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 F_{254} 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 ($CDCl_3$: δ = 7.26 ppm (^{1}H), δ = 77.16 (^{13}C); d_6-DMSO: δ = 2.50 ppm (^{1}H), δ = 39.52 (^{13}C)). 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^n 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 Δ*vif* 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 pEXG2Δ*vif.*

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

Primer sequences 5' – 3'	
LA *vif* fwd	GGAAGCATAAATGTAAAGCACGCATGCAGCAGTTCGCC
LA *vif* rev	TCCCGCACCACAGGCTTCTTCCCTGGTGC
RA vif fwd	AAGAAGCCTGTGGTGCGGGATGAATACC
RA vif rev	GGAAATTAATTAAGGTACCGTACGCAGGATGTCGTGGC

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 pEXG2Δ*slp*) and acceptor (QS1027) strain cultures were diluted to an OD_{600} = 0.1 and grown to OD_{600} = 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.

Primer sequences 5'–3'	
KOC LA *vif* fwd	GTTCGTGACCAGGCCTACTG
KOC LA *vif* rev	CCTCGGGGGCTTCGTATTCA
KOC RA *vif fwd*	GGGTTGGCGAATGCACAGTT
KOC RA *vif rev*	ATCCGCCGTGGATCGAAATG

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 Na_2SO_4, 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 Δ*vif* deletion mutant

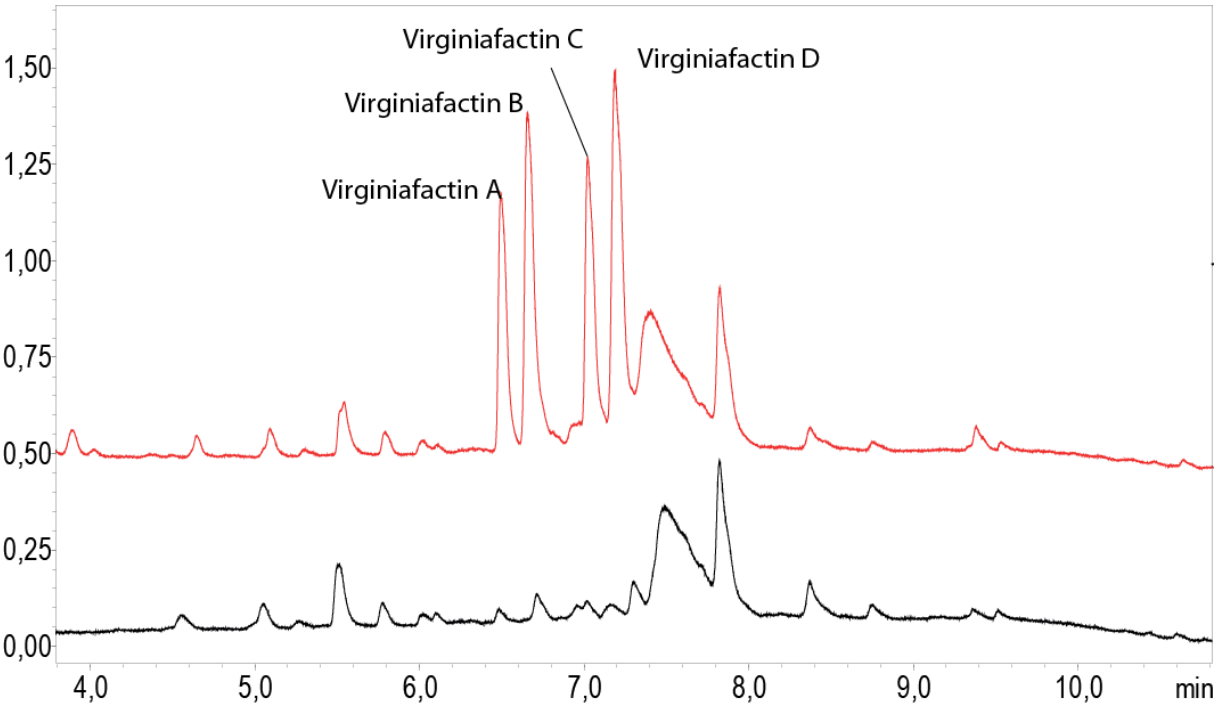


Figure S1: HPLC-traces of extracts from the QS1027 WT (red) and the Δ*vif* deletion mutant (black)

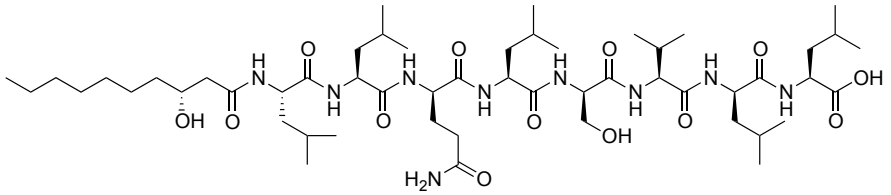
Isolation of Virginiafactins A–D

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 Na_2SO_4 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; 5 – 14 min: 70% – 90% MeCN in water containing 0.1% formic acid) to yield three linear lipopeptides as white solids: virginiafactin B: t_R = 10.6 min; virginiafactin C: t_R = 12.5 min; virginiafactin D: t_R = 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 compounds as white solids.

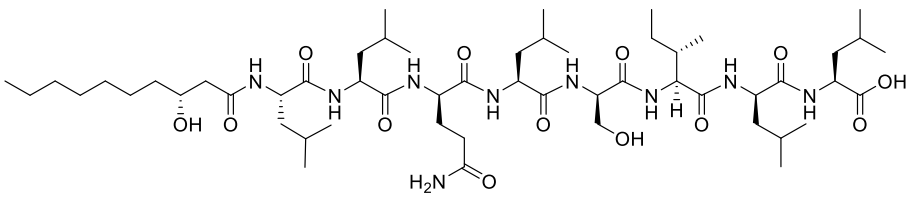
For the isolation of virginiafactin A, a similar method was used as above only with Kings Medium B as a cultivation medium. Extracts were obtained analougously and the residue was fractionated using a HyperSep™ C18 Cartridge (Thermo Scientific) and eluted with a mixture of 30%, 50%, 75%, and 100% (v/v) 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 : t_R = 10.1 min.

Virginiafactin A



Yield: 2.4 mg; ^{1}H NMR (600 MHz, *d*$_4$-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); ^{13}C NMR (150 MHz, *d*$_4$-MeOH): δ = 177.7, 176.0, 175.6, 175.76 175.0, 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, 23.6, 23.5, 23.4, 22.0, 21.9, 21.9, 21.8, 21.7, 19.8, 18.8, 14.5. HRMS (ESI-) calcd for $[C_{53}H_{97}N_9O_{13}\text{-H}]^-$ 1066.7135, found 1066.7135, $[\alpha]_D^{25}$= –6.0 (c = 0.1, MeOH).

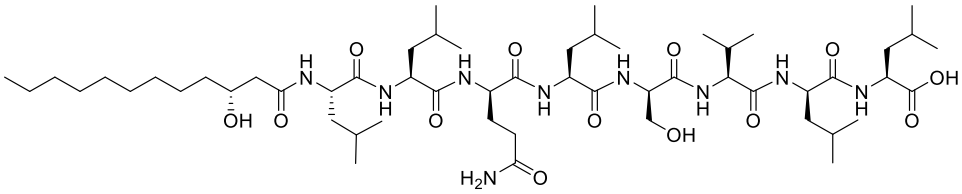
Virginiafactin B



Yield: 1.4 mg; ^{1}H NMR (600 MHz, *d*$_4$-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),

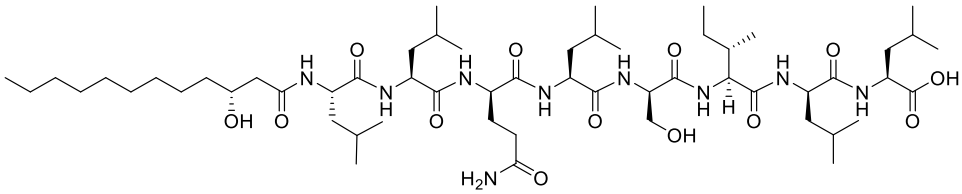
3.90 – 3.80 (m, 2H), 2.47 (dd, $^{3}J_{H,H}$= 14.2, 4.5, 1H), 2.39 – 2.28 (m, 3H), 2.21 – 2.13 (m, 1H), 2.09 – 2.01 (m, 1H), 1.98 – 1.90 (m, 1H), 1.78 – 1.55 (m, 13H), 1.54 – 1.42 (m, 4H), 1.39 – 1.23 (m, 10H), 1.21-1.11 (m, 2H), 1.00-0.85 (m, 39H); ^{13}C NMR (150 MHz, *d*$_4$-MeOH): δ = 177.7, 175.7, 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, 53.7, 53.1, 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, 25.9, 23.7, 23.6, 23.6, 23.4, 22.0, 22.0, 21.9, 21.8, 21.7, 16.0, 14.5, 11.6; t_r = 3.6 min; HRMS (ESI+) calcd for $[C_{54}H_{99}N_9O_{13}+H]^+$ 1082.7435, found 1082.7442, $[\alpha]_D^{25}$ = –15.1 (c = 0.20, MeOH).

Virginiafactin C



Yield: 1.5 mg; ^{1}H NMR (600 MHz, *d*$_4$-MeOH): δ = 4.49 (dd, $^{3}J_{H,H}$=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, $^{3}J_{H,H}$= 9.6, 4.7, 1H), 4.20 (d, $^{3}J_{H,H}$= 6.9, 1H), 4.01 – 3.96 (m, 1H), 3.85 (dq, $^{3}J_{H,H}$= 11.4, 5.5, 2H), 2.47 (dd, $^{3}J_{H,H}$= 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*$_4$-MeOH): δ = 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, 53.7, 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, 21.9, 21.8, 21.7, 19.8, 18.8, 14.5; t_r = 4.1 min; HRMS (ESI+) calcd for $[C_{55}H_{101}N_9O_{13}+H]^+$ 1096.7592 found 1096.7599, $[\alpha]_D^{25}$ = –17.2 (c = 0.21, MeOH).

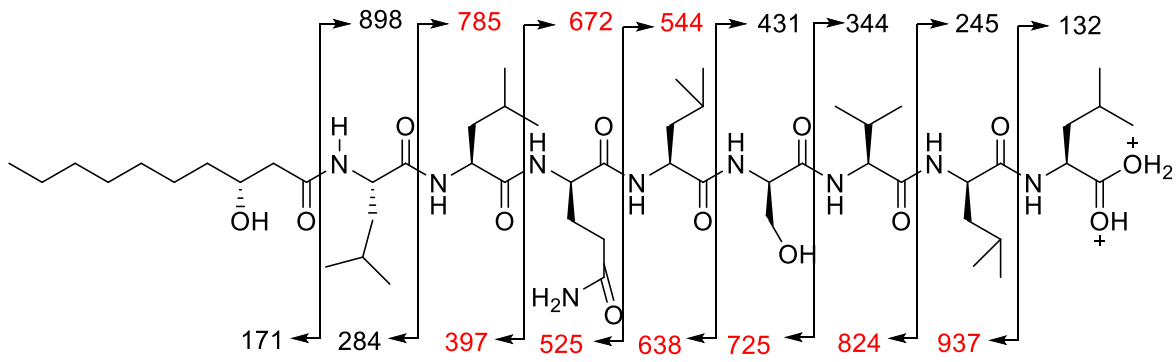
Virginiafactin D



Yield: 2.4 mg; ^{1}H NMR (600 MHz, *d*$_4$-MeOH: δ = 4.50 (dd, $^{3}J_{H,H}$= 9.9, 4.8, 1H), 4.45 – 4.41 (m, 1H), 4.40 – 4.35 (m, 3H), 4.32(t, $^{3}J_{H,H}$= 7.2, 1H), 4.27 – 4.22 (m, 2H), 4.02 – 3.96 (m, 1H), 3.90 – 3.81 (m, 2H), 2.47 (dd, $^{3}J_{H,H}$= 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*$_4$-MeOH): δ = 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, 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; t_r = 4.4 min; HRMS (ESI+) calcd for $[C_{56}H_{103}N_9O_{13}+H]^+$ 1110.7748, found 1110.7755, $[\alpha]_D^{25}$ = –14.2 (c = 0.24, MeOH).

Sequence Analysis of Virginiafactins A – D using ESI-MS^2

Virginiafactin A



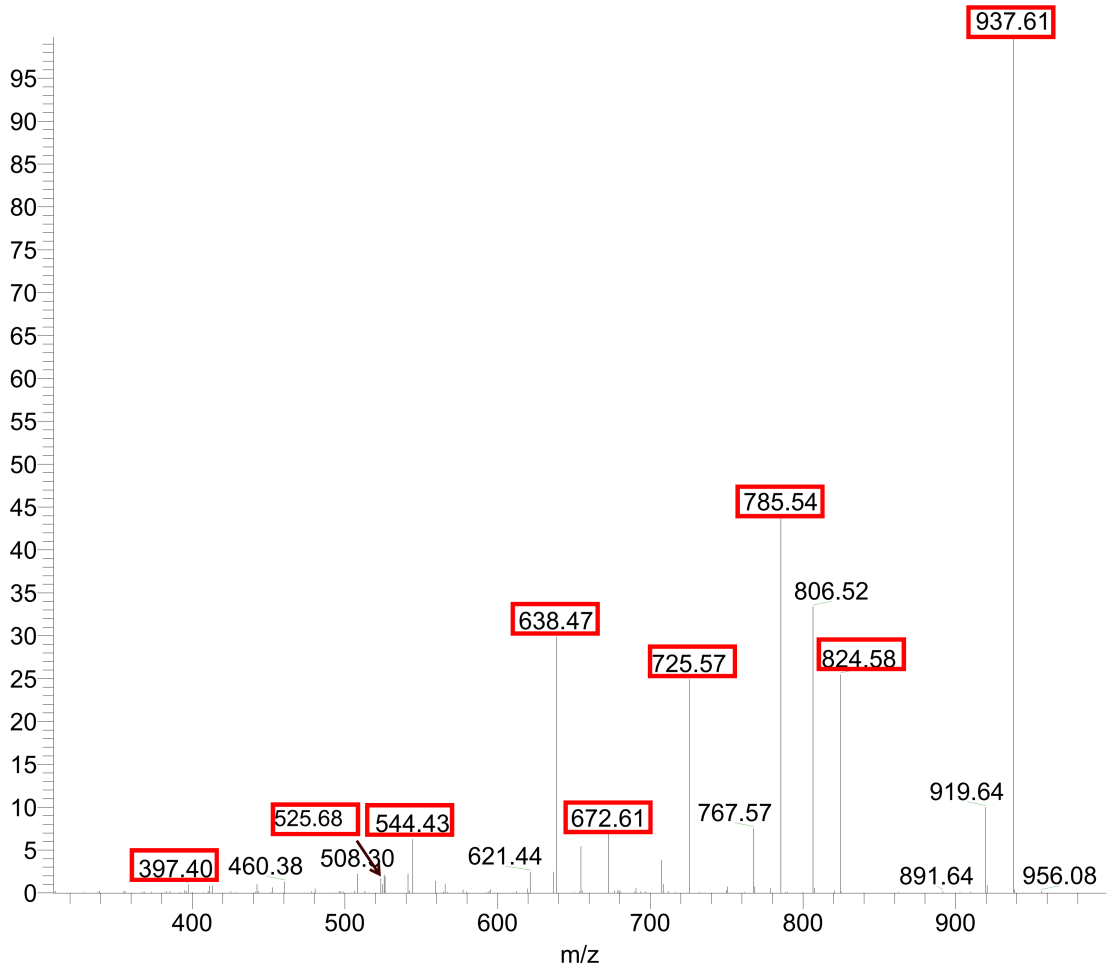


Figure S2: MS^2-fragmentation of virginiafactin A. Colored numbers correspond to the *m/z* ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.

Virginiafactin B

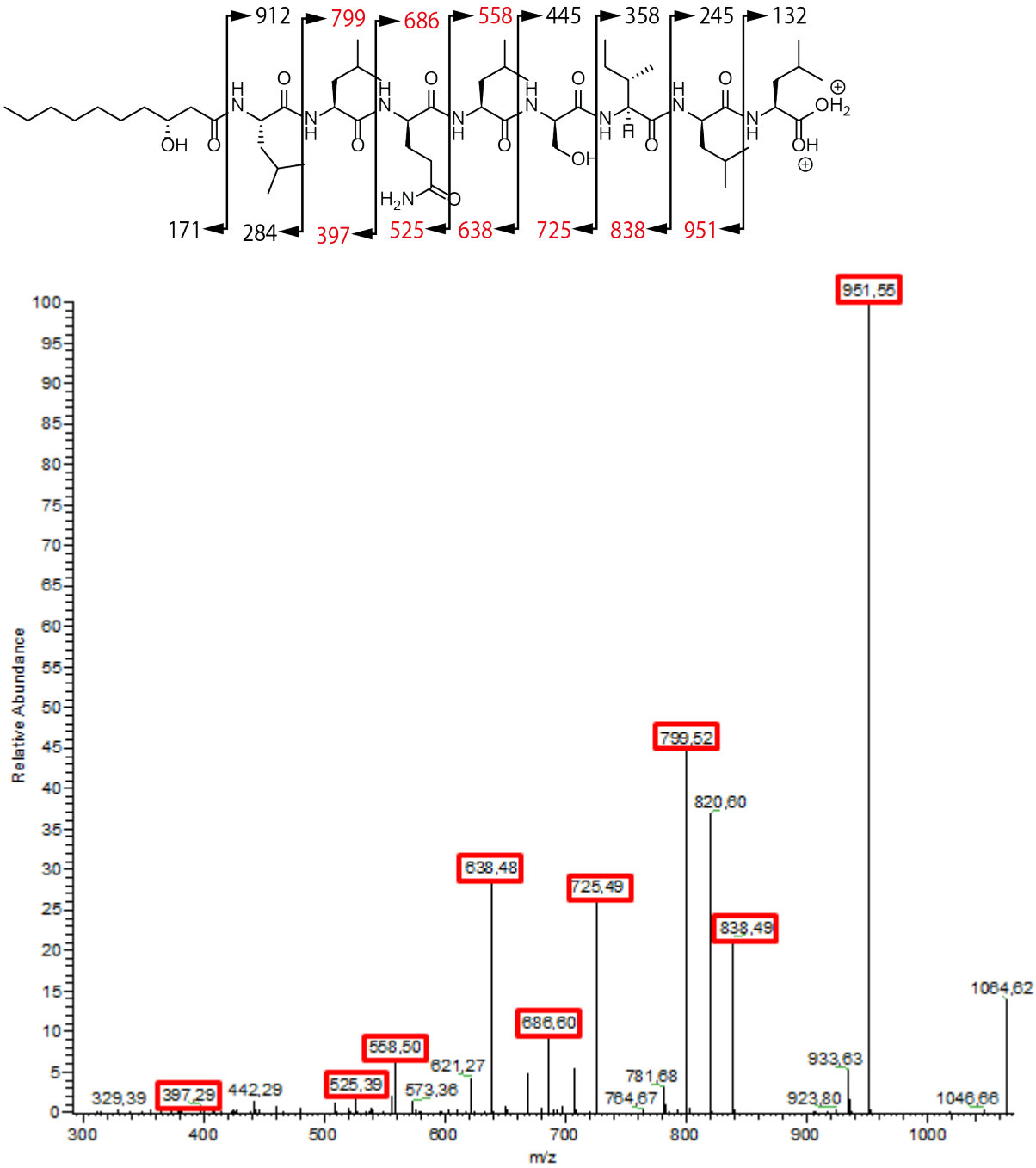


Figure S3: MS^2-fragmentation of virginiafactin B. Colored numbers correspond to the *m/z* ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.

Virginiafactin C

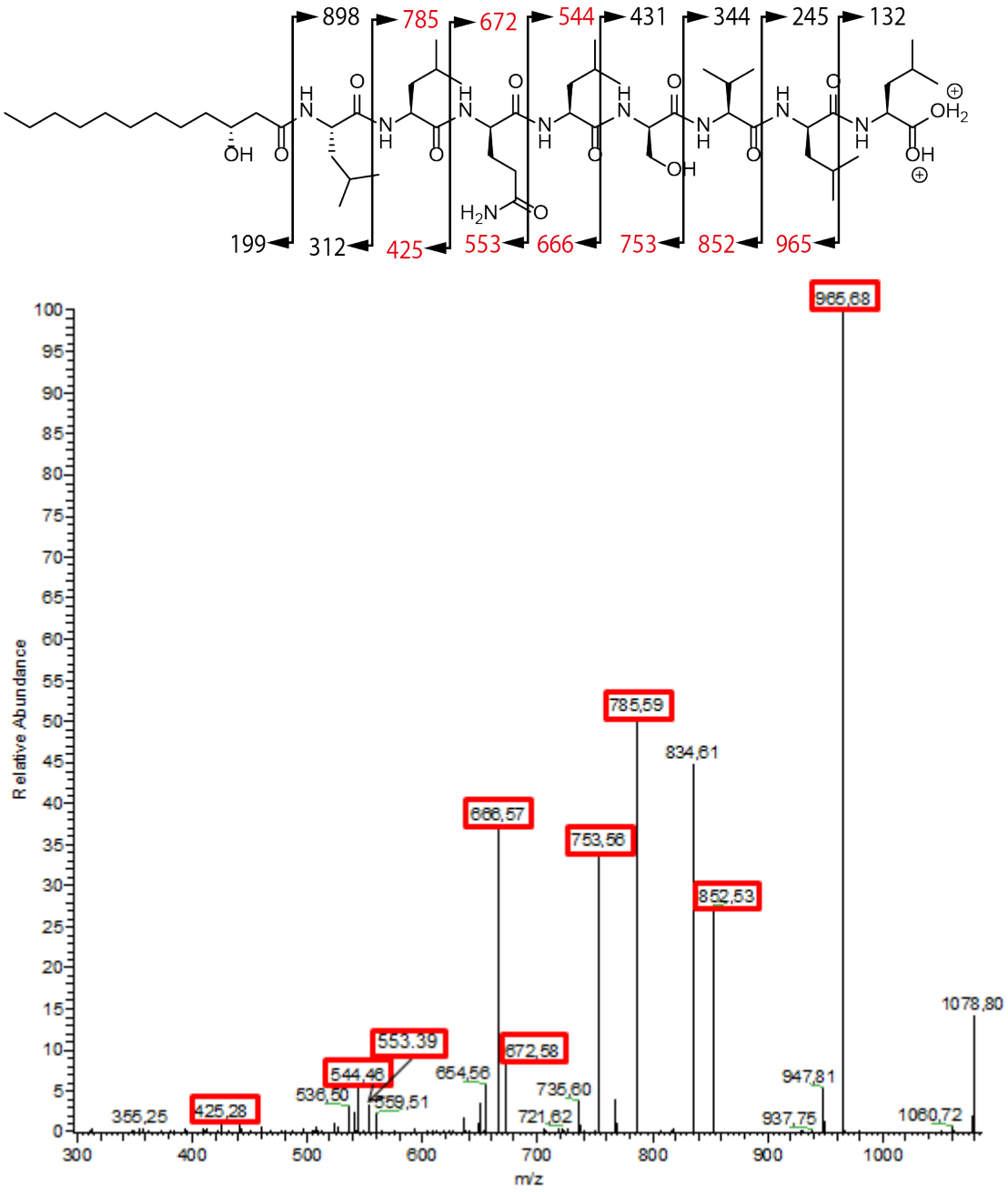
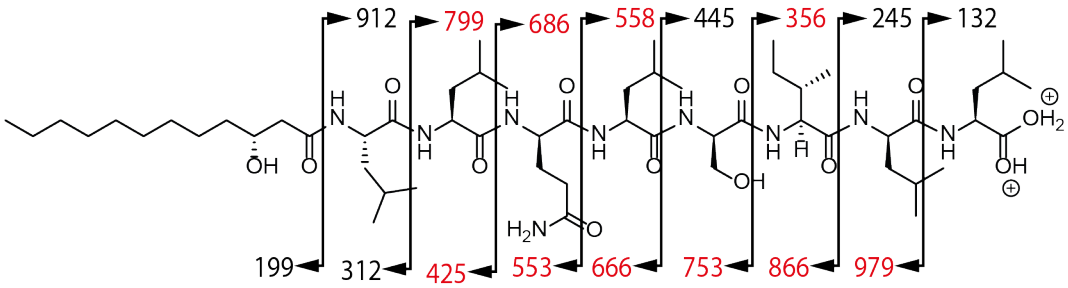


Figure S4: MS^2-fragmentation of virginiafactin C. Colored numbers correspond to the *m/z* ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.

Virginiafactin D



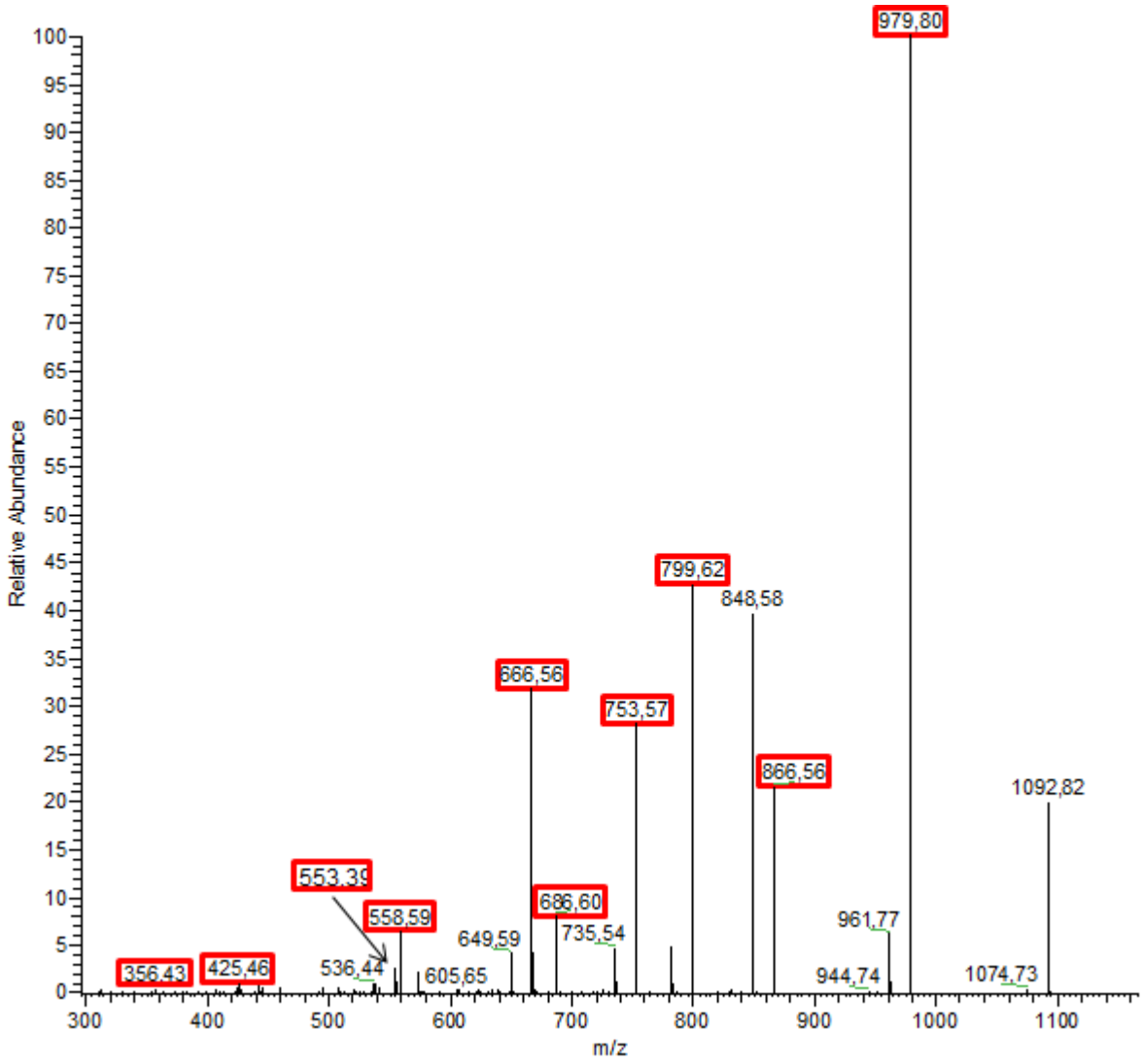


Figure S5: MS^2-fragmentation of virginiafactin D. Colored numbers correspond to the *m/z* ratio of fragments that were unambiguously identified; fragments labeled in black could not be detected in the spectrum.

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 *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. $NaHCO_3$ 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-*allo*Thr (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

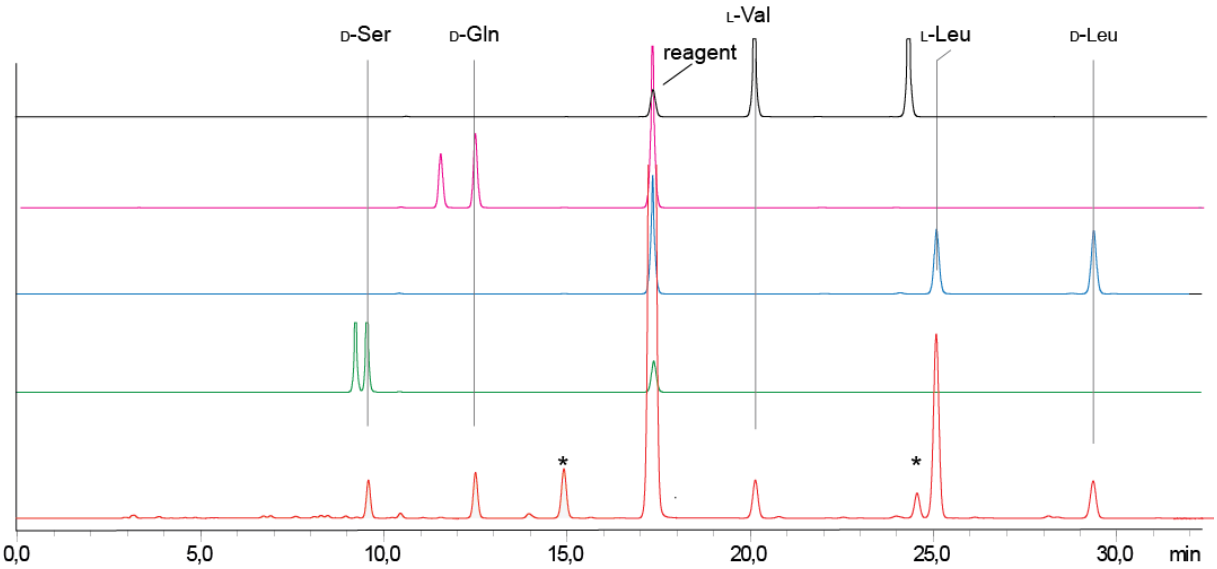


Figure S6: HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin A (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Val (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from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

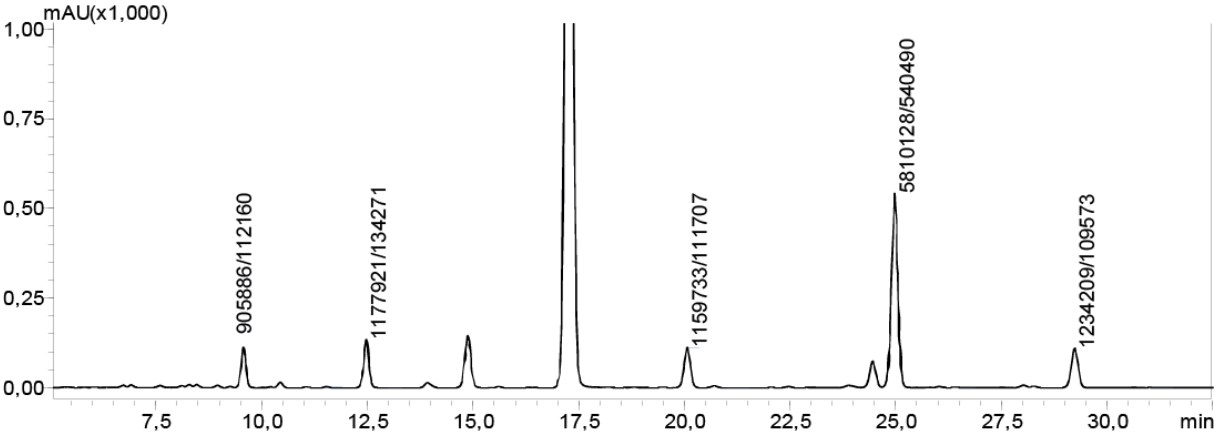


Figure S7: HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin A (λ = 330 nm).

Table S3: Integration areas for peaks from **Fig. S7**

Peak # / amino acid	$T_{ret.}$ /[min]	area
1 / D-Ser	9.56	905886
2 / D-Gln	12.49	1177921
3 / L-Val	20.07	1159733
4 / -L-Leu	25.12	5810128
5 / D-Leu	29.42	1234209

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

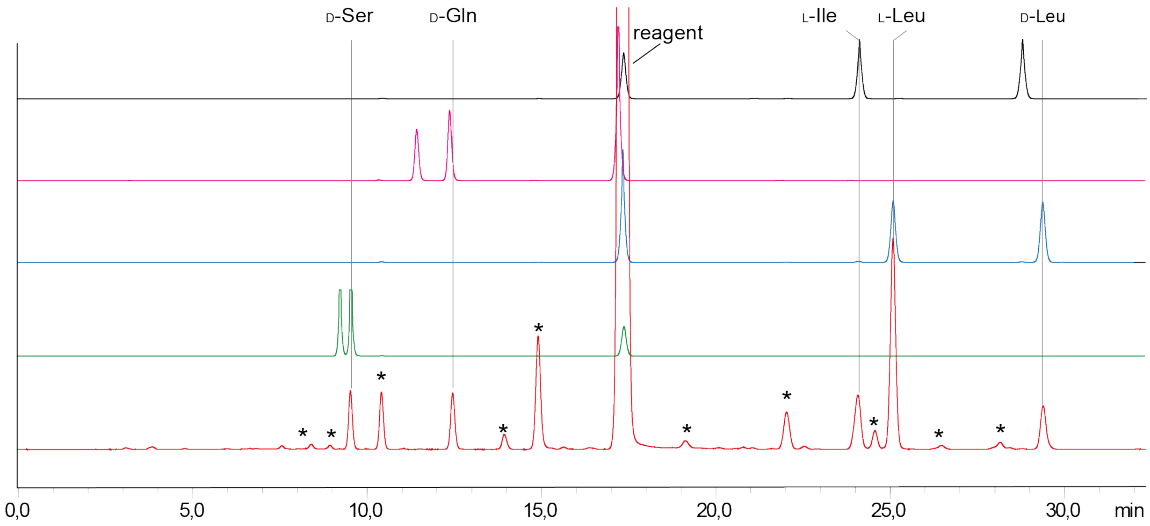


Figure S8: HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin B (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Ile (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from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

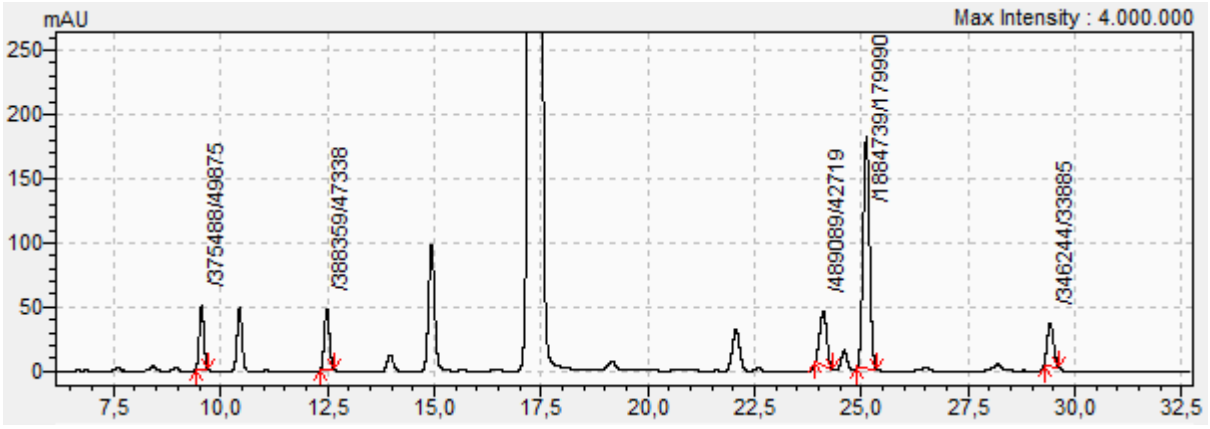


Figure S9: HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin B (λ = 330 nm).

Table S4: Integration areas for peaks from **Fig. S9**

Peak # / amino acid	$T_{ret.}$ /[min]	area
1 / D-Ser	9.556	375488
2 / D-Gln	12.487	388359
3 / L-Ile	24.109	489089
4 / -L-Leu	25.114	1884739
5 / D-Leu	29.420	346244

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

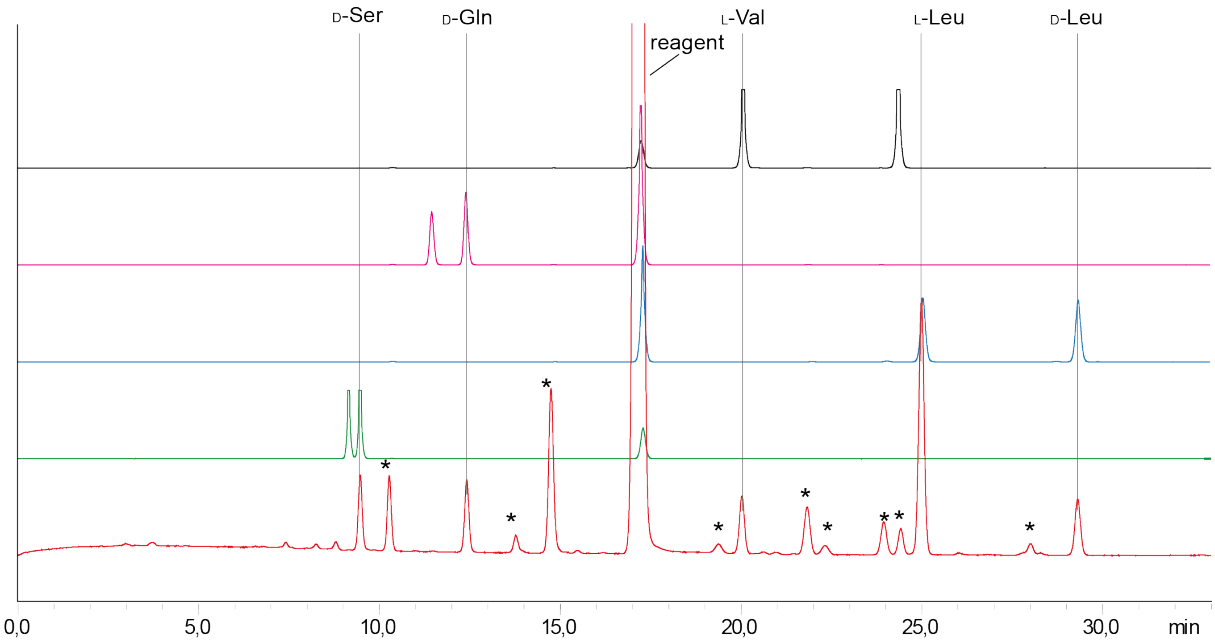


Figure S10: HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin C (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Ile (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from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

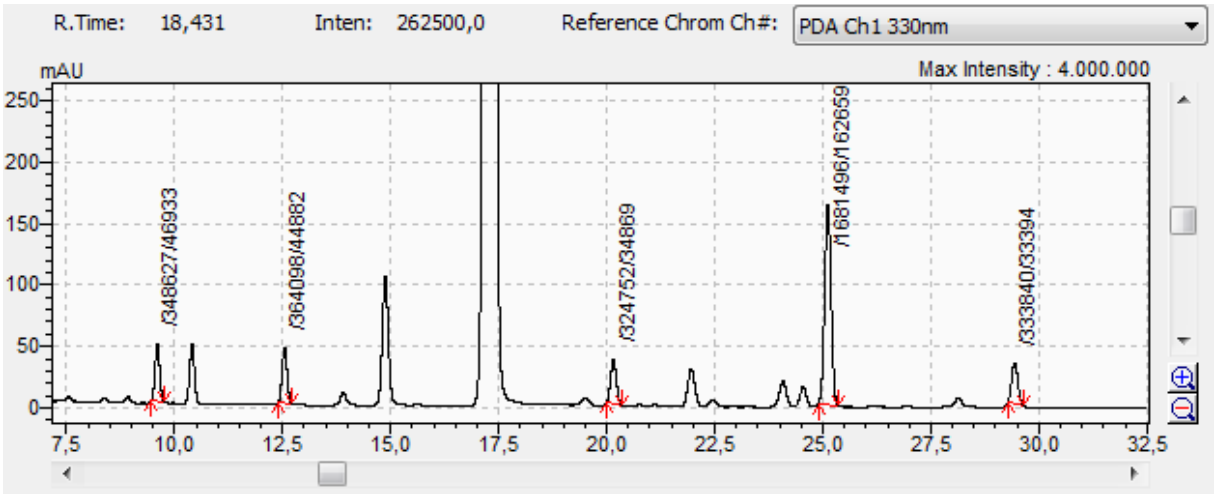


Figure S11: HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin C (λ = 330 nm).

Table S5: Integration areas for peaks from **Fig. S11**.

Peak # / amino acid	$T_{ret.}$ /[min]	area
1 / D-Ser	9.60	348627
2 / D-Gln	12.54	364098
3 / L-Val	20.15	324752
4 / -L-Leu	25.12	1681496
5 / D-Leu	29.44	333840

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

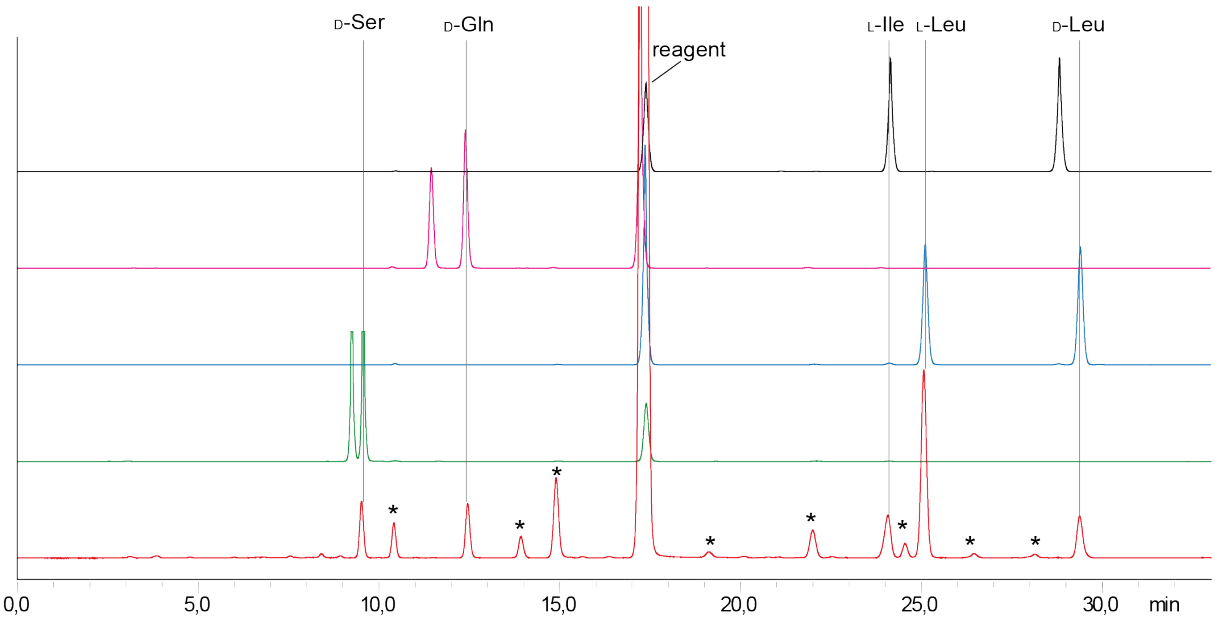


Figure S12: HPLC profiles of the hydrolyzed and FDAA-derivatized virginiafactin D (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Ile (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from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

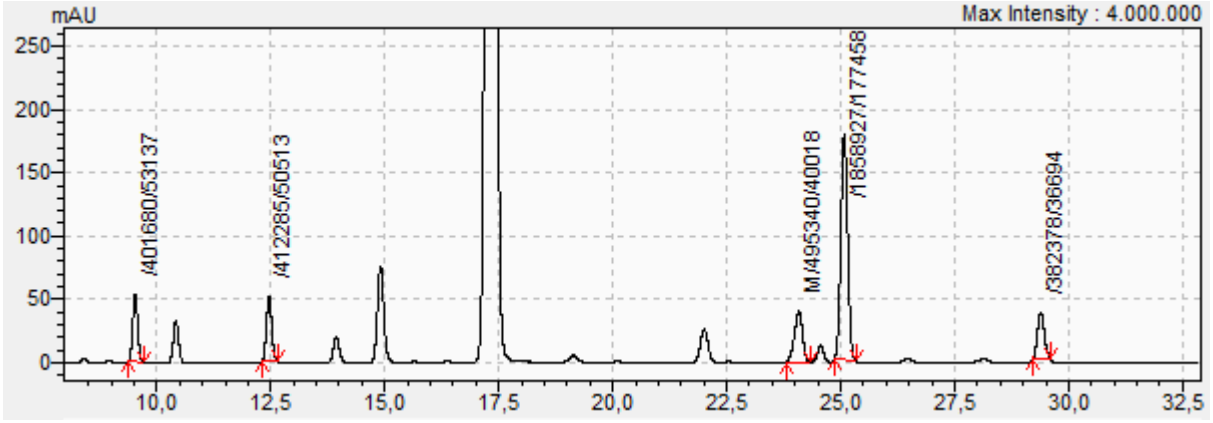


Figure S13: HPLC profile of the hydrolyzed and FDAA derivatized virginiafactin D (λ = 330 nm).

Table S6: Integration areas for peaks from **Fig. S13**.

Peak # / amino acid	$T_{ret.}$ /[min]	area
1 / D-Ser	9.52	401680
2 / D-Gln	12.487	412285
3 / L-Ile	24.08	495340
4 / -L-Leu	25.07	1858927
5 / D-Leu	29.389	382378

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

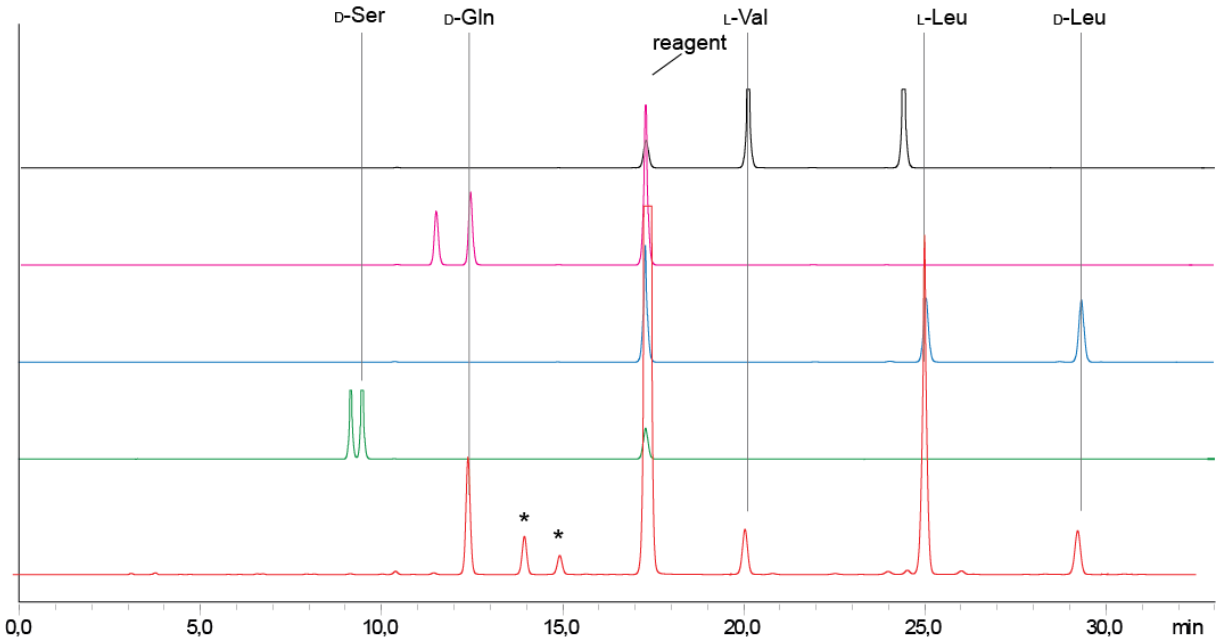


Figure S14: HPLC profiles of the hydrolyzed and FDAA-derivatized cichofactin A (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Val (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA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

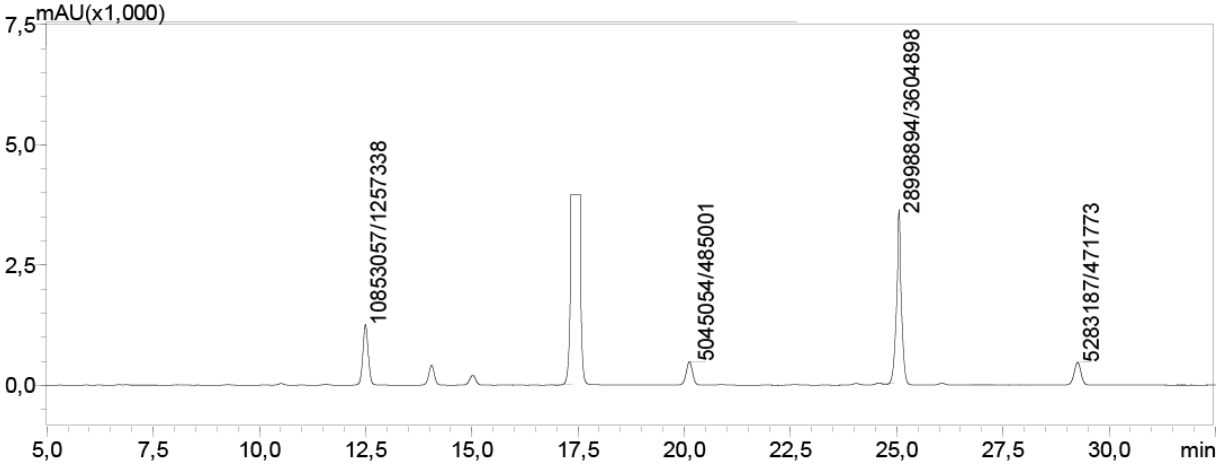


Figure S15: HPLC profile of the hydrolyzed and FDAA derivatized cichofactin A (λ = 330 nm).

Table S7: Integration areas for peaks from **Fig. S15**.

Peak # / amino acid	$T_{ret.}$ /[min]	area
1 / D-Gln	12.499	10853057
2 / L-Val	20.12	5045054
3 / -L-Leu	25.053	28998894
4 / D-Leu	29.26	5283187

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

Cichofactin B

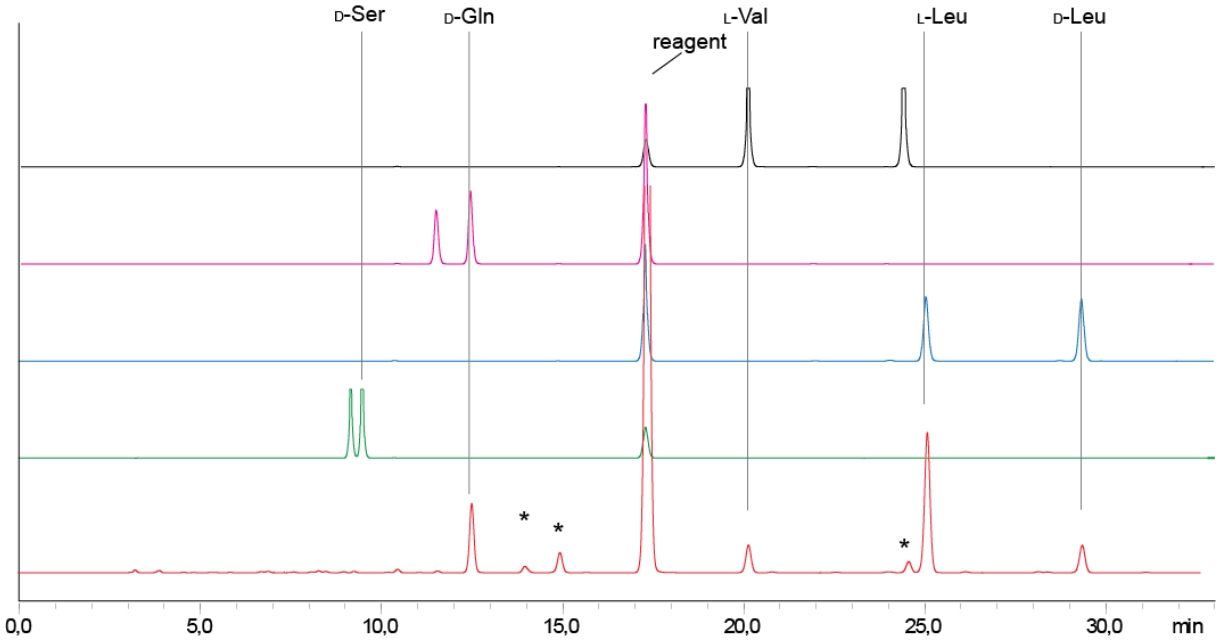


Figure S16: HPLC profiles of the hydrolyzed and FDAA-derivatized cichofactin B (red) and derivatized reference amino acids: L/D-Ser (green), L/D-Leu (blue), L/D-Gln (pink) and L/D-Val (black) (all chromatograms detected at λ = 330 nm). Annotated peaks are labeled. Asterisks indicate impurities from FDAA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

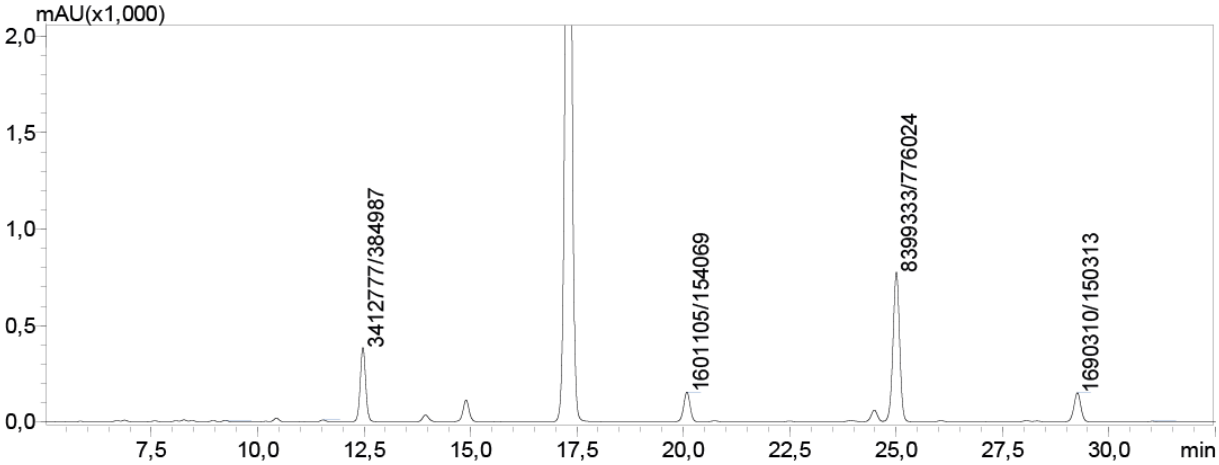


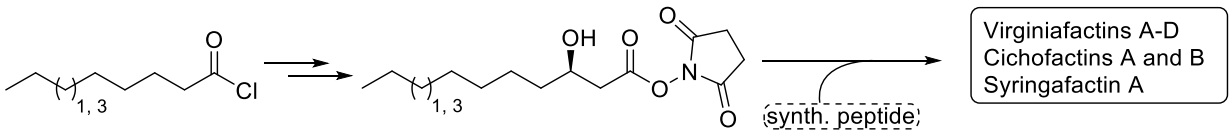
Figure S17: HPLC profile of the hydrolyzed and FDAA derivatized cichofactin B D (λ = 330 nm).

Table S8: Integration areas for peaks from **Fig. S17**

Peak # / amino acid	$T_{ret.}$ /[min]	area
1 / D-Gln	12.475	3412777
2 / L-Val	20.083	1601105
3 / -L-Leu	25.004	8399333
4 / D-Leu	29.262	1690310

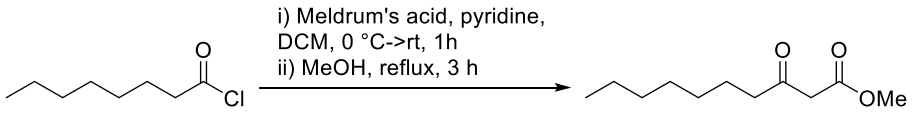
Result: 2.1 x D-Gln; 1.0 x L-Val, 5.2 x L-Leu, 1.0 x D-Leu

Synthesis of Lipo-octapeptides



The fatty acid moieties were synthesized following previous published procedures (Bauer J, *et al. Chem. Eur. J.* **2006**, *12*, 7116; Oikawa Y, *et. al. J. Org. Chem.* **1978**, *43*, 2087.).

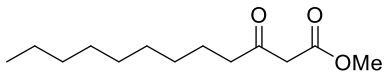
Synthesis of methyl 3-oxodecanoate **1a**



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.

^{1}H NMR (300 MHz, $CDCl_3$, only signals of the predominant keto tautomer are given): δ = 3.68 (s, 3H), 3.39 (s, 2H), 2.47 (t, 2H, $^3J_{H,H}$= 7.4), 1.54 (dt, 2H, $^3J_{H,H}$= 14.1, 7.1), 1.32 – 1.15 (m, 8H), 0.82 (distorted t, 3H, $^3J_{H,H}$= 6.7). ^{13}C NMR (75.5 MHz, $CDCl_3$, only signals of the predominant keto tautomer are given): δ = 202.8, 167.7, 52.3, 49.0, 43.1, 31.7, 29.0, 29.0, 23.5, 22.6, 14.0. HRMS (ESI+): $[C_{11}H_{21}O_3+H]^+$, calc.: 201.1845, found: 201.1487. R_f = 0.79 (9:1 (v/v), hexanes/ethyl acetate). The analytical data is in accordance with previously published data.

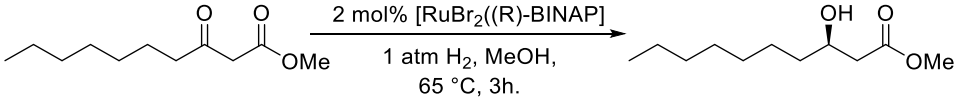
Methyl 3-oxododecanoate **1b**



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

Scale: 10 mmol; yield 720 mg (2.79 mmol, 28%; 40% brsm). ^{1}H NMR (300 MHz, $CDCl_3$, only signals of the predominant keto tautomer are given): δ = 3.64 (s, 3H), 3.36 (s, 2H), 2.44 (t, 2H, $^3J_{H,H}$= 7.4), 1.56– 1.43 (m, 2H), 1.28 – 1.09 (m, 12H), 0.79 (distorted t, 3H, $^3J_{H,H}$= 6.7). ^{13}C NMR (75.5 MHz, $CDCl_3$, only signals of the predominant keto tautomer are given): δ = 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+): $[C_{13}H_{24}O_3+H]^+$, calcd: 229.1798, found: 229.1802. R_f = 0.70 (9:1 (v/v), hexanes/ethyl acetate).

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



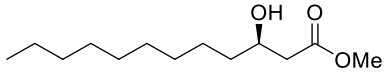
The catalyst was freshly prepared: 10.5 mg (0.0168 mmol, 0.044 eq.) (R)-BINAP and 4.4 mg (0.014 mmol, 0.02 eq.) (COD)Ru(2-methylallyl)$_2$ were placed together with 700 µL degassed acetone in a 10 mL *Schlenk*-tube, equipped with a magnetic stirring bar. 180 µL methanolic

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, $^3J_{H,H}$= 16.5, 3.1), 2.40 (dd, 1H, $^3J_{H,H}$= 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, $^3J_{H,H}$= 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. $[\alpha]_D^{22}$= –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.

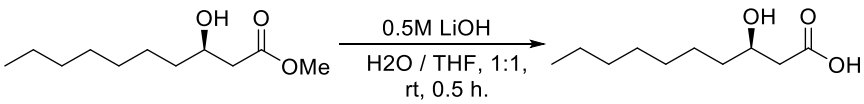
Methyl (*R*)-3-hydroxydodecanoate **2b**



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, $^3J_{H,H}$= 16.4, 3.3), 2.40 (dd, 1H, $^3J_{H,H}$= 16.4, 8.9), 1.59 – 1.17 (m, 16H), 0.87 (distorted t, 3H, $^3J_{H,H}$= 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).

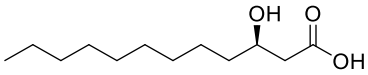
Synthesis of (*R*)-3-hydroxydecanoic acid **3a**



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_2SO_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, $^3J_{H,H}$= 16.7, 3.3), 2.47 (dd, 1H, $^3J_{H,H}$= 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, $^3J_{H,H}$= 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-): $[C_{10}H_{20}O_3-H]^-$, calcd: 187.1340, found: 187.1329. $[\alpha]_D^{22}$= –19.7 (c=0.5,DCM). R_f = 0.57 (4:1 (v/v), hexanes/ethyl acetate).

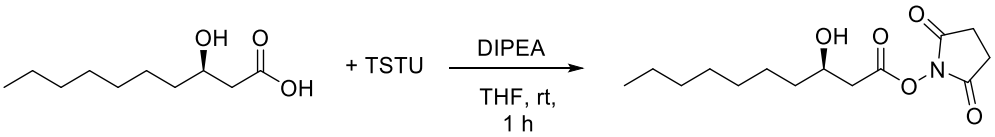
(*R*)-3-hydroxydodecanoic acid **3b**



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, $CDCl_3$): δ = 4.08 – 3.98 (m, 1H), 2.58 (dd, 1H, $^{3}J_{H,H}$= 16.6, 3.3), 2.47 (dd, 1H, $^{3}J_{H,H}$= 16.6, 8.8), 1.61 – 1.19 (m, 16H), 0.88 (distorted t, 3H, $^{3}J_{H,H}$= 6.7). ^{13}C (75.5 MHz, $CDCl_3$): δ = 176.7, 68.0, 40.9, 36.5, 31.9, 29.6, 29.5, 29.5, 29.3, 25.4, 22.7, 14.1. HRMS (ESI-): $[C_{12}H_{24}O_3\text{-}H]^-$, calcd: 215.1653, found: 215.1647. R_f = 0.67 (1:1 (v/v), hexanes/ethyl acetate + 0.1% formic acid).

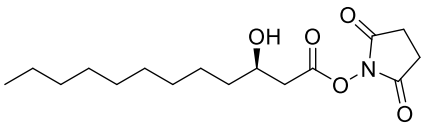
Synthesis of succinimidyl (*R*)-3-hydroxydecanoate **4a**



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, $CDCl_3$): δ = 4.20 – 4.11 (m, 1H), 2.93 – 2.82 (s, 4H), 2.80 (dd, 1H, $^{3}J_{H,H}$= 15.3, 3.4), 2.70 (dd, 1H, $^{3}J_{H,H}$= 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, $^{3}J_{H,H}$= 7.0). ^{13}C (125.8 MHz, $CDCl_3$): δ = 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+): $[C_{14}H_{23}NO_5\text{+}H]^+$, 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, $CDCl_3$): δ = 4.18 – 4.06 (m, 1H), 2.85 (s, 4H), 2.79 (dd, 1H, $^{3}J_{H,H}$= 15.3, 3.7), 2.69 (dd, 1H, $^{3}J_{H,H}$= 15.3, 8.6), 1.66 – 1.18 (m, 16H), 0.87 (distorted t, 3H, $^{3}J_{H,H}$= 6.7). ^{13}C (75.5 MHz, $CDCl_3$): δ = 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+): $[C_{16}H_{27}NO_5\text{+}H]^+$, 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.

Product	Peptide sequence
Virginiafactin S1	(D-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Ile)-(L-Leu)(L-Leu)
Virginiafactin S2	(L-Leu)-(D-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Ile)-(L-Leu)(L-Leu)
Virginiafactin S3 and S5	(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Val)-(D-Leu)(L-Leu)
Virginiafactin S4 and S6	(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Ser)-(L-Ile)-(D-Leu)(L-Leu)
Cichofactin S1	(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Gln)-(L-Val)-(D-Leu)(L-Leu)
Cichofactin S2	(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-Gln)-(L-Val)-(D-Leu)(L-Leu)
Syringafactin S1	(L-Leu)-(L-Leu)-(D-Gln)-(L-Leu)-(D-*allo*Thr)-(L-Val)-(D-Leu)(L-Leu)

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_{10}-fatty acid chain: 65% MeCN in water containing 0.1% formic acid, isocratic; for compounds with C_{12}-fatty acid chain: 75% MeCN in water containing 0.1% formic acid, isocratic).

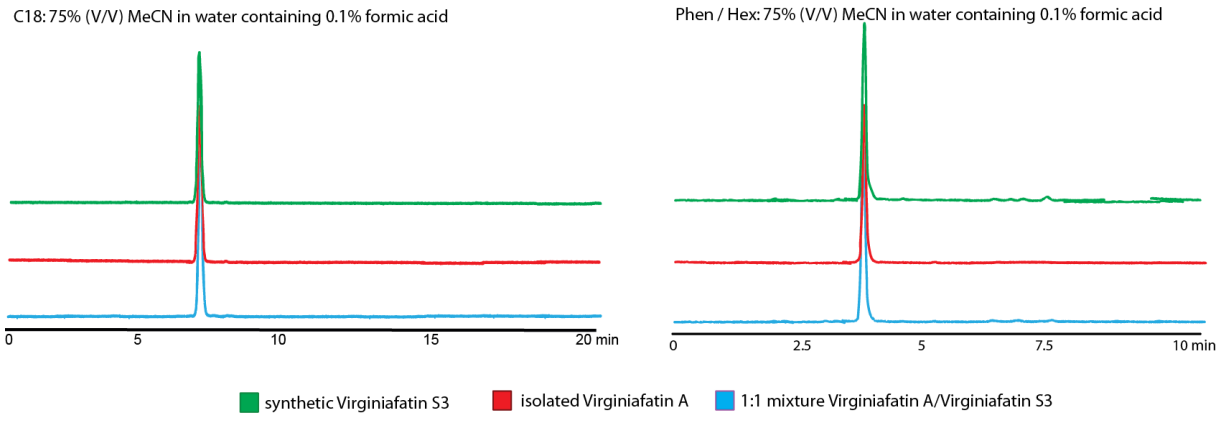


Figure S18: HPLC profile of virginiafactin A, virginiafactin S3 and their mixture under different conditions

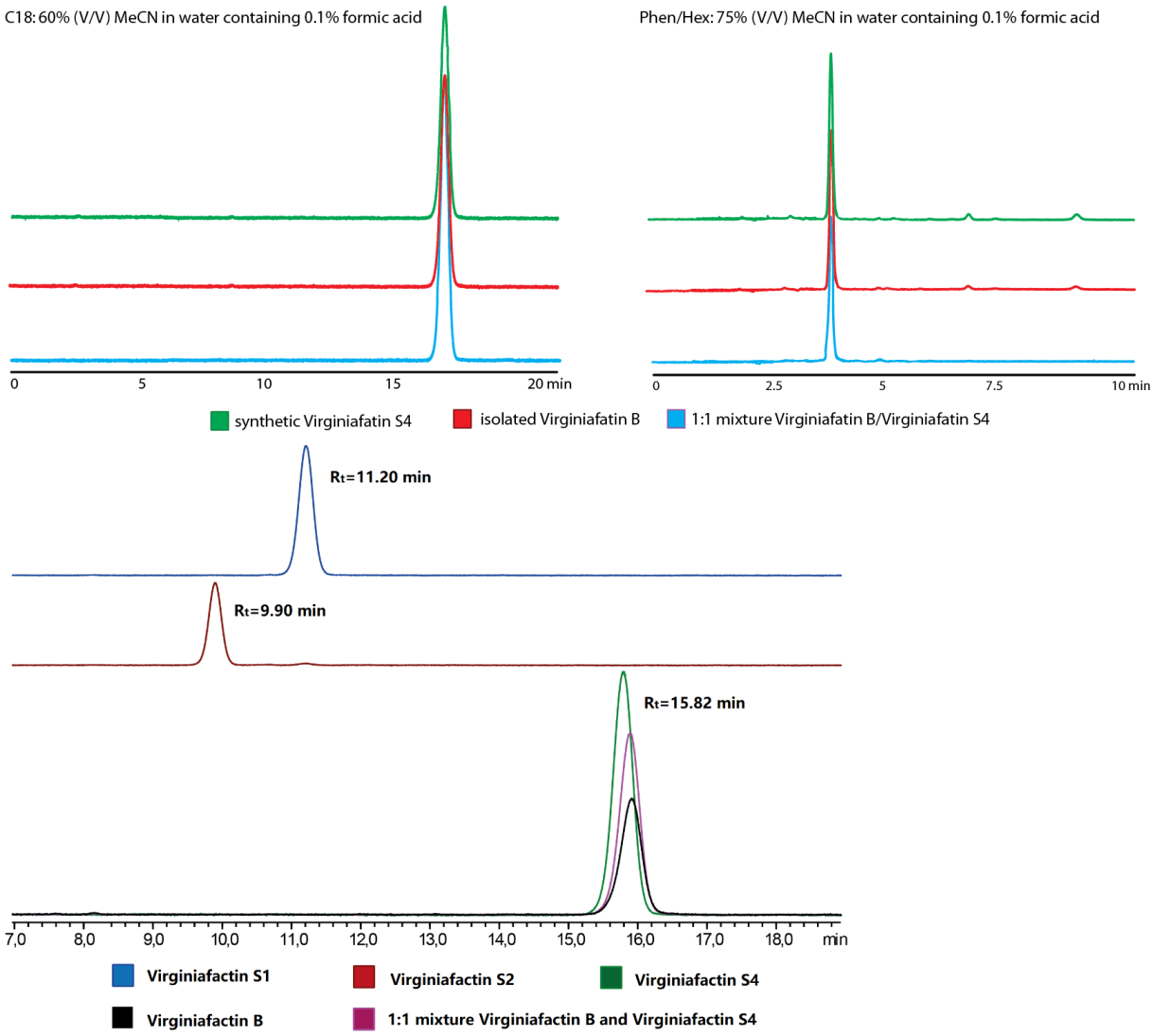


Figure S19: Top: HPLC profile of virginiafactin B, virginiafactin S4 and their mixture under different conditions, Bottom: HPLC profile of virginiafactin B, virginiafactin S1, virginiafactin S2, and virginiafactin S4 (C18 60% v/v MeCN in water containing 0.1 % formic acid).

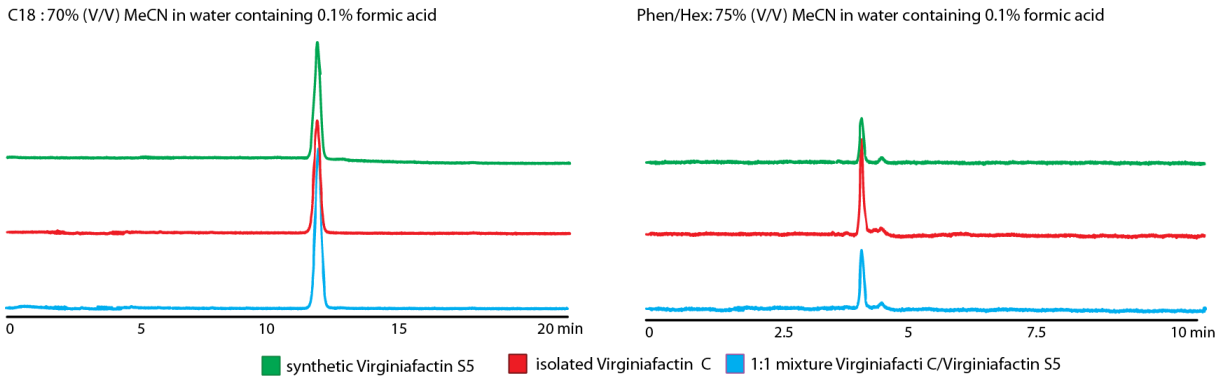


Figure S20: HPLC profile of virginiafactin C, virginiafactin S5 and their mixture under different conditions

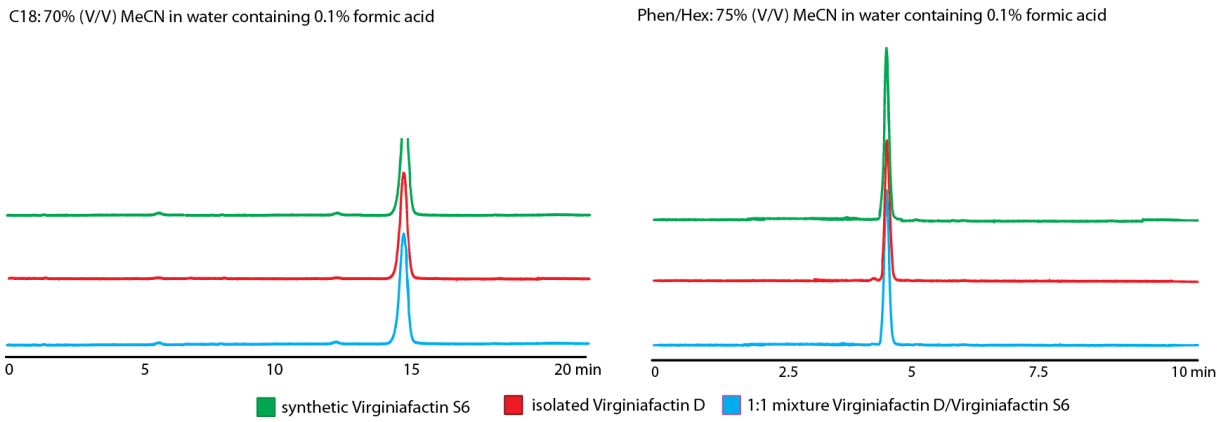


Figure S21: HPLC profile of virginiafactin D, virginiafactin S6 and their mixture under different conditions

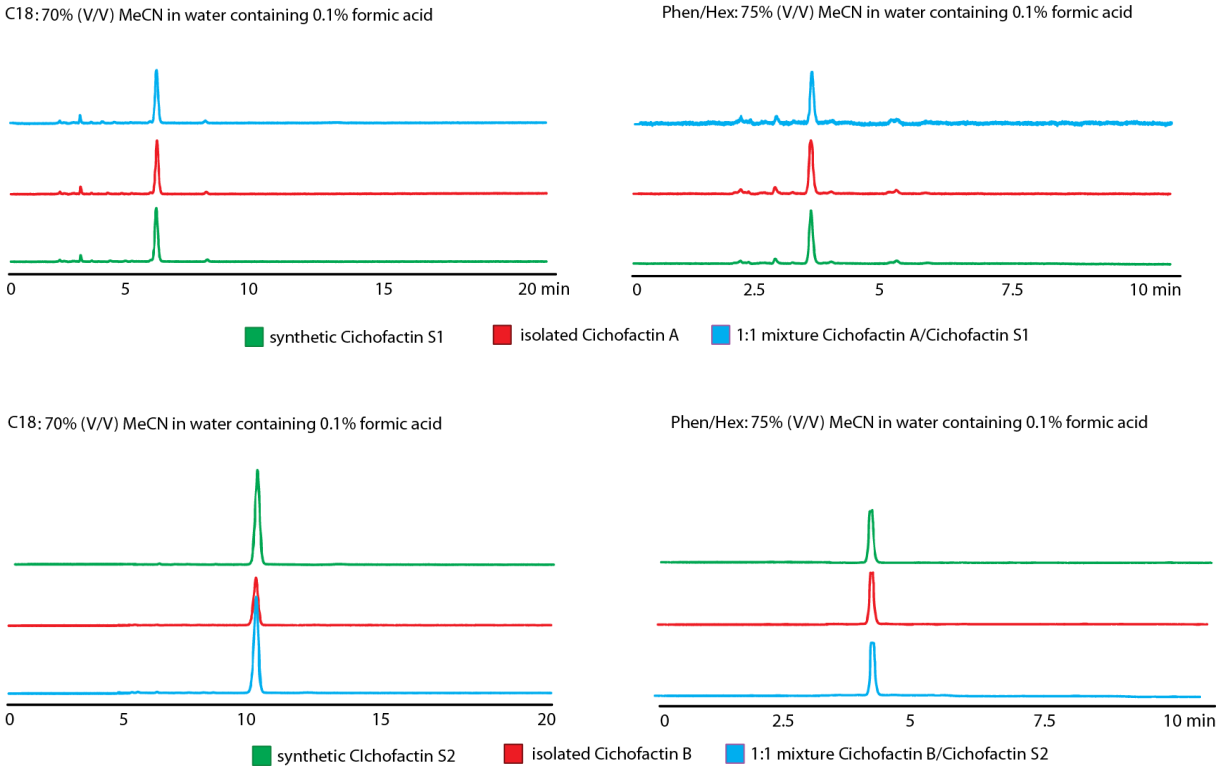


Figure S22: Top: HPLC profile of cichofactin A, cichofactin S1 and their mixture under different conditions. Bottom HPLC profile of cichofactin B, cichofactin S2 and their mixture under different conditions

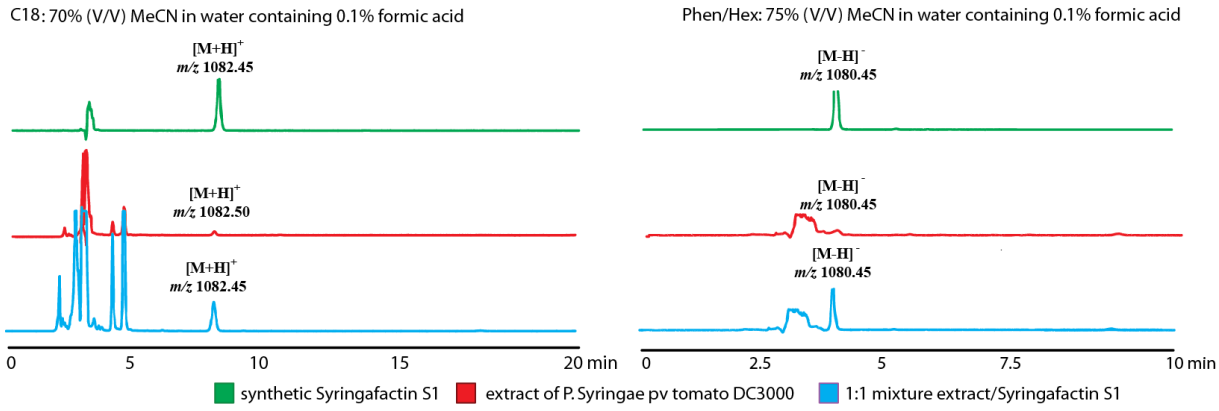
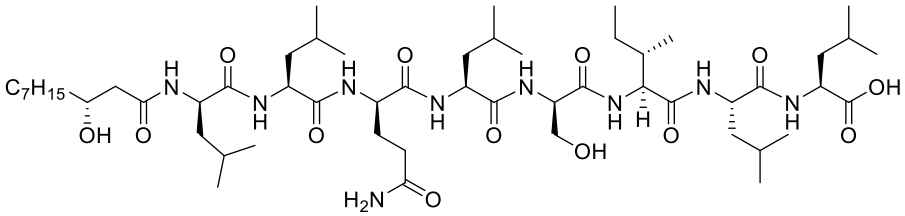


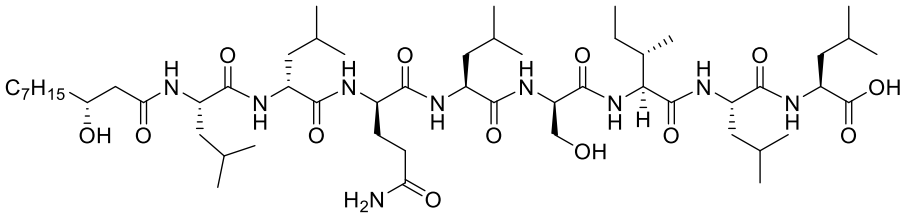
Figure S23: HPLC profile of extract of *P. syringae* pv. *tomato* DC3000, syringafactin S1 and the corresponding mixture under different conditions

Virginiafactin S1



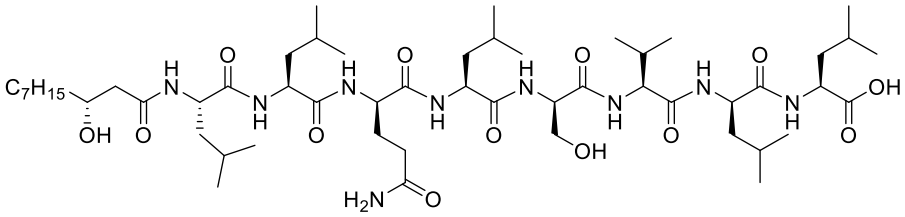
Scale: 0.0054 mmol; yield 2.0 mg (0.0018 mmol, 34%). ^{1}H NMR (600 MHz, CD_3OD): δ = 4.44 (dd, $^{3}J_{H,H}$= 9.5, 5.2, 1H), 4.40 (t, $^{3}J_{H,H}$= 5.2, 1H), 4.37 (dd, $^{3}J_{H,H}$= 9.5, 5.0, 1H), 4.35 –.4.30 (m, 2H), 4.27 (d, $^{3}J_{H,H}$= 7.2, 1H), 4.25 (dd, $^{3}J_{H,H}$= 9.6, 5.0, 1H), 3.97 – 3.95 (m, 1H), 3.87 – 3.82 (qd, $^{3}J_{H,H}$= 11.5, 5.0, 2H), 3.65 – 3.64 (m, 1H), 2.40 (dd, $^{3}J_{H,H}$= 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, 14H), 1.54 – 1.46 (m, 3H), 1.35 – 1.25 (m, 14H), 0.98 – 0.88 (m, 36H). ^{13}C(150 MHz, CD_3OD): δ = 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, 33.1, 33.0, 32.6, 30.8, 30.7, 30.4, 28.0, 26.6, 26.0, 26.0, 26.0, 26.0, 25.9, 25.8, 23.7, 23.6, 23.6, 23.5, 23.4, 22.1, 22.1, 22.0, 21.8, 21.6, 16.0, 14.4, 11.6. HRMS (ESI-): $[C_{54}H_{99}N_9O_{13}\text{-}H]^-$, calcd: 1080.7279, found: 1080.7284. $[\alpha]_D^{25}$= –3.0 (c = 0.1, MeOH).

Virginiafactin S2



Scale: 0.0054 mmol; yield 2.2 mg (0.0020 mmol, 37%). ^{1}H NMR (600 MHz, CD_3OD): δ = 4.46 (dd, $^{3}J_{H,H}$= 9.5, 5.2, 1H), 4.39 (t, $^{3}J_{H,H}$= 5.2, 1H), 4.34 – 4.32 (m, 2H), 4.31 – 4.27 (m, 2H), 4.22 (dd, $^{3}J_{H,H}$= 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, $^{3}J_{H,H}$= 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_3OD): δ = 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, 25.9, 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-): $[C_{54}H_{99}N_9O_{13}\text{-}H]^-$, calc.: 1080.7279, found: 1080.7283. $[\alpha]_D^{25}$= –3.0 (c = 0.1, MeOH).

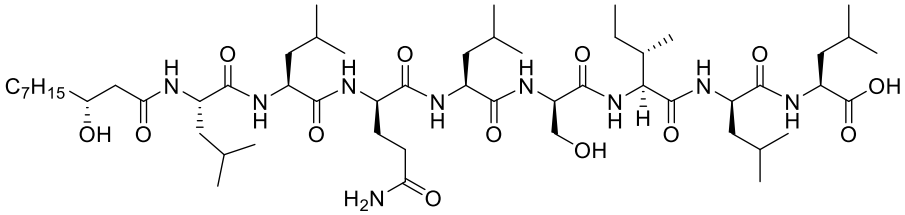
Virginiafactin S3



Scale: 0.0049 mmol; yield 3.95 mg (0.0037 mmol, 75%). ^{1}H NMR (600 MHz, CD_3OD): δ = 4.48 (dd, $^{3}J_{H,H}$= 9.5, 5.2, 1H), 4.42 (t, $^{3}J_{H,H}$= 7.1, 1H), 4.40 – 4.36 (m, 2H), 4.33 (dd, $^{3}J_{H,H}$= 9.5, 5.1, 1H), 4.26 (dd, $^{3}J_{H,H}$= 9.6, 4.9, 1H), 4.00 – 3.95 (m, 1H), 3.85 (qd, $^{3}J_{H,H}$= 11.5, 5.0, 2H), 2.46

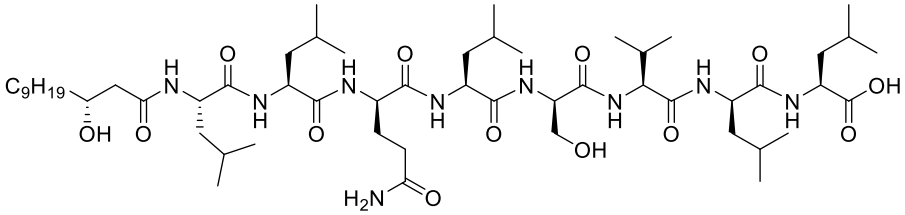
(dd, $^3J_{H,H}$= 14.2, 4.6, 1H), 2.38 – 2.25 (m, 4H), 2.22 – 2.11 (m, 2H), 2.10 – 2.00 (m, 2H); 1:75 – 1.54 (m, 15H), 1.54 – 1.40 (m, 4H), 1.40 – 1.08 (m, 24H), 1.06 – 0.72 (m, 36H). ^{13}C(150 MHz, CD_3OD): δ = 177.7, 175.7, 175.6, 175.0, 174.9, 174.3, 174.2, 173.5, 172.6, 70.0, 62.9, 60.7, 57.4, 55.2, 53.8, 53.7, 53.2, 52.7, 44.7, 42.2, 41.8, 41.6, 41.4, 41.1, 38.4, 33.03, 32.8, 31.2, 30.7, 30.68, 30.5, 30.2, 28.2, 26.8, 26.0, 25.9, 23.7, 23.6, 23.4, 22.0, 21.9, 21.86, 21.8, 21.7, 19.8, 18.8, 14.4. HRMS (ESI+): $[C_{53}H_{97}N_9O_{13}+H]^+$, calc.: 1068.7279, found: 1068.7267. $[\alpha]_D^{25}$= –3.0 (c = 0.1, MeOH).

Virginiafactin S4



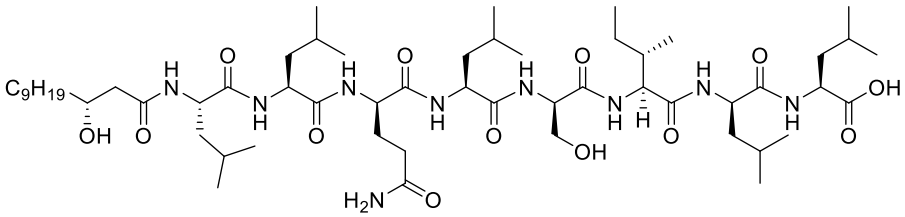
Scale: 0.0049 mmol; yield 3.95 mg (0.0037 mmol, 75%). ^{1}H NMR (600 MHz, CD_3OD): δ = 4.49 (dd, $^3J_{H,H}$= 9.6, 5.0, 1H), 4.43 (t, $^3J_{H,H}$= 6.5, 1H), 4.40 – 4.34 (m, 3H), 4.32 (dd, $^3J_{H,H}$= 8.7, 6.1, 1H), 4.28 – 4.22 (m, 2H), 4.01 – 3.96 (m, 1H), 3.90 – 3.80 (m, 2H), 2.47 (dd, $^3J_{H,H}$= 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*$_4$-MeOH): δ = 177.7, 175.7, 175.6, 175.0, 174.9, 174.4, 174.2, 173.5, 172.6, 70.0, 62.9, 59.8, 57.4, 55.2, 53.9, 53.8, 53.7, 53.1, 52.4, 44.7, 41.9, 41.7, 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, 23.7, 23.6, 23.6, 23.4, 22.0, 21.9, 21.9, 21.8, 21.0, 16.1, 14.5, 11.7. HRMS (ESI+): $[C_{54}H_{99}N_9O_{13}+H]^+$, calcd: 1082.7435, found: 1082.7464. $[\alpha]_D^{25}$= –5.9 (c = 0.1, MeOH).

Virginiafactin S5



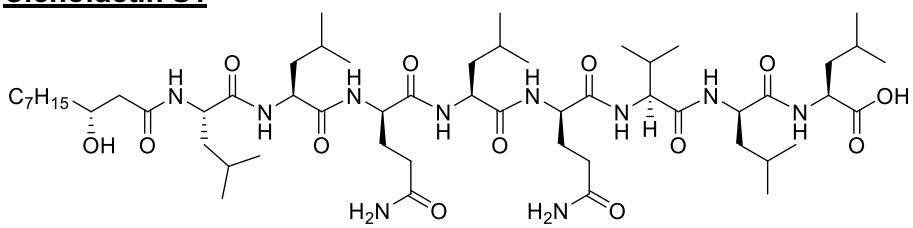
Scale: 0.0049 mmol; yield 3.92 mg (0.0036 mmol, 72%). ^{1}H NMR (600 MHz, *d*$_4$-MeOH): δ = 4.49 (dd, $^3J_{H,H}$=9.6, 5.2, 1H), 4.43 (t, $^3J_{H,H}$=7.0, 1H), 4.40 – 4.35 (m, 3H), 4.33 (dd, $^3J_{H,H}$=9.3, 5.4, 1H), 4.26 (dd, $^3J_{H,H}$= 9.5, 4.8, 1H), 4.19 (d, $^3J_{H,H}$= 6.9, 1H), 4.01 – 3.96 (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*$_4$-MeOH): δ = 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, 53.8, 53.7, 53.2, 52.5, 44.7, 42.0, 41.9, 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, 14.5. HRMS (ESI+): $[C_{55}H_{101}N_9O_{13}+H]^+$, calcd: 1096.7592, found: 1096.7574. $[\alpha]_D^{25}$= –12.5 (c = 0.1, MeOH).

Virginiafactin S6



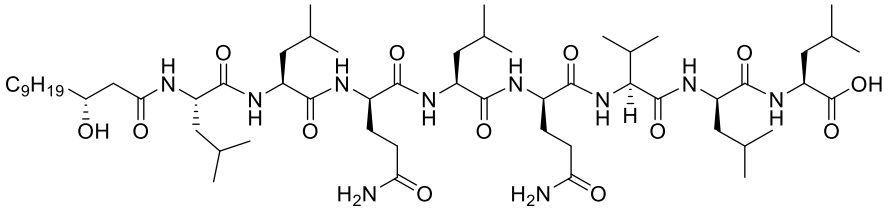
Scale: 0.0057 mmol; yield 4.73 mg (0.0043 mmol, 75%). ^{1}H NMR (600 MHz, *d*$_4$-MeOH: δ = 4.49 (dd, $^{3}J_{H,H}$= 9.9, 4.9, 1H), 4.43 (t, $^{3}J_{H,H}$= 6.5, 1H), 4.40 – 4.35 (m, 3H), 4.32 (dd, $^{3}J_{H,H}$= 8.7, 6.1, 1H), 4.27 – 4.22 (m, 2H), 4.02 – 3.96 (m, 1H),3.85 (qd, , $^{3}J_{H,H}$= 9.2, 6.4, 2H), 2.47 (dd, $^{3}J_{H,H}$= 14.1, 4.5, 1H), 2.39 – 2.25 (m, 3H), 2.21 – 2.13 (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, 14H), 1.21 – 1.13 (m, 2H), 1.00 – 0.83 (m, 39H); ^{13}C NMR (150 MHz, *d*$_4$-MeOH): δ = 177.7, 176.7, 175.6, 175.0, 174.9, 174.4,174.2, 173.5, 172.6, 70.0, 62.9, 59.8, 57.4, 55.2, 53.9, 53.8, 53.7, 53.1, 52.4, 44.7, 41.9, 41.7, 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, 25.9, 23.4, 22.0, 21.9, 21.9, 21.8, 21.7, 16.1, 14.6, 11.7. HRMS (ESI+) calcd for $[C_{56}H_{103}N_9O_{13}+H]^+$ 1110.7748, found 1110.7725, $[\alpha]_D^{25}$= –6.6 (c = 0.1, MeOH)

Cichofactin S1



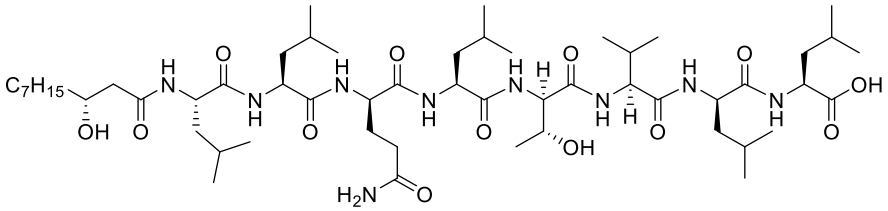
Scale: 0.0049 mmol; yield 3.37 mg (0.0030 mmol, 62%). ^{1}H NMR (500 MHz, *d*$_4$-MeOH: δ = 4.50 (dd, $^{3}J_{H,H}$= 9.9, 4.9, 1H), 4.44 – 4.40 (m, 1H), 4.40 – 4.30 (m, 4H), 4.16 (d, $^{3}J_{H,H}$= 7.1, 1H), 4.00 – 3.95 (m, 1H), 4.02 – 3.96 (m, 1H), 2.47 – 2.44 (dd, $^{3}J_{H,H}$= 14.1, 4.5, 1H), 2.38 – 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*$_4$-MeOH): δ = 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, 42.0, 41.7, 41.4, 41.1, 38.4, 33.1, 32.9, 32.6, 31.3, 30.8, 30.7, 30.5, 28.5, 28.3, 26.8, 26.0, 26.0, 26.0, 25.9, 25.9, 23.7, 23.6, 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 $[C_{55}H_{100}N_{10}O_{13}+H]^+$ 1109.7544, found 1109.7542, $[\alpha]_D^{25}$= –5.2 (c = 0.1, MeOH).

Cichofactin S2



Scale: 0.0050 mmol; yield 3.98 mg (0.0035 mmol, 70%). ^{1}H NMR (600 MHz, *d*$_4$-MeOH: δ = 4.50 (dd, $^{3}J_{H,H}$= 9.9, 4.9, 1H), 4.43 – 4.40 (m, 2H), 4.38 – 4.30 (m, 4H), 4.16 (d, $^{3}J_{H,H}$= 7.1, 1H), 3.99 – 3.97 (m, 1H), 2.45 (dd, $^{3}J_{H,H}$= 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*$_4$-MeOH): δ = 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, 30.8, 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, 23.6, 23.5, 22.0, 22.0, 21.9, 21.8, 21.8, 21.8, 19.9, 18.8, 14.5. HRMS (ESI-) calcd for $[C_{57}H_{104}N_{10}O_{13}-H]^-$ 1135.7714, found 1135.7714, $[\alpha]_D^{25}$= –7.1 (c = 0.1, MeOH).

Syringafactin S1



Scale: 0.0055 mmol; yield 4.43 mg (0.0041 mmol, 74%). ^{1}H NMR (600 MHz, *d*$_4$-MeOH: δ = 4.50 (dd, $^{3}J_{H,H}$= 9.9, 4.9, 1H), 4.45 – 4.41 (m, 2H), 4. (dd, $^{3}J_{H,H}$= 9.9, 5.0, 1H), 4.33 (d, $^{3}J_{H,H}$= 3.7, 1H), 4.31 – 4.23 (m, 3H), 4.17 (d, $^{3}J_{H,H}$= 7.1, 1H), 4.01 – 3.97 (m, 1H), 2.48 (dd, $^{3}J_{H,H}$= 14.1, 4.5, 1H), 2.37 – 2.31 (m, 3H), 2.21 – 2.14 (m, 2H), 2.06 – 2.00 (m, 1H), 1.77 – 1.56 (m, 15H), 1.50 – 1.45 (m, 3H), 1.32 – 1.30 (m, 10H), 1.18 (d, $^{3}J_{H,H}$= 6.5, 3H), 0.98 – 0.89 (m, 38H); ^{13}C NMR (150 MHz, *d*$_4$-MeOH): δ = 177.7, 175.9, 175.6, 175.4, 175.0, 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, 23.6, 23.5, 23.5, 23.5, 23.4, 21.9, 21.9, 21.9, 21.9, 21.8, 20.2, 19.9, 18.9, 14.4. HRMS (ESI-) calcd for $[C_{54}H_{99}N_9O_{13}\text{-}H]^-$ 1080.7292, found 1080.7288, $[\alpha]_D^{25}$= –4.3 (c = 0.1, MeOH).

Phylogenetic Analyses

In order to shed more light on the directionality of the transfer *vif*-A$_{Ser}$ ↔ *jes*$_{QS}$-A$_{Ser}$, we intended to add another *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 *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 *Pseudomonas* sp. MWU13-2860, we designated this cluster *jes*$_{MWU}$. As expected from the high similarity between the *jes*$_{MWU}$ and *jes*$_{QS}$ clusters, most of the *jes* domains cluster according to their positions within the assembly line. Surprisingly, however, both *jes*$_{MWU}$-A9$_{Ser}$ and *jes*$_{MWU}$-A18$_{Ser}$ regions formed their own clade descending from an ancestral *jes*-A18$_{Ser}$ 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 *jes*$_{MWU}$ BGC after the triplication in the ancestor of the *Pseudomonas* strain.

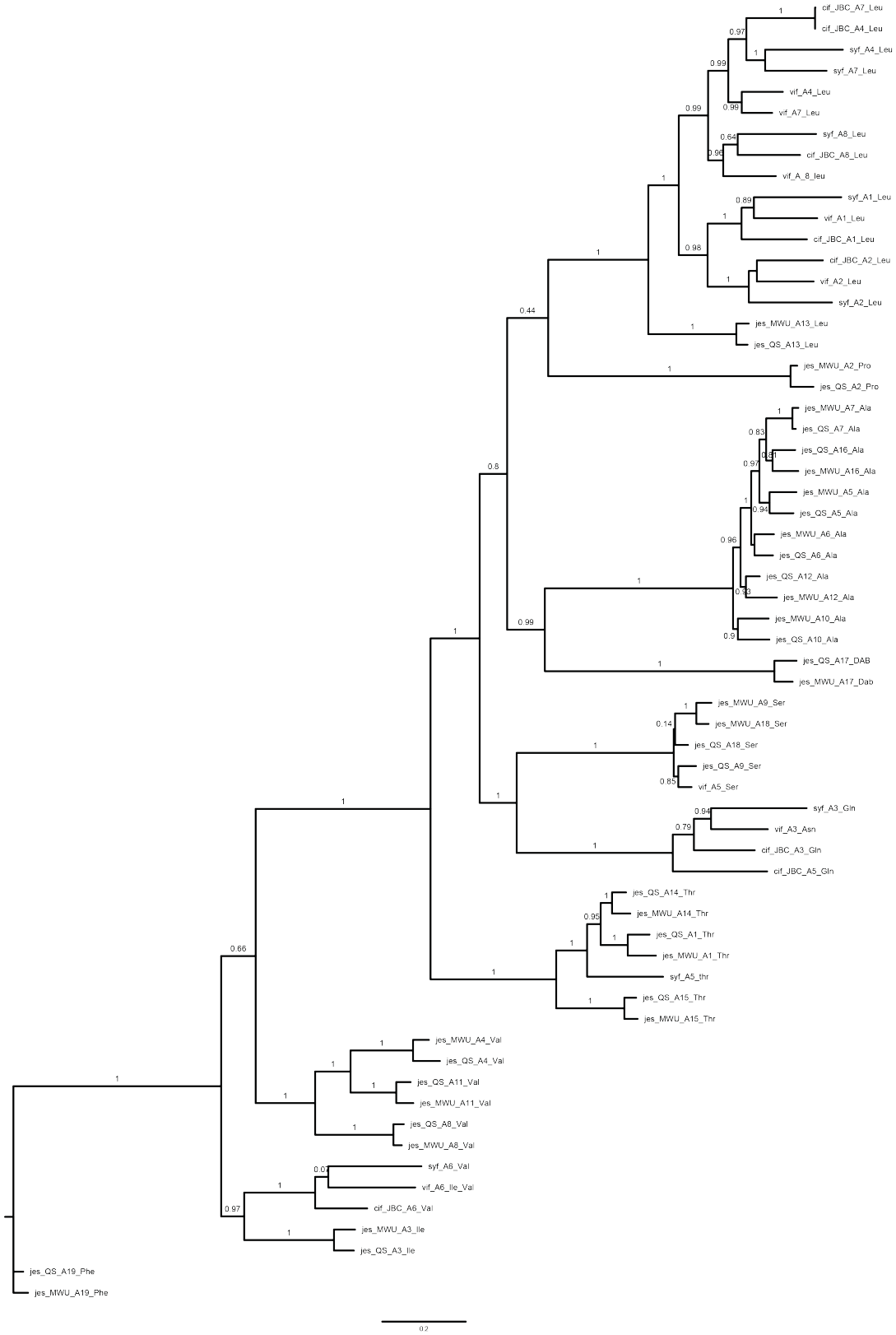


Figure S24: Phylogenetic tree of the coding regions of 62 A domains of the BGC *vif*, *cif*, *syf*, jes_{QS}, and jes_{MWU}. Tree inference was performed using Maximum Likelihood estimation implemented in PhyML 3.0 using the GTR model with a gamma distribution of rates. Support values (Shimodaira-Hasegawa-like 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 –37421.72274. Scale bar indicates substitutions per site.

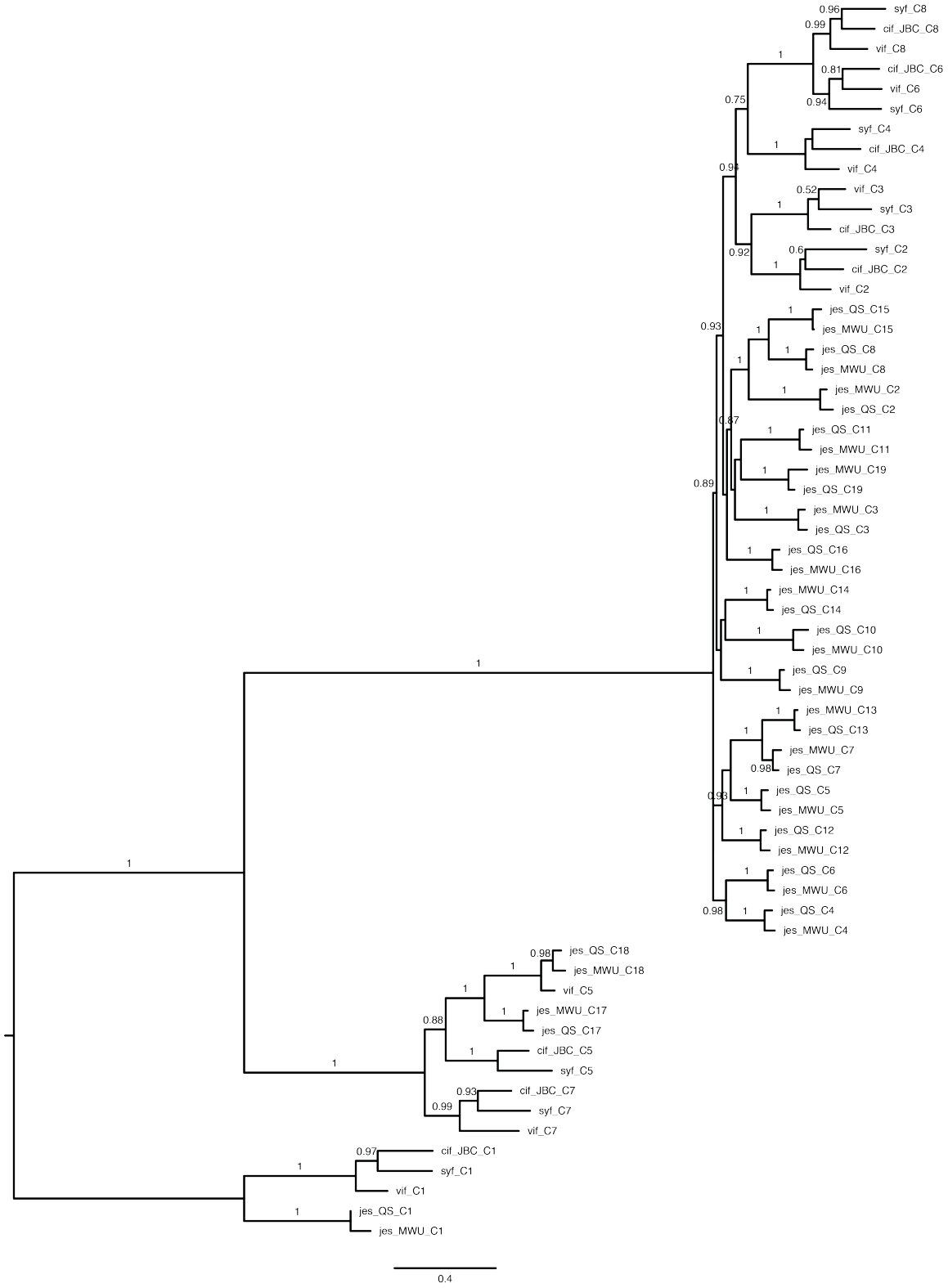


Figure S25: Phylogenetic tree of the coding regions of 62 C domains of the BGC *vif*, *cif*, *syf*, jes_{QS}, and jes_{MWU}. Tree inference was performed using Maximum Likelihood estimation implemented in PhyML 3.0 using the GTR model with a gamma distribution of rates. Support values (Shimodaira-Hasegawa-like 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

Primer	**Sequences 5'→3'**
cif_A4_F	TTTGAACGAGCCATGGAGCA
cif_A4_R	CAACTGCTCCAGCAAGGC
cif_A7_F	GTTACGTTCATTACTTCGAGCAACT
cif_A7_R	AGACAGTCAACAGTTCGGCAC

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

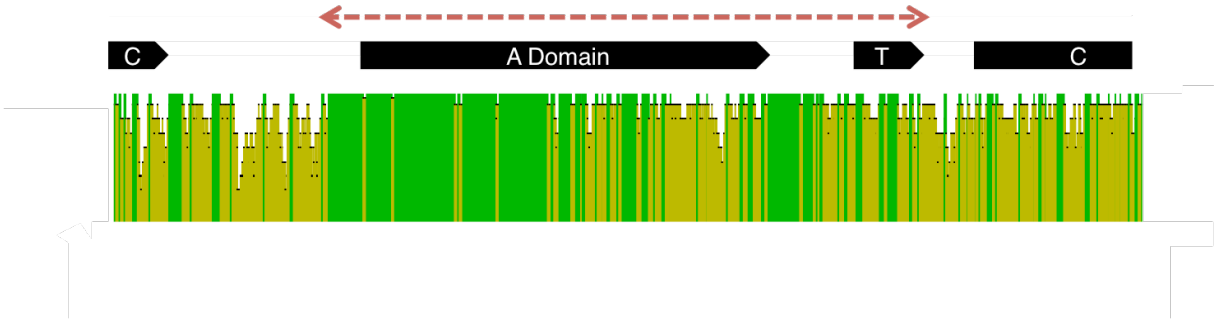


Figure S26. Sequence alignment of *jes* module 6 and 12. Shown are the mean pairwise identities green: 100%, green-brown: 30 – 100%, red: < 30%. Sliding window size: 10 nt. The red dashed bar on top shows the repeated sequence with identity cut-off = 93%

Analysis of the Virginifactin BGC

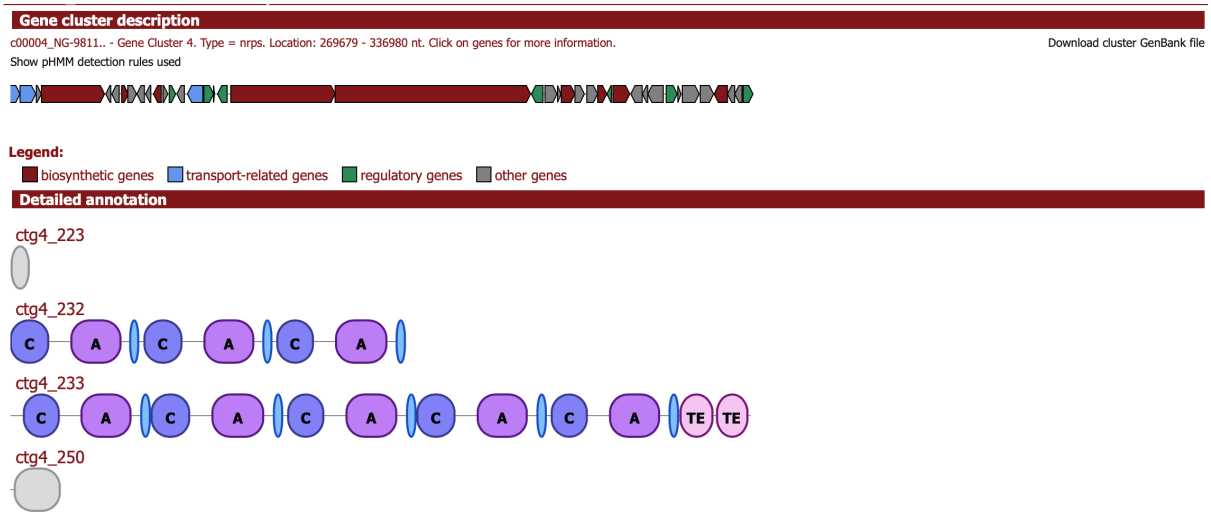


Figure S27. Screenshot of the antiSMASH analysis of the *vifA* and *vifB* genes to visualize the boundaries of this BGC. According to our analysis no further tailoring enzymes are involved in the biosynthesis of the virginiafactins.

Sequence Information

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cif M4   80474 CCGTGCTGACCATCCATCAGCGCATCGAACGTCAGGCCCTCGACCAGCCGGACGCCATCG 80415
                 G  C  ACCATCCAT      ATCGA    CAGGC  T  A C  CC GA GCCATCG
cif M7   71021 --GGTCAAACCATCCATGGTTTGATCGAGGCGCAGGCTGTTCAACGACCTGATGCCATCG 70964

cif M4   80414 CCTCGCAAGTGGGCGATCAGCACCTGAGCTACAGCGAACTGAATAGCAAGGCCAATGCCT 80355
               CCTCGCAAGTGGGCGATCAGCACCTGAGCTACAGCGAACTGAATAGCAAGGCCAATGCCT
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cif M4   80354 TGGCCCATCACCTGATCAGCCTGGGCGTGCGCCCGGATGATCGTGTGGCCGTGGTCGCTC 80295
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Figure S28. Nucleotide sequence alignment of *cif*-M4 and *cif*-7M.

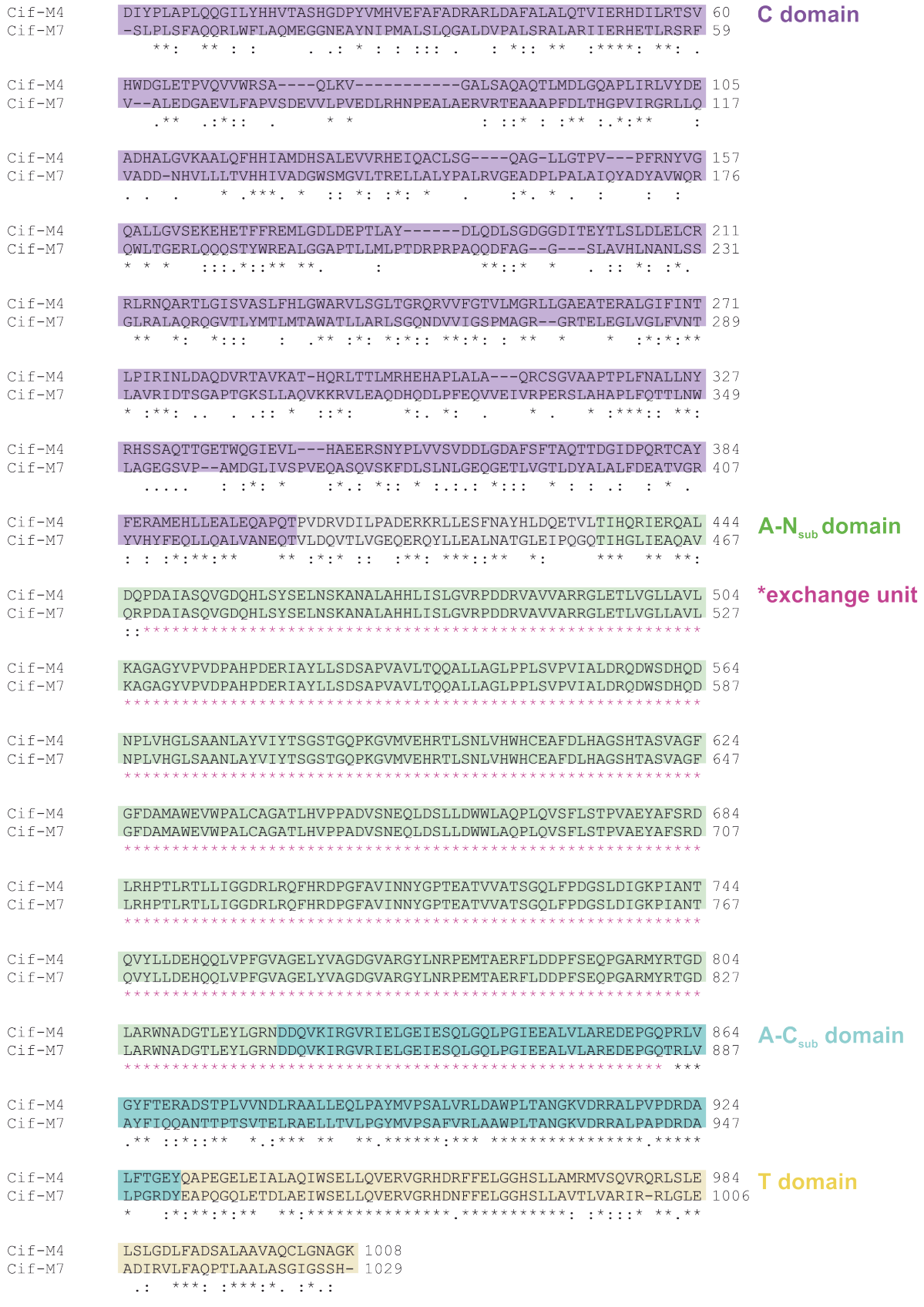

Figure S29. Protein sequence alignment of Cif-M4 and Cif-7M.

NMR Spectra

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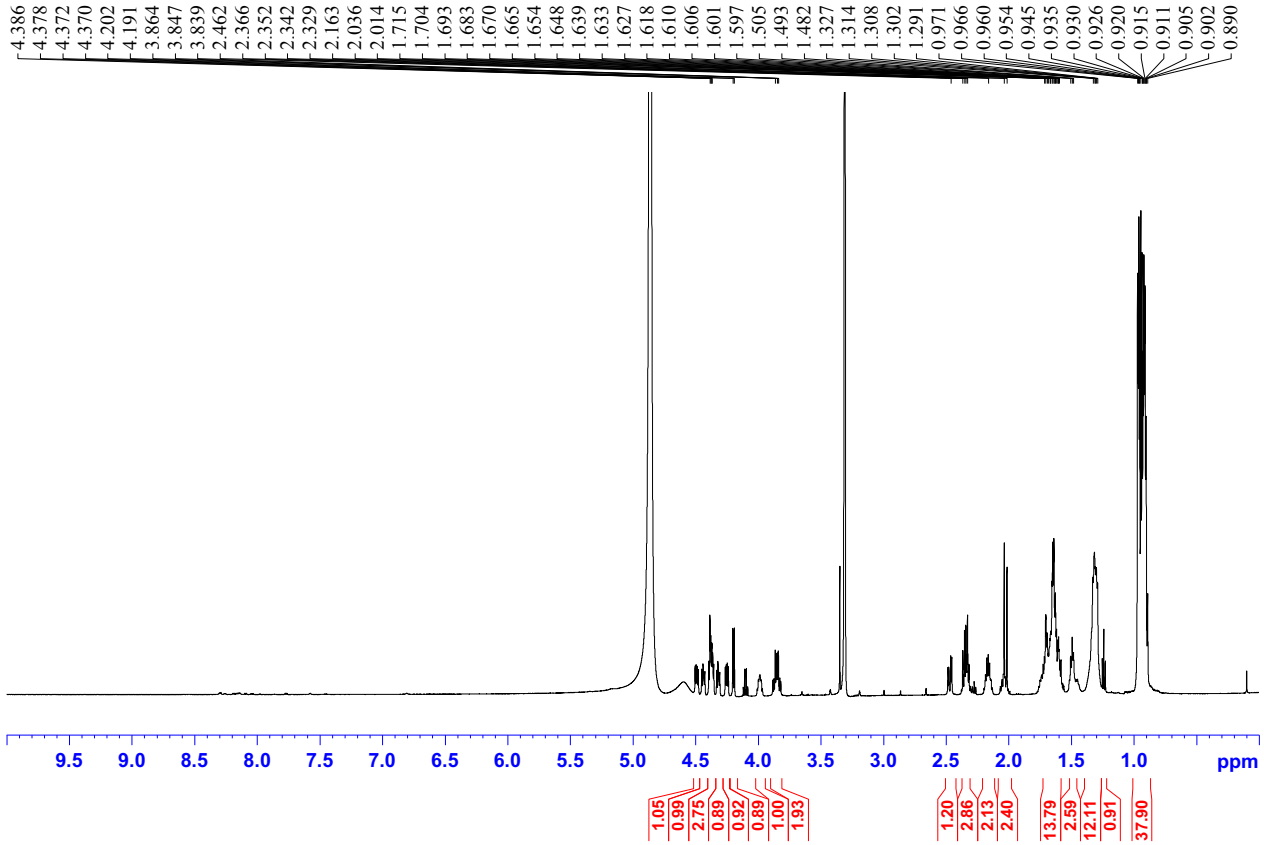

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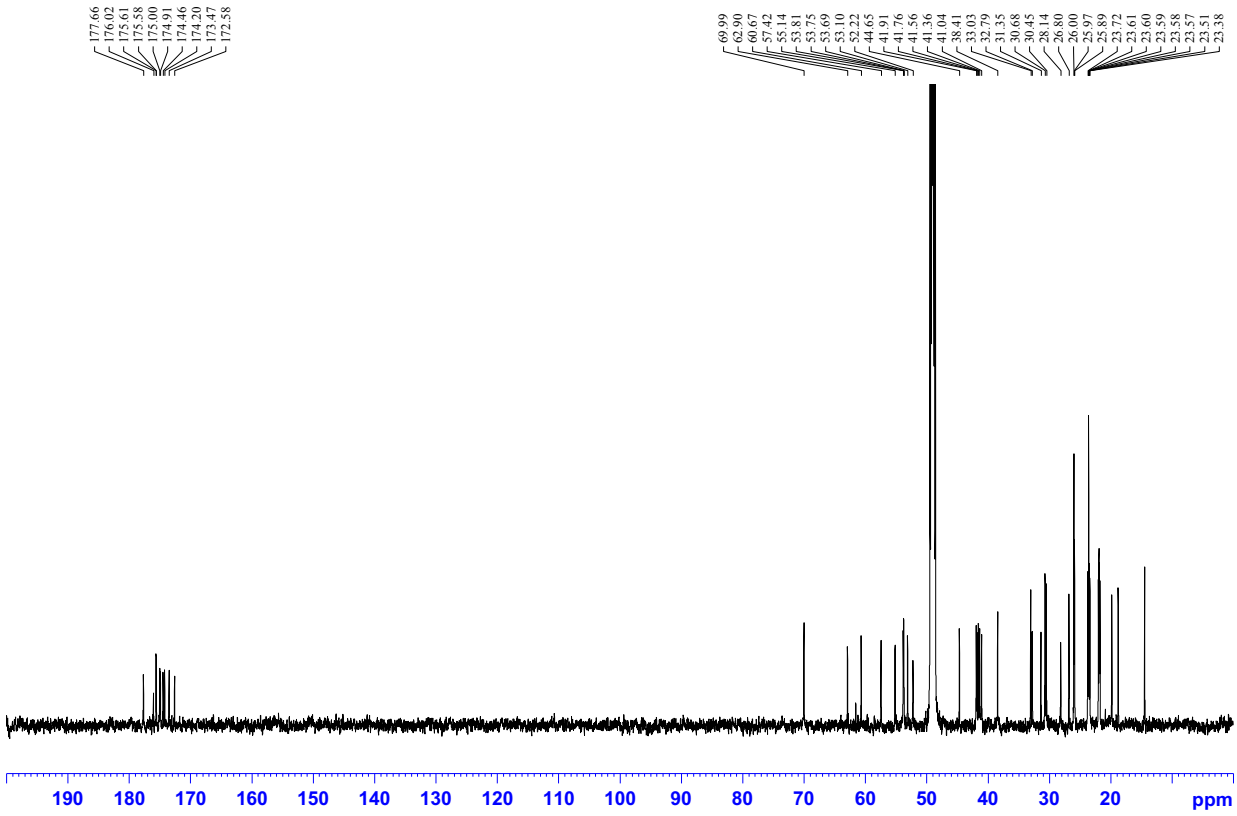
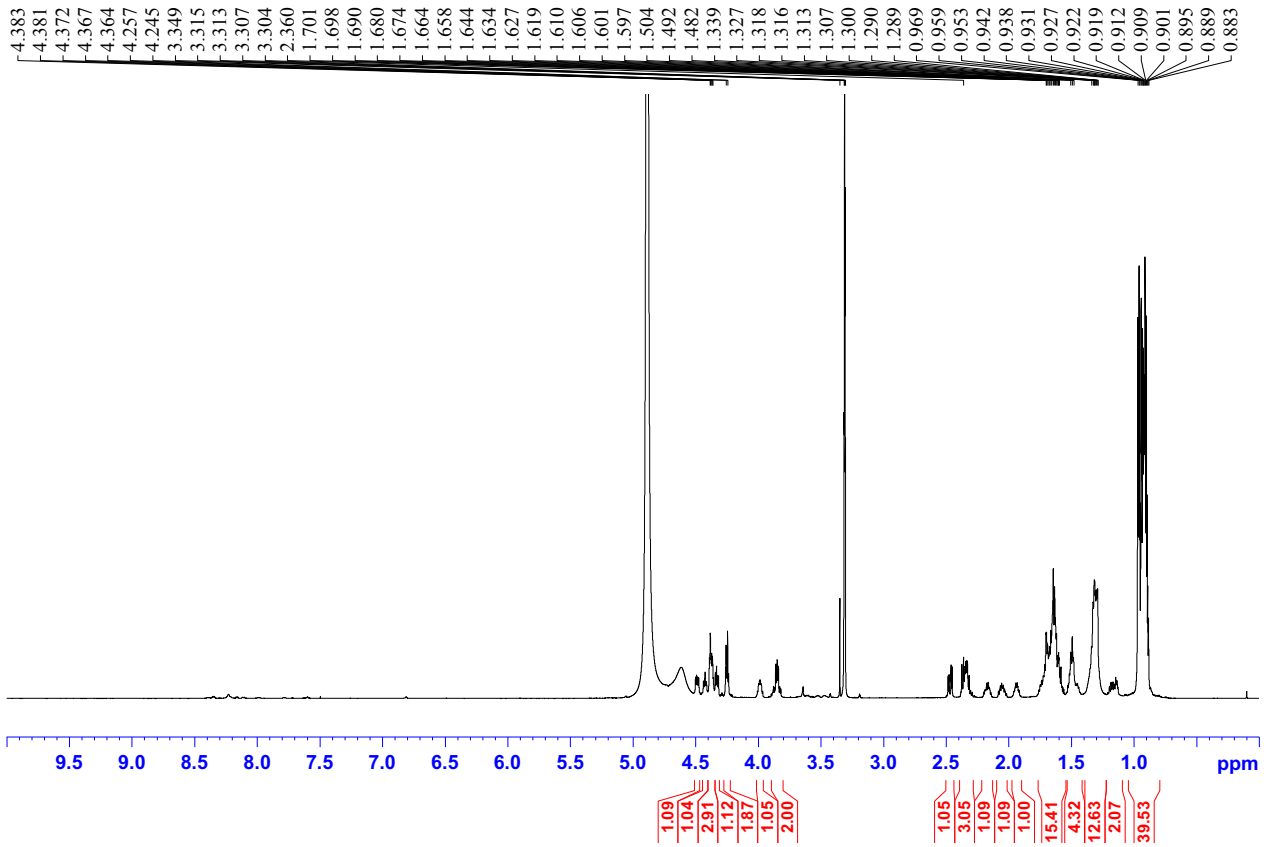


^{1}H (600 MHz, methanol-d_4): Virginiafactin B



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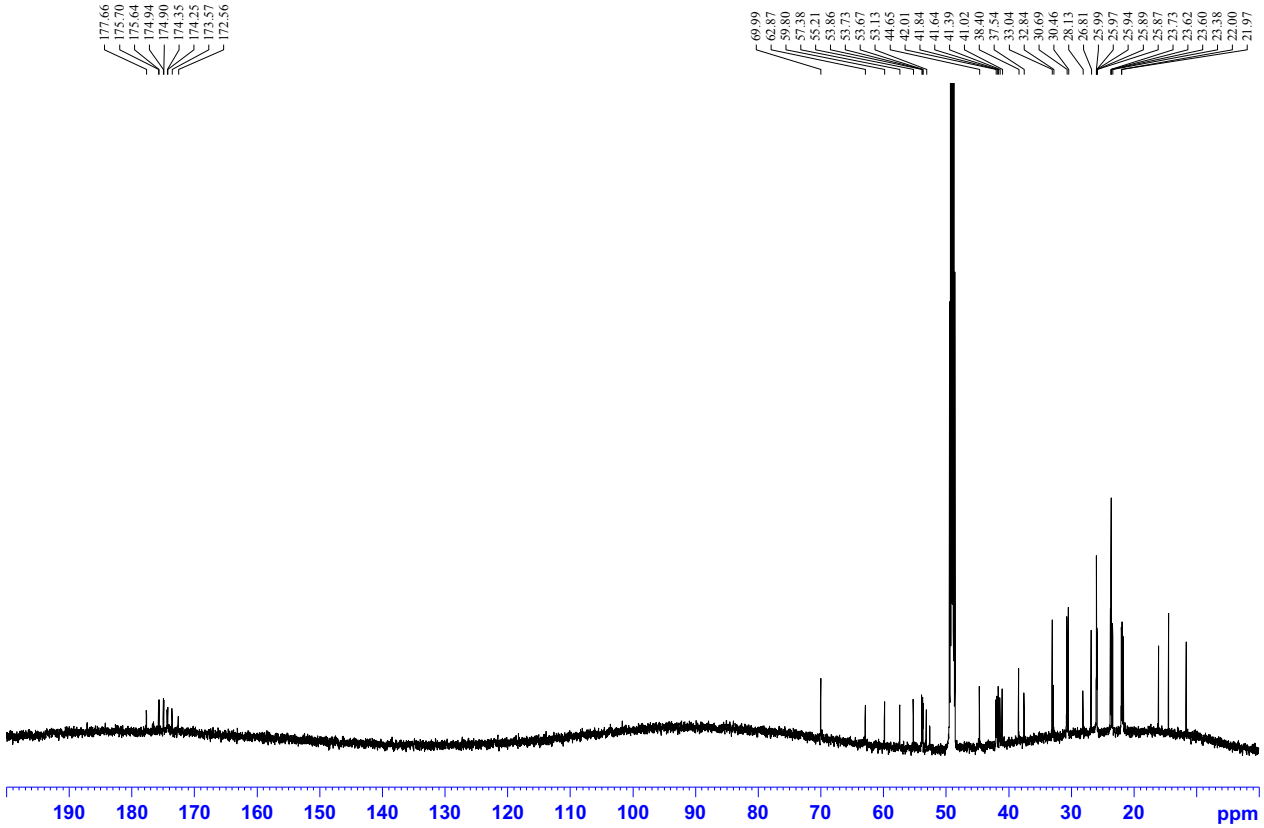
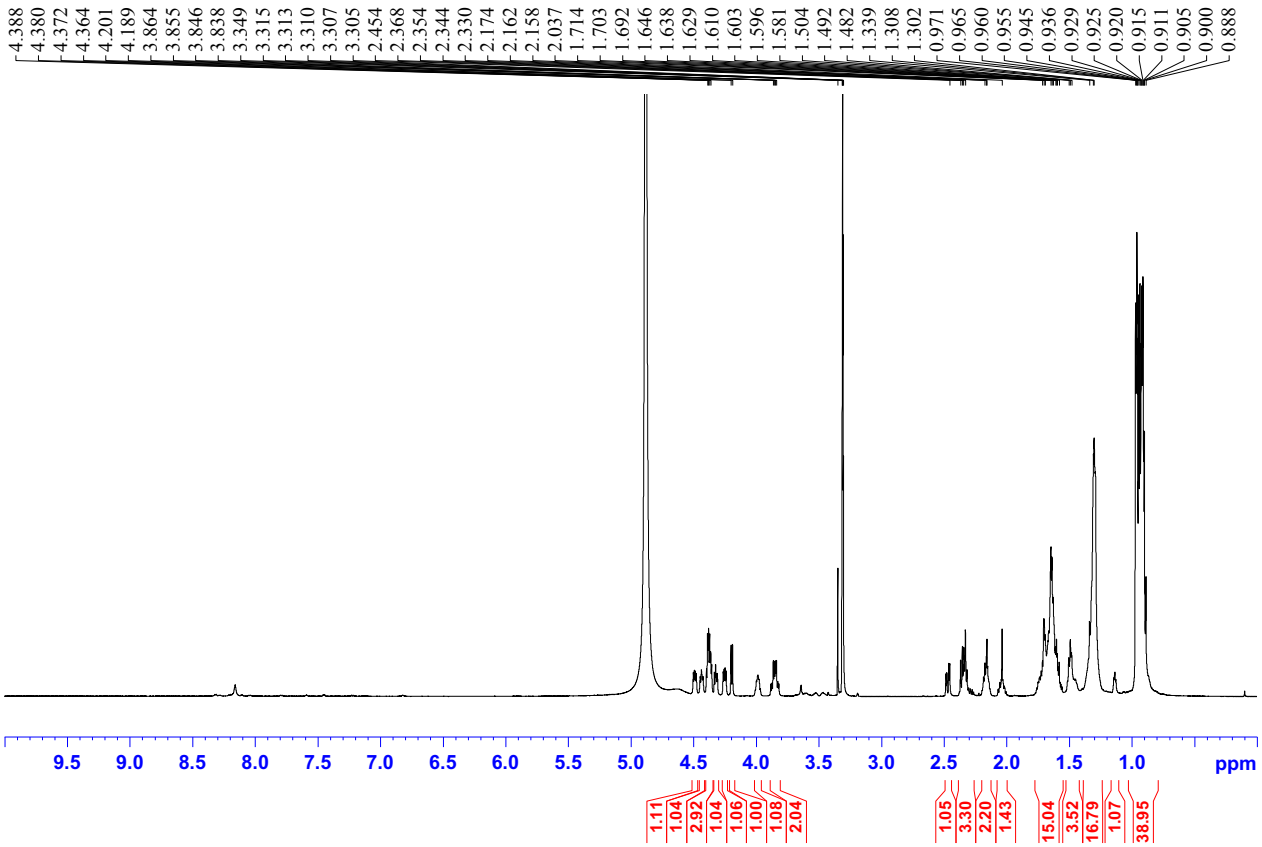


^{1}H (600 MHz, methanol-d_4): Virginiafactin C



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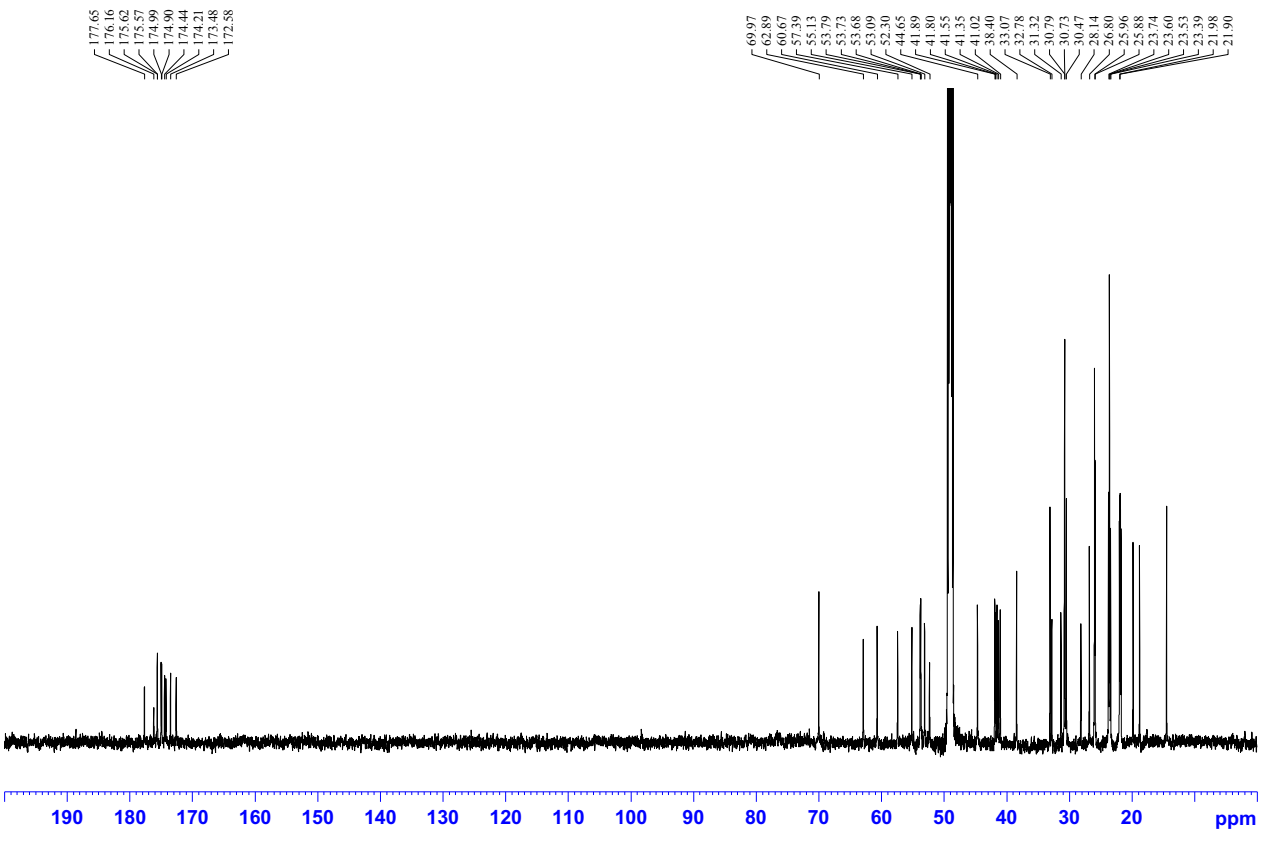

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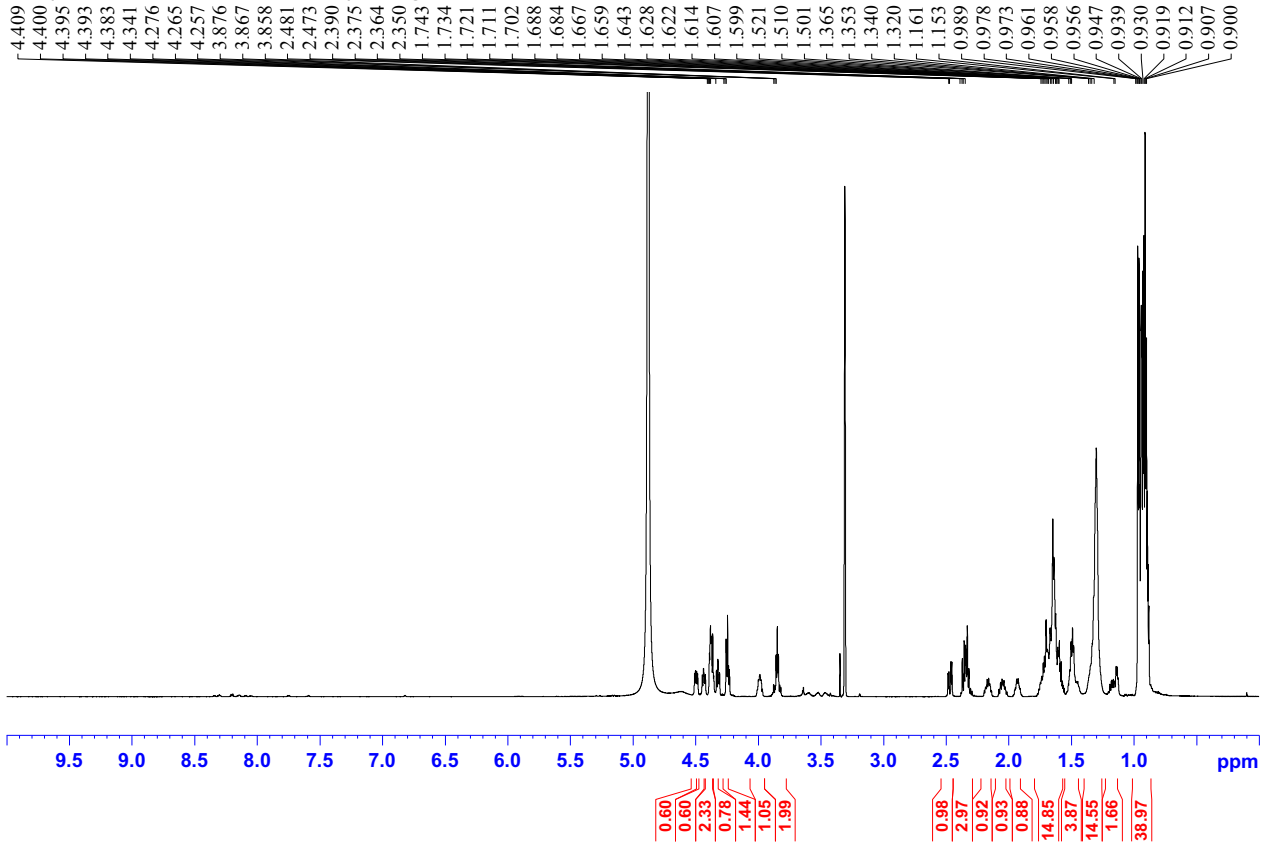
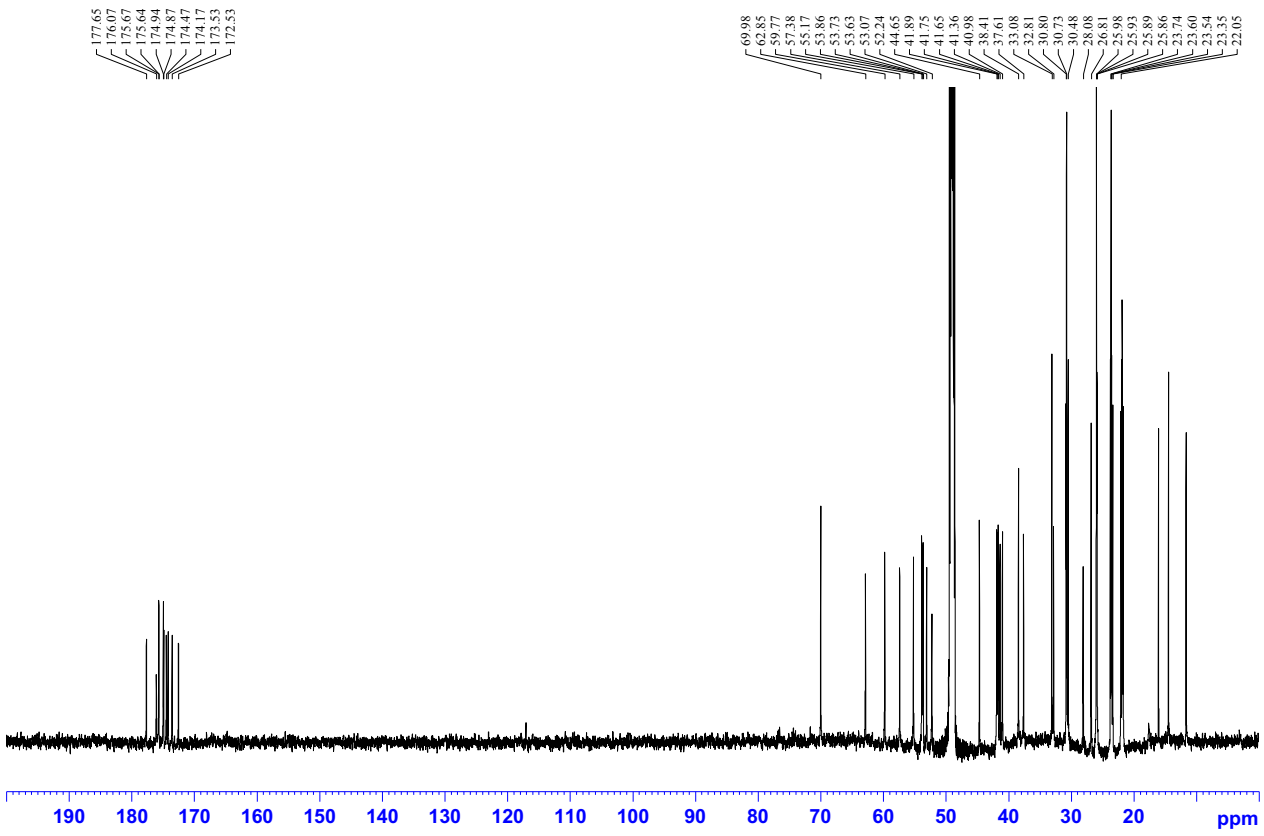


^{13}C (150 MHz, methanol-d_4): Virginiafactin D



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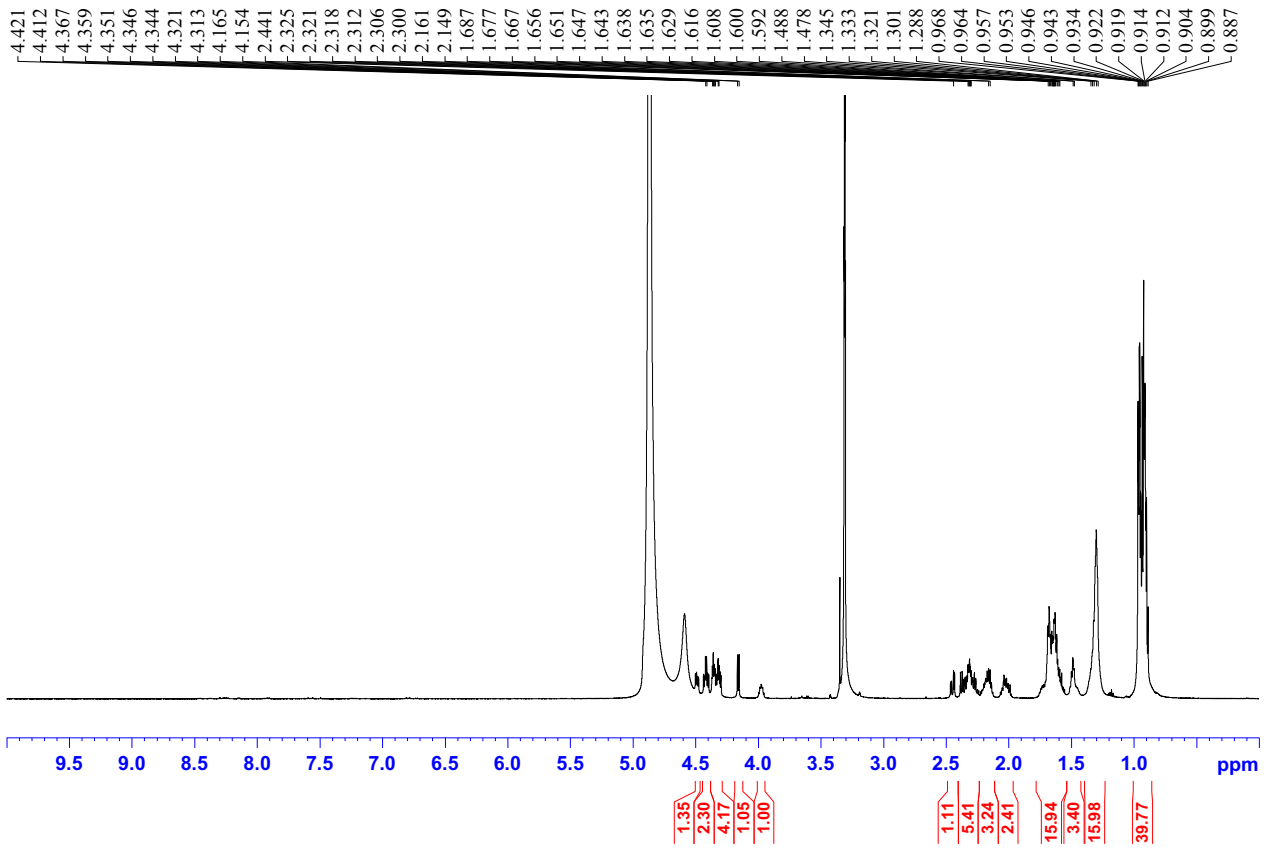

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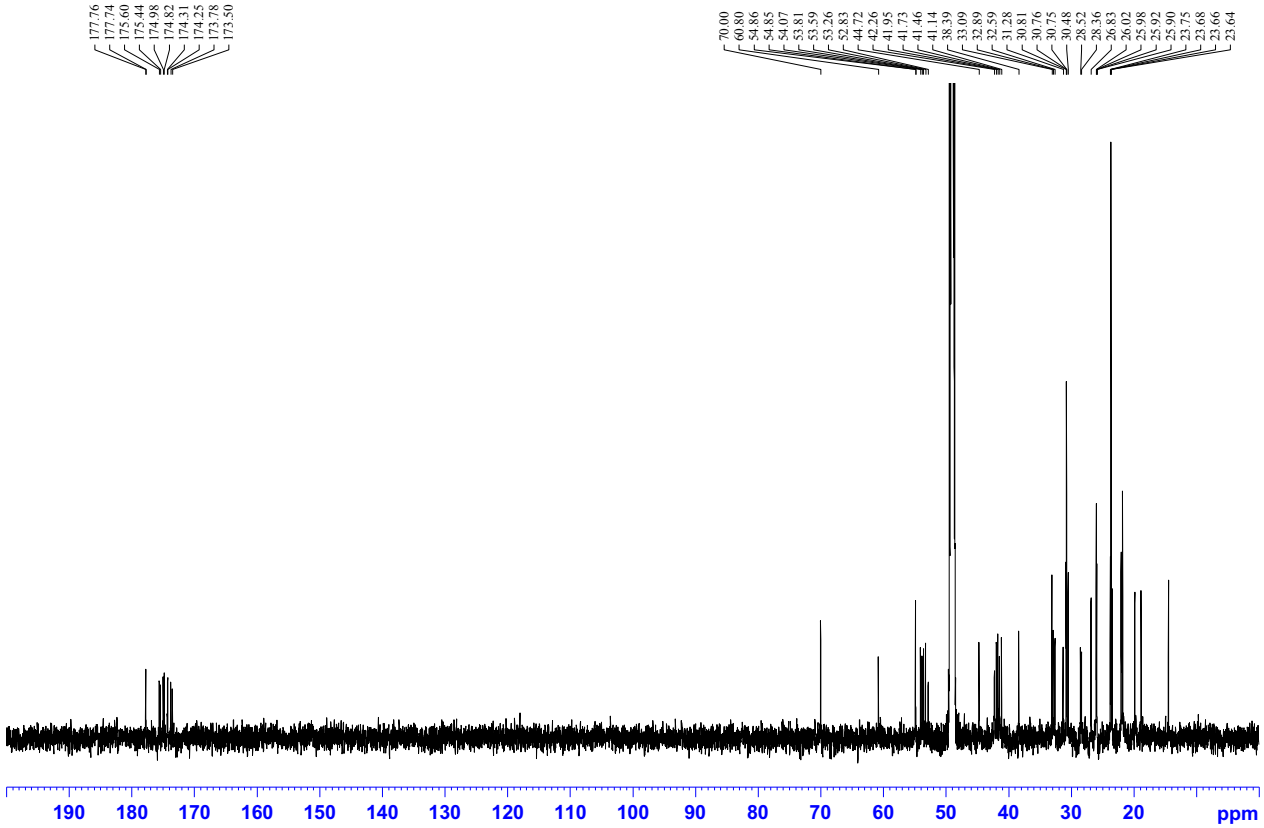

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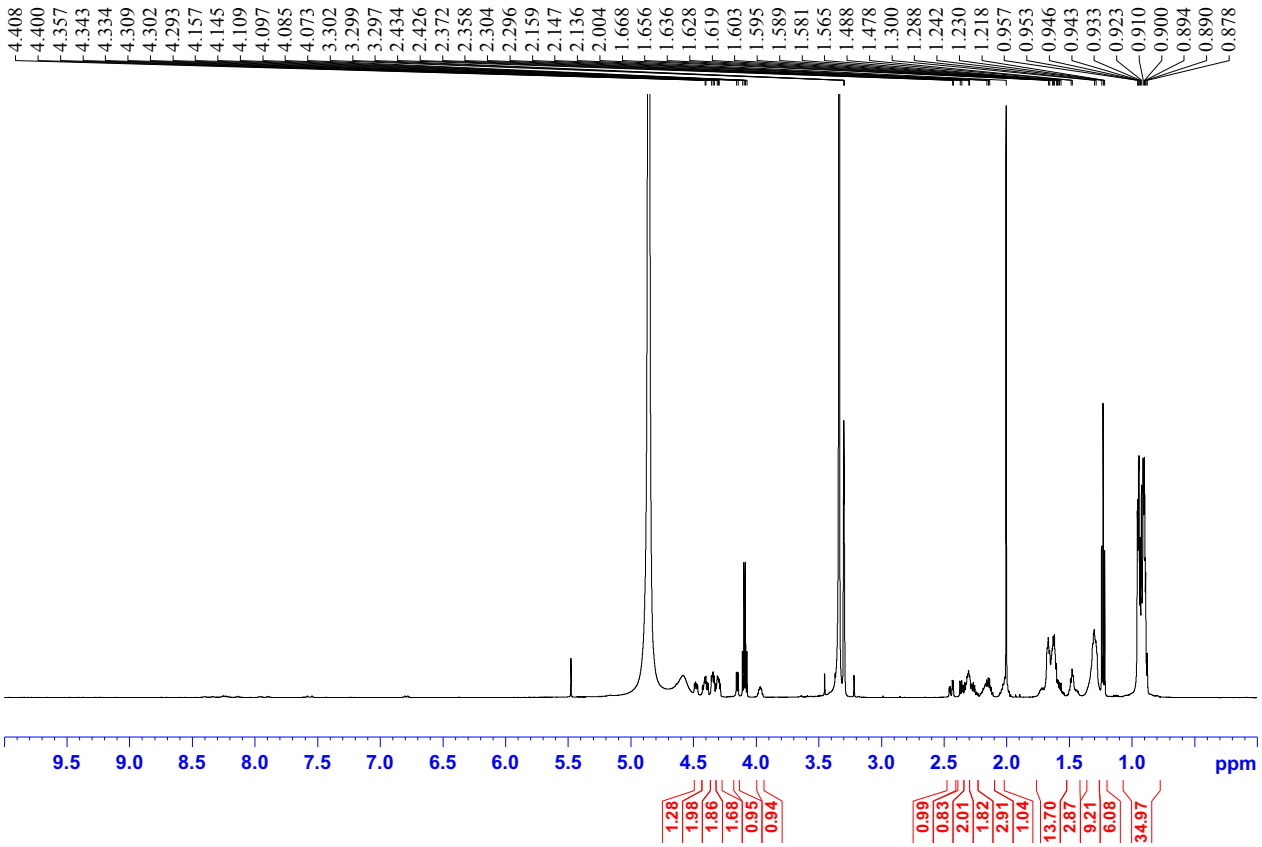
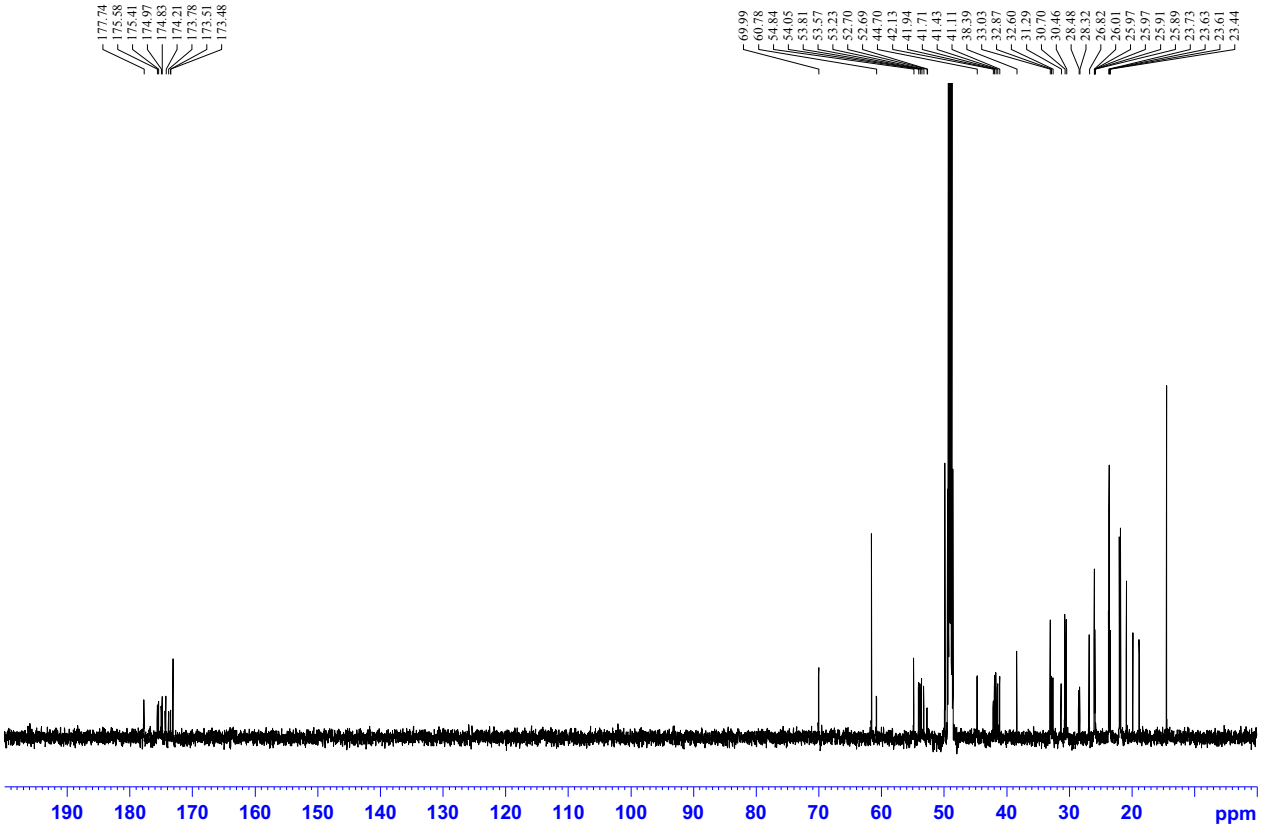
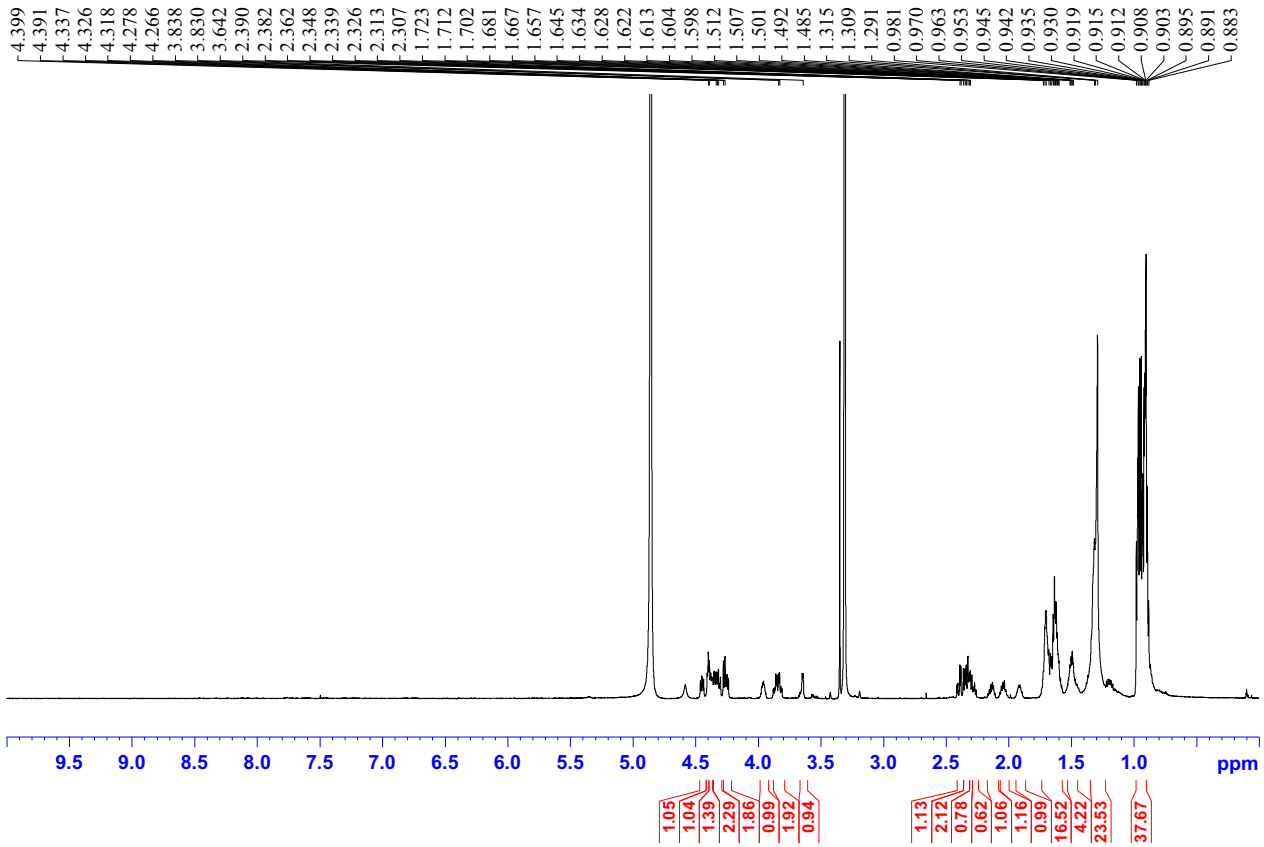


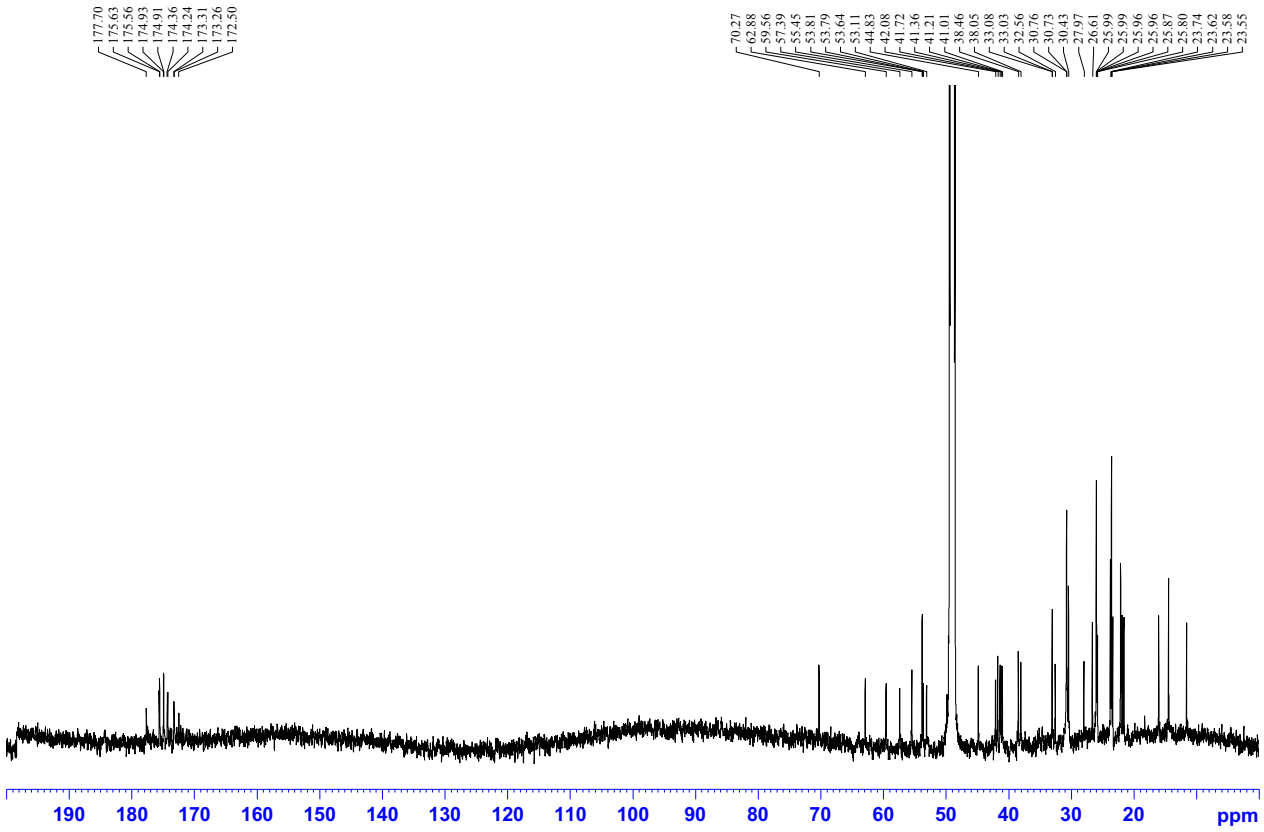
^{13}C (150 MHz, methanol-d_4): Cichofactin B



^{1}H (600 MHz, methanol-d_4): Virginiafactin S1



^{13}C (150 MHz, methanol-d_4): Virginiafactin S1



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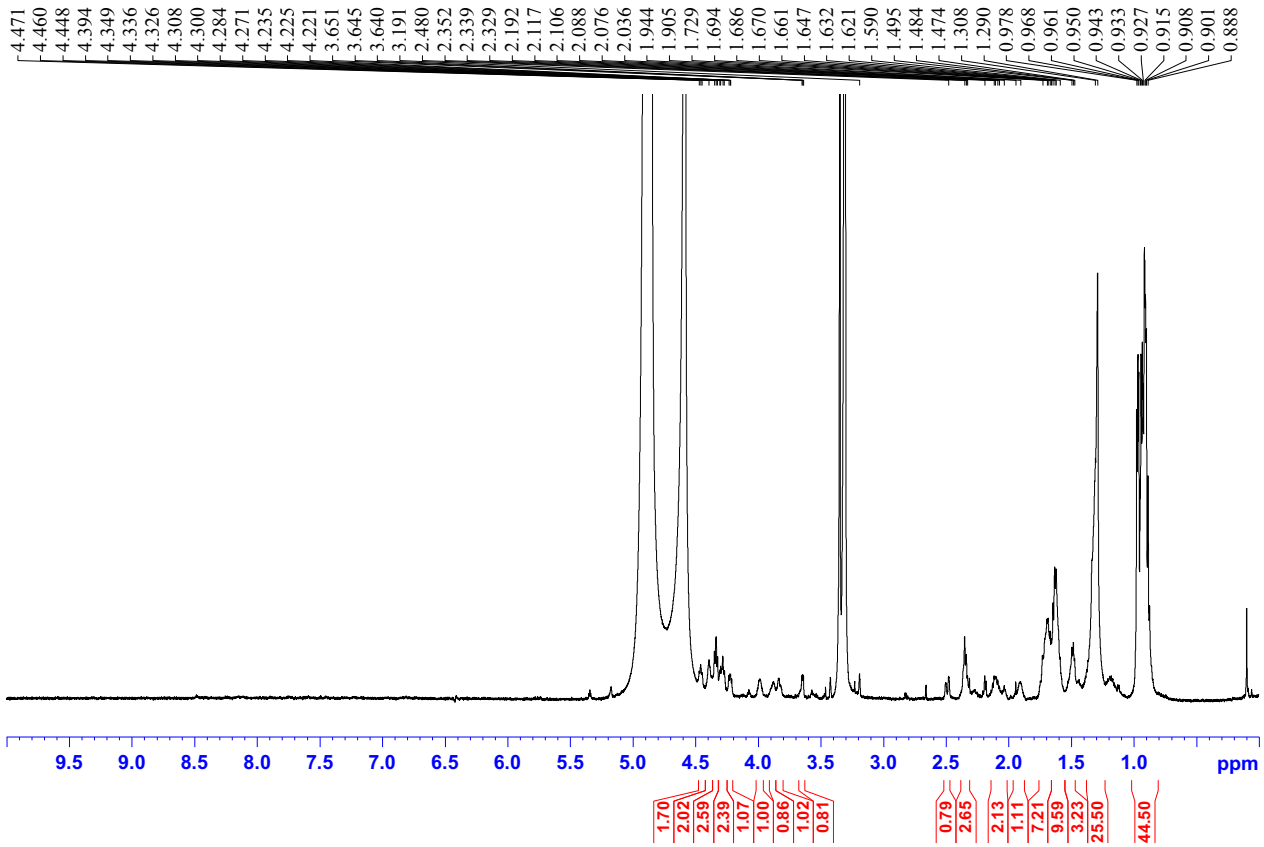

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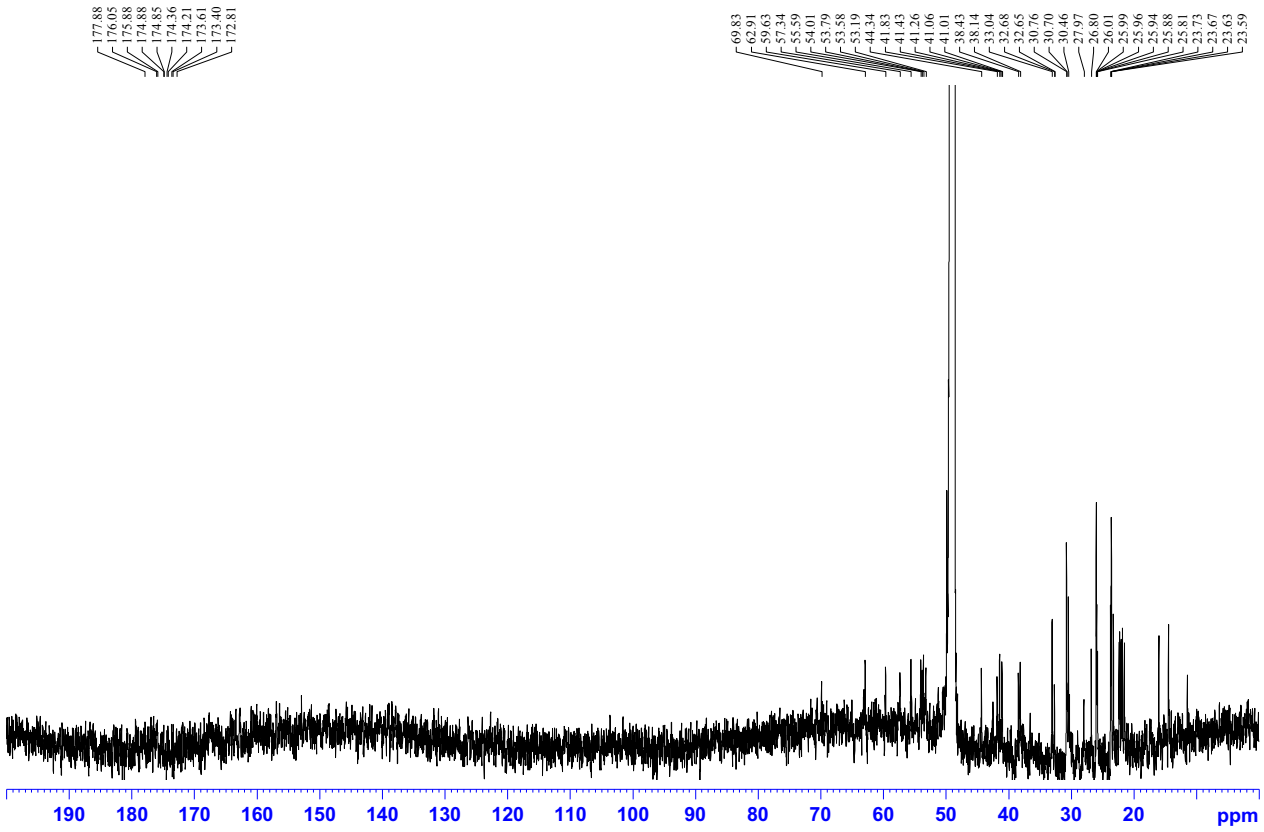
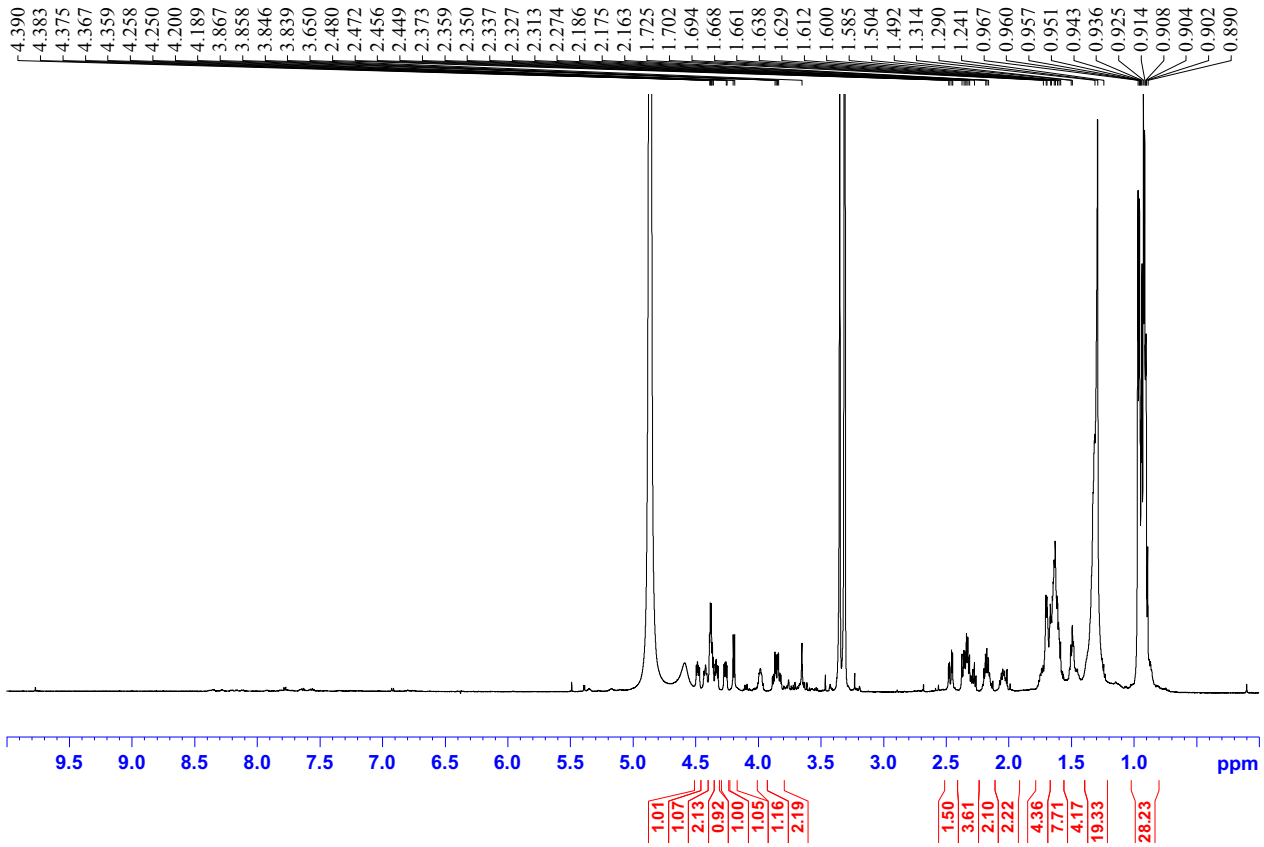
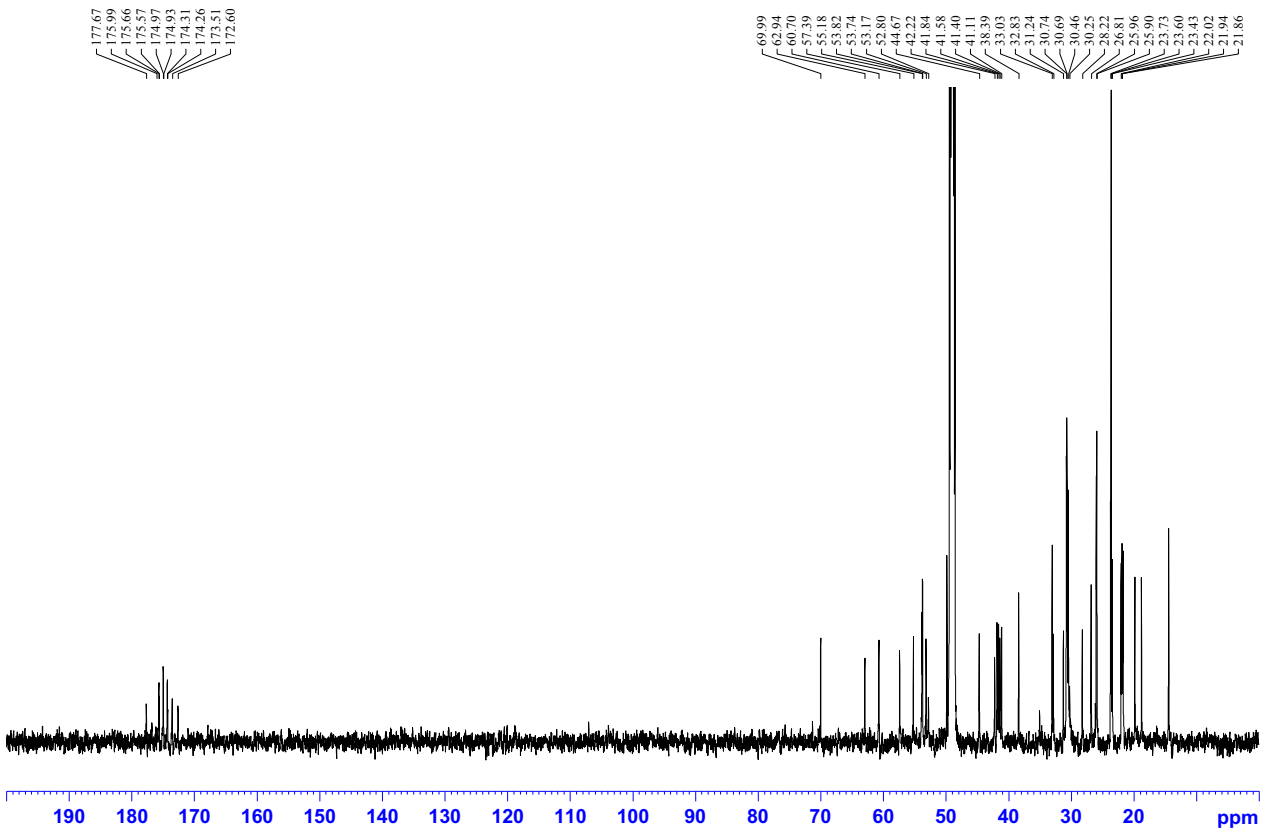


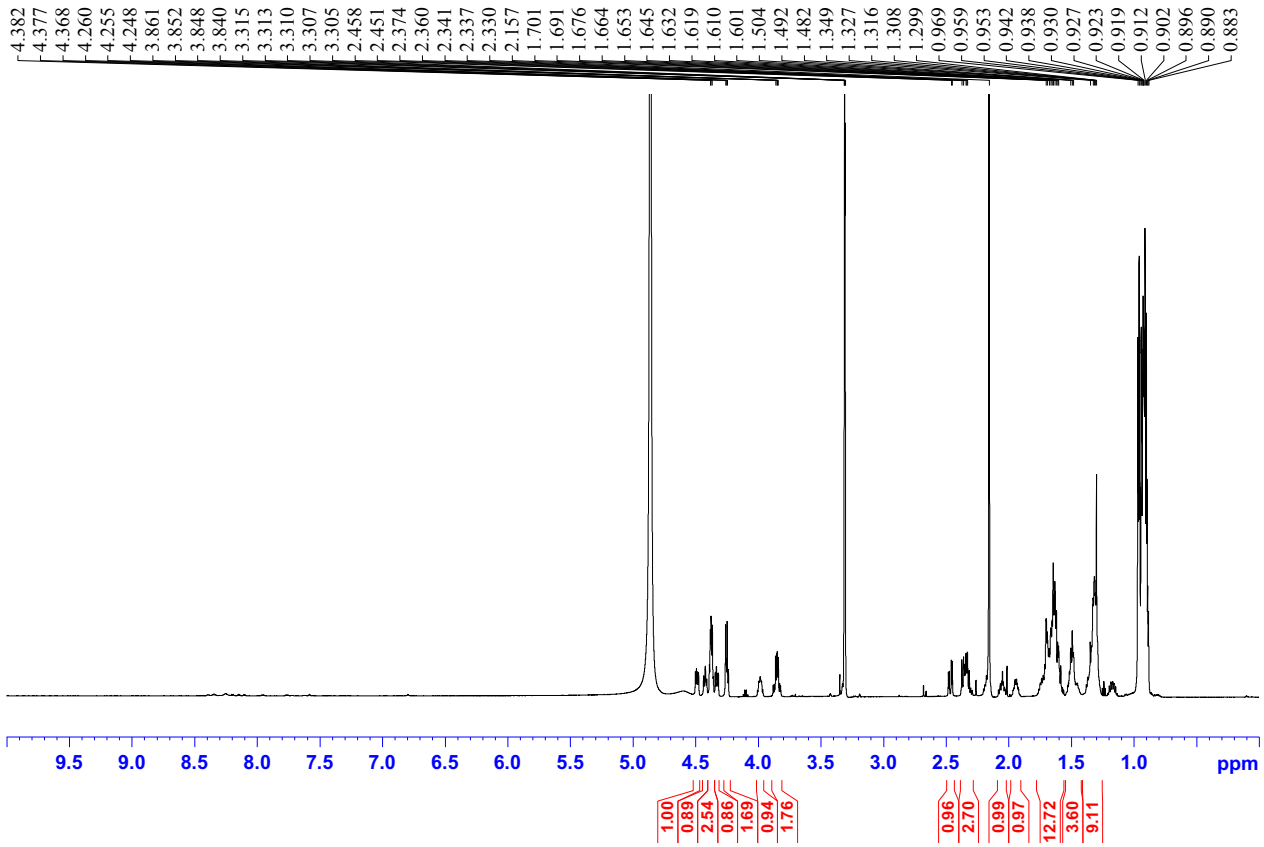
^{1}H (600 MHz, methanol-d_4): Virginiafactin S3



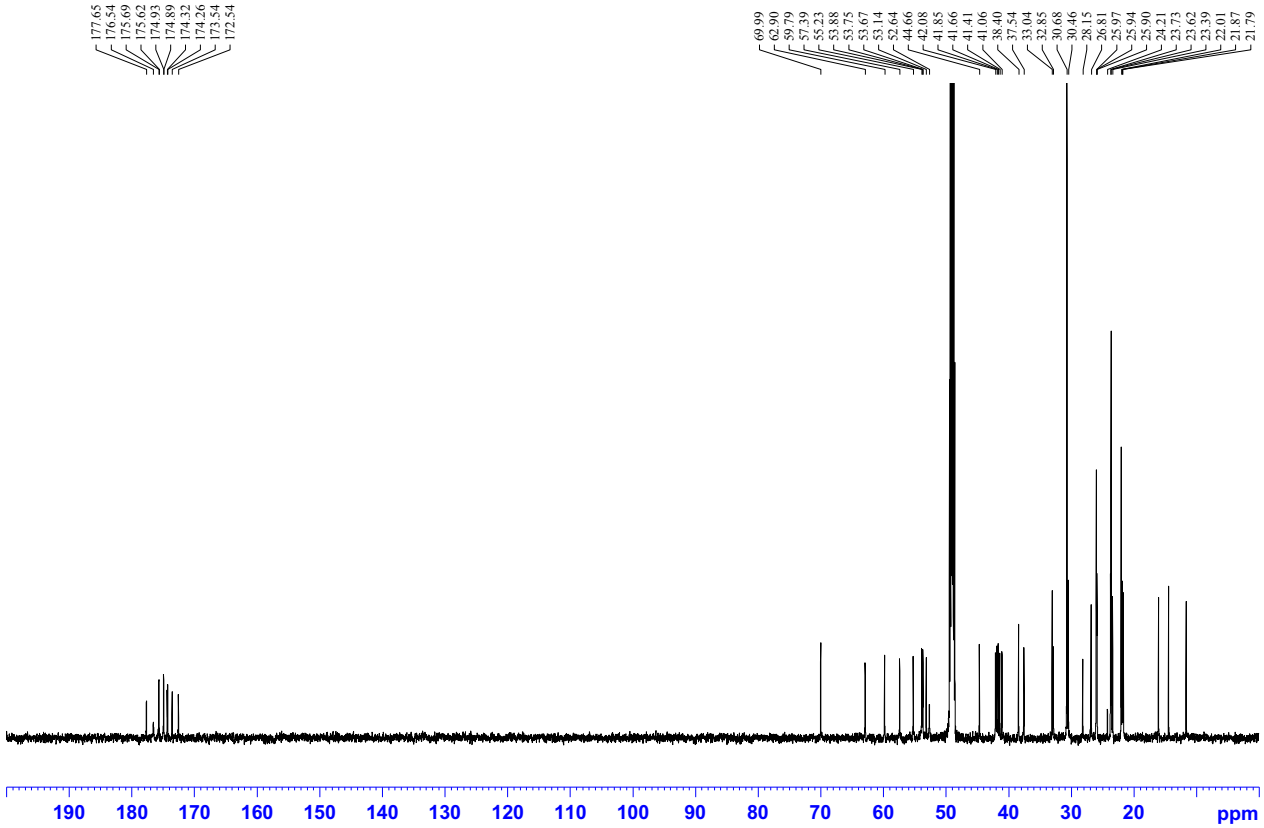
^{13}C (150 MHz, methanol-d_4): Virginiafactin S3



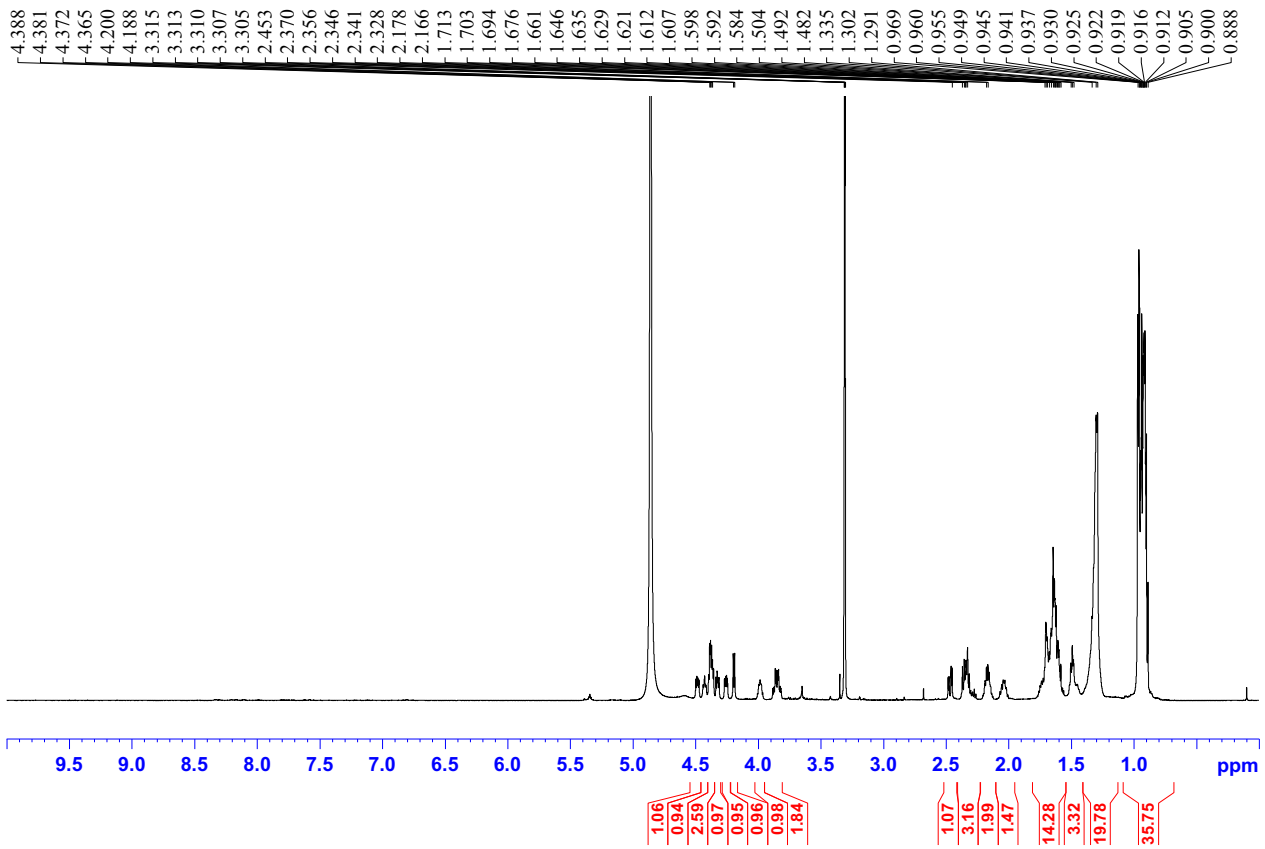
^{1}H (600 MHz, methanol-d_4): Virginiafactin S4



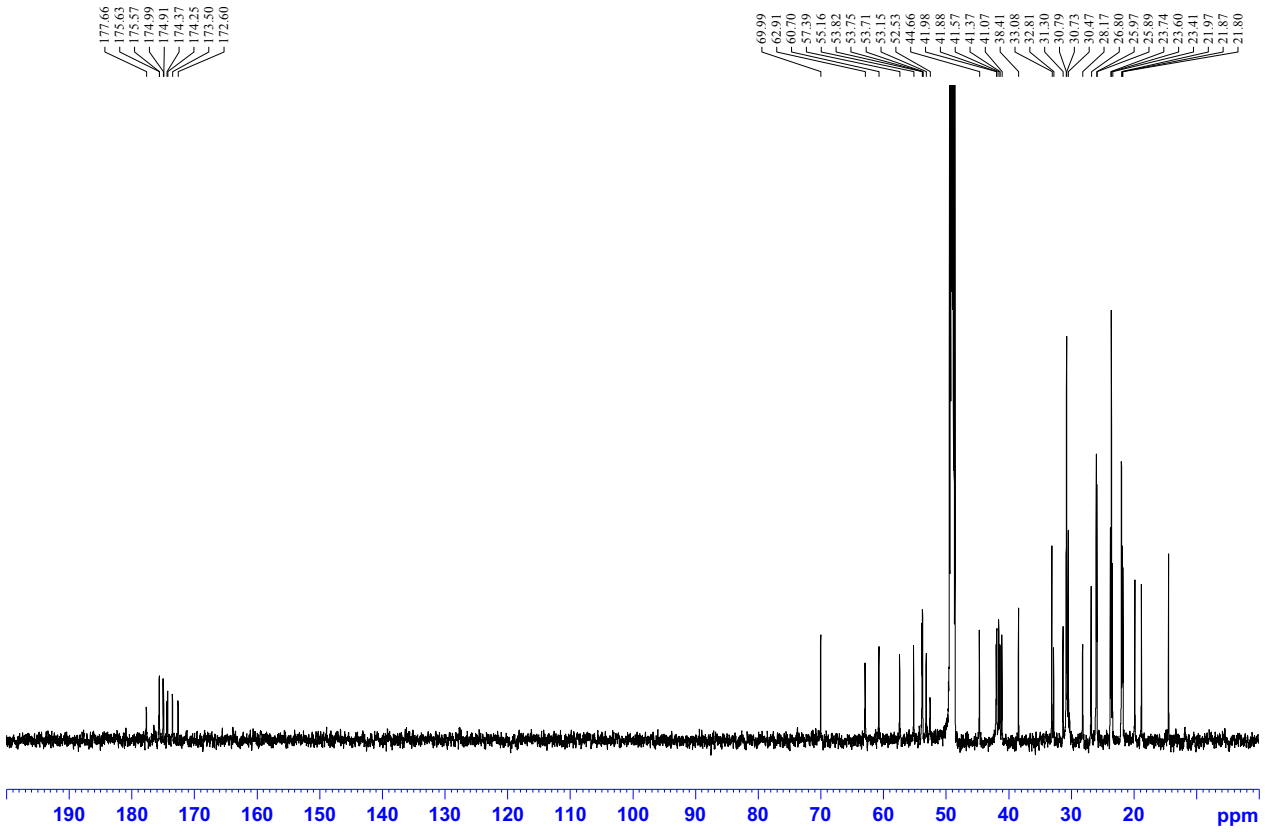
^{13}C (150 MHz, methanol-d_4): Virginiafactin S4



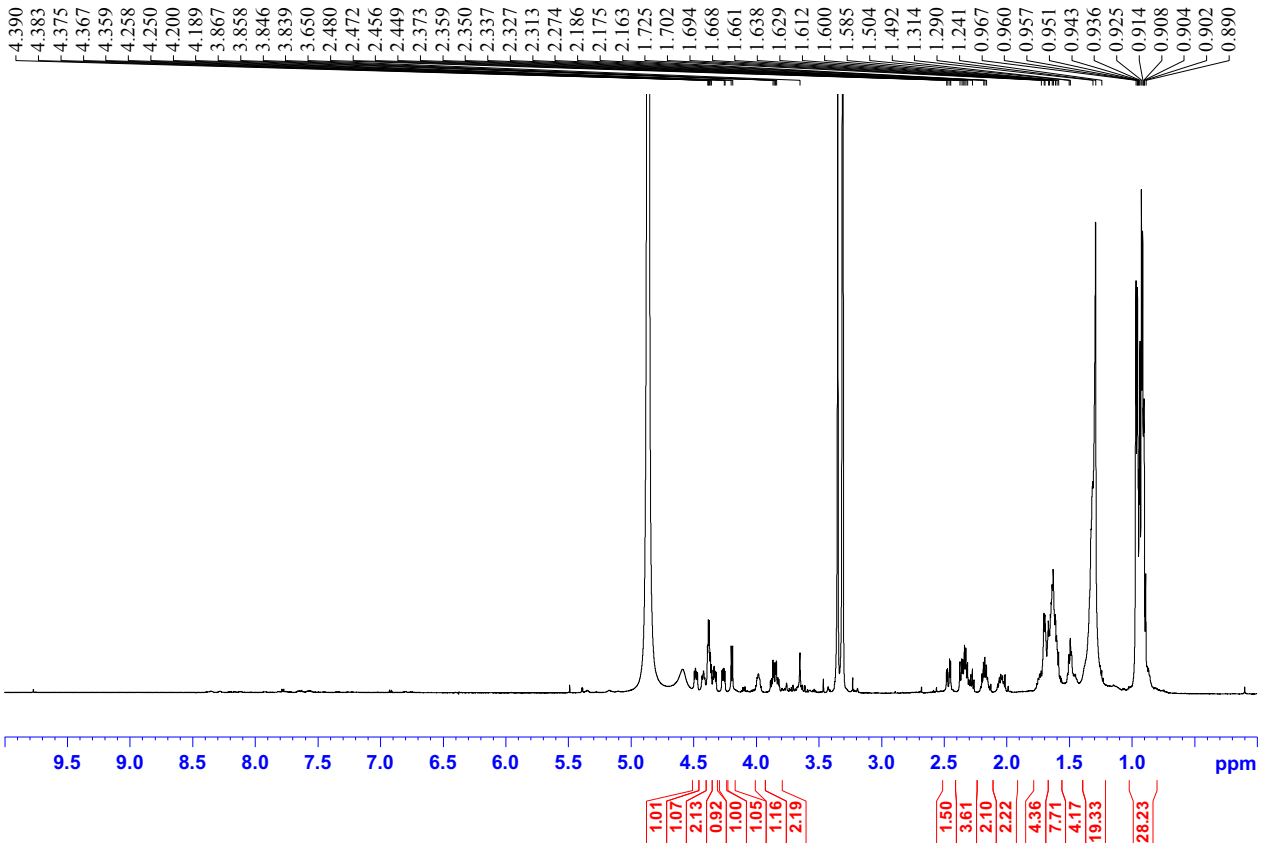
^{1}H (600 MHz, methanol-d_4): Virginiafactin S5



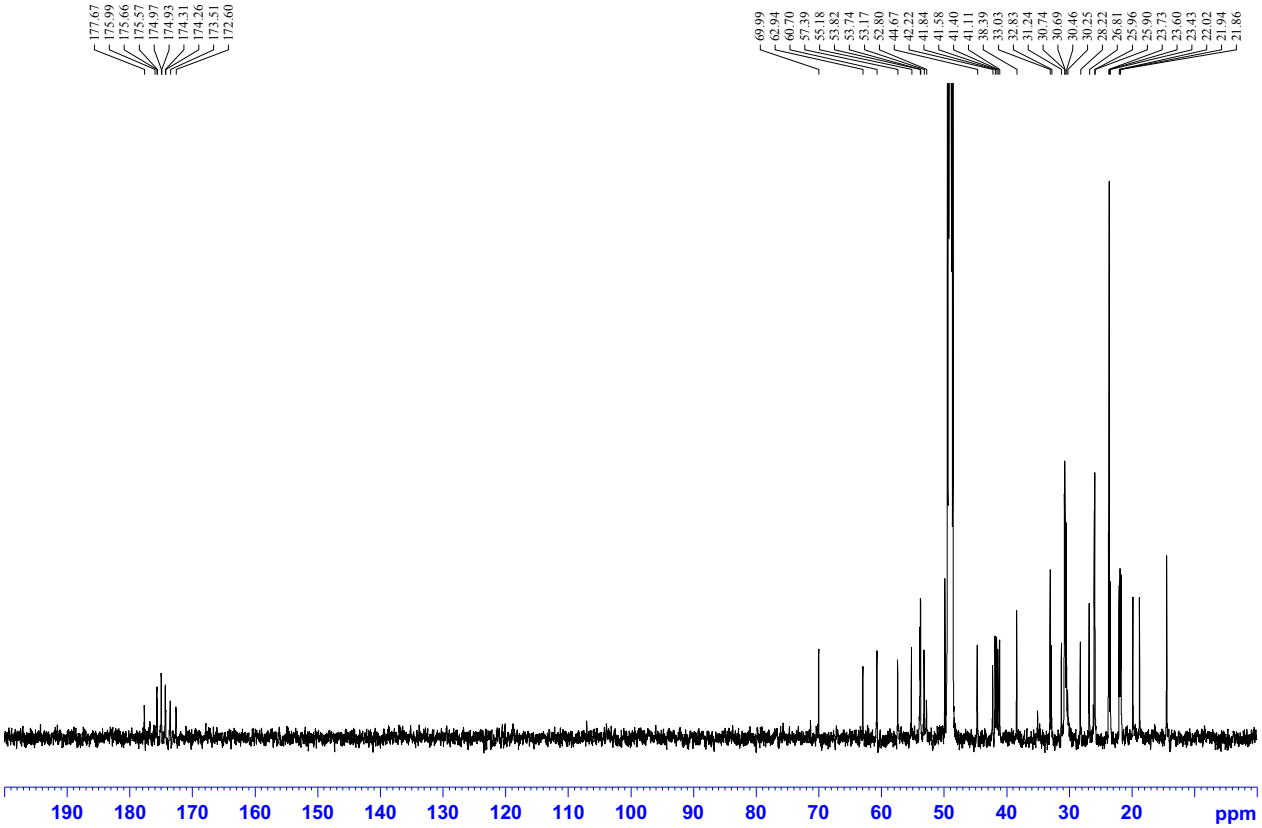
^{13}C (150 MHz, methanol-d_4): Virginiafactin S5



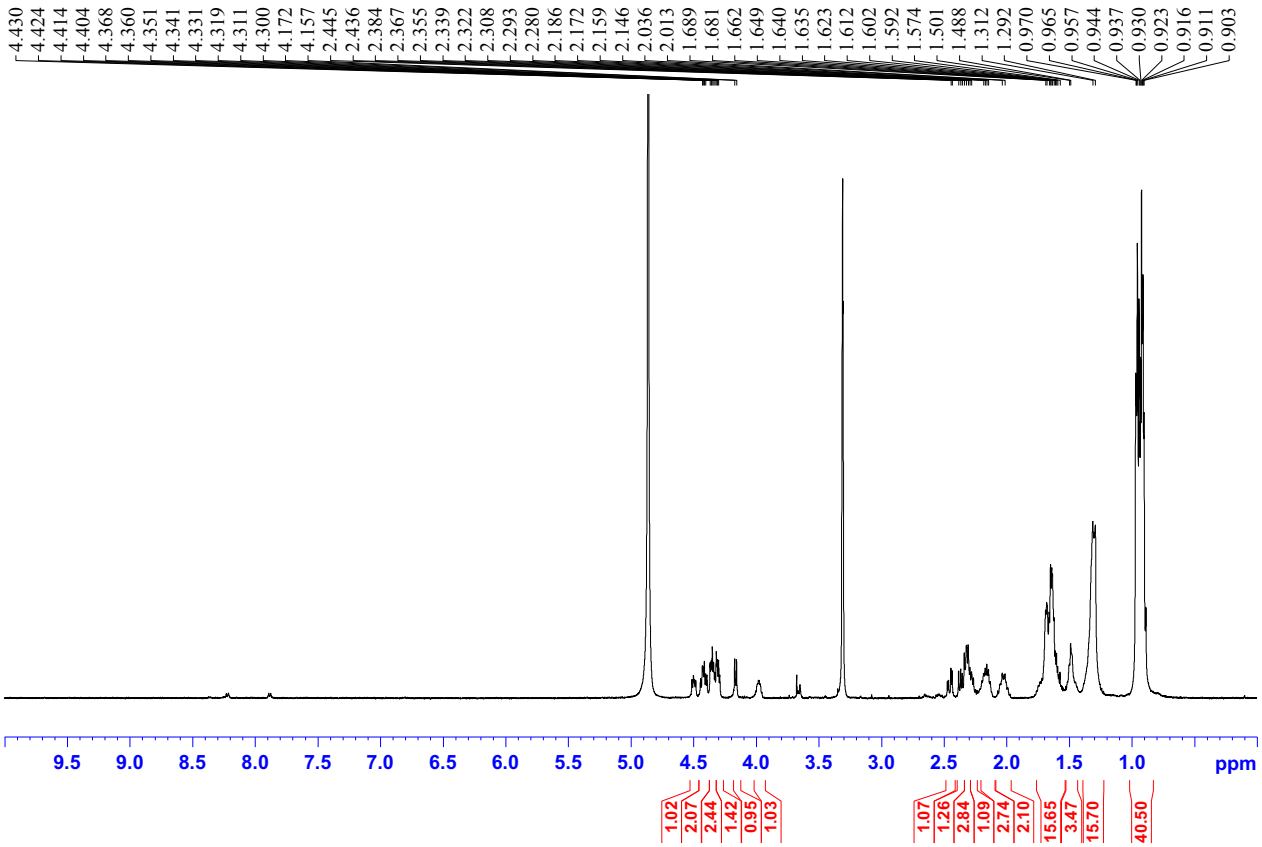
^{1}H (600 MHz, methanol-d_4): Virginiafactin S6



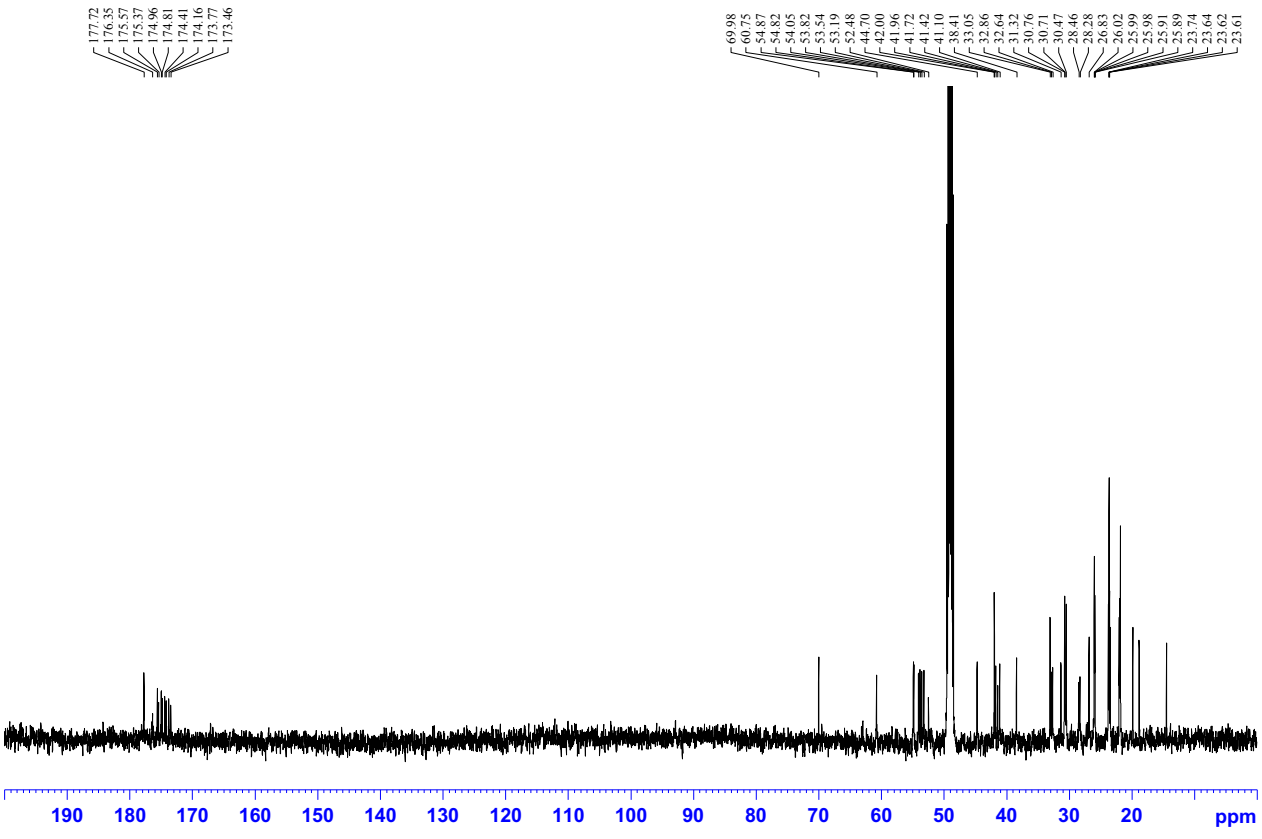
^{13}C (150 MHz, methanol-d_4): Virginiafactin S6



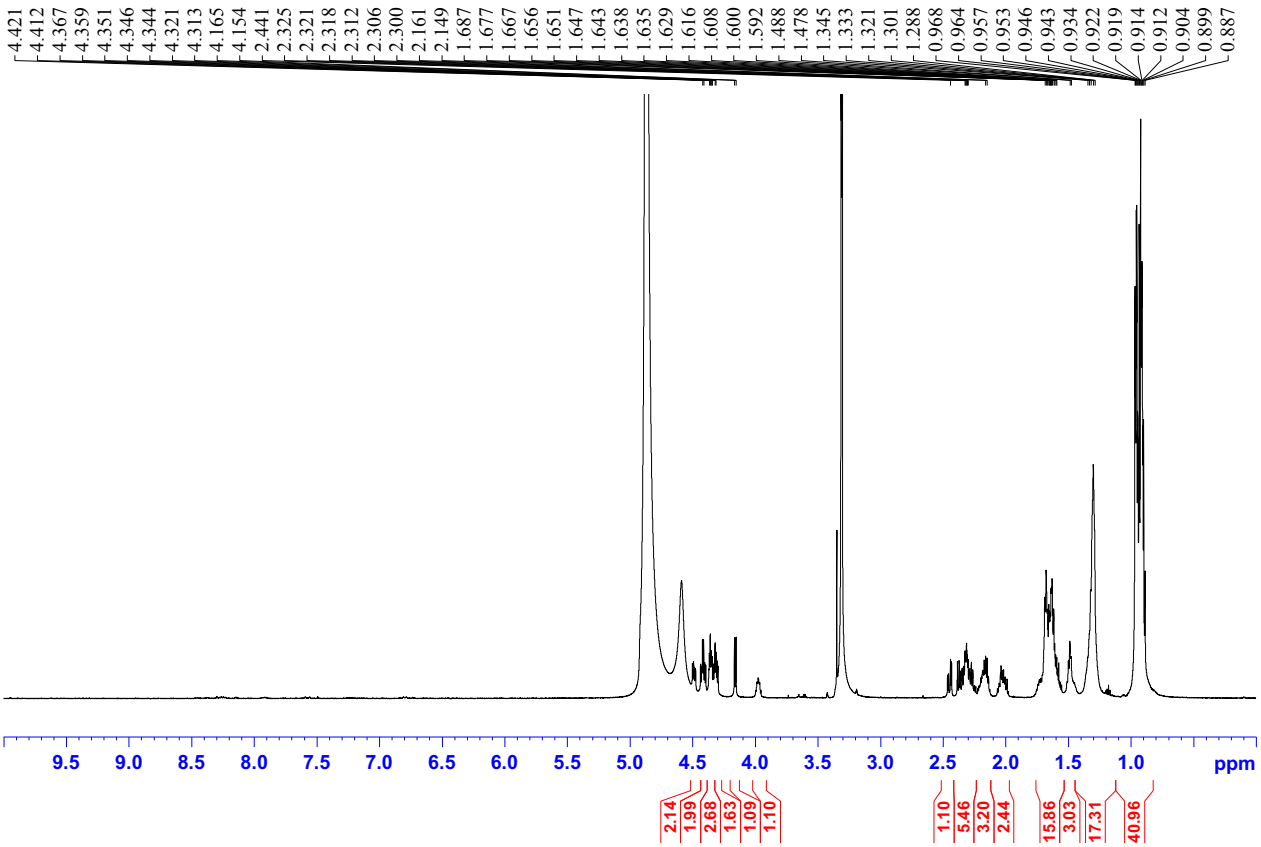
^{1}H (600 MHz, methanol-d_4): Cichofactin S1



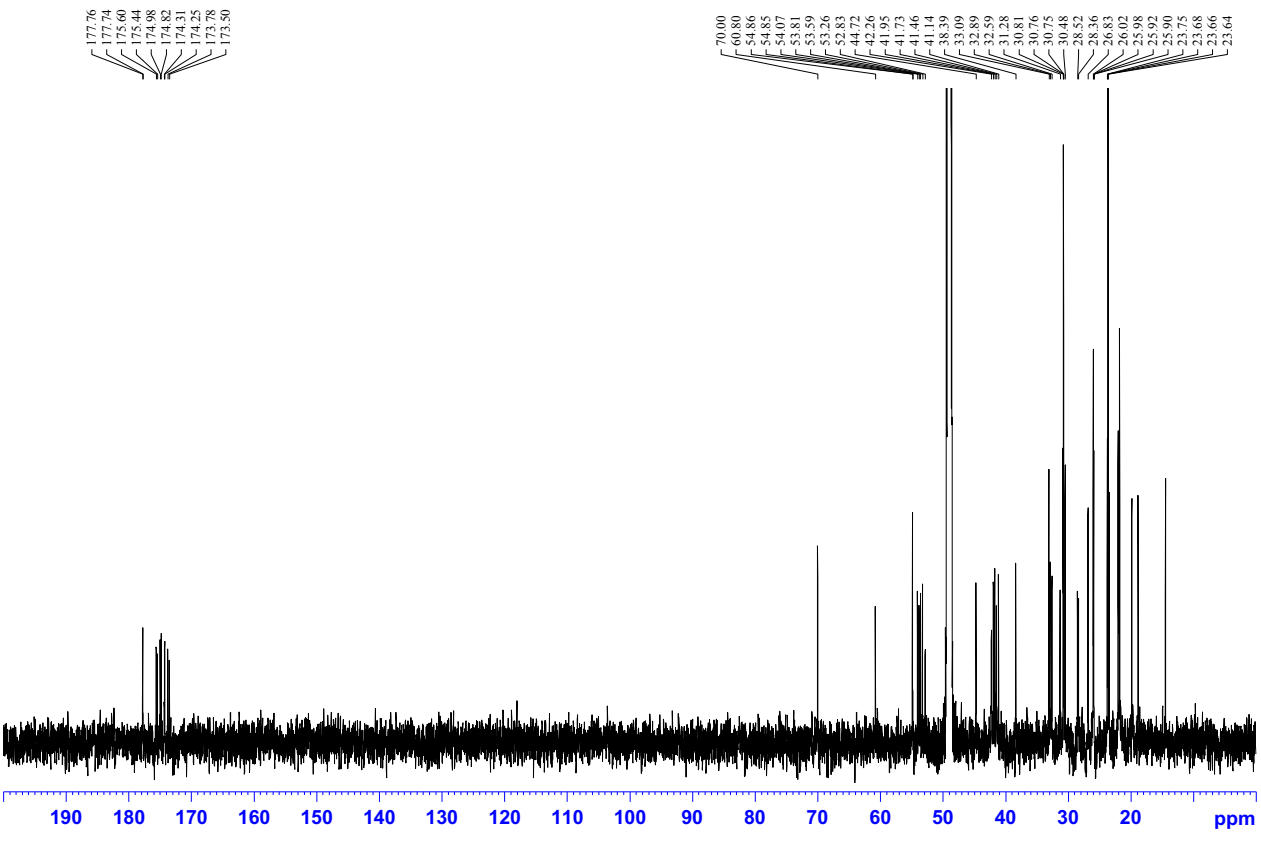
^{13}C (150 MHz, methanol-d_4): Cichofactin S1



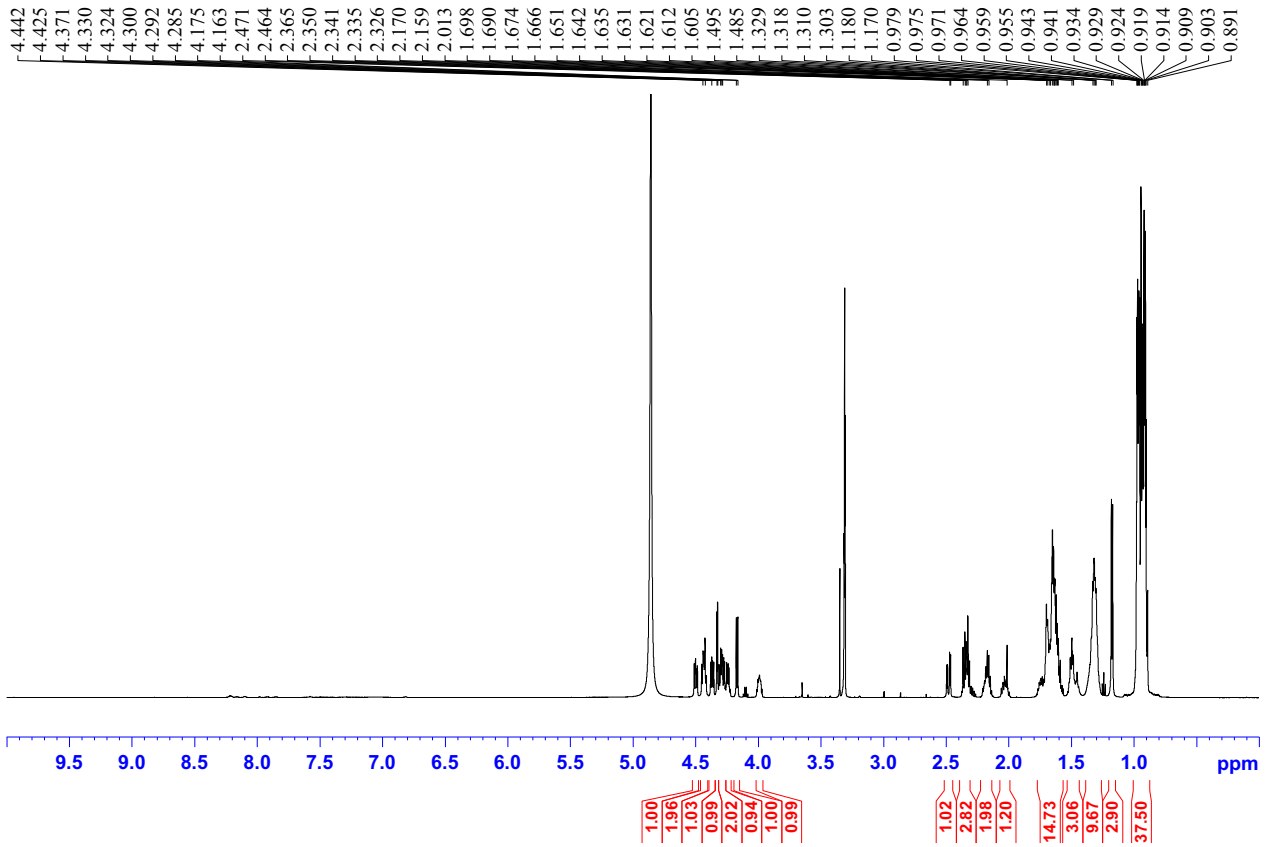
^{1}H (600 MHz, methanol-d_4): Cichofactin S2



^{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