Published in final edited form as:

Biochemistry. 2016 December 06; 55(48): 6696–6704. doi:10.1021/acs.biochem.6b01040.

Substitution of a Single Amino Acid Reverses the Regiospecificity of the Baeyer-Villiger Monooxygenase PntE in the Biosynthesis of the Antibiotic Pentalenolactone

Ke Chen^{‡,1}, Shiwen Wu^{‡,1}, Lu Zhu^{‡,1}, Chengde Zhang¹, Wensheng Xiang⁴, Zixin Deng¹, Haruo Ikeda³, David E. Cane^{*,2}, and Dongqing Zhu^{*,1}

¹The Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Ministry of Education), Wuhan University, Wuhan, Hubei Province, 430071, China

²Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912-9108, United States

³Laboratory of Microbial Engineering, Kitasato Institute for Life Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Minami-ku, Kanagawa 252-0373, Japan

⁴School of Life Science, Northeast Agricultural University, Harbin, Heilongjiang Province, 150030, China

Abstract

In the biosynthesis of pentalenolactone (**1**), PenE and PntE, orthologous proteins from *Streptomyces exfoliatus* and *S. arenae*, respectively, catalyze the flavin-dependent Baeyer-Villiger oxidation of 1-deoxy-11-oxopentalenic acid (**4**) to the lactone pentalenolactone D (**5**), in which the less-substituted methylene carbon has migrated. By contrast, the paralogous PtlE enzyme from *S. avermitilis* catalyzes the oxidation of **4** to neopentalenolactone D (**6**), in which the more substituted methane substitution has undergone migration. We report the design and analysis of 13 single and multiple mutants of PntE mutants in order to identify the key amino acids that contribute to the regiospecificity of these two classes of Baeyer-Villiger monooxygenases. The L185S mutation in PntE reversed the observed regiospecificity of PntE such that all recombinant PntE mutants harboring this L185S mutation acquired the characteristic regiospecificity of PtlE, catalyzing the conversion of **4** to **6** as the major product. The recombinant PntE mutant harboring R484L exhibited reduced regiospecificity, generating a mixture of lactones containing more than 17% of **6**. These *in vitro* results were corroborated by analysis of the complementation of the *S.*

*Corresponding Author: Dongqing Zhu, The Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Ministry of Education), Wuhan University, Wuhan, Hubei Province, 430071, China; Tel: +86-27-68752471; dzhu2011@whu.edu.cn; David E. Cane, Department of Chemistry, Box H, Brown University, Providence, RI 02912-9108, USA; Tel: +1-401-863-3588; David_Cane@brown.edu.

‡These authors contributed equally.

SUPPORTING INFORMATION AVAILABLE

Table of strains, plasmids and primers, alignment of amino acid sequences of proteins, SDS-PAGE analysis of proteins purified, and GC-MS data are available free of charge at the ACS Publications website at DOI: 10.1021/acs.biochem.*****

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

avermitilis *ptlED* double deletion mutant with *pntE* mutants, such that *pntE* mutants harboring L185S produced **6** as the major product, while complementation of the *ptlED* deletion mutant with *pntE* mutants carrying the R484L mutation gave **6** as more than 33% of the total lactone product mixture.

Graphical abstract

Keywords

Baeyer-Villiger monooxygenase; regiospecificity; pentalenolactone; *Streptomyces*

Baeyer-Villiger oxidations are regiospecific and stereospecific reactions in which ketones are oxidized by peracids or H₂O₂ to form esters or lactones, with preferred insertion of the oxygen atom between the ketone carbonyl and the more substituted carbon substituent, with retention of configuration at the migrating carbon atom. The first example of a biological Baeyer-Villiger reaction catalyzed by a Baeyer-Villiger monooxygenase (BVMO) was reported in 1948.¹ Since then, many BVMOs have been isolated from a variety of microorganisms, typically associated with catabolic pathways, including cyclopentanone monooxygenase (CPMO),² cyclohexanone monooxygenase (CHMO),³ cyclododecanone monooxygenase (CDMO),⁴ cyclopentadecanone monooxygenase (CPDMO),⁵ 4-hydroxyacetophenone monooxygenase (HAPMO),⁶ 4-sulfoacetophenone monooxygenase (SAPMO),⁷ phenylacetone monooxygenase (PAMO),⁸ and steroid monooxygenase (SMO).⁹ More recently, the first examples of BVMOs involved in anabolic pathways have been reported. These include the orthologous proteins PenE and PntE which are involved in pentalenolactone biosynthesis in *Streptomyces exfoliatus* and *S. arenae*, respectively,¹⁰ as well as the closely related paralog PtlE implicated in neopentalenolactone biosynthesis in *S. avermitilis*.¹¹ Additional anabolic BVMOs include CcsB for cytochalasin biosynthesis in *Aspergillus clavatus*,^{12, 13} and MtmOIV in mithramycin biosynthesis in *S. argillaceus*.¹⁴ The overwhelming majority of these oxygenases are classified as Type I BVMOs, which are NADPH- and FAD-dependent, while Type II BVMOs, such as the 2,5-diketocamphane 1,2-monooxygenase (2,5-DKCMO) which is involved in oxidative degradation of camphor by *Pseudomonas putida*, are NADH- and FMN-dependent.¹⁵

The first Baeyer-Villiger monooxygenase for which the structure was determined was PAMO from *Thermobifida fusca*.^{16, 17} Structures of several other BVMOs have subsequently been reported, including those of CHMO,^{18–20} MtmOIV,^{21, 22} OTEMO,²³

SMO,²⁴ and SAFMO.²⁵ The X-ray structure analysis has helped clarify the detailed mechanism of BVMO-catalyzed reactions, while assisting protein engineering or directed evolution to broaden the application of BVMOs in synthetic chemistry. For example, while wild-type PAMO has a very narrow substrate range, this thermophilic enzyme has been engineered to expand its substrate scope.^{26–30} Alternatively, although CHMO (as well as CPMO) has an intrinsically broader substrate range, it is less thermostable than PAMO, leading to protein engineering efforts to enhance its oxidative stability and thermostability.³¹

Pentalenolactone (**1**) is an oxidized sesquiterpene antibiotic that has been isolated from more than 30 species of *Streptomyces*.^{32–35} Its antibiotic action against bacteria, fungi and protozoa is due to the presence of an electrophilic epoxylactone moiety that alkylates the active site cysteine of the target glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).^{36–38} Two homologous pentalenolactone biosynthetic gene clusters have been characterized in detail, the *pen* cluster from *S. exfoliatus* UC5319 and the closely related *pnt* cluster from *S. arenae* TU469,³⁹ and the detailed biochemical role of each of the biosynthetic enzymes encoded by both clusters has been established.⁴⁰ BLAST searches reveal at least 10 additional highly likely pentalenolactone biosynthetic gene clusters in Streptomycete genomes. The first step in the biosynthesis of pentalenolactone is the cyclization of the universal acyclic precursor farnesyl diphosphate (**2**, FPP) by the PenA or PntA gene products to give the tricyclic hydrocarbon pentalenene (Scheme 1).^{41, 42} Six sets of redox enzymes - the P450s PenI and PntI,⁴³ the non-heme iron, α -ketoglutarate-dependent dioxygenases PenH and PntH,^{44, 45} the dehydrogenases PenF and PntF,⁴⁵ the flavin-dependent Baeyer-Villiger monooxygenases PenE and PntE,¹⁰ the α -ketoglutarate-dependent dioxygenases PenD and PntD,¹⁰ and the cytochrome P450s PenM and PntM⁴⁶ are then responsible for the multistep oxidative conversion of pentalenene to **1** in *S. exfoliatus* and *S. arenae*, respectively. PenR and PntR, which belong to the MarR/SlyA family MarR/SlyA family of transcriptional regulators, are responsible for regulation of the pentalenolactone biosynthesis in *S. exfoliatus* and *S. arenae*, respectively.⁴⁷ The biosynthesis of the isomeric metabolite neopentalenolactone in *S. avermitilis* is controlled by the closely related *ptl* gene cluster.^{11, 40, 48} The two pathways diverge at the stage of the Baeyer-Villiger type oxidation of 1-deoxy-11-ketopentalenic acid (**4**), with PenE and PntE giving exclusively pentalenolactone D (**5**, PL-D), in which the less-substituted C-12 methylene has undergone migration, while PtlE converts **4** to the isomeric neopentalenolactone D (**6**, neoPL-D), with migration of the more substituted C-9 methine carbon (Scheme 1), as would be observed for nonenzymatic Baeyer-Villiger oxidations.

PenE, and PntE have very high levels of sequence similarity and identity over 584–589 amino acids (PenE/PntE: 93% identity, 96% similarity) while the pairwise sequence identity and similarity of each to PtlE (594 aa) is only slightly lower (PenE/PtlE: 70% identity, 81% similarity; PntE/PtlE: 69% identity, 82% similarity). We have previously reported experiments with hybrid PntE/PtlE proteins that indicated that the regiospecificity of these BVMOs was most strongly influenced by the N-terminal regions of each protein, particularly the region harboring the presumptive FAD-binding motif.¹⁰ In order to determine the key amino acid residues that control the striking differences in regiospecificity between PenE and PntE on the one hand and PtlE on the other, we have now generated a

series of PntE mutants, using as a guide the reported crystal structures of PAMO and CHMO, as well as the reported results of protein engineering of these two catabolic BVMOs.^{49, 50} Selected amino acids in PntE were systematically replaced by the corresponding amino acids found in PtlE and the products obtained by incubating each PntE mutant with the common α -methylcyclopentanone-containing substrate **4** were analyzed by GC-MS. It was found that mutation of Leu185 of PntE to the corresponding Ser that is found in PtlE (L185S) essentially completely reversed the intrinsic regiospecificity of PntE, resulting in the oxidation of **4** to the isomeric neopentalenolactone D (**6**) as the major product. The alternative PntE R484L mutant gave a mixture of both **6** (17–37%) and **5** (83–63%), as measured by either *in vitro* or *in vivo* analysis. We also examined the orthologous BVMO, SBI_09676, which is found in the putative pentalenolactone biosynthetic gene cluster of *S. bingchenggensis*, and established that SBI_09676 catalyzed the expected oxidation of **4** to **5**, as observed for both PenE and PntE.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Streptomyces and *E. coli* strains, and plasmids used in this work, are listed in Table S1 (Supporting Information). Primer sequences are listed in Table S2.

Materials

Reagents and solvents purchased from Sigma-Aldrich were of the highest quality available and were used without further purification. Restriction enzymes, T4 DNA ligase and DNA polymerase were purchased from New England Biolabs and used according to the manufacturer's specifications. Ni-NTA affinity columns were purchased from GE Healthcare. Amicon Ultra Centrifugal Filter Units were purchased from Millipore. DNA primers were synthesized by GenScript, Nanjing, China.

Methods

Growth media and conditions used for *E. coli* and *Streptomyces* strains and standard methods for handling *E. coli* and *Streptomyces in vivo* and *in vitro* were as described previously,^{51, 52} unless otherwise noted. All DNA manipulations were performed following standard procedures.⁵² DNA sequencing was carried out at GenScript, Nanjing, China. All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford, using a PerkinElmer Lambda 25 UV/vis spectrophotometer with bovine serum albumin as the standard.⁵³ Protein purity was estimated using SDS-PAGE and visualized using Coomassie Brilliant Blue stain. GC-MS analyses were carried out on an Agilent 7890A/5975C-GC/MSD at 70 eV electron impact (EI) operating in positive ion mode, using a HP5MS capillary column (30 m $\times$ 0.25 mm) with a solvent delay of 3 min and a temperature program of 60 °C for 2 min, followed by a temperature gradient of 60–280 °C for 11 min at 20 °C/min and a hold at 280 °C for 2 min.

Construction of *pntE* Mutants

The construction of plasmids harboring each *pntE* mutant gene is listed in Table S1, illustrated by the example of the PntE(P127A) mutant. In order to introduce mutations into

site A of *pntE*, four primers were designed: ZLF3 containing an NdeI site and the start codon, ZLR3a containing the desired site A mutation, and the complementary ZLF3a, and ZLR3 containing the stop codon and an EcoRI site. A 378-bp DNA fragment harboring 5'-partial *pntE* was amplified with primer pair ZLF3/ZLR3a and digested with NdeI, while a 1392-bp DNA fragment harboring 3'-partial *pntE* was amplified with primer pair ZLF3a/ZLR3 and digested with EcoRI. The two DNA fragments were phosphorylated with T4 polynucleotide kinase and inserted into the NdeI and EcoRI sites of pET28a to generate plasmid pWHU1623. A 1770-bp NdeI+EcoRI DNA fragment harboring *pntE* with the site-specific P127A mutation from pWHU1623 was inserted into the corresponding site of pIB139 to generate plasmid pWHU1601. Analogous methods were used in the construction of the additional *pntE* mutants. All plasmids were sequenced to confirm that the resulting clones contained the desired mutations and that no additional mutations had been introduced during PCR amplification. pET28a-derivative plasmids were used for expression of PntE mutant proteins. pIB139-derivative plasmids were used in the complementation of the *S. avermitilis ptiED* double deletion mutant.

Expression and Purification of Recombinant PntE Mutant Proteins

Overexpression and purification of recombinant PntE and PntE mutants carrying an N-terminal His₆-tag were as described previously.¹⁰ *E. coli* BL21(DE3) harboring the corresponding plasmids was grown in Luria-Bertani (LB) medium supplemented with 50 µg/mL kanamycin at 37 °C until the OD₆₀₀ reached 0.6–0.8. IPTG was added to a final concentration of 0.4 mM, and the culture was further incubated at 18 °C overnight. The cells were then harvested by centrifugation at 5000 *g* for 15 min and resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 7.4). After cell disruption by sonication, the cell debris was removed by centrifugation at 20000 *g* for 60 min, and the supernatant was loaded into a Ni-NTA column pre-equilibrated with lysis buffer. After collecting the flow-through and washing with 20 mM imidazole in lysis buffer at a flow rate of 2 mL/min, the protein was eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 100 mM imidazole, pH 7.4) at a flow rate of 1 mL/min. The fractions were analyzed by SDS-PAGE. The pooled PntE protein was concentrated and buffer-exchanged into storage buffer (50 mM NaH₂PO₄, 0.1 mM DTT, pH 8.0, containing 10% glycerol) using an Amicon Ultra Centrifugal Filter (Ultracel-30K). The yield of purified recombinant PntE and PntE mutants was typically ca. 35 mg/L of culture.

Incubation of PntE Mutants with 1-deoxy-11-oxopentalenic acid (4)

Enzyme-coupled incubation of 1-deoxy-11β-hydroxypentalenic acid (**3**) with PtiF and PntE or PntE mutants has been described previously^{10, 11} as has the preparation of **3**.^{11, 44, 45} The recombinant 1-deoxy-11β-hydroxypentalenic acid dehydrogenase PtiF was prepared as described previously.⁴⁵ The sample of **3** (0.1 mM) was incubated at room temperature with recombinant PtiF (14.4 µM) and β-NAD⁺ (1.6 mM) in 1 mL of Tris buffer (100 mM Tris-HCl, 1.5 mM DTT, 2% DMSO, pH 8.0) to generate **4**. After 1 h, purified PntE or PntE mutant protein (1.7 µM) was added along with β-NADPH (1 mM) and FAD (50 µM). After an additional 2.5 h, the reaction mixture was quenched by addition of 10% HCl to adjust the pH to 2.0, followed by extraction with 3×1 mL of dichloromethane. The combined organic extracts were dried over anhydrous Na₂SO₄ and concentrated on a rotovap. The residue was

resuspended in dichloromethane containing 10% methanol and treated with trimethylsilyldiazomethane (TMS-CHN₂) to yield pentalenolactone D methyl ester (**5**-Me) and/or *neo*-pentalenolactone D methyl ester (**6**-Me), which were identified by direct GC-MS comparison with authentic samples. All incubations were carried out under the same conditions. The regiospecificity of wild-type PntE and of PntE mutants is expressed as the ratio of **5**-Me or **6**-Me to total product (**5**-Me plus **6**-Me). Since the specific enzyme activity of individual PntE mutants tended to decrease to different degrees during the time period of the extended preparative incubations prior to GC-MS analysis, the relative total activity (RA) was used to compare the enzyme activity of PntE mutants to that of wild-type PntE under the same conditions (Table 1). The rate of conversion of **4** oxidized by wild-type PntE was defined as 100%.

Complementation of *S. avermitilis* *ptlED* Double Deletion Mutant with *pntE* mutants, *ptlE* mutants, or *SBI_09676*

The construction of the *S. avermitilis* *ptlED* in-frame double deletion mutant SUKA16::pKU462-*ermEp1ptl_cluster-ptlED* and the *S. avermitilis* *ptlED* mutant complemented with *pntE* have been previously described.^{10, 11} The recombinant pIB139-derived plasmids harboring mutant *pntE*, *ptlE*, mutant *ptlE*, or *SBI_09676* (Table S1) recovered from *E. coli* DH10B were transformed into *E. coli* ET12567/pUZ8002, and the unmethylated plasmids were conjugated into *S. avermitilis* *ptlED* mutant using apramycin to select the respective exconjugants. The complementational strains of each *ptlED* mutant carrying mutant *pntE* genes were confirmed by PCR using the primer pair ZLF3 and ZLR3, to give 1770-bp PCR products. The complementational strains of *ptlED* mutant harboring *ptlE* or mutant *ptlE* genes were confirmed by PCR using the primer pair WSW12F and WSW12R to give 1785-bp PCR products. The complementational strain of the *ptlED* mutant with *SBI_09676* was confirmed by PCR using the primer pair ZLF4 and ZLR4 to give 1775-bp products.

Analysis of Products from Complementation of the *S. avermitilis* *ptlED* Double Deletion Mutants

The cultivation of complemented strains and the isolation of the resulting products were as previously described.^{10, 11, 48} For analysis of the ratio of **5** and **6** isolated from the *S. avermitilis* *ptlED* double mutant carrying mutant *pntE*, mutant *ptlE*, or the *SBI_09676* gene, cultures of each strain were centrifuged to obtain the fermentation broth which was adjusted to pH 2.0 with 2 N HCl and extracted with chloroform. The organic layers were dried over anhydrous Na₂SO₄, concentrated, and methylated with TMS-CHN₂. The extracts were analyzed by GC-MS under the conditions described above.

RESULTS

Choice of Mutant Sites in PntE

Since to date there have been no reported crystal structures for either PtlE, PenE or PntE, we used the structures of PAMO and CHMO, two crystallographically characterized and extensively studied BVMO homologs, to predict the key amino acid residues that influence the regioselectivity of PtlE, PenE, and PntE. There are 35 amino acid residues in PAMO

(*Thermobifida fusca* YX, GenBank WP_011291921, AAZ55526), highlighted in Figure S1 with solid green arrows, that are either known to be located within the FAD, NADP or substrate binding sites, or for which mutants of these amino acid residues expand the substrate scope, increase enantiomeric excess (*E*-values), and/or increase enzyme activity.^{16, 26–29} According to the crystal structure of CHMO (*Rhodococcus* sp. HI-31; GenBank BAH56677) as well as the results of directed evolution, 17 amino acid residues, illustrated in Figure S1 with hollow blue arrows, are close to the active site and contribute to the enantioselectivity.^{19, 20, 54–56}

A BLASTP search of the NCBI nonredundant (nr) protein database with the PtlE, PenE, and PntE sequences revealed 18 sequence records of predicted *Streptomyces* proteins with high degrees of sequence similarity and identity from 9 known species and 6 unassigned species (Table S3). Of these proteins, the majority were clearly orthologous to PenE and PntE, while only B446_29680 (AGS72742.1) of *S. collinus* more closely resembled PtlE, suggesting that only B446_29680 was likely to convert ketone **4** to *neo*-pentalenolactone D (**6**), while all of the remaining predicted gene products most likely would catalyze the oxidation of **4** to pentalenolactone D (**5**).

Sequence alignment of PtlE, PenE, PntE and their homologues with PAMO and CHMO was used to aid in selection of appropriate sites for mutation in PntE. Both PtlE and B446_29680 share 6 common residues that correspond to key amino acids in both PAMO and CHMO and which differ from those at homologous sites found in PntE and PntE and their homologues, corresponding to P127, L185, M186, S238, R484, I488 in PntE (shown in Figure S1 as solid red rhombuses). Based on this analysis, we prepared 4 PntE mutants in which selected groups of residues were replaced by the amino acids found at the corresponding positions in PtlE, as shown in Figure 1. We also prepared 5 multiple-site PntE mutants containing combinations of the mutant sets shown in Figure 1 (Table 1).

Biochemical Characterization of PntE Mutants

Each of these 9 PntE mutants were screened *in vitro* by incubation with *in situ* generated 1-deoxy-11-oxopentalenic acid (**4**), and the methylated products were analyzed by GC-MS to determine the regiospecificity of the oxidative conversion to pentalenolactone D (**5**) or neopentalenolactone D (**6**). pET28a derivatives harboring *pntE* mutant genes were transformed and expressed in *E. coli* BL21(DE3) to give recombinant PntE mutant proteins carrying an N-terminal His₆-tag. Purification by metal ion affinity chromatography using a Ni²⁺-NTA resin gave recombinant PntE mutant proteins, >90% pure by SDS-PAGE (Figure S2). Each recombinant enzyme was incubated with **4**, which was generated *in situ* from 1-deoxy-11β-hydroxypentalenic acid (**3**) using recombinant PtlF dehydrogenase, due to the tendency of **4** to undergo facile epimerization at C-9 during attempted purification (Scheme 1). The reduced flavin cofactor was continuously regenerated using excess NADPH and catalytic quantities of FAD. The organic extracts were treated with TMS-CHN₂ and the derived methyl esters were analyzed by capillary GC-MS and direct comparison with authentic samples by both retention time and mass spectrum (Table 1 and Figure S3 A–L). A control incubation with boiled PtlF and boiled PntE gave only **3**-Me, the control with active PtlF and boiled PntE gave only unoxidized **4**-Me and 9-*epi*-**4**-Me, the control with PtlF and

PntE gave 96% **5**-Me and 4% **6**-Me, all as previously reported (Figure S3A, B, and C; Table 1).¹⁰ Of the incubations with each of the nine PntE mutants, all mutants that harbored the adjacent L185S and M186L mutations oxidized **4** exclusively to *neo*-pentalenolactone (**6**) (Figure S3 E, H, J, K, L; Table 1), while mutants harboring the combined V480A, A482S and R484L mutations, alone or in combination with the P127A mutation, generated a mixture of both lactones **5** and **6** (Figure S3F and I; Table 1). All other mutant combinations examined had little or no effect on the observed regiospecificity of the Baeyer-Villiger oxidation (Table 1). The relative activity of the various PntE mutants did decrease to different degrees relative to wild-type PntE, but with no obvious correlation between the magnitude of this decrease in total Baeyer-Villiger monooxygenase activity and the observed regiospecificity of each mutant.

Complementation of the *S. avermitilis* *ptlED* Double Deletion Mutant by *pntE* Mutant Genes

Since the low activity of several PntE mutants made detection of minor oxidation products difficult, we therefore also carried out parallel *in vivo* incubation experiments in which the genes for each *pntE* mutant were used to complement the previously described *S. avermitilis* SUKA16 *ptlED* double deletion mutant which accumulates the intermediate **4** (Table 1, Figure S4). The individual *pntE* mutant genes under control of the constitutive *ermEp* promoter were each conjugated into *S. avermitilis* *ptlED*. GC-MS analysis of the samples extracted from cultures complemented with *pntE* carrying the combined L185S and M186L mutations confirmed the formation of *neo*-pentalenolactone D methyl ester (**6**-Me) (Figure S4E, H, J, K, and L), as observed for cultures of the control strain complemented with wild type *ptlE* (Figure S4B). The sample from a culture complemented with the triple mutant *pntE*(V480A/A482S/R484L) produced 60–70% pentalenolactone D (**5**) and 40–30% *neo*-pentalenolactone D (**6**) (Figure S4F and I). The regioselectivity of the Baeyer-Villiger reaction catalyzed by each mutant *pntE* gene product from each complementation experiment conformed to the product distribution observed in the corresponding *in vitro* experiment. None of the complementation strains accumulated either the intermediate α -methylcyclopentanone substrate **4** or *epi*-**4**, as evidenced by the absence of either **4**-Me or *epi*-**4**-Me among the incubation products detected by GC-MS analysis.

Identification of Amino Acid Residues Affecting the Regiospecificity of PntE

Of the 9 PntE mutants subjected to both *in vitro* and *in vivo* analysis, those with mutations in L185S and M186L changed their intrinsic regiospecificity of oxidation from that of wild-type PntE, which produces pentalenolactone D (**5**),¹⁰ to that of PtlE, which produces the isomeric *neo*-pentalenolactone D (**6**).¹¹ The triple V480A/A482S/R484L mutation had a similar but more modest effect on the oxidative regiospecificity, giving mixtures of both **5** and **6**. In order to identify the specific amino acid residues that are responsible for the observed changes in the regiospecificity of the biochemical Baeyer-Villiger oxidation of **4**, four additional PntE single-site mutants were constructed: PntE(L185S), PntE(M186L), PntE(A482S) and PntE(R484L).

pET28a derivatives harboring each of these single mutant *pntE* genes were transformed and expressed in *E. coli* BL21(DE3) to give recombinant PntE mutants carrying an N-terminal

His₆-tag. Purification by metal ion affinity chromatography using a Ni²⁺-NTA resin gave the corresponding recombinant PntE mutant proteins, >90% pure by SDS-PAGE (Figure S2). Each recombinant enzyme was incubated with **4**, which was generated *in situ*, as described above. PntE(L185S) gave a mixture of 93% *neo*-pentalenolactone D (**6**) and 7% pentalenolactone D (**5**) (Figure S3M). PntE(M186L), PntE(A482S) and PntE(R484L) gave mixtures of 83–93% **5** and 17–7% **6** (Figure S3N, O, and P). Unlike the PntE mutants harboring multiple mutations, the four PntE single mutants each retained 78–96% oxidative enzyme activity compared to wild-type PntE (Table 1).

For *in vivo* complementation analysis, pIB139 derivatives harboring the individual *pntE* single-site mutant genes were each conjugated into the *S. avermitilis* *ptlED* double deletion mutant. GC-MS analysis established that *S. avermitilis* *ptlED::pntE*(L185S) formed 92% *neo*-pentalenolactone D (**6**) and 8% pentalenolactone D (**5**) (Figure S4M). By contrast, strain *ptlED::pntE*(M186L) produced a mixture of 88% **5** and only 12% **6** (Figure S4N). *ptlED::pntE*(R484L) produced a mixture with only 37% **6**, somewhat higher than that observed by *in vitro* analysis (Figure S4P). Strain *ptlED::pntE*(A482S) produced a mixture of 95 % **5** and only 5% **6** (Figure S4O).

Complementation of the *S. avermitilis* *ptlED* Deletion Mutant with *ptlE* Mutant Genes

Based on the above observations, we also attempted to reverse engineer PtlE so as to favor Baeyer-Villiger formation of pentalenolactone D (**5**). We therefore expected that both PtlE(S189L) and PtlE(L487R) might display significant reversals in regiospecificity compared to wild-type PtlE. Although the *E. coli* protein expression system produced mutant PtlE proteins only as insoluble inclusion bodies, *in vivo* complementation experiments allowed analysis of the regiospecificity of each mutant. Derivatives of pIB139 harboring each mutant *ptlE* gene were constructed and conjugated into *S. avermitilis* *ptlED*, as described above. Both *S. avermitilis* *ptlED::ptlE* and *ptlED::ptlE*(S485A), which served as controls, each formed exclusively *neo*-pentalenolactone D (**6**) (Figure S4B and R), based on GC-MS analysis of the methylated products. Interestingly, the *ptlED::ptlE*(S189L) exconjugant also produced only the native **6**, with no pentalenolactone D (**5**) detected in the extract (Figure S4Q). The levels of **4**-Me and 9-*epi*-**4**-Me that were observed in the culture extracts were almost triple the amount of **6**-Me, indicating that mutant PtlE(S189L) had low intracellular activity. Similarly, the *ptlED::ptlE*(L487R) exconjugant also produced only **6**, accompanied by very minor (<1%) amounts of **4**-Me (Figure S4S). Thus neither the S189L nor the L487R mutations in PtlE were able to alter the intrinsic regiospecificity of wild-type PtlE for formation of *neo*-pentalenolactone D (**6**).

Complementation of the *S. avermitilis* *ptlED* Deletion Mutant with the *SBI_09676* Gene

Based on sequence similarity, SBI_09676 (ADI12794.1) from *S. bingchenggensis* BCW-1 was predicted to catalyze the oxidation of **4** to pentalenolactone D (**5**), analogous to the behavior of both PntE and PenE. To test this prediction, the corresponding *SBI_09676* gene was therefore amplified by PCR and inserted into pIB139. The resulting pIB139 derivative harboring the *SBI_09676* gene was then conjugated into *S. avermitilis* *ptlED*. GC-MS analysis of the methylated culture extracts showed that *S. avermitilis* *ptlED::SBI_09676* formed a mixture of 93% pentalenolactone D (**5**) and 7% *neo*-pentalenolactone D (**6**),

confirming that SBI_09676 has the same intrinsic regiospecificity for Baeyer-Villiger oxidation of **4** as both PntE and PenE (Figure S4 T).

DISCUSSION

PntE(L185S) mutant

We have previously reported the construction of a progressive series of *ptlE/ptnE* hybrid genes and carried out *in vivo* analysis that indicated that the 100–400 aa region, which harbors the FAD-binding motif, has the most important influence on the regiospecificity of Baeyer-Villiger oxidation of the intermediate ketone **4** to give either pentalenolactone D (**5**) or *neo*-pentalenolactone D (**6**).¹⁰ As described above, the significant reversal in the observed regiospecificity of oxidation observed for the PntE L185S mutant is fully consistent with our earlier conclusion that the regiospecificity of oxidation is under control of residues in the 100–400 aa region, and significantly refines the likely locus of oxidative regiospecificity.

To date no crystal structure of a BVMO harboring a Criegee-type ketone substrate–peroxyflavin adduct has been reported. The exact protein molecular basis for the substrate specificity, regioselectivity, and enantioselectivity of a BVMO is therefore still unclear. In non-enzyme-catalyzed Baeyer-Villiger reactions, steric, conformational and electronic effects lead to the migration of the more substituted carbon, a result that is similar to those for most biochemical reactions catalyzed by BVMOs. A small number of BVMOs, however, have been found to generate the less-favored, so-called “abnormal”, lactones in which the less-substituted carbon has migrated. For example, wild-type PAMO oxidizes racemic bicyclo[3.2.0]hept-2-en-6-one to produce a 3:1 mixture of the “normal” and “abnormal” lactone products, with a high enantiomeric excess (*ee*) of the (1*S*,5*R*)-enantiomer. By contrast, the Q152F mutant of PAMO generates both the “normal” and “abnormal” lactone in a ratio of about 1:3, with the (1*R*,5*S*)-enantiomer of “abnormal” lactone as the main product.²⁹ PtlE and the mutant PntE(L185S) both generate *neo*-pentalenolactone D (**6**) by Baeyer-Villiger oxidation of α -methylketone **4**, corresponding to migration of the more substituted, methyl-bearing carbon, C9, thus resulting in what would be considered the “normal” lactone product. Both PtlE and PntE(L185S) carry a Ser residue at the site homologous to Gln152 of PAMO. Both Gln and Ser are neutral polar amino acid residues. The Q152F mutant of PAMO, which carries the neutral nonpolar Phe residue at this site, generates the “abnormal” lactone as the main product. Interestingly, PntE, PenE and the orthologous SBI_09676, which all generate the “abnormal” pentalenolactone D (**5**) from **4** by migration of the less-substituted C11 methylene, also carry a neutral nonpolar amino acid residue, Leu185, at the homologous site. These results indicate that the polarity of the amino acid at this key site strongly influences the regioselectivity of biochemical BVMOs.

Gln152 of PAMO, which is one of 17 amino acids that are within 5 Å of the substrate and contribute to the substrate specificity,²⁷ interacts with the FAD cofactor through two hydrogen bonds.^{16, 17, 57} It is likely that the positioning of the ketone substrate relative to the FAD cofactor shifts in the Q152F mutant of PAMO due to the absence of the corresponding hydrogen bonds, resulting in the observed change in oxidative regioselectivity. By contrast, it is conceivable that the L185S mutation in PntE introduces a new H-bond interaction between the Ser185 sidechain hydroxyl and the phosphodiester group of the ribose

phosphate linker of the FAD cofactor, thereby altering the key conformation of the substrate–peroxyflavin adduct (Scheme 2). In non-enzyme-catalyzed Baeyer-Villiger reactions, the more substituted ketone substituent undergoes migration, possibly due to differential stabilization of a partial positive charge on the migrating carbon center. By contrast, the products of the biochemical Baeyer-Villiger oxidation are under strict stereoelectronic control, in which the migrating substituent is expected to be antiperiplanar to the peroxy O-O bond of the intermediate Criegee adduct. The less-substituted C11 methylene of the corresponding Criegee intermediate in wild-type PntE would therefore be in the correct position (**7a**) so as to generate pentalenolactone D (**5**). In the PntE L185S mutant, the postulated additional hydrogen bond conceivably shifts the position of the bound FAD cofactor, with the displacement of the isoalloxazine toward C11 and rotation about the peroxy O-O bond of the Criegee adduct so that this critical bond would now be antiperiplanar to the more substituted C11 methine carbon (**7b**), resulting in the generation of *neo*-pentalenolactone D (**6**). This suggested stereoelectronic model remains to be validated by eventual comparison of the three-dimensional structures of PntE and PntE(L185S) with bound substrate and cofactor.

The homologous residue in CHMO, Leu145, which does not directly interact with FAD,¹⁸ has been shown to occupy an ideal position for formation of the Criegee intermediate in the structure of CHMO_{Rotated},¹⁹ and it is also in close proximity to the product ϵ -caprolactone in the structures of both CHMO_{Tight} and CHMO_{Loose}.²⁰ Notably, the CHMO L145N mutant with a neutral polar Asn residue at this site, showed a significant decrease in activity,¹⁹ although the regiospecificity of the reactions catalyzed by CHMO (L145N) was not reported. This site has been proposed to contribute to the difference in oxidative regiospecificity between CHMO and CPMO (Phe156 of CPMO corresponding residue of Leu145 of CHMO).^{20, 54} The same phenomenon was also further studied in a close homologue, CHMO_{Arthro} from *Arthrobacter* sp., which catalyzes the regiospecific oxidation of (+)-trans-dihydrocarvone to 100% abnormal lactone. The mutant CHMO_{Arthro} containing L196F (the site homologous to Leu145 of CHMO) produced the normal lactone as the major product at a decreased overall rate.⁵⁸

PtIE(S189L) mutant

Although based on these trends, the corresponding PtIE(S189L) mutant was predicted to have a reversed regiospecificity corresponding to that of PntE, the *S. avermitilis* exconjugant carrying the corresponding mutation, *ptIED::ptIE*(S189L), did not produce any pentalenolactone D (**5**), giving rise only to decreased quantities of the normal PtIE product *neo*-pentalenolactone D (**6**). The observed accumulation in the culture extracts of *ptIED::ptIE*(S189L) of the intermediate **4** which serves as the BVMO substrate indicated that the S189L mutation in PtIE had resulted in substantial loss of activity. A similar result has been reported for the PAMO Q152F mutant, which decreased in activity by 83% in comparison with wild-type PAMO.²⁹ By contrast, PntE(L185S) retained substantial activity both *in vivo* and *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was supported by the National Natural Science Foundation of China (NSFC 81373305, NSFC 31401057) (D.Z.), and by U. S. National Institutes of Health Grant GM030301 (D.E.C.).

ABBREVIATIONS

BV	Baeyer-Villiger
BVMO	Baeyer-Villiger monooxygenase
DTT	1,4-dithiothreitol
FAD	flavin adenine dinucleotide
FPP	farnesyl diphosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
LB	Luria-Bertani medium
NAD⁺	β -nicotinamide adenine dinucleotide
NADPH	β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate
IPTG	isopropyl- β -D-thiogalactopyranoside

References

1. Turfitt GE. The microbiological degradation of steroids: 4. Fission of the steroid molecule. *Biochem J.* 1948; 42:376–383.
2. Iwaki H, Hasegawa Y, Wang S, Kayser MM, Lau PC. Cloning and characterization of a gene cluster involved in cyclopentanol metabolism in *Comamonas* sp. strain NCIMB 9872 and biotransformations effected by *Escherichia coli*-expressed cyclopentanone 1,2-monooxygenase. *Appl Environ Microbiol.* 2002; 68:5671–5684. [PubMed: 12406764]
3. Sheng D, Ballou DP, Massey V. Mechanistic studies of cyclohexanone monooxygenase: chemical properties of intermediates involved in catalysis. *Biochemistry.* 2001; 40:11156–11167. [PubMed: 11551214]
4. Kostichka K, Thomas SM, Gibson KJ, Nagarajan V, Cheng Q. Cloning and characterization of a gene cluster for cyclododecanone oxidation in *Rhodococcus ruber* SC1. *J Bacteriol.* 2001; 183:6478–6486. [PubMed: 11591693]
5. Iwaki H, Wang S, Grosse S, Bergeron H, Nagahashi A, Lertvorachon J, Yang J, Konishi Y, Hasegawa Y, Lau PC. *Pseudomonas* cyclopentadecanone monooxygenase displaying an uncommon spectrum of Baeyer-Villiger oxidations of cyclic ketones. *Appl Environ Microbiol.* 2006; 72:2707–2720. [PubMed: 16597975]
6. Kamerbeek NM, Olsthoorn AJ, Fraaije MW, Janssen DB. Substrate specificity and enantioselectivity of 4-hydroxyacetophenone monooxygenase. *Appl Environ Microbiol.* 2003; 69:419–426. [PubMed: 12514023]

7. Weiss M, Denger K, Huhn T, Schleheck D. Two enzymes of a complete degradation pathway for linear alkylbenzenesulfonate (LAS) surfactants: 4-sulfoacetophenone Baeyer-Villiger monooxygenase and 4-sulfophenylacetate esterase in *Comamonas testosteroni* KF-1. *Appl Environ Microbiol.* 2012; 78:8254–8263. [PubMed: 23001656]
8. Fraaije MW, Wu J, Heuts DP, van Hellemond EW, Spelberg JH, Janssen DB. Discovery of a thermostable Baeyer-Villiger monooxygenase by genome mining. *Appl Microbiol Biotechnol.* 2005; 66:393–400. [PubMed: 15599520]
9. Morii S, Sawamoto S, Yamauchi Y, Miyamoto M, Iwami M, Itagaki E. Steroid monooxygenase of *Rhodococcus rhodochrous*: sequencing of the genomic DNA, and hyperexpression, purification, and characterization of the recombinant enzyme. *J Biochem.* 1999; 126:624–631. [PubMed: 10467180]
10. Seo MJ, Zhu D, Endo S, Ikeda H, Cane DE. Genome mining in *Streptomyces*. Elucidation of the role of Baeyer-Villiger monooxygenases and non-heme iron-dependent dehydrogenase/oxygenases in the final steps of the biosynthesis of pentalenolactone and neopentalenolactone. *Biochemistry.* 2011; 50:1739–1754. [PubMed: 21250661]
11. Jiang J, Tetzlaff CN, Takamatsu S, Iwatsuki M, Komatsu M, Ikeda H, Cane DE. Genome mining in *Streptomyces avermitilis*: A biochemical Baeyer-Villiger reaction and discovery of a new branch of the pentalenolactone family tree. *Biochemistry.* 2009; 48:6431–6440. [PubMed: 19485417]
12. Qiao K, Chooi YH, Tang Y. Identification and engineering of the cytochalasin gene cluster from *Aspergillus clavatus* NRRL 1. *Metab Eng.* 2011; 13:723–732. [PubMed: 21983160]
13. Hu Y, Dietrich D, Xu W, Patel A, Thuss JA, Wang J, Yin WB, Qiao K, Houk KN, Vederas JC, Tang Y. A carbonate-forming Baeyer-Villiger monooxygenase. *Nat Chem Biol.* 2014; 10:552–554. [PubMed: 24838010]
14. Gibson M, Nur-e-alam M, Lipata F, Oliveira MA, Rohr J. Characterization of kinetics and products of the Baeyer-Villiger oxygenase MtmOIV, the key enzyme of the biosynthetic pathway toward the natural product anticancer drug mithramycin from *Streptomyces argillaceus*. *J Am Chem Soc.* 2005; 127:17594–17595. [PubMed: 16351075]
15. Kadow M, Sass S, Schmidt M, Bornscheuer UT. Recombinant expression and purification of the 2,5-diketocamphane 1,2-monooxygenase from the camphor metabolizing *Pseudomonas putida* strain NCIMB 10007. *AMB Express.* 2011; 1:13. [PubMed: 21906366]
16. Malito E, Alfieri A, Fraaije MW, Mattevi A. Crystal structure of a Baeyer-Villiger monooxygenase. *Proc Natl Acad Sci U S A.* 2004; 101:13157–13162. [PubMed: 15328411]
17. Orru R, Dudek HM, Martinoli C, Torres Pazmino DE, Royant A, Weik M, Fraaije MW, Mattevi A. Snapshots of enzymatic Baeyer-Villiger catalysis: oxygen activation and intermediate stabilization. *J Biol Chem.* 2011; 286:29284–29291. [PubMed: 21697090]
18. Mirza IA, Yachnin BJ, Wang S, Grosse S, Bergeron H, Imura A, Iwaki H, Hasegawa Y, Lau PC, Berghuis AM. Crystal structures of cyclohexanone monooxygenase reveal complex domain movements and a sliding cofactor. *J Am Chem Soc.* 2009; 131:8848–8854. [PubMed: 19385644]
19. Yachnin BJ, Sprules T, McEvoy MB, Lau PC, Berghuis AM. The substrate-bound crystal structure of a Baeyer-Villiger monooxygenase exhibits a Criegee-like conformation. *J Am Chem Soc.* 2012; 134:7788–7795. [PubMed: 22506764]
20. Yachnin BJ, McEvoy MB, MacCuish RJ, Morley KL, Lau PC, Berghuis AM. Lactone-bound structures of cyclohexanone monooxygenase provide insight into the stereochemistry of catalysis. *ACS Chem Biol.* 2014; 9:2843–2851. [PubMed: 25265531]
21. Beam MP, Bosserman MA, Noinaj N, Wehenkel M, Rohr J. Crystal structure of Baeyer-Villiger monooxygenase MtmOIV, the key enzyme of the mithramycin biosynthetic pathway. *Biochemistry.* 2009; 48:4476–4487. [PubMed: 19364090]
22. Bosserman MA, Downey T, Noinaj N, Buchanan SK, Rohr J. Molecular insight into substrate recognition and catalysis of Baeyer-Villiger monooxygenase MtmOIV, the key frame-modifying enzyme in the biosynthesis of anticancer agent mithramycin. *ACS Chem Biol.* 2013; 8:2466–2477. [PubMed: 23992662]
23. Leisch H, Shi R, Grosse S, Morley K, Bergeron H, Cygler M, Iwaki H, Hasegawa Y, Lau PC. Cloning, Baeyer-Villiger biooxidations, and structures of the camphor pathway 2-oxo- (3)-4,5,5-trimethylcyclopentenylacetyl-coenzyme A monooxygenase of *Pseudomonas putida* ATCC 17453. *Appl Environ Microbiol.* 2012; 78:2200–2212. [PubMed: 22267661]

24. Franceschini S, van Beek HL, Pennetta A, Martinoli C, Fraaije MW, Mattevi A. Exploring the structural basis of substrate preferences in Baeyer-Villiger monooxygenases: insight from steroid monooxygenase. *J Biol Chem*. 2012; 287:22626–22634. [PubMed: 22605340]
25. Hwang WC, Xu Q, Wu B, Godzik A. Crystal structure of a Baeyer-Villiger flavin-containing monooxygenase from *Staphylococcus aureus* MRSA strain MU50. *Proteins*. 2014; doi: 10.1002/prot.24661
26. Reetz MT, Wu S. Greatly reduced amino acid alphabets in directed evolution: making the right choice for saturation mutagenesis at homologous enzyme positions. *Chem Commun (Camb)*. 2008; 44:5499–5501.
27. Reetz MT, Wu S. Laboratory evolution of robust and enantioselective Baeyer-Villiger monooxygenases for asymmetric catalysis. *J Am Chem Soc*. 2009; 131:15424–15432. [PubMed: 19807086]
28. Wu S, Acevedo JP, Reetz MT. Induced allostery in the directed evolution of an enantioselective Baeyer-Villiger monooxygenase. *Proc Natl Acad Sci U S A*. 2010; 107:2775–2780. [PubMed: 20133612]
29. Dudek HM, de Gonzalo G, Pazmino DE, Stepniak P, Wyrwicz LS, Rychlewski L, Fraaije MW. Mapping the substrate binding site of phenylacetone monooxygenase from *Thermobifida fusca* by mutational analysis. *Appl Environ Microbiol*. 2011; 77:5730–5738. [PubMed: 21724896]
30. van Beek HL, de Gonzalo G, Fraaije MW. Blending Baeyer-Villiger monooxygenases: using a robust BVMO as a scaffold for creating chimeric enzymes with novel catalytic properties. *Chem Commun (Camb)*. 2012; 48:3288–3290. [PubMed: 22286124]
31. Opperman DJ, Reetz MT. Towards practical Baeyer-Villiger-monooxygenases: design of cyclohexanone monooxygenase mutants with enhanced oxidative stability. *Chembiochem*. 2010; 11:2589–2596. [PubMed: 21080396]
32. Koe BK, Sobin BA, Celmer WD. PA 132, a new antibiotic. I. Isolation and chemical properties. *Antibiot Annu*. 1956:672–675. [PubMed: 13425447]
33. Keller-Schlerlein W, L J, Nyfeler R, Zähler H. Metabolic products of microorganisms. 105. Arenacemycin E, D and C. *Arch Mikrobiol*. 1970; 84:301–316.
34. Takeuchi S, Ogawa Y, Yonehara H. The structure of pentalenolactone (PA-132). *Tetrahedron Lett*. 1969; 32:2737–2740.
35. Martin DG, Slomp G, Mizsak S, Duchamp DJ, Chidester CG. The structure and absolute configuration of pentalenolactone (PA 132). *Tetrahedron Lett*. 1970; 33:4901–4904.
36. Cane DE, Sohng JK. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by pentalenolactone: kinetic and mechanistic studies. *Arch Biochem Biophys*. 1989; 270:50–61. [PubMed: 2930199]
37. Hartmann S, Neeff J, Heer U, Mecke D. Arenacemycin (pentalenolactone): a specific inhibitor of glycolysis. *FEBS Lett*. 1978; 93:339–342. [PubMed: 361434]
38. Duzenko M, Balla H, Mecke D. Specific inactivation of glucose metabolism from eucaryotic cells by pentalenolactone. *Biochim Biophys Acta*. 1982; 714:344–350. [PubMed: 7034785]
39. Seo MJ, Zhu D, Endo S, Ikeda H, Cane DE. Genome mining in *Streptomyces*. Elucidation of the role of Baeyer-Villiger monooxygenases and non-heme iron-dependent dehydrogenase/oxygenases in the final steps of the biosynthesis of pentalenolactone and neopentalenolactone. *Biochemistry*. 2011; 50:1739–1754. [PubMed: 21250661]
40. Cane DE, Ikeda H. Exploration and mining of the bacterial terpenome. *Acc Chem Res*. 2012; 45:463–472. [PubMed: 22039990]
41. Cane DE, Abell C, Harrison PH, Hubbard BR, Kane CT, Lattman R, Oliver JS, Weiner SW. Terpenoid biosynthesis and the stereochemistry of enzyme-catalysed allylic addition-elimination reactions. *Philos Trans R Soc Lond B Biol Sci*. 1991; 332:123–129. [PubMed: 1678531]
42. Cane DE, Sohng JK, Lamberson CR, Rudnicki SM, Wu Z, Lloyd MD, Oliver JS, Hubbard BR. Pentalenene synthase. Purification, molecular cloning, sequencing, and high-level expression in *Escherichia coli* of a terpenoid cyclase from *Streptomyces* UC5319. *Biochemistry*. 1994; 33:5846–5857. [PubMed: 8180213]

43. Quaderer R, Omura S, Ikeda H, Cane DE. Pentalenolactone biosynthesis. Molecular cloning and assignment of biochemical function to PtlI, a cytochrome P450 of *Streptomyces avermitilis*. J Am Chem Soc. 2006; 128:13036–13037. [PubMed: 17017767]
44. You Z, Omura S, Ikeda H, Cane DE. Pentalenolactone biosynthesis. Molecular cloning and assignment of biochemical function to PtlH, a non-heme iron dioxygenase of *Streptomyces avermitilis*. J Am Chem Soc. 2006; 128:6566–6567. [PubMed: 16704250]
45. You Z, Omura S, Ikeda H, Cane DE. Pentalenolactone biosynthesis: Molecular cloning and assignment of biochemical function to PtlF, a short-chain dehydrogenase from *Streptomyces avermitilis*, and identification of a new biosynthetic intermediate. Arch Biochem Biophys. 2007; 459:233–240. [PubMed: 17178094]
46. Zhu D, Seo MJ, Ikeda H, Cane DE. Genome mining in *Streptomyces*. Discovery of an unprecedented P450-catalyzed oxidative rearrangement that is the final step in the biosynthesis of pentalenolactone. J Am Chem Soc. 2011; 133:2128–2131. [PubMed: 21284395]
47. Zhu D, Wang Y, Zhang M, Ikeda H, Deng Z, Cane DE. Product-mediated regulation of pentalenolactone biosynthesis in *Streptomyces* by the MarR/SlyA Family activators PenR and PntR. J Bacteriol. 2013; 195:1255–1266. [PubMed: 23316039]
48. Tetzlaff CN, You Z, Cane DE, Takamatsu S, Omura S, Ikeda H. A gene cluster for biosynthesis of the sesquiterpenoid antibiotic pentalenolactone in *Streptomyces avermitilis*. 2006:6179–6186.
49. Balke K, Kadow M, Mallin H, Sass S, Bornscheuer UT. Discovery, application and protein engineering of Baeyer-Villiger monooxygenases for organic synthesis. Org Biomol Chem. 2012; 10:6249–6265. [PubMed: 22733152]
50. Zhang ZG, Parra LP, Reetz MT. Protein engineering of stereoselective Baeyer-Villiger monooxygenases. Chemistry. 2012; 18:10160–10172. [PubMed: 22807240]
51. Kieser, T.; Bibb, MJ.; Buttner, MJ.; Chater, KF.; Hopwood, DA. Practical *Streptomyces* genetics. The John Innes Foundation; Norwich, United Kingdom: 2000.
52. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y: 1989.
53. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]
54. Mihovilovic MD, Rudroff F, Winninger A, Schneider T, Schulz F, Reetz MT. Microbial Baeyer-Villiger oxidation: stereopreference and substrate acceptance of cyclohexanone monooxygenase mutants prepared by directed evolution. Org Lett. 2006; 8:1221–1224. [PubMed: 16524308]
55. Reetz MT, Brunner B, Schneider T, Schulz F, Clouthier CM, Kayser MM. Directed evolution as a method to create enantioselective cyclohexanone monooxygenases for catalysis in Baeyer-Villiger reactions. Angew Chem Int Ed Engl. 2004; 43:4075–4078. [PubMed: 15300699]
56. Kayser MM, Clouthier CM. New bioorganic reagents: evolved cyclohexanone monooxygenase—why is it more selective? J Org Chem. 2006; 71:8424–8430. [PubMed: 17064015]
57. Martinoli C, Dudek HM, Orru R, Edmondson DE, Fraaije MW, Mattevi A. Beyond the protein matrix: Probing cofactor variants in a Baeyer-Villiger oxygenation reaction. ACS Catal. 2013; 3:3058–3062. [PubMed: 24443704]
58. Balke K, Schmidt S, Genz M, Bornscheuer UT. Switching the regioselectivity of a cyclohexanone monooxygenase toward (+)-trans-dihydrocarvone by rational protein design. ACS Chem Biol. 2016; 11:38–43. [PubMed: 26505211]

Figure 1.

Alignment of seven BVMO protein partial sequences in 4 mutation targets within PntE.

A, B, C, D, 4 mutant target sites within PntE. B446_29680 (GenBank AGS72742.1) from *S. collinus* Tu 365, Pt1E from *S. avermitilis* NRRL8165, PntE from *S. arenae* TU469, PenE from *S. exfoliatus* UC5319, SBI_09676 (GenBank ADI12797.1) from *S. bingchengensis* BCW-1, CHMO (GenBank BAH56677) from *Rhodococcus* sp. HI-31, PAMO (GenBank WP_011291921 or AAZ55526) from *Thermobifida fusca* YX. Red solid rhombuses, the positions of mutation target amino acids of PntE; Solid green arrows, important amino acids of PAMO, Hollow blue arrows, the important amino acids of CHMO.

Scheme 1. Biosynthesis of Neopentalenolactone D (**6**) and Pentalenolactone (**1**)

Scheme 2.

Proposed stereoelectronic model for the Baeyer-Villiger Oxidation of **4** to **5** or **6** by Fragmentation of Discrete Conformers of the Common Criegee Adduct **7**

Regioselectivity of PntE Mutants, PntE Mutants and SBI_09676 in the Baeyer-Villiger Oxidation of 1-deoxy-11-oxopentalenic acid (4)

Table 1

Protein (mutant site)	<i>in vitro</i> (%)			<i>in vivo</i> (%)		
	RA ^a	5 : 6 ^b	Fig. S3 ^c	4 ^a	5 : 6 ^b	Fig. S4 ^c
I ^e						
PntE(WT)	100	96:4	C	0	100:0	C
PntE(A)	69	100:0	D	0	100:0	D
PntE(B)	23	0:100	E	0	0:100	E
PntE(C)	79	69:31	F	0	61:39	F
PntE(D)	90	93:7	G	0	95:5	G
PntE(AB)	34	2:98	H	0	0:100	H
PntE(AC)	37	66:34	I	0	68:32	I
PntE(BC)	7	0:100	G	0	0:100	G
PntE(ABC)	6	0:100	K	trace	0:100	K
PntE(ABCD)	6	0:100	L	trace	0:100	L
II						
PntE(L185S)	91	7:93	M	trace	8:92	M
PntE(M186L)	84	87:13	N	trace	88:12	N
PntE(A482S)	96	93:7	O	trace	95:5	O
PntE(R484L)	78	83:17	P	trace	63:37	P
III						
PntE(WT)	ND ^f	ND		trace	0:100	B
PntE(S189L) ^g	ND	ND		74	0:26	Q
PntE(S485A) ^g	ND	ND		8	0:92	R
PntE(L487R)	ND	ND		0	1:99	S
IV						
SBI_09676(WT)	ND	ND		0	93:7	T

^aRA, relative activity. For a fair comparison, all incubations were carried out under the same conditions and relative activities are expressed as the rate of conversion normalized to that obtained with wild-type PntE based on GC-MS analysis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

^gRatio of compound PL-D (5) to *neo*PL-D (9) based on GC-MS analysis.

^cThe corresponding mutant codes of samples in the SI Figures for GC-MS analysis

^dTrace, compound 4 observed in MS and without peak in TIC.

^eMutant groups A, B, C, and D, correspond to mutations described in Figure 1.

^fND, not determined.

^gRatio of compound 4 to *neo*-pentalenolactone D (6) based on GC-MS analysis. Pentalenolactone D (5) was not detected.