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Targeted Rediscovery and Biosynthesis of the Farnesyl-Transferase Inhibitor Pepticinnamin E

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Dedicated to Prof. Christopher T. Walsh on the occasion of his 75th birthday.

The natural product pepticinnamin E potently inhibits protein farnesyl transferases and has potential applications in treating cancer and malaria. Pepticinnamin E contains a rare N-terminal cinnamoyl moiety as well as several nonproteinogenic amino acids, including the unusual 2-chloro-3-hydroxy-4-methoxy-N-methyl-L-phenylalanine. The biosynthesis of pepticinnamin E has remained uncharacterized because its original producing strain is no longer available. Here we identified a gene cluster (*pcm*) for this natural product in a new producer, *Actinobacteria bacterium* OK006, by means of a targeted rediscovery strategy. We demonstrated that the *pcm* cluster is responsible for the biosynthesis of pepticinnamin E, a nonribosomal peptide/polyketide hybrid. We also characterized a key *O*-methyltransferase that modifies 3,4-dihydroxy-L-phenylalanine. Our work has identified the gene cluster for pepticinnamins for the first time and sets the stage for elucidating the unique chemistry required for biosynthesis.

Living organisms synthesize an astounding number of natural products, many of which have important uses in medicine and biotechnology. Although tens of thousands of biosynthetic gene clusters have been identified from the genomes of bacteria, relatively few have been characterized in terms of the natural products they produce. Additionally, although many bioactive natural products have been isolated, their biosynthetic gene clusters remain undetermined. Examples include the neurotoxin tetrodotoxin,[1] the psychoactive drug ibotenic acid,[2] and the anticancer agent acivicin,[3] isolated from marine animals, mushrooms, and bacteria, respectively. The lack of knowledge relating to the biosynthesis of these natural products limits the ability to study and engineer these compounds.

The pepticinnamins were discovered in 1992 as farnesyl-transferase inhibitors.[4] Farnesyl-transferase inhibitors have been explored both for cancer therapy and as potential treatments for malaria and trypanosomiasis.[5] Six pepticinnamin variants, A–F, have been isolated; among them, pepticinnamin E is the most abundant and thoroughly characterized. Pepticinnamin E is composed of a cinnamoyl tail, a highly modified tripeptide core, and an ester-linked diketopiperazine unit (Scheme 1).[6] The tripeptide core contains three nonproteinogenic aromatic amino acids: D-tyrosine, *N*-methyl-2-chloro-4-*O*-methyl-3,4-dihydroxy-L-phenylalanine (*N*-Me-2-Cl-4-*O*-Me-L-DOPA), and *N*-methyl-L-phenylalanine (*N*-Me-L-Phe). The diketopiperazine unit consists of a D-serine and a glycine. Total synthesis of pepticinnamin E and its epimer revealed that the second amino acid in the natural product possesses *S* stereochemistry and is *N*-Me-2-Cl-4-*O*-Me-L-DOPA.[7] Mode of action studies revealed that pepticinnamin E acts as a natural bisubstrate inhibitor of farnesyl transferases and several analogues induce apoptosis in tumor cells.[8]

The unique structure of pepticinnamin E suggests that its biosynthesis requires unusual enzymatic chemistry. The 2-Cl-4-*O*-Me-L-DOPA component, for example, is a rare nonproteinogenic amino acid in nonribosomal peptides. Diketopiperazine formation is an unusual strategy for release of peptide natural products from nonribosomal peptide synthetases (NRPSs). Identifying the biosynthetic gene cluster for a given natural product generally requires access to the producing microbe or the genome sequence of the producer. However, the original strain from which pepticinnamin was isolated—*Streptomyces* sp. OH-4652—has not been sequenced and is not readily accessible, thus posing a significant challenge for studying the biosynthesis of pepticinnamin E.

To overcome this challenge, we set out to identify new pepticinnamin producers by bioinformatics. Many natural products have been identified from more than one bacterial strain, due to horizontal gene transfer of their biosynthetic gene clusters. This phenomenon has caused substantial natural product rediscovery problems.[9] We reasoned, though, that we could take advantage of this phenomenon and of the growing microbial genome databases to identify the biosynthetic gene clusters for natural products for which the original producers are unavailable. Using this targeted rediscovery strategy, we identified a new bacterial producer for pepticinnamin E—*Actinobacteria bacterium* OK006—and determined the biosynthetic gene cluster responsible for its biosynthesis. Additionally, we reconstituted the activity of the methyltransferase responsible for the formation of 2-Cl-4-*O*-Me-L-DOPA and characterized the substrate scope of this enzyme.

Analysis of the pepticinnamin E structure revealed that it possesses an N-terminal cinnamoyl lipid moiety similar to that present in the natural product skyllamycin A and identical to that found in the natural product WS9326A (Scheme 1).[10] The biosynthetic gene clusters for skyllamycin A and WS9326A have been identified and are both polyketide synthase (PKS)-

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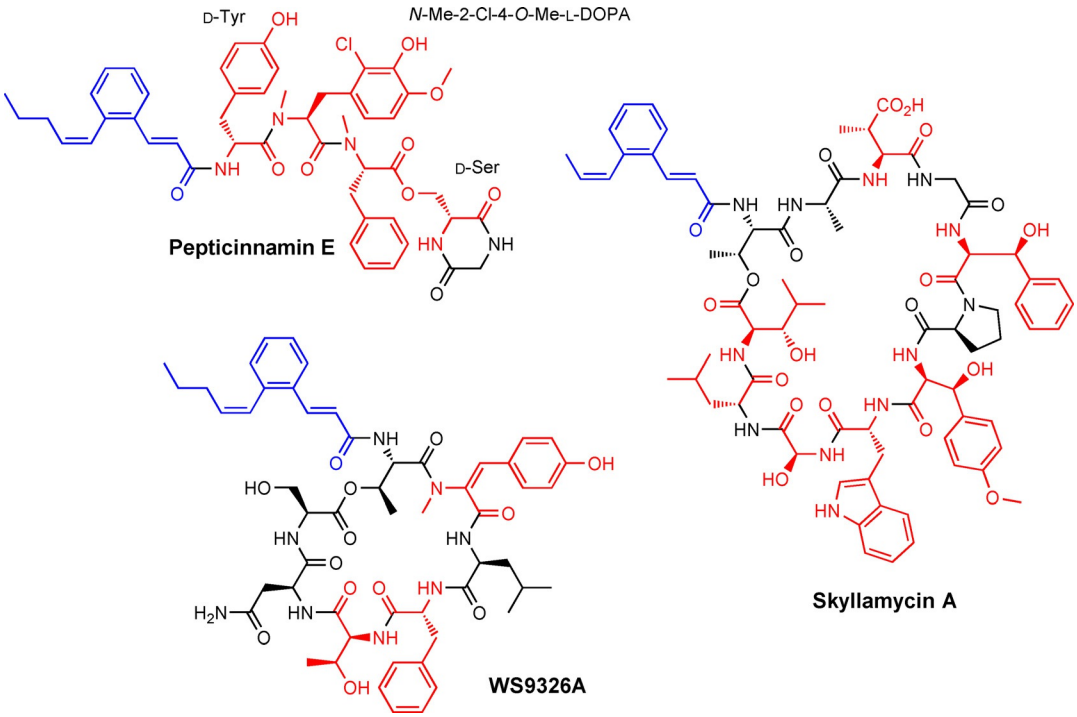
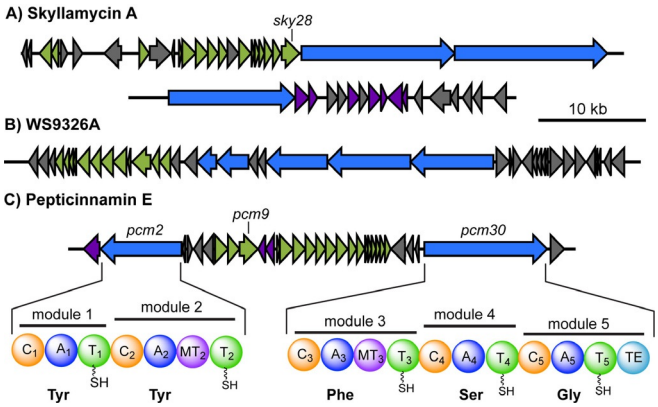


Scheme 1. Structures of PKS-NRPS hybrid natural products containing cinnamoyl moieties. Cinnamoyl moieties are highlighted in blue, and nonproteinogenic amino acids in red.

NRPS hybrids (Scheme 2A and B).[11] It has been proposed that the formation of the benzene ring in the cinnamoyl moiety of skyllamycin A is catalyzed either by the putative oxidoreductase Sky4 or by the phytoene-dehydrogenase-like enzyme Sky28.[11a] Therefore, we used Sky28 as a biosynthetic "hook" to identify gene clusters that might encode enzymes for the synthesis of the cinnamoyl moiety. Specifically, Sky28 (GenBank accession No. AEA30271.1) was used as a query for a BLASTP search against the Joint Genomic Institute's Integrated Microbial Genome Database. The top 500 hits were inspected for proximity of NRPS genes, and further analyzed by antiSMASH 3.0 and MultiGeneBlast against the MiBiG database.[12]

Bo Li earned her Ph.D. in biochemistry from the University of Illinois at Urbana-Champaign in 2009. As a graduate student, she worked with Prof. Wilfred van der Donk, studying the enzymatic transformations responsible for making lanthionine-containing peptides. She conducted postdoctoral research in the laboratory of Prof. Christopher T. Walsh at Harvard Medical School as a Jane Coffin Childs Fellow, characterizing the biosynthesis of disulfide-containing natural products. She joined the faculty of the Department of Chemistry at UNC in 2013. Li's group integrates chemistry and bacterial genomics to identify new antimicrobial natural products, biosynthetic pathways, and new mechanisms to overcome antibiotic resistance.

This analysis enabled us to identify a putative 45 kb gene cluster for pepticinnamin biosynthesis—*pcm*—in *A. bacterium* OK006, a *Streptomyces* strain isolated from the root endosphere of a poplar tree.[13] This cluster encodes a gene product—Pcm9—that is 58% identical to Sky28 (Scheme 2C). It also harbors a number of NRPS and PKS genes. The core PKS genes in this cluster share high sequence identity with those in the clusters for skyllamycin and WS9326 that are involved in



Scheme 2. Identification of a candidate biosynthetic gene cluster for pepticinnamin E by bioinformatics. By using signature genes for the cinnamoyl moieties in the gene clusters for A) skyllamycin (*sky28*), and B) WS9326A, the C) pepticinnamin gene cluster was identified that contains *pcm9*, a *sky28* homologue. Genes are colored by function as follows: NRPS in blue, PKS in green, tailoring enzymes in purple, and others in gray. Predicted domain structure of the NRPS genes in the *pcm* cluster, *pcm2* and *pcm30*, is shown in (C). C: condensation. A: adenylation. T: thiolation or peptidyl carrier protein (PCP). MT: *N*-methyltransferase. TE: thioesterase.

biosynthesis of the cinnamoyl moiety (Scheme 2C, Table S1 in the Supporting Information).

We conducted further analysis of the two NRPSs—Pcm2 and Pcm30—in this cluster using NRPSpredictor2,[14] and identified a total of five modules: two in Pcm2 and three in Pcm30. Substrate prediction based on bioinformatics suggested that the adenylation (A) domains in modules 1–5 prefer tyrosine, tyrosine, phenylalanine, serine, and glycine, respectively (Scheme 2C, Table S2 in the Supporting Information). The predicted substrate preference matches the composition of the core peptide in pepticinnamin E. Additionally, module 2 in Pcm2 and module 3 in Pcm30 each contain a methyltransferase domain (Scheme 2C), consistent with the *N*-methyl groups present in the peptide backbone of pepticinnamin E at the second and third amino acids.

We also identified three potential tailoring enzymes for the formation of 2-Cl-4-*O*-Me-L-DOPA in pepticinnamin E: Pcm1 (a flavin-dependent halogenase), Pcm11 (a biopterin-dependent hydroxylase), and Pcm10 [a methyltransferase dependent on *S*-adenosyl methionine (SAM)]. These enzymes are of particular interest because halogenation of aromatic residues typically occurs at the 3- and 4-postions rather than at the 2-position as found in 2-Cl-4-*O*-Me-L-DOPA. Additionally, hydroxylations in bacterial nonribosomal peptide pathways are often catalyzed by cytochrome P450 or non-heme iron/α-ketoglutarate-dependent enzymes;[15] biopterin-dependent hydroxylases, essential for the metabolism of aromatic amino acids and the synthesis of neurotransmitters in mammals, are relatively rare in bacteria.[16] On the basis of these bioinformatic evidence, we hypothesized that this cluster encodes biosynthetic enzymes for pepticinnamin production. We set out to test this hypothesis and to characterize the unusual tailoring enzymes.

Having identified a candidate biosynthetic gene cluster for pepticinnamin E from *A. bacterium* OK006, we first examined the ability of this strain to produce pepticinnamin E. The bacterium was cultured in different media to elicit pepticinnamin production, and the resulting cell pellets were extracted with methanol. The extracts were then analyzed by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) analysis. We identified peaks with mass-to-charge ratios (*m/z*) of 908.356 and 930.345, corresponding to $[M+H]^+$ and $[M+Na]^+$ adducts, respectively, of pepticinnamin E. In-source mass fragmentation yielded product ions with *m/z* of 603.223 and 547.191, which correspond to the *b* and *y* ions, respectively, at the 2-Cl-4-*O*-Me-L-DOPA residue (Figures 1A and S1). ^{1}H NMR analysis of isolated pepticinnamin E also supports the reported structure (Figure S2 and Table S3), but we did not determine the stereochemistry of the isolated product. These results demonstrated that *A. bacterium* OK006 is a new producer of pepticinnamin E.

To validate the hypothesis that the *pcm* cluster is responsible for the biosynthesis of pepticinnamin E, we disrupted *pcm2* by generating insertional mutants, each with a single-crossover apramycin resistance cassette. Attempts at double-crossover mutants were unsuccessful. Growth and extraction of the mutant strains under the same conditions as used for the wild-type bacterium yielded no pepticinnamin E detectable by UV

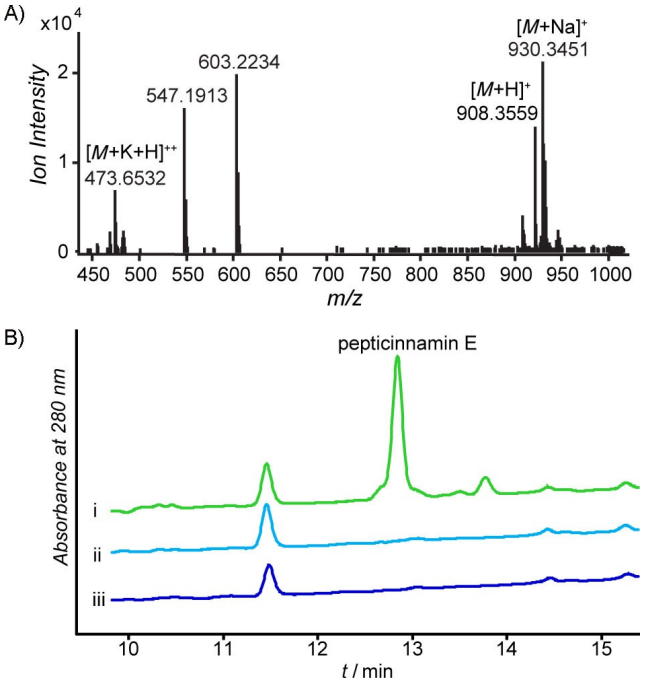
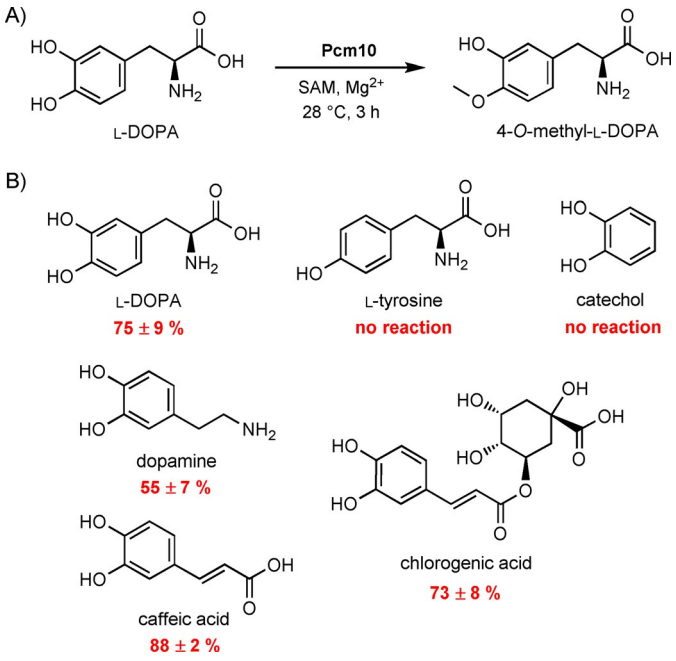


Figure 1. The *pcm* cluster is responsible for pepticinnamin E biosynthesis. A) High-resolution mass spectrum of pepticinnamin E in the culture extract of *A. bacterium* OK006. In-source fragmentation ions were also observed (Figure S1). B) Pepticinnamin E is produced by wild-type *A. bacterium* OK006, but not by the *pcm2* insertional mutants. The UV traces at $A_{280\,nm}$ of cell extracts are shown for i) wild-type *A. bacterium* OK006 and ii), iii) two independent *pcm2::apr*^{R} mutant strains.

absorption or MS (Figure 1B). This result confirms that *pcm* is the biosynthetic gene cluster for pepticinnamin E.

Pcm10 shares 58% sequence identity with SafC, a characterized catechol 4-*O*-methyltransferase involved in the biosynthesis of saframycin MX1.[17] Therefore, we hypothesized that Pcm10 might catalyze *O*-methylation of L-DOPA and of derivatives of L-DOPA. To characterize the function of Pcm10, we cloned, expressed, and purified a His-tagged version in *E. coli*. The activity of Pcm10 was reconstituted in a buffered solution containing SAM and L-DOPA at 28 °C. A methylated L-DOPA product was detected by LC-HRMS, and its production was abolished in assay mixtures containing boiled Pcm10. This result indicated that Pcm10 converts L-DOPA into methylated L-DOPA. To identify the position of methylation, the methylated L-DOPA product from a 10 mL overnight Pcm10 reaction was purified and subjected to ^{1}H NMR analysis (Figure S3). The ^{1}H shifts are consistent with those reported for 4-methyl-L-DOPA,[18] but differ from those of 3-methyl-L-DOPA.[19] This result indicated that Pcm10 methylates L-DOPA at the 4-hydroxy position, consistent with the structure of pepticinnamin E. No methyltransfer activity was observed when Pcm10 was incubated with L-tyrosine, thus suggesting that hydroxylation of L-tyrosine occurs prior to 4-methylation.

To probe the substrate scope of Pcm10, we tested catechol and its derivatives, including dopamine, caffeic acid, and chlorogenic acid, as substrates for Pcm10. Pcm10 did not modify catechol, but converted ≈50% of dopamine and more than 70% of caffeic acid and chlorogenic acid into their methy-



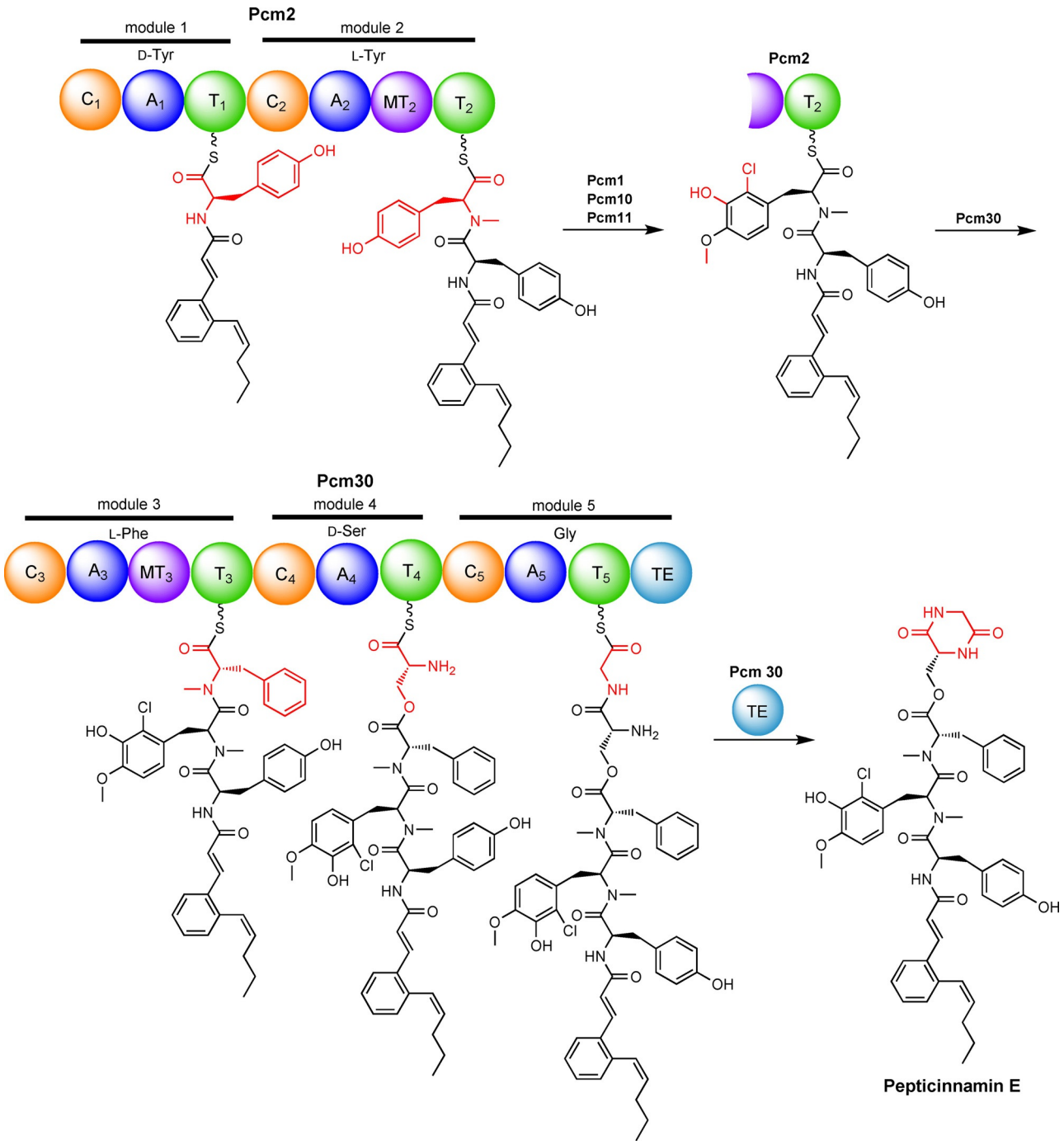
Scheme 3. Pcm10 is a promiscuous *O*-methyltransferase that modifies catechol derivatives. A) Pcm10 regioselectively *O*-methylates L-DOPA at the 4-hydroxy position. B) Pcm10 converts a variety of catechol derivatives into their *O*-methylated products.

lated products during the incubation period (Scheme 3, Figures S4 and S5). The activity of Pcm10 toward these substrates is comparable with that towards L-DOPA as a substrate under the same conditions. The regiochemistry of the methylated products in the cases of dopamine, caffeic acid, or chlorogenic acid was not determined. The shapes of the product peaks for these non-native substrates suggest that Pcm10 may not be regioselective toward these substrates (Figure S4). These results indicate that Pcm10 is a promiscuous methyltransferase of modified catechols. SafC can also methylate catechol derivatives and is regioselective for L-DOPA, but not the non-native substrates.[17] These observations are explained by a recent structural study that revealed the active site of SafC to be plastic; it can accommodate a variety of catechol substrates, and the regioselectivity of SafC is largely substrate-dependent.[20]

We conducted additional bioinformatic analyses to characterize the timing of hydroxylation and other tailoring reactions in the generation of 2-Cl-4-*O*-Me-L-DOPA. The amino acid precursor is likely activated by the second A domain of Pcm2 (Pcm2-A2). Pcm2-A2 shares 56% sequence identity with the previous A domain (Pcm2-A1) that likely activates Tyr1 of pepticinnamin E. It also possesses a 10-residue specificity code nearly identical to that in Pcm2-A1 and another Tyr-activating A domain in the NRPS NosD,[21] suggesting that L-Tyr is the preferred substrate for Pcm2-A2. Further, phylogenetic analysis of Pcm1 revealed that it groups with flavin-dependent halogenases SgcC3 and BhaA,[22] which prefer thiolation-domain (T)-tethered substrates, rather than with those that prefer free substrates (Figure S6). Lastly, the promiscuity of Pcm10 toward modified catechols suggests that methylation through the action of Pcm10 could occur with substrates (L-DOPA or 2-chloro-L-DOPA) loaded onto Pcm2 as T-tethered thioesters. Therefore, in the biosynthesis of 2-Cl-4-*O*-Me-L-DOPA, L-Tyr is likely first loaded onto the second T domain of Pcm2, followed by hydroxylation through the action of Pcm11, chlorination through that of Pcm1, and *O*-methylation through that of Pcm10. Modification of T-domain-tethered substrates has also been reported in many NRPS biosynthetic pathways.[23] This strategy might control the flux of the amino acid precursor into natural product biosynthesis and reduce the formation of unnecessary intermediates.

From our experimental results and bioinformatic analyses, we propose a pathway for pepticinnamin E biosynthesis (Scheme 4). Each module is responsible for the activation and loading of the corresponding amino acid, condensation with the downstream amino acid, and methylation of the amide NH group in the cases of the second and third amino acids. Besides the canonical NRPS chemistry, a few domains in the pathway may catalyze unusual chemistry. Two out of the five amino acids in pepticinnamin E—Tyr1 and Ser4—exhibit D stereochemistry. Although D-amino acids in nonribosomal peptides often results from the epimerase activity of condensation (C) domains, the C domains in the *pcm* pathway exhibit no significant similarity to those with dual epimerase/condensation functions (Figure S7). In fact, phylogenetic analysis revealed that C domains in modules 2–5 all group with LCL domains that catalyze amide formation between two L-configured amino acids, whereas the first C domain groups with starter domains (Figure S7). Additionally, *N*-Me-Phe3 and Ser4 are connected through an ester linkage. A standalone C domain, SgcC5, in the C-1027 gene cluster has been shown to catalyze ester bond formation between the benzoxazolinate and the enediyne core.[24] Other C domains have also been associated with nonribosomal peptide chain extension through ester bonds in the pathways for kutzneride,[25] valinomycin,[26] and cereulide.[27] Thus, the fourth C domain (C4) in Pcm30 may directly catalyze ester bond formation or facilitate the rearrangement from an amide to an ester. However, like in the case of SgcC5, no significant changes in the conserved residues are observed in that of Pcm30-C4. The ester linkage allows the amine moiety of Ser4 to condense with Gly5, forming the C-terminal diketopiperazine. Diketopiperazine formation may be catalyzed by the terminal thioesterase (TE) domain of Pcm30. However, sequence analysis of Pcm30-TE identified the conserved GxSxG motif but did not suggest any unusual features. Further, phylogenetic analysis revealed no distinct grouping with any subtypes of TE domains (Figure S8). The functions and mechanisms of the C and TE domains in the pepticinnamin pathway remain to be characterized.

In summary, we have employed a bioinformatic approach to identify the biosynthetic gene cluster of pepticinnamin E and to discover a new producing bacterium for this natural product. We also identified and characterized an *O*-methyltransferase enzyme in the biosynthesis of the rare nonproteinogenic amino acid 2-Cl-4-*O*-Me-L-DOPA and demonstrated that this enzyme is promiscuous toward a variety of catechol derivatives. Our work sets the stage for elucidating the biosynthetic chemistry of pepticinnamin E, especially the timing and mech-



Scheme 4. Proposed biosynthetic pathway to pepticinnamin E.

anisms of biopterin-dependent hydroxylation, chlorination, and diketopiperazine formation.

Experimental Section

Cultivation of *A. bacterium* OK006 and extraction of pepticinnamin E: *A. bacterium* OK006 was cultivated on ISP Medium No. 4 (ISP4) plates (BD Difco) at 28 °C. Liquid cultures were grown in tryptic soy broth (TSB, BD Difco) at 28 °C for 24 h with aeration. For production of pepticinnamin E, a sample of this TSB liquid culture (1 mL) was used to inoculate pepticinnamin production medium [D-glucose (20 g L^{-1}), asparagine (10 g L^{-1}), magnesium sulfate heptahydrate (0.2 g L^{-1}), potassium phosphate dibasic (anhydrous, 0.5 g L^{-1}), iron(II) sulfate heptahydrate (0.02 g L^{-1}), sodium chloride (5.84 g L^{-1}), 200 mL], and grown for five days at 28 °C with shaking at 250 rpm. To extract pepticinnamin E, cultures were centrifuged at 4000 *g* for 1 h to harvest bacterial cells. The cell pellets were re-suspended in methanol and gently rocked for 2 h. After centrifugation at 4500 *g* for 30 min, the methanol extract was filtered through 0.2 μm syringe filters and concentrated to dryness under reduced pressure. The resulting extracts were dissolved in methanol for analysis by LC-HRMS. For LC-HRMS analysis, a sample (10 μL) was analyzed with an Agilent Technologies 1260 Infinity II

LC system coupled to an Agilent Technologies 6520 accurate-mass Q-TOF instrument. Samples were separated on a Gemini NX-C_{18} column (Phenomenex, 110 Å, 5 μm, 50 mm × 2 mm) at a flow rate of 0.4 mL min^{-1} over a linear gradient of 2 to 98% acetonitrile containing 0.1% formic acid over 20 min. Electrospray ionization mass spectrometry (ESI-MS) was conducted in positive ion mode with the following parameters: gas temperature 350 °C, drying gas 12 L min^{-1}, nebulizer 50 lb in^{-2}, fragmentor 175 V, skimmer 65 V, capillary cap 3500 V, octopole RF 750 V.

Reconstitution of Pcm10 methyltransferase activity and analysis by LC-HRMS: The activity of Pcm10 was reconstituted as follows. Each 100 μL reaction mixture contained Tris (pH 8.3, 50 mM), NaCl (200 mM), $MgCl_2$ (1 mM), SAM (1.6 mM), and substrate (1 mM). Reactions were initiated by addition of Pcm (104 μL) to a final concentration of 9.2 μM, and the mixtures were incubated for 1.5 h at 28 °C and 750 rpm and quenched by the addition of 1 volume of trichloroacetic acid (10%) to precipitate proteins in the assay. Reaction mixtures were stored at −20 °C prior to LC-HRMS analysis, at which point the precipitated proteins were removed by centrifugation at 21 700 *g* for 5 min. The supernatant was diluted 1:2 in H_2O, and the diluted sample (10 μL) was analyzed with an Agilent Technologies 6520 Accurate-Mass Q-TOF LC-HRMS. Samples were separated on a Kinetex C_{18} column (Phenomenex, 100 Å, 5 μm, 150 mm × 4.6 mm) at a flow rate of 0.4 mL min^{-1} over a linear gradient of 5 to 95% acetonitrile containing 0.1% formic acid over 25 min. ESI-MS was conducted in positive ion mode with the following parameters: gas temperature 350 °C, drying gas 12 L min^{-1}, nebulizer 50 lb in^{-2}, fragmentor 175 V, skimmer 65 V, capillary cap 3500 V, octopole RF 750 V. Assay mixtures containing catechol as a substrate were analyzed by ESI-MS in negative ion mode with the following parameters: gas temperature 350 °C, drying gas 5 L min^{-1}, nebulizer 20 lb in^{-2}, fragmentor 175 V, skimmer 65 V, capillary cap 3500 V, octopole RF 750 V.

Analysis of the substrate scope of Pcm10 was performed under the same conditions with the different substrates (1 mM). After 3 h incubation, the reaction mixtures were quenched with 1 volume of trichloroacetic acid (10%) and stored at −20 °C. Prior to HPLC analysis, samples were centrifuged at 21 000 *g* for 5 min to remove the protein precipitants and neutralized with NaOH solution. A sample (20 μL) was injected onto a Kinetex C_{18} column (Phenomenex, 100 Å, 5 μm, 150 mm × 4.6 mm) and analyzed by Shimadzu analytical HPLC with use of a gradient of 2.5→20% solvent B over 10 min for L-DOPA and L-Tyr, and a gradient of 2.5→50% solvent B over 10 min for catechol, caffeic acid, chlorogenic acid, and dopamine (solvent A: water containing 0.1% trifluoroacetic acid; solvent B: acetonitrile containing 0.1% trifluoroacetic acid). Substrate consumption and product formation in the reaction was calculated from the integrated peak areas of UV absorbance at 280 nm at specific retention times.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioinformatics · genome mining · natural products · nonribosomal peptides · transferases

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