Supporting Information

Structure Elucidation of the Syringafactin Lipopeptides Provides Insight in the Evolution of Nonribosomal Peptide Synthetases

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General Methods

Cultivation of Microorganisms

*Pseudomonas* strains were propagated in Luria Bertani (LB, Carl Roth, Germany) medium (liquid or solid, supplemented with 1.5% w/w agar) or SM/5 (Formedium™, UK) medium (liquid or solid) at 28 or 22 °C. Glycerol stocks of bacterial strains were prepared by mixing 1 mL overnight culture of the respective strain with 0.5 mL of 60% (v/v) aq. glycerol and stored at –80 °C.

Reagents and Solvents

Chemicals used during this study were purchased from ABCR, Sigma-Aldrich, TCI, Alfa Aesar, or Carl Roth and were used without further purification. Solvents were purchased from VWR as HPLC grade. Anhydrous solvents were purchased either from Acros or Alfa Aesar.

Flash-Chromatography

Normasil 60 silica gel (40 – 63 µm particle size) was used as stationary phase for normal phase flash chromatography. Fractions were analyzed by thin layer chromatography (TLC).

Thin Layer Chromatography (TLC)

As stationary phase silica 60 with fluorescence indicator F254 on aluminium foil (Merck) was used. Compounds were detected either by UV absorption (254 nm) or by staining using cerium-ammonium-molybdate (CAM).

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were recorded on Bruker Avance II 300, Avance III 500 and Avance III 600 machines. Deuterated NMR solvents were purchased from VWR. Chemical shift δ are reported in ppm, coupling constants J in Hz. The residue proton signal (1) of the respective solvent were used as internal standards (CDCl3: δ = 7.26 ppm (1H), δ = 77.16 (13C); d6-DMSO: δ = 2.50 ppm (1H), δ = 39.52 (13C)). The signal fine structures are described, using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet) as well as combinations of these. Spectra were analyzed using Bruker TopSpin software.

High Resolution Mass Spectrometry (HRMS)

LC-ESI-HRMS measurements were carried out on an Accela UPLC system (Thermo Scientific) equipped with an Accucore C18 column (100 x 2.1 mm, particle size 2.6 µm) coupled with a Q-Exactive mass spectrometer (Thermo Scientific) with an electrospray ionization (ESI) source.

Tandem Mass Spectrometric Analysis Using Electrospray Ionization (ESI-MS/MS)

ESI-MS/MS measurements were performed using the LC-MS/MS system Dionex UltiMate 3000 binary RSLC HPLC (Thermo Fisher Scientific, Dreieich, Germany) and a LTQ XL Linear Ion Trap mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany) equipped with an electrospray ion source. The HPLC was equipped with an ACCUCORE RP-MS column (150 x 4.6 mm, 2.6 µm, 80 Å, Thermo Fischer Scientific, flow rate = 1 mL/min, method: 0 – 6 min: 35% MeCN in water containing 0.1% formic acid; 6 – 12 min: linear gradient 35 – 100% MeCN in water containing 0.1% formic acid). The ESI was used in positive mode with the capillary temperature set at 400 °C, the source voltage at 4 kV and the capillary voltage at 30 V. The ion trap was set using the standard scan rate and for MS<sup>n</sup> experiments the normalized collision energy was 35 eV using CID. Data was analyzed using ChemBioDraw (PerkinElmer, Waltham, Massachusetts, USA) and Mass Frontier 7.0 (Thermo Fisher Scientific, Dreieich, Germany).

Liquid Chromatography Coupled With Mass Spectrometric Detection (LC-MS)

LC-MS measurements were performed on a Shimadzu LCMS-2020 system equipped with single quadrupole mass spectrometer using a Kinetex C18 column (50 x 2.1 mm, particle size 1.7 µm, pore diameter 100 Å, Phenomenex). Column oven was set to 40 °C; scan range of MS was set to m/z 150 to 2,000 with a scan speed of 10,000 u/s and event time of 0.25 s under positive and negative mode. Desolvation line temperature was set to 250 °C with an interface temperature of 350 °C and a heat block temperature of 400 °C. The nebulizing gas flow was
set to 1.5 L/min and dry gas flow to 15 L/min. If not otherwise stated a standard LC-method was used: flow rate = 0.7 mL/min; 0 – 0.5 min: 10% (v/v) MeCN in water containing 0.1% formic acid; 0.5 – 8.5 min: linear gradient 10 – 100% MeCN in water containing 0.1% formic acid; 8.5 – 11.5 min: 100% MeCN in water containing 0.1% formic acid).

Optical Rotation

Optical rotation was measured on a JASCO P-1020 polarimeter equipped with a JASCO MCB-100 mini circulating bath, set to 25 °C.

Generation of Gene Deletion Mutant Δvif in P. sp QS1027

For the generation of markerless genomic deletion mutants a gentamicin-resistance (gentR) selection and sucrose counterselection (sacB) process was used. The corresponding pEXG2-based suicide vectors (Rietsch A, et al. Proc. Nat. Acad. Sci. U.S.A. 2005, 102, 8006. and Stallforth P, et al. Proc. Nat. Acad. Sci. U.S.A. 2013, 110, 14528.) were constructed using the Gibson Assembly method. The parent plasmid pEXG2 was linearized by HindIII and EcoRI restriction. Left and right homology arms (LA, RA) were PCR amplified from genomic DNA using primer pairs LA vif fwd/LA vif rev and RA vif fwd/RA vif rev, respectively (see table underneath). These primers include a sequence of about 20 bp complementary to the adjacent PCR fragment and the linearized vector (the primers were designed using: http://nebuilder.neb.com). The LA and RA were ligated into the pEXG2 vector using the standard Gibson Assembly protocol (New England Biolabs) to yield the respective deletion construct pEXG2Δvif.

Table S1: Primers used for constructing gene deletion plasmids by Gibson assembly.

| Primer sequences 5’ – 3’       | TCCGCACACACGGCTTCTTCTCCCTGCTG       | LA vif rev          |
| LA vif fwd                 | GGAAGCATAAATGTAAGCAGCATGCAGCAGTTCGCC | RA vif fwd          |
| RA vif rev                 | GGAAATTACGTTACGCGTACGCAGGATGCTG GCC | RA vif rev          |

The vectors were transformed into chemically competent E. coli Top10 cells via heat shock at 42 °C. Plasmids were purified using the QIAprep® Spin Miniprep Kit (Qiagen) and sequenced. For subsequent intergenic conjugation, chemically competent E. coli ET12567 pUZ8002 was transformed with the respective deletion vector constructs. Biparental mating was performed using a standard protocol. Briefly, overnight donor (E. coli ET12567 pUZ8002 pEXG2Δslp) and acceptor (QS1027) strain cultures were diluted to an OD600 = 0.1 and grown to OD600 = 0.6. The cultures were mixed in 1:1 (v/v, QS1027:E. coli), 1:2, and 1:3 ratios. The mixed cultures were washed with sterile, deionized water. Mating spots (30 μL) were placed on dry LB agar plates and incubated at 28 °C overnight. The mating spots were then suspended in LB medium (200 μL) and plated on LB plates (100 μL, 15 μg/mL gentamicin and 100 μg/mL ampicillin). Single transformants were selected and used to inoculate LB medium cultures. Overnight cultures were plated on 5% sucrose LB plates (without NaCl) for selection of double crossover mutants. Deletion mutants were identified by PCR using primer pairs including both up- and downstream regions of the homology arms: KOC LA vif fwd/KOC LA vif rev and KOC RA vif fwd/KOC RA vif rev (see table underneath).
Table S2: Gene deletion control primers.

<table>
<thead>
<tr>
<th>Primer sequences 5’–3’</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOC LA vif fwd</td>
<td>GTTCGTGACCAGGCCTACTG</td>
</tr>
<tr>
<td>KOC LA vif rev</td>
<td>CCTCGGGGGCTTCGTATTCA</td>
</tr>
<tr>
<td>KOC RA vif fwd</td>
<td>GGGTTGCGAATGCACAGTT</td>
</tr>
<tr>
<td>KOC RA vif rev</td>
<td>ATCCGCGTGATCGAAATG</td>
</tr>
</tbody>
</table>

The vif deletion mutant was created by a clean deletion of the first leucine adenylation (A_{Leu}) domain in the virginiafactin biosynthetic gene cluster.

**Metabolic Profiling via LC-MS**

**Preparation of Samples**

Cultures of the respective bacterial strain were inoculated from cryo stocks in 10 mL LB-medium and cultivated in 50 mL Erlenmeyer flasks on a gyratory shaker (180 rpm) at 22 °C for 24 h. 5 mL of the culture were extracted with 10 mL ethyl acetate, the organic phase was dried over Na₂SO₄, decanted and the solvent was removed *in vacuo*. The residue was dissolved in 200 µL MeOH, filtered through a 0.2 µm PTFE syringe filter and further analyzed *via* LC-MS.

**Detection of Virginiafactin A–D in QS1027 and the Δvif deletion mutant**

![Figure S1: HPLC-traces of extracts from the QS1027 WT (red) and the Δvif deletion mutant (black)](image)

**Isolation of Virginiafactins A–D**

A 5 mL LB liquid culture was prepared from a single colony of *Pseudomonas* sp. QS1027 and incubated overnight at 22 °C while shaking on a gyratory shaker at 180 rpm. A 200 mL LB liquid culture was inoculated from the 5 mL pre-culture and shaken for 12 h at 22 °C. A 20 L batch fermenter of LB medium was inoculated with the 200 mL pre-culture and fermented for 24 h at 25 °C. The bacterial culture was centrifuged for 12 min. at 5,000 g and the supernatant was acidified to pH 2 using 6 N aqueous HCl. The supernatant was extracted with 20 L ethyl...
acetate, the organic phase was dried over Na₂SO₄ and solvents were removed in vacuo. The residue (866 mg) was fractionated using a HyperSep™ C18 Cartridge (Thermo Scientific) and eluted with a mixture of 15%, 50%, 75%, and 100% (v/v) MeCN in water containing 0.1% formic acid. LC-MS analysis showed that the 75% fraction contained linear lipopeptides and was further purified using a semi-preparative HPLC (Shimadzu) equipped with a Luna® C18(2) column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, flow rate = 5 mL/min, method: 0 – 1 min: 10% (v/v) MeCN in water containing 0.1% formic acid; 1–5 min: linear gradient 10% – 70% MeCN in water containing 0.1% formic acid) to yield three linear lipopeptides as white solids: virginiafactin B: tᵣ = 10.6 min; virginiafactin C: tᵣ = 12.5 min; virginiafactin D: tᵣ = 13.2 min.

All three lipopeptides were further purified using a semi-preparative HPLC (Shimadzu) equipped with a Luna® Phenyl-Hexyl column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, flow rate = 5 mL/min, method: 0 – 5 min: 75% (v/v) MeCN in water containing 0.1% formic acid) to yield three linear lipopeptides as white solids.

For the isolation of virginiafactin A, a similar method was used as above only with Kings Medium B as a cultivation medium. Extracts were obtained analogously and the residue was fractionated using a HyperSep™ C18 Cartridge (Thermo Scientific) and eluted with a mixture of 30%, 50%, 75%, and 100% (v/v) MeCN in water; LC-MS analysis showed that the 30% and 50% fractions contained the linear lipopeptides and was further purified using a semi-preparative HPLC (Shimadzu) equipped with a Luna® C18(2) column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, flow rate = 5 mL/min, method: 0–1 min: 10% (v/v) MeCN in water containing 0.1% formic acid; 1–5 min: linear gradient 10%–70% MeCN in water containing 0.1% formic acid; 5 – 14 min: 70%–90% MeCN in water containing 0.1% formic acid) to yield linear lipopeptide as beige solid: Virginiafactin A: tᵣ = 10.1 min.

**Virginiafactin A**

![Structure of Virginiafactin A]

Yield: 2.4 mg; ¹H NMR (600 MHz, d₄-MeOH): δ = 4.49 (dd, ³J_H,H= 9.9, 4.8, 1H), 4.44 (dd, ³J_H,H= 9.5, 5.4, 1H), 4.40 – 4.35 (m, 3H), 4.32 (dd, ³J_H,H= 9.5, 5.4, 1H), 4.5 (dd, ³J_H,H= 9.5, 4.9, 1H), 4.20 (d, ³J_H,H= 6.9, 1H), 4.00 – 3.98 (m, 1H), 3.88 – 3.82 (m, 2H), 2.47 (dd, ³J_H,H= 14.2, 4.5, 1H), 2.37 – 2.31 (m, 3H), 2.19 – 2.13 (m, 2H), 2.07 – 2.00 (m, 1H), 1.73 – 1.58 (m, 13H), 1.50 – 1.48 (m, 2H), 1.32 – 1.29 (m, 12H), 0.97 – 0.89 (m, 39H); ¹³C NMR (150 MHz, d₄-MeOH): δ = 177.7, 176.0, 175.6, 175.7, 175.0, 174.9, 174.5, 174.2, 173.5, 172.6, 70.0, 62.9, 60.7, 57.4, 55.1, 53.8, 53.8, 53.7, 53.1, 52.2, 44.6, 41.9, 41.8, 41.6, 41.4, 41.0, 38.4, 33.0, 32.8, 31.4, 30.7, 30.5, 28.1, 26.8, 26.0, 26.0, 25.9, 23.7, 23.6, 23.6, 23.6, 23.6, 23.5, 23.4, 22.0, 21.9, 21.9, 21.8, 21.7, 19.8, 18.8, 14.5. HRMS (ESI-) calcd for [C₉₃H₇₃N₉O₁₃·H]⁺ 1066.7135, found 1066.7135, [α]D²⁵ = -6.0 (c = 0.1, MeOH).

**Virginiafactin B**

![Structure of Virginiafactin B]

Yield: 1.4 mg; ¹H NMR (600 MHz, d₄-MeOH): δ = 4.49 (dd, ³J_H,H= 9.9, 4.8, 1H), 4.42 (t, ³J_H,H= 7.2, 1H), 4.40 – 4.35 (m, 3H), 4.35 – 4.31 (m, 1H), 4.28 – 4.22 (m, 2H), 4.01 – 3.96 (m, 1H),
3.90 – 3.80 (m, 2H), 2.47 (dd, $^3J_{HH} = 14.2, 4.5, 1H)$, 2.39 – 2.28 (m, 3H), 2.21 – 2.13 (m, 1H), 2.09 – 2.01 (m, 1H), 1.98 – 1.90 (m, 1H), 1.78 – 1.55 (m, 13H), 1.54 – 1.42 (m, 4H), 1.39 – 1.23 (m, 10H), 1.21–1.11 (m, 2H), 1.00-0.85 (m, 39H); $^{13}$C NMR (150 MHz, $d_6$-MeOH): $\delta =$ 177.7, 175.7, 175.4, 175.0, 174.9, 174.3, 174.2, 173.6, 172.6, 70.0, 62.9, 59.8, 57.4, 55.2, 53.9, 53.7, 53.1, 52.3, 44.7, 42.0, 41.8, 41.6, 41.4, 41.0, 38.4, 37.5, 33.0, 32.8, 30.7, 30.5, 28.1, 26.8, 26.0, 26.0, 25.9, 25.9, 23.7, 23.6, 23.4, 22.0, 22.0, 21.9, 21.8, 21.7, 16.0, 14.5, 11.6; $\mathrm{t} =$ = 3.6 min; HRMS (ESI+) calcd for $[\text{C}_{54}\text{H}_{98}\text{N}_3\text{O}_{13}+\text{H}]^+$ 1082.7435, found 1082.7442, $[\alpha]_D^{25} =$ −15.1 (c = 0.20, MeOH).

**Virginiadactin C**

![Chemical Structure of Virginiadactin C]

Yield: 1.5 mg; $^1$H NMR (600 MHz, $d_6$-MeOH); $\delta =$ 4.49 (dd, $^3J_{HH} = 9.8, 5.1, 1H$), 4.46 – 4.42 (m, 1H), 4.40 – 4.35 (m, 3H), 4.34 – 4.30 (m, 1H), 4.25 (dd, $^3J_{HH} = 9.6, 4.7, 1H$), 4.20 (d, $^3J_{HH} = 6.9, 1H$), 4.01 – 3.96 (m, 1H), 3.85 (dq, $^3J_{HH} = 11.4, 5.5, 2H$), 2.47 (dd, $^3J_{HH} = 14.2, 4.5, 1H$), 2.39 – 2.26 (m, 3H), 2.20 – 2.13 (m, 2H), 2.08 – 2.00 (m, 1H), 1.78 – 1.55 (m, 13H), 1.53 – 1.42 (m, 3H), 1.39 – 1.24 (m, 14H), 1.16 – 1.12 (m, 1H), 1.00 – 0.85 (m, 39H); $^{13}$C NMR (150 MHz, $d_6$-MeOH): $\delta =$ 177.7, 176.2, 175.6, 175.6, 175.0, 174.9, 174.4, 174.2, 173.5, 172.6, 70.0, 62.9, 60.7, 57.4, 55.1, 53.8, 53.7, 53.1, 52.3, 44.7, 41.9, 41.8, 41.6, 41.4, 41.0, 38.4, 33.1, 32.8, 31.3, 30.8, 30.7, 30.5, 28.1, 26.8, 26.0, 25.9, 23.7, 23.6, 23.5, 23.4, 22.0, 21.9, 21.8, 21.7, 19.8, 18.8, 14.5; $\mathrm{t} =$ = 4.1 min; HRMS (ESI+) calcd for $[\text{C}_{55}\text{H}_{101}\text{N}_3\text{O}_{13}+\text{H}]^+$ 1096.7592 found 1096.7599, $[\alpha]_D^{25} =$ −17.2 (c = 0.21, MeOH).

**Virginiadactin D**

![Chemical Structure of Virginiadactin D]

Yield: 2.4 mg; $^1$H NMR (600 MHz, $d_6$-MeOH); $\delta =$ 4.50 (dd, $^3J_{HH} = 9.9, 4.8, 1H$), 4.45 – 4.41 (m, 1H), 4.40 – 4.35 (m, 3H), 4.32 (t, $^3J_{HH} = 7.2, 1H$), 4.27 – 4.22 (m, 2H), 4.02 – 3.96 (m, 1H), 3.90 – 3.81 (m, 2H), 2.47 (dd, $^3J_{HH} = 14.2, 4.5, 1H$), 2.39 – 2.28 (m, 3H), 2.21 – 2.13 (m, 1H), 2.09 – 2.01 (m, 1H), 1.97 – 1.89 (m, 1H), 1.78 – 1.55 (m, 13H), 1.54 – 1.42 (m, 4H), 1.39 – 1.23 (m, 14H), 1.21 – 1.11 (m, 2H), 1.00 – 0.85 (m, 39H); $^{13}$C NMR (150 MHz, $d_6$-MeOH); $\delta =$ 177.7, 176.1, 175.7, 175.6, 174.9, 174.9, 174.5, 174.2, 173.5, 172.5, 70.0, 62.9, 59.8, 57.4, 55.2, 53.9, 53.7, 53.6, 53.1, 52.2, 44.6, 41.9, 41.7, 41.6, 41.4, 41.0, 38.4, 37.6, 33.1, 32.8, 30.8, 30.7, 30.5, 28.1, 26.8, 26.0, 25.9, 25.9, 23.7, 23.6, 23.5, 23.4, 22.1, 21.9, 21.9, 21.8, 21.7, 16.1, 14.6, 11.7; $\mathrm{t} =$ = 4.4 min; HRMS (ESI+) calcd for $[\text{C}_{56}\text{H}_{103}\text{N}_3\text{O}_{13}+\text{H}]^+$ 1110.7748, found 1110.7755, $[\alpha]_D^{25} =$ −14.2 (c = 0.24, MeOH).
Sequence Analysis of Virginiafactins A – D using ESI-MS²

Virginiafactin A

Figure S2: MS²-fragmentation of virginiafactin A. Colored numbers correspond to the m/z ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.
Figure S3: MS²-fragmentation of virginiafactin B. Colored numbers correspond to the m/z ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.
Figure S4: MS²-fragmentation of virginiafactin C. Colored numbers correspond to the m/z ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.
Figure S5: MS²-fragmentation of virginiafactin D. Colored numbers correspond to the m/z ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.
Determination of the Configuration of the Amino Acids in Lipopeptides using Marfey's Reagent

In general, 250 µL 6 N aq. HCl were added to 100 – 250 µg of the respective lipopeptide. The reaction vessel was heated to 100 °C and shaken at that temperature for 16 h. Solvent was removed \textit{in vacuo} and the residue was dissolved in 100 µL water. 200 µL Marfey’s Reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA, 1% solution in acetone, Thermo Scientific) and 40 µL 1 M aq. NaHCO₃ solution were added. The reaction was shaken for 1 h at 40 °C. Then, the reaction was neutralized with 20 µL 2 N aq. HCl and filtered through a PTFE filter (0.2 µm). Analysis was performed on a semi-preparative HPLC (Shimadzu) equipped with a Luna® C18(2) column (250 × 4.6 mm, 5 µm, 100 Å, Phenomenex, flow rate = 1 mL/min, method: 0 – 1 min: 25% (v/v) MeCN in water containing 0.1% formic acid; 1 – 46 min: linear gradient 25 – 65% MeCN in water containing 0.1% formic acid). Amino acids (5 µmol) for comparison were dissolved in 100 µL water and treated in the same manner.

Standard Retention Times (in min.):
L-Ser ($t_R = 9.16$); D-Ser ($t_R = 9.48$); L-Thr ($t_R = 10.05$); D-alloThr ($t_R = 11.1$); L-Gln ($t_R = 11.50$); D-Gln ($t_R = 12.45$); L-Val ($t_R = 20.12$); D-Val ($t_R = 24.41$); L-Ile ($t_R = 24.16$); D-Ile ($t_R = 28.38$); L-Leu ($t_R = 25.05$); D-Leu ($t_R = 29.23$).

Virginiafactin A

![Figure S6](image)

Figure S6: HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin A (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Val (black) (all chromatograms detected at $\lambda = 330$ nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.

![Figure S7](image)

Figure S7: HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin A ($\lambda = 330$ nm).
Table S3: Integration areas for peaks from Fig. S7

<table>
<thead>
<tr>
<th>Peak # / amino acid</th>
<th>t&lt;sub&gt;ret&lt;/sub&gt; /[min]</th>
<th>area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / d-Ser</td>
<td>9.56</td>
<td>905886</td>
</tr>
<tr>
<td>2 / d-Gln</td>
<td>12.49</td>
<td>1177921</td>
</tr>
<tr>
<td>3 / L-Val</td>
<td>20.07</td>
<td>1159733</td>
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<tr>
<td>4 / L-Leu</td>
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<tr>
<td>5 / d-Leu</td>
<td>29.42</td>
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</tbody>
</table>

Result: 0.8 x d-Ser, 1.0 x d-Gln, 1.3 x L-Val, 4.9 x L-Leu, 1.0 x d-Leu

**Virginiafactin B**

![HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin B (red) and derivatized reference amino acids: L-/D-Ser (green), L-/D-Leu (blue), L-/D-Gln (pink) and L-/D-Ile (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.](image)

**Figure S8:** HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin B (red) and derivatized reference amino acids: L-/D-Ser (green), L-/D-Leu (blue), L-/D-Gln (pink) and L-/D-Ile (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.

![HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin B (λ = 330 nm).](image)

**Figure S9:** HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin B (λ = 330 nm).
**Table S4:** Integration areas for peaks from Fig. S9

<table>
<thead>
<tr>
<th>Peak # / amino acid</th>
<th>( T_{rel.} [/{\text{min}}] )</th>
<th>area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / D-Ser</td>
<td>9.556</td>
<td>375488</td>
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<tr>
<td>2 / D-Gln</td>
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<td>3 / L-Ile</td>
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<td>4 / L-Leu</td>
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<td>1884739</td>
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<tr>
<td>5 / D-Leu</td>
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<td>346244</td>
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</tbody>
</table>

Result: 1.0 x D-Gln; 1.0 x D-Ser, 1.3 x L-Ile, 4.6 x L-Leu, 0.9 x D-Leu

**Virginiafactin C**

**Figure S10:** HPLC profiles of the hydrolyzed and FDAA-derivated virginiafactin C (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Ile (black) (all chromatograms detected at \( \lambda = 330 \) nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.

**Figure S11:** HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin C (\( \lambda = 330 \) nm).
Table S5: Integration areas for peaks from Fig. S11.

<table>
<thead>
<tr>
<th>Peak # / amino acid</th>
<th>$T_{ref.}$/[min]</th>
<th>area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / d-Ser</td>
<td>9.60</td>
<td>348627</td>
</tr>
<tr>
<td>2 / d-Gln</td>
<td>12.54</td>
<td>364098</td>
</tr>
<tr>
<td>3 / l-Val</td>
<td>20.15</td>
<td>324752</td>
</tr>
<tr>
<td>4 / l-Leu</td>
<td>25.12</td>
<td>1681496</td>
</tr>
<tr>
<td>5 / d-Leu</td>
<td>29.44</td>
<td>333840</td>
</tr>
</tbody>
</table>

Result: 1.0 x d-Gln; 1.0 x d-Ser, 0.9 x l-Val, 4.6 x l-Leu, 0.9 x d-Leu.

Virginiafactin D

Figure S12: HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin D (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Ile (black) (all chromatograms detected at $\lambda = 330$ nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.

Figure S13: HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin D ($\lambda = 330$ nm).
Table S6: Integration areas for peaks from Fig. S13.

<table>
<thead>
<tr>
<th>Peak # / amino acid</th>
<th>T_{ret}, [min]</th>
<th>area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / D-Ser</td>
<td>9.52</td>
<td>401680</td>
</tr>
<tr>
<td>2 / D-Gln</td>
<td>12.487</td>
<td>412285</td>
</tr>
<tr>
<td>3 / L-Ile</td>
<td>24.08</td>
<td>495340</td>
</tr>
<tr>
<td>4 / L-Leu</td>
<td>25.07</td>
<td>1858927</td>
</tr>
<tr>
<td>5 / D-Leu</td>
<td>29.389</td>
<td>382378</td>
</tr>
</tbody>
</table>

Result: 1.0 x D-Gln; 1.0 x D-Ser, 1.2 x L-Ile, 4.5 x L-Leu, 0.9 x D-Leu

Cichofactin A

Figure S14: HPLC profiles of the hydrolyzed and FDAA-derivatized cichofactin A (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Val (black) (all chromatograms detected at \( \lambda = 330 \) nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.

Figure S15: HPLC profile of the hydrolyzed and FDAA derivatized cichofactin A (\( \lambda = 330 \) nm).
Table S7: Integration areas for peaks from Fig. S15.

<table>
<thead>
<tr>
<th>Peak # / amino acid</th>
<th>( T_{ret.} / \text{[min]} )</th>
<th>area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / d-Gln</td>
<td>12.499</td>
<td>10853057</td>
</tr>
<tr>
<td>2 / L-Val</td>
<td>20.12</td>
<td>5045054</td>
</tr>
<tr>
<td>3 / L-Leu</td>
<td>25.053</td>
<td>28998894</td>
</tr>
<tr>
<td>4 / d-Leu</td>
<td>29.26</td>
<td>5283187</td>
</tr>
</tbody>
</table>

Result: 2.2 x d-Gln; 1.0 x L-Val, 5.7 x L-Leu, 1.0 x d-Leu

Cichofactin B

Figure S16: HPLC profiles of the hydrolyzed and FDAA-derivatized cichofactin B (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Val (black) (all chromatograms detected at \( \lambda = 330 \text{ nm} \)). Annotated peaks are labeled. Asterisks indicate impurities from FDAA or byproducts from the hydrolysis reaction.

Figure S17: HPLC profile of the hydrolyzed and FDAA derivatized cichofactin B D (\( \lambda = 330 \text{ nm} \)).
**Table S8:** Integration areas for peaks from **Fig. S17**

<table>
<thead>
<tr>
<th>Peak # / amino acid</th>
<th>$T_{ret.}$ [min]</th>
<th>area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / d-Gln</td>
<td>12.475</td>
<td>3412777</td>
</tr>
<tr>
<td>2 / L-Val</td>
<td>20.083</td>
<td>1601105</td>
</tr>
<tr>
<td>3 / L-Leu</td>
<td>25.004</td>
<td>8399333</td>
</tr>
<tr>
<td>4 / d-Leu</td>
<td>29.262</td>
<td>1690310</td>
</tr>
</tbody>
</table>

Result: 2.1 x d-Gln; 1.0 x L-Val, 5.2 x L-Leu, 1.0 x d-Leu
Synthesis of Lipo-octapeptides


Synthesis of methyl 3-oxodecanoate 1a

720.5 mg (5.0 mmol, 1 eq.) of Meldrum’s acid (2,2-dimethyl-1,3-dioxane-4,6-dione) and 807 µL (10 mmol, 2 eq.) anhydrous pyridine were solved in 20 mL anhydrous DCM. While stirring the solution was cooled to 0 °C. 940 µL (5.5 mmol, 1.1 eq.) octanoyl chloride were added dropwise. When the addition was complete, the reaction was allowed to warm to room temperature and was stirred for an additional hour. 5 mL of toluene were added to the reaction mixture and the solvents were removed in vacuo. The crude extract was re-dissolved in 20 mL anhydrous MeOH and heated to reflux while stirring. After 3 h the reaction was cooled down to room temperature and the solvent was removed in vacuo. The crude product was purified by normal phase flash chromatography (98:2 to 90:10 (v/v) hexanes/ethyl acetate) to yield 660 mg (3.3 mmol, 66%) of title compound as a colorless liquid.

\[ \text{H NMR (300 MHz, CDCl}_3, \text{only signals of the predominant keto tautomer are given): } \delta = 3.68 (s, 3H), 3.39 (s, 2H), 2.47 (t, 2H, } \text{J}_{H,H} = 7.4), 1.54 (dt, 2H, } \text{J}_{H,H} = 14.1, 7.1), 1.32 – 1.15 (m, 8H), 0.82 (distorted t, 3H, } \text{J}_{H,H} = 6.7). \]

\[ \text{C NMR (75.5 MHz, CDCl}_3, \text{only signals of the predominant keto tautomer are given): } \delta = 202.8, 167.7, 52.3, 49.0, 43.1, 31.7, 29.0, 29.0, 23.5, 22.6, 14.0. \]

HRMS (ESI+): [\text{C}_{11}\text{H}_{21}\text{O}_3+H]^+, \text{calcd: } 201.1845, \text{found: } 201.1487. \text{R}_f = 0.79 (9:1 (v/v), hexanes/ethyl acetate). The analytical data is in accordance with previously published data.

Methyl 3-oxododecanoate 1b

Compound 1b was synthesized analogously to compound 1a using the same protocol.

Scale: 10 mmol; yield 720 mg (2.79 mmol, 28%; 40% brsm). \[ \text{H NMR (300 MHz, CDCl}_3, \text{only signals of the predominant keto tautomer are given): } \delta = 3.68 (s, 3H), 3.39 (s, 2H), 2.47 (t, 2H, } \text{J}_{H,H} = 7.4), 1.56 – 1.43 (m, 2H), 1.28 – 1.09 (m, 12H), 0.79 (distorted t, 3H, } \text{J}_{H,H} = 6.7). \]

\[ \text{C NMR (75.5 MHz, CDCl}_3, \text{only signals of the predominant keto tautomer are given): } \delta = 202.7, 167.6, 52.1, 48.9, 42.9, 31.8, 29.3, 29.3, 29.2, 28.9, 23.4, 22.6, 14.0. \]

HRMS (ESI+): [\text{C}_{13}\text{H}_{24}\text{O}_3+H]^+, \text{calcld: } 229.1798, \text{found: } 229.1802. \text{R}_f = 0.70 (9:1 (v/v), hexanes/ethyl acetate).

Synthesis of methyl (R)-3-hydroxydecanoate (R)-2a

The catalyst was freshly prepared: 10.5 mg (0.0168 mmol, 0.044 eq.) (R)-BINAP and 4.4 mg (0.014 mmol, 0.02 eq.) (COD)Ru(2-methylallyl)_2 were placed together with 700 µL degassed acetone in a 10 mL Schlenk-tube, equipped with a magnetic stirring bar. 180 µL methanolic
HBr (3.5 µL of 48% HBr diluted in 177 µL anhydrous, degassed MeOH) were added to the solution. The reaction was kept under argon and stirred at room temperature for 30 min. The solvent was removed in vacuo and the brownish precipitate was used as hydrogenation catalyst.

A solution of the β-keto ester 1a (140 mg, 0.7 mmol, 1 eq.) in 1.4 mL anhydrous, degassed MeOH was added to the flask containing the catalyst. A balloon filled with hydrogen gas was connected and the reaction vessel was flushed with hydrogen gas (five times). Then, the reaction was heated to 55 °C while stirring and it was kept at this temperature for 3 h. The reaction mixture was cooled to room temperature, filtered through a pad of Celite® and the solvent was removed in vacuo. After normal phase flash chromatography (4:1 (v/v), hexanes/ethyl acetate) 73.0 mg (0.36 mmol, 51%) of title compound was obtained as colorless oil.

$^1$H NMR (500 MHz, CDCl$_3$): δ = 4.02 – 3.95 (m, 1H), 3.71 (s, 3H), 2.50 (dd, 1H, $^3$J$_{HH}$ = 16.5, 3.1), 2.40 (dd, 1H, $^3$J$_{HH}$ = 16.4, 9.1), 1.57 – 1.47 (m, 1H), 1.47 – 1.37 (m, 2H), 1.37 – 1.20 (bs, 9H), 0.87 (distorted t, 3H, $^3$J$_{HH}$ = 6.9).

$^{13}$C (125.8 MHz, CDCl$_3$): δ = 173.7, 68.2, 51.9, 41.2, 36.7, 31.9, 29.6, 29.4, 25.6, 22.8, 14.2. HRMS (ESI+): [C$_{11}$H$_{22}$O$_3$+H]$^+$, calc.: 203.1642, found: 203.1644.

[α]$^2$D = –17.02 (c=1, CHCl$_3$). R$_f$ = 0.48 (4:1 (v/v), hexanes/ethyl acetate).

The analytical data is in accordance with previously published data.

**Methyl (R)-3-hydroxydodecanoate 2b**

![Methyl (R)-3-hydroxydodecanoate](image)

Compound 2b was synthesized analogously to compound 2a using the same protocol.

Scale: 1.23 mmol; yield 159 mg (0.69 mmol, 56%). $^1$H NMR (300 MHz, CDCl$_3$): δ = 4.05 – 3.95 (m, 1H), 3.71 (s, 3H), 2.51 (dd, 1H, $^3$J$_{HH}$ = 16.4, 3.3), 2.40 (dd, 1H, $^3$J$_{HH}$ = 16.4, 8.9), 1.59 – 1.17 (m, 16H), 0.87 (distorted t, 3H, $^3$J$_{HH}$ = 6.7).

$^{13}$C (75.5 MHz, CDCl$_3$): δ = 173.6, 68.2, 51.9, 41.2, 36.7, 32.0, 29.7, 29.7, 29.6, 29.4, 25.6, 22.8, 14.2. HRMS (ESI+): [C$_{13}$H$_{26}$O$_3$+H]$^+$, calc.: 231.1955, found: 231.1958. R$_f$ = 0.44 (4:1 (v/v), hexanes/ethyl acetate).

**Synthesis of (R)-3-hydroxydecanoic acid 3a**

![Synthesis of (R)-3-hydroxydecanoic acid](image)

17.4 mg (0.09 mmol, 1 eq.) of methyl ester 2a were solved in 2.5 mL THF. While stirring a 2.5 mL of an 1M aqueous LiOH-solution were added. After 0.5 h complete conversion of the starting material was detected by TLC. The reaction mixture was neutralized by addition of 2.5 mL 1M aqueous HCl, followed by extraction using 3x10 mL ethyl acetate. The organic phases were pooled, dried over anhydrous Na$_2$SO$_4$, filtered and the solvent was removed in vacuo. 15.2 mg (0.081 mmol, 90%) of the title compound were obtained as a colorless solid and was used in following reactions without further purification.

$^1$H NMR (500 MHz, CDCl$_3$): δ = 4.06 – 4.00 (m, 1H), 2.57 (dd, 1H, $^3$J$_{HH}$ = 16.7, 3.3), 2.47 (dd, 1H, $^3$J$_{HH}$ = 16.6, 9.0), 1.60 – 1.51 (m, 1H), 1.51 – 1.40 (m, 2H), 1.37 – 1.23 (bs, 9H), 0.87 (distorted t, 3H, $^3$J$_{HH}$ = 6.9).

$^{13}$C (125.8 MHz, CDCl$_3$): δ = 177.7, 68.2, 41.2, 36.7, 31.9, 29.6, 29.3, 25.6, 22.8, 14.2. HRMS (ESI-): [C$_{10}$H$_{20}$O$_3$]$,^-$ calc.: 187.1340, found: 187.1329. [α]$^2$D = –19.7 (c=0.5, DCM). R$_f$ = 0.57 (4:1 (v/v), hexanes/ethyl acetate).
(R)-3-hydroxydodecanoic acid 3b

Compound 3b was synthesized analogously to compound 3a using the same protocol.

Scale: 0.6 mmol; yield 121.2 mg (0.56 mmol, 94%). $^1$H NMR (300 MHz, CDCl₃): $\delta = 4.08 - 3.98$ (m, 1H), 2.58 (dd, 1H, $^3$J₉,₈ = 16.6, 3.3), 2.47 (dd, 1H, $^3$J₉,₈ = 16.6, 8.8), 1.61 – 1.19 (m, 16H), 0.88 (distorted t, 3H, $^3$J₉,₈ = 6.7). $^{13}$C (75.5 MHz, CDCl₃): $\delta = 176.7$, 68.0, 40.9, 36.5, 31.9, 29.6, 29.5, 29.3, 25.4, 22.7, 14.1. HRMS (ESI-): [C₁₂H₂₀O₃-H]$,^-$ calcd: 215.1653, found: 215.1647. $R_f = 0.67$ (1:1 (v/v), hexanes/ethyl acetate + 0.1% formic acid).

Synthesis of succinimidyl (R)-3-hydroxydecanoate 4a

8.5 mg (0.045 mmol, 1 eq.) of (R3)-hydroxydecanoic acid and 14 mg (0.06 mmol, 1.03 eq.) of N,N,N',N'-tetramethyl-O-(N-succinimidyl)uroniumtetrafluoroborat (TSTU) were solved in 0.5 mL anhydrous THF. To the stirred mixture 10 µL (0.06 mmol, 1.3 eq.) N,N-diisopropylethylamine (DIPEA) were added and it was stirred at room temperature. After 30 min. complete conversion of the starting material was observed by TLC. The crude reaction mixture was subsequently subjected to column chromatography (2:1 (v/v) hexanes/ethyl acetate). 5.9 mg (0.02 mmol, 46%) of the title compound were isolated as a colorless solid.

$^1$H NMR (500 MHz, CDCl₃): $\delta = 4.20 – 4.11$ (m, 1H), 2.93 – 2.82 (s, 4H), 2.80 (dd, 1H, $^3$J₉,₈ = 15.3, 3.4), 2.70 (dd, 1H, $^3$J₉,₈ = 15.5, 8.9), 1.64 – 1.50 (m, 2H), 1.01 – 1.43 (m, 1H), 1.40 – 1.23 (m, 9H), 0.88 (distorted t, 3H, $^3$J₉,₈ = 7.0). $^{13}$C (125.8 MHz, CDCl₃): $\delta = 169.3$, 167.6, 68.4, 39.6, 36.8, 31.9, 29.5, 29.3, 25.7, 25.6, 22.8, 14.2. HRMS (ESI+): [C₁₄H₂₃NO₅+H]$^+$, calcd: 286.1649, found: 286.1645. $R_f = 0.3$ (1:1 (v/v), hexanes/ethyl acetate).

Succinimidyl (R)-3-hydroxydodecanoate 4b

Scale: 0.36 mmol; yield 31.1 mg (0.1 mmol, 28%). $^1$H NMR (300 MHz, CDCl₃): $\delta = 4.18 – 4.06$ (m, 1H), 2.85 (s, 4H), 2.79 (dd, 1H, $^3$J₉,₈ = 15.3, 3.7), 2.69 (dd, 1H, $^3$J₉,₈ = 15.3, 8.6), 1.66 – 1.18 (m, 16H), 0.87 (distorted t, 3H, $^3$J₉,₈ = 6.7). $^{13}$C (75.5 MHz, CDCl₃): $\delta = 169.4$, 167.5, 68.3, 39.5, 36.8, 32.0, 29.6, 29.5, 29.4, 25.7, 25.6, 22.8, 14.2. HRMS (ESI+): [C₁₆H₂₇NO₅+H]$^+$, calcd: 314.1962, found: 314.1961. $R_f = 0.47$ (1:1 (v/v), hexanes/ethyl acetate).

General Method for Synthesis of Lipopeptides

Lipopeptides were synthesized by coupling of the activated fatty acids 4a and 4b with synthetic octa-peptides (GenScript). Synthetic peptides are denoted with an additional S in their names.
**Table S9:** Sequences of synthetic peptides used in lipopeptide syntheses. Differences are highlighted in red.

<table>
<thead>
<tr>
<th>Product</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virginiafactin S1</td>
<td>(D-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Ile)-(L-Leu)(L-Leu)</td>
</tr>
<tr>
<td>Virginiafactin S2</td>
<td>(L-Leu)-(D-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Ile)-(L-Leu)(L-Leu)</td>
</tr>
<tr>
<td>Virginiafactin S3 and S5</td>
<td>(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Val)-(D-Leu)(L-Leu)</td>
</tr>
<tr>
<td>Virginiafactin S4 and S6</td>
<td>(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Ile)-(D-Leu)(L-Leu)</td>
</tr>
<tr>
<td>Cichofactin S1</td>
<td>(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Gln)-(L-Val)-(D-Leu)(L-Leu)</td>
</tr>
<tr>
<td>Cichofactin S2</td>
<td>(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Gln)-(L-Val)-(D-Leu)(L-Leu)</td>
</tr>
<tr>
<td>Syringafactin S1</td>
<td>(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-alloThr)-(L-Val)-(D-Leu)(L-Leu)</td>
</tr>
</tbody>
</table>

The individual peptide fragments were dissolved in anhydrous DMF to give a 0.01M solution. To this solution 2.5 eq. of the respective activated fatty acid derivative 4a or 4b as well as 3 eq. DIPEA were added and the mixture was stirred at room temperature. The reaction progress was monitored using LC-MS. All reactions showed complete turnover to the desired product after 30 min. The crude reaction mixtures were directly subjected to purification using semi preparative HPLC (Shimadzu) equipped with a Luna® C18(2) column (250 × 10 mm, 5 µm, 100 Å, Phenomenex, flow rate = 5 mL/min, method: for compounds with C\textsubscript{10}-fatty acid chain: 65% MeCN in water containing 0.1% formic acid, isocratic; for compounds with C\textsubscript{12}-fatty acid chain: 75% MeCN in water containing 0.1% formic acid, isocratic).

**Figure S18:** HPLC profile of virginiafactin A, virginiafactin S3 and their mixture under different conditions.
Figure S19: Top: HPLC profile of virginiafactin B, virginiafactin S4 and their mixture under different conditions, Bottom: HPLC profile of virginiafactin B, virginiafactin S1, virginiafactin S2, and virginiafactin S4 (C18 60% v/v MeCN in water containing 0.1% formic acid).

Figure S20: HPLC profile of virginiafactin C, virginiafactin S5 and their mixture under different conditions.
Figure S21: HPLC profile of virginiafactin D, virginiafactin S6 and their mixture under different conditions

Figure S22: Top: HPLC profile of cichofactin A, cichofactin S1 and their mixture under different conditions. Bottom: HPLC profile of cichofactin B, cichofactin S2 and their mixture under different conditions

Figure S23: HPLC profile of extract of P. syringae pv. tomato DC3000, syringaefactin S1 and the corresponding mixture under different conditions
**Virginiafactin S1**

![Chemical Structure](image)

Scale: 0.0054 mmol; yield 2.0 mg (0.0018 mmol, 34%). $^1$H NMR (600 MHz, CD$_3$OD): $\delta = 4.44$ (dd, $^3J_{H,H} = 9.5, 5.2, 1H$), 4.40 (t, $^3J_{H,H} = 5.2, 1H$), 4.37 (dd, $^3J_{H,H} = 9.5, 5.0, 1H$), 4.35 – 4.30 (m, 2H), 4.27 (d, $^3J_{H,H} = 7.2, 1H$), 4.25 (dd, $^3J_{H,H} = 9.6, 5.0, 1H$), 3.97 – 3.95 (m, 1H), 3.87 – 3.82 (qd, $^3J_{H,H} = 11.5, 5.0, 2H$), 3.65 – 3.64 (m, 1H), 2.40 (dd, $^3J_{H,H} = 14.2, 4.6, 1H$), 2.36 – 2.26 (m, 4H), 2.15 – 2.12 (m, 1H), 2.06 – 2.02 (m, 1H); 1.93 – 1.90 (m, 1H); 1.72 – 1.60 (m, 1H), 1.54 – 1.46 (m, 3H), 1.35 – 1.25 (m, 14H), 0.98 – 0.88 (m, 36H). $^{13}$C(150 MHz, CD$_3$OD): $\delta = 177.7, 175.6, 175.6, 174.9, 174.9, 174.4, 174.2, 173.3, 173.3, 172.5, 70.3, 62.9, 59.6, 57.4, 55.4, 53.8, 53.8, 53.6, 53.1, 44.8, 42.1, 41.7, 41.4, 41.2, 41.0, 38.5, 38.0, 33.3, 33.0, 32.6, 30.8, 30.7, 30.4, 28.0, 26.6, 26.0, 26.0, 26.0, 25.9, 25.8, 23.7, 23.6, 23.6, 23.4, 22.1, 22.1, 22.0, 21.8, 21.6, 16.0, 14.4, 11.6. HRMS (ESI-): [C$_{54}$H$_{96}$N$_2$O$_{13}$]$^-$, calcd: 1080.7279, found: 1080.7284. [α]$_{D}^{25}$ = −3.0 (c = 0.1, MeOH).

**Virginiafactin S2**

![Chemical Structure](image)

Scale: 0.0054 mmol; yield 2.2 mg (0.0020 mmol, 37%). $^1$H NMR (600 MHz, CD$_3$OD): $\delta = 4.46$ (dd, $^3J_{H,H} = 9.5, 5.2, 1H$), 4.39 (t, $^3J_{H,H} = 5.2, 1H$), 4.34 – 4.32 (m, 2H), 4.31 – 4.27 (m, 2H), 4.22 (dd, $^3J_{H,H} = 9.6, 5.0, 1H$), 4.01 – 3.96 (m, 1H), 3.92 – 3.81 (m, 2H), 3.67 – 3.63 (m, 1H), 2.49 (dd, $^3J_{H,H} = 14.2, 4.6, 1H$), 2.35 – 2.31 (m, 3H), 2.12 – 2.03 (m, 2H); 1.94 – 1.90 (m, 1H); 1.72 – 1.59 (m, 14H), 1.49 – 1.47 (m, 4H), 1.38 – 1.25 (m, 14H), 0.98 – 0.89 (m, 36H). $^{13}$C(150 MHz, CD$_3$OD): $\delta = 177.9, 176.0, 175.9, 174.9, 174.8, 174.4, 174.2, 173.6, 173.4, 172.8, 69.8, 62.9, 59.6, 57.3, 55.6, 54.0, 53.8, 53.6, 53.2, 44.3, 41.8, 41.4, 41.3, 41.1, 41.0, 38.4, 38.1, 33.0, 32.7, 32.6, 30.8, 30.7, 30.5, 28.0, 26.8, 26.0, 26.0, 26.0, 25.9, 25.8, 23.7, 23.7, 23.6, 23.6, 23.3, 22.3, 22.3, 22.0, 21.8, 21.5, 16.0, 14.5, 11.4. HRMS (ESI-): [C$_{54}$H$_{96}$N$_2$O$_{13}$]$^-$, calcd: 1080.7279, found: 1080.7283. [α]$_{D}^{25}$ = −3.0 (c = 0.1, MeOH).

**Virginiafactin S3**

![Chemical Structure](image)

Scale: 0.0049 mmol; yield 3.95 mg (0.0037 mmol, 75%). $^1$H NMR (600 MHz, CD$_3$OD): $\delta = 4.48$ (dd, $^3J_{H,H} = 9.5, 5.2, 1H$), 4.42 (t, $^3J_{H,H} = 7.1, 1H$), 4.40 – 4.36 (m, 2H), 4.33 (dd, $^3J_{H,H} = 9.5, 5.1, 1H$), 4.26 (dd, $^3J_{H,H} = 9.6, 4.9, 1H$), 4.00 – 3.95 (m, 1H), 3.85 (qd, $^3J_{H,H} = 11.5, 5.0, 2H$), 2.46
(dd, $^3J_{H,H}$ = 14.2, 4.6, 1H), 2.38 – 2.25 (m, 4H), 2.22 – 2.11 (m, 2H), 2.10 – 2.00 (m, 2H); 1.75 – 1.54 (m, 15H), 1.54 – 1.40 (m, 4H), 1.40 – 1.08 (m, 24H), 1.06 – 0.72 (m, 36H). $^{13}$C (150 MHz, CD$_3$OD): $\delta$ = 177.7, 175.7, 175.6, 175.0, 174.9, 174.3, 174.2, 173.5, 172.6, 70.0, 62.9, 60.7, 57.4, 55.2, 53.8, 53.7, 53.2, 52.7, 44.7, 42.2, 41.8, 41.6, 41.4, 41.1, 38.4, 33.0, 32.8, 31.2, 30.7, 30.6, 30.5, 30.2, 28.2, 26.8, 26.0, 25.9, 23.7, 23.6, 23.4, 22.0, 21.9, 21.8, 21.7, 19.8, 18.8, 14.4. HRMS (ESI+): [C$_{55}$H$_{97}$N$_9$O$_{13}$+H]$^+$, calc'd: 1068.7279, found: 1068.7267. $[\alpha]_{D}^{25}$ = −3.0 (c = 0.1, MeOH).

**Virginiafactin S4**

![Chemical Structure](image)

Scale: 0.0049 mmol; yield 3.95 mg (0.0037 mmol, 75%). $^1$H NMR (600 MHz, CD$_3$OD): $\delta$ = 4.49 (dd, $^3J_{H,H}$ = 9.6, 5.0, 1H), 4.43 (t, $^3J_{H,H}$ = 6.5, 1H), 4.40 – 4.34 (m, 3H), 4.32 (dd, $^3J_{H,H}$ = 8.7, 6.1, 1H), 4.28 – 4.22 (m, 2H), 4.01 – 3.96 (m, 1H), 3.90 – 3.80 (m, 2H), 2.47 (dd, $^3J_{H,H}$ = 14.6, 5.0, 1H), 2.38 – 2.25 (m, 3H), 2.21 – 2.12 (m, 1H), 2.09 – 2.01 (m, 1H), 1.97 – 1.90 (m, 1H), 1.78 – 1.55 (m, 13H), 1.54 – 1.42 (m, 4H), 1.39 – 1.23 (m, 10H), 1.21 – 1.11 (m, 2H), 0.99 – 0.85 (m, 39H). $^{13}$C NMR (150 MHz, d$_6$-MeOH): $\delta$ = 177.7, 175.7, 175.6, 175.0, 174.9, 174.4, 174.2, 173.5, 172.6, 70.0, 62.9, 60.7, 55.2, 53.8, 53.7, 53.2, 52.7, 44.7, 42.2, 41.8, 41.6, 41.4, 41.1, 38.4, 33.1, 32.8, 31.3, 30.8, 30.7, 30.5, 28.2, 26.8, 26.0, 25.9, 23.7, 23.6, 23.4, 22.0, 21.9, 21.8, 21.7, 19.8, 18.8, 14.7. HRMS (ESI+): [C$_{54}$H$_{96}$N$_9$O$_{12}$+H]$^+$, calc'd: 1082.7435, found: 1082.7464. $[\alpha]_{D}^{25}$ = −5.9 (c = 0.1, MeOH).

**Virginiafactin S5**

![Chemical Structure](image)

Scale: 0.0049 mmol; yield 3.92 mg (0.0036 mmol, 72%). $^1$H NMR (600 MHz, d$_6$-MeOH): $\delta$ = 4.49 (dd, $^3J_{H,H}$ = 9.6, 5.2, 1H), 4.43 (t, $^3J_{H,H}$ = 7.0, 1H), 4.40 – 4.35 (m, 3H), 4.33 (dd, $^3J_{H,H}$ = 9.3, 5.4, 1H), 4.26 (dd, $^3J_{H,H}$ = 9.5, 4.8, 1H), 4.19 (d, $^3J_{H,H}$ = 6.9, 1H), 4.01 – 3.96 (m, 1H), 3.85 (dq, $^3J_{H,H}$ = 11.4, 5.0, 2H), 2.47 (dd, $^3J_{H,H}$ = 14.2, 4.6, 1H), 2.39 – 2.26 (m, 3H), 2.20 – 2.12 (m, 2H), 2.08 – 2.00 (m, 1H), 1.77 – 1.56 (m, 13H), 1.52 – 1.42 (m, 3H), 1.39 – 1.24 (m, 14H), 1.16 – 1.12 (m, 1H), 1.00 – 0.85 (m, 39H); $^{13}$C NMR (150 MHz, d$_6$-MeOH): $\delta$ = 177.7, 175.6, 175.6, 175.0, 174.9, 174.4, 174.3, 173.5, 172.6, 70.0, 62.9, 60.7, 57.4, 55.2, 53.8, 53.7, 53.2, 52.5, 44.2, 42.7, 41.9, 41.6, 41.4, 41.1, 38.4, 33.1, 32.8, 31.3, 30.8, 30.7, 30.5, 26.2, 26.8, 26.0, 25.9, 23.7, 23.6, 23.4, 22.0, 21.9, 21.8, 21.7, 19.8, 18.8, 14.5. HRMS (ESI+): [C$_{55}$H$_{97}$N$_9$O$_{13}$+H]$^+$, calc'd: 1096.7592, found: 1096.7574. $[\alpha]_{D}^{25}$ = −12.5 (c = 0.1, MeOH).

**Virginiafactin S6**
Cichofactin S1

Scale: 0.0049 mmol; yield 3.37 mg (0.0030 mmol, 62%). \(^1\)H NMR (500 MHz, \(d_6\)-MeOH): \(\delta = 4.50\) (dd, \(3J_{HH} = 9.9, 4.9, 1H\)), 4.44 – 4.40 (m, 1H), 4.40 – 4.30 (m, 4H), 4.16 (d, \(3J_{HH} = 7.1, 1H\)), 4.00 – 3.95 (m, 1H), 4.02 – 3.96 (m, 1H), 2.47 – 2.44 (dd, \(3J_{HH} = 14.1, 4.5, 1H\)), 2.38 – 2.25 (m, 5H), 2.20 – 2.13 (m, 3H), 2.05 – 1.99 (m, 2H), 1.76 – 1.53 (m, 12H), 1.50 – 1.45 (m, 4H), 1.39 – 1.23 (m, 14H), 0.97 – 0.90 (m, 36H); \(^{13}\)C NMR (125 MHz, \(d_6\)-MeOH): \(\delta = 177.7, 176.4, 175.6, 175.4, 175.0, 174.8, 174.4, 174.2, 173.8, 173.5, 70.0, 60.8, 54.9, 54.8, 54.1, 53.8, 53.5, 53.2, 52.5, 44.7, 42.0, 41.7, 41.4, 41.1, 38.4, 33.1, 32.9, 32.6, 31.3, 30.8, 30.7, 30.5, 28.1, 26.8, 26.0, 26.0, 25.9, 25.9, 23.7, 23.6, 23.6, 23.6, 23.4, 22.0, 22.0, 21.9, 21.8, 21.8, 19.8, 18.3, 14.5. HRMS (ESI+) calcd for [C\(_{56}\)H\(_{103}\)N\(_{20}\)O\(_{13}\)+H]\(^+\) 1110.7748, found 1110.7725, [\(\alpha\)]\(_D\)\(^{25}\) = −6.6 (c = 0.1, MeOH).

Cichofactin S2

Scale: 0.0050 mmol; yield 3.98 mg (0.0035 mmol, 70%). \(^1\)H NMR (600 MHz, \(d_6\)-MeOH): \(\delta = 4.50\) (dd, \(3J_{HH} = 9.9, 4.9, 1H\)), 4.43 – 4.40 (m, 2H), 4.38 – 4.30 (m, 4H), 4.16 (d, \(3J_{HH} = 7.1, 1H\)), 3.99 – 3.97 (m, 1H), 2.45 (dd, \(3J_{HH} = 14.1, 4.5, 1H\)), 2.39 – 2.24 (m, 5H), 2.22 – 2.11 (m, 3H), 2.01 – 1.98 (m, 2H), 1.76 – 1.55 (m, 16H), 1.50 – 1.48 (m, 4H), 1.34 – 1.29 (m, 14H), 0.97 – 0.90 (m, 36H); \(^{13}\)C NMR (150 MHz, \(d_6\)-MeOH): \(\delta = 177.8, 177.7, 175.6, 175.4, 175.0, 174.8, 174.3, 174.3, 173.8, 173.5, 70.0, 60.8, 54.9, 54.9, 54.1, 53.8, 53.6, 53.3, 52.8, 44.7, 42.3, 42.0, 41.7, 41.5, 41.1, 38.4, 33.1, 32.9, 32.6, 31.3, 30.8, 30.8, 30.5, 28.5, 28.4, 26.8, 26.0, 26.0, 25.9, 25.9, 23.8, 23.7, 23.7, 23.6, 23.6, 23.5, 22.0, 22.0, 21.9, 21.8, 21.8, 21.8, 19.9, 18.8, 14.5. HRMS (ESI-) calcd for [C\(_{57}\)H\(_{104}\)N\(_{31}\)O\(_{13}\)-H]\(^-\) 1135.7714, found 1135.7714, [\(\alpha\)]\(_D\)\(^{25}\) = −7.1 (c = 0.1, MeOH).
Syringafactin S1

\[
\text{C}_2\text{H}_5\text{N} = \text{O} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{OH} \\
\text{H}_2\text{N} \text{CO} \\
\text{OH}
\]

Scale: 0.0055 mmol; yield 4.43 mg (0.0041 mmol, 74%). \( ^1\)H NMR (600 MHz, \( d_4\)-MeOH): \( \delta = 4.50 \text{ (dd, } ^3J_{H,H} = 9.9, 4.9, 1H), 4.45 - 4.41 \text{ (m, 2H)}, 4. \text{ (dd, } ^3J_{H,H} = 9.9, 5.0, 1H), 4.33 \text{ (d, } ^3J_{H,H} = 3.7, 1H), 4.31 - 4.23 \text{ (m, 3H)}, 4.17 \text{ (d, } ^3J_{H,H} = 7.1, 1H), 4.01 - 3.97 \text{ (m, 1H)}; \)

\( ^{13}C \) NMR (150 MHz, \( d_4\)-MeOH): \( \delta = 177.7, 175.9, 175.6, 175.4, 175.0, 174.5, 174.0, 173.5, 172.8, 70.0, 68.3, 60.9, 60.6, 54.9, 53.9, 53.7, 53.7, 53.1, 52.1, 44.7, 41.9, 41.7, 41.6, 41.5, 41.0, 38.5, 33.0, 32.8, 31.3, 30.7, 30.5, 28.4, 26.8, 26.0, 26.0, 25.9, 25.9, 23.7, 23.6, 23.5, 23.5, 23.4, 21.9, 21.9, 21.9, 21.8, 20.2, 19.9, 18.9, 14.4). HRMS (ESI-)
calcd for \([\text{C}_{54}\text{H}_{99}\text{N}_{9}\text{O}_{13} - \text{H}]^+\) 1080.7292, found 1080.7288, \([\text{a}]_D^{25} = -4.3 \text{ (c = 0.1, MeOH)}\).

**Phylogenetic Analyses**

In order to shed more light on the directionality of the transfer \( \text{vif-A}_{\text{Ser}} \leftrightarrow \text{jesQS-A}_{\text{Ser}} \), we intended to add another \( \text{jes-like} \) BGC to our phylogenetic analysis as an outgroup that might not have undergone domain transfers. A search in the NCBI database for BGC related to the jessenipeptin BGC using BLAST led us to the draft genome of \( \text{Pseudomonas} \) sp. MWU13-2860. The cluster was distributed over two contigs (NCBI accession numbers PPYB02000007 and PPYB02000026), but comparison of the contig ends allowed merging the contigs to yield a complete BGC that aligned very well with the jessenipeptin BGC (93% identity over a length of 64 kb). The overall domain structure was identical and antiSMASH predicted the same nonribosomally synthetized peptide backbone. Since the BGC was found in \( \text{Pseudomonas} \) sp. MWU13-2860, we designated this cluster \( \text{jes}_{\text{MWU}} \). As expected from the high similarity between the \( \text{jes}_{\text{MWU}} \) and \( \text{jes}_{\text{QS}} \) clusters, most of the \( \text{jes} \) domains cluster according to their positions within the assembly line. Surprisingly, however, both \( \text{jes}_{\text{MWU}} \text{-A}_{9\text{Ser}} \) and \( \text{jes}_{\text{MWU}} \text{-A}_{18\text{Ser}} \) regions formed their own clade descending from an ancestral \( \text{jes-}\text{A}_{18\text{Ser}} \) domain in the subtree. Thus, they do not help to disentangle the directionality of domain exchanges. Rather, this finding is an indication for yet another exchange event that took place within the \( \text{jes}_{\text{MWU}} \) BGC after the triplication in the ancestor of the \( \text{Pseudomonas} \) strain.
Figure S24: Phylogenetic tree of the coding regions of 62 A domains of the BGC vif, cif, syf, jesQS, and jesMWU. Tree inference was performed using Maximum Likelihood estimation implemented in PhyML 3.0 using the GTR model with a gamma distribution of rates. Support values (Shimodaira-Hasegawa-like branch test) are shown above branches. The tree was rooted using the clade of Phe-activating A domains as outgroup. The log-likelihood of the final tree was computed as −37421.7274. Scale bar indicates substitutions per site.
Figure S25: Phylogenetic tree of the coding regions of 62 C domains of the BGC vif, cif, syf, jesQS, and jesMWU. Tree inference was performed using Maximum Likelihood estimation implemented in PhyML 3.0 using the GTR model with a gamma distribution of rates. Support values (Shimodaira-Hasegawa-like branch test) are shown above branches. The tree was rooted using the clade C starter domains as outgroup. The log-likelihood of the final tree was computed as –30249.59945. Scale bar indicates substitutions per site.
PCR Amplification and Sanger Sequencing of cif A4 and A7

Table S10: Primers for amplification and sequencing of *Pseudomonas cichorii* JBC1 / SF1-54 cif-A4 domain and *Pseudomonas cichorii* JBC1 / SF1-54 cif-A7 domain

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>cif_A4_F</td>
<td>TTTGAACGAGCCATGGAGCA</td>
</tr>
<tr>
<td>cif_A4_R</td>
<td>CAACTGCTCCAGCAAGGC</td>
</tr>
<tr>
<td>cif_A7_F</td>
<td>GTTACGTTCATTACTTCCAGCAGC</td>
</tr>
<tr>
<td>cif_A7_R</td>
<td>AGACAGTCAACAGTTCGGCAC</td>
</tr>
</tbody>
</table>

Genomic DNA was isolated from 3 mL bacterial overnight culture in LB medium using the QIAamp DNA Mini Kit (Qiagen). One pair of primers was used for amplification of the A4 / A7 domain of the respective strain’s gDNA. Primers bind outside of the A domain sequence in unconserved regions. PCR reactions were carried out using Q5® High-Fidelity 2x Master Mix (New England Biolabs) and a touchdown PCR protocol (mid-annealing temperature 60 °C). PCR reaction products (1515bp each) were purified for subsequent Sanger sequencing (LGC Genomics, Berlin) by gel extraction using GeneJET Gel Extraction Kit (Thermo Scientific). Sequences were mapped to gDNA reference sequences using Geneious 11.0.3. Sanger sequencing confirmed both gDNA sequences for A4 and A7 domains of both strains.

Sequence Alignment of jes Modules 6 and 12

Figure S26. Sequence alignment of *jes* module 6 and 12. Shown are the mean pairwise identities green: 100%, green-brown: 30 – 100%, red: < 30%. Sliding window size: 10 nt. The red dashed bar on top shows the repeated sequence with identity cut-off = 93%
Analysis of the Virginifactin BGC

Figure S27. Screenshot of the antiSMASH analysis of the vifA and vifB genes to visualize the boundaries of this BGC. According to our analysis no further tailoring enzymes are involved in the biosynthesis of the virginifactins.

Sequence Information

| cif M4  | 80474 | CCGTGTGACCATCACCACCATGCACTACATGCACTGACATGCGACCTCGACACCCGACCGACGATCG 80415 |
| cif M7  | 71021 | --GGTCACAACATCCATGTTTGGATCGAGGCCAGGCTGTGTCACGCTGATGCCACATCG 70964 |
| cif M4  | 80414 | CCTGCGAAGTGGCCGATACCAATGCACTGATGCACTTACAGCAGATCAGCGATACAGCGATACG 80355 |
| cif M7  | 70963 | CCTGCGAAGTGGCCGATACCAATGCACTGATGCACTTACAGCAGATCAGCGATACG 70904 |
| cif M4  | 80354 | TGCCGCAATCACCCTAGACTGCGCCGACCATGCACTGACATGCGACCTCGACACCCGACCGACGATCG 80295 |
| cif M7  | 70903 | TGCCGCAATCACCCTAGACTGCGCCGACCATGCACTGACATGCGACCTCGACACCCGACCGACGATCG 70844 |
| cif M4  | 80294 | GTCGTGCTGCTGAAACACTGTCGGTTGGGTGTGCTGATGCCACATCG 80235 |
| cif M7  | 70843 | GTCGTGCTGCTGAAACACTGTCGGTTGGGTGTGCTGATGCCACATCG 70784 |
| cif M4  | 80234 | TGCCGACACCCGCTCAATCGACATGCACTTACAGCAGATCAGCGATACAGCGATACG 80175 |
| cif M7  | 70783 | TGCCGACACCCGCTCAATCGACATGCACTTACAGCAGATCAGCGATACG 70724 |
| cif M4  | 80174 | CGGTGCCGATCTTGACACTGCACACATGCACTTACAGCAGATCAGCGATACG 80115 |
| cif M7  | 70723 | CGGTGCCGATCTTGACACTGCACACATGCACTTACAGCAGATCAGCGATACG 70664 |
| cif M4  | 80114 | TGATTTGCTTGGATGCTCGGATCGCTGACATGCACTTACAGCAGATCAGCGATACG 80055 |
| cif M7  | 70663 | TGATTTGCTTGGATGCTCGGATCGCTGACATGCACTTACAGCAGATCAGCGATACG 70604 |
| cif M4  | 80054 | TGACGCCCGCAACCTGCTGACATGCACTTACAGCAGATCAGCGATACG 70544 |
| cif M7  | 70603 | TGACGCCCGCAACCTGCTGACATGCACTTACAGCAGATCAGCGATACG 7044 |
| cif M4  | 79994 | CGGTGCGCTGACGATGCGACCCGACCGACTGACATGCACTTACAGCAGATCAGCGATACG 79935 |
Figure S28. Nucleotide sequence alignment of cif-M4 and cif-7M.
Figure S29. Protein sequence alignment of Cif-M4 and Cif-M7.
NMR Spectra

$^1$H (600 MHz, methanol-$d_4$): Virginiafactin A

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin A
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin B

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin B
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin C

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin C
\(^1\)H (600 MHz, methanol-\(d_4\)): Virginiafactin D

\(^{13}\)C (150 MHz, methanol-\(d_4\)): Virginiafactin D
$^1$H (600 MHz, methanol-$d_4$): Cichofactin A

$^{13}$C (150 MHz, methanol-$d_4$): Cichofactin A
$^1$H (600 MHz, methanol-$d_4$): Cichofactin B

$^{13}$C (150 MHz, methanol-$d_4$): Cichofactin B
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin S1

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin S1
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin S2

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin S2


$^1\text{H}$ (600 MHz, methanol-$d_4$): Virginiafactin S3

$^{13}\text{C}$ (150 MHz, methanol-$d_4$): Virginiafactin S3
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin S4

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin S4
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin S5

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin S5
$^1$H (600 MHz, methanol-$d_4$): Virginiafactin S6

$^{13}$C (150 MHz, methanol-$d_4$): Virginiafactin S6
$^1$H (600 MHz, methanol-$d_4$): Cichofactin S1

$^{13}$C (150 MHz, methanol-$d_4$): Cichofactin S1
$^1$H (600 MHz, methanol-$d_4$): Cichofactin S2

$^{13}$C (150 MHz, methanol-$d_4$): Cichofactin S2
$^{1}H$ (500 MHz, methanol-$d_{4}$): Syringafactin S1

$^{13}C$ (125 MHz, methanol-$d_{4}$): Syringafactin S1