

Genome Mining in *Streptomyces avermitilis*: A Biochemical Baeyer–Villiger Reaction and Discovery of a New Branch of the Pentalenolactone Family Tree[†]

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Received May 5, 2009; Revised Manuscript Received May 31, 2009

ABSTRACT: Incubation of 1-deoxy-11-oxopentalenic acid (**12**) with recombinant PtlE protein from *Streptomyces avermitilis* in the presence of NADPH and catalytic FAD gave the Baeyer–Villiger oxidation product, the previously unknown compound neopentalenolactone D (**13**), representing a new branch of the pentalenolactone biosynthetic pathway. The structure and stereochemistry of the derived neopentalenolactone D methyl ester (**13-Me**) were fully assigned by a combination of GC-MS and NMR analysis and confirmed by X-ray crystallography. Neopentalenolactone D (**13**) was also isolated from engineered cultures of *S. avermitilis* from which the *ptlD* gene within the 13.4-kb (*neo*)-*ptl* biosynthetic gene cluster had been deleted. The Δ *ptlE* Δ *ptlD* double deletion mutant accumulated **12**, the substrate for the *ptlE* gene product, while the corresponding single Δ *ptlE* mutant produced **12** as well as the related oxidation products **14** and **15**. Engineered strains of *S. avermitilis*, SUKA5 and pKU462::ermRp-*ptl* cluster, harboring the complete (*neo*)-*ptl* cluster produced the oxidized lactone **18** and the closely related seco acid hydrolysis products **16** and **17**.

The sesquiterpenoid antibiotic pentalenolactone (**1**) occurs widely in *Streptomyces*, having been isolated from more than 30 Streptomyces species since first being reported in 1957 (1–5). Pentalenolactone is active against both Gram-positive and Gram-negative strains of bacteria as well as pathogenic and saprophytic fungi (6). The electrophilic epoxide moiety of the antibiotic selectively and irreversibly inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH¹) by alkylation of the active site cysteine (7–10). Self-resistance in the pentalenolactone producer *Streptomyces arenae* is due to an inducible, pentalenolactone-insensitive isozyme of GAPDH (11–14). Pentalenolactone is also a potent and specific antiviral agent, inhibiting the replication of DNA viruses, such as the causal agent of herpes simplex HSV-1 and HSV-2 (15), and can inhibit vascular smooth muscle cell proliferation (16).

Isotopic incorporation experiments using intact cultures of *Streptomyces exfoliatus* UC5319 have established that pentalenene (**2**) is the parent sesquiterpene hydrocarbon for

pentalenolactone (**1**) and related metabolites (17, 18) (Scheme 1). Pentalenene synthase from *S. exfoliatus* UC5319 has been cloned and expressed in *E. coli* (19), and its crystal structure has been determined (20). Extensive studies have elucidated the mechanism and stereochemistry of the pentalenene synthase-catalyzed cyclization of farnesyl diphosphate (**3**) to pentalenene (**2**) (21–23).

A variety of plausible intermediates in the conversion of pentalenene (**2**) to pentalenolactone (**1**) have been isolated (Scheme 1), including 1-deoxypentalenic acid (**4**) (4), pentalenolactone D (**5**) (24), pentalenolactone E (**6**) (25), and pentalenolactone F (**7**) (24, 26), as well as several closely related shunt metabolites such as pentalenic acid (**8**) (21, 27) and *epi*-pentalenolactone F (**28**), in addition to pentalenolactones G (26, 29), H (27), A (24), B (24), P (30), and O (30).

Streptomyces avermitilis is a Gram-positive soil bacterium that produces a variety of secondary metabolites, one of which, the anthelmintic agent avermectin, is widely used in human and veterinary medicine. Within the 9.03-Mb linear chromosome, 30 or more gene clusters are related to secondary metabolism, with at least six of them encoding putative terpenoid biosynthetic pathways (31, 32). Among the latter, an ~13.4-kb gene cluster centered at 3.75 Mb in the *S. avermitilis* genome containing 13 unidirectionally transcribed open reading frames (ORFs) has been implicated in the biosynthesis of pentalenolactones (Figure 1) (33).

Although pentalenolactone itself has not been detected in the organic extract of *S. avermitilis*, we have isolated the shunt metabolite pentalenic acid (**8**) from these cultures (33). Deletion of the 13.4-kb operon from *S. avermitilis* abolished pentalenic acid production, while transfer of the entire gene cluster to

[†]This work was supported by U.S. National Institutes of Health Grant GM30301 to D.E.C., by Grant-in-Aid for Scientific Research on Priority Areas “Applied Genomics” from the Ministry of Education, Culture, Sports, Science and Technology, Japan to H.I., and by Grant-in-Aid for Scientific Research of the Japan Society for the Promotion of Science No. 20310122 to H.I.

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¹Abbreviations: BVMO, Baeyer–Villiger monooxygenase; DTT, 1,4-dithiothreitol; FPP, farnesyl diphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl- β -D-thiogalactopyranoside; NAD⁺, β -nicotinamide adenine dinucleotide; ORF, open reading frame.

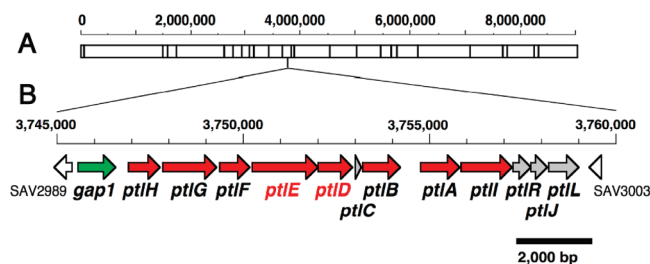
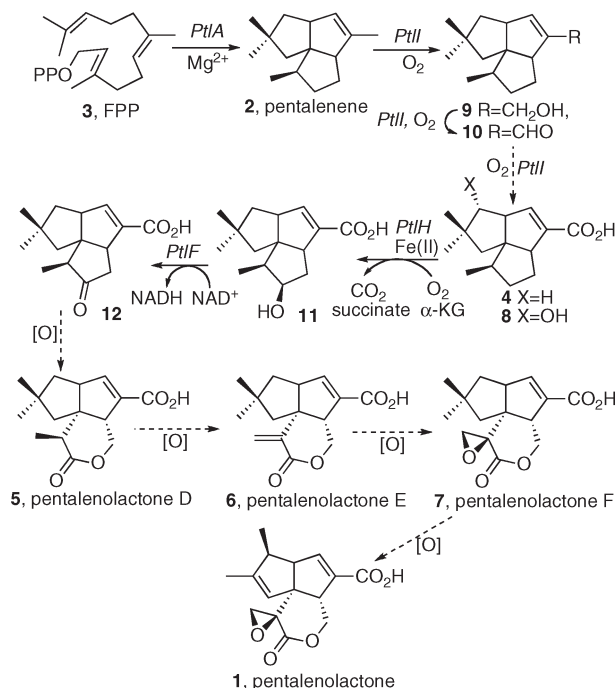


FIGURE 1: Proposed gene cluster for pentalenolactone biosynthesis in *S. avermitilis*. (A) *AseI*-physical map of the linear chromosome of *S. avermitilis*. (B) Region implicated in (neo)pentalenolactone biosynthesis.

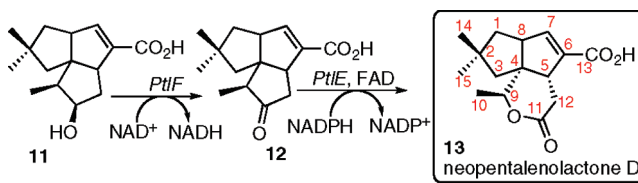
Scheme 1: Proposed Biosynthesis of Pentalenolactone



S. lividans 1326, a strain that normally does not produce pentalenolactones, resulted in generation of **8**.

In parallel with these molecular genetic investigations, we have been systematically expressing in *E. coli* the individual genes from the 13.4-kb biosynthetic cluster in order to identify the natural substrates and intrinsic biochemical reactions for each ORF. The furthest upstream gene, *gap1* (SAV2990), was shown to encode an inducible, pentalenolactone-insensitive GAPDH (33). By contrast, a second constitutive GAPDH isozyme encoded by *gap2* (SAV6296), which is located outside the biosynthetic cluster, was efficiently inactivated by pentalenolactone. The presence of the pentalenolactone-resistance gene (*gap1*) as part of the biosynthetic gene cluster is commonly observed for microbial antibiotics and is analogous to the demonstrated mode of resistance in *S. arenae* (11–14). Further direct evidence for the function of the 13.4-kb cluster came from an expression of *ptlA* (SAV2998), which was demonstrated to encode a pentalenene synthase that catalyzed the cyclization of farnesyl diphosphate (**3**) to the parent sesquiterpene pentalenene (**2**) (Scheme 1) (19, 21, 33). We also established that the *ptlI* gene (SAV2999) encodes a cytochrome P450 that catalyzes the two-step oxidation of pentalenene (**2**) to 1-deoxypentalenol (**9**) and 1-deoxypentalenal (**10**). The latter compound is most likely further oxidized to 1-deoxypentalenic acid (**4**) by the same enzyme (34), although

Scheme 2: PtlE-Catalyzed Baeyer–Villiger Oxidation of **12** to Neopentalenolactone D (**13**)



this has yet to be demonstrated conclusively. The *ptlH* (SAV2991) gene product, a nonheme iron/α-ketoglutarate-dependent dioxygenase (35), then catalyzes the hydroxylation of 1-deoxypentalenic acid (**4**) to 1-deoxy-11β-hydroxypentalenic acid (**11**). We have reported the crystal structure of PtlH in a variety of complexes with the cofactors Fe(II), α-ketoglutarate, and the nonreactive enantiomer of the substrate, *ent*-1-deoxypentalenic acid (36). PtlF (SAV2993), a typical NAD⁺-dependent short chain dehydrogenase/reductase, then catalyzes the oxidation of **11** to 1-deoxy-11-oxopentalenic acid (**12**) (37).

Of the remaining ORFs in the cluster, *ptlB* apparently encodes a typical farnesyl diphosphate synthase, while *ptlR* and *ptlG* have been assigned on the basis of sequence similarity as a putative transcriptional regulator and a transmembrane efflux protein, respectively (31–33). The *ptlJ* (SAV3001) knockout mutant of *S. avermitilis* produced the same family of pentalenolactone-like metabolites as the wild-type, ruling out *ptlJ* as a biosynthetic gene (unpublished results). The predicted *ptlC* (SAV2996) gene product is exceptionally small (58 amino acids), with no similarity to other known proteins, and is therefore unlikely to serve as a catalyst in the pentalenolactone biosynthetic pathway.

Only three gene products in the *S. avermitilis* biosynthetic gene cluster thus remain to be assigned: *ptlE* (SAV2994), *ptlD* (SAV2995), and *ptlL* (SAV3002). We hypothesized that PtlE, a putative flavin-dependent monooxygenase with a high level of sequence similarity to more than two dozen known or proposed Baeyer–Villiger type enzymes, would catalyze the oxidation of 1-deoxy-11-oxopentalenic acid (**12**) to the corresponding lactone, pentalenolactone D (**5**). Pentalenolactone D might conceivably be oxidized by PtlD, a putative α-ketoglutarate-dependent dioxygenase, and/or by PtlL to pentalenolactone F (**7**) and perhaps to pentalenolactone (**1**). We now report that recombinant PtlE does indeed catalyze a Baeyer–Villiger oxidation of **12**, but that the regiochemistry of the oxygen insertion reaction is opposite to that expected. The resulting product, which has been named neopentalenolactone D (**13**) (Scheme 2), represents a new branch of the pentalenolactone family tree.

EXPERIMENTAL PROCEDURES

Materials and Methods. General methods were as previously described (33). *S. avermitilis* cosmid CL_216_D07 was from the *S. avermitilis* genomic library (<http://avermitilis.lsc.kitasato-u.ac.jp>) (32). NMR experiments were carried out on 300 and 400 MHz (Bruker) and 500 MHz (JEOL) spectrometers. MALDI-TOF measurements were performed on an Applied Biosystems Voyager DE PRO MALDI-TOF benchtop mass spectrometer. GC-MS analyses were carried out either on a Hewlett-Packard Series 2 GC-MSD, at 70 eV electron impact (EI), operating in positive ion mode, using a DB5 capillary column (30 m × 0.25 mm) with a temperature program of 50–280 °C, 20 °C/min, or on a Shimadzu GC-17A, 70 eV EI, operating in positive ion mode, using a neutral bond-5 capillary

column (5% phenylmethylsilicon; 30 m \times 0.25 mm) with a temperature program of 50–280 °C, 20 °C/min. Tryptic in-gel digestion and LC-MS/MS tests were conducted at Yale Cancer Center Mass Spectrometry Resource and the W. M. Keck Foundation Biotechnology Resource Laboratory on a Waters Q-TOF Ultima mass spectrometer. LC-MS/MS spectra were analyzed using the automated Mascot algorithm against the NCBI-nr database. DNA sequencing was carried out on an ABI 3730 DNA sequencer using Big Dye Terminator V3.0 sequencing chemistry at the U. C. Davis Automated DNA Sequencing Facility.

Expression and Purification of PtlE Protein. The *ptlE* gene (SAV2994) was amplified by PCR with template DNA from *S. avermitilis* cosmid CL_216_D07 (<http://avermitilis.ls.kitasato-u.ac.jp>) (32) using the forward (5'-CCGCGCGCCATATGGATATCGAGGAGTGAGAGCGAAG-3') and reverse primer (5'-GGCCGGCTCGAGACTAGTCAATTGCTACCCTTCACGTAATTCGAGGTCG-3') to introduce *Nde*I and *Xho*I restrictions sites (**bold**) flanking the normal start and stop codons, respectively. The initial denaturation step (95 °C, 2 min) was followed by 25 cycles of touchdown PCR using a decreasing annealing temperature (95 °C, 0.5 min; 55–30 °C (–1 °C/step), 0.5 min; 72 °C, 2.5 min) followed by 15 cycles using an annealing temperature of 63 °C (95 °C, 0.5 min; 63 °C, 0.5 min; 72 °C, 2.5 min), and then a final incubation at 72 °C (10 min). The resulting amplicon was purified, digested with *Nde*I, and *Xho*I, and ligated into the corresponding sites of pET28E (33) with T4 DNA ligase at 4 °C overnight, then cloned into electrocompetent *E. coli* XL-1 Blue with selection by ampicillin. This vector proved to be unsuitable due to poor yields of soluble protein, and as a result, the pET28EMOX vector was digested with *Nde*I and *Xho*I, and ligated into the corresponding sites of pET31b with T4 DNA ligase at 4 °C overnight, then cloned into electrocompetent *E. coli* XL-1 Blue with selection by ampicillin. The DNA sequence of the insert was verified by dideoxy sequencing, and the resulting plasmid pET31bMOX was used to transform *E. coli* BL21(DE3).

Glycerol stocks (2 μ L) of *E. coli* BL21(DE3)/pET31bMOX were used to inoculate 5 mL of LB media supplemented with 50 μ g/mL ampicillin at 37 °C and 250 rpm overnight. The seed culture was then transferred into 500 mL of LB media with the same concentration of antibiotic and grown at 37 °C and 250 rpm until OD₆₀₀ was \sim 0.6. The culture was induced by the addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) followed by incubation at 18 °C overnight. The cells were harvested by centrifugation (7500g, 15 min, 4 °C), and the cell pellet was stored at –80 °C until needed.

For protein purification, the cell pellet from a 1-L culture of BL21(DE3)/pET31bMOX was resuspended in 30 mL of lysis buffer (20 mM Tris-HCl, 0.1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride, 0.1% Triton X-100, and 10% glycerol at pH 8.0). After the addition of lysozyme (2 mg/mL), the mixture was shaken at 4 °C for 30 min before being sonicated in an ice bath for 3 cycles. After centrifugation, the cell lysate was purified by 25% (\sim 1 M) ammonium sulfate (AS) precipitation, and the resultant supernatant (containing 1 M AS) was further purified on a 25-mL *n*-butyl-sepharose column that had been pre-equilibrated in buffer A (1 M ammonium sulfate, 50 mM NaH₂PO₄, and 0.1 mM DTT at pH 7.0). After loading of the supernatant onto the column, the resin was washed with 60 mL of buffer A followed by a 180-mL linear gradient from buffer A to buffer B (50 mM NaH₂PO₄ and 0.1 mM DTT at pH 7.0).

The purified PtlE protein eluted in buffer B showed a single band at M_r of 64 kDa on SDS–PAGE (Figure S1, Supporting Information), which was shown to consist of two proteins by MALDI-TOF MS. The 64-kDa band from the SDS–PAGE gel was excised and subjected to in-gel tryptic digestion and LC-MS/MS analysis.

Preparation of PtlH and PtlF proteins. Expression and purification of PtlH and PtlF proteins were carried out as previously described (35, 37).

1-Deoxy-11 β -hydroxypentalenic Acid (11). Preparative-scale incubation of PtlH and (\pm)-1-deoxypentalenic acid (4) (\sim 12 mg) was performed as described (35, 37), and the resulting product was purified by preparative TLC as the corresponding methyl ester, yielding 3 mg of methyl 1-deoxy-11 β -hydroxypentalenate (**11-Me**). Hydrolysis of **11-Me** in 2 mL of 5% aqueous K₂CO₃ plus 4 mL of methanol at reflux for 2 days gave 2 mg of 1-deoxy-11 β -hydroxypentalenic acid (**11**), which was stored at –80 °C.

Coupled Incubation of 11 with PtlF and PtlE. A reaction mixture containing 1-deoxy-11 β -hydroxypentalenic acid (**11**) (0.2 mM), PtlF (1.1 μ M), and β -NAD⁺ (1.6 mM) in 0.5 mL of assay buffer (100 mM Tris-HCl, 1.5 mM DTT, and 2% DMSO at pH 8.2) was incubated at room temperature for 10 min. The increase in NADH concentration was monitored at 340 nm. β -NADPH (1 mM) and a mixture of 5 μ M PtlE and 50 μ M FAD were then added. After an additional 1 h, the reaction was quenched with 30 μ L of 10% HCl, followed by extraction with 3 \times 1 mL of dichloromethane. The organic extract was dried over anhydrous MgSO₄, concentrated on a rotovap, and treated with 5 μ L of trimethylsilyldiazomethane (TMS-CHN₂) to yield the crude methyl esters, which were analyzed directly by GC-MS. A new peak, retention time (r.t.), 13.81 min (m/z 278), was detected (Figure S2A, Supporting Information). This peak was not observed in control experiments from which either PtlE or 1-deoxy-11 β -hydroxypentalenic acid (**11**) was omitted.

Neopentalenolactone D (13). To each of four 500-mL Erlenmeyer flasks was added a mixture containing 1-deoxy-11 β -hydroxypentalenic acid (**11**) (0.2 mM), PtlF (1.1 μ M), and β -NAD⁺ (1.6 mM) in 10 mL of assay buffer. The flasks were covered with aluminum foil and shaken at 50 rpm at room temperature. After 10 min, 1 mM β -NADPH, 5 μ M PtlE (10 mL), and 50 μ M FAD were added, and shaking was continued at 50 rpm overnight. The contents of the four flasks were combined and acidified with 10% HCl to pH 1–2, then extracted with dichloromethane (3 \times 100 mL). The organic extract was dried over anhydrous MgSO₄ and then concentrated on a rotovap to \sim 3 mL. The crude product was filtered through a cotton plug and concentrated further to 20 μ L. The residue was then dissolved in 2 mL of 1:1 methanol and benzene, and treated with TMS-CHN₂ until no more N₂ bubbles were generated. The reaction was left at room temperature for an additional 30 min, and the solution was then concentrated. The resulting yellow residue was applied to a preparative TLC plate (20 cm \times 20 cm, 500 μ m thick, with fluorescence indicator) and developed in 2:1 *n*-hexane/ethyl acetate. The band corresponding to the desired product (R_f = 0.45) was excised and immersed in 5 mL of ethyl acetate for 10 min before filtration through a cotton plug. The silica gel was washed with an additional 10 mL of ethyl acetate. The combined organic solutions were concentrated to give neopentalenolactone D methyl ester **13-Me** (0.7 mg). ¹H NMR (400.13 MHz, CDCl₃): δ 6.80 (1H, H-7, t), 4.39 (1H, H-9, m), 3.78 (3H, OCH₃, s), 3.31 (1H, H-5, m), 3.15 (1H, H-12 β , dd), 3.03 (1H, H-8, m), 2.40 (1H, H-12 α , m), 1.99 (1H, H-3 α , d), 1.70

(1H, H-1 α , m), 1.58 (1H, H-1 β , m), 1.49 (1H, H-3 β , d), 1.40 (3H, H-10, d), 1.08 (3H, H-14, s), 1.05 (3H, H-15, s). ^{13}C NMR (75.47 MHz, CDCl_3): δ 172.9 (C-11), 164.5 (C-13), 148.4 (C-7), 135.6 (C-6), 78.0 (C-9), 57.8 (C-4), 55.1 (C-8), 52.1 (C-5), 51.7 (OCH_3), 47.7 (C-3), 44.2 (C-1), 41.4 (C-2), 33.1 (C-12), 31.1 (C-14), 29.1 (C-15), 15.1 (C-10).

Construction of *ptlD*, *ptlE*, and *ptlD-ptlE* Deletion Mutants. The deletion mutants were prepared using the entire *ptl*-cluster clone (14.9-kb *AvrII-SnaBI*), which was cloned into the *XbaI/Ecl136I* sites of the actinophage-based integrating vector pKU462 as described previously (33), using as host strain *S. avermitilis* SUKA16 from which the entire *ptl*-cluster as well as genes for the other major polyketide and terpenoid metabolites had been deleted (38). To increase production, the *ermE* promoter was introduced upstream of the *ptlH* gene in the recombinant plasmid, designated as pKU462::ermEp-*ptl*-cluster. The derived *ptl*-cluster is thus transcribed as two operons, *ptlH-ptlG-ptlF-ptlE-ptlD-ptlC-ptlB* and *ptlA-ptlI-ptlR-ptlJ-ptlL*, and the resultant transformants harboring the *ermE* promoter exhibited enhanced production of pentalenic acid (**8**) in *S. lividans* 1326. Each in-frame deletion segment was constructed using the λ RED recombination system. An antibiotic resistance gene, *aad(3'')*, was amplified for each targeted deletion mutant using the designated primer pairs. In-frame Δ *ptlD*-deletion mutant: forward primer, 5'-GACGACCTCGCCGACCTCGAATTACGTGAA-**GGGTAGGTG**GCCAGTGAGTTCGAGCGACT-3' (*ptlD*-fwd; bold characters indicate the upstream region of *ptlD* on the chromosome, and underlined characters correspond to the start codon of *ptlD*) and the reverse primer, 5'-TGACGCGC-GAGTGACACCGCCTCGGCCCGCGCGGCATC**ACCCCG**-GGTACCGAGCGAAC-3' (*ptlD*-rev; bold characters indicate the downstream region of *ptlD* on the chromosome, and underlined characters correspond to the stop codon of *ptlD*) primers for the construction of the in-frame Δ *ptlD*-deletion. In-frame Δ *ptlE*-deletion mutant: forward primer, 5'-AACCCACCG-CACCGATATCTTTATGAGGGAGATACCGTGGCCAGTG-AGTTCGAGCGACT-3' (*ptlE*-fwd; bold characters indicate the upstream region of *ptlE* on the chromosome, and underlined characters correspond to the start codon of *ptlE*) and the reverse primer, 5'-ACCAGGTATCCGCGTAATGTCCATTGCTCTT-GACACCTACCCCGGTACCGAGCGAAC-3' (*ptlE*-rev; bold characters indicate the downstream region of *ptlE* on the chromosome, and underlined characters correspond to the stop codon of *ptlE*). In-frame Δ *ptlE* Δ *ptlD* deletion mutant: *ptlE*-fwd and *ptlD*-rev primers. Each pair of primers was used for the amplification of *loxP-aad(3'')-loxP* segment using pKU473 (pULwL::aad(3'')) as a template. The initial denaturation step (95 °C, 5 min) was followed by 5 cycles of amplification (95 °C, 0.5 min; 50 °C, 0.5 min; 72 °C, 1.0 min) followed by 25 cycles using an annealing temperature of 65 °C, and then a final incubation at 72 °C (10 min). The plasmid pKU462::ermEp-*ptl* cluster, carrying the entire *ptl* cluster under control of the *ermE* promoter, was introduced into *E. coli* BW25113 carrying pKD46 (39) by selecting for kanamycin-resistance (50 $\mu\text{g/mL}$). Transformants carrying the pKU462::ermEp-*ptl* cluster were grown in LB containing 100 $\mu\text{g/mL}$ of ticarcillin and 50 $\mu\text{g/mL}$ of kanamycin and induced λ -RED operon (γ , β , exo) at an early logarithmic phase by the addition of 10 mM L-arabinose (40). After ca. of 1 h cultivation, competent cells for electroporation were prepared, and each amplified PCR segment, after treatment with 0.1 unit of *DpnI* to remove any traces of template DNA, was directly introduced into these cells. The desired transformants, in

which the target gene(s) on the pKU462::ermEp-*ptl* cluster had been replaced by the PCR-fragment containing each 40-bp homologous sequence at both ends by λ RED system-based homologous recombination, were selected by kanamycin (50 $\mu\text{g/mL}$) and streptomycin (50 $\mu\text{g/mL}$)/spectinomycin (100 $\mu\text{g/mL}$) resistance. After confirmation of the replacement region, in which a target gene had been replaced by *loxP-aad(3'')-loxP*, on the recombinant plasmids, each replacement derivative (0.5 μg) was treated with 2 units of Cre recombinase (New England Biolabs, USA) according to manufacturer's procedures for the excision of the *loxP-aad(3'')-loxP* segment. The reaction mixture was directly transformed into *E. coli* DH5 α , and the in-frame deletion derivatives were selected by kanamycin-resistance and streptomycin/spectinomycin-sensitive phenotypes. The resultant recombinant plasmids were confirmed by restriction enzyme digestion. The desired in-frame deletion plasmids, pKU462::ermEp-*ptl* cluster Δ *ptlD*, pKU462::ermEp-*ptl* cluster Δ *ptlE*, and pKU462::ermEp-*ptl* cluster Δ *ptlE-ptlD*, were introduced into *S. avermitilis* SUKA16 by conjugation, as previously described (38).

Isolation of Products from In-Frame Deletion Mutants. The cultivation of in-frame deletion mutants was performed as previously described (33). The culture (500 mL) was separated by centrifugation (3,000 rpm for 10 min), and the supernatant was treated with 2.5 g of synthetic SP825 resin (Mitsubishi Chemical Co., Japan) to adsorb the products. The resin was washed with 25 mL of deionized water, and the products were eluted by 20 mL of methanol. Sedimented mycelium was extracted with 100 mL of methanol. After removal of the mycelium, the methanol extract was combined with the methanol eluate from the supernatant fraction, and methanol was evaporated under reduced pressure. The brownish solution was diluted with water, acidified to pH 2.5 with 2 N HCl, and extracted twice with a half volume of ethyl acetate. The organic phase was collected and evaporated to dryness. The brownish residue was methylated by TMS- CHN_2 . Methylated products were separated by silica gel column chromatography (10 ϕ \times 200 mm; developed with 20:1 chloroform/methanol). Finally, each component was purified by a preparative ODS-HPLC (Pegasil 5 μm ; 20 ϕ \times 250 mm) developing with 50% acetonitrile in water at a flow rate 9.9 mL/min and detection at 225 nm. In all purification steps, the products were monitored by GC-MS.

The Δ *ptlE* mutant produced two major components, **14** (**14-Me**; ret. time 10.00 min, m/z 278 [M^+]) and **15** (**15-Me**; ret. time 10.67 min, m/z 280 [M^+]), and one trace component, as determined by GC-MS analysis. The trace peak was identified as 1-deoxy-11-oxopentalenic acid methyl ester (**12-Me**) by direct comparison with an authentic sample. Each component was purified from a large scale culture of the Δ *ptlE* mutant. 1-Deoxy-9 β -hydroxy-11-oxo-pentalenic acid methyl ester (**14-Me**). HR-MS (EI) 278.1521 (calculated for $\text{C}_{16}\text{H}_{22}\text{O}_4$ 278.1518), ^1H NMR (500 MHz, CDCl_3): δ 6.76 (1H, H-7, s), 3.75 (3H, $-\text{OCH}_3$, s), 3.38 (1H, H-5, m), 3.17 (1H, H-8, m), 2.93 (1H, H-12 β , dd, J_1 = 18.8 Hz, J_2 = 11.0 Hz), 2.24 (1H, H-12 α , dd, J_1 = 18.8 Hz, J_2 = 5.5 Hz), 2.20 (1H, H-3 α , d, J = 13.7 Hz), 1.73 (1H, H-1 α , dd, J_1 = 12.8 Hz, J_2 = 8.7 Hz), 1.50 (1H, H-1 β , dd, J_1 = 12.8 Hz, J_2 = 5.0 Hz), 1.45 (1H, H-3 β , d, J = 13.7 Hz), 1.23 (3H, H-10, s), 1.05 (3H, H-14, s), 1.03 (3H, H-15, s). ^{13}C NMR (125 MHz, CDCl_3): δ 216.7 (C-11), 165.0 (C-13), 148.6 (C-7), 136.9 (C-6), 79.3 (C-9), 63.3 (C-4), 53.3 (C-8), 51.6 ($-\text{OCH}_3$), 50.9 (C-5), 48.5 (C-3), 45.8 (C-1), 40.2 (C-2), 38.1 (C-12), 30.0 (C-14), 29.9 (C-15), 20.2 (C-10). 1-Deoxy-9 β ,11 α -dihydroxypentalenic acid methyl

ester (**15-Me**). HRMS (EI) 280.1678 (calculated for $C_{16}H_{24}O_4$ 280.1675). 1H NMR (500 MHz, $CDCl_3$): δ 6.62 (1H, H-7, s), 3.88 (1H, H-11 β , brs), 3.65 (3H, $-OCH_3$, s), 3.43 (1H, H-8, m, $J_1=9.6$ Hz, $J_2=5.5$ Hz, $J_3=2.7$ Hz), 3.27 (1H, H-5, m, $J_1=10.0$ Hz, $J_2=4.1$ Hz, $J_3=1.4$ Hz), 2.51 (1H, H-12 β , m, $J_1=15.1$ Hz, $J_2=10.0$ Hz, $J_3=5.0$ Hz), 1.94 (1H, H-3 α , d, $J=13.3$ Hz), 1.64 (1H, H-12 α , m), 1.61–1.52 (3H, H-1 β , H-3 α , H-3 β m), 1.32 (3H, H-10, s), 1.05 (3H, H-14, s), 0.97 (3H, H-15, s). ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.2 (C-13), 148.5 (C-7), 138.3 (C-6), 84.4 (C-9), 81.8 (C-11), 66.7 (C-4), 56.8 (C-5), 55.2 (C-8), 51.4 ($-OCH_3$), 50.2 (C-3), 45.2 (C-1), 39.4 (C-2), 37.4 (C-12), 31.1 (C-15), 28.7 (C-14), 18.0 (C-10).

X-ray Crystallography of Neopentalenolactone D Methyl Ester (13-Me). The Δ ptlD mutant produced neopentalenolactone D (**13**), which was converted to the corresponding methyl ester **13-Me**. **13-Me** (1.2 mg) was crystallized in benzene/*n*-hexane (1:4). The colorless prismatic crystal having an approximate dimension of $0.2 \times 0.15 \times 0.20$ mm³ was mounted on a glass fiber. All measurements were made on a Rigaku AFC5R diffractometer with graphite monochromated Cu K α radiation ($\lambda = 1.54178$ Å) and a rotating anode generator. Crystal data for **13-Me**: empirical formula, $C_{16}H_{22}O_4$; molecular weight, 278.35; crystal system, orthorhombic; lattice type, primitive; space group, $P2_12_12_1$; lattice parameters, $a = 10.068(3)$ Å, $b = 24.219(3)$ Å, $c = 6.109(4)$ Å, $V = 1489(1)$ Å³; Z value, 4; D_{calc} , 1.241 g/cm³; temperature, 23.0 °C. The crystal structure was solved by the direct method with SIR-92. Refinement was performed by full-matrix least-squares. Final R and R_w were 0.119 and 0.154, respectively, for 1538 observed reflections. Crystallographic data for the structure of **13-Me** have been deposited with the Cambridge Crystallographic Data Center, deposition No. CCDC-729357 (Figure S9, Supporting Information).

Isolation of Seco Acids 16 and 17 from the S. avermitilis Deletion Mutant Carrying Overexpressed ptlH-G-F-E-D-C-B. A recombinant plasmid pKU462::ermEp-ptl cluster, in which the *ermE* promoter controls the transcription of the *ptlH-ptlG-ptlF-ptlE-ptlD-ptlC-ptlB* operon, while the other operon, *ptlA-tpiI-ptlR-ptlL-ptlJ*, was transcribed by its own promoter that was located upstream of *ptlA*, was introduced into *S. avermitilis* SUKA16. The cultivation, extraction, methylation, and purification utilized the same procedures described for isolation of products from the in-frame deletion mutants. Two peaks were detected at retention times of 10.4 and 12.0 min by GC-MS analysis as m/z 308 [M^+] and m/z 324 [M^+], respectively. Finally, each component was purified by preparative ODS-HPLC (Pegasil 5 μ m; $20\phi \times 250$ mm, developed with 50% aqueous acetonitrile) and analyzed by 1H and ^{13}C NMR. The faster eluting compound was the keto seco-acid methyl ester **16-Me**, and the slower eluting compound was the hydroxyketo seco-acid methyl ester **17-Me**. **16-Me**: HRMS (EI) 308.1636 (calculated for $C_{17}H_{24}O_5$ 308.1624). 1H NMR (500 MHz, $CDCl_3$): δ 6.73 (1H, H-7, dd, $J_1=2.3$ Hz, $J_2=1.4$ Hz), 3.79 (1H, H-8, ddd, $J_1=10.1$ Hz, $J_2=5.3$ Hz, $J_3=2.3$ Hz), 3.72 (3H, $-OCH_3$, s), 3.63 (3H, $-OCH_3$, s), 3.51 (1H, H-5, m), 2.54 (1H, H-12 α , dd, $J_1=16.0$ Hz, $J_2=5.5$ Hz), 2.35 (1H, H-3 α , d, $J=14.0$ Hz), 2.26 (1H, H-12 β , dd, $J_1=16.0$ Hz, $J_2=7.8$ Hz), 1.78 (1H, H-1 α , ddd, $J_1=13.1$ Hz, $J_2=10.1$ Hz, $J_3=1.4$ Hz), 1.71 (1H, H-3 β , d, $J=14.0$ Hz), 1.41 (1H, H-1 β , dd, $J_1=13.1$ Hz, $J_2=5.3$ Hz), 1.02 (3H, H-14, s), 0.83 (3H, H-15, s), ^{13}C NMR (125 MHz, $CDCl_3$): δ 210.8 (C-9), 172.4 (C-11), 164.8 (C-13), 148.9 (C-7), 133.6 (C-6), 70.8 (C-4), 51.67 (C-8), 51.64 ($-OCH_3$), 51.57 ($-OCH_3$), 51.56 (C-3), 50.4 (C-5), 44.6 (C-1), 39.4 (C-2), 35.5 (C-12), 30.2 (C-14),

29.1 (C-15), 28.4 (C-10). **17-Me**: HR-FABMS (positive ion) 325.1649 (calculated for $C_{17}H_{24}O_6$ 324.1573). 1H NMR (500 MHz, $CDCl_3$): δ 6.72 (1H, H-7, dd, $J_1=2.3$ Hz, $J_2=1.4$ Hz), 4.45 (1H, H-10 α , d, $J=18.8$ Hz), 4.37 (1H, H-10 β , d, $J=18.8$ Hz), 3.81 (1H, H-8, ddd, $J_1=10.4$ Hz, $J_2=5.5$ Hz, $J_3=2.3$ Hz), 3.73 (3H, $-OCH_3$, s), 3.64 (3H, $-OCH_3$, s), 3.15 (1H, 10 $-OH$, s), 2.65 (1H, H-12 α dd, $J_1=17.0$ Hz, $J_2=4.6$ Hz), 2.30 (1H, H-3 α , d, $J=14.2$ Hz), 2.24 (1H, H-12 β , dd, $J_1=17.0$ Hz, $J_2=8.7$ Hz), 1.78 (1H, H-1 α , ddd, $J_1=13.3$ Hz, $J_2=10.4$ Hz, $J_3=1.4$ Hz), 1.75 (1H, H-3 β , d, $J=14.2$ Hz), 1.44 (1H, H-1 β , dd, $J_1=13.3$ Hz, $J_2=5.5$ Hz), 1.05 (3H, H-14, s), 0.85 (3H, H-15, s), ^{13}C NMR (75 MHz, $CDCl_3$): δ 213.1 (C-9), 172.6 (C-11), 164.5 (C-13), 148.2 (C-7), 133.6 (C-6), 67.6 (C-10), 67.5 (C-4), 52.4 (C-8), 51.8 ($-OCH_3$), 51.7 ($-OCH_3$), 51.7 (C-5), 50.5 (C-3), 44.6 (C-1), 39.6 (C-2), 34.9 (C-12), 30.1 (C-14), 29.5 (C-15).

Isolation of 18 from the S. avermitilis Deletion Mutant Carrying the Intact ptl Cluster. Another large deletion mutant of *S. avermitilis* SUKA5 (Δ 79,460–1,595,563 nt Δ olm), the parental strain of SUKA16 but in which the entire *ptl* cluster was still intact, was grown in the vegetative medium, and then a portion of the culture was inoculated into four 500-mL flasks containing 100 mL of the production medium (2.0 g of glucose, 1.0 g of malt extract, and 0.4 g of yeast extract per 100 mL (pH 7.4)). The mutant was grown with shaking at 200 rpm for 6 days at 28 °C. The culture was centrifuged, and the supernatant (ca. 400 mL) was subjected to purification on an ODS (50–100 μ m; $10\phi \times 100$ mm) column. The column was washed with deionized water, and then products were eluted by 20–100% acetonitrile in water. The fractions containing the desired component were combined and were further purified by a preparative ODS-HPLC (Pegasil 5 μ m; $10\phi \times 250$ mm; developing with 20% acetonitrile in 0.1% trifluoroacetate; flow rate 4 mL/min; and detection at 210 nm). A major peak eluting at 23 min was harvested and concentrated to give 3.79 mg of neopentalenolactone (**18**) with m/z 278 [M^+], which was analyzed by NMR. **18**: HRMS (EI) 278.1160 (calculated for $C_{15}H_{18}O_5$ 278.1154). 1H NMR (400 MHz, $CDCl_3$): δ 6.71 (1H, H-7, d, $J=2.0$ Hz), 4.51 (1H, H-10 α , d, $J=18.1$ Hz), 4.42 (1H, H-10 β d, $J=18.1$ Hz), 3.83 (1H, H-8, m), 3.47 (1H, H-5, m), 2.53 (1H, H-12 α , dd, $J_1=12.2$ Hz, $J_2=3.5$ Hz), 2.35 (1H, H-3 α , d, $J=14.0$ Hz), 2.27 (1H, H-12 β , dd, $J_1=12.2$ Hz, $J_2=6.1$ Hz), 1.81 (1H, H-1 α , dd, $J_1=16.3$ Hz, $J_2=0.9$ Hz), 1.70 (1H, H-3 β d, $J=16.3$ Hz), 1.45 (1H, H-1 β , dd, $J_1=16.3$ Hz, $J_2=6.2$ Hz), 1.04 (3H, H-14, s), 0.86 (3H, H-15, s). ^{13}C NMR (75 MHz, $CDCl_3$): δ 213.0 (C-9), 174.3 (C-13), 172.0 (C-11), 147.9 (C-7), 134.4 (C-6), 68.0 (C-4), 66.7 (C-10), 52.2 (C-8), 51.5 (C-5), 50.8 (C-3), 44.2 (C-1), 39.0 (C-2), 34.5 (C-12), 29.1 (C-14), 28.4 (C-15).

RESULTS

Formation of Neopentalenolactone D by a PtlE-Catalyzed Baeyer–Villiger Reaction. Expression of plasmid pET31bMOX in *E. coli* BL21(DE3) cells gave soluble recombinant PtlE, which was purified by ammonium sulfate precipitation and *n*-butyl-sepharose chromatography. Although SDS–PAGE showed a single band at ~64 kDa (Figure S1, Supporting Information), MALDI-TOF MS analysis showed that the 594-amino acid PtlE (m/z 65887 \pm 70 Da; predicted 65826 Da) was accompanied by a roughly equal quantity of a second protein (m/z 57166 \pm 60 Da) that was identified as the 57329-Da *E. coli* GroEL chaperonin (4I) by in-gel tryptic digestion and LC-MS/MS analysis. Since all attempts to remove this 57-kDa contaminant were unsuccessful, and control experiments showed that the

chaperonin did not affect the course of the PtlE-catalyzed reaction, the PtlE protein preparation was used without further purification.

The presumed substrate for PtlE, 1-deoxy-11-oxopentalenic acid (**12**), undergoes facile epimerization of the secondary C-9 methyl group adjacent to the ketone during purification of the corresponding methyl ester. We therefore tested the activity of PtlE using a coupled assay in which the requisite substrate **12** was generated *in situ* from 1-deoxy-11 β -hydroxypentalenic acid (**11**) using recombinant PtlF dehydrogenase (Scheme 2). After treatment of the organic extracts with TMS-CHN₃, GC-MS analysis of the derived methyl esters revealed a single product **13-Me**, m/z 278, consistent with the addition of a single oxygen atom to **12** as would be expected for the generation of a Baeyer–Villiger oxidation product. Direct comparison of the retention time and mass spectrum of **13-Me** with those of authentic pentalenolactone D methyl ester (5-Me) (**24**), however, showed that the two compounds, while very similar, were not identical. Coinjection of pentalenolactone D methyl ester with the enzymatic reaction product gave two distinct peaks, each of m/z 278: pentalenolactone D methyl ester (**5-Me**), r.t. 13.70 min, and the new product **13-Me**, r.t. 13.81 min (Figure S2, Supporting Information). The MS spectra of these two compounds indicated that while they share a similar fragmentation pattern for $m/z \leq 192$, suggesting that they might share a common substructure consisting of two fused five-member-rings, differences in the $192 < m/z < 278$ range indicate that they differ in the precise structure of the lactonic component. (Figure S2, Supporting Information).

Control incubations with PtlF and a crude extract of BL21 (DE3)/pET31b in place of recombinant PtlE gave only the ketone **12-Me**, the methyl ester of the product of the PtlF-catalyzed oxidation of **11**, and its epimer (9-*epi*-**11-Me**), with no m/z 278 products, as determined by GC-MS (37). Additional controls confirmed that PtlE-catalyzed formation of **13** required both 1-deoxy-11 β -hydroxypentalenic acid (**11**) and FAD. Under no conditions could pentalenolactone D methyl ester (**5-Me**) be detected.

To determine the structure of **13-Me**, a preparative-scale incubation was carried out with PtlF, PtlE, and 1-deoxy-11 β -hydroxypentalenic acid (**11**). The resulting product was converted to the methyl ester, which was purified by preparative TLC and analyzed by a combination of ¹H and ¹³C NMR, as well as COSY, HSQC, HMBC, and NOESY NMR. In the ¹³C NMR spectrum of **13-Me**, the peak at δ 172.9 (C-11) confirmed the presence of a lactone, similar to C-11 (δ 175.4) in pentalenolactone D (**5-Me**) (42). This lactonic carbonyl signal is consistent with the observed increase of 16 amu from the m/z 262 of the ketone **12-Me**. Compared to pentalenolactone D (**5-Me**), C-9 and H-9 of **13-Me** are shifted downfield, while both C-12 and H-12 are shifted upfield, indicating that the oxygen atom has been inserted between C-9 and C-11, rather than between C-11 and C-12 as in **5-Me**. The isomeric lactone structure was supported by the HMBC spectrum of **13-Me** in which H-9 (δ 4.39) showed cross-peaks with both C-5 (δ 52.1) and C-3 (δ 47.7), while H-12 β (δ 3.15) was correlated with C-11 (Figures 2A and S7 (Supporting Information)). By contrast, there is no correlation between H-9 (δ 4.39) and C-11 (δ 172.9), nor between H-10 (δ 1.40) and C-11. The configuration of **13-Me** was readily established by the NOESY spectrum, which shows cross-peaks between H-9 (δ 4.39) and both H-8 (δ 3.03) and H-12 α (δ 2.40) (Figures 2A and S8 (Supporting Information)). The new product generated by PtlE-catalyzed oxidation of **12** has been given the name neopentalenolactone D (**13**).

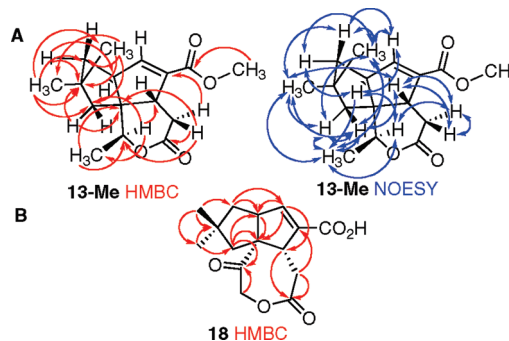


FIGURE 2: HMBC and NOESY Correlations. (A) Neopentalenolactone D methyl ester (**13-Me**). (B) Neopentalenoketolactone (**18**).

Analysis of Products from *S. avermitilis* Mutants. As a complement to the *in vitro* experiments with recombinant PtlE, we have also investigated the formation of neopentalenolactone D by *S. avermitilis*, including the role of the *ptlE* and *ptlD* genes. To this end, we used the large deletion mutant of *S. avermitilis* SUKA16 from which the gene clusters for biosynthesis of the major metabolites of *S. avermitilis* had all been deleted by successive rounds of homologous recombination, including the clusters for the polyketides avermectin, oligomycin, and filipin, and those for the terpenoids geosmin, the carotenes, and the entire *ptl* cluster. This multiple deletion mutant, which does not produce significant levels of secondary metabolites, has been found to be very useful for the analysis of transformants generated by introduction of terpenoid biosynthetic gene clusters, such as, for example, the two-gene operon for 2-methylisoborneol biosynthesis (38). We have previously reported the cloning of a 14.9-kb *AvrII*-*SnaBI* segment of the *S. avermitilis* genome harboring the entire *ptl* cluster (33). Since *ptlD* and *ptlE* are located in an operon consisting of *ptlH*-*ptlG*-*ptlF*-*ptlE*-*ptlD*-*ptlC*-*ptlB*, in-frame deletions of *ptlD* or *ptlE* must be used in order to avoid polar effects on the expression of the downstream genes in the polycistronic transcript. The required in-frame deletion fragments could be constructed using the λ RED system for homologous recombination in which short homologous sequences (~40-bp) from the targeted gene flank the selectable insertion sequences, followed by precise excision of the selectable marker gene by Cre-loxP site-specific recombination. The recombinant plasmid pKU462::ermEp-*ptl* cluster, carrying the in-frame deletion Δ *ptlD*, Δ *ptlE*, or Δ *ptlD*-*ptlE*, was then introduced by conjugation into the large deletion mutant SUKA16. After selection of the individual exoconjugants by neomycin-resistance, the products of preparative-scale incubations of each deletion mutant were extracted from both the supernatant and mycelium and methylated using TMS-CHN₃. The derived methyl esters were then purified by a combination of silica gel column chromatography and preparative ODS-HPLC.

GC-MS analysis of the *S. avermitilis* Δ *ptlE* mutant revealed the formation of 1-deoxy-11-oxopentalenic acid (**12**) as a minor component and two new compounds, **14** (**14-Me**; m/z 278 [M^+]) and **15** (**15-Me**; m/z 280 [M^+]) (Figure 3). Each of these major metabolites was purified from a preparative-scale culture of the Δ *ptlE* mutant, and the structures of the derived methyl esters were determined to be 1-deoxy-9 α -hydroxy-11-oxo-pentalenic acid methyl ester (**14-Me**) and 1-deoxy-9 α ,11 β -dihydroxypentalenic acid methyl ester (**15-Me**). The double deletion mutant, Δ *ptlE*- Δ *ptlD*, did not produce either **14** or **15**, yielding only 1-deoxy-11-oxopentalenic acid (**12**).

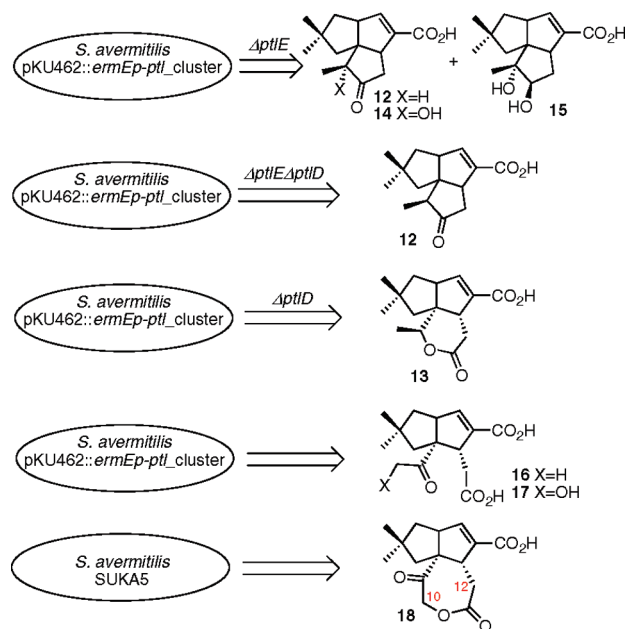


FIGURE 3: Neopentalenolactone metabolites produced by *S. avermitilis* mutants.

Although the major product of the $\Delta ptlD$ mutant displayed a molecular ion peak of m/z 278 that corresponded to that predicted for pentalenolactone D methyl ester (**5-Me**), the observed GC-MS retention time differed from that of an authentic sample of **5-Me**. NMR analysis revealed that this new metabolite was instead identical to enzymatically generated neopentalenolactone D methyl ester (**13-Me**). The structure and stereochemistry of **13-Me** was unambiguously confirmed by single crystal X-ray crystallographic analysis (Figure S9, Supporting Information).

While wild-type *S. avermitilis* had earlier appeared to produce derivatives of pentalenolactone (33), in the present study the level of production of these metabolites was significantly reduced, with only small amounts of pentalenic acid (**8**) being detected. To overcome these low titers and to try to identify the actual antibiotic end-product of the biosynthetic pathway encoded by the *S. avermitilis* *ptl* cluster, we employed two complementary experimental approaches. The first approach utilized the *S. avermitilis* chromosomal *ptl*-cluster deletion mutant (38) that had been derived from the recombinant plasmid pKU462::*ermEp-ptl* cluster and that had also been used for the construction of the various in-frame deletion mutants. Extraction and GC-MS analysis of the derived methyl esters revealed the formation of two major, antibiotically inactive, components, each of which was purified by silica gel column chromatography and preparative ODS-HPLC: the keto seco-acid methyl ester **16-Me** and hydroxyketo seco-acid methyl ester **17-Me**. The second approach utilized the *S. avermitilis* strain SUKA5 that lacked the gene clusters for the biosynthesis of the major metabolites avermectin, oligomycin, and filipin but retained the entire chromosomal *ptl* cluster. The culture filtrate was directly absorbed to ODS and fractionated by stepwise elution using 20–100% acetonitrile in water. The structure of the purified major component neopentalenoketolactone (**18**) was elucidated by ESI-MS and ^1H , ^{13}C NMR spectroscopic studies including COSY, HSQC, and HMBC (Figure 2B). The large coupling constants (18.1 and 12.2 Hz) of the methylenes at H-10 and H-12 and the ^{13}C NMR chemical shift (66.7 ppm) at C-10 indicates the presence of

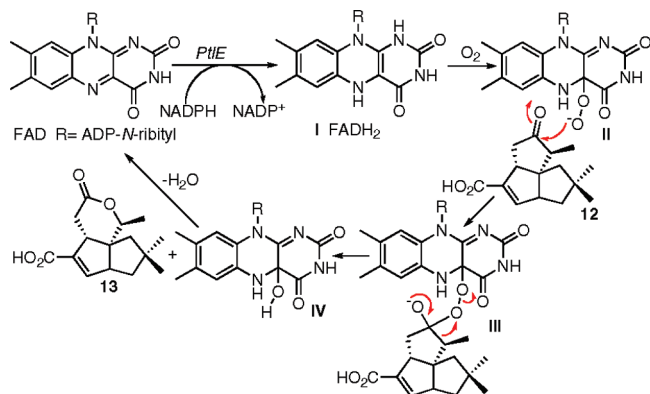
a 7-membered ketolactone ring moiety. The lactone underwent slow hydrolytic decomposition to the corresponding hydroxy-keto seco-acid **17**.

DISCUSSION

Neither neopentalenolactone D (**13**) nor any derived metabolites have previously been isolated from any pentalenolactone-producing *Streptomyces* species or from any other source. The apparent absence of pentalenolactone (**1**) or related metabolites such as **5–7** in *S. avermitilis* suggests that this organism harbors a previously undetected branch of the classical pentalenolactone family tree. (Although we previously reported the isolation from *S. avermitilis* of a compound initially thought to be pentalenolactone F (**7**) (33), we have been unable to reproduce this result.)

PtlE catalyzes the oxidative conversion of 1-deoxy-11-oxopentalenic acid (**12**) to neopentalenolactone D (**13**), the regioisomer of the predicted Baeyer–Villiger oxidation product, pentalenolactone D (**5**) (Scheme 2). The formation of neopentalenolactone D (**13**) is not an artifact of the *in vitro* reaction conditions resulting from misfolding of recombinant PtlE or the absence of some required protein or small molecule cofactors. Thus, an engineered strain of *S. avermitilis* harboring an integrated copy of the 13.4-kb *ptl* cluster from which *ptlD* had been deleted and under control of the *ermE* promoter accumulates neopentalenolactone D (Figure 3). Neither pentalenolactone D (**5**) nor any derived metabolites could be detected in the extracts of this $\Delta ptlD$ mutant strain. The corresponding $\Delta ptlE$ deletion mutant produced neither neopentalenolactone D (**13**) nor the isomeric lactone **5**, instead accumulating only 1-deoxy-11-oxopentalenic acid (**12**), the substrate for PtlE, and the closely related shunt metabolites, 1-deoxy-9 α -hydroxy-11-oxopentalenic acid (**14**) and 1-deoxy-9 α ,11 β -dihydroxypentalenic acid (**15**). The $\Delta ptlE\Delta ptlD$ double mutant strain accumulated primarily **12**, without the formation of either shunt metabolite **14** or **15**. Were there a distinct monooxygenase in *S. avermitilis* capable of converting **12** directly to pentalenolactone D (**5**), the $\Delta ptlE$ and the $\Delta ptlE\Delta ptlD$ mutants would have been expected to produce either pentalenolactone D or a derived metabolite. In fact, cyclopentanone **12** is oxidized *in vivo* by the *S. avermitilis* PtlE protein exclusively to neopentalenolactone D (**13**). In both the parent wild-type organism or the engineered strain harboring the full 13.4-kb cluster under the control of the *ermE* promoter, the intermediate neopentalenolactone D apparently undergoes further oxidation mediated by PtlD.

PtlE is a member of a large family of FAD-dependent, type I Baeyer–Villiger monooxygenases (BVMOs) that catalyze the oxidation of a ketone to an ester or lactone, with NADPH serving as the ultimate source of reducing equivalents to recycle the enzyme-bound flavin. According to the generally accepted mechanism, the first step would be the reaction of molecular oxygen with the reduced flavin FADH_2 (**I**) to give the 4a-peroxyanion **II** or the corresponding 4a-hydroperoxyflavin intermediate, which then attacks the 11-oxo substrate **12** to give the Criegee-type adduct **III** (43) (Scheme 3). Compound **III** would then undergo ring expansion and migration of the secondary methyl substituent, C-10, to give the corresponding Baeyer–Villiger lactone **13**, with concomitant formation of 4a-hydroxyflavin **IV**. Dehydration of **IV** then regenerates the FAD cofactor. In nonenzymatic Baeyer–Villiger reactions involving peracid oxidants, the more substituted alkyl substituent generally exhibits the higher migratory aptitude. Interestingly, many enzyme-catalyzed Baeyer–Villiger reactions also exhibit a preference for migration of the

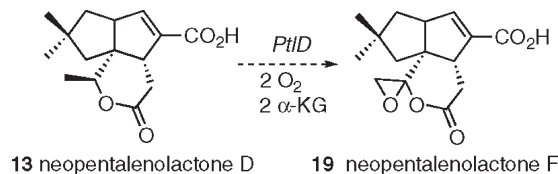
Scheme 3: PtlE-Catalyzed Baeyer–Villiger Oxidation of **12** to Neopentalenolactone D (**13**)

more electron-rich substituent. Nevertheless, there are notable exceptions. For example, racemic fused cyclobutanones undergo differential bio-oxidation whereby the antipodal substrates give rise to isomeric Baeyer–Villiger products, presumably due to differences in the active site binding of the individual enantiomers of the substrate (44, 45).

The prototype of the type I BVMO is cyclohexanone monooxygenase (CHMO, P12015) (EC 1.14.13.22) from *Acinetobacter* sp. NCIB 9871 (46). Among the numerous other members of this class are ketosteroid monooxygenase (EC 1.14.13.54) from *Rhodococcus rhodochrous* (47), cyclopentadecanone monooxygenase (CpdB) from *Pseudomonas* sp. strain HI-70 (48), and cyclododecanone monooxygenase (CddA) from *Rhodococcus ruber* strain SC1 (49). The crystal structures of phenylacetone monooxygenase (EC 1.14.13.92) (50) and cyclohexanone monooxygenase have been determined (51). In these proteins, the FAD- and NADPH-binding domains are connected by a linker carrying the highly conserved sequence motif **FXGXXXH-XXXW(D/P)** (52). In phenylacetone monooxygenase, a conserved Arg337 residue located close to the 4 α -carbon of the flavin cofactor was found to be essential for enzymatic catalysis, possibly by stabilizing the 4 α -flavin peroxide **II**. Indeed, site-directed mutagenesis of this residue eliminated monooxygenase activity (53).

Several motifs are strictly conserved in the type I Baeyer–Villiger monooxygenase family. Besides the fingerprint sequence **FXGXXXHXXXW(D/P)**, there are two typical Rossmann fold sequences, **GXGXXG** and **GTG**, for binding of the FAD cofactor and the NADPH coenzyme, respectively, and two other conserved motifs, **GG** and **DXXXXA(S/T)G** (52, 54–56).

A BLAST search using PtlE as the query reveals many other putative members of this same family of Baeyer–Villiger monooxygenases (Figure S10, Supporting Information). Thus, the 594-aa *S. avermitilis* Baeyer–Villiger monooxygenase PtlE has significant similarity to A7HSA3 (cyclohexanone monooxygenase, 59% identity, and 73% similarity) from *Parvibaculum lavamentivorans* DS-1; Q1D8E0 (53% identity and 69% similarity) from *Myxococcus xanthus* DK 1622; Q89NI1 (53% identity, 68% similarity) from *Bradyrhizobium japonicum* USDA 110; and Q1T7B5 (cyclopentadecanone monooxygenase, CpdB, 52% identity, and 68% similarity) from *Pseudomonas* sp. HI-70 (48) as well as A0AD32 (SAMR0677, 52% identity, and 66% similarity) from *S. ambifaciens* ATCC 23877; SCAB11301 (51% identity and 64% similarity) from *S. scabies* 87.22; B1 VPM8 (SGR6949, 48% identity, and 60% similarity) from *S. griseus* IFO 13350; and Q9RL17 (SCO0300, 48% identity,

Scheme 4: Proposed Oxidation of Neopentalenolactone D (**13**) to Neopentalenolactone F (**19**)

and 60% similarity) from *S. coelicolor* A3(2), all of which retain the conserved BVMO-specific motifs. Significantly, while the majority of known or likely bacterial BVMO enzymes are probably opportunistic catabolic enzymes with broad substrate specificity, PtlE is one of the few biochemically characterized members of the type I BVMO class that has been assigned a specific biosynthetic role, having both a defined substrate, 1-deoxy-11-oxopentalenic acid (**12**), and a strictly controlled reaction specificity (57, 58).

Neopentalenolactone D (**13**), which does not show any detectable antibiotic activity, lacks the epoxide pharmacophore required for the inactivation of GAPDH by classical pentalenolactones. Nonetheless, the *S. avermitilis* *neo-ptl* cluster harbors an inducible, pentalenolactone-insensitive GAPDH encoded by *sav2990*, that presumably affords self-resistance to an antibiotically active end-product of the newly discovered branch of the neopentalenolactone biosynthetic pathway. The fact that the Δ *ptlD* mutant of *S. avermitilis* accumulates neopentalenolactone D (**13**) suggests that **13** is likely to be the natural substrate for the *ptlD* gene product. Interestingly, PtlD has 35% amino acid sequence identity to a wide variety of bacterial taurine dioxygenases, TauD/TfdD, as well as a strong similarity to related members of this family of enzymes, suggesting that it is a nonheme iron, α -ketoglutarate-dependent dioxygenase. PtlD, may therefore convert neopentalenolactone D (**13**) to the as yet unidentified neopentalenolactone F (**19**) (Scheme 4). The acyloxyepoxide **19** would be expected to inhibit GAPDH by a mechanism analogous to that of pentalenolactone. Notably, the α -ketoglutarate-dependent dioxygenase hyoscyamine 6 β -hydroxylase catalyzes a related two-step epoxidation of hyoscyamine to scopolamine (59). A similar conversion of an alcohol to an epoxide in the biosynthesis of fosfomycin is also mediated by another nonheme iron-dependent hydroxylpropylphosphonate epoxidase, Fom4 (60, 61). Although we have yet to isolate neopentalenolactone F (**19**), consistent with the predicted formation of **19** is the isolation of its presumptive rearrangement product neopentalenoketolactone (**18**) and the derived hydroxy-keto seco-acid **17** as well as the abortive oxidation product **16** from engineered *S. avermitilis* strains harboring the complete 13.4 kb *neo-ptl* cluster. The proposed hydrolysis of neopentalenolactone F (**19**) to **17** is in fact preceded by the reported isolation of pentalenolactone O, the epoxide hydrate of pentalenolactone itself (30). Direct confirmation of the specific biochemical role of PtlD and the identification of the ultimate metabolite in the *S. avermitilis* neopentalenolactone biosynthetic pathway are under active investigation.

ACKNOWLEDGMENT

We thank T.-L. Shen and R. Hopson of the Department of Chemistry, Brown University for assistance with GC-MS and NMR analysis, respectively, and Z. You of Brown University for helpful advice. We thank the *E. coli* Genetic Stock Center at Yale University for distribution of *E. coli* BW25113 and pKD46, and

I. Saito of The Institute for Medical Sciences, The University of Tokyo for distribution of pULwL (*loxP* resource). We also thank T. Hirose, Kitasato Institute for Life Sciences, Kitasato University for measuring the X-ray crystallographic data of neopentalenolactone D methyl ester.

SUPPORTING INFORMATION AVAILABLE

SDS–PAGE of recombinant PtlE, MS and NMR spectra, and ORTEP figure for neopentalenolactone D, and CLUSTALW alignment of PtlE and Baeyer–Villiger monooxygenases. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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