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Supporting Information

Biosynthesis of the Fungal Nonribosomal Peptide Penilumamide A and Biochemical Characterization of a Pterin-Specific Adenylation Domain

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Experimental Methods

Strains

The fungal strain *Aspergillus flavipes* CNL-338 was obtained from Professor William Fenical at Scripps Institution of Oceanography. The strain was isolated as an endophyte from a red alga *Laurencia* sp. collected in the Bahamas[1]. Originally classified as a *Penicillium* sp., the strain identification was corrected upon morphological observation and fungal ITS sequence comparison (Accession number MT579592). *E. coli* BL21(DE3) was used to express the NRPS A domains from *plm*A, *plm*J and *plm*K. *Saccharomyces cerevisiae* strain BJ5464-NpgA (*MATα ura3-52 trp1 leu2-Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*)[2] was used to reconstitute and express *plm*A, *plm*J and *plm*K. This yeast strain contains the constitutive gene *npg*A, encoding a phosphopantetheine transferase from *Aspergillus nidulans,* integrated into the genome.[3]

General molecular biology procedures

PCR reactions were carried out using AccuPrime Taq DNA polymerase (Invitrogen) and Phusion DNA polymerase (New England Biolabs). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA) (Table S1). pCR®-blunt (Invitrogen) was used to construct recombinant DNA products, and DNA sequencing was performed by GeneWiz (South Plainfield, NJ, USA). DNA manipulation using standard techniques was performed in *E. coli* TOP10 (Invitrogen).

Chemicals and spectroscopic procedures

All solvents were purchased as HPLC grade or higher from Fisher Scientific (Waltham, MA, USA). Reverse phase LC-MS was performed using an Acquity Arc UHPLC/MS (Waters, Milford, MA, USA) in positive mode electrospray ionization with a Waters XBridge BEH C18 column (2.1 mm x 100 mm, 2.5 μm) fitted with the appropriate Waters VanGuard filter cartridge. ESI-HR TOF-MS was performed on an Eksigent Ekspert™ nanoLC 425 chromatography system connected to a Bruker maXis II™ ETD QToF mass spectrometer fitted with an Eksigent C18 column (75 μm x 15 cm, 5 μm). The HRMS method used a gradient of 5–95% MeCN with 0.1% formic acid:H_2O with 0.1% formic acid over 33 minutes followed by 95% MeCN with 0.1% formic acid for 10 minutes at a flow rate of 10 μL/min.

Fermentation conditions

For genomic DNA extraction, wild-type *A. flavipes* CNL-338 and the Δ*plm*A mutant were cultured in 10 mL YPM media (0.2% yeast extract, 0.2% peptone, 0.4% mannitol) with 3.3% artificial sea salt (Instant Ocean, USA) in 10 x 35 mm petri dishes for 4 days at 30 °C under static conditions. For the Δ*plm*A mutant, 300 μg/mL of zeocin was added for antibiotic selection. For chemotype analysis between wild-type *A. flavipes* CNL-338 and the Δ*plm*A mutant, cultures were grown in 5 mL of YPM media with or without zeocin in 10 mL borosilicate glass tubes at 30 °C for 10 days at 170 rpm. For the isolation of a penilumamide A standard, wild-type CNL-338 was grown in 2 x 1 L of YPM media (0.2% yeast extract, 0.2% peptone, 0.4% mannitol) with 3.3% artificial sea salt (Instant Ocean, USA) for 10 days at 30 °C and 180 rpm.

*Isolation of penilumamide A (**1**)*

Whole cell extraction of the 2 x 1 L cultures was performed by partitioning with a 1:1 volume of ethyl acetate three times and drying *in vacuo* to obtain the crude extract (300 mg). The crude extract was resuspended in 500 μL methanol before semi-preparative HPLC purification using a mobile phase gradient of MeOH:H_2O with 0.1% TFA (30-100% methanol over 30 minutes, 4 mL/min) on a reverse phase Phenomenex Luna 5 μm C18 column (100 Å, 250 x 10 mm). Fractions containing **1** were pooled and concentrated before a second round of semi-preparative HPLC using isocratic 55% methanol (4 mL/min) on the same column used previously to yield 6.3 mg of pure **1**. The identity of **1** was confirmed by UV/Vis λ_{max} values, ^{1}H NMR, and ESI-HR TOF-MS data (Table S2, Figures S1 and S2).

Isolation of fungal genomic DNA

Fungal cells were collected from the static culture into sterile 1.5 mL microcentrifuge tubes and lyophilized overnight. Cells were then broken into a fine powder by mechanical crushing. Lysis buffer (700 μL) (10 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5% SDS, 0.1 M LiCl) was added to each sample and inverted to afford a slurry, which was left at room temperature for 5 minutes. Phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0 (700 μL)

was added to each sample, inverted to mix, and allowed to sit at room temperature for five minutes. Samples were centrifuged to remove cell debris at 21,000 x g for 10 minutes at 4 °C. The aqueous top layer was transferred to a fresh microcentrifuge tube, where 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0 was added. Samples were centrifuged again, as described above, and the aqueous top layer was again transferred to a new microcentrifuge tube. Ethanol (1 mL of 95%) was added, tubes were inverted gently for five minutes at room temperature and centrifuged again. The supernatant was aspirated and 400 μL of 70% ethanol was added to wash the pellet. After incubation at room temperature for five minutes, the samples were centrifuged at 21,000 x g for 2 minutes at room temperature. The supernatant was aspirated and pellets were dried at room temperature for ~15 minutes. Pellets were resuspended in 50 μL 10 mM Tris-HCl pH 8.0 and treated with RNase A (0.5 mg/mL) by incubating at 50 °C for 30 minutes. Genomic DNA (gDNA) was stored at 4 °C until needed.

Genome sequencing, assembly, and mining

The genome of *A. flavipes* CNL-338 was sequenced at the High-Throughput Genomics Core at the Huntsman Cancer Institute at the University of Utah. A 180 bp PCR-free DNA library was constructed and sequenced using Illumina HiSeq (125 cycle paired-end) resulting in >250X coverage. Genome assembly was performed on the FutureSystems server using the SOAPdenovo (k-mer = 89) and IDBA-UD (k-mer = iterative) software packages[4,5]. Assembly of the 33 Mbp genome resulted in 1,899 contigs with an N_{50} value of 109,851 bp. Automated annotation was carried out using antiSMASH software[6], which revealed 51 biosynthetic clusters, of which 23 are predicted to be nonribosomal peptide-related. The assembled genome was also used to create a local BLAST database using Blast+ software (NCBI-BLAST®), and manual annotation of each putative NRPS-related gene cluster confirmed the presence of a single cluster containing three nonribosomal peptide synthetases, comprising four modules, as well as eight genes dedicated to pterin/lumazine biosynthesis. This resulting *plm* biosynthetic gene cluster was annotated and submitted to GenBank (Accession number ON2974468) (Figure 2A and Table S3).

Construction of gene inactivation cassette for plmA

To confirm the *plm* cluster's involvement in penilumamide A biosynthesis, the NRPS encoding *plm*A was targeted for gene inactivation using a disruption cassette (Figure S3). The zeocin resistance gene *Shble* and the constitutive tryptophan promoter PtrpC were designed by fusion PCR to disrupt *plm*A by antisense insertion[7]. The knockout cassette was constructed by attaching 2 kb of flanking homologous DNA located upstream and downstream of the targeted gene to the resistance marker. For construction of the *plm*A cassette, primer pairs plmA_KO_P1 and plmA_KO_P3 were used for the upstream region, while plmA_KO_P4 and plmA_KO_P6 were used for the downstream region (Table S1). The disruption cassette for inactivation of *plm*A was ligated into pCR®-blunt plasmid at 16 °C overnight and used to transform *E. coli* TOP10. Its identity was confirmed by restriction enzyme digestion and DNA sequencing, and the cassette was amplified from pCR®-blunt using primers plmA_KO_P2 and plmA_KO_P5.

Transformation and verification of disruption cassette in A. flavipes CNL-338

A. flavipes CNL-338 protoplasts were transformed with linearized inactivation cassette (10 μg) as described previously[8,9]. Transformants were grown on stabilized minimal agar medium (1.2 M sorbitol, 1.5% agar, 1% dextrose, 5% nitrate salts, 0.1% trace elements) supplemented with 300 μg/mL zeocin. To confirm correct integration of the disruption cassette into the genome, gDNA was extracted and used as a template for PCR using primer pairs plmA_KO_P0 with PtrpC_R and zeocin_F with plmA_KO_P7 (Figure S3). These pieces both use an internal primer that anneals to either the promoter (PtrpC_R) or the resistance gene (zeocin_F) and an external primer that anneals either upstream or downstream of the region that was manipulated (Table S1). This effectively verifies not only the presence of the cassette in the mutant strain, but also the genomic location in the cluster. Wild-type (WT) *A. flavipes* CNL-338 gDNA was used as a control.

LC-MS analysis of the ΔplmA mutant

The Δ*plm*A *A. flavipes* CNL-338 mutant strain was cultured in 5 mL of YPM media with or without zeocin in 10 mL test tubes for 10 days at 170 rpm and extracted with two rounds of 1:1 volume ethyl acetate. Crude extracts were dried on a rotary evaporator, and the pellet was resuspended in 100 μL methanol. Extracts of the Δ*plm*A mutant were analyzed by LC-MS with a linear gradient of 5–95% MeCN:H_2O with 0.1% formic acid over

5 minutes followed by 95% MeCN for 1 minute at a flow rate of 0.6 mL/min. Mass data of the Δ*plm*A strain was compared by extracted ion chromatogram (EIC) to the wild-type *A. flavipes* CNL-338 as well as an authentic standard of penilumamide A (Figure 2B).

Cloning, expression and purification of Plm NRPSs in yeast

*plm*A was predicted to contain two introns and thus was cloned in three overlapping pieces for intron-free reconstitution (4038 bp). Piece two (2154 bp) did not contain any introns and was amplified from gDNA using primers PlmA_F2 and PlmA_R2. Piece one (1116 bp) and piece three (1053 bp) were amplified from cDNA to remove predicted introns. RNA was extracted from a 3-day old YPM culture of WT *A. flavipes* CNL-338 using the RiboPure Yeast kit (Ambion). The manufacturer's instructions were followed except contaminating gDNA was digested with DNase (2 U/μL) (Invitrogen) at 37 °C for 4 hours. cDNA was synthesized from total RNA for pieces one and three using SuperScript II Reverse Transcriptase (Invitrogen) with the reverse primers oligo_dT and PlmA_R3, respectively. The cDNA was used as a PCR template to amplify pieces one and three using primer pairs PlmA_F1 with PlmA_R1 and PlmA_F3 with PlmA_R3, respectively. All pieces were subcloned into pCR®-blunt (Invitrogen) for sequence verification. Piece two was re-amplified from the pCR-blunt construct with the same primer pairs as mentioned above, whereas pieces one and three were amplified using primer pairs PlmA_F1 with PlmA_yeast_R and PlmA_yeast_F with PlmA_R3, respectively, so that the amplified DNA contained the necessary overhangs for yeast recombination. A 2μ expression vector was linearized with PmlI and NdeI overnight at 37 °C. *S. cerevisiae* BJ5464-NpgA was co-transformed with the three intron-free pieces of *plm*A and the linear vector using the *S. c.* EasyComp™ Transformation Kit (Invitrogen) (Figure S4). The resulting expression plasmid pSHw_plmA, which places *plm*A under control of the ADH2 promoter, was sequence verified from transformants using primers pADH2, tADH2 and PlmA_ver1-PlmA_ver5.

A single confirmed transformant was inoculated into 3 mL SD_{ct} media (0.5% bacto cassamino acids technical grade, 2% dextrose) supplemented with adenine (0.02 mg/mL), tryptophan (0.02 mg/mL) and yeast nitrogen base solution (0.17% nitrogen base without amino acids, 0.5% ammonium sulfate) and grown for 3 days at 28 °C and 180 rpm. A 1 mL aliquot of this seed culture was used to inoculate 1 L of YPD media (1% yeast extract, 2% peptone) supplemented with 1% dextrose, and the culture was shaken at 28 °C and 180 rpm for 4 days. Yeast cells were harvested by centrifugation (3285 x g at 4 °C for 15 mins) and the pellet was resuspended in 30 mL lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 10 mM imidazole, pH 8.0). Cells were sonicated on ice in one-minute intervals until homogenized. The lysate was cleared in two steps: first, the mixture was centrifuged at 37,156 x g and 4 °C for 1 hour, then the supernatant was passed through a 0.45 μm PVDF syringe filter. Ni-NTA agarose resin (2 mL) was added to the cleared lysate, and the solution was incubated at 4 °C for 16 hours. Soluble PlmA (149.49 kDa) was purified by gravity-flow column chromatography using increasing concentration of imidazole in buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM-250 mM imidazole, pH 7.9). Purified protein was concentrated and buffer exchanged into buffer B (50 mM Tris-HCl, 2 mM EDTA, 2 mM DTT, 100 mM NaCl, pH 8.0) using an Amicon Ultracel 100,000 MWCO centrifugal filter (Merck Millipore Inc.) and stored in 10% glycerol at -80 °C until needed (Figure S4). The protein concentration was calculated to be 1.4 mg/L by Bradford assay using BSA as a standard.

*plm*J was also predicted to contain two introns but due to its size (7128 bp), was cloned in four overlapping pieces for intron-free reconstitution. Piece two (2934 bp) and piece three (2614 bp) did not contain any introns and were amplified from gDNA using primers pairs PlmJ_F2 with PlmJ_R2 and PlmJ_F3 with PlmJ_R3. Piece one (1149 bp) and piece four (1042 bp) were amplified from cDNA to remove predicted introns. RNA extraction and cDNA synthesis were performed as described above for *plm*A except reverse primers PlmJ_R1 and oligo_dT were used for pieces one and four, respectively. Pieces one and four were then amplified from the cDNA template using primer pairs PlmJ_F1 with PlmJ_R1 and PlmJ_F4 with PlmJ_R4, respectively. All pieces were subcloned into pCR®-blunt (Invitrogen) for sequence verification. Pieces two and three were re-amplified with the same primers from pCR-blunt, whereas pieces one and four were amplified to contain the necessary overhangs for yeast recombination using primer pairs PlmJ_yeast_F with PlmJ_R1 and PlmJ_F4 with PlmJ_yeast_R, respectively. The co-transformation with linear expression vector was performed as described above for *plm*A. The resulting expression plasmid pSHw_plmJ was sequence verified from transformants using primers pADH2, tADH2 and PlmJ_ver1-PlmJ_ver10. Protein purification of PlmJ (261.74 kDa) was performed as described above for PlmA. The protein concentration was calculated to be 0.9 mg/L by Bradford assay using BSA as a standard.

*plm*K was predicted to contain one intron and thus was cloned in two overlapping pieces for intron-free reconstitution (3921 bp). Piece two (3121 bp) did not contain any introns and was amplified from gDNA using the primer pair PlmK_F2 with PlmK_R2. Piece one (1041 bp) was amplified from cDNA to remove the predicted intron. RNA extraction and cDNA synthesis were performed as described above for *plm*A except the reverse primer PlmK_R1 was used. All pieces were subcloned into pCR®-blunt (Invitrogen) for sequence verification. Both pieces of *plm*K were re-amplified with the same primers already containing the necessary overhangs for recombination. The co-transformation with linear expression vector was performed as described above for *plm*A. The resulting expression plasmid pSHw_plmK was sequence verified from transformants using primers pADH2, tADH2 and PlmK_ver1-PlmK_ver4. Protein purification of PlmK (144.97 kDa) was performed as described above for PlmA. The protein concentration was calculated to be 0.7 mg/L by Bradford assay using BSA as a standard.

In vitro NRPS assays

The NRPS machinery responsible for biosynthesizing penilumamide A was reconstituted *in vitro* using an established assay method from the Marahiel group[10]. In brief, each 100 μL reaction contained 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM pterine-6-carboxylic acid, 1 mM L-methionine, 1 mM anthranilic acid, and 100 nM concentrations of PlmJ, PlmK and PlmA. The pterine-6-carboxylic acid substrate was used as a commercially available alternative to the highly functionalized 1,3-dimethyl-lumazine-6-carboxylic acid building block produced by *A. flavipes* CNL-338. PlmA, PlmJ and PlmK NRPSs were each incubated independently and in various combinations to determine the minimum number of modules required for tripeptide biosynthesis. Assays were incubated at 25 °C for 12 hours before extraction with a 1:1 volume of 99% ethyl acetate containing 1% acetic acid. The organic layer was dried, resuspended in 50 μL methanol, and analyzed by LC-MS with a linear gradient of 5–95% MeCN:H_2O with 0.1% formic acid over 5 minutes followed by 100% MeCN for 1 minute at a flow rate of 0.6 mL/min. Mass data was compared by extracted ion chromatogram (EIC) for the expected tripeptide product **2** across all assay conditions and compared to a no enzyme control reaction (Figure 3A). UV/Vis λ_{max} values and ESI-HR TOF-MS data for compound **2** can be found in Figure S1, panel B.

Cloning, expression, and purification of Plm A domains in E. coli

PlmA, PlmJ and PlmK were analyzed using the Pfam 34.0 webtool[11] to determine domain boundaries. All four adenylation (A) domains were encoded in genetic regions uninterrupted by introns. The sequences coding for PlmA A and PlmJ A2 were amplified from *A. flavipes* CNL-338 gDNA. Amplification of *plm*A A (1485 bp) used the primer pair PlmA_A_F and PlmA_A_R, which contained EcoRI and NotI restriction sites, respectively. Amplification of *plm*J A2 (1531 bp) used the primer pair PlmJ_A2_F and PlmJ_A2_R, which contained NcoI and NotI restriction sites, respectively. The two PCR products were independently subcloned into pCR®-blunt (Invitrogen) for sequence verification. The digested A domain gene sequences were then ligated into the pHis8 expression vector[12], which was prepared by digestion with the same corresponding restriction enzymes. The resulting expression plasmids pSHw_plmA_A and pSHw_plmJ_A2 were used to transform chemically competent *E. coli* TOP10 cells for sequence verification and *E. coli* BL21 (DE3) cells for protein production. *plm*J A1 (1511 bp) and *plm*K A (1524 bp) were codon optimized, synthesized by GeneWiz (South Plainfield, NJ, USA), and cloned independently into the pHis8 expression vector. The resulting expression plasmids pSHw_plmJ_A1 and pSHw_plmK_A, respectively, were used to transform chemically competent *E. coli* BL21 (DE3) cells for protein production.

A single colony of each construct was used to inoculate 5 mL LB media (0.5% yeast extract, 1% peptone, 0.5% NaCl) supplemented with 50 μg/mL kanamycin and grown overnight at 37 °C and 170 rpm. These seed cultures were used to inoculate 1 L each of Terrific Broth (TB) (2.4% yeast extract, 1.2% tryptone, 0.004% glycerol) supplemented with 50 mg/L kanamycin. The cultures were incubated at 37 °C and 170 rpm until an OD_{600} of 1.0 was reached. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration of 0.1 mM, at which point the cultures were incubated at 16 °C for a further 16 hours. Bacteria cells were harvested by centrifugation (3285 xg at 4 °C for 15 mins) and the pellet was resuspended in 20 mL lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 7.9). Cells were sonicated on ice in 30 second intervals until homogenized. The lysate was cleared by centrifugation at 32,914 x g and 4 °C for 30 minutes. Ni-NTA agarose resin (1 mL) was added to the supernatant, and the solution was incubated on a rotator at 4 °C for 4 hours. Soluble PlmA A (56 kDa), PlmJ A1 (58 kDa), PlmJ A2 (58 kDa) and PlmK A (57 kDa) were purified by gravity-flow column chromatography using increasing concentrations of

imidazole in buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM-250 mM imidazole, pH 7.9) followed by fast protein liquid chromatography (FPLC) using an ÄKTApurifier FPLC system (GE) equipped with a HiLoad™ Superdex™ size exclusion column (16/600, 75 pg). An isocratic flow of 1 mL/min of 50 mM Tris-HCl pH 8.0 was used for elution during a 120-minute method. Purified proteins were concentrated and buffer exchanged into buffer B (50 mM Tris-HCl, 2 mM DTT, pH 8.0) using Amicon Ultracel 50,000 MWCO centrifugal filters (Merck Millipore Inc.) and stored in 10% glycerol at -80 °C until needed (Figure S5). The protein concentrations were calculated by Bradford assay using BSA as a standard (PlmA A = 17.3 mg/L, PlmJ A1 = 6.2 mg/L, PlmJ A2 = 12.8 mg/L and PlmK A = 11.5 mg/L).

In vitro A domain assays via PPi release

The substrate loading of all four Plm A domains was first interrogated by an established, colorimetric *in vitro* method from the Garneau-Tsodikova group that indirectly measures PPi release[13]. This protocol uses the Malachite Green Phosphate Assay Kit (cat # POMG-25H) from BioAssay Systems (Hayward, CA, USA). In brief, all assays were performed in 96-well plates, and each 40 μL reaction contained 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 15 mM $MgCl_2$, 2.25 mM ATP, 0.2 U/mL inorganic pyrophosphatase, 3 mM of each substrate and 1 μM enzyme. The full panel of 20 proteinogenic amino acids were tested as substrates, along with formic acid, anthranilic acid, pterine-6-carboxylic acid, L-methionine sulfoxide, and L-methionine sulfone. PlmA A, PlmJ A1, PlmJ A2 and PlmK A were independently incubated with all substrates at 25 °C for 1 hour before addition of 10 μL of the malachite green reagent. Color was allowed to develop for 15 minutes before absorbance was measured at 600 nm using a Molecular Devices SpectraMax M5 microplate reader. All assays were performed in triplicate and normalized to boiled enzyme controls. Negative controls included reactions with no amino acids added for all enzymes and boiled enzyme reactions with all substrates (Figures 3B and S6).

In vitro A domain assays via hydroxylamine trapping

To further validate the A domain assay results obtained by pyrophosphate release, a complementary method was used as described by the Challis group involving hydroxylamine release of aminoacyl-adenylates and direct detection as iron complexes[14]. All assays were performed in 96-well plates, and each 30 μL reaction contained 50 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$, 2.25 mM ATP, 150 mM hydroxylamine, 3 mM of each substrate and 2.5 μM enzyme. The full panel of 20 proteinogenic amino acids were tested as substrates, along with formic acid, anthranilic acid, pterine-6-carboxylic acid, L-methionine sulfoxide, and L-methionine sulfone. PlmA A, PlmJ A1, PlmJ A2 and PlmK A were independently incubated with all substrates at 30 °C for 6 hours before addition of 30 μL of stopping solution (10% (w/v) $FeCl_3 \bullet 6H_2O$, 3.3% trichloroacetic acid in 0.7 M HCl). Absorbance was measured immediately at 540 nm using a Molecular Devices SpectraMax M5 microplate reader. All assays were performed in triplicate and normalized to boiled enzyme controls. Negative controls included reactions with no amino acids added for all enzymes and boiled enzyme reactions with all substrates (Figures 3C and S7).

Bioinformatic analyses of Plm enzyme domains

PlmA, PlmJ and PlmK were analyzed with the Pfam 34.0 webtool[11] to determine the boundaries of all corresponding adenylation (A), thiolation (T) and condensation (C) domains. Sequence alignments were made using the ClustalW algorithm and visualized using the ClustalX software to interrogate various motifs (Figure S8). An in-depth bioinformatic comparison of all Plm domains was used in proposing the biosynthesis of penilumamide A (**1**) (Figures 4 and S8-S11, and Tables S5, S7, and S9). All phylogenetic trees were constructed and visualized using MEGA 6.0 software with the JTT model of amino acid substitution.

Pfam annotations indicated that across the four Plm NRPS modules, there were 5 predicted C domains, which were added to an existing but modified dataset of canonical bacterial C domains[15,16] along with several other unique fungal C domains. A maximum-likelihood phylogenetic tree was assembled containing a total of 200 C domain sequences (Figure S11 and Table S10). Due to a lack of clarity from the large bacterial C domain tree, a smaller and more relevant phylogeny was generated containing 58 C domain sequences, of which 34 are of fungal origin (Table S8 and Figure S10). Further, condensation domain residues from the region containing the canonical HHxxxDG motif were compared to ensure all predicted C domains were active (Table S9). The first C domain in PlmA (PlmA C1) is predicted to be inactive, as it does not contain this motif, including the catalytic histidine. Interestingly, it seems that fungal C domains tend to replace the first histidine of the HHxxxDG motif with either serine or threonine (16 out of 57 sequences in Table S9).

It was also predicted that each of the four Plm NRPS modules contained an A domain and a T domain, which were aligned and compared to other bacterial and fungal domains. A maximum-likelihood phylogenetic tree was constructed containing 44 NRPS A domains, of which 32 are of fungal origin (Table S4 and Figure S9). PlmA_A clades closest to the alanine-loading domain TqaA_A3 from tryptoquialanine biosynthesis[17] but is also closely related to three tryptophan-incorporating domains. PlmJ_A1 and PlmK_A both clade with a distinct group of anthranilate-activating enzymes, and PlmJ_A2 is most closely related to the phenylalanine-loading BenZ_A2 from benzomalvin biosynthesis[18] and the valine-loading PsyA_A2 from psychrophilin biosynthesis[19]. Thiolation domain residues near the active site serine were also compared to ensure all predicted T domains were active (Tables S6 and S7). With the exception of NpsP4_T and NpsP5_T, which are uncharacterized T domains from napsamycin biosynthesis[20], all T domains maintain the active site serine required for phosphopantetheinylation.

In an effort to better understand substrate specificity in fungal A domains, all 44 sequences also had their 10 amino acid ”specificity codes” tabulated (Table S5)[21,22]. While not absolutely required, the first position of the code is usually aspartate, but it appears that at least in fungi, a significant portion of A domains (14 out of 44 sequences in Table S5) have substitutions to glycine, alanine, serine, threonine, and even proline.

Homology models of Plm condensation domains

It was not clear from bioinformatic analyses which of the four Plm NRPS modules might be inactive or skipped to account for tripeptide production, so homology models of all 4 intact condensation domains were generated using AlphaFold2[23] and compared to the crystal structure of VibH, a standalone C domain[24]. C domains are known to have a solvent-accessible channel going through their center and passing the catalytic histidine, dividing the protein into N- and C-terminal lobes. The homology model of PlmJ C1 showed the lowest RMSD when aligned with VibH (10.312), and when its HHxxxDR motif, comprising H125, H126, D130 and R131, was modeled into VibH, the side chain of R131 protruded into the substrate channel (Figure S12). Position 131 (A.A. 8) is rarely an arginine in C domains (Table S9), but it has been observed that position 126 (A.A. 3) is an arginine in enzyme-recruiting X domains such as Tcp12 C2[25].

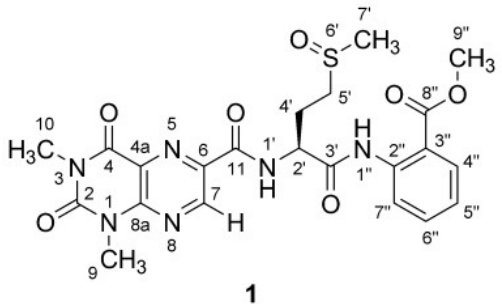
Table S1. Primers used in this work.

Primer name	Sequence (shown 5'-3')
plmA KO P0	cttcactacatgctacaggcc
plmA KO P1	tctcctccaaacgcatgtctggtg
plmA KO P2	cacgtacctcaatggacatgtggtcc
plmA KO P3	ttcaatatcatcttctgtcgacggaaggatatttcaaggctcctgag
plmA KO P4	gaaggctttaatttgcaagctgatgggctgaacatagccaaagcac
plmA KO P5	gtcgcactgatcacatagcttgtagg
plmA KO P6	cattcgaagcagcggtctagatgtgg
plmA KO P7	tgccatgggtcagctt
PtrpC R	gtcgacagaagatgatattg
zeocin F	agcttgcaaattaaagcctt
oligo_dT	ttttttttttttttt
PlmA_F1	catagcttccacatccacg
PlmA_R1	ctaaccagttgaagattggg
PlmA_F2	ccgtgaatatgtgtgttc
PlmA_R2	gtagaggcatatcctgc
PlmA_F3	atggctaccaagaaatcagc
PlmA_R3	ctgaagatgtcctgccag
PlmA_yeast_F	tcaactatcaactattaactatatcgtaataccatatggctaccaagaaatcagc
PlmA_yeast_R	tgtcatttaaattagtgatggtgatggtgatgcacaccagttgaagattgggcca
PlmJ_F1	atgggttcgaatgaatcccag
PlmJ_R1	ggaatctggaattgactctgctg
PlmJ_F2	tgccatcggcatccataa
PlmJ_R2	cggtcataatcagtcagcatg
PlmJ_F3	ctcgtggtaacatgtcaac
PlmJ_R3	ctatgtcgttgagcagtacgg
PlmJ_F4	actcccagatttcatcc
PlmJ_R4	gatgaattctaccatg
PlmJ_yeast_F	tcaactatcaactattaactatatcgtaataccatatgggttcgaatgaatcccag
PlmJ_yeast_R	tgtcatttaaattagtgatggtgatggtgatgcacaactatattgccactgttc
PlmK_F1	tcaactatcaactattaactatatcgtaataccatatgacgctttcagacttgtcg
PlmK_R1	cctggcaatgtgaagataagatg
PlmK_F2	ctggtacatttgtcccactctg
PlmK_R2	tgtcatttaaattagtgatggtgatggtgatgcactgtatcttcatctcgaattgtcgca
pADH2_F	gcaaaacgtaggggcaaacaa
tADH2_R	gagctcggtaccctcga
PlmA_ver1	gtatgatctcgtactatccgag
PlmA_ver2	aagcataggaggagaactgc
PlmA_ver3	gcctcaatgccaatcacacg
PlmA_ver4	gattcaatcgacgcgatgaagg
PlmA_ver5	ctagggttgccagtgtgattc
PlmJ_ver1	ggggatacacactacaaacaag
PlmJ_ver2	gatcctctcagagatgcaaacc
PlmJ_ver3	catcggtaggcctacaaatg
PlmJ_ver4	gcctttgaactgtgtgctcc
PlmJ_ver5	ggctcagcaagagaagattg
PlmJ_ver6	caaggaaacttgagcggc
PlmJ_ver7	ttactctcgctggacagttg
PlmJ_ver8	ctatttcgctaggtgacagcttg
PlmJ_ver9	atccgaagctgtctacgttg
PlmJ_ver10	gttcactgtctgaccgagaatgag
PlmK_ver1	gcagtctcactctatcctatcg

PlmK_ver2	cgaagtctactgcaccctga
PlmK_ver3	cggtaggtgtcattgttgc
PlmK_ver4	atggaccgaatggtgcgat
PlmA_A_F	**gaattc**cctgatgcaccggctgtttgtg
PlmA_A_R	**gcggccgc**ctagcggaggccaccccgatccacttt
PlmJ_A2_F	**ccatgg**caattgcagcaggtctggtcatc
PlmJ_A2_R	**gcggccgc**ctatcggaggaatttgcggtc

EcoRI (GAATTC), NotI (GCGGCCG) and NcoI (CCATGG) restriction recognition sites are bolded and underlined

Table S2. ^{1}H NMR resonances of **1**, isolated from *A. flavipes* CNL-338. [a] ^{1}H chemical shifts are referenced to the DMSO-d_6 2.50 ppm signal.



1

penilumamide A (**1**) 500 MHz , DMSO-d_6[a]	
Position	δ (^{1}H)
1	–
2	–
3	–
4	–
4a	–
5	–
6	–
7	9.29 (d, *J* = 8.2 Hz)
8	–
8a	–
9	3.61 (s)
10	3.37 (s)
11	–
1’	9.32 (s)
2’	4.85 (m)
3’	–
4’	2.30–2.38 (m)
	2.42–2.49 (m)
5′	2.73–2.82 (m)
	2.89–2.95 (m)
6’	–
7′	2.56 (s)
1”	11.04 (s)
2”	–
3”	–
4”	7.64 (ddd, 8.4, 7.3, 1.1 Hz)
5”	7.22 (dd, 7.6, 7.5 Hz)
6”	7.90 (dd, *J* = 7.8, 1.2 Hz
7”	8.33 (d, *J* = 8.5 Hz)
8”	–
9”	3.68 (s)

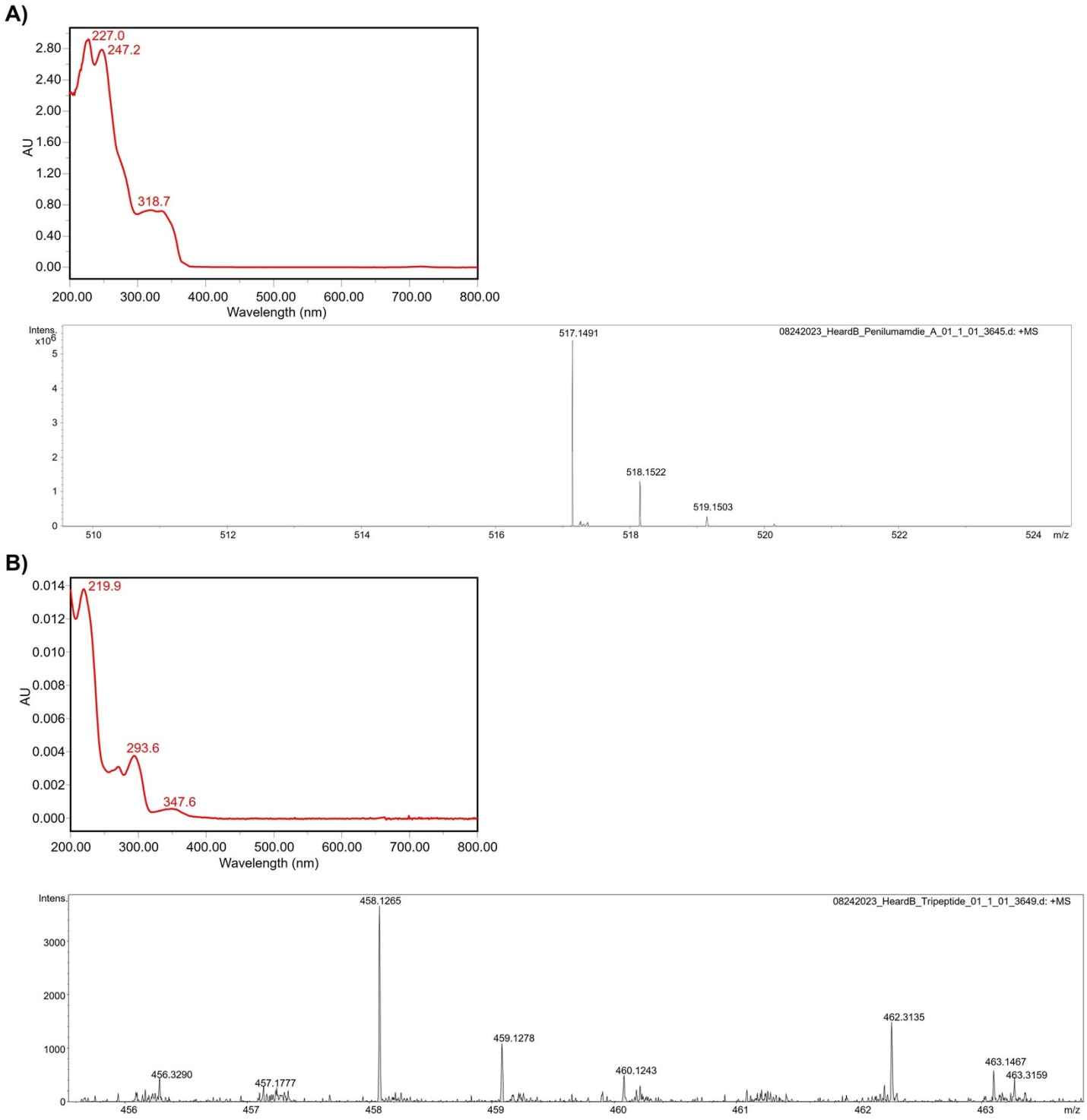


Figure S1. Identification of penilumamide A (**1**) from *A. flavipes* CNL-338 and demethyl-pterin-penilumamide (**2**) from NRPS assays. A) UV profile and HR-ESI-MS of penilumamide A (**1**) $C_{22}H_{24}N_6O_7S$ [HRMS *m/z*: $[M+H]^+$ calcd for $C_{22}H_{25}N_6O_7S$ 517.1505; found 517.1491]. B) UV profile and HR-ESI-MS of **2** $C_{19}H_{19}N_7O_5S$ generated from the NRPS assay in Figure 3A [HRMS *m/z*: $[M+H]^+$ calcd 458.1247 for $C_{19}H_{20}N_7O_5S$; found 458.1265].

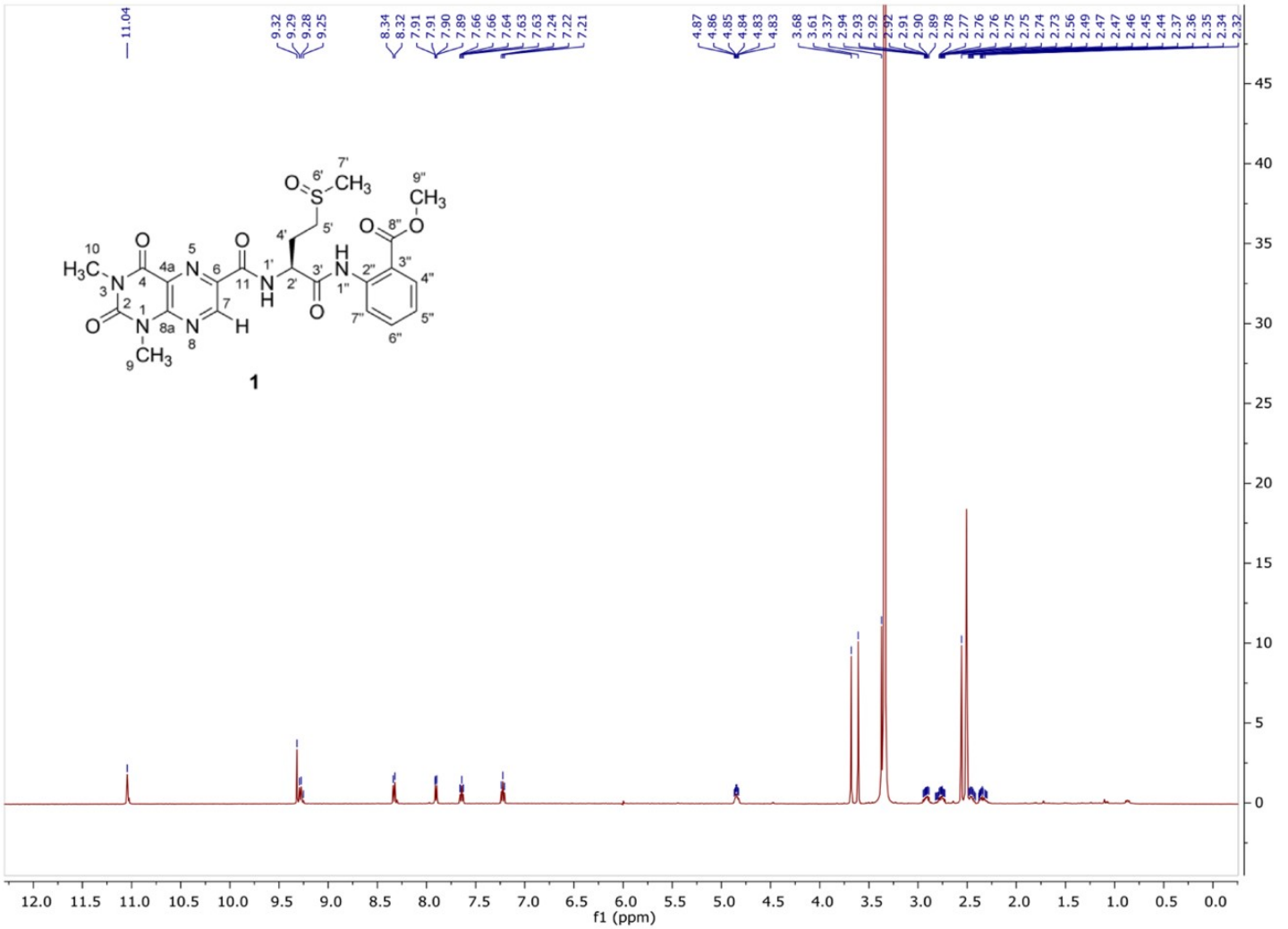


Figure S2. ^{1}H NMR spectrum of **1**, isolated from *A. flavipes* CNL-338. Data was acquired at 500 MHz in DMSO-d_6.

Table S3. Annotation of the *plm* gene cluster in *Aspergillus flavipes* sp. CNL-338. NRPS = nonribosomal peptide synthetase, C = condensation, A = adenylation, T = thiolation, GMC = glucose-methanol-choline, GTP = guanosine triphosphate, FAD = flavin adenine dinucleotide, SAM = S-adenosyl-L-methionine.

Gene product	Total amino acids	Proposed function	Sequence similarity (origin)	Identity/ similarity (%)	Accession number
PlmA	1342	NRPS (C_1-A-T-C_2)	*Aspergillus pseudoviridinutans*	73/83	GIJ86599.1
PlmB	591	GMC oxidoreductase	*Penicillium* sp. *'occitanis'*	44/61	PCG91499.1
PlmC	249	GTP cyclohydrolase I	*Aspergillus pseudoviridinutans*	80/86	GIJ86601.1
PlmD	247	Cytidine deaminase-like	*Aspergillus alliaceus*	48/65	XP_031896599.1
PlmE	487	Aldehyde dehydrogenase	*Aspergillus avenaceus*	51/67	KAE8153438.1
PlmF	446	FAD-dependent oxidoreductase	*Aspergillus flavus* AF70	45/64	KOC15531.1
PlmG	313	Dihydroneopterin aldolase/epimerase	*Penicillium roqueforti* FM164	51/72	CDM28775.1
PlmH	244	SAM-dependent methyltransferase	*Aspergillus flavus*	40/57	KAB8251571.1
PlmI	257	SAM-dependent methyltransferase	*Aspergillus parasiticus*	42/60	KAB8205443.1
PlmJ	2375	NRPS (A_1-T_1-C_1-A_2-T_2-C_2)	*Aspergillus udagawae*	42/60	GFF56995.1
PlmK	1313	NRPS (A-T-C)	*Aspergillus udagawae*	40/59	GFF23642.1

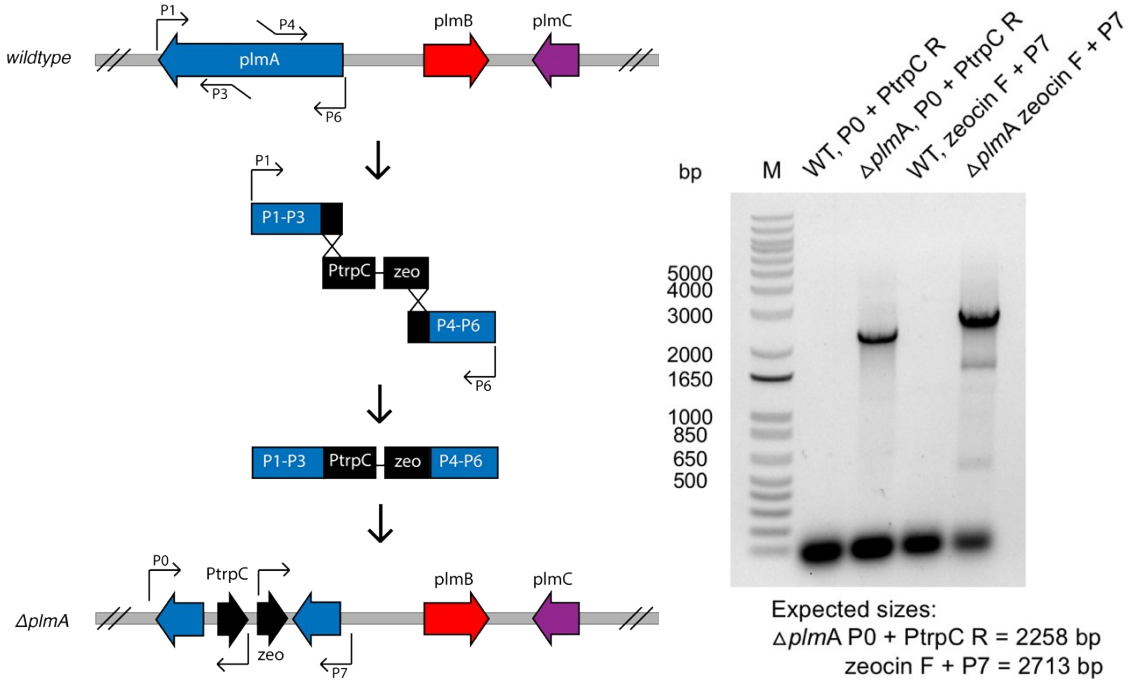


Figure S3. Generation of the *plm*A NRPS gene inactivation cassette and integration into the genome by PCR verification. Wild-type (WT) *A. flavipes* CNL-338 gDNA was used as a negative control.

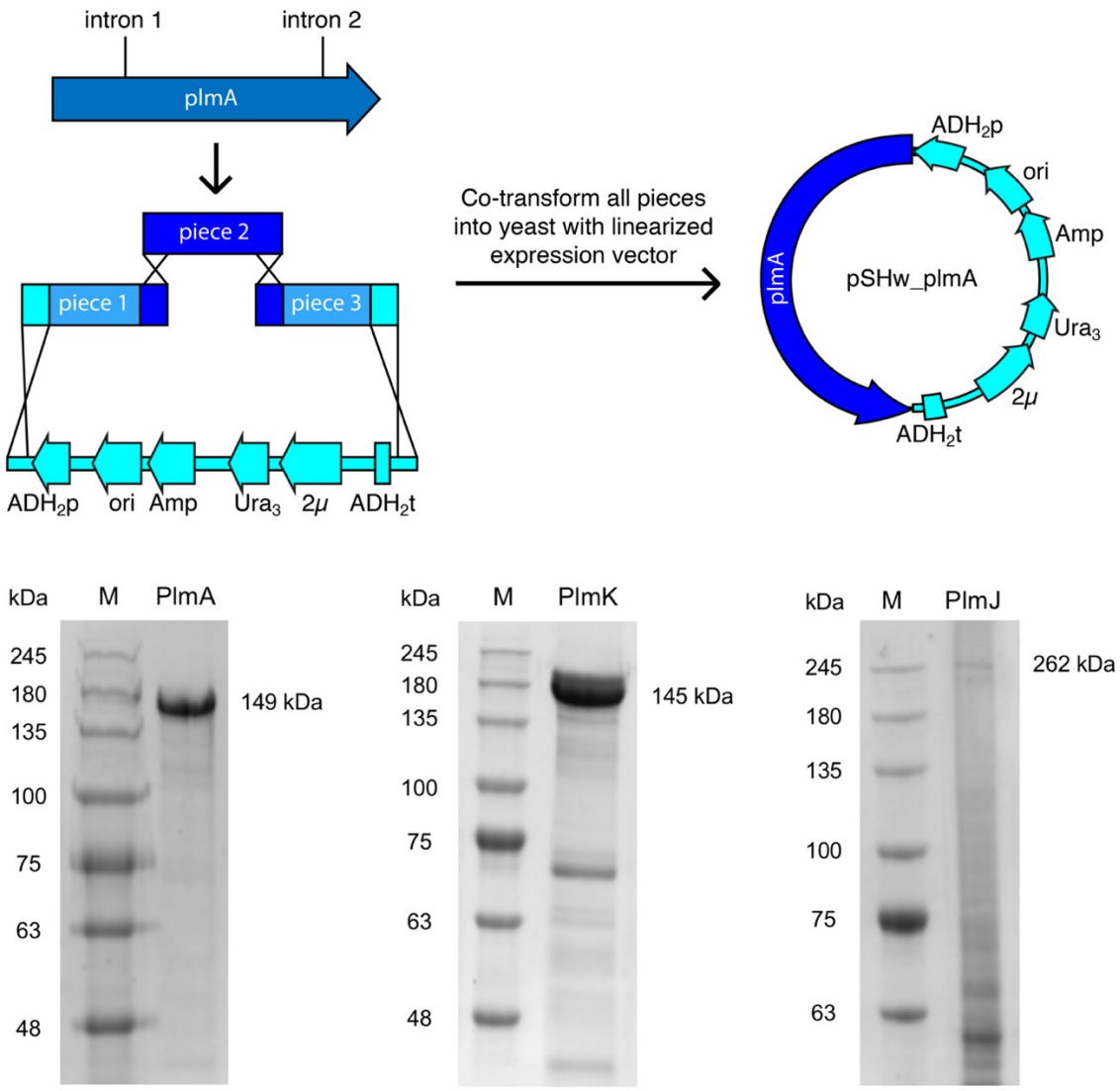


Figure S4. Reconstituting *plm*A, *plm*J and *plm*K for expression in *S. cerevisiae* BJ5464-NpgA. Overlapping regions between two neighboring DNA segments ranged from 140-187 bp and overlapping regions with the vector backbone were 35 bp. A representation for the reconstitution of intron-free *plm*A is shown. PlmA, PlmJ and PlmK were produced as C-terminal hexahistidyl-tagged proteins in *S. cerevisiae* BJ5464-NpgA, purified by Ni-NTA affinity chromatography to yield between 0.7-1.4 mg/L, and analyzed for purity using an 8 % SDS-PAGE gel. Bluestain protein ladder 11-245 kDa (GoldBio) was used for all gels.

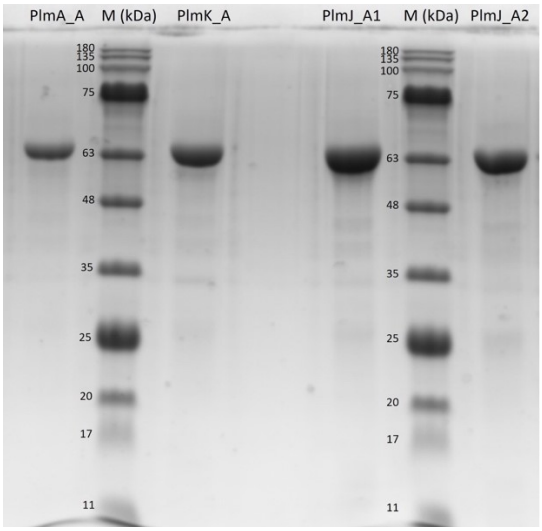


Figure S5. SDS-PAGE of recombinant NRPS adenylation domains purified from *E. coli* BL21 (DE3). Each dissected adenylation domain was expressed as an N-terminal octahistidyl-tagged protein, purified by Ni-NTA agarose affinity resin to yield between 6-17 mg/L, and analyzed for purity using a 12 % SDS-PAGE gel. Bluestain protein ladder 11-245 kDA (GoldBio) was used for all gels.

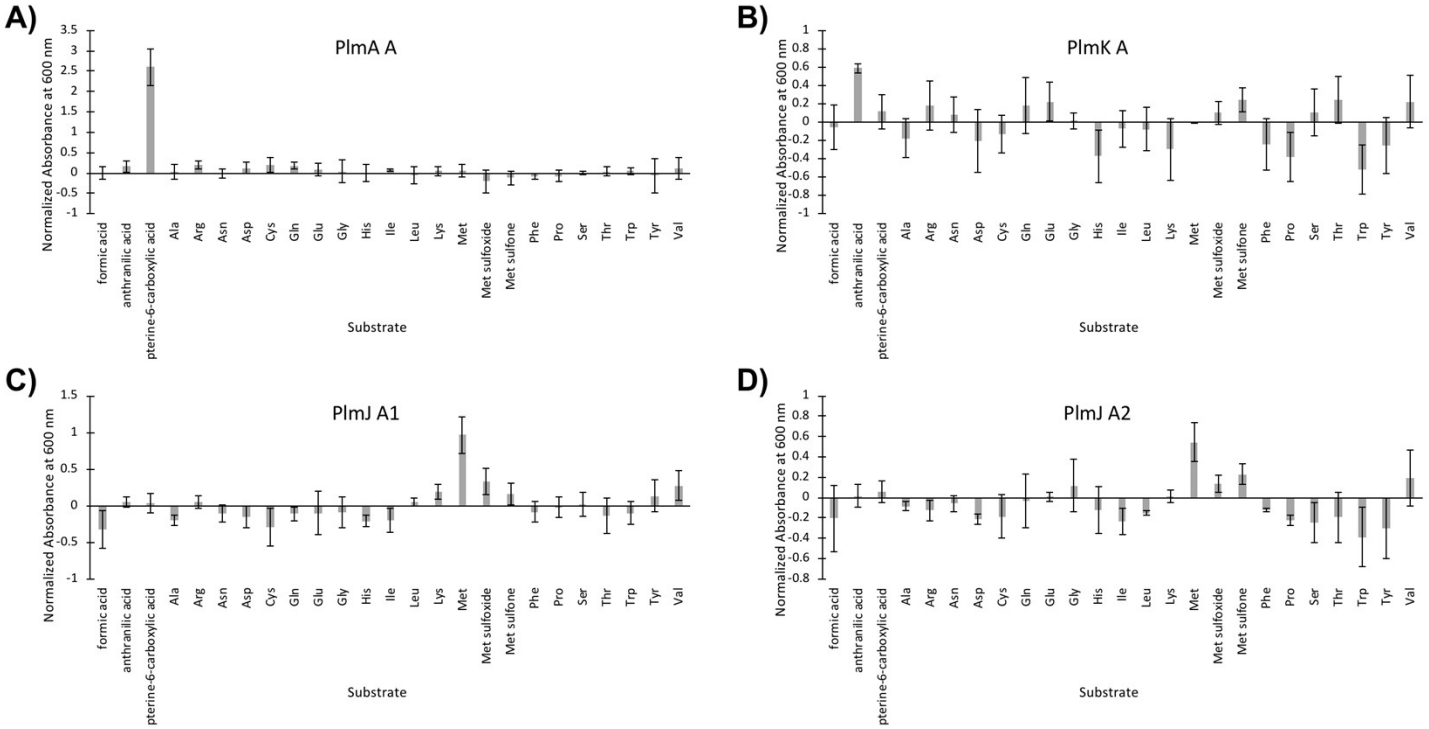


Figure S6. *In vitro* adenylation assays to determine substrate loading of the four Plm adenylation domains via PPi release. Data represents the mean of three replicates, and error bars represent the standard deviation. Adenylation activity was determined through the malachite green/phosphate detection method,[13] measured in triplicate as absorbance at 600 nm, and normalized to boiled enzyme controls for each substrate. It should be noted that pterine-6-carboxylic acid was used as a commercially available alternative to the highly functionalized 1,3-dimethyllumazine-6-carboxylic acid building block.

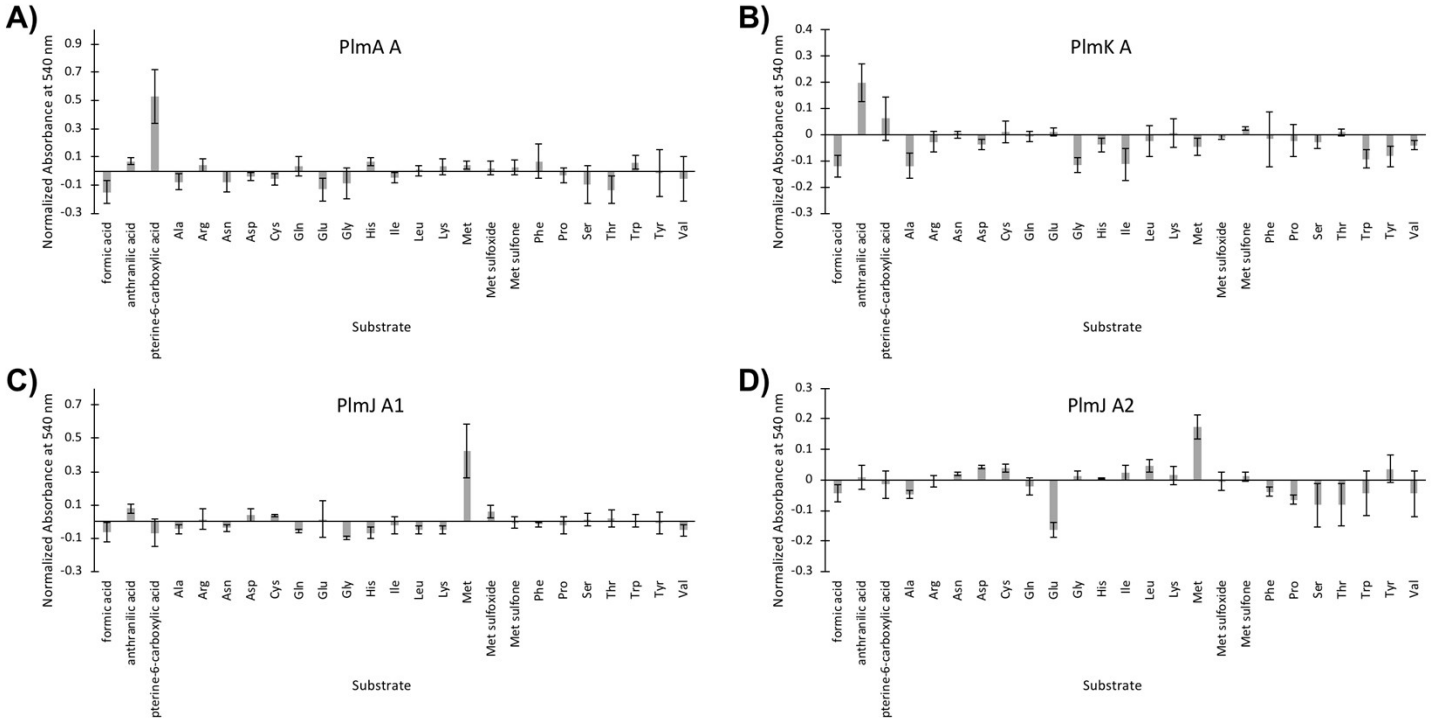
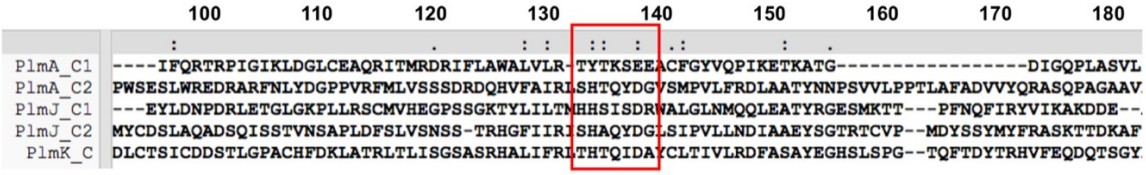


Figure S7. *In vitro* adenylation assays to determine substrate loading of the four Plm adenylation domains via hydroxylamine trapping. Data represents the mean of three replicates, and error bars represent the standard deviation. Adenylation activity was determined through the hydroxylamine release/iron complex method,[14] measured in triplicate at 540 nm, and normalized to boiled enzyme controls for each substrate. It should be noted that pterine-6-carboxylic acid was used as a commercially available alternative to the highly functionalized 1,3-dimethyllumazine-6-carboxylic acid building block.

A)

B)



C)

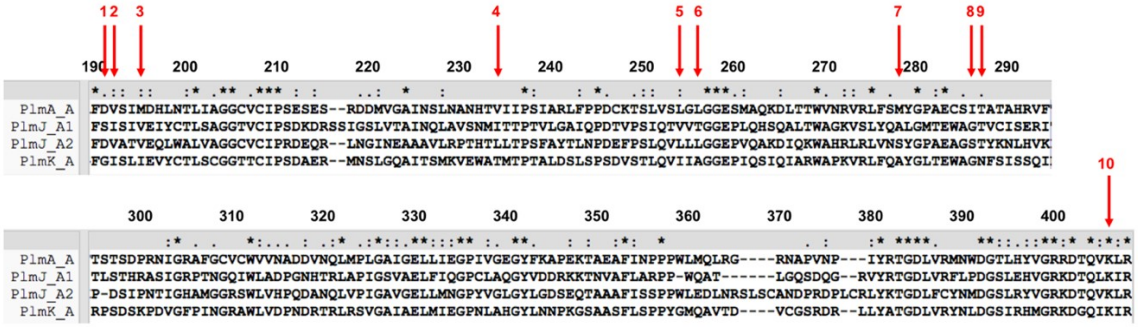


Figure S8. Sequence alignments of thiolation, condensation and adenylation domains found in PlmA, PlmJ and PlmK. A) Four thiolation (T) domains identified in the three *plm* encoding NRPSs with the conserved DxFFxLGGHSL motif highlighted. The amino acids are also listed in Table S7. B) Partial sequence of the five condensation (C) domains identified in the three *plm* encoding NRPSs with the core HHxxxDG motif highlighted. The amino acids are also listed in Table S9. C) Alignment of the four adenylation (A) domains with the 10 amino acid ”specificity code” denoted as 1-10. The amino acids are also listed in Table S5. The ClustalW algorithm was used for all alignments, and the ClustalX software was used for visualization.

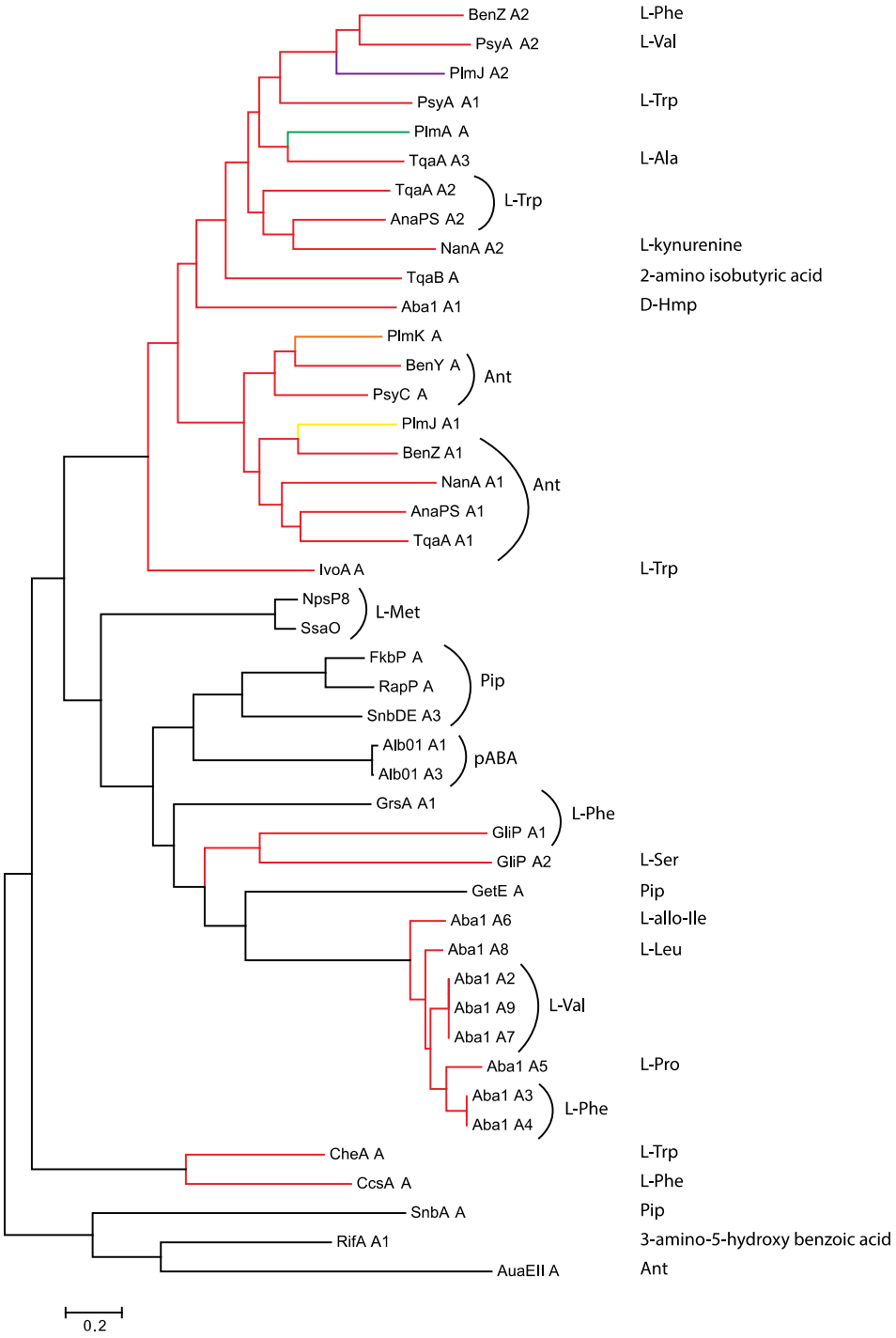


Figure S9. Maximum-likelihood phylogenetic tree of 44 NRPS A domains based on substrate selectivity. Black lines are bacterial A domains, and red lines are fungal A domains. The four *plm* encoding A domains are in green (PlmA A), yellow (PlmJ A1), purple (PlmJ A2), and orange (PlmK A). The maximum-likelihood tree was generated using MEGA 6.0 software with the JTT model of amino acid substitution. The scale bar represents the average number of amino acid substitutions. D-Hmp = D-2-hydroxy-3-methylpentanoic acid, Ant = anthranilic acid, Pip = pipecolate, pABA = para-aminobenzoic acid.

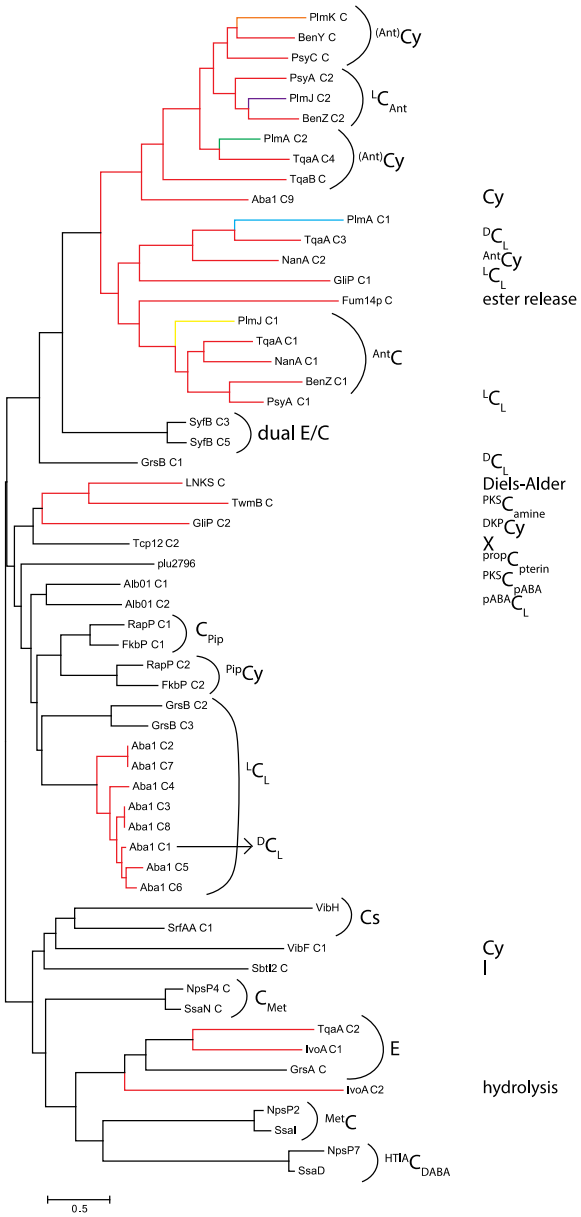


Figure S10. Maximum-likelihood phylogenetic tree of 58 NRPS C domains based on function. Black lines are bacterial C domains, and red lines are fungal C domains. The five *plm* encoding C domains are in blue (PlmA C1), green (PlmA C2), yellow (PlmJ C1), purple (PlmJ C2), and orange (PlmK C). The maximum-likelihood tree was generated using MEGA 6.0 software with the JTT model of amino acid substitution. The scale bar represents the average number of amino acid substitutions. Cs = starter C domain, Cy = terminal cyclizing C domain, E = epimerization domain, I = interfacing domain, X = interfacing domain that recruits Oxy enzymes, and dual E/C = bifunctional C domain that also epimerizes the donor substrate. $^{X}C_{Y}$ notation denotes the donor substrate as X and the acceptor substrate as Y. L/D = proteinogenic amino acid stereochemistry, PKS = polyketide synthase, pABA = para-aminobenzoic acid, DKP = diketopiperazine, prop = propionate, Pip = pipecolate, Met = methionine, Ant = anthranilic acid, HTIA = 6-OH-tetrahydro-isoquinoline carboxylate, DABA = diaminobutyric acid.

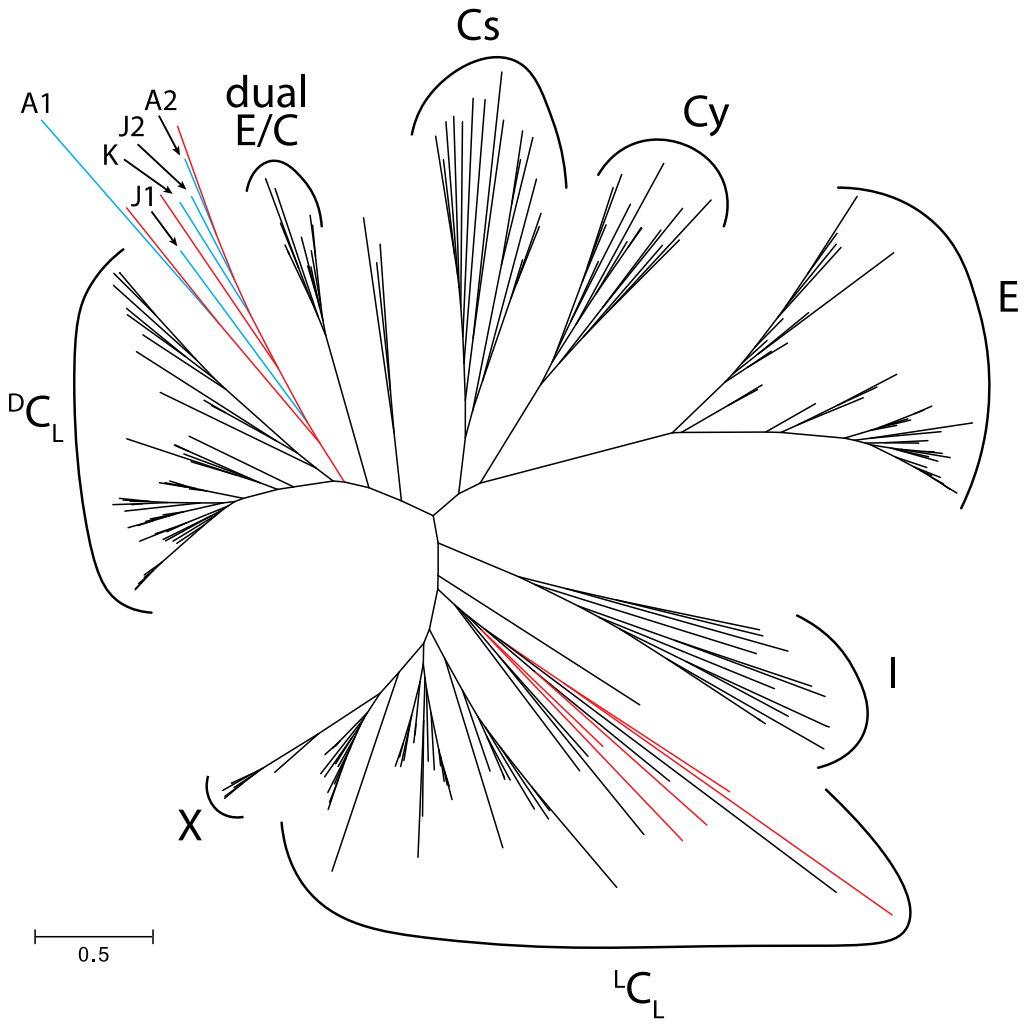


Figure S11. Maximum-likelihood phylogenetic tree of 200 NRPS C domains based on function. Modified from refs. 15 and 16. Black lines are bacterial C domains, red lines are fungal C domains, and blue lines are the five *plm* C domains: PlmA C1 (A1), PlmA C2 (A2), PlmJ C1 (J1), PlmJ C2 (J2), and PlmK C (K). The phylogenetic tree was generated using MEGA 6.0 software with the JTT model of amino acid substitution. The scale bar represents the average number of amino acid substitutions. Cs = starter C domain, Cy = terminal cyclizing C domain, E = epimerization domain, I = interfacing domain, $^{L}C_{L}$ = C domain that condenses two L-amino acid substrates, X = interfacing domain that recruits P450 enzymes, $^{D}C_{L}$ = C domain that condenses a D-amino acid donor with an L-amino acid acceptor, dual E/C = bifunctional C domain that also epimerizes the donor substrate.

Table S4. Adenylation domain amino acid sequences analyzed in Figure S9.

Protein (domains)	Enzyme class	Function	Origin	Accession No.
PlmA (A)	NRPS	Penilumamide synthetase I	*Aspergillus flavipes* CNL-338 (this work)	ON297638
PlmJ (A1,A2)	NRPS	Penilumamide synthetase II	*Aspergillus flavipes* CNL-338 (this work)	ON297638
PlmK (A)	NRPS	Penilumamide synthetase III	*Aspergillus flavipes* CNL-338 (this work)	ON297638
Aba1 (A1-9)	NRPS	Aureobasidin A1 complex	*Aureobasidium pullulans*	ACJ04424.1
Alb01 (A1,A3)	PKS-NRPS	Albicidin synthetase 1	*Xanthomonas albilineans*	CAE52339.1
AnaPS (A1,A2)	NRPS	Acetylaszonalenin synthetase	*Aspergillus fischeri* NRRL 181	A1DN09.1
AuaEII (A)	NRPS	anthranilate-CoA ligase	*Stigmatella aurantiaca Sg a15*	CCA65703.1
BenY (A)	NRPS	Benzomalvin synthetase Y	*Aspergillus terreus*	P9WEU8.1
BenZ (A1,A2)	NRPS	Benzomalvin synthetase Z	*Aspergillus terreus*	P9WEU9.1
CcsA (A)	PKS-NRPS	Cytochalasin synthetase A	*Aspergillus clavatus* NRRL 1	A1CLY8
CheA (A)	PKS-NRPS	Chaetoglobosin synthetase A	*Penicillium expansum*	CAO91861.1
FkbP (A)	NRPS	FK506-binding protein	*Streptomyces hygroscopicus* subsp. *ascomyceticus*	AAF86395.1
GetE (A)	NRPS	GE81112 adenylation domain	*Streptomyces* sp. L-49973	CBL93716.1
GliP (A1,A2)	NRPS	Gliotoxin synthetase	*Aspergillus fumigatus*	AAW03307.1
GrsA (A1)	NRPS	Gramicidin S synthetase A	*Brevibacillus brevis*	P0C062.1
IvoA (A)	NRPS	Ivory mutation-related protein A	*Aspergillus nidulans* FGSC A4	C8V7P4.1
NanA (A1,A2)	NRPS	Nanangelenin synthetase A	*Aspergillus nanangensis*	QIQ51365.1
NpsP8	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76684.1
PsyA	NRPS	Psychrophilin	*Penicillium* sp. YT-	AMQ36132.1

(A1,A2)		synthetase A	2016	
PsyC (A)	NRPS	Psychrophilin synthetase C	*Penicillium* sp. YT-2016	AMQ36134.1
RapP (A)	NRPS	Pipecolate incorporating enzyme	*Streptomyces hygroscopicus*	Q54298.1
RifA (A1)	PKS-NRPS	Rifamycin polyketide synthase A	*Amycolatopsis mediterranei*	O54666
SnbA (A)	NRPS	Pristinamycin I synthetase 1	*Streptomyces pristinaespiralis*	P95819
SnbDE (A3)	NRPS	Pristinamycin I synthase 3 and 4	*Streptomyces pristinaespiralis*	O07944
SsaO	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4NDI3
TqaA (A1,A2,A3)	NRPS	Tryptoquialanine synthetase A	*Penicillium aethiopicum*	ADY16697.1
TqaB (A)	NRPS	Tryptoquialanine synthetase B	*Penicillium aethiopicum*	ADY16689.1

Table S5. Comparative analysis of adenylation domain residues that mediate amino acid specificity. Black text is bacterial C domains, and red text is fungal C domains. Residue positions are numbered according to AnaPS A2. Ant = anthranilic acid, Pip = pipecolate, L-Met^{SO} = L-methionine sulfoxide, pABA = para-aminobenzoic acid, L-Ky = L-kynurenine, AHBA = 3-amino-5-hydroxybenzoic acid, D-Hmp = D-2-hydroxy-3-methylpentanoic acid, AIB = 2-amioisobutyric acid.

Protein	Position 1 A.A. 190	Position 2 A.A. 191	Position 3 A.A. 194	Position 4 A.A. 231	Position 5 A.A. 251	Position 6 A.A. 253	Position 7 A.A. 275	Position 8 A.A. 283	Position 9 A.A. 284	Position 10 A.A. 392	Substrate
PlmA A	D	V	M	V	L	L	M	I	T	K	pterin
PlmJ A1	S	I	V	I	V	T	A	G	T	K	Met
PlmJ A2	D	V	V	L	L	L	S	S	T	K	Met
PlmK A	G	I	I	T	I	A	A	G	N	K	Ant
AnaPS A2	D	V	M	F	S	L	E	V	A	K	L-Trp
CheA A1	D	M	I	I	T	W	C	A	A	K	L-Trp
PsyA A1	G	A	T	F	L	L	G	S	A	K	L-Trp
TqaA A2	G	G	M	H	L	S	G	A	V	K	L-Trp
IvoA A	D	V	D	L	L	T	V	S	V	K	L-Trp
GrsA A1	D	A	W	T	I	A	A	I	C	K	L-Phe
CcsA A1	D	M	S	E	S	W	C	F	C	K	L-Phe
BenZ A2	G	M	N	V	L	L	G	G	V	K	L-Phe
Aba1 A3	D	A	W	V	L	S	G	I	Q	K	L-Phe
Aba1 A4	D	A	W	V	L	S	G	I	Q	K	L-Phe
GliP A1	D	G	G	I	I	L	A	T	C	K	L-Phe
AnaPS A1	G	A	L	F	L	I	A	G	V	K	Ant
AuaEII A	A	F	G	Y	C	S	G	H	I	K	Ant
BenZ A1	D	I	N	F	I	T	A	G	T	K	Ant
BenY A	D	M	F	I	V	T	L	G	M	K	Ant
PsyC A	D	I	I	L	I	S	A	G	I	K	Ant
TqaA A1	G	V	I	F	I	V	A	G	V	K	Ant
NanA A1	D	I	I	L	L	L	V	G	V	K	Ant
FkbP A	D	Y	Q	Y	L	Q	H	L	I	K	Pip

GetE A	D	V	Q	D	I	S	H	M	V	K	Pip
RapP A	D	Y	Q	Y	L	Q	H	L	V	K	Pip
SnbA A	P	F	P	S	L	V	V	L	T	K	Pip
SnbDE A3	D	F	Q	F	I	Q	V	A	V	K	Pip
PsyA A2	D	M	V	F	L	L	L	G	I	K	L-Val
Aba1 A2	G	A	W	M	L	A	A	I	L	K	L-Val
Aba1 A7	D	A	W	M	L	A	A	I	L	K	L-Val
Aba1 A9	D	A	W	M	L	A	A	I	L	K	L-Val
NpsP8	T	G	L	I	V	V	I	C	V	K	L-Met
SsaO	D	G	L	I	A	L	I	C	V	K	L-Met^{SO}
Aba1 A5	D	V	W	V	M	S	A	I	Q	K	L-Pro
Aba1 A6	D	A	L	V	L	I	V	V	L	K	L-*allo*-Ile
Aba1 A8	D	A	W	M	L	L	A	V	I	K	L-Leu
GliP A2	D	Y	N	S	V	A	A	S	I	K	L-Ser
TqaA A3	D	M	V	I	I	L	G	S	A	K	L-Ala
Alb01 A1	S	V	K	Y	V	T	N	N	D	K	pABA
Alb01 A3	A	V	K	Y	V	T	N	N	D	K	pABA
NanA A2	G	A	G	M	L	L	G	T	V	K	L-Ky
RifA A1	D	L	I	A	G	A	A	G	A	K	AHBA
Aba1 A1	D	A	L	L	V	L	I	T	V	K	D-Hmp
TqaB A	D	L	F	M	V	L	G	G	C	K	AIB

Table S6. Protein sequences used for the thiolation domain comparison in Table S7.

Protein (domains)	Enzyme class	Function	Origin	Accession No.
PlmA (T)	NRPS	Penilumamide synthetase I	*Aspergillus flavipes* CNL-338 (this work)	ON297683
PlmJ (T1,T2)	NRPS	Penilumamide synthetase II	*Aspergillus flavipes* CNL-338 (this work)	ON297683
PlmK (T)	NRPS	Penilumamide synthetase III	*Aspergillus flavipes* CNL-338 (this work)	ON297683
Aba1 (T1-9)	NRPS	Aureobasidin A1 complex	*Aureobasidium pullulans*	ACJ04424.1
Alb01 (T1-4)	PKS-NRPS	Albicidin synthetase 1	*Xanthomonas albilineans*	CAE52339.1
BenY (T)	NRPS	Benzomalvin synthetase Y	*Aspergillus terreus*	P9WEU8.1
BenZ (T1,T2)	NRPS	Benzomalvin synthetase Z	*Aspergillus terreus*	P9WEU9.1
BnvB	NRPS	Bonnevillamide synthetase B	*Streptomyces* sp. UTZ13	QVT76735.1
BnvD (T1-4)	NRPS	Bonnevillamide synthetase D	*Streptomyces* sp. UTZ13	QVT76739.1
BnvF (T)	NRPS	Bonnevillamide synthetase F	*Streptomyces* sp. UTZ13	QVT76742.1
FkbP (T)	NRPS	FK506-binding protein	*Streptomyces hygroscopicus* subsp. *ascomyceticus*	AAF86395.1
GliP (T1,T2)	NRPS	Gliotoxin synthetase	*Aspergillus fumigatus*	AAW03307.1
GrsA (T)	NRPS	Gramicidin S synthetase A	*Brevibacillus brevis*	P0C062.1
GrsB (T1-4)	NRPS	Gramicidin S synthetase B	*Brevibacillus brevis*	CAA43838.1
IvoA (T)	NRPS	Ivory mutation-related protein A	*Aspergillus nidulans* FGSC A4	C8V7P4.1
NanA (T1-3)	NRPS	Nanangelenin synthetase A	*Aspergillus nanangensis*	QIQ51365.1
NpsP1	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76660.1
NpsP3 (T)	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76664.1

NpsP4 (T)	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76666.1
NpsP5 (T)	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76667.1
PsyA (T1,T2)	NRPS	Psychrophilin synthetase A	*Penicillium* sp. YT-2016	AMQ36132.1
PsyC (T)	NRPS	Psychrophilin synthetase C	*Penicillium* sp. YT-2016	AMQ36134.1
RapP (T)	NRPS	Pipecolate incorporating enzyme	*Streptomyces hygroscopicus*	Q54298.1
SbtI2 (T)	NRPS	Serobactin synthetase 2	*Herbaspirillum seropedicae*	QDD64765.1
SrfAA (T1-3)	NRPS	Surfactin synthetase A	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_388230.2
SsaH	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4N7P4
SsaL (T)	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4N7U3
SsaN (T)	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4NDH3
SsaP (T)	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4N8U7
SyfB (T1-5)	NRPS	Syringafactin synthetase B	*Pseudomonas syringae* group *genomosp.* 3	WP_011104220.1
Tcp12 (T)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	Q70AZ6
TqaA (T1-3)	NRPS	Tryptoquialanine synthetase A	*Penicillium aethiopicum*	ADY16697.1
TqaB (T)	NRPS	Tryptoquialanine synthetase B	*Penicillium aethiopicum*	ADY16689.1
TwmB (T)	PKS-NRPS	Wortmanamide synthetase B	*Talaromyces wortmannii*	QBC19710.1
VibF (T)	NRPS	Vibriobactin synthetase F	*Vibrio cholerae*	WP_001923521.1

Table S7. Comparative analysis of thiolation domain residues. Black text is bacterial T domains, and red text is fungal T domains.

Protein	A.A. 1	A.A. 2	A.A. 3	A.A. 4	A.A. 5	A.A. 6	A.A. 7	A.A. 8	A.A. 9	A.A. 10*	A.A. 11
PlmA T	D	H	F	F	H	L	G	G	D	S	I
PlmJ T1	Q	N	F	F	H	L	G	G	D	S	I
PlmJ T2	D	N	F	F	H	L	G	G	D	S	I
PlmK T	D	D	F	F	A	L	G	G	D	S	L
Aba1 T1	S	K	F	F	D	L	N	G	D	S	I
Aba1 T2	D	N	F	F	H	L	G	G	H	S	L
Aba1 T3	D	N	F	F	D	L	G	G	H	S	L
Aba1 T4	D	N	F	F	H	L	G	G	H	S	L
Aba1 T5	D	D	F	F	E	L	G	G	H	S	L
Aba1 T6	D	N	F	F	D	L	G	G	H	S	L
Aba1 T7	D	N	F	F	H	L	G	G	H	S	L
Aba1 T8	D	N	F	F	D	L	G	G	H	S	L
Aba1 T9	D	N	F	F	H	L	G	G	H	S	L
Alb01 T1	M	D	F	F	A	V	G	G	H	S	V
Alb01 T2	D	N	F	F	A	L	G	G	H	S	L
Alb01 T3	D	N	F	F	E	L	G	G	H	S	V
Alb01 T4	D	N	F	F	A	L	G	G	H	S	L
BenY T	D	D	I	L	A	L	G	A	D	S	I
BenZ T1	D	N	F	F	H	L	G	G	D	S	I
BenZ T2	D	N	F	I	Q	L	G	G	D	S	I
BnvB	T	D	F	E	Q	L	L	H	E	S	L
BnvD T1	E	S	F	F	D	L	G	G	H	S	L
BnvD T2	D	N	F	F	D	L	G	G	H	S	L
BnvD T3	D	S	F	F	D	L	G	G	H	S	L
BnvD T4	D	A	F	F	E	L	G	G	H	S	L

BnvF T	D	D	F	F	E	L	G	G	Y	S	L
FkbP T	D	D	F	F	T	L	G	G	H	S	L
GliP T1	K	D	F	F	A	M	G	G	N	S	L
GliP T2	D	D	F	R	A	L	G	G	H	S	V
GrsA T	D	N	F	Y	A	L	G	G	D	S	I
GrsB T1	D	N	F	F	S	L	G	G	H	S	L
GrsB T2	D	N	F	F	E	L	G	G	H	S	L
GrsB T3	D	D	F	F	T	I	G	G	H	S	L
GrsB T4	D	N	F	F	E	L	G	G	H	S	L
IvoA T	Q	S	F	I	R	N	G	G	D	S	I
NanA T1	D	D	F	F	Q	L	G	G	N	S	M
NanA T2	D	N	F	F	H	I	G	G	D	S	V
NanA T3	D	D	F	F	R	L	G	G	D	S	I
NpsP1	A	D	L	F	E	K	N	G	S	S	L
NpsP3 T	E	G	F	F	A	L	G	G	D	S	L
NpsP4 T	A	G	P	A	T	D	A	G	T	P	S
NpsP5 T	D	D	F	F	A	H	G	G	G	A	L
PsyA T1	D	N	F	F	H	F	G	G	D	S	I
PsyA T2	D	N	F	F	D	V	G	G	D	S	I
PsyC T	D	D	F	H	V	L	G	G	D	S	V
RapP T	D	D	F	F	A	L	G	G	H	S	L
Sbtl2 T	E	D	F	F	E	L	G	A	H	S	L
SrfAA T1	D	N	F	F	E	T	G	G	H	S	L
SrfAA T2	D	H	F	F	D	I	G	G	H	S	L
SrfAA T3	D	N	F	F	S	L	G	G	D	S	I
SsaH	D	-	L	F	E	N	D	G	S	S	L
SsaL T	E	G	F	F	S	L	G	G	D	S	L
SsaN T	D	D	F	F	Q	V	G	G	Y	S	L

SsaP T	D	D	F	F	A	F	G	G	G	S	L
SyfB T1	D	R	F	F	E	L	G	G	H	S	L
SyfB T2	D	N	F	F	E	L	G	G	H	S	L
SyfB T3	D	H	F	F	D	M	G	G	H	S	L
SyfB T4	D	H	F	F	E	L	G	G	H	S	L
SyfB T5	D	S	F	F	E	L	G	G	H	S	L
Tcp12 T	D	A	F	H	D	L	G	G	S	S	A
TqaA T1	H	N	F	F	A	L	G	G	D	S	V
TqaA T2	D	N	F	Y	H	L	G	G	D	S	I
TqaA T3	D	N	F	F	F	R	G	G	H	S	I
TqaB T	D	N	Y	F	S	L	G	G	T	S	L
TwmB T	A	M	R	L	D	Q	M	G	D	S	L
VibF T	D	D	F	F	D	F	G	G	H	S	L

Table S8. Protein sequences used for the condensation domain comparison in Figure S10.

Protein (domains)	Enzyme class	Function	Origin	Accession No.
PlmA (C1,C2)	NRPS	Penilumamide synthetase I	*Aspergillus flavipes* CNL-338 (this work)	ON297683
PlmJ (C1,C2)	NRPS	Penilumamide synthetase II	*Aspergillus flavipes* CNL-338 (this work)	ON297683
PlmK (C)	NRPS	Penilumamide synthetase III	*Aspergillus flavipes* CNL-338 (this work)	ON297683
Aba1 (C1-9)	NRPS	Aureobasidin A1 complex	*Aureobasidium pullulans*	ACJ04424.1
Alb01 (C1,C2)	PKS-NRPS	Albicidin synthetase 1	*Xanthomonas albilineans*	CAE52339.1
BenY (C)	NRPS	Benzomalvin synthetase Y	*Aspergillus terreus*	P9WEU8.1
BenZ (C1,C2)	NRPS	Benzomalvin synthetase Z	*Aspergillus terreus*	P9WEU9.1
FkbP (C1,C2)	NRPS	FK506-binding protein	*Streptomyces hygroscopicus* subsp. *ascomyceticus*	AAF86395.1
Fum14p (C)	NRPS	Fumonisin biosynthesis protein 14	*Fusarium verticillioides*	AAN74817.2
GliP (C1,C2)	NRPS	Gliotoxin synthetase	*Aspergillus fumigatus*	AAW03307.1
GrsA (C)	NRPS	Gramicidin S synthetase A	*Brevibacillus brevis*	P0C062.1
GrsB (C1-3)	NRPS	Gramicidin S synthetase B	*Brevibacillus brevis*	CAA43838.1
IvoA (C1,C2)	NRPS	Ivory mutation-related protein A	*Aspergillus nidulans* FGSC A4	C8V7P4.1
LNKS (C)	PKS-NRPS	Lovastatin nonaketide synthase (LovB)	*Aspergillus terreus*	Q9Y8A5.1
NanA (C1,C2)	NRPS	Nanangelenin synthetase A	*Aspergillus nanangensis*	QIQ51365.1
NpsP2	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76661.1
NpsP4 (C)	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76666.1
NpsP7	NRPS	Napsamycin biosynthesis protein	*Streptomyces* sp. DSM 5940	ADY76678.1

plu2796 (C)	dehydrogenase-NRPS	Pepteridine synthetase	*Photorhabdus laumondii* subsp. *laumondii* TTO1	CAE15170.1
PsyA (C1,C2)	NRPS	Psychrophilin synthetase A	*Penicillium* sp. YT-2016	AMQ36132.1
PsyC (C)	NRPS	Psychrophilin synthetase C	*Penicillium* sp. YT-2016	AMQ36134.1
RapP (C1,C2)	NRPS	Pipecolate incorporating enzyme	*Streptomyces hygroscopicus*	Q54298.1
SbtI2 (C)	NRPS	Serobactin synthetase 2	*Herbaspirillum seropedicae*	QDD64765.1
SrfAA (C1)	NRPS	Surfactin synthetase A	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_388230.2
SsaI	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4NDG7
SsaN (C)	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4NDH3
SsaD	NRPS	Sansanmycin biosynthesis protein	*Streptomyces* sp. SS	M4N8V1
SyfB (C3,C5)	NRPS	Syringafactin synthetase B	*Pseudomonas syringae* group *genomosp.* 3	WP_011104220.1
Tcp12 (C2)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	Q70AZ6
TqaA (C1-4)	NRPS	Tryptoquialanine synthetase A	*Penicillium aethiopicum*	ADY16697.1
TqaB (C)	NRPS	Tryptoquialanine synthetase B	*Penicillium aethiopicum*	ADY16689.1
TwmB (C)	PKS-NRPS	Wortmanamide synthetase B	*Talaromyces wortmannii*	QBC19710.1
VibF (C1)	NRPS	Vibriobactin synthetase F	*Vibrio cholerae*	WP_001923521.1
VibH	NRPS	Vibriobactin synthetase H	*Vibrio cholerae*	AAD48879.1

Table S9. Comparative analysis of condensation domain catalytic residues based on canonical functions. Black text is bacterial C domains, and red text is fungal C domains. Cs = starter C domain, Cy = terminal cyclizing C domain, E = epimerization domain, I = interfacing domain, X = interfacing domain that recruits Oxy enzymes, and dual E/C = bifunctional C domain that also epimerizes the donor substrate. $^{X}C_{Y}$ notation denotes the donor substrate as X and the acceptor substrate as Y, where L/D = proteinogenic amino acid stereochemistry.

Protein	A.A. 1	A.A. 2*	A.A. 3*	A.A. 4	A.A. 5	A.A. 6	A.A. 7*	A.A. 8*	A.A. 9	A.A. 10	Domain Type
PlmA C1	-	T	Y	T	K	S	E	E	A	C	
PlmA C2	L	S	H	T	Q	Y	D	G	V	S	
PlmJ C1	M	H	H	S	I	S	D	R	W	A	Other
PlmJ C2	I	S	H	A	Q	Y	D	G	L	S	
PlmK C	L	T	H	T	Q	I	D	A	Y	C	
Aba1 C2	M	H	H	T	I	Y	D	G	W	S	
Aba1 C3	M	H	H	I	I	S	D	G	W	S	
Aba1 C4	M	H	H	I	I	S	D	G	W	S	
Aba1 C5	M	H	H	I	I	S	D	G	W	S	
Aba1 C6	M	H	H	I	I	S	D	G	W	S	
Aba1 C7	M	H	H	T	I	Y	D	G	W	S	$^{L}C_{L}$
Aba1 C8	M	H	H	I	I	S	D	G	W	S	
GliP C1	A	H	H	S	L	I	D	G	Y	S	
GrsB C2	M	H	H	I	I	S	D	G	V	S	
GrsB C3	M	H	H	I	I	S	D	G	V	S	
PsyA C1	M	H	H	A	L	Y	D	A	W	S	
Aba1 C1	M	H	H	I	I	S	D	G	W	S	
GrsB C1	F	H	H	I	L	M	D	G	W	C	$^{D}C_{L}$
TqaA C3	M	S	H	A	I	M	D	R	T	T	
SrfAA C1	V	H	H	V	I	S	D	G	I	S	Cs
VibH	A	H	H	I	V	L	D	G	Y	G	
Aba1 C9	L	S	H	A	L	Y	D	G	L	S	Cy

BenY C	L	S	H	S	Q	Y	D	G	L	S	
FkbP C2	L	H	H	L	A	G	D	G	W	S	
GliP C2	T	S	H	A	V	A	D	L	N	S	
NanA C2	I	S	H	A	L	W	D	G	G	P	
PsyC C	L	S	H	A	Q	H	D	G	F	S	Cy
RapP C2	L	H	H	I	A	G	D	G	W	S	
TqaA C4	I	S	H	S	Q	W	D	G	V	S	
TqaB C	T	T	H	A	Q	F	D	A	M	T	
VibF C1	A	D	M	I	A	C	D	A	Q	S	
GrsA C	I	H	H	L	V	V	D	G	I	S	
IvoA C1	V	H	H	L	V	V	D	F	V	S	E
TqaA C2	I	H	H	L	V	V	D	L	V	S	
SyfB C3	Y	H	H	V	I	M	D	H	I	A	dual E/C
SyfB C5	F	H	H	L	I	M	D	H	V	A	
Sbtl2 C	C	S	P	L	V	A	D	Y	R	S	I
Tcp12 C2	V	H	R	I	A	A	D	D	D	S	X
Alb01 C1	I	H	H	I	I	S	D	G	W	S	
Alb01 C2	I	H	H	I	V	F	D	G	W	S	
BenZ C1	M	H	H	S	V	I	D	G	L	S	
BenZ C2	L	S	H	A	Q	Y	D	G	W	C	
FkbP C1	V	H	H	V	A	G	D	G	W	S	Other
Fum14p C	L	D	H	T	H	C	D	A	F	S	
IvoA C2	F	S	H	T	I	I	D	A	A	S	
LNKS C	Y	H	R	L	V	G	D	G	S	T	
NanA C1	I	H	H	A	I	C	D	L	W	A	

NpsP2	I	D	H	I	I	N	D	L	A	S	
NpsP4 C	E	H	H	L	V	H	D	G	R	S	
NpsP7	L	D	H	A	L	V	D	E	H	S	
PsyA C2	L	S	H	A	Q	Y	D	G	V	S	
RapP C1	V	H	H	I	A	G	D	G	W	S	Other
SsaD	L	D	H	A	L	V	D	E	Q	S	
SsaI	I	D	H	I	I	N	D	L	A	S	
SsaN C	E	H	H	L	V	H	D	G	R	S	
TqaA C1	M	H	H	C	I	C	D	R	W	A	
TwmB C	V	H	P	L	V	L	D	A	T	S	

Table S10. Protein sequences used for the condensation domain comparison in Figure S11.

Protein (domains)	Enzyme class	Function	Origin	Accession No.
PlmA (C1,C2)	NRPS	Penilumamide synthetase I	*Aspergillus flavipes* CNL-338	ON297683
PlmJ (C1,C2)	NRPS	Penilumamide synthetase II	*Aspergillus flavipes* CNL-338	ON297683
PlmK (C)	NRPS	Penilumamide synthetase III	*Aspergillus flavipes* CNL-338	ON297683
Aba1 (C9)	NRPS	Aureobasidin A1 complex	*Aureobasidium pullulans*	ACJ04424.1
AcmB (C1,C2,C4)	NRPS	Actinomycin synthetase II	*Streptomyces anulatus*	O68487
AcmC (C1-3)	NRPS	Actinomycin synthetase III	*Streptomyces anulatus*	Q9L8H4
AebF (C)	NRPS	Enterobactin synthetase F	*Vibrio campbellii*	KGR33264.1
AltG (C)	NRPS	Bromoalterochromide interfacing domain	*Pseudoalteromonas luteoviolacea*	WP_063365585.1
AltI (C)	NRPS	Bromoalterochromide interfacing domain	*Pseudoalteromonas luteoviolacea*	WP_063365570.1
AmbE (C)	NRPS	AMB synthetase E	*Pseudomonas aeruginosa* PAK	VUY44935.1
ArfA (C1,C2)	NRPS	Arthrofactin synthetase A	*Pseudomonas* sp. MIS38	BAC67534.2
ArfC (C2,C4)	NRPS	Arthrofactin synthetase C	*Pseudomonas* sp. MIS38	Q84BQ4
Bamb_5915 (C)	NRPS	Enacyloxin synthetase	*Burkholderia ambifaria* AMMD	ABI91460.1
BpsA (C1-3)	NRPS	Balhimycin synthetase A	*Amycolatopsis balhimycina*	Q939Z1
BpsB (C1-4)	NRPS	Balhimycin synthetase B	*Amycolatopsis balhimycina*	Q939Z0
BpsC (C1,C2)	NRPS	Balhimycin synthetase C	*Amycolatopsis balhimycina*	Q939Y9
CdaPS1 (C1-4,C7)	NRPS	CDA synthetase 1	*Streptomyces coelicolor*	WP_011028842.1
CdaPS2 (C1-4)	NRPS	CDA synthetase 2	*Streptomyces coelicolor*	WP_011028843.1
CroK (C2)	NRPS	Crochelin synthetase	*Chondromyces crocatus*	AIR74925.1
CrpD (C2)	NRPS	Cryptophycin synthetase D	*Nostoc* sp. ATCC 53789	QHG20896.1

Dbv16 (C1,C2)	NRPS	Glycopeptide synthetase X domain	*Nonomuraea gerenzanensis*	Q7WZ75
Dbv17 (C1-4)	NRPS	Glycopeptide synthetase E domain	*Nonomuraea gerenzanensis*	Q7WZ74
Dbv25 (C1,C2)	NRPS	Glycopeptide synthetase E domain	*Nonomuraea gerenzanensis*	Q7WZ66
Dbv26 (C)	NRPS	Glycopeptide synthetase	*Nonomuraea gerenzanensis*	Q7WZ65
DhbF (C1)	NRPS	Bacillibactin synthetase F	*Bacillus cereus*	WP_011198679.1
EntF (C)	NRPS	Enterobactin synthetase F	*Escherichia coli*	CAD6019783.1
ErcD (C5)	NRPS	Erythrochelin synthetase	*Saccharopolyspora erythraea* NRRL 2338	CAM02313.1
Fum14p (C)	NRPS	Fumonisin biosynthesis protein 14	*Fusarium verticillioides*	AAN74817.2
GliP (C2)	NRPS	Gliotoxin synthetase	*Aspergillus fumigatus*	AAW03307.1
GrsA (C)	NRPS	Gramicidin S synthetase A	*Brevibacillus brevis*	P0C062.1
GrsB (C1)	NRPS	Gramicidin S synthetase B	*Brevibacillus brevis*	P0C064.2
GrsB (C1-3)	NRPS	Gramicidin S synthetase B	*Brevibacillus brevis*	CAA434838.1
HcsF (C)	NRPS	Interfacing domain	*Pseudomonas thivervalensis*	WP_053122086.1
HcsI (C)	NRPS	Interfacing domain	*Pseudomonas thivervalensis*	WP_053122092.1
HMWP2 (C1)	NRPS	Yersiniabactin synthetase	*Pseudomonas syringae* group *genomosp.* 3	WP_011104107.1
IcoA (C3,C4)	NRPS	Icosalide synthetase	*Burkholderia gladioli*	AYA44686.1
IvoA (C2)	NRPS	Ivory mutation-related protein A	*Aspergillus nidulans* FGSC A4	C8V7P4.1
LicA (C1,C4)	NRPS	Lichenysin synthetase A	*Bacillus licheniformis*	WP_011197536.1
LicB (C4)	NRPS	Lichenysin synthetase B	*Bacillus licheniformis*	WP_011197537.1
LNKS (C)	PKS-NRPS	Lovastatin nonaketide synthase (LovB)	*Aspergillus terreus*	Q9Y8A5.1

MbtB (C)	NRPS	Mycobactin synthetase B	*Mycobacterium avium*	WP_010949506.1
MbtB (C)	NRPS	Mycobactin synthetase B	*Mycobacterium tuberculosis*	WP_010950705.1
McyA (C)	NRPS	Microcystin synthetase A	*Microcystis aeruginosa* PCC 7806	CAO90227.1
MloJ (C2)	PKS-NRPS	Malonomycin synthetase J	*Streptomyces rimosus* subsp. *paromomycinus*	AYJ71721.1
NanA (C2)	NRPS	Nanangelenin synthetase A	*Aspergillus nanangensis*	QIQ51365.1
NbtF (C)	NRPS	Mycobactin synthetase F	*Nocardia farcinica*	WP_011207299.1
NocB (C)	NRPS	Nocardicin synthetase B	*Nocardia uniformis* subsp. *tsuyamanensis*	AAT09805.1
NosA (C2,C3)	NRPS	Nostopeptolide synthetase A	*Nostoc* sp. GSV224	Q9RAH4
NosC (C1,C2)	NRPS	Nostopeptolide synthetase C	*Nostoc* sp. GSV224	Q9RAH2
NosD (C1,C2)	NRPS	Nostopeptolide synthetase D	*Nostoc* sp. GSV224	Q9RAH1
NRPS (C1,C2)	NRPS	Nonribosomal peptide synthetase	*Pectobacterium atrosepticum*	WP_011093071.1
NRPS (C1)	NRPS	Nonribosomal peptide synthetase	*Pseudomonas aeruginosa* PAO1	NP_252017.1
NRPS (C1)	NRPS	Nonribosomal peptide synthetase	*Nocardia farcinica*	WP_011209327.1
NRPS (C1)	NRPS	Nonribosomal peptide synthetase	*Photobacterium profundum*	WP_011218392.1
NRPS (C1)	NRPS	Nonribosomal peptide synthetase	*Yersinia pseudotuberculosis*	WP_011193026.1
NRPS (C2)	NRPS	Nonribosomal peptide synthetase	*Yersinia pseudotuberculosis*	WP_011193027.1
NRPS (C2)	NRPS	Nonribosomal peptide synthetase	*Streptomyces coelicolor*	WP_011031827.1
NRPS (C1,C3)	NRPS	Nonribosomal peptide synthetase	*Chromobacterium violaceum*	WP_011136349.1
NRPS (C1,C3)	NRPS	Nonribosomal peptide synthetase	*Burkholderia pseudomallei*	WP_011205655.1
NRPS (C4)	NRPS	Nonribosomal peptide synthetase	*Pseudomonas protegens*	WP_011062464.1
OfaA (C1)	NRPS	Orfamide A synthetase A	*Pseudomonas protegens*	WP_011060446.1
OfaB (C2,C4)	NRPS	Orfamide A synthetase B	*Pseudomonas protegens*	WP_011060447.1

OrbJ (C2)	NRPS	Ornibactin synthetase J	*Burkholderia cepacia*	AUD11993.1
PchE (C)	NRPS	Pyochelin synthetase E	*Pseudomonas protegens*	ABW70809.1
PchE (C)	NRPS	Pyochelin synthetase E	*Pseudomonas aeruginosa* PAO1	NP_252916.1
PchF (C1)	NRPS	Pyochelin synthetase F	*Burkholderia pseudomallei*	WP_011205430.1
PchF (C1)	NRPS	Pyochelin synthetase F	*Pseudomonas protegens*	WP_011061772.1
PCZA363.3 (C1-3)	NRPS	Vancomycin group synthetase	*Amycolatopsis orientalis*	O52819
PCZA363.4 (C1-4)	NRPS	Vancomycin group synthetase	*Amycolatopsis orientalis*	O52820
PCZA363.5 (C1,C2)	NRPS	Vancomycin group synthetase	*Amycolatopsis orientalis*	O52821
PfbI (C)	NRPS	Interfacing domain	*Alcanivorax pacificus*	WP_052269209.1
plu2320 (C1)	NRPS	Yersiniabactin synthetase	*Photorhabdus laumondii*	WP_011146562.1
plu2670 (C6,C9)	NRPS	Nonribosomal peptide synthetase	*Photorhabdus laumondii*	WP_011146892.1
plu2796 (C)	dehydrogenase-NRPS	Pepteridine synthetase	*Photorhabdus laumondii* subsp. *laumondii* TTO1	CAE15170.1
PpsA (C1-3)	NRPS	Plipastatin synthetase A	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_389716.2
PpsB (C2,C3)	NRPS	Plipastatin synthetase B	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_389715.1
PpsC (C1-3)	NRPS	Plipastatin synthetase C	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_389714.1
PpsD (C2,C4)	NRPS	Plipastatin synthetase D	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_389713.2
PpsE (C)	NRPS	Plipastatin synthetase E	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_389712.1
PSEEN_RS 14935 (C)	NRPS	Interfacing domain	*Pseudomonas entomophila*	WP_011534377.1
PS1 (C1,C2)	NRPS	Peptide synthetase	*Streptomyces lavendulae*	Q93N86
PS2 (C1-3,C6)	NRPS	Peptide synthetase	*Streptomyces lavendulae*	Q93N87
PS3 (C1,C2)	NRPS	Peptide synthetase	*Streptomyces lavendulae*	Q93N88
PS4 (C1,C2)	NRPS	Peptide synthetase	*Streptomyces lavendulae*	Q93N89

Pvd2 (C2)	NRPS	Pyoverdine synthetase 2	*Pseudomonas syringae* group *genomosp.* 3	WP_011103871.1
Pvd4 (C2)	NRPS	Pyoverdine synthetase 4	*Pseudomonas syringae* group *genomosp.* 3	WP_011103873.1
PvdD (C3)	NRPS	Pyoverdine synthetase D	*Pseudomonas protegens*	WP_011062374.1
PvdI (C4)	NRPS	Pyoverdine synthetase I	*Pseudomonas protegens*	WP_011062376.1
PvdJ (C)	NRPS	Pyoverdine synthetase J	*Pseudomonas taiwanensis*	AJW67534.1
PvdK (C)	NRPS	Pyoverdine synthetase K	*Pseudomonas fluorescens*	WP_011333311.1
RapP (C1,C2)	NRPS	Pipecolate incorporating enzyme	*Streptomyces rapamycinicus* NRRL 5491	BCH36730.1
SbtI2 (C)	NRPS	Serobactin synthetase 2	*Herbaspirillum seropedicae*	QDD64765.1
SbtI2 (C)	NRPS	Serobactin synthetase 2	*Herbaspirillum seropedicae*	WP_048348543.1
SfmC (C)	NRPS	Saframycin synthetase C	*Streptomyces lavendulae*	ABI22133.1
SgcC5	NRPS	C-1027 condensation domain	*Streptomyces* sp. CB02366	ANY94448.1
SnbC (C1-3)	NRPS	Pristinamycin I synthetase 2	*Streptomyces pristinaespiralis*	Q54959
SnbDE (C1-3)	NRPS	Pristinamycin I synthase 3 and 4	*Streptomyces pristinaespiralis*	O07944
SrfAA (C1,C2,C4)	NRPS	Surfactin synthetase A	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_388230.2
SrfAB (C1,C2,C4)	NRPS	Surfactin synthetase B	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_388231.2
SrfAC (C)	NRPS	Surfactin synthetase C	*Bacillus subtilis* subsp. *subtilis* str. 168	NP_388233.2
StaA (C1,C2)	NRPS	Staurosporine synthetase A	*Streptomyces toyocaensis*	Q8KLL3
StaB (C1,C2)	NRPS	Staurosporine synthetase B	*Streptomyces toyocaensis*	Q8KLL4
StaC (C1-4)	NRPS	Staurosporine synthetase C	*Streptomyces toyocaensis*	Q8KLL5
StaD (C1,C2)	NRPS	Staurosporine synthetase D	*Streptomyces toyocaensis*	Q8KLL6
SyfA (C1,C2)	NRPS	Syringafactin synthetase A	*Pseudomonas syringae* group *genomosp.* 3	WP_011104219.1

SyfB (C3,C5)	NRPS	Syringafactin synthetase B	*Pseudomonas syringae* group *genomosp.* 3	WP_011104220.1
SyrE (C5,C6)	NRPS	Syringomycin synthetase	*Pseudomonas syringae* pv. *syringae*	O85168
TaiE (C)	PKS-NRPS	Thailandamide synthetase E	*Cupriavidus taiwanensis*	WP_012356046.1
Tcp9 (C1,C2)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	Q70AZ9
Tcp10 (C)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	Q70AZ8
Tcp11 (C1-4)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	Q70AZ7
Tcp12 (C1,C2)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	Q70AZ6
Tcp12 (C2)	NRPS	Teicoplanin synthetase	*Actinoplanes teichomyceticus*	CAE53353.1
TqaA (C4)	NRPS	Tryptoquialanine synthetase A	*Penicillium aethiopicum*	ADY16697.1
TwmB (C)	PKS-NRPS	Wortmanamide synthetase B	*Talaromyces wortmannii*	QBC19710.1
TycB (C1)	NRPS	Tyrocidine synthetase B	*Brevibacillus brevis*	QDS34188.1
Var5 (C)	NRPS	Interfacing domain	*Variovorax paradoxus*	ALG65340.1
VarH (C)	NRPS	Interfacing domain	*Variovorax boronicumulans*	WP_062469880.1
VibF (C1)	NRPS	Vibriobactin synthetase F	*Vibrio cholerae*	WP_001923521.1
VibH	NRPS	Vibriobactin synthetase H	*Vibrio cholerae*	AAD48879.1
Zmn19	NRPS	Zeamine condensation domain	*Serratia plymuthica* RVH1	CCM44339.1

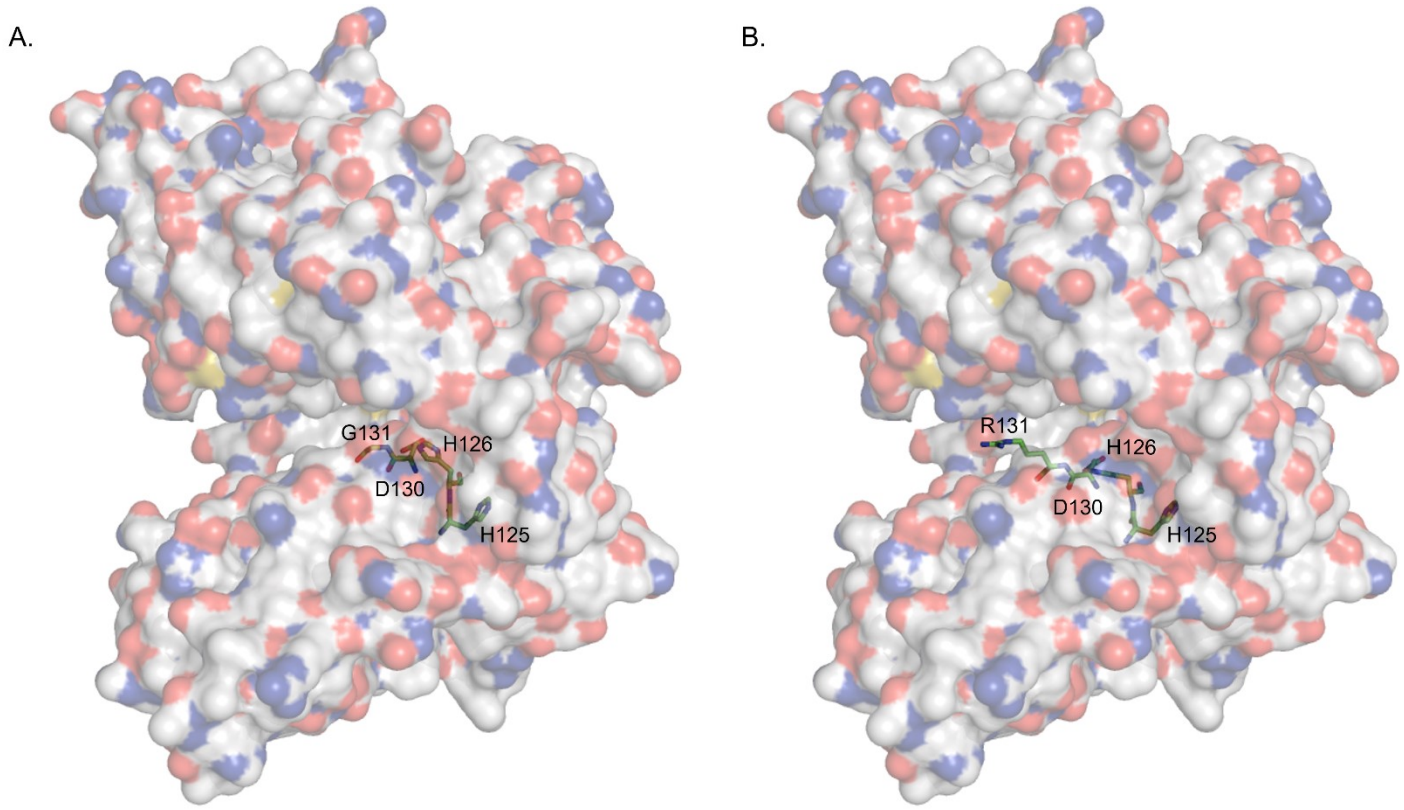


Figure S12. Structural comparison of VibH and PlmJ_C1. A) Electrostatic surface representation of VibH (PDB: 1L5A)[24] showing the HHxxxDG motif, comprising residues H125, H126, D130 and G131, as sticks. B) A homology model of PlmJ_C1 was generated using AlphaFold2[23], and it was aligned to the crystal structure of VibH using PyMol software (RMSD = 10.312). The HHxxxDR motif of PlmJ_C1, comprising H125, H126, D130 and R131, is indicated as sticks.

Supplementary References

(1) Meyer, S. W.; Mordhorst, T. F.; Lee, C.; Jensen, P. R.; Fenical, W.; Köck, M. Penilumamide, a Novel Lumazine Peptide Isolated from the Marine-Derived Fungus, Penicillium Sp. CNL-338. *Org Biomol Chem* **2010**, *8* (9), 2158–2163. https://doi.org/10.1039/b910629d.

(2) Lee, K. K. M.; Da Silva, N. A.; Kealey, J. T. Determination of the Extent of Phosphopantetheinylation of Polyketide Synthases Expressed in Escherichia Coli and Saccharomyces Cerevisiae. *Anal Biochem* **2009**, *394* (1), 75–80. https://doi.org/10.1016/j.ab.2009.07.010.

(3) Keszenman-Pereyra, D.; Lawrence, S.; Twfieg, M.-E.; Price, J.; Turner, G. The NpgA/ CfwA Gene Encodes a Putative 4'-Phosphopantetheinyl Transferase Which Is Essential for Penicillin Biosynthesis in Aspergillus Nidulans. *Curr Genet* **2003**, *43* (3), 186–190. https://doi.org/10.1007/s00294-003-0382-7.

(4) Luo, R.; Liu, B.; Xie, Y.; Li, Z.; Huang, W.; Yuan, J.; He, G.; Chen, Y.; Pan, Q.; Liu, Y.; Tang, J.; Wu, G.; Zhang, H.; Shi, Y.; Liu, Y.; Yu, C.; Wang, B.; Lu, Y.; Han, C.; Cheung, D. W.; Yiu, S.-M.; Peng, S.; Xiaoqian, Z.; Liu, G.; Liao, X.; Li, Y.; Yang, H.; Wang, J.; Lam, T.-W.; Wang, J. SOAPdenovo2: An Empirically Improved Memory-Efficient Short-Read de Novo Assembler. *GigaScience* **2012**, *1*, 18. https://doi.org/10.1186/2047-217X-1-18.

(5) Peng, Y.; Leung, H. C. M.; Yiu, S. M.; Chin, F. Y. L. IDBA-UD: A de Novo Assembler for Single-Cell and Metagenomic Sequencing Data with Highly Uneven Depth. *Bioinforma Oxf Engl* **2012**, *28* (11), 1420–1428. https://doi.org/10.1093/bioinformatics/bts174.

(6) Blin, K.; Shaw, S.; Kloosterman, A. M.; Charlop-Powers, Z.; van Wezel, G. P.; Medema, M. H.; Weber, T. AntiSMASH 6.0: Improving Cluster Detection and Comparison Capabilities. *Nucleic Acids Res* **2021**, *49* (W1), W29–W35. https://doi.org/10.1093/nar/gkab335.

(7) Szewczyk, E.; Nayak, T.; Oakley, C. E.; Edgerton, H.; Xiong, Y.; Taheri-Talesh, N.; Osmani, S. A.; Oakley, B. R.; Oakley, B. Fusion PCR and Gene Targeting in Aspergillus Nidulans. *Nat Protoc* **2006**, *1* (6), 3111–3120. https://doi.org/10.1038/nprot.2006.405.

(8) Shibuya, I.; Gomi, K.; Iimura, Y.; Takahashi, K.; Tamura, G.; Hara, S. Molecular Cloning of the Glucoamylase Gene of Aspergillus Shirousami and Its Expression in Aspergillus Oryzae. *Agric Biol Chem* **1990**, *54* (8), 1905–1914.

(9) Kawai, S.; Hashimoto, W.; Murata, K. Transformation of Saccharomyces Cerevisiae and Other Fungi. *Bioeng Bugs* **2010**, *1* (6), 395–403. https://doi.org/10.4161/bbug.1.6.13257.

(10) Bloudoff, K.; Fage, C. D.; Marahiel, M. A.; Schmeing, T. M. Structural and Mutational Analysis of the Nonribosomal Peptide Synthetase Heterocyclization Domain Provides Insight into Catalysis. *Proc Natl Acad Sci U S A* **2017**, *114* (1), 95–100. https://doi.org/10.1073/pnas.1614191114.

(11) Mistry, J.; Chuguransky, S.; Williams, L.; Qureshi, M.; Salazar, G. A.; Sonnhammer, E. L. L.; Tosatto, S. C. E.; Paladin, L.; Raj, S.; Richardson, L. J.; Finn, R. D.; Bateman, A. Pfam: The Protein Families Database in 2021. *Nucleic Acids Res* **2021**, *49* (D1), D412–D419. https://doi.org/10.1093/nar/gkaa913.

(12) Jez, J. M.; Ferrer, J. L.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. Dissection of Malonyl-Coenzyme A Decarboxylation from Polyketide Formation in the Reaction Mechanism of a Plant Polyketide Synthase. *Biochemistry* **2000**, *39* (5), 890–902. https://doi.org/10.1021/bi991489f.

(13) McQuade, T. J.; Shallop, A. D.; Sheoran, A.; Delproposto, J. E.; Tsodikov, O. V.; Garneau-Tsodikova, S. A Nonradioactive High-Throughput Assay for Screening and Characterization of Adenylation Domains for Nonribosomal Peptide Combinatorial Biosynthesis. *Anal Biochem* **2009**, *386* (2), 244–250. https://doi.org/10.1016/j.ab.2008.12.014.

(14) Kadi, N.; Challis, G. L. Chapter 17. Siderophore Biosynthesis a Substrate Specificity Assay for Nonribosomal Peptide Synthetase-Independent Siderophore Synthetases Involving Trapping of Acyl-Adenylate Intermediates with Hydroxylamine. *Methods Enzymol* **2009**, *458*, 431–457. https://doi.org/10.1016/S0076-6879(09)04817-4.

(15) Reitz, Z. L.; Hardy, C. D.; Suk, J.; Bouvet, J.; Butler, A. Genomic Analysis of Siderophore β-Hydroxylases Reveals Divergent Stereocontrol and Expands the Condensation Domain Family. *Proc Natl Acad Sci U S A* **2019**, *116* (40), 19805–19814. https://doi.org/10.1073/pnas.1903161116.

(16) Rausch, C.; Hoof, I.; Weber, T.; Wohlleben, W.; Huson, D. H. Phylogenetic Analysis of Condensation Domains in NRPS Sheds Light on Their Functional Evolution. *BMC Evol Biol* **2007**, *7*, 78. https://doi.org/10.1186/1471-2148-7-78.

(17) Gao, X.; Chooi, Y.-H.; Ames, B. D.; Wang, P.; Walsh, C. T.; Tang, Y. Fungal Indole Alkaloid Biosynthesis: Genetic and Biochemical Investigation of the Tryptoquialanine Pathway in Penicillium Aethiopicum. *J Am Chem Soc* **2011**, *133* (8), 2729–2741. https://doi.org/10.1021/ja1101085.

(18) Clevenger, K. D.; Ye, R.; Bok, J. W.; Thomas, P. M.; Islam, M. N.; Miley, G. P.; Robey, M. T.; Chen, C.; Yang, K.; Swyers, M.; Wu, E.; Gao, P.; Wu, C. C.; Keller, N. P.; Kelleher, N. L. Interrogation of Benzomalvin Biosynthesis Using Fungal Artificial Chromosomes with Metabolomic Scoring (FAC-MS): Discovery of a Benzodiazepine Synthase Activity. *Biochemistry* **2018**, *57* (23), 3237–3243. https://doi.org/10.1021/acs.biochem.8b00076.

(19) Zhao, M.; Lin, H.-C.; Tang, Y. Biosynthesis of the α-Nitro-Containing Cyclic Tripeptide Psychrophilin. *J Antibiot (Tokyo)* **2016**, *69* (7), 571–573. https://doi.org/10.1038/ja.2016.33.

(20) Kaysser, L.; Tang, X.; Wemakor, E.; Sedding, K.; Hennig, S.; Siebenberg, S.; Gust, B. Identification of a Napsamycin Biosynthesis Gene Cluster by Genome Mining. *Chembiochem Eur J Chem Biol* **2011**, *12* (3), 477–487. https://doi.org/10.1002/cbic.201000460.

(21) Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. The Specificity-Conferring Code of Adenylation Domains in Nonribosomal Peptide Synthetases. *Chem Biol* **1999**, *6* (8), 493–505. https://doi.org/10.1016/S1074-5521(99)80082-9.

(22) Challis, G. L.; Ravel, J.; Townsend, C. A. Predictive, Structure-Based Model of Amino Acid Recognition by Nonribosomal Peptide Synthetase Adenylation Domains. *Chem Biol* **2000**, *7* (3), 211–224. https://doi.org/10.1016/s1074-5521(00)00091-0.

(23) Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S. A. A.; Ballard, A. J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A. W.; Kavukcuoglu, K.; Kohli, P.; Hassabis, D. Highly Accurate Protein Structure Prediction with AlphaFold. *Nature* **2021**, *596* (7873), 583–589. https://doi.org/10.1038/s41586-021-03819-2.

(24) Keating, T. A.; Marshall, C. G.; Walsh, C. T.; Keating, A. E. The Structure of VibH Represents Nonribosomal Peptide Synthetase Condensation, Cyclization and Epimerization Domains. *Nat Struct Biol* **2002**, *9* (7), 522–526. https://doi.org/10.1038/nsb810.

(25) Haslinger, K.; Peschke, M.; Brieke, C.; Maximowitsch, E.; Cryle, M. J. X-Domain of Peptide Synthetases Recruits Oxygenases Crucial for Glycopeptide Biosynthesis. *Nature* **2015**, *521* (7550), 105–109. https://doi.org/10.1038/nature14141.