deoxypentalenic acid (**4**) to a new biosynthetic intermediate, 11β-hydroxy-1-deoxypentalenic acid (**5**).[5] Although several presumptive intermediates of pentalenolactone biosynthesis have been isolated from a wide variety of *Streptomyces* species,[3,4] PtlH is the only enzyme linking pentalenene (**3**) to pentalenolactone (**1**) that has been characterized to date. Here we describe the biochemical characterization of PtlI, the cytochrome P450 that is shown to catalyze the conversion of pentalenene (**3**) to pentalen-13-al (**7**) by stepwise oxidation via pentalen-13-ol (**6**).

According to the proposed biosynthetic pathway (Scheme 1), the enzymes responsible for the conversion of pentalenene (**3**) to pentalenolactone (**1**) must first oxidize pentalenene to the corresponding unsaturated carboxylic acid **4**. Cytochrome P450s are known to catalyze numerous oxygenation reactions of nonactivated hydrocarbons.[6] Among these reactions is the three-step oxidation of a methyl group to a carboxylic acid.[7−9] We therefore speculated that PtlI might be responsible for all or part of the allylic oxidation of pentalenene (**3**) to 1-deoxypentalenic acid (**4**) (Scheme 2).

PtlI was amplified by polymerase chain reaction (PCR) from DNA of *S. avermitilis* cosmid CL_216_D07 and cloned between the *Nde*I and *Xho*I sites of the vector pET31b. The resulting construct pET31b-*PtlI* was transformed into *Escherichia coli* BL21-(DE3). After induction with IPTG, the expressed PtlI protein, carrying a C-terminal His_6-tag, was purified to homogeneity by Ni−NTA chromatography.[10] MALDI-TOF MS of purified protein showed subunit M_D *m*/*z* 51667 ± 50 (calcd 51723 for apo-protein) and *m*/*z* 52078 ± 50 (calcd 52339 for holo-protein). Treatment of

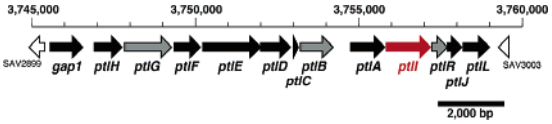
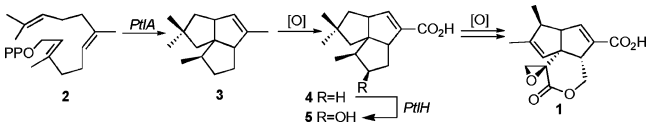
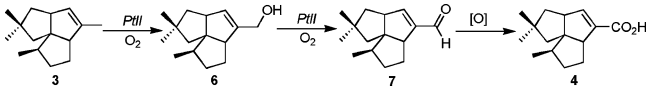


Figure 1. Pentalenolactone biosynthetic gene cluster from *S. avermitilis* (see http://avermitilis.ls.kitasato-u.ac.jp/).

Scheme 1



Scheme 2



the sodium dithionite-reduced protein with carbon monoxide gave the characteristic P450 UV difference spectrum.[11]

Titration of PtlI with pentalenene (**3**)[12] resulted in the typical blue-shift from 420 to 390 nm (type I binding).[13] The dissociation constant for **3** was determined by nonlinear fitting of the UV-difference spectra to give $K_D = 1.44 \pm 0.14$ μM. By contrast, the control sesquiterpene (−)-*trans*-caryophyllene showed no type I binding when added to PtlI.

A mixture of recombinant PtlI (0.57 μM), *E. coli* flavodoxin (Fld, 3.9 μM),[14,15a] *E. coli* flavodoxin reductase (Fdr, 6.3 μM),[14,15b] NADPH (0.45 mM), and a NADPH regeneration system [glucose-6-phosphate (3.1 mM) and glucose-6-phosphate dehydrogenase (10 u)] in 3.0 mL of 50 mM phosphate buffer, 10% glycerol (v/v), pH 7.4, was incubated with (±)-(**3**) (1.1 mM) plus 0.8% DMSO for 16 h at room temperature. GC−MS analysis of the pentane extract revealed exclusively two new peaks with *m*/*z* 218 (retention time 10.96 min) and 220 (retention time 11.03 min), identical to authentic pentalen-13-al (**7**) and pentalen-13-ol (**6**), respectively (Figure 2 and Supporting Information).[12] The ^{1}H NMR spectrum of the crude neutral extract also showed the characteristic aldehydic and olefinic signals at δ 9.71 and 6.704 (d, $J = 0.8$ Hz), respectively, for **7** (Figure S8). Chiral GC−MS analysis, under conditions in which individual enantiomers of (±)-pentalen-13-ol (**6**) and (±)-pentalen-13-al (**7**) were well resolved, confirmed that enzymatically produced **6** and **7** were each single enantiomers. Preparative-scale incubation with (±)-pentalenene (**3**) gave a mixture containing **6** and **7**, which was dissolved in methanol and treated with sodium borohydride to give alcohol **6**, identical by ^{1}H NMR to chemically synthesized pentalen-13-ol (**6**). Incubation using alcohol **6** as substrate confirmed that PtlI catalyzes the oxidation of **6** to aldehyde **7**.[16] By contrast, only trace amounts of 1-deoxypentalenic acid **4** could be detected under a wide variety of incubation conditions.

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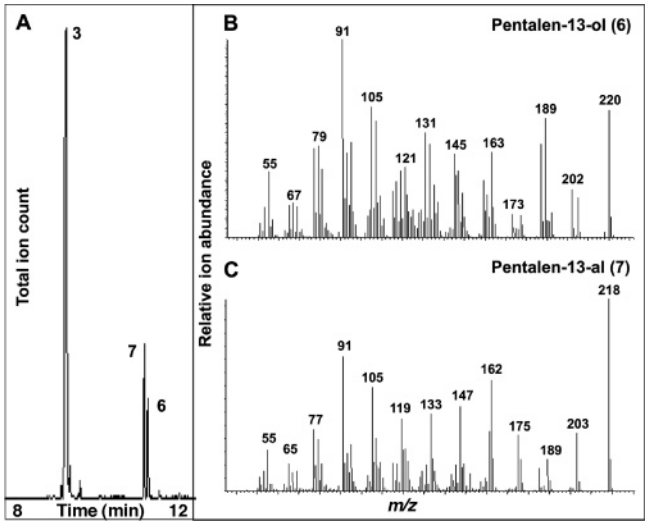

Figure 2. GC−MS analysis of incubation of PtlI with (±)-pentalenene (**3**). (A) GC trace of pentane extract. (B) MS of **6** from PtlI-catalyzed oxidation of **3**. (C) MS of **7** from PtlI-catalyzed oxidation of **3**.

PtlI showed a pH optimum of 8.0 for the oxidation of pentalenene to pentalen-13-ol. The apparent steady-state kinetic parameters for the first oxidation step were determined by carrying out a series of 10 min incubations with 4−40 μM of (±)-pentalenene (**3**) and quantitation of the product pentalen-13-ol (**6**) by GC−MS. Under these conditions, further oxidation of **6** was negligible.[8b] Fitting of the initial velocities to the Michaelis−Menten equation gave $k_{cat} = 0.503 \pm 0.006$ min^{-1} and a K_m of 3.33 ± 0.62 μM for the active enantiomer of **3**.

These results establish that the *ptlI* gene product can catalyze the two-step oxidation of pentalenene (**3**) to pentalen-13-al (**7**) (Scheme 2). At this point, it remains an open question how aldehyde **7** gets converted to 1-deoxypentalenic acid (**4**). Although it remains possible that PtlI might support the latter oxidation under the appropriate conditions,[17] by analogy to other P450s,[7−9] it is also conceivable that another redox enzyme from within the biosynthetic gene cluster could be responsible for this conversion. The work reported here sheds new light on the biosynthetic gap between pentalenene (**3**), generated by PtlA-catalyzed cyclization of FPP, and 11β-hydroxy-1-deoxypentalenic acid (**5**), the product of PtlH-catalyzed hydroxylation of 1-deoxypentalenic acid. Biochemical characterization of the remaining ORFs of the pentalenolactone biosynthetic gene cluster is in progress.

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Supporting Information Available: Expression of recombinant PtlI, binding spectra of **3** to PtlI, CO difference spectrum, mass spectra of products, chiral GC traces, NMR data, kinetic assays, and full citation for ref 14b. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Omura, S.; Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M.; Takahashi, Y.; Horikawa, H.; Nakazawa, H.; Osonoe, T.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12215−12220. (b) Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S. *Nat. Biotechnol.* **2003**, *21*, 526−531.

(2) Lamb, D. C.; Ikeda, H.; Nelson, D. R.; Ishikawa, J.; Skaug, T.; Jackson, C.; Omura, S.; Waterman, M. R.; Kelly, S. L. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 610−619.

(3) Tetzlaff, C. N.; You, Z.; Cane, D. E.; Takamatsu, S.; Omura, S.; Ikeda, H. *Biochemistry* **2006**, *45*, 6179−6186.

(4) (a) Cane, D. E.; Sohng, J. K.; Lamberson, C. R.; Rudnicki, S. M.; Wu, Z.; Lloyd, M. D.; Oliver, J. S.; Hubbard, B. R. *Biochemistry* **1994**, *33*, 5846−5857. (b) Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Abell, C.; Hubbard, B. R.; Kane, C. T.; Lattman, R. *J. Am. Chem. Soc.* **1990**, *112*, 4513−4524.

(5) (a) You, Z.; Omura, S.; Ikeda, H.; Cane, D. E. *J. Am. Chem. Soc.* **2006**, *128*, 6566−6567. (b) The 1-deoxypentalenic acid (**4**) has been isolated as the glucuronate ester: Takahashi, S.; Takeuchi, M.; Arai, M.; Seto, H.; Otake, N. *J. Antibiot.* **1983**, *36*, 226−228.

(6) Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841−2887.

(7) Gibberellin biosynthesis: (a) Helliwell, C. A.; Poole, A.; Peacock, W. J.; Dennis, E. S. *Plant Physiol.* **1999**, *119*, 507−510. (b) Helliwell, C. A.; Chandler, P. M.; Poole, A.; Dennis, E. S.; Peacock, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2065−2070.

(8) Bile acid biosynthesis: (a) Cali, J. J.; Russell, D. W. *J. Biol. Chem.* **1991**, *266*, 7774−7778. (b) Pikuleva, I. A.; Babiker, A.; Waterman, M. R.; Bjorkhem, I. *J. Biol. Chem.* **1998**, *273*, 18153−18160.

(9) Artemisinic acid biosynthesis: (a) Ro, D. K.; Paradise, E. M.; Ouellet, M.; Fisher, K. J.; Newman, K. L.; Ndungu, J. M.; Ho, K. A.; Eachus, R. A.; Ham, T. S.; Kirby, J.; Chang, M. C. Y.; Withers, S. T.; Shiba, Y.; Sarpong, R.; Keasling, J. D. *Nature* **2006**, *440*, 940−943. (b) Teoh, K. H.; Polichuk, D. R.; Reed, D. W.; Nowak, G.; Covello, P. S. *FEBS Lett.* **2006**, *580*, 1411−1416.

(10) The nonionic detergent Triton X-100 (0.1%) was a required component of the lysis buffer in order for PtlI to bind to the Ni−NTA resin, suggesting that PtlI may be membrane-associated.

(11) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370−2378.

(12) Compounds (±)-**3**, (±)-**6**, (±)-**7**, and (±)-**4** were synthesized as previously described: (a) Ohfune, Y.; Shirahama, H.; Matsumoto, T. *Tetrahedron Lett.* **1976**, *17*, 2869−2872. (b) Ohtsuka, T.; Shirahama, H.; Matsumoto, T. *Tetrahedron Lett.* **1983**, *24*, 3851−3854. (c) Cane, D. E.; Tillman, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 122−124. (d) Tillman, A. M. Ph.D. Thesis, Brown University, Providence, RI, 1984; pp 115−163.

(13) Jefcoate, C. R. *Methods Enzymol.* **1978**, *52*, 258−279.

(14) The natural electron transport pair for PtlI is unknown. *S. avermitilis* harbors six putative ferredoxin reductases and nine ferredoxins (cf. ref 2). Numerous attempts to observe PtlI-catalyzed oxidation of pentalenene (**3**) using typical redox pairs, such as spinach ferredoxin and NADPH: ferredoxin oxidoreductase or putidaredoxin and putidaredoxin reductase, were uniformly unsuccessful. By contrast, incubations of **3** and NADPH with unpurified cell-free extracts of *E. coli* PtlI expression cultures indicated that the endogenous *E. coli* flavodoxin (Fld) and flavodoxin reductase (Fdr) supported activity of PtlI. Although *S. avermitilis* does not harbor a native flavodoxin, similar observations have been previously reported for other P450s. (a) Jenkins, C. M.; Waterman, M. R. *J. Biol. Chem.* **1994**, *269*, 27401−27408. (b) Zhao, B.; et al. *J. Biol. Chem.* **2005**, *280*, 11599−11607.

(15) (a) FldA, Swissprot P61949: Jenkins, C. M.; Pikuleva, I.; Kagawa, N.; Waterman, M. R. *Arch. Biochem. Biophys.* **1997**, *347*, 93−102. (b) Fdr, Swissprot P28861: Jenkins, C. M.; Waterman, M. R. *Biochemistry* **1998**, *37*, 6106−6113.

(16) Some background oxidation of (±)-**6** to pentalen-13-al (**7**) could also be detected. Under typical incubation conditions, the ratio of P450-catalyzed oxidation to oxidation in the absence of PtlI was ~3:1, as judged by GC−MS [PtlI (1.4 μM), Fld (6.0 μM), Fdr (3.4 μM), NADPH (0.53 mM), glucose-6-phosphate (0.53 mM), and glucose-6-phosphate dehydrogenase (5 u) in 2.9 mL of 50 mM Tris-HCl buffer, 10% glycerol (v/v), pH 8, was incubated with (±)-**6** (0.1 mM) for 2 h at 25 °C]. This finding was consistent with chiral GC−MS analysis of an incubation of (±)-**6** with PtlI, which revealed formation of both enantiomers of aldehyde **7** in a ratio of 2.6:1 natural **7** to enantio-**7**.

(17) Incubations carried out with PtlI, ferredoxin FdxD (SAV3129), and ferredoxin reductase FprD (SAV5675), the most abundant electron carriers in *S. avermitilis*, showed no enhancement in pentalenene oxidation and no detectable formation of 1-deoxypentalenic acid (**4**).

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