Supporting Information

Sequential Allylic Alcohol Formation by a Multifunctional Cytochrome P450 Monooxygenase with Rare Redox Partners

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Supplementary General Material and Procedure

1.1. Materials
All organic reaction related chemicals and solvents were purchased from Sigma-Aldrich (Darmstadt), Carl Roth (Karlsruhe), Tokyo Chemical Industry (TCI Deutschland GmbH, Frankfurt am Main), VWR international GmbH (Dresden), and Alfa Aesar (Karlsruhe), and used without further purification unless otherwise specified. Taq and Phusion DNA polymerase and DNA modifying enzymes (restriction digestion and ligation) were purchased from New England Biolabs (NEB, Frankfurt am Main). DNA sequencing was performed at Eurofins Genomics (Ebersberg). Primers were purchased from Eurofins Genomics. Pre-stained protein markers were purchased from NEB. DNA markers were obtained from ThermoFisher Scientific (Dreieich).

1.2. General and analytic procedure
Analytic HPLC was performed on a Shimadzu Prominence HPLC system; controller CBM-20A, pumps LC 20AT, auto sampler SIL-20AC HT, column oven CTO-20A, and PDA detector SPD M20A using a reverse phase column (Nucleodur C18 gravity 100 Å, 5 µm, 250 × 4 mm, Macherey-Nagel) at a flow rate 1 mL·min⁻¹. HPLC grade acetonitrile and deionized water with 0.1% (v/v) trifluoroacetic acid (TFA) were used as mobile phase for HPLC. The gradient elution was acetonitrile/water with 0.1% TFA, 0.5/99.5 to 100/0 for 30 min, acetonitrile 100% for 10 min. Preparative HPLC was performed on Shimadzu Prominence HPLC system; controller CBM-20A, pumps LC-8A, and PDA detector SPD-M20A. LC-HRMS measurements were performed using an QExactive Orbitrap High Performance Benchtop LC-MS with an electrospray ion source and an Accela HPLC system (ThermoFisher Scientific, Bremen). NMR spectra were recorded on Bruker AVANCE III 500 and 600 MHz equipped with a Bruker Cryo platform.

Supplementary Experimental Section

2.1. Bacterial strains, cultivation and genomic DNA purification
Trinickia caryophylli DSM50341, which was obtained from DSMZ (Braunschweig), was cultivated on potato dextrose agar (PDA, Carl Roth) at 30 °C for 1 day. The overnight pre-cultured cells were inoculated in potato dextrose broth (PDB, Carl Roth) and then cultured at 30 °C with orbital shaking (110 rpm) for 3 days. Genomic DNA from pure culture of T. caryophylli was obtained using MasterPure™ DNA purification kit (Epicentre Biotechnologies, Madison). Pseudomonas protegens Pf-5, which was obtained from DSMZ, was cultivated on a LB agar plate at 30 °C for 2 days. The overnight pre-cultured cells were inoculated in a LB broth and then cultured at 30 °C with orbital shaking (150 rpm) until an OD₆₀₀ of 4–5. The bacterial culture was centrifuged (2,800 × g, 5 min) and then the precipitated cells were washed with tris-acetate-phosphate (TAP) medium two times. P. protegens Pf-5 cells were incubated in TAP media at 30 °C with orbital shaking (120 rpm) for 3 days. Genomic DNA from pure culture of P. protegens Pf-5 was acquired using the same purification kit as T. caryophylli.
2.2. Knockout plasmid preparation and insertional knockout of cayG, cayK, cayL, and cayKL

The primers used in this study are listed in Table S1. For inactivation of the cayG, cayK, cayL, and cayKL genes, these genes were partially replaced with the apramycin cassette. Two gene fragments containing the targeting genes were amplified by Phusion DNA polymerase using a corresponding primer pair with the genomic DNA from T. caryophylli. The apramycin resistance cassette (Apr$^R$) gene was amplified by Phusion DNA polymerase using Apr$_f$ and Apr$_rv$ primers with pIJ773 as a template and resulting amplicon was restricted with NheI and PacI. This restricted Apr$^R$ gene was ligated with NheI-restricted gene fragment followed by with PacI-restricted gene fragment. Three gene fused fragment was ligated into pJET1.2 blunt vector to yield knockout plasmids for cayG, cayK, cayL, and cayKL genes.

E. coli TOP 10 competent cells were transformed using electroporator (Eporator®, Eppendorf) at 2,200 V with vectors and the combined gene fragments and grown at 37 °C in LB agar containing apramycin (50 µg·mL$^{-1}$) and ampicillin (100 µg·mL$^{-1}$). After selecting the colony containing the corresponding mutated genes (Figure S1), they were inoculated into LB medium with apramycin and ampicillin and incubated at 37 °C overnight. The resulting plasmids were purified by NEB Monarch® Plasmid Miniprep Kit, and transformed into T. caryophylli using electroporator at 2,200 V. These transformants were incubated on PDA plates at 30 °C. The colonies containing the corresponding mutation (Figure S1) were inoculated into PDB medium with apramycin and ampicillin and incubated for 12 h. They were transferred into PDA plates at 30 °C, and further incubated for 3 days. The PDA containing the bacterial cells were collected and soaked in ethyl acetate for 1 h. The ethyl acetate extract was filtered. This process was repeated two times. The combined ethyl acetate extracts were dried over sodium sulfate and concentrated under reduced pressure to a final volume of 5 mL. After addition of dimethylsulfoxide (DMSO, 5 mL), the solution was concentrated to a final volume of 4 mL.

2.3. Heterologous expression of cayG, cayK and cayL in P. protegens Pf-5

The primers used in this study are listed in Table S2. The corresponding genes in the caryoynencin BGC were PCR amplified from the genomic DNA of T. caryophylli. After the amplicon was restricted, the DNA fragment was ligated into the pJB861 vector, which was restricted with corresponding sites, by T4 DNA ligase. E. coli TOP 10 cells were transformed using electroporator at 2,500 V with the resulting plasmid and grown at 37 °C in LB medium containing kanamycin (50 µg·mL$^{-1}$). The resulting plasmid was purified by NEB Monarch® Plasmid Miniprep Kit and then introduced into P. protegens Pf-5 using electroporator at 2,500 V. Antibiotic resistance colonies were inoculated into LB medium with kanamycin. The overnight culture was used to incubate the same medium at 30 °C until the OD$_{600}$ reached 0.4–0.6. The cells were collected with centrifugation at 2,800 × g for 10 min. The collected cells were washed with TAP medium two times and diluted until OD$_{600}$ reached 0.4–0.6. After m-toluic acid was added (the final concentration was reached to 1 mM), the strain was incubated at 30 °C for 1 day. The solution was extracted with ethyl acetate three times. The combined ethyl acetate solutions were dried over sodium sulfate. After 5 mL of DMSO was added, the solution was concentrated using rotary evaporator to a final volume of 4 mL.

2.4. Production of N-His$_6$ CayL, N-His$_6$ CayG, and MalE-CayK proteins in E. coli.

The primers used in this study are listed in Table S3. The cayL, cayG, and cayK in the caryoynencin BGC were amplified as a template of the genomic DNA of T. caryophylli, and ligated into pET28b (+) with corresponding restriction enzyme sites. The cayK gene fragment
was ligated into pMAL-c2x. Expression of recombinant proteins were performed as described below. The resulting plasmids were transformed onto *E. coli* BL21 (DE3) cells. Transformants were grown at 37 °C in LB medium containing kanamycin (50 µg·mL⁻¹) for pET28b-cayG and -cayL, and ampicillin (50 µg·mL⁻¹) for pMAL-c2x-cayK. The overnight cultures were inoculated into 1 L of LB medium (1 mM of 5-aminolevulinic acid was supplemented for pET28b-cayG) in a 100-fold dilution. These cultures were incubated at 37 °C until OD₆₀₀ reached 0.4–0.6. Protein expression were induced by the addition of isopropyl-β-thiogalactoside (IPTG) to a final concentration of 0.5 mM for CayL and CayK, 0.02 mM for CayG. After overnight incubation at 16 °C, the cells were harvested by centrifugation at 4,500 × g for 8 min at 4 °C, resuspended in 20 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl, and disrupted the cells by sonication. Cell debris were removed by centrifugation at 20,000 × g for 30 min, and the supernatants (CayL, CayG) were mixed by slow agitation with 2 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin for 30 min at 4 °C. The slurries were transferred to columns and washed with 50 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. The proteins were eluted with 10 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. The collected proteins were analyzed with SDS-PAGE and were dialyzed three times against 1 L of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl. Amicon Ultra-15 (Merck KGaA, Darmstadt) were used for protein concentration. Glycerol (10–20%) was added prior to storage at –80 °C.

For purification of MalE fused CayK, cells were resuspended with 20 mL of 100 mM Tris buffer (pH 8.0) containing 200 mM NaCl, 1 mM EDTA, and disrupted the cells by sonication. Cell debris were removed by centrifugation at 9,300 × g for 40 min at 4 °C. The supernatant was mixed by slow agitation with 2 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin for 30 min at 4 °C. The slurries were transferred to columns and washed with 50 mL of 50 mM Tris buffer (pH 8.0) containing 200 mM NaCl and 20 mM imidazole. The proteins were eluted with 10 mL of 100 mM Tris buffer (pH 8.0), 200 mM NaCl, 1 mM EDTA, and 10 mM (D)-(+) maltose. The collected proteins were analyzed with SDS-PAGE and were dialyzed three times against 1 L of 50 mM potassium phosphate (pH 7.0) at 4 °C. The dialyzed protein was applied to a Hitrap Q HP (GE Healthcare Life Science) anion exchange chromatography column (5 mL) equilibrated with the 50 mM potassium phosphate (pH 7.0). Column was washed with the same buffer. MalE-CayK was eluted with a linear gradient of 0–1 M NaCl in 15 CV. The collected proteins were analyzed with SDS-PAGE. Amicon Ultra-15 (NMWL: 50 KDa) was used for protein concentration. 10% glycerol was added to storage at –80 °C.

Protein concentrations were determined by NanoDrop One Microvolume Spectrophotometer (ThermoFisher Scientific).

### 2.5. Production of N-His₆ flavodoxin reductase protein in *E. coli*.

*E. coli* flavodoxin reductase (FDR) was amplified using primers (Ecoli_FDR_f / Ecoli_FDR_r) in the colony PCR with *E. coli* BL21(DE3) cells. Obtained amplicon was ligated into pET28b (+) with restriction enzyme sites. The resulting plasmids were transformed into *E. coli* BL21 (DE3). Transformants were grown at 37 °C in LB medium containing kanamycin (50 µg·mL⁻¹) for pET28b-FDR. The overnight precultures were inoculated in to 1 L of LB medium in a 100-fold dilution and incubated at 37 °C until OD₆₀₀ reached 0.4–0.6. The FDR gene expression was induced by the addition of IPTG to a final concentration of 0.5 mM. After overnight cultivation at 16 °C, the cells were harvested by centrifugation at 4,500 × g for 8 min at 4 °C, resuspended in 20 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl, and disrupted the cells by sonication. Cell debris were removed by centrifugation at 20,000 × g for 30 min, and the supernatant was mixed by slow agitation with 2 mL of Ni-NTA resin for 30 min at 4 °C.
The slurries were transferred to columns and washed with 50 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. The proteins were eluted with 10 mL of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. The collected proteins were analyzed with SDS-PAGE and were dialyzed three times against 1 L of 100 mM Tris buffer (pH 8.0) containing 300 mM NaCl. Amicon Ultra-15 were used for protein concentration. 10–20% glycerol prior to storage at –80 °C.

2.6. In Vitro Assay of CayG, CayL, and CayK

In vitro assays for CayG were performed in 100 µL reaction containing of 4 mM NADPH, 0.1 mM E. coli flavodoxin reductase, 10 mM glucose-6-phosphate, and 1 unit mL⁻¹ baker’s yeast glucose-6-phosphate dehydrogenase, 10 µL of the substrate (in DMSO) in addition to 50 µM N-His₆-CayG, 100 µM N-His₆-CayL, and 20 µM N-MalE-CayK proteins. The reaction mixture was incubated at 30 °C and shaken at 180 rpm for 2 h and stopped by extracted with ethyl acetate (2 × 120 µL). The combined organic layers were concentrated and redissolved in 100 µL methanol.

2.7. Isolation and modification of polyyne compounds

P. protegens Pf-5 culture broth (1 L) was extracted with ethyl acetate (1 L) three times and the extracts were dried over Na₂SO₄. After 2 mL of DMSO were added to the extracts, the solvent was concentrated under the reduced pressure. The remining DMSO solution was subjected to preparative HPLC using reverse phase column (Luna 100Å C18(2) AXIA, 10 µm, 250 × 21.2 mm, Phenomenex) at a flow rate 18 mL·min⁻¹ with a gradient system, solvent A (water with 0.1% TFA), solvent B (acetonitrile), 0.5% B for 1 min, to 80% B for 4 min, to 92% B for 20 min, to 100% B for 0.5 min and kept for 2.5 min.

The structures of unstable polyyne compounds were determined by the synthetic derivatization using click reaction with benzyl azide, copper sulfate, and ascorbic acid or further derivatization using trimethylsilyldiazomethane. A 1 L culture of P. protegens Pf-5 was extracted with 1 L of ethyl acetate three times. The combined organic layers were concentrated to 20 mL under reduced pressure and 20 mL of DMF was added. The solution was concentrated to final volume of 18 mL. Aqueous solution of copper (II) sulfate (1 mL, 0.8 M), ascorbic acid (1 mL, 1.1 M) and benzyl azide (0.2 mL) were added to the polyyne solution in DMF. The mixture was stirred at room temperature until the starting material disappeared. The solution was diluted with ethyl acetate (50 mL), washed with water (3 × 50 mL) and saturated sodium chloride solution, dried with sodium sulfate, and concentrated under reduced pressure. The pure triazole compound was obtained after the purification using preparative HPLC described in section S1.2. The further methylation was performed by addition of methanol (100 µL) and trimethylsilyldiazomethane (2.0 M in diethyl ether, portionwise (5 × 1 µL)). The solvent was removed by reduced pressure, and the residue was purified by flash column chromatography on silica gel (Hex:EtOAc = 4:1 ~ 1:1).

![Chemical structure of Compound 14](image)

Compound 14. (Original compound 7)
\(^1\)H NMR (600 MHz; DMSO-\(d_6\)): \(\delta\) (ppm) = 1.57–1.63 (m, 2H, H2), 2.16 –2.22 (m, 4H, H2 and H4), 5.64 (s, 2H, benzyl), 5.81 (d, \(J = 16.1\) Hz, 1H, H6), 6.57 (dt, \(J = 15.9, 7.1\) Hz, 1H, H5), 7.30–7.38 (m, 5H, phenyl), 8.76 (s, 1H, H14), 11.98 (br, 1H, H1). \(^{13}\)C NMR (125 MHz; DMSO-\(d_6\)): \(\delta\) (ppm) = 29.0 (1C, C3), 32.2 (1C, C4), 32.9 (1C, C2), 53.2 (1C, benzyl), 64.4, 67.4, 68.6, 72.0, 76.6, 79.8 (6C, C7–C12), 107.7 (1C, C6), 127.5 (1C, C13), 128.0 (2C, phenyl), 128.4 (1C, phenyl), 128.9 (2C, phenyl), 131.0 (1C, C14), 135.2 (1C, phenyl), 152.6 (1C, C5), 174.8 (1C, C1). HRMS (ESI): \(\text{C}_{21}\text{H}_{18}\text{N}_3\text{O}_2\) [M–H]\(^-\) calc. 342.1248, obs. 342.1248.

Compound 15. (Original compound 8)

\(^1\)H NMR (600 MHz; DMSO-\(d_6\)): \(\delta\) (ppm) = 1.24–1.30 (m, 2H, H4), 1.37–1.40 (m, 2H, H5), 1.48–1.51 (m, 2H, H3), 2.17–2.21 (m, 4H, H2 and H6), 5.65 (s, 2H, benzyl), 5.82 (s, 1H, H8), 7.31–7.41 (m, 5H, phenyl), 8.78 (s, 1H, H16), 11.85 (br, 1H, H1). \(^{13}\)C NMR (125 MHz; DMSO-\(d_6\)): \(\delta\) (ppm) = 24.7 (1C, C4), 27.9 (1C, C5), 28.5 (1C, C6), 33.2 (1C, C2), 53.8 (1C, benzyl), 64.8, 68.0, 69.1, 72.4, 77.1, 80.4 (6C, C7–C12), 108.1 (1C, C8), 128.0 (1C, C15), 128.5 (2C, phenyl), 128.8 (1C, phenyl), 129.3 (2C, phenyl), 131.5 (1C, C16), 135.8 (1C, phenyl), 154.2 (1C, C7), 174.8 (1C, C1). HRMS (ESI): \(\text{C}_{23}\text{H}_{22}\text{N}_3\text{O}_2\) [M–H]\(^-\) calc. 370.1561, obs. 370.1555.

Compound 16. (Original compound 9)

\(^1\)H NMR (600 MHz; DMSO-\(d_6\)): \(\delta\) (ppm) = 1.24–1.30 (m, 2H, H4), 1.38–1.42 (m, 4H, H5 and H7), 2.15–2.20 (m, 4H, H4 and H8), 5.65 (s, 2H, benzyl), 5.76 (d, \(J = 15.6, 1\)H, H2), 5.82 (d, \(J = 16.0\) Hz, 1H, H10), 6.60 (dt, \(J = 15.9, 7.1\) Hz, 1H, H9), 6.80 (dt, \(J = 15.6, 7.1\) Hz, 1H, H3), 7.31–7.40 (m, 5H, phenyl), 8.78 (s, 1H, H18). \(^{13}\)C NMR (125 MHz; DMSO-\(d_6\)): \(\delta\) (ppm) = 27.7 (1C, C7), 27.9 (1C, C5), 28.5 (1C, C6), 31.7 (1C, C8), 33.2 (1C, C4), 53.8 (1C, benzyl), 64.9, 68.1, 69.2, 72.5, 77.2, 80.5 (6C, C7–C12), 107.8 (1C, C10), 122.6 (1C, C2), 128.1 (1C, C17), 128.6 (2C, phenyl), 128.9 (1C, phenyl), 129.4 (2C, phenyl), 131.6 (1C, C18), 135.9 (1C, phenyl), 149.1 (1C, C3), 154.0 (1C, C9), 167.9 (1C, C1). HRMS (ESI): \(\text{C}_{25}\text{H}_{24}\text{N}_3\text{O}_2\) [M–H]\(^-\) calc. 396.1711, obs. 396.1718.
Compound 17. (Original compound 10)

\[ \text{H NMR (600 MHz; acetone-}\text{d}_6): \delta (ppm) = 1.21–1.33 (m, 4H, H6, H7), 1.34–1.48 (m, 2H, H5), 1.56–1.59 (m, 2H, H4), 2.20–2.28 (m, 2H, H8), 2.36–2.46 (m, 2H, H2), 3.62 (s, 3H methyl), 3.95–3.98 (m, 2H, H3), 5.70 (s, 2H, benzyl), 5.90 (dt, J = 15.9, 1.6 Hz, 1H, H10), 6.56 (dt, J = 15.9, 7.2 Hz, 1H, H9), 7.35–7.41 (m, 5H, phenyl), 8.45 (s, 1H, H18). \]

\[ \text{C NMR (125 MHz; acetone-}\text{d}_6): \delta (ppm) = 25.7 (1C, C5), 26.1 (1C, C7), 29.1 (1C, C6), 34.0 (1C, C8), 37.9 (1C, C4), 43.1 (1C, C2), 51.6 (1C, methyl), 54.7 (1C, benzyl), 65.2 (1C, C11–C16), 68.2 (1C, C11–C16), 68.6 (1C, C3), 68.6 (1C, C11–C16), 73.0 (1C, C11–C16), 77.4 (1C, C11–C16), 80.1 (1C, C11–C16), 108.4 (1C, C10), 129.1 (2C, phenyl), 129.5 (1C, C17), 129.9 (2C, phenyl), 130.9 (1C, C18), 153.6 (1C, C9), 174.9 (1C, C1). \]

HRMS (ESI): C_{26}H_{27}N_{3}O_{3} \[ [M+H]^+ \text{calc.} 430.2125, \text{obs.} 430.2118. \]

\[ \text{Compound 18. (Original compound 4)} \]

\[ \text{H NMR (600 MHz; DMSO-}\text{d}_6): \delta (ppm) = 1.24–1.30 (m, 6H, H4–H6), 1.31–1.35 (m, 2H, H7), 1.47–1.49 (m, 2H, H3), 2.18–2.20 (m, 2H, H2), 5.08–5.10 (m, 2H, H8), 5.65 (s, 2H, benzyl), 5.90 (dd, J = 15.9, 1.8 Hz, 1H, H10), 6.64 (dd, J = 15.9, 4.7 Hz, 1H, H9), 7.31–7.40 (m, 5H, phenyl), 8.78 (s, 1H, H18). \]

\[ \text{C NMR (125 MHz; DMSO-}\text{d}_6): \delta (ppm) = 24.7 (1C, C3), 28.5, 28.6, 29.0 (3C, C4–C6), 33.6 (1C, C2), 36.4 (1C, C7), 53.3 (1C, benzyl), 64.6, 67.5, 68.7 (3C, C7–C12), 70.0 (1C, C8), 73.0, 77.3, 79.7 (3C, C7–C12), 105.0 (1C, C10), 127.5 (1C, C17), 128.0 (2C, phenyl), 128.4 (1C, phenyl), 128.9 (2C, phenyl), 131.0 (1C, C18), 135.3 (1C, phenyl), 156.2 (1C, C9), 174.5 (1C, C1). \]

HRMS (ESI): C_{25}H_{24}N_{3}O_{3} [M-H]^- \text{calc.} 414.1823, \text{obs.} 414.1821.

Compound 6. (Original compound 18)

\[ \text{H NMR (600 MHz; acetone-}\text{d}_6): \delta (ppm) = 1.32–1.38 (m, 6H, H3–H5), 1.35–1.51 (m, 2H, H6), 1.56–1.61 (m, 2H, H7), 2.31 (m, 2H, H2), 3.62 (s, 3H Methyl), 4.18–4.26 (m, 1H, H8), 5.72 (s, 2H, benzyl), 5.94 (dd, J = 15.9, 1.8 Hz, 1H, H10), 6.63 (dd, J = 15.9, 4.8 Hz, 1H, H9), 7.37–7.43 (m, 5H, phenyl), 8.47 (s, 1H, H18). \]

HRMS (ESI): C_{26}H_{27}N_{3}O_{3} [M+H]^+ \text{calc.} 430.2125, \text{obs.} 430.2119.

2.8. Determination of the absolute configuration of compound 6
The stereochemical assignment of the C-8 hydroxyl group was determined by the Mosher method.\cite{3} 4-Dimethylaminopyridine in dichloromethane (2.3 M, 10 μL) was added to the solution of compound 6 in dry dichloromethane (100 μL). Portions of (R)- and (S)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) (n × 1 μL) were added at room temperature until the reaction became complete. The mixture was purified by flash column chromatography on silica gel (Hex:EtOAc = 4:1) to give the desired compounds 19a and 19b, respectively.

**Compound 19a.**

$^1$H NMR (600 MHz; acetone-$d_6$): δ (ppm) = 1.19–1.43 (m, 6H, H4–H6), 1.54–1.61 (m, 2H, H3), 1.62 (m, 1H, H7), 1.74 (m, 1H, H7), 2.26–2.30 (m, 2H, H2), 3.63 (2, 3H, methyl), 5.68 (m, 1H, H8), 5.72 (s, 2H, benzyl), 6.06 (dd, $J = 16.0, 1.2$ Hz, 1H, H10), 6.60 (dd, $J = 16.0, 6.9$ Hz, 1H, H9), 7.38–7.55 (m, 10H, phenyl), 8.49 (s, 1H, H18). HRMS (ESI): $C_{36}H_{34}F_3N_3O_5$ [M+H]$^+$ calc. 646.2523, obs. 646.2513.

**Compound 19b.**

$^1$H NMR (600 MHz; acetone-$d_6$): δ (ppm) = 1.19–1.38 (m, 6H, H4–H6), 1.58–1.68 (m, 2H, H3), 1.66 (m, 1H, H7), 1.81 (m, 1H, H7), 2.28–2.32 (m, 2H, H2), 3.62 (2, 3H, methyl), 5.63 (m, 1H, H8), 5.72 (s, 2H, benzyl), 5.82 (dd, $J = 16.0, 1.2$ Hz, 1H, H10), 6.49 (dd, $J = 16.0, 6.5$ Hz, 1H, H9), 7.38–7.77 (m, 10H, phenyl), 8.49 (s, 1H, H18). HRMS (ESI): $C_{36}H_{34}F_3N_3O_5$ [M+H]$^+$ calc. 646.2523, obs. 646.2515.

### 2.9. Synthesis of S-acetylcytamine (SNAC) thioester of compound 2

To a solution of compound 2 (1 mg, 2.5 μmol) in dichloromethane (1 mL), N-acetyl cysteamine (399 μL, 3.75 μmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (959 μg, 5.01 μmol), and 4-dimethylaminopyridine (30 μg, 0.25 μmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for additional 12 h. The solution was concentrated and purified by flash column chromatography on silica gel (Hex:EtOAc = 1:1) to provide the compound 13. The structure of 13 was confirmed with NMR spectral data of compound 20 obtained by above-mentioned (2.7) click reaction.
Compound 13. (Original compound 2)

\[
\text{Compound 20. (Original compound 13)}
\]

\(^1\text{H NMR (600 MHz; DMSO-}d_6\text{): } \delta (ppm) = 1.21-1.29 (m, 6H, H4–H6), 1.32-1.40 (m, 2H, H7), 1.52-1.55 (m, 2H, H3), 2.13 (s, 3H, methyl), 2.15–2.20 (m, 2H, H8), 2.54–2.57 (m, 2H, H2), 2.86–2.89 (m, 2H, SCH_2CH_2) 3.13–3.16 (m, 2H, SCH_2C_2), 5.65 (s, 2H, benzyl), 5.80 (d, J =15.9 Hz, 1H, H10), 6.58 (dt, J =15.9, 7.2 Hz, 1H, H9), 7.31–7.40 (m, 5H, phenyl), 8.78 (s, 1H, H18).

\(^1\text{3C NMR (125 MHz; DMSO-}d_6\text{): } \delta (ppm) = 21.0 (1C, methyl), 25.0 (1C, C3), 28.1 (1C, C7), 28.1, 28.2, 29.3 (3C, C4–C6), 28.1 (1C, SCH_2CH_2), 32.8 (1C, C8), 38.2 (1C, SCH_2CH_2), 43.4 (1C, C2), 53.2 (1C, benzyl), 64.3, 67.6, 68.6, 71.9, 76.9, 79.9 (6C, C11–C16), 107.2 (1C, C10), 127.5 (1C, C17), 128.0 (2C, phenyl), 128.4 (1C, phenyl), 128.8 (2C, phenyl), 131.0 (1C, C18), 135.3 (1C, phenyl), 153.5 (1C, C9), 172.0 (1C, C(=O)CH_3), 198.5 (1C, C1). HRMS (ESI): C_{29}H_{32}N_4O_2S [M+H]^+ calc. 501.2319, obs. 501.2312.

2.10. Phylogenetic analysis of CayG and other P450 proteins in bacteria.

To select Actinomycetes cytochrome P450 proteins, reviews by Liu,\(^4\) Shen and coworkers\(^5\) were referred. Other proteins were collected by BlastP (https://blast.ncbi.nlm.nih.gov/) using database protein data bank (PDB), UniProtKB/Swiss-Prot, and Non-redundant protein sequences. CYP numbers were determined by using BLAST tool in Biocatnet CYP v6.0 server (https://cyped.biocatnet.de/).\(^6\) Alignment of 87 amino acid sequences was performed by MAFFT ver. 7 (https://mafft.cbrc.jp/alignment/software/)\(^7\) using the default setting. Obtained data were analyzed by IQ tree (http://www.iqtree.org/)\(^8\) with automatic model selection mode (ModelFinder),\(^9\) where the LG+F+R5 was selected, generating the unrooted fast maximum-likelihood-based tree. Phylogenetic bootstrap analysis was performed by ultrafast approximate bootstrap with 1,000 bootstrap replicates. The obtained tree was displayed using MEGA7.\(^10\)

2.11. Sequence similarity network and Genome Neighborhood analysis for CayG.

A sequence similarity network was produced by using the sequence similarity tool through EFI-EST\(^11\) and visualized by Cytoscape.\(^12\) The network was produced by using the UniProt 2021-02 and the InterPro86 databases, and consisted of 1,000 sequences with CayG. Final network shown is alignment score of 100 and E-value cutoff of 10\(^{-5}\), with each node representing sequences with 100% sequence identity. To search neighbor genes of cayG in genomes, the datum obtained by EFI-EST was subjected to EFI-GNT and generating Genomic Neighborhood Diagrams including CayG homologues and orthologues were analyzed.
## Supplementary Tables and Figures

### Table S1. Primers used for insertional knockout of cayG, cayK, cayL, and cayKL in T. caryophylli DSM50341

| Primers       | sequence (Underline; restriction enzyme sites)                                                                 |
|---------------|----------------------------------------------------------------------------------------------------------------|
| KOcayG_f1     | 5’-AGCGATCTTTTCGACACCCGAG-3’                                                                                   |
| KOcayG_r1     | 5’-GCTACTTTAATGCTAGCTAGCTAGCTAGATCGCAAGGAATGCAAAGGCC-3’ (Nhel)                                                |
| KOcayG_f2     | 5’-GCTACGCTAGCTTTAATGACATAGTTCCGGTGCTGATCG-3’ (Pci)                                                          |
| KOcayG_r2     | 5’-GTATCGACGTTGCTAG-3’                                                                                        |
| KOcayK_f1     | 5’-ATCCCCAATGCTCGAGTC-3’                                                                                      |
| KOcayK_r1     | 5’-GCTACTTTAATGCTAGCTAGCTAGCTAGATCGCAAGGAATGCAAAGGCC-3’ (Nhel)                                                |
| KOcayK_f2     | 5’-GCTACGCTAGCTTTAATGACATAGTTCCGGTGCTGATCG-3’ (Pci)                                                          |
| KOcayK_r2     | 5’-GTCCGCATCGACGGGGCTTTG-3’                                                                                   |
| KOcayL_f1     | 5’-ACCCGACGAACCTGCATGTCAAC-3’                                                                                  |
| KOcayL_r1     | 5’-GCTACTTAATTAAAGCTAGCTAGCTAGCTAGATCGCAAGGAATGCAAAGGCC-3’ (Nhel)                                            |
| KOcayL_f2     | 5’-GCTACGCTAGCTTTAATGACATAGTTCCGGTGCTGATCG-3’ (Pci)                                                          |
| KOcayL_r2     | 5’-GATCGACGTTGCTAG-3’                                                                                        |
| KOcayKL_f1    | same sequence as KOcayK_f1                                                                                     |
| KOcayKL_r1    | same sequence as KOcayK_r1 (Nhel)                                                                               |
| KOcayKL_f2    | same sequence as KOcayL_f2 (Pci)                                                                                |
| KOcayKL_r2    | same sequence as KOcayL_r2                                                                                     |
| Apra_f        | 5’-GCTCGCTAGCTAGCTCGAGTCGACATCGGACACATCTACG-3’ (Nhel)                                                         |
| Apra_r        | 5’-GCTACCTTTAATGATCGGAGTCGTCGATTCCCGGACC-3’ (Pci)                                                            |

### Table S2. Primers used for heterologous expression plasmid preparation using cayG, cayK, and cayL in P. protegens Pf-5

| Primers   | sequence (Undertline; restriction enzyme sites)                                                                 |
|-----------|----------------------------------------------------------------------------------------------------------------|
| cayG_Pf5_f| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayG_Pf5_r| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_f| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_r| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_f| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_r| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_f| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_r| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_f| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
| cayK_Pf5_r| 5’-AAAAAAGCCGCAATCCACCAGTCCCGAGC-3’ (BamHl)                                                                   |
Table S3. Primers used for productions of N-His\textsubscript{6} CayL, N-His\textsubscript{6} CayG, and MalE-CayK protein in \textit{E. coli}

| Primers        | sequence (Underline; restriction enzyme sites)                                                                 |
|----------------|---------------------------------------------------------------------------------------------------------------|
| cayL\textsubscript{f}  | 5'-AAAAAAA CATATG TCCGACATCGCAGATCGAATAC-3' (Ndel)                                                         |
| cayL\textsubscript{r}  | 5'-AAAAAAA AGCTTT CAAGATCGAACACCATTACCGAG-3' (HindIII)                                                       |
| cayG\textsubscript{f}  | 5'-ACAGCACATG CATGACAGCCTTTC-3' (Ndel)                                                                       |
| cayG\textsubscript{r}  | 5'-AAAAAAA AGCTTT CAAGATCGAACACCATTACCGAG-3' (HindIII)                                                       |
| cayK\textsubscript{f}  | 5'-GGTTGGA TTCCAGTCTATCAGCTTACAG-3' (BamHI)                                                                  |
| cayK\textsubscript{r}  | 5'-GGTAA AGCTTT CAAGATCGAACCCCGG-3' (HindIII)                                                                |
| Ecoli\_FDR\textsubscript{f} | 5'-AAAAAGACTAGCATGCGATGGGAAGACAGGC-3' (Nhel)                                                                |
| Ecoli\_FDR\textsubscript{r} | 5'-AAAAAGATCCCTTACCAGTACTGCGCTCG-3' (BamHI)                                                                |

Table S4. Plasmids used in this study.

| Plasmids        | Function                                      | Source or reference                  |
|-----------------|-----------------------------------------------|--------------------------------------|
| pJET1.2 blunt   | Blunt end cloning vector for knockout          | Thermo Fisher Scientific             |
| pET28b (+)      | N-His\textsubscript{6}-tagged protein expression vector | Novagen (Merck KGaA)                |
| pMal c2x        | N-MalE-tagged protein expression vector        | New England Biolabs                  |
| pJ773           | Source of apramycin resistance gene (Apr\textsuperscript{R}) | Gust et al.\textsuperscript{[13]}  |
| pJB861          | Broad-host range expression vector            | Blatny et al.\textsuperscript{[14]} |

Table S5. Bacterial strains used in this study.

| Strains         | Function                                      | Source or reference                  |
|-----------------|-----------------------------------------------|--------------------------------------|
| \textit{Escherichia coli} |                                              |                                      |
| XL1 Blue        | Strain for general cloning                    | Invitrogen                           |
| TOP10           | Strain for general cloning                    | Invitrogen                           |
| BL21 (DE3)      | Strain for heterologous expression            | NEB                                  |
| pET28b-cayG     | N-His\textsubscript{6}-CayG protein production | This study                           |
| pET28b-cayL     | N-His\textsubscript{6}-CayL protein production | This study                           |
| pET28b-FDR      | N-His-fravodoxin reductase production         | This study                           |
| pMal c2x-cayK   | MalE-CayK protein production                  | This study                           |
| \textit{Trinckia caryophylli} |                                              |                                      |
| DSM50341        | Wild-type for mutagenesis                     | DSMZ                                 |
| \Delta cayG     | Apr\textsuperscript{R} cassette is partially inserted in cayG gene | This study                           |
| \Delta cayK     | Apr\textsuperscript{R} cassette is partially inserted in cayK gene | This study                           |
| \Delta cayL     | Apr\textsuperscript{R} cassette is partially inserted in cayL gene | This study                           |
| \Delta cayKL    | Apr\textsuperscript{R} cassette is partially inserted in cayKL gene | This study                           |
| \textit{Psuedomonas protegens} |                                              |                                      |
| Pf-5 (ATCC BAA-477) | Wild-type for mutagenesis                    | Howell et al.\textsuperscript{[15]} |
| pJB861          | Empty plasmid as negative control             | This study                           |
| pJB861-cayG     | \textit{cayG} heterologous expression         | This study                           |
| pJB861-cayKG    | \textit{cayK} and G heterologous expression   | This study                           |
| pJB861-cayLG    | \textit{cayL} and G heterologous expression   | This study                           |
| pJB861-cayKLG   | \textit{cayK}, L, and G heterologous expression | This study                           |
| pJB861-cayK     | \textit{cayK} heterologous expression         | This study                           |
| pJB861-cayKL    | \textit{cayK} and L heterologous expression   | This study                           |
Figure S1. PCR confirmation of insertional gene knockout for cayKL, cayK, cayL, and cayG in *T. caryophylli* Pf-5. **A)** Knockout strategy using double-crossover event of target genes with an apramycin resistance cassette (*Apr^R*) insertion. **B)** Agarose gel analysis of PCR products as a template of (a) *T. caryophylli* cayKL (wild type), ΔcayKL (mutant), using a primer pair KOcayKL_f1 and KOcayKL_r2, (b) *T. caryophylli* cayK (wild-type), ΔcayK (mutant), using a primer pair KOcayK_f1 and KOcayK_r2, (c) *T. caryophylli* cayL (wild type), ΔcayL (mutant), using a primer pair KOcayL_f1 and KOcayL_r2, (d) *T. caryophylli* cayG (wild type), ΔcayG (mutant), using a primer pair KOcayG_f1 and KOcayG_r2. **M** (Molecular Marker), 1; 1000 bp, 2; 2000 bp, 3; 3000 bp.
Figure S2. Amino acid sequence alignment of CayK, ferritin, and other ferritin-like proteins. Red and magenta shaded amino acids indicate the conserved defined/potential metal ligands and potentially conserved amino acids, respectively. FtnA; ferritin, BrfB; bacterioferritin, Ruer; ruberythrin, Eryt; erythrin, Rrsc; ribonucleotide reductase small chain. BrfB_Caje; 1KRQ, *Campylobacter jejuni* subsp. *jejuni* NCTC 11168, BrfB_Esco; 1EUM, *Escherichia coli*, BrfB_Hepy; Q9ZL11.1, *Helicobacter pylori* J99, BrfB_Mytu; 3QD8, *Mycobacterium tuberculosis*, CayK_Bugl; KAF1061804.1, *Burkholderia gladioli* LvStA, CayK_Bupl; WP_042627244.1, *Burkholderia plantarii*, CayK_Trca; WP_085229150.1, *Trinickia caryophylli*, Eryt_Pyab; Q9V026, *Pyrococcus abyssi*, Eryt_Pyfu; Q8U1L6, *Pyrococcus furiosus*, Eryt_Thma; Q9WXM4, *Thermotoga maritima*, FtnA_Esco; 1EUM, *Escherichia coli*, FtnA_Psae; 3R2L, *Pseudomonas aeruginosa*, FtnA_Pyfu; 2JD7, *Pyrococcus furiosus*, Rrsc_Mytu; 3OMJ, *Geobacillus kaustophilus* HTA426, Rrsc_Mytu; 3EE4, *Mycobacterium tuberculosis*, Rrsc_Suca; 6QRZ, *Sulfolobus acidocaldarius*, Ruer_Bups; 4D10, *Burkholderia pseudomallei* 1710b, Ruer_Devu; 1S30, *Desulfovibrio vulgaris*, Ruer_Pyfu; 1NNQ, *Pyrococcus furiosus*.
Figure S2 continued. Amino acid sequence alignment of CayK, ferritin, and other ferritin-like proteins.
Figure S2 continued. Amino acid sequence alignment of CayK, ferritin, and other ferritin-like proteins.
Figure S3. Amino acid sequence alignment of CayL and other rubredoxins. Conserved motifs are shown over the sequences. Iron binding cysteines are highlighted as red. CayL; TRX17445.1, *Trinickia caryophylli*, Rub_Clpa; 2PVE, *Clostridium pasteurianum*, Rub_Devu1; 1RDV, *Desulfovibrio vulgaris* str. 'Miyazaki F', Rub_Devu2; 1RB9, *Desulfovibrio vulgaris* str. Hildenborough, Rub_Mytu1; 2KN9, *Mycobacterium tuberculosis*, Rub_Mytu2; 7A9A, *Mycobacterium tuberculosis* H37Rv, Rub_Psae; 2V3B, *Pseudomonas aeruginosa* PAO1, Rub_Pyab; 1YK5, *Pyrococcus abyssi*, Rub_Pyfu; 1BRF, *Pyrococcus furiosus*, Rub_Trca1; TRX20258.1, *Trinickia caryophylli*, Rub_Trca2; TRX19883.1, *Trinickia caryophylli*. 
Figure S4. Selected polyyne biosynthetic gene clusters. Color codes indicate homologous enzyme genes.
Table S6. CayG-neighbour CayK and CayL proteins obtained through EFI-EST (Enzyme similarity tool) followed by GNT (Genome neighbourhood tool).

| Polyenyne | Strain | CayK   | CayL   | CayG   |
|-----------|--------|--------|--------|--------|
| Caryoynencin | Trinickia caryophylli DSM50341 | TRX17444.1<sup>a</sup> | TRX17445.1<sup>a</sup> | TRX17446.1<sup>a</sup> |
| Caryoynencin | Burkholderia gladioli Lv-SIA | KAF1061804.1<sup>c</sup> | KAF1061803.1<sup>c</sup> | KAF1061802.1<sup>c</sup> |
| Caryoynencin<sup>b</sup> | Burkholderia gladioli BSR3 | AEA60657.1<sup>d</sup> | AEA60656.1<sup>c</sup> | AEA60655.1<sup>d</sup> |
| Caryoynencin<sup>b</sup> | Burkholderia gladioli Co14 | AYQ88359.1<sup>e</sup> | AYQ88358.1<sup>c</sup> | AYQ88357.1 |
| Caryoynencin<sup>b</sup> | Burkholderia gladioli A1 | WP_025100203.1<sup>c</sup> | WP_013697988.1<sup>c</sup> | WP_025100202.1<sup>c</sup> |
| Caryoynencin<sup>b</sup> | Burkholderia plantarii ATCC 43733 | ALK32875.1<sup>f</sup> | ALK32874.1<sup>f</sup> | ALK32873.1<sup>f</sup> |
| Caryoynencin<sup>b</sup> | Burkholderia plantarii FDAARGOS_922 | MBI0330365.1 | MBI0330366.1 | MBI0330367.1 |
| Caryoynencin<sup>b</sup> | Burkholderia sp. SJZ089 | TWC73896.1<sup>g</sup> | TWC73895.1<sup>c</sup> | TWC73894.1<sup>c</sup> |

<sup>a</sup>Identical proteins with those of T. caryophylli Ballard 720, HAMBI_2159, LMG2155.
<sup>b</sup>No experimental evidence.
<sup>c</sup>Identical proteins with those of B. gladioli AU0368, C101, BCC1668, BCC1669, BCC1700, BCC1714, BCC1754, BCC1755, BCC1775, BCC1800, BCC1806, BCC1836, BCC1842, BCC1851, BCC1866, BCC1872.
<sup>d</sup>Identical proteins with those of B. gladioli 3834s-5, BCC1675, BCC1735, BCC1780, BCC1821, GSRB05.
<sup>e</sup>Identical proteins with those of B. gladioli 3723STDY6437373, Ap-962, Ax-1720, BCC1650, BCC1661, BCC1665, BCC1686, BCC1689, BCC1692, BCC1697, BCC1710, BCC1733, BCC1812, BCC1819, BCC1829, BCC1880, Cy637, Cy647, ISTR5, Tr860.
<sup>f</sup>Identical proteins with those of B. plantarii LMG9035, PG1, ZJ171.
<sup>g</sup>Identical proteins with those of Burkholderia sp. SJZ091, SJZ115, UCD-UG_CHAPALOTE.
<sup>h</sup>Identical proteins with those of Xylella fastidiosa Salento-1, Salento-2, CoDiRO, OLS0478, CoF0407, OLS0479, Xylella fastidiosa subsp. pauca De Donno, PD7202.
Figure S5. SDS-PAGE analysis of purified proteins. A) N-His$_6$-tagged CayG, B) N-His$_6$-tagged CayL, C) MalE-CayK, D) N-His$_6$-tagged flavodoxin reductase (FDR). M; Molecular marker.
### Table S7. Reducing conditions for *in vitro* CayG-CayK system.

| Assay No. | Components (final concentration) |
|-----------|----------------------------------|
| 1         | 1 mM Ascorbic acid               |
| 2         | 1 mM L-Cysteine                  |
| 3         | 1 mM Dithiothreitol (DTT)        |
| 4         | 0.1 mM Flavodoxin reductase, 10 mM glucose-6-phosphate (G6P), 1 U mL⁻¹ baker’s yeast G6P dehydrogenase, 4 mM NADPH |
| 5         | 20 mM L-Glutathion               |
| 6         | 0.294 – 294 mM Hydrogen peroxide |
| 7         | 1 mM α-Ketoglutaric acid (KG)    |
| 8         | 50 μM phenazine methosulfate (PMS), 500 μM NADH |
| 9         | 50 μM phenazine methosulfate (PMS), 500 μM NADPH |
| 10        | 20 mM Sodium dithionite          |
| 11        | 0.1 U Spinach ferredoxin reductase, 10 mM glucose-6-phosphate (G6P), 1 U mL⁻¹ baker’s yeast G6P dehydrogenase, 4 mM NADPH |
| 12        | 1 mM Tris(2-carboxyethyl)phosphine (TCEP) |

with 50 μM CayG, and 20 μM CayK in 50 mM Tris HCl, pH 8.0, at 30 °C for 2 h.
Figure S6. Structure elucidation of compound 5 E/Z isomers. A) Structure of 5, B) $^1$H and C) $^1$H-$^1$H COSY NMR spectrum (5.4–7.4 ppm range) after CuAAC and methylation reactions with compound 3 obtained from the in vitro assay of CayG with protegencin (2).

Table S8. $^1$H NMR assignments of H7–H10 of 5.

| C7-C8/C9-C10 | E/E     | E/Z     | Z/E     |
|--------------|---------|---------|---------|
| H7           | 6.09 (d, $J = 15.1$, 7.1 Hz) | 6.18 (d, $J = 15.1$, 7.1 Hz) | 5.78 (m) |
| H8           | 6.30 (dd, $J = 15.0$, 10.8 Hz) | 6.64 (dd, $J = 15.1$, 11.2 Hz) | 6.21 (t, $J = 11.3$ Hz) |
| H9           | 6.95 (dd, $J = 15.6$, 10.8 Hz) | 6.80 (t, $J = 11.0$ Hz) | 7.32 (dd, $J = 15.5$, 11.6 Hz) |
| H10          | 5.76 (d, $J = 15.6$ Hz) | 5.54 (d, $J = 10.7$ Hz) | 5.85 (d, $J = 15.4$ Hz) |
Figure S7. $^1$H NMR spectrum of compound 18.

Figure S8. $^{13}$C NMR spectrum of compound 18.
Figure S9. $^1$H-$^1$H COSY NMR spectrum of compound 18.

Figure S10. HSQC NMR spectrum of compound 18.
Figure S11. HMBC NMR spectrum of compound 18.
Figure S12. $^1$H NMR spectrum of compound 6.
Figure S13. $^1$H NMR spectrum of compound 19a.

Figure S14. $^1$H-$^1$H COSY NMR spectrum of compound 19a.
Figure S15. $^1$H NMR spectrum of compound 19b.

Figure S16. $^1$H-$^1$H COSY NMR spectrum of compound 19b.
Figure S17. UV-Vis spectral data of compounds 7–11 (a–e).
Figure S18. HPLC-DAD profiles of polyyne compounds. a), c), e), g) and i) polyyne compounds 7, 8, 9, 10 and 11 obtained from *P. protegens* Pf-5, respectively, and b), e), f), h) and j) their modified compounds 14, 15, 16, 17 and 11 after CuAAC reactions, respectively.
Figure S19. $^1$H NMR spectrum of compound 14.

Figure S20. $^{13}$C NMR spectrum of compound 14.
Figure S21. $^1$H-$^1$H COSY NMR spectrum of compound 14.

Figure S22. HSQC NMR spectrum of compound 14.
Figure S23. HMBC NMR spectrum of compound 14.
Figure S24. $^1$H NMR spectrum of compound 15.

Figure S25. $^{13}$C NMR spectrum of compound 15.
Figure S26. $^1$H-$^1$H COSY NMR spectrum of compound 15.

Figure S27. HSQC NMR spectrum of compound 15.
Figure S28. HMBC NMR spectrum of compound 15.
Figure S29. $^1$H NMR spectrum of compound 16.

Figure S30. $^{13}$C NMR spectrum of compound 16.
Figure S31. $^1$H-$^1$H COSY NMR spectrum of compound 16.

Figure S32. HSQC NMR spectrum of compound 16.
Figure S33. HMBC NMR spectrum of compound 16.
Figure S34. $^1$H NMR spectrum of compound 17.

Figure S35. $^{13}$C NMR spectrum of compound 17.
Figure S36. $^1$H-$^1$H COSY NMR spectrum of compound 17.

Figure S37. HSQC NMR spectrum of compound 17.
Figure S38. HMBC NMR spectrum of compound 17.
Figure S39. $^1$H NMR spectrum of compound 20.

Figure S40. $^{13}$C NMR spectrum of compound 20.
Figure S41. $^1$H-$^1$H COSY NMR spectrum of compound 20.

Figure S42. HSQC NMR spectrum of compound 20.
Figure S43. HMBC spectrum of compound 20.
Figure S44. UV-Vis and HRMS spectral data from the *in vitro* assay of CayG with compound 8.
Figure S45. UV-Vis and HRMS spectral data from the in vitro assay of CayG with compound 9.
Figure S46. UV-Vis and HRMS spectral data from the *in vitro* assay of CayG with compound 10.
Figure S47. Maximum likelihood phylogenetic tree of CayG and other bacterial P450 proteins. The CYPxxx indicates CYP clans. CayG homologous proteins are highlighted as yellow. Gray colors are used for separation of clans. Colored marks revealed functions. The numbers at the nodes indicate the ultrafast bootstrap score (1,000 replicates, shown value: %) for reliability of the different groups.
Table S9. P450 proteins used in phylogenetic analysis. CYP No. was obtained by BLAST tool in Biocatnet CYP v6.0 server (https://cyped.biocatnet.de/).

| Protein | Compound | Function | Source | CYP No. | Accession No. |
|---------|----------|----------|--------|---------|---------------|
| AmphL   | Amphotericin | Hydroxylation | Streptomyces nodosus | 107E | AAK73504.1 |
| AmphN   | Amphotericin | Hydroxylation/oxidation | Streptomyces nodosus | 105H | AAK73509.1 |
| AurH    | Aureothin | Hydroxylation/ether formation | Streptomyces thioluteus HKI-227 | 151A | 3P3L |
| AveE    | Avemectin | Hydroxylation | Streptomyces avermitilis NBRC 14893 | 117A | BAC68651.1 |
| AziB1   | Azinomycin | Hydroxylation | Streptomyces sahachiroi | 107-likelike | B4XY99.1 |
| BonL    | Bongkrekic acid | Hydroxylation/oxidation | Burkholderia gladioli DMSZ11318 | 107H | AFN27475.1 |
| C158A1  | Biflaviolin | Biaryl ring coupling | Streptomyces coelicolor A3(2) | 158A | 2DKK |
| C158A2  | Biflaviolin | Biaryl ring coupling | Streptomyces coelicolor A3(2) | 158A | 1SE6 |
| CayG_Bugl1 | Caryoynencin | Desaturation/hydroxylation | Burkholderia gladioli Lv-stA | 113-likelike | KAF1061802.1 |
| CayG_Bugl2 | Caryoynencin | Desaturation/hydroxylation | Burkholderia gladioli BCC1694 | 113-likelike | WP_186166357.1 |
| CayG_Bupl | Caryoynencin | Desaturation/hydroxylation | Burkholderia plantarii LMG 9035 | 113-likelike | WP_042627242.1 |
| CayG_Trca | Caryoynencin | Desaturation/hydroxylation | Trinickia caryophylli DSM50341 | 113-likelike | AIG53832.1 |
| ChmP1   | Chalcomycin | Hydroxylation | Streptomyces bikinensis | 107B | AAS79447.1 |
| ChmPll  | Chalcomycin | Hydroxylation | Streptomyces bikinensis | 107B | AAS79446.1 |
| CloG_Cofu | Collimonin | Hydroxylation/oxidation | Collimonas fungivorans Ter331 | 107H | AEK64077.1 |
| CYP51_MeBF | unknown | unknown | Methylcoccus spp. BF19-07 | 51-likelike | WP_198324057.1 |
| CYP51_Meca | unknown | unknown | Methylcoccus capsulatus str. Bath | 51-likelike | WP_010961920.1 |
| CYP105P1 | Filipin | Hydroxylation | Streptomyces avermitilis | 105P | 3ABA |
| CYP105P2 | Flavone | Hydroxylation | Streptomyces peucetius | 105P | 5IT1 |
| CYP137_Myau | unknown | unknown | Mycolicibacterium austroafricanum | 137A | WP_109489531.1 |
| CYP137_Myva | unknown | unknown | Mycolicibacterium vanbaalenii | 137A | WP_049778016.1 |
| CYP199A4 | Methoxybenzoate | Demethylation | Rhodopseudomonas palustris HaA2 | 208A | 4DNZ |
| CYP-sb21 | Cyclosporin | Hydroxylation | Nonomuraea dietziae | 107Z | 4M4S |
| EpnI    | Eponemycin | Epoxidation | Streptomyces hygroscopicus ATT53709 | 107B-likelike | AHB38510.1 |
| EpxC    | Epoxomicin | Epoxidation | Goodfellowiella coeruleoviolacea ATT53904 | 107B-likelike | AHB38496.1 |
| EryK    | Erythromycin | Hydroxylation | Saccharopolyspora erythraea NRRL 2338 | 113A-likelike | 2JJN |
Table S9. Continued. P450 proteins used in phylogenetic analysis.

| Protein  | P450 Name | Microcompound                                      | Strain/Species                              | Accession   |
|----------|-----------|----------------------------------------------------|---------------------------------------------|-------------|
| FkbD_Sthy| FK506/FK520| Hydroxylation/oxidation                            | Streptomyces hygroscopicus subsp. ascymyceticus | 122A AAF86397.1 |
| FkbD_Stts| FK506/FK520| Hydroxylation/oxidation                            | Streptomyces tsukubensis                     | 122A TAJ41673.1 |
| FR9R     | Splicostatin| Hydroxylation/epoxidation                          | Burkholderia spp., FERM BP-3421              | 136-1 AIC32704.1 |
| FscP     | Candidin  | Hydroxylation/oxidation                            | Streptomyces spp., FR-008                   | 105H AAA82557.1 |
| GdmP     | Geldanamycin| Desaturation                                      | Streptomyces hygroscopicus 17997            | 105U ABI93790.1 |
| GilOIII  | Gilvocarcin| Desaturation                                      | Streptomyces griseoflavus Goe 3592          | 217A AAP69584.1 |
| GrhO3    | Griseorrhodin| Epoxidation                                        | Streptomyces graminofaciens                | 105G AAM33670.1 |
| GsfF     | Antibiotic FD-891| Hydroxylation                                      | Streptomyces graminofaciens                | 105A BAJ16472.1 |
| HerG     | Herboxidiene| Hydroxylation                                      | Streptomyces chromofuscus                  | 107B AEZ54507.1 |
| HedR     | Hedermycin | Epoxidation                                        | Streptomyces griseoruber                    | 105-1 AAP85338.1 |
| HmtN     | Himastatin| Hydroxylation                                      | Streptomyces himastatinicus ATCC 53653      | 113A 4E2P   |
| HmtS     | Himastatin| Hydroxylation                                      | Streptomyces himastatinicus ATCC 53653      | 113A CBZ42153.1 |
| HmtT     | Himastatin| Decarboxylation                                    | Streptomyces himastatinicus ATCC 53653      | 113A 4GGV   |
| JulI     | Julichrome| Bially ring coupling                               | Streptomyces sampsonii                     | 105A QNL10608.1 |
| LnmA     | Leinamycin| Hydroxylation                                      | Streptomyces atroolivaceus S-140            | 107B 4Z5P   |
| LnmZ     | Leinamycin| Hydroxylation                                      | Streptomyces atroolivaceus S-140            | 107B 4Z5Q   |
| MeiE     | Meilingmycin| Hydroxylation                                     | Streptomyces nanchangensis                 | 117A AAM97314.1 |
| MycCI    | Mycincinim VIII| Hydroxylation                                     | Micromonospora griseorubida                | 105U Q83WF5.3 |
| MycG     | Mycinicin IV| Hydroxylation/epoxidation                          | Micromonospora griseorubida A11725         | 107B Q59523.1 |
| NikQ     | Nikkomycin| Hydroxylation on PCP                               | Streptomyces tendae                         | 163B CAC11139.1 |
| NorH     | Neoaureothin| Hydroxylation/ether formation                      | Streptomyces orinoci                       | 151A CAO85895.1 |
| NorH-Stsc| Neoaureothin| Hydroxylation/ether formation                      | Streptomyces scabrisporus DSM 41855        | 151A WP_063744948.1 |
| NovI     | Novobiocin| Hydroxylation on PCP                               | Streptomyces niveus                         | 163B Q9L9F9.1 |
| NysL     | Nystatin  | Hydroxylation                                      | Streptomyces noursei ATCC 11455            | 107E AAF71768.1 |
| NysN     | Nystatin  | Hydroxylation/oxidation                            | Streptomyces albulus                        | 105H AVX51103.1 |
Table S9. Continued. P450 proteins used in phylogenetic analysis.

| Protein | Substrate                  | Reaction                          | Source                                    | Accession |
|---------|----------------------------|-----------------------------------|-------------------------------------------|-----------|
| OleP    | Oleandomycin               | Hydroxylation/epoxidation         | *Streptomyces antibioticus*               | 107B 4XE3 |
| OxyA    | Vancomycin                 | Biaryl ring coupling              | *Amycolatopsis orientalis*                | 165B Q8RN05.1 |
| OxyB    | Vancomycin                 | Biaryl ring coupling              | *Amycolatopsis orientalis*                | 165B Q8RN04.1 |
| OxyC    | Vancomycin                 | Biaryl ring coupling              | *Amycolatopsis orientalis*                | 165B Q8RN03.1 |
| OxyD    | Vancomycin                 | Hydroxylation                     | *Amycolatopsis orientalis*                | 146A CCD33151.1 |
| P450terf| Terfenadine                | Hydroxylation                     | *Streptomyces platensis*                  | 107L CBX53644.1 |
| PikC    | Narbomycin                 | Hydroxylation/oxidation           | *Streptomyces venezuelae*                 | 107L 2BVJ |
| PimD    | Pimaricin                  | Epoxidation                       | *Streptomyces natalensis*                 | 107E 2XBJ |
| PimG    | Pimaricin                  | Hydroxylation/oxidation           | *Streptomyces natalensis*                 | 105H CAC20928.1 |
| PldB    | Pladienolide               | Hydroxylaiton                     | *Streptomyces platensis*                  | 107B-like BAH02272.1 |
| PteC    | Filipin                    | Hydroxylation                     | *Streptomyces avermitilis*                | 183-like Q821Y3.1 |
| PtlI    | Pentalenene                | Hydroxylation/oxidation           | *Streptomyces avermitilis*                | 107B 3WVS |
| RapJ    | Rapamycin                  | Hydroxylation                     | *Streptomyces rapamycinicus*              | 183-like Q821Y3.1 |
| RapN    | Papamycin                  | Hydroxylation                     | *Streptomyces rapamycinicus*              | 105H AAR16519.1 |
| RavOIII  | Ravidomycin               | Desaturation                      | *Streptomyces ravus*                      | 107H CAC68123.1 |
| RevI    | Reveromycin                | Hydroxylation                     | *Streptomyces avendii*                    | 107H 3WVS |
| RhiH    | Rhizoxin                   | Epoxidation                       | *Mycetohabitans rhizoxinica*              | 117B CBW75250.1 |
| RhiH_Myen| Rhizoxin                  | Epoxidation                       | *Mycetohabitans endofungorum*             | 117B WP_104077490.1 |
| RhiH_Raso| Rhizoxin                  | Epoxidation                       | *Ralstonia solanacearum*                  | 117B CBJ53768.1 |
| RimG    | Rimocidin                  | Hydroxylation/oxidation           | *Streptomyces diastaticus*                | 105H AAR16519.1 |
| saAcmM  | Oxo-Pro                    | Oxidation                         | *Streptomyces antibioticus*               | 107Z 5NWS |
| ScnD    | Pimaricin                  | Epoxidation                       | *Streptomyces chattanoogensis*            | 107E ADX66473.1 |
| SoyC    | Xenobiotics                | Hydroxylation/epoxidation/Dealkylation/desaturation | *Streptomyces griseus*                   | 105A P26911.1 |
| SpcP    | Indolocarbazole            | Biaryl ring coupling/ decarboxylation | *Streptomyces sanyensis*                     | 105A P26911.1 |
| SpcH_Stsp| Spectinabilin             | Hydroxylation/ether formation     | *Streptomyces spectabilis*                 | 151A WP_144322036.1 |
| SpcH_Stfl| Spectinabilin             | Hydroxylation/ether formation     | *Streptomyces flavufunginii*              | 151A WP_190118357.1 |
### Table S9. Continued. P450 proteins used in phylogenetic analysis.

| Protein | Accession | Activity/Modification          | Organism                        | Accession  |
|---------|-----------|--------------------------------|---------------------------------|------------|
| StaF    | A47934    | Biaryl ring coupling on PCP    | *Streptomyces toyocaensis*      | 165B 5EX8  |
| StaH    | A47934    | Biaryl ring coupling on PCP    | *Streptomyces toyocaensis*      | 165B 5EX6  |
| StaP    |           | Biaryl ring coupling/ decarboxylation | *Streptomyces spp. TP-A0274*   | 245A 2Z3T |
| Taml    |           | Hydroxylation/oxidation/ epoxidation | *Streptomyces spp. 307-9*       | 107B 6XA2 |
| ThII    | Tylosin   | Hydroxylation                  | *Streptomyces fradiae* 107B-like | AAA21341.1 |
| TrdI    | Triadamycin | Hydroxylation/oxidation/ epoxidation | *Streptomyces spp. 17944* 107B-like | AGN29328.1 |
| TstR    | Thailanstatin | Hydroxylation/epoxidation   | *Burkholderia thailandensis MSMB43* 136-like | AGN11891.1 |
| TxtC    | Thaxtomin | Hydroxylation                  | *Streptomyces acidiscabies* 105A | AAL36838.1 |
| TylHI   | Tylosin   | Hydroxylation                  | *Streptomyces fradiae* 105U 6B11 |           |
| ZbmVIIc | Zorbamycin | Hydroxylation on PCP          | *Streptomyces flavoviridis* 185-like | ACG60779.1 |
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