**Supporting Information**

**Nine Posttranslational Modifications During the Biosynthesis of Cinnamycin**

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**Materials**

Oligonucleotide primers for mutagenesis were synthesized by Operon Technologies. Taq and Platinum Pfx DNA polymerases, DpnI, restriction endonucleases, and bacteriophage T4 DNA ligase were purchased from Invitrogen. Phusion DNA polymerase was purchased from New England Biolabs. Cloning vectors (pET and pDuet) were obtained from Novagen. Gel extraction, plasmid mini-prep, and PCR purification kits were purchased from Qiagen. All strains were grown in media acquired from Difco laboratories. Other items procured include isopropyl-1-thio-D-galactopyranoside (IPTG, CalBiochem), iodoacetamide (IAA, Acros Organics), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Aldrich), and dithiothreitol (DTT, Sigma). Endoproteinase LysC was purchased from Roche Applied Science.

**General Methods**

Molecular biological manipulations were carried out using standard techniques.1 PCR was performed using an automatic thermocycler (PTC 150, MJ Research) and DNA sequencing was performed at the Biotechnology Center of the University of Illinois at Urbana-Champaign (UIUC). *Escherichia coli* DH5α cells (UIUC Cell Media Facility) and *E. coli* BL21 (DE3) cells (Stratagene) were used for plasmid preparation and protein expression, respectively. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager-DE-STR (Applied Biosystems). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). GC/MS-MS analysis (Agilent HP 5973 mass spectrometer and a Supelco SPB-1701 30 m x 0.25 mm fused silica capillary column) was performed at the Metabolomics Center of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign.

**Cloning of cinA, cinM, cinX and cinorf7 and construction of expression plasmids**

The genes *cinA*, *cinM*, *cinX* and *cinorf7* were cloned from *S. cinnamoneous* genomic DNA. The amplification of *cinA* was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (55 °C for 30 s), and extending (72 °C for 135 s) using CinA_wt_FP_NdeI_pET15b and CinA_wt_RP_BamHI_pET15b as forward and reverse primers (Table S1). The PCRs (50 μL) included 1x HF buffer (Finnzymes), DMSO (4%), Phusion DNA Polymerase (Finnzymes) (0.02 unit/μL), dNTPs (1 mM), *S. cinnamoneous* genomic DNA, and primers (1 μM each). Amplification of the final PCR product was confirmed by 2% agarose gel electrophoresis, and
the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragment and the pET15b vector were digested in 1 x NEBuffer 4 (New England Biolabs) with NdeI (for 15 h) and BamHI at 37 ºC (for 3 h). The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit (QIAGEN). The resulting DNA insert was ligated with the digested pET15b vector at 16 ºC for 15 h using T4 DNA ligase.

The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin agar plates, and grown at 37 ºC for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicillin medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing.

Similarly, cinM, cinX and cinorf7 were amplified using appropriate primers (Table S1) by PCR. The reactions contained 1xFailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). Amplification of the final PCR products were confirmed by 1% agarose gel electrophoresis and the products were purified using QIAquick PCR purification kits. The resulting DNA fragments and the pET16b and pET28b vectors were digested in 1 x NEBuffer 4 with NdeI and XhoI at 37 ºC (for 15 h). The digested products were purified by agarose gel electrophoresis followed using a QIAquick gel extraction kit. The resulting DNA inserts were ligated with the digested corresponding vectors at 16 ºC for 15 h using T4 DNA ligase to generate cinM-pET16b, cinX-pET28b and cinorf7-pET28b. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin (for pET16b) and LB-kanamycin (for pET28b) agar plates, and grown at 37 ºC for 15 h. Colonies were picked and incubated in 5 mL of LB-ampicillin/kanamycin medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.

**Construction of the CinA(A−1K)**

Site-directed mutagenesis of CinA was performed by multistep overlap extension PCR. First, the amplification of cinA was conducted via 30 cycles of denaturing (94 ºC for 20 s), annealing (55 ºC for 30 s), and extending (72 ºC for 120 s) using the CinA-NdeI-FP primer and CinA-K_RP_(A−1K)_pET15b reverse primer (see Table S1) to yield a 5’ fragment of the mutated CinA(A−1K) gene (FP reaction). The PCR mixtures included 1x FailSafe PreMix G, DMSO (4%), Platinum Pfx DNA polymerase (0.025 unit/μL), Taq DNA polymerase (0.05 unit/μL), and primers (1 μM each). In parallel, a PCR using CinA-K_FP_(A−1K)_pET15b forward primer and the CinA_wt_RP_BamHI_pET15b primer was also conducted to produce the 3’ fragment of the mutated CinA(A−1K) gene using the same PCR conditions that were used for the FP reaction (RP reaction). The overlapping products from the FP reaction and RP reaction were combined in equal amounts and extended by five cycles of denaturing, annealing, and extending in a solution containing 1x FailSafe PreMix G, DMSO (4%), and Platinum Pfx DNA polymerase (0.025 unit/μL). Following the extension, the CinA_NdeI_FP and CinA_wt_RP_BamHI_pET15b primers were added (final concentration of 2 μM), and the reaction mixture was incubated for 25 additional cycles of denaturing, annealing, and extending.

Amplification of the final PCR product was confirmed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits. The
resulting DNA fragment and the pET28 vector were digested in 1x NEBuffer 4 with NdeI (15 h) and BamHI (3 h) at 37 ºC. The digested products were purified by agarose gel electrophoresis followed using a QIAquick gel extraction kit. The resulting DNA insert was ligated with the digested pET15b vector at 16 ºC for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with the ligation product via heat shock, plated on LB-ampicillin agar plates, and grown at 37 ºC for 15 h. Three colonies were picked and incubated in 5 mL of LB-ampicillin medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.

**Generation of a co-expression system in E. coli**

The genes encoding CinA(A–1K), CinM, CinX and Cinorf7 were amplified using appropriate primers (Table S1) using CinA(A–1K)-pET15b, CinM-pET16b, CinX-pET28b and Cinorf7-pET28b as templates following the protocols described above. The gene encoding CinA(A–1K) with an N-terminal FLAG-tag (DYKDDDDKF) was placed into multiple cloning site I (MCSI) of pRSFDuet-1 by using Ascl and HindIII restriction sites, whereas cinM was placed into MCSII of the same vector by using EcoRV and KpnI restriction sites. Double digestion for the corresponding gene and the corresponding vector was carried out with Ascl and HindIII-HF enzymes by use of 1x NEBuffer 4 for 10 h at 37 ºC. Similarly, double digestion for the corresponding gene and the corresponding vector was carried out with EcoRV-HF and KpnI-HF enzymes by use of 1x NEBuffer 4 for 10 h at 37 ºC. In addition, two pACYCDuet-1 vectors were constructed, one containing only cinX in MCSII, and one containing cinorf7 in MCSI and cinX in MSCII. NcoI and HindIII restriction sites were used to introduce cinorf7 whereas EcoRV and XhoI restriction sites were used to place cinX into pACYCDuet-1. For NcoI and HindIII, a double digest reaction was carried out in 1x NEBuffer 2 at 37 ºC for 10 h, whereas for EcoRV and XhoI, a double digest reaction was carried out in the presence of 1x NEBuffer 3 together with 1x BSA (NEB) for 10 h at 37 ºC. The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction kit. The resulting DNA inserts were ligated with the digested corresponding vectors at 16 ºC for 15 h using T4 DNA ligase. The ligation reaction mixture was diluted 5 times with water prior to transformation. E. coli DH5α cells were transformed with 10 µL of the ligation product via heat shock, plated on LB-kanamycin plates for pRSFDuet-1 vector or LB-chloramphenicol agar plates for pACYCDuet-1 vector, and grown at 37 ºC for 15 h. Three colonies were picked and incubated in 5 mL of LB-kanamycin/chloramphenicol medium at 37 ºC for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit. The sequences of the resulting plasmids were confirmed by DNA sequencing.

**Overexpression and purification of His6-CinA, His6-CinA(A–1K), and His6-CinA(A–1K/D15A)**

Peptides were overexpressed and purified from E. coli BL21 (DE3) cultures as described for other His6-LanA peptides. Briefly, purification was accomplished by solubilizing the included bodies from the overexpression experiments using 4 M guanidine hydrochloride and loading this solution onto a 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare). The desired peptides were eluted in 1-2 column volumes of elution buffer containing 4 M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.5, 100 mM NaCl, and 1 M imidazole. Desalting was achieved by preparative reverse phase high performance liquid chromatography
(RP-HPLC) on a Waters system using a C4 PrepLC column. Solvents for preparative RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile/20% water). A gradient of 2 – 100 % of solvent B over 45 min was executed with a flow rate of 8 mL/min. Peptides were detected by absorbance at 220 nm. Lyophilized powder was analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-ToF MS) and desired products were stored at – 20 ºC. MALDI-ToF MS was carried out on a Voyager-DE-STR mass spectrometer (Applied Biosystems).

**Overexpression of His10-CinM and His6-CinX**

*E. coli* BL21 (DE3) cells were transformed with pET16b-CinM and pET28b-CinX. Single colony transformants were grown in a 37 ºC shaker for 12-15 h in 50 mL of Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin (for pET16b-CinM) or 50 μg/mL kanamycin (for pET28b-CinX). A 20 mL aliquot was centrifuged at 5000×g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB containing the corresponding antibiotic, and the culture was grown aerobically at 37 ºC until the A600 was ~ 0.6 to 0.8. IPTG was added to a final concentration of 1 mM and the culture was transferred to 18 ºC for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000×g for 20 min at 4 ºC. The cell paste (~ 4 g) was stored at –80 ºC until use.

**Purification of His10-CinM and His6-CinX**

Both CinM and CinX were kept at 4 ºC for the duration of the purification process. For each enzyme, the cell paste was resuspended in ~ 20 mL of LanM Start Buffer (20 mM Tris, pH 7.6, 500 mM NaCl, 10 % glycerol) and lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 s pause, 15 min). The sample was centrifuged at 23,700×g for 45 min (Beckman JA-20 rotor) and the supernatant was clarified through 0.45 μm syringe filters (Corning). The sample was then loaded onto a 5 mL HisTrap HP column (GE Healthcare Life Sciences). The column was washed with 20 mL each of start buffer containing 25 and 50 mM imidazole and then 10 mL each of start buffer containing 100, 200, and 500 mM imidazole. The eluent was collected in several fractions, which were analyzed by Tris-SDS-PAGE (4-20% acrylamide gradient). The fractions containing the desired protein (200-500 mM imidazole) were combined and concentrated using an Amicon Ultra-15 centrifugal filter unit (30 kDa molecular mass cutoff for His6-CinM, 10 kDa molecular mass cutoff for His6-CinX, Millipore) to less than 2 mL. Buffer exchange of the concentrated proteins with Final Buffer (20 mM Tris, pH 7.6, 500 mM KCl, 10% glycerol) was conducted twice via size exclusion chromatography (GE Healthcare Life Sciences). The resulting protein samples were aliquoted, frozen with liquid nitrogen, stored at –80 °C. Protein concentrations were determined using A280 measurements and were calculated with theoretical extinction coefficient obtained from the ProtParam function on the ExPASy Proteomics Server (His6-CinM, ε280 = 158,180 M⁻¹cm⁻¹, His6-CinX, ε280 = 62,005 M⁻¹cm⁻¹). For CinM, typical protein yields ranged from 6 -10 mg per liter of overexpressed cells, whereas for CinX protein yields ranged from 1.7 mg to 2 mg per liter of overexpressed cells.

An anion exchange column was required to obtain highly pure CinM for PAR analysis or other sensitive experiments. After the CinM-containing fractions from the nickel column were concentrated to ~ 2 mL as described above, the overall salt concentration was reduced by repeated dilution with buffer (50 mM HEPES, pH 7.5, 10% glycerol) and re-concentration with the centrifugal filtering device. Dilution to ~ 10 mL and re-concentration to ~ 2 mL was repeated
twice. A HiTrap Q Sepharose anion exchange column (GE Healthcare) was equilibrated with 10 mL of buffer containing 100 mM NaCl. After loading the filtered, salt-reduced CinM sample, the resin was washed with 10 mL each of wash buffers containing 100, 200, 300, 400, 500, 600, 800, and 1000 mM NaCl. The majority of CinM eluted from the column in 2 fractions between 400 and 500 mM NaCl.

**PAR Assay Metal Analysis for His10-CinM**

The procedure used for CinM metal analysis is a variation of previously published protocols. All flasks, beakers, syringes, pipet tips, tubes, concentrating devices, and cuvettes were pre-rinsed immediately prior to use with Millipore water or MOPS Dialysis Buffer (MDB, 50 mM MOPS, pH 7.6, 500 mM KCl, 10% glycerol) that was stirred with 3% (w/v) Chelex-100 (Bio-Rad) multivalent metal ion scavenging ion exchange resin for at least 3 h. Glass and metal were avoided where possible and all reagents used during the assay were treated with 3% (w/v) Chelex-100. The high-purity CinM fractions from IMAC and anion exchange chromatography were dialyzed using a Slide-A-Lyzer Dialysis Cassette with a 10 kDa MWCO and 0.5-3.0 mL capacity (Pierce/Thermo Scientific). Dialysis was carried out with three changes of MDB (1:1000 v/v each) pre-treated with 30 g/L Chelex-100 over a total time of ~ 30 h at 4 °C. The protein was then concentrated using a centrifugal filtering device (Millipore) presoaked in MDB treated with 3% (w/v) Chelex-100. CinM concentration was determined using A$_{280}$ as discussed above. The zinc content for CinM was analyzed by a spectroscopic assay based on the absorption change at 500 nm associated with the formation of a zinc complex with 4-(2-pyridylazo)resorcinol (PAR, Sigma). A sample of 50 μL of 9 μM protein was incubated with 4 M guanidine hydrochloride to denature the protein in the presence of MDB containing 0.1 M PAR. Titration of the protein mixture with 1 mM of the thiol-modifying reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Acros) resulted in the gradual release of cysteine-bound metal, which formed a complex with PAR. The absorbance at 500 nm was measured, and the titration was considered complete when no more significant increase in absorbance was observed. The addition of DTNB caused a minimal background increase in absorbance at 500 nm, which was measured using an identical titration of sample lacking protein and subtracted from the experimental value obtained in the presence of protein. The concentration of Zn$^{2+}$ was determined using a zinc atomic absorption standard that was treated under the same conditions to obtain a standard curve.

**Detection of lysinoalanine (Lal) in CinA by Gas Chromatography-Mass Spectrometry Analysis (GC-MSMS)**

The presence of Lal in CinA modified with CinM and CinX followed by LysC protease cleavage and alkaline treatment was confirmed via hydrolysis and derivatization of the peptide, followed by analysis by Gas Chromatography Mass Spectrometry (GC/MS) with comparison to Lal in authentic cinnamycin (Novacta) treated in the same way. Modified CinA (~ 0.9 mg) prepared in vitro as described above was dissolved in 6 M HCl (3 mL) and heated with stirring at 110 °C in a high-pressure, sealed vessel for 22 h. The reaction was cooled and concentrated under reduced pressure. Methanol (3 mL) was chilled in an ice-water bath, and acetyl chloride (1 mL) was added dropwise. This solution was added to the hydrolysate, and the mixture was heated at 110 °C for 45 min. The reaction was again allowed to cool and concentrated under reduced pressure to dryness, then chilled in an ice-water bath. Dichloromethane (2.5 mL) and pentafluoroproionic anhydride (1 mL) were added, and the mixture was heated at 110 °C for 15
min. The reaction was allowed to cool and was dried under a stream of nitrogen. The residue was taken up in 100 μL of methanol, transferred to a clean vial and dried under a stream of nitrogen. Similarly, cinnamycin (Novacta; 0.9 mg) was hydrolyzed and derivatized in the same way. The sample and the standard were analyzed individually by GC/MS using an Agilent HP 5973 mass spectrometer and a Supelco SPB-1701 30 m x 0.25 mm fused silica capillary column. Samples were dissolved in methanol and introduced to the instrument via splitless injection and a flow rate of 1.5 mL/min. The temperature gradient used was 100 ºC for 5 min, then 100 ºC to 280 ºC over 18 min, then held at 280 ºC for 1 min.
List of Primers From 5’ to 3’

CinA wt FP Ndel pET15b: GGGAAATTCCATATGACCGCTTCGATTTCTTCAAGCAG
CinA wt RP BamHI pET15b: TATAAACCGGATCCCTACTTGTTTGAGGCTGCACG
CinM FP Ndel pET16b: GGGAAATTCCATATGAGTGGTAACACGTATCCC
CinM RP XhoI pET16b: TATATAACCCGCTCGAGCTACTTGCTTCTTCAAGCAG
CinX Ndel FP pET28b: GGAATTCTAATGACCTGAATTGACCTGGAAGCTG
CinX XhoI RP pET28b: CCGCTCAAGTACCCGCATGATGATGCTGCTGCTG
Cinor7 FP Ndel pET28b: GCCCTGTTGCGCCGCGGCGAGCCTATAGAAGGTGGCAAAGGAGCCGACGATCTACC
Cinor7 RP XhoI pET28b: GGGCCCGCTTATGACCCGAGCTCCGAGTGCTGCTTCTTCAAGCAG
CinA-K FP (A-1K) pET15b: CACCGAAGCTTCTAGTGCCCGCGACAGCTG
CinA-K RP (A-1K) pET15b: CAGCTCTGGCCGCACTTGAAGGCTTCTTCAAGCAG

pDuet System Cloning

CinA-K Ascl pRSFDuet-1 MCSI FP: TGGCATATGAGCGTCGAGCTACGCTCCTCTTCTTCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCT
**Cinnamycin**  
*S. cinnamoneus*

![Diagram of the gene cluster for cinnamycin production, regulation, and immunity from *Streptomyces cinnamoneus cinnamoneus* DSM 40005.](image)

**Figure S1.** The gene cluster for cinnamycin production, regulation, and immunity from *Streptomyces cinnamoneus cinnamoneus* DSM 40005.5.

![Tris-HCl 4-20% Gradient SDS-PAGE](image)

**Figure S2.** Tris-HCl 4-20% Gradient SDS-PAGE for (A) His10-CinM (MW calculated 121,312 Da) and (B) His6-CinX (MW calculated 38,859 Da) after purification by IMAC chromatography.
Figure S3. MALDI-TOF mass spectra of CinA peptide modified by CinX before (black line) and after incubation with CinM (red line). Samples were cleaved with LysC prior to MS analysis. A peptide fragment arising from LysC cleavage of CinX (CinA A25-51) causes suppression of the ionization of the peptides of interest.

Figure S4. Importance of the leader sequence for enzymatic activity of CinM and CinX. (A) MALDI mass spectrum of CinA1-19 treated with His_{10}-CinM, (B) MALDI mass spectrum of CinA1-19 treated with His_{6}-CinX.
Figure S5. Tandem MS/MS analysis of CinA1-19 treated with His₆-CinX in vitro. The $y''4$ and $y''5$ ions define Asp15 as the site of hydroxylation, which is also consistent with all other observed fragment ions.
Figure S6. Detection of lysinoalanine. (A) His$_6$-CinA(A–1K) was processed with His$_{10}$-CinM and His$_6$-CinX in vitro followed by increase of the pH and LysC protease treatment. After subsequent acid hydrolysis and derivatization, the sample was analyzed by GC-MS. (B) GC-MS analysis of cinnamycin standard (Novacta) after acidic hydrolysis and derivatization (C) Mass spectrum of the lysinoalanine (Lal) peaks of the sample in A, (D) Mass spectrum of the lysinoalanine (Lal) peaks of cinnamycin standard (Novacta). All samples were treated with 6 M HCl and derivatized as described in the Methods section.

The major peaks observed in panels C and D are 190 Da (CF$_3$CF$_2$CONHC$_2$H$_4$), 230 Da (CF$_3$CF$_2$CONHC$_5$H$_8$), 465 Da (M – CF$_3$CF$_2$CONHCHCO- OCH$_3$), 405 Da (465 Da-CH$_3$OCO), 433 Da (465 Da-CH$_3$OH), 640 (M – CH$_3$OCO), M is derivatized Lal (see text): 699 Da.
Figure S7. MALDI-ToF MS analysis of His$_6$-CinA(A–1K) modified by CinM and CinX in *E. coli*. (A) His$_6$-CinA(A–1K) modified by CinM and CinX in *E. coli* and treated with LysC; (B) Iodoacetamide (IAA) treatment of the co-expression product followed by LysC cleavage. The ion marked with an asterisk is CP – 4 H$_2$O. CP, CinA core peptide.
Figure S8. (A, top) CinA(A–1K) modified in E. coli by CinM, CinX, and Cinorf7 followed by in vitro LysC cleavage; (A, middle) The peptide from the top spectrum was analyzed by the OPA assay as described in the text; (A, bottom) Authentic cinnamycin was analyzed by the OPA assay as a positive control. (B) The outer ring of spots shows various concentrations of authentic cinnamycin, the inner ring of spots shows LysC-cleaved co-expression product (CinA(A–1K), CinM, CinX, Cinorf7). Concentrations: spot 1: 60 μg/mL, 2: 30 μg/mL, 3: 15 μg/mL, 4: 10 μg/mL, 5: 7.5 μg/mL, 6: 5 μg/mL, 7: 2.5 μg/mL, 8: 1 μg/mL. CP, CinA core peptide.
**Figure S9.** A. Two other genes with high sequence similarity to *cinorf7* were identified in the protein databases. The ortholog from *Lyngbya majuscula* (ZP_08425231) has two possible precursor peptides close by in the genome. These two peptides are shown in panel B (ZP_08425235_02840 and ZP_08425237_02860). The second ortholog was found in *Frankia* Sp. It has one possible precursor peptide nearby (ZP_06416733_6431 in panel B). B. The sequences of the predicted leader peptide of the three putative precursor peptides are not well conserved, but the core region show very high sequence identity to the cinnamycin group of peptides. The predicted start of the core peptide is indicated with a vertical black line.
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