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Supporting Information for

Helper Bacteria Halt and Disarm Mushroom Pathogens by Linearizing Structurally Diverse Cyclolipopeptides

Ron Hermenau, Susann Kugel, Anna J. Komor, and Christian Hertweck*

Corresponding author: Christian Hertweck

Email: Christian.hertweck@leibniz-hki.de

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Materials and Methods

Bacterial strains and culture conditions

The strains used in this study, *Mycetocola tolaasinivorans* DSM15179, *Mycetocola lacteus* DSM15177, and *Pseudomonas tolaasii* DSM19342, were obtained from the Jena Microbial Resource Collection (JMRC); aliquots from glycerol stocks were streaked out on nutrient agar or Kings B-agar plates. These cultures were used to inoculate seed cultures (25 °C, 200 rpm, ON) in 2 mL Kings B medium (per L: 20 g proteose peptone #3 (Difco); 1.5 g K_2HPO_4; 1.5 g $MgSO_4 \cdot 7\ H_2O$; 10 mL glycerol), which were used for inoculation of larger scale cultures.

Pathogenicity assays

Seed cultures (2 mL) were grown overnight in Kings B medium and centrifuged (2 min, 5,000 rpm). The obtained pellet was washed with 0.85 % aqueous NaCl and centrifuged again. OD_{600} of the washed cells was adjusted to 1 by dilution with 0.85 % aqueous NaCl. Equal volumes of bacterial suspensions were then mixed with either 0.85 % NaCl (for single strain inoculation) or a second bacterial suspension (for co-inoculation). Aliquots (15 µL) of the resulting mixtures were used for inoculating potato tuber slices or mushroom cubes placed in petri dishes with a water reservoir. Pictures were taken following incubation at room temperature (20–22 °C) for 41 h (Figures S1 and S2).

Swarming assay

Seed cultures of *M. tolaasinivorans*, *M. lacteus*, and *P. tolaasii* were centrifuged, and the cell pellets were washed twice with 0.9 % NaCl prior to adjusting OD_{600} to 1. For co-cultures equal volumes of culture suspensions were mixed; for axenic cultures cell suspensions were diluted with an equal volume of 0.85 % aqueous NaCl. Aliquots (3 µL) of each culture (or the respective mixed culture) were spotted in the center of Kings B soft agar (0.6 % agar). Plates were sealed and incubated at 25 °C for 24 h. Pictures were taken, and swarming areas were determined using Photoshop CC2018 (Adobe, scale bar photographed together with petri dishes served as reference value; Figures S3–S4).

Liquid cultures and LC-HRMS analyses

Overnight seed cultures were pelleted and washed with 0.85 % aqueous NaCl. The OD_{600} of resuspended cells was adjusted to 5 by dilution with 0.85 % aqueous NaCl. Liquid Kings B medium (30 mL in 100 mL baffled Erlenmeyer flasks) was inoculated with the obtained suspensions in a ratio of 1:100 (300 µL). Cultures were placed on a rotary shaker at 25 °C and 160 rpm. Samples taken after 16 h were dried in a vacuum concentrator and the residue was extracted with an equal volume of methanol. The supernatant obtained after centrifugation was diluted 1:1 with distilled water and analyzed using LC-HRMS. LC-HRMS(/MS) measurements were carried out on a Thermo Fisher Scientific QExactive Orbitrap equipped with an electrospray ion source using an Accucore C18 column (100 × 2.1 mm; 2.6 µm; Thermo Scientific) and an elution gradient [solvent A: H_2O + 0.1 % HCOOH, solvent B: acetonitrile + 0.1 % HCOOH, gradient: 5 % to 98 % B in 10 min, 98 % B for 4 min, flow rate: 0.2 mL min^{-1}, injection: 3 µL].

Imaging mass spectrometry

Conductive indium tin oxide coated glass slides were autoclaved and covered with a thin layer of Kings B agar (1.5 % agar). Overnight seed cultures were pelleted, washed with 0.85 % aqueous NaCl and resuspended in 0.85 % aqueous NaCl to a final OD_{600} of 0.5. Cell suspensions were streaked on the solidified agar and mixed in the middle of the inoculation zone using an inoculation loop. After incubation at 25 °C for 25 h they were placed overnight in a 37 °C incubator to dry the agar. Samples were sprayed with 2.5 mL of universal MALDI matrix (1:1 mixture of 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid) dissolved at 20 mg mL^{-1} in a mixture of acetonitrile, methanol and water (70:25:5), using an ImagePrep device 2.0 (Bruker Daltonics) with 60 consecutive cycles (a 180° rotation of the sample after 30 cycles was performed) of 31 seconds (1 s spraying, 10 s incubation time, and 20 s of active drying). MALDI-TOF-MS data were acquired using an ultrafleXtreme spectrometer from Bruker Daltonics (MS scan range: 500–3,000 *m/z* reflector positive mode) equipped with an ultraviolet laser (smartbeam-II laser of 1,000 Hz, laser power: 30–50 %). Desorption and ionization were performed with the assistance of a matrix as described above. Calibration of the acquisition method in reflector positive mode was performed using Peptide Calibration Standard II (Bruker Daltonics) containing bradykinin1–7, angiotensin II, angiotensin I, substance P, bombesin, ACTH clip1–17, ACTH clip18–39, and somatostatin 28. Spectra were processed with baseline

subtraction in flexAnalysis 3.3 and visualized in SciLS (Bruker). Images were obtained using root mean square normalization.

Isolation of tolaasin

P. tolaasii was cultured in 2 L Kings B medium (1 L Erlenmeyer flasks containing 400 mL medium) for 72 h on a rotary shaker (25 °C, 130 rpm). Cells were removed by centrifugation and the obtained supernatant was filtered through a 0.22 µm membrane yielding a cell free broth. Based on BASSARELLO *et al.* (1) the solution was acidified to pH 3 using concentrated HCl and 148.2 g $CaCl_2$ were added prior to stirring the suspension for 10 min at room temperature. The precipitate formed after 4 h of storage at 4 °C was collected by centrifugation and lyophilized yielding 1.66 g crude extract which was dissolved in 50 mL methanol. The supernatant obtained after centrifugation was evaporated to dryness and suspended in 12.5 mL methanol. Solids were removed by centrifugation and the supernatant was fractionated using an Agilent 1260 Infinity HPLC with an Agilent Eclipse XDB-C8 column (9.4 × 250 mm; 5 µm) and an elution gradient (A: H_2O + 0.1 % TFA; B: acetonitrile; 4 mL min^{-1}; 40 % B to 52 % B in 25 min; 200 µL injections). Fractions were collected automatically based on peak slope and fractions containing the main peak were pooled and lyophilized yielding 80 mg of a white powder. LC-MS analysis showed that the main component was tolaasin I with minor impurities of other tolaasins.

Isolation of pseudodesmin A and elucidation of absolute configuration of Leu-5

P. tolaasii was cultured in 2 L Kings B medium (1 L Erlenmeyer flasks containing 400 mL medium) for 72 h on a rotary shaker (25 °C, 130 rpm). Cells were harvested by centrifugation and the obtained pellet was extracted three times with 250 mL acetone and the solvent was evaporated to dryness yielding 3.168 g of crude extract. The residue was suspended in 200 mL 60 % methanol (in water) and applied to an open column containing LiChroPrep C18 (equilibrated in the same solvent). Elution was performed stepwise with 80 % aqueous methanol and then with pure methanol. The pure methanol fraction was evaporated to dryness and the resulting residue (2.013 g) was suspended in 50 mL 1 M HCl and extracted four times with 50 mL ethyl acetate. The organic phases were combined, dried over Na_2SO_4 and evaporated to dryness, yielding 623 mg extract. Final purification was performed using preparative HPLC (Shimadzu LC-8A pumps controlled by CBM-20A and equipped with SPD-M20A DAD) with a Phenomenex Luna 10u C18(2) 100A column (250 × 21.2 mm; with guard column) and an isocratic flow of 83 % aqueous acetonitrile (elution time: 9.9–14 min) yielding 297 mg pseudodesmin A as a white lyophilized powder.

The obtained material was submitted to 2D NMR experiments and HSQC signals were compared with data published by GEUDENS *et al.* (2). A substantial difference was found in the ^{1}H and ^{13}C shifts in α-position of Leu5 dependent on the absolute configuration. While for L-Leu5 in viscosinamide the shifts are δ_H = 3.66 ppm and δ_C = 53.90 ppm, the respective values for pseudodesmin A and D-Leu5 were reported to be δ_H = 3.97 ppm and δ_C = 55.74 ppm (3). For the compound isolated in this study, we determined the shifts to be δ_H = 3.98 ppm and δ_C = 55.79 ppm. The absolute configuration of Leu-5 was therefore deduced to be D and the peptide was identified as pseudodesmin A (Figures S6 and S7).

NMR spectra were measured on Bruker Avance DRX 600 MHz spectrometers with cryo probe in acetonitrile-d_3. Spectra were referenced to the residual solvent peak.

Hydrolysis of cyclic peptides

The isolated tolaasin mixture (0.41 mg, 0.206 µmol), and pseudodesmin A (0.61 mg, 0.542 µmol) were individually dissolved in methanol (200 µL), and 103 µL (1.03 µmol, 5 eq) or 271 µL (2.71 µmol, 5 eq) 0.01 M aq. LiOH were added. The solutions were stirred at room temperature for 3 h. 100 µL samples of the reaction mixtures were quenched with 10 µL 1 M HCl and further diluted with 50 µL methanol prior to analysis via LC-HRMS/MS (Figures S8–S13).

Generation of deletion mutants

Plasmid construction was planned based on a method applied by KLAPPER *et al.* (4) using NEBuilder® (New England Biolabs, https://nebuilder.neb.com). Homologous regions (750 bp) up- and downstream of the area to delete were amplified using Q5® High-Fidelity polymerase (Thermo Scientific) and primer pairs Tol-LA-fw/Tol-LA-rev and Tol-RA-fw/Tol-RA-rev for tolA, and Pse-LA-fw/Pse-LA-rev and Pse-RA-fw/Pse-RA-rev for *pseB*. Amplicons were purified via gel electrophoresis and extracted using the illustra™ GFX™ PCR DNA and gel band purification kit (GE Healthcare). Restriction digest of plasmid pEXG2 was carried out using EcoRI-HF and HindIII-HF (New England Biolabs) in CutSmart®

buffer (New England Biolabs). Construction of plasmids pEXG2-Tol and pEXG2-Pse was carried out using NEBuilder® HiFi DNA assembly master mix (New England Biolabs). *E. coli* TOP10 electro competent cells were transformed with the obtained plasmids and resulting clones were screened via colony PCR using DreamTaq Green PCR master mix (2 x) (Thermo Scientific). Positive clones were used for plasmid isolation using the Monarch® plasmid miniprep kit (New England Biolabs). Plasmids were sequenced to check for amplification errors (Eurofins Genomics) and further used to transform *E. coli* S17-1 λpir chemical competent cells. The obtained clones were used for biparental mating: overnight cultures of donor (*E. coli* S17-1 λpir pEXG2-Tol or *E. coli* S17-1 λpir pEXG2-Pse) and acceptor (*P. tolaasii*) were mixed in two different ratios (1:1 and 9:1) and washed with deionized sterile water. A sample (30 µL) of the resulting suspension was spotted on dried LB agar and incubated at 30 °C overnight. Spots were suspended in 500 µL LB medium and 200 µL of a 1:10 dilution were plated on LB agar plates (20 µg mL^{-1} gentamicin, 100 µg mL^{-1} ampicillin) and incubated for two days at 30 °C. Colonies were picked and used to inoculate fresh LB medium (20 µg mL^{-1} gentamicin, 100 µg mL^{-1} ampicillin). After washing the cells with antibiotic free LB medium, they were plated on 5 % sucrose LB agar plates (without NaCl). Obtained potential double crossover mutants were screened via colony PCR. Successful deletion of the targeted areas was further checked by sequencing from one homologous region to the other, proving the generation of marker-free in-frame deletion mutants (Figure S14 and S15).

Bioactivity-guided fractionation

A mixture of *M. tolaasinivorans* and *M. lacteus* cells was cultivated for 48 h at 25 °C in a 400 mL to 4 L scale in Kings B medium. Cells were harvested by centrifugation and suspended in 2 mL lysis buffer (30 mM Tris pH 7.5, 100 mM NaCl, 5 % glycerol) per 100 mL culture. The obtained suspension was lysed using a sonotrode, and cellular debris was removed by centrifugation. Ground $(NH_4)_2SO_4$ was added stepwise to the stirred supernatant (saturation concentrations calculated using an online tool provided by EnCor Biotechnology; http://www.encorbio.com/protocols/AM-SO4.htm). Precipitated proteins were collected by centrifugation and redissolved in assay buffer (1 mL g^{-1} pellet, 50 mM Na_2HPO_4, 150 mM NaCl, 5 % glycerol; pH 7.5) and used for bioactivity assays. One volume of a solution containing tolaasin (1 mg mL^{-1} in 50 mM Na_2HPO_4, 150 mM NaCl, pH 6.5) was mixed with two volumes of the respective protein fraction. Heat-denatured fractions and protein-free buffer were used as negative controls. Assays were stopped with one reaction volume methanol prior to analysis via LC-MS. Active fractions (20–50 % saturation with $(NH_4)_2SO_4$) were pooled and dialyzed with assay buffer (ratio 1:100) using a standard regenerated cellulose dialysis membrane (MWCO 3,500 Da; Spectra/Por®) for 16 h at 4 °C. Supernatant obtained after centrifugation of the resulting suspension was further fractionated by anion exchange chromatography using an ÄKTA pure25 FPLC equipped with a HiTrap Q HP 5 mL or a 20 mL HiPrep Q FF 16/10 column (GE Healthcare). In each case, the dialyzed solution was filtered (1.22 µm pore size), diluted 1:20 with 20 mM Tris pH 7.5 and loaded to the equilibrated column. An optimized gradient of the elution buffer (20 mM Tris pH 7.5, 1 M NaCl) was applied. Eluted fractions were concentrated using Amicon Ultra-15 centrifugal filter units (MWCO 3,000 Da; Merck KGaA) and dialyzed with assay buffer at 22 °C prior to performing activity assays. Active fractions were pooled and further separated based on surface hydrophobicity. Therefore, the concentrated protein solutions were added to 40 mL of a buffer solution (50 mM Na_2HPO_4, 1 M $(NH_4)_2SO_4$, pH 7.0). The supernatant obtained after centrifugation was loaded onto a HiTrap Butyl HP 5 mL column and an optimized gradient with elution buffer (50 mM Na_2HPO_4, pH 7.0) was used to elute proteins, monitored by a UV detector (220/280 nm). Fractions containing proteins were concentrated using Amicon Ultra-15 centrifugation filter units (MWCO 3,000 Da; Merck, KGaA) and Nanosep® centrifugal devices (MWCO 10,000 Da, Pall Corporation) and were subsequently used in activity assays. Active fractions were further analyzed by SDS-PAGE and gel bands were excised for tryptic digestion using a commercially available Trypsin/Lys-C mix (Promega). MALDI-TOF-MS/MS measurements were carried out and identified peptides were mapped to proteins predicted *in silico* based on the genomes of both species. Whole protein content of active fractions was analyzed using LC-MS. Samples were run into an SDS gel for approx. 0.5 cm and the band containing all proteins was excised and used for tryptic digestion applying a protocol based on SHEVCHENKO *et al.* (5) using commercially available Trypsin/Lys-C mix (Promega).

LC-MS/MS analysis of tryptic peptides was performed on an Ultimate 3000 RSLC nano instrument coupled to a QExactive Plus or a QExactive HF mass spectrometer (Thermo Fisher Scientific). Tryptic peptides were trapped for 4 min on an Acclaim Pep Map 100 column (2 cm × 75 µm, 3 µm) at a flow-rate of 5 µL min^{-1}. The peptides were then separated on an Acclaim Pep Map column (15 cm × 75 µm, 2 µm; 25 cm × 75 µm, 2 µm; or 50 cm × 75 µm, 2 µm) using different binary gradients (A: H_2O + 0.1 %

HCOOH; B: 0.1 % HCOOH in 90:10 (ACN/H_2O): 0–4 min at 4 % B, 10 min at 7 % B, 40 min at 10 % B, 60 min at 15 % B, 80 min at 25 % B, 90 min at 30 % B, 110 min at 50 % B, 115 min at 60 % B, 120–125 min at 96 % B, 125.1–150 min at 4 % B; or: 0–4 min at 4 % B, 8 min at 6 % B, 40 min at 11 % B, 60 min at 19 % B, 75 min at 28 % B, 80 min at 34 % B, 87 min at 41 % B, 91 min at 50 % B, 93 min at 60 % B, 95–99 min at 96 % B, 100–120 min at 4 % B; or: 0–4 min at 4 % B, 10 min at 6 % B, 60 min at 12 % B, 100 min at 20 % B, 120 min at 25 % B, 140 min at 35 % B, 160 min at 60 % B, 170–175 min at 96 % B, 175.1–200 min at 4 % B; or: 0–4 min at 4 % B, 5 min at 6 % B, 25 min at 8 % B, 65 min at 20 % B, 80 min at 30 % B, 90 min at 50 % B, 95–100 min at 96 % B, 100.1–120 min at 4 % B. Positively charged ions were generated by a Nanospray Flex Ion Source (Thermo Fisher Scientific) using a stainless steel emitter with 2.2 kV spray voltage. Ions were measured in data-dependent MS^2 Top10 (QExactive Plus) or Top15 (QExactive HF) mode: Precursor ions were scanned at m/z 300–1,500; R: 70,000 FWHM (QExactive Plus) or 120,000 FWHM (QExactive HF), AGC target: 1·106, max. IT: 100 ms (QExactive HF) or 120 ms (QExactive Plus). Fragment ions generated in the HCD cell at 30 % normalized collision energy using N2 were scanned at R: 15,000 FWHM (QExactive HF) or 17,500 FWHM (QExactive Plus), AGC target: 2·105, max. IT: 100 ms (QExactive HF) or 120 ms (QExactive Plus) using a dynamic exclusion of 30 s.
MS/MS data were searched against the In-House database of *Mycetocola tolaasinivorans* and *Mycetocola lacteus* proteins using Proteome Discoverer 1.4 and the algorithms of Mascot 2.4.1, Sequest HT (PD1.4), and MS Amanda 1.0. Two missed cleavages were allowed for tryptic peptides. The precursor mass tolerance was set to 10 ppm and the fragment mass tolerance was set to 0.02 Da. Dynamic modifications were set as oxidation of Met. The static modification was set to carbamidomethylation of Cys. At least two peptides per protein and a strict target false discovery (FDR) rate of <1 % (compared against a reverse decoy database) were required for positive protein hits.
Fractionation was repeated several times using different gradients to improve separation of protein fractions. In total, results from four MALDI-TOF-MS/MS measurements and five LC-MS/MS analyses were pooled and filtered for proteins with an annotated hydrolytic activity (hit lists can be found in Supplemental Table). Of seven candidates (1182, 1671, 2172, 2821, 5596, 4841, 6011) occurring in four to six of the analyses five were identified as proteins from *M. lacteus* (1182 (homologue of 6011), 1671 (homologue of 2172), 2821, 5596, 4841) and were selected for heterologous production in *E. coli*.

Heterologous protein production
Genes coding for proteins identified during bioactivity guided fractionation were amplified using Phusion® High Fidelity DNA Polymerase or KAPA HiFi DNA Polymerase (Kapa Biosystems) using primer pairs as listed in Table S2 and gDNA of the respective strain as a template. Obtained amplicons were purified using gel electrophoresis and extracted using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). DNA fragments were then ligated into pJET1.2/blunt vectors using the CloneJet PCR Cloning Kit (Thermo Scientific) and subsequently used for electroporation of *E. coli* TOP10. Resulting mutants were screened by colony PCR using DreamTaq Green PCR Master Mix (2x) (Thermo Scientific) and plasmids of positive mutants were isolated using the Monarch® Plasmid Miniprep Kit (New England Biolabs). Plasmids were sequenced to exclude errors incorporated during amplification and plasmids with correct sequences were submitted to restriction digests using the appropriate restriction enzymes NdeI and BamHI, or HindIII (New England Biolabs), respectively (see Table S2). Digested DNA fragments were then ligated into pET-28c vectors linearized using the same restriction enzymes as the fragments. Transformation of *E. coli* TOP10 electro competent cells with the obtained plasmids was performed and resulting mutants were used for plasmid preparation using the Monarch® Plasmid Miniprep Kit (New England Biolabs). *E. coli* BL21 (DE3) electro competent cells were transformed with the obtained plasmids and the resulting mutants were cultivated in 30 mL LB medium (50 µg mL^{-1} kanamycin) up to an OD_{600} of 0.6–0.8 prior to induction with IPTG (final concentration 0.2 mM). Cultures were incubated at 16 °C on a rotary shaker overnight. Cells were harvested by centrifugation and suspended in 2 mL assay buffer (50 mM Na_2HPO_4, 150 mM NaCl, 5 % glycerol; pH 7.5) and lysed using a sonotrode. Supernatants obtained by centrifugation were analyzed for the presence of the desired protein by SDS-PAGE (Figure S16A). 50 µL of the lysate were further mixed with 10 µL of a 1 mg mL^{-1} solution of tolaasin (in assay buffer containing 10 % methanol) and incubated at room temperature for 5 h. Conversion of tolaasin I (**1**) to tolaasin C (**2**) was evaluated using LC-HRMS after diluting the assay mixture 1:20 with methanol.

Results are shown in Figure S17. Protein with tag 2821 was found active in cleaving tolaasin I (**1**) and was named TdfL (**t**olaasin **d**egrading **f**actor in *M. **l**acteus*). The sequence of TdfL was then blasted against the *in silico* proteome of *M. tolaasinivorans* yielding a hit with 83.3 % identity and a query coverage of 99 %. The protein, named TdfT (**t**olaasin **d**egrading **f**actor in *M. **t**olaasinivorans*) was produced in same way and showed hydrolytic activity towards tolaasin I (**1**).

Purification of TdfL and TdfT and activity assays

LB medium (800 mL, in 1 L baffled Erlenmeyer flasks with 400 mL medium each, supplemented with 50 µg mL^{-1} kanamycin) was inoculated with overnight cultures of *E. coli* BL21 (DE3) pET-28-2821 (TdfL) or *E. coli* BL21 (DE3) pET-28-3804 (TdfT) and cultivated to an OD_{600} of 1 prior to induction with IPTG (final concentration 0.2 mM). Subsequent cultivation was performed on a rotary shaker at 16 °C overnight. Cells were then harvested by centrifugation and resuspended in 10 mL binding buffer (50 mM Na_2HPO_4, 150 mM NaCl, 5 % glycerol, 10 mM imidazole; pH 7.5) per gram cell pellet. 2 µL Benzonase® (≥250 U $µL^{-1}$; Merck KGaA), and 6 mg lysozym (≥35,000 FIP U mg^{-1}, Carl Roth) per gram cell pellet were added prior to addition of $MgCl_2$ to a final concentration of 1 mM. Cells were lysed using a sonotrode and cell debris was removed by centrifugation. Obtained lysates were incubated with TALON® metal affinity resin (Takara Bio) for 1 h at 4 °C before resin beads were collected in open columns. Beads were washed with binding buffer and eluted with elution buffer (50 mM Na_2HPO_4, 150 mM NaCl, 5 % glycerol, 150 mM imidazole; pH 7.5). Purity of fractions was analyzed by SDS PAGE (Figure S16B). The eluates were further concentrated and washed repeatedly with storage buffer (50 mM Na_2HPO_4, 150 mM NaCl, 20 % glycerol, pH 7.5) using Amicon Ultra-15 filter units (MWCO 50,000, Merck KGaA). Protein concentrations of obtained enzyme solutions were estimated using absorbance at λ = 280 nm and adjusted to a stock concentration of 5 µM. Protein aliquots were stored at –80 °C until needed.

Heat-inactivated protein samples were obtained by incubation of aliquots at 95 °C for 10 min. Final concentrations of substrates were adjusted to 0.5 mM for tolaasin and 0.1 mM for pseudodesmin A, respectively. TdfL and TdfT were used at a final concentration of 1.25 µM. Assays were incubated at room temperature as specified and stopped by addition of 19 volume equivalents of methanol. Levels of tolaasin C (**2**) and pseudodesmin C (**5**) were analyzed by LC-HRMS (Figure S18). Activity of the enzymes was also assayed in 2 mL cultures containing a final concentration of 1.25 µM TdfT or TdfL in Kings B. A mix of both enzymes was heat inactivated as described before and used next to plain Kings B medium as a negative control. *P. tolaasii* was inoculated to an initial OD_{600} of 0.1 using cells of an overnight seed culture washed with 0.85 % aqueous NaCl. Culture tubes were placed overnight on a rotary shaker at 25 °C. After 16 h of incubation, aliquots of cultures were lyophilized, and the residue extracted with methanol. Obtained extracts were analyzed using LC-HRMS (Figure 4F).

Analysis of TdfL, TdfT, and TdfS

The amino acid sequence of TdfL was blasted against proteins derived from the genome sequence of *Mycetocola saprophilus,* a third member of the genus reported to detoxify tolaasin (6). We refer to the putative enzyme identified as a homologue of TdfL and TdfT as TdfS (**t**olaasin **d**egrading **f**actor in *M. **s**aprophilus*). To test whether these three enzymes belong to the class of serine proteases (as annotated by BLAST), we aligned the amino acid sequences of the proteins with that of P23687, a characterized prolyl endopeptidase from *Sus scrofa* (pig) using Geneious 11.0.3 and the built-in alignment algorithm with default settings. All three active site residues (Ser, Asp, His) were found conserved in the three proteins (Figure S19).

Hydrolytic activity of heat-inactivated *Mycetocola* cultures

M. tolaasinivorans and *M. lacteus* were cultured overnight in Kings B medium (30 mL, 25 °C, 150 rpm). 5 mL of each culture were autoclaved (121 °C, 15 min) separately prior to use as heat-inactivated samples. Samples (10 µL) of tolaasin I or pseudodesmin A solution (2.5 µg $µL^{-1}$ in methanol) were added to 400 µL of an untreated cell suspension (alive) or an autoclaved cell suspension (HI) and incubated at room temperature for 22 h. samples of the mixtures (100 µL) were then dried in a vacuum concentrator and the residue was extracted with an equal volume of methanol. The supernatant obtained after centrifugation was diluted 1:1 with distilled water and analyzed using LC-HRMS and the conditions described above. While the untreated cells of both strains were able to

hydrolyze both **1** and **4**, no activity was observed for the autoclaved cultures (Figure S20). It can be concluded that **4** is also hydrolyzed by an enzyme produced by *M. tolaasinivorans* and *M. lacteus*.

Isolation of pseudodesmin C (5)

Three 1 L baffled Erlenmeyer flasks containing each 400 mL King's B medium were inoculated with each 4 mL of an overnight seed culture of *M. lacteus* and 4 mL of an overnight seed culture of *P. tolaasii* Δ*tolA* (to avoid contamination with tolaasins). After cultivation for 29 h at 25 °C and 120 rpm on a rotary shaker, cells were removed by centrifugation and the supernatant was acidified to pH 2 using concentrated HCl. The obtained supernatant was extracted four times with equal volumes ethyl acetate. Organic phases were combined, dried over Na_2SO_4 and evaporated to dryness to yield 990 mg of an orange oil. The crude extract was suspended in 3 mL methanol and 7 mL water prior to application on an open column filled with LiChroprep RP18 (equilibrated in 30 % aqueous methanol). The column was rinsed stepwise with increasing methanol concentrations and fractions were checked for the presence of pseudodesmin C via LC-MS. Up to 70 % aqueous methanol, no pseudodesmin C (**5**) could be detected. Fractions containing **5** (eluted with 85 % and 100 % methanol) were pooled and evaporated to dryness, yielding 129 mg crude pseudodesmin C. Further purification was performed using the preparative LC system described above equipped with a Phenomenex Synergi Fusion RP 80A (250 × 21.2 mm) with a guard column (solvent A: H_2O + 0.1 % TFA; solvent B: HPLC grade acetonitrile; gradient: 0–4 min 40 % B; 4–25 min 40–80 % B; flow: 20 mL min^{-1}). Fractions containing **5** were pooled (R_t = 16.3 min) and lyophilized to yield 29.1 mg of **5** as a white powder. UV, IR, and NMR spectra together with the assigned signals can be found in Figures S21–30 and Table S5

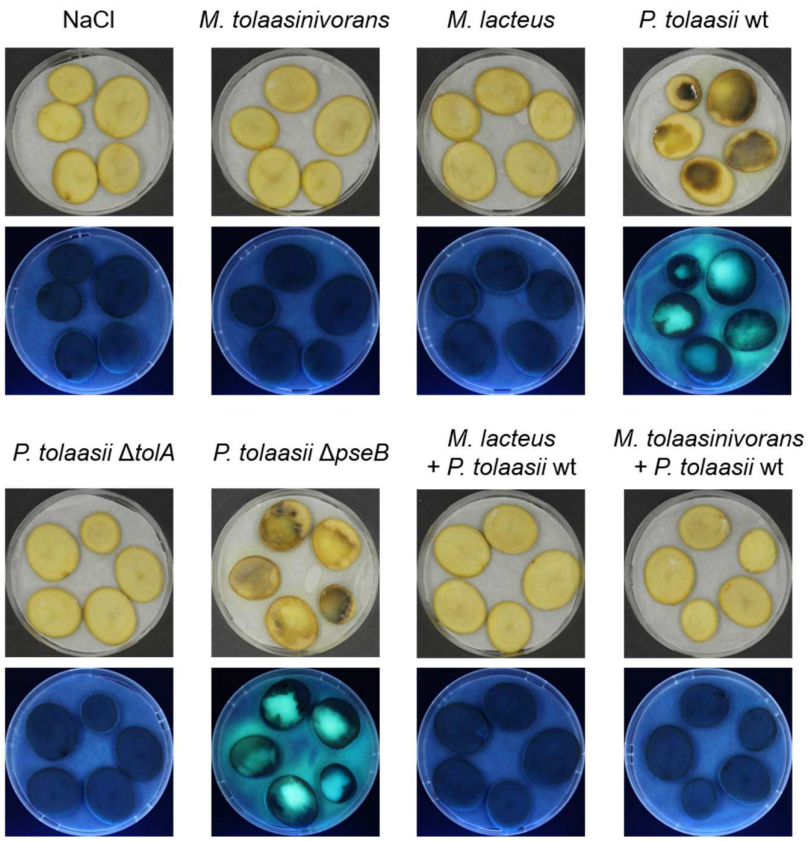


Fig. S1. Pathogenicity assays using potato slices. Potato slices inoculated with the designated strains under bright light and UV light (λ = 366 nm) 42 h past inoculation. Fluorescence caused by pyoverdin produced by *P. tolaasii* indicates successful colonization of the potato slices by the pathogen.

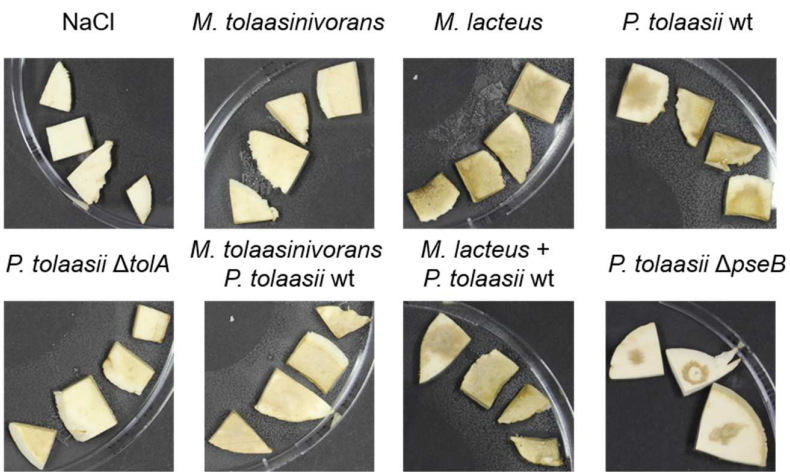


Fig. S2. Pathogenicity assays using *A. bisporus* cubes. Mushroom pieces inoculated with the designated strains 42 h past inoculation.

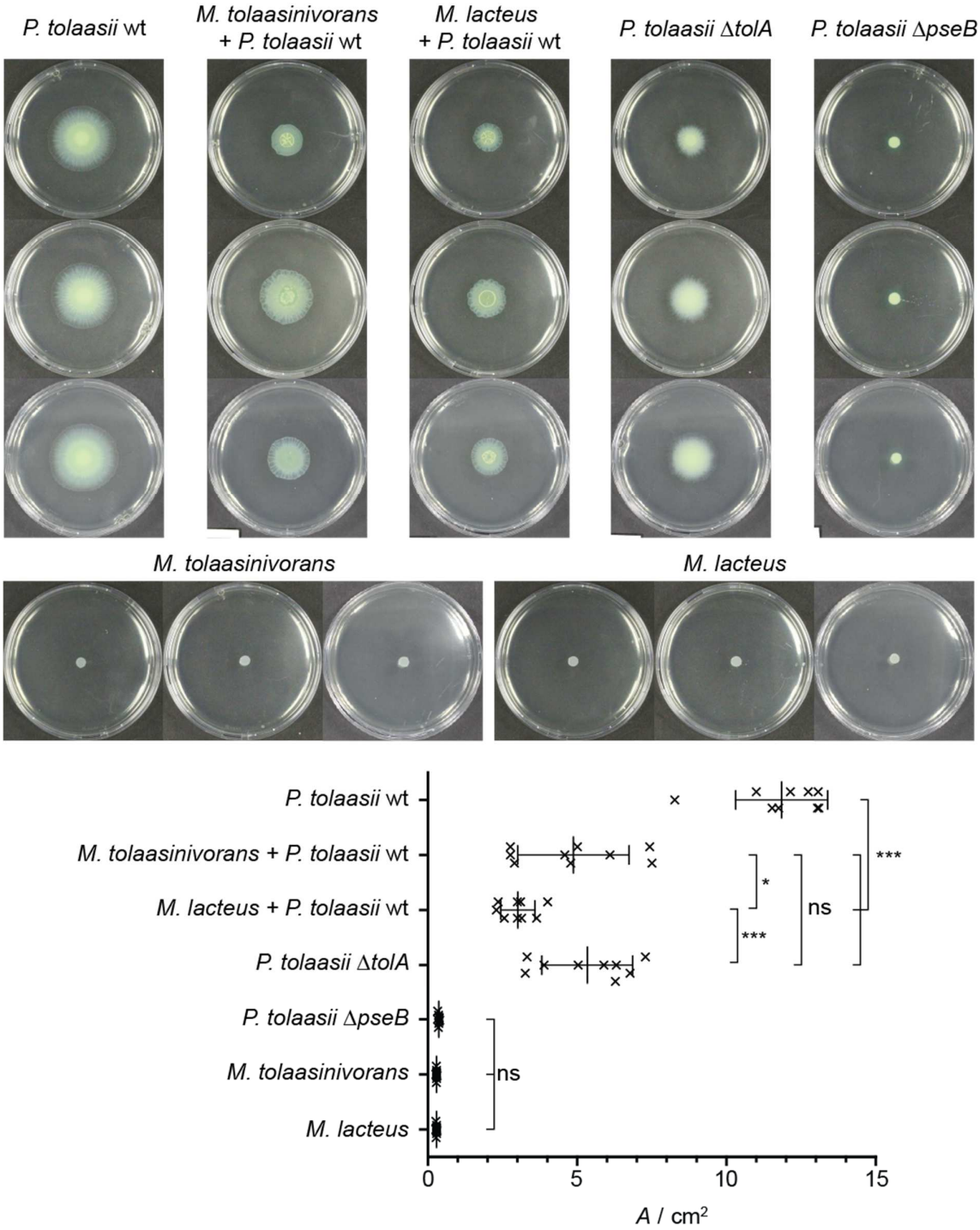


Fig. S3. Swarming assays using *M. lacteus*, *M. tolaasinivorans*, and *P. tolaasii* wt and deletion mutants. Plot represents means with individual data points and standard deviation. Swarming areas were determined using Photoshop and a scale bar in the photo as reference. Pictures were taken 24 h past inoculation on soft agar (0.6 %). Data originates from three individual experiments with three technical replicates each. Photos show representative images from each individual experiment. One way ANOVA with Tukey's multiple comparison test was performed with GraphPad Prism 5. ***: $p < 0.001$; *: $p < 0.05$; ns: not significant.

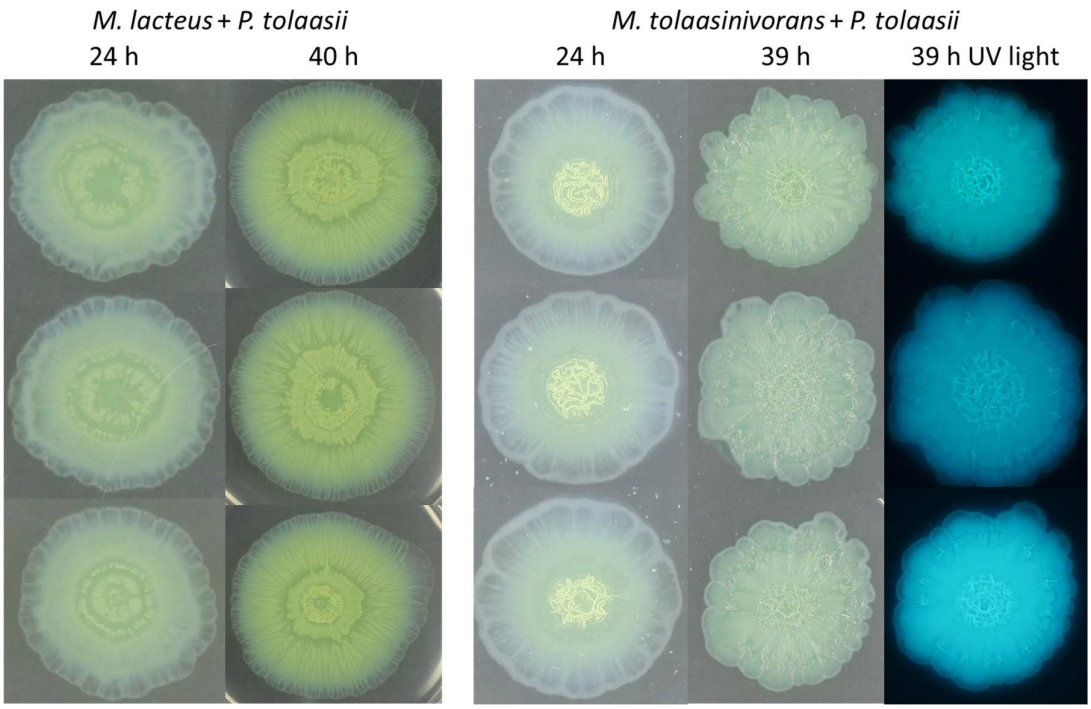
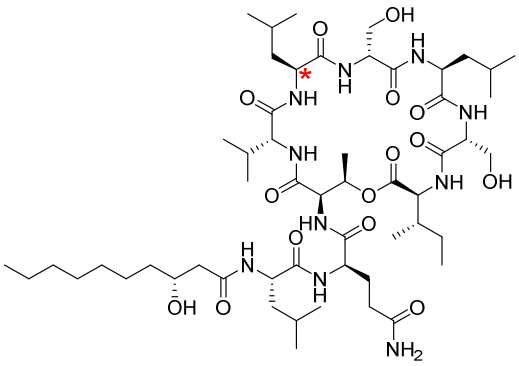
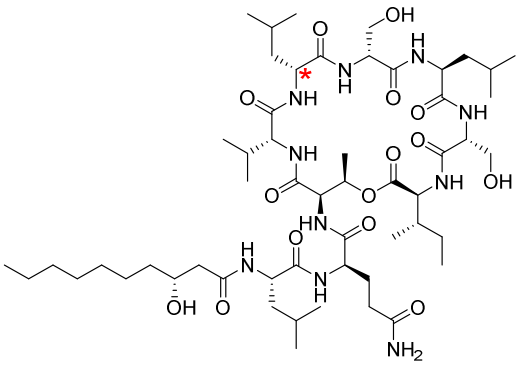


Fig. S4. Magnification of mixed cultures in swarming assays. Pictures were taken from the bottom of the agar plate for all but *M. tolaasinivorans* + *P. tolaasii* (39 h pictures). 3-dimensional structures in the colony were observed only in mixed cultures but not in axenic ones. Biggest structures were found at the point of inoculation. Fluorescence under UV light (λ = 366 nm) can be attributed to production of pyoverdin by *P. tolaasii*.



viscosinamide A (3)



pseudodesmin A (4)

Fig. S5. Structures of viscosinamide A (**3**) and pseudodesmin A (**4**). Asterisk marks stereogenic center at Leu-5.

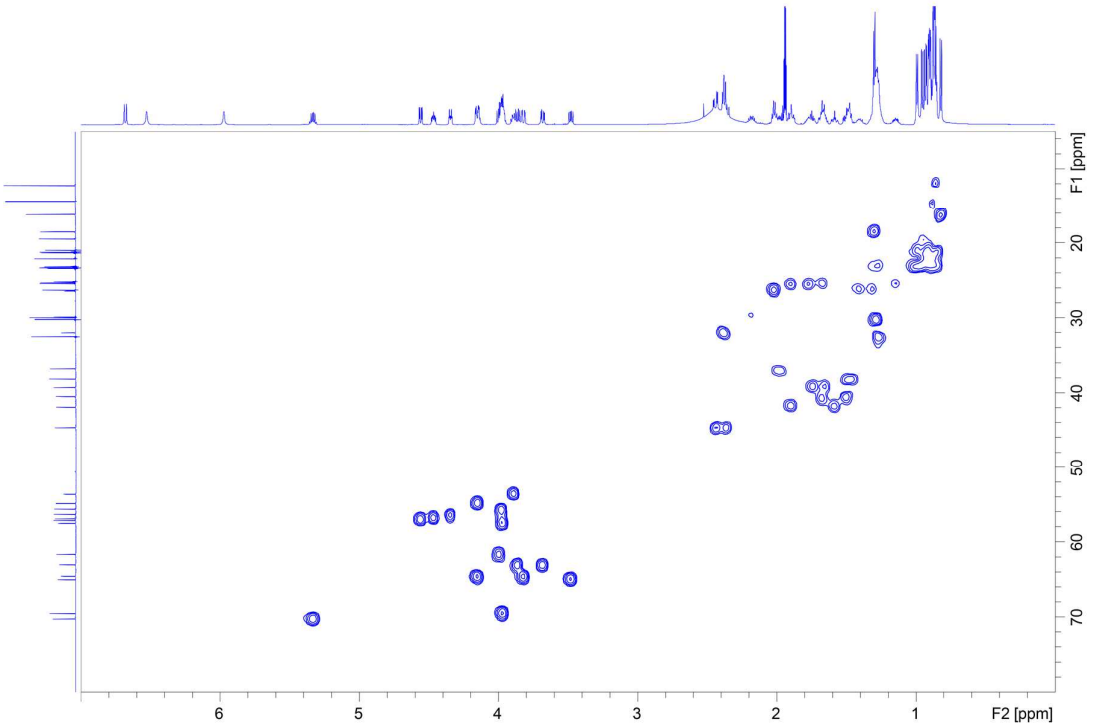


Fig. S6. HSQC spectrum of pseudodesmin A (**4**) (600 MHz, 298 K, acetonitrile-d_3), isolated from *P. tolaasii*

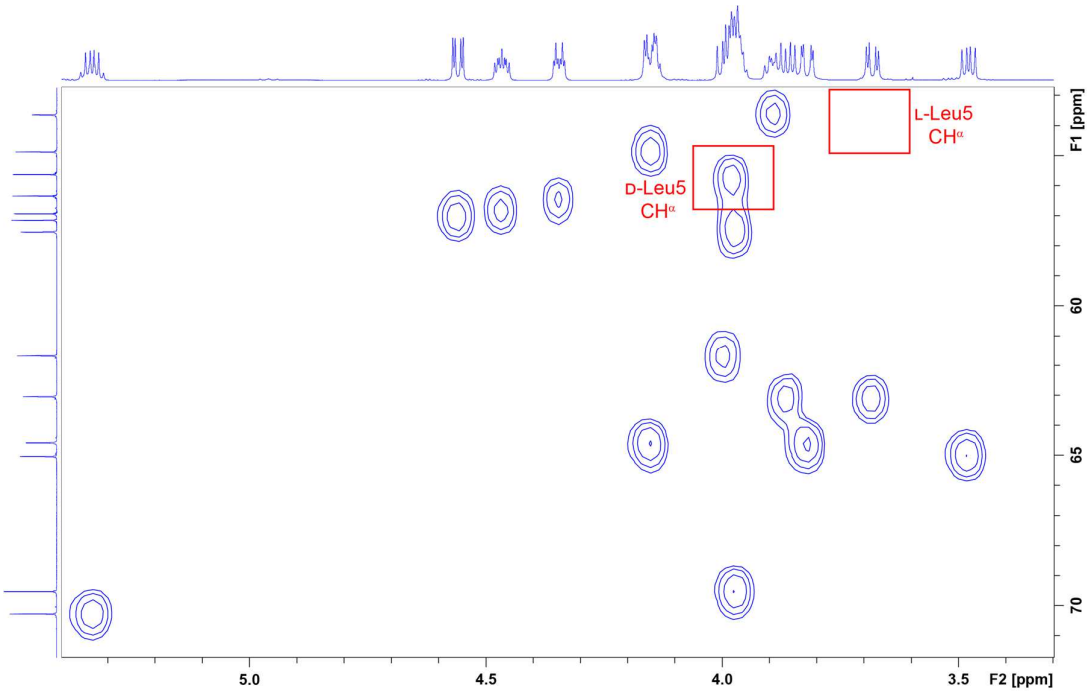


Fig. S7. HSQC spectrum showing region of α-protons in pseudodesmin A (**4**). Expected location of CH^{α} correlations of Leu5 dependent on absolute configuration are highlighted based on data published by GEUDENS *et al.* (2)

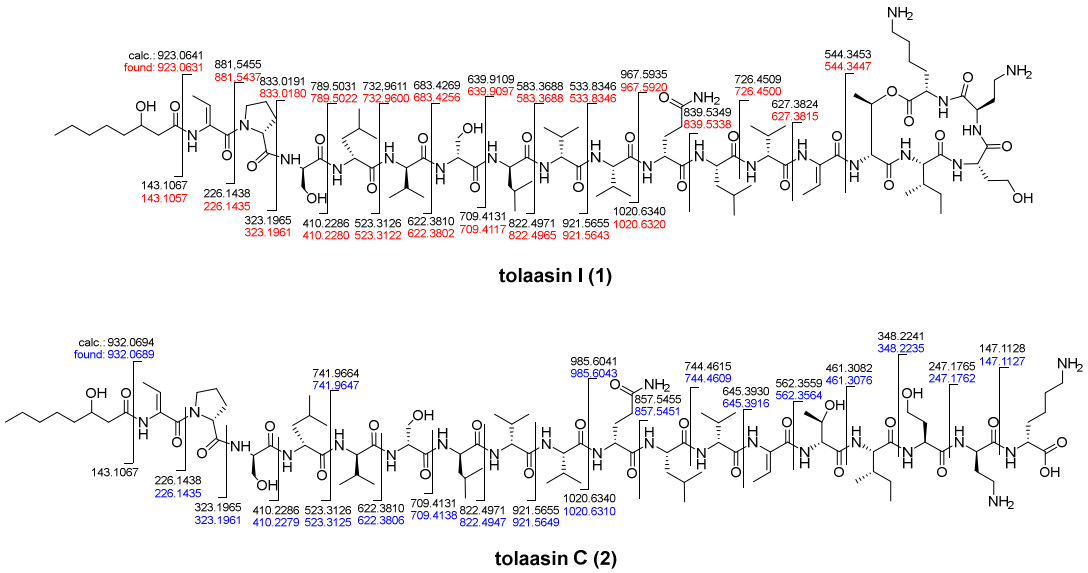


Fig. S8. MS/MS analysis of tolaasin I (**1**) and tolaasin C (**2**) detected in detoxification assays. Masses correspond to single or double charged fragments. Values represent calculated (black) and found (red or blue) *m/z* values.

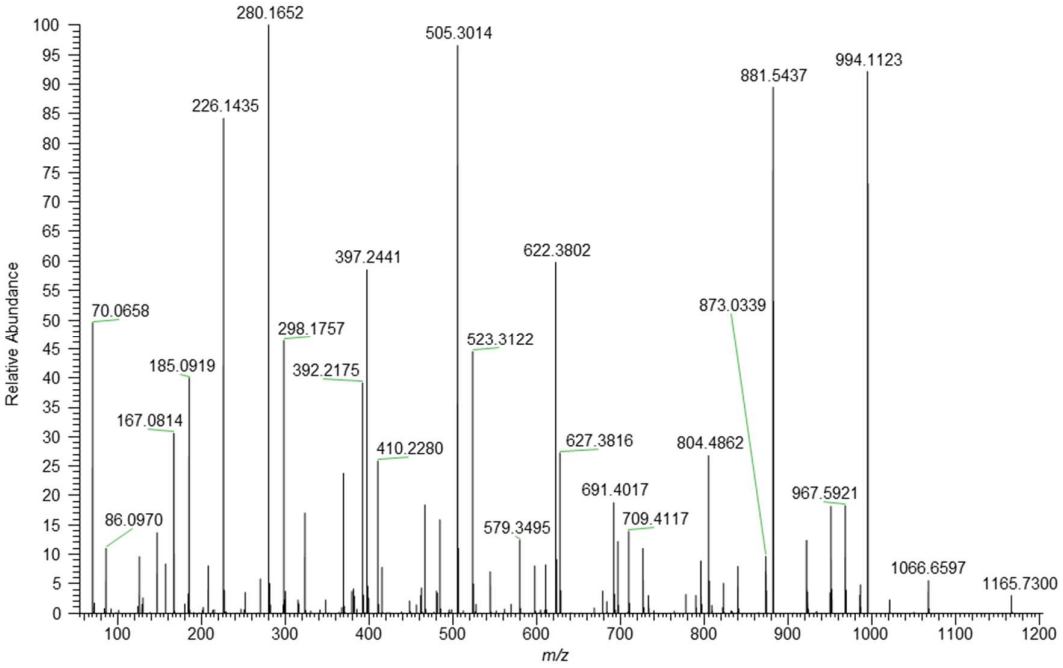


Fig. S9. HRMSMS spectrum of tolaasin I (**1**). *m/z* 994.10 $[M + 2H]^{2+}$ was used as parent ion.

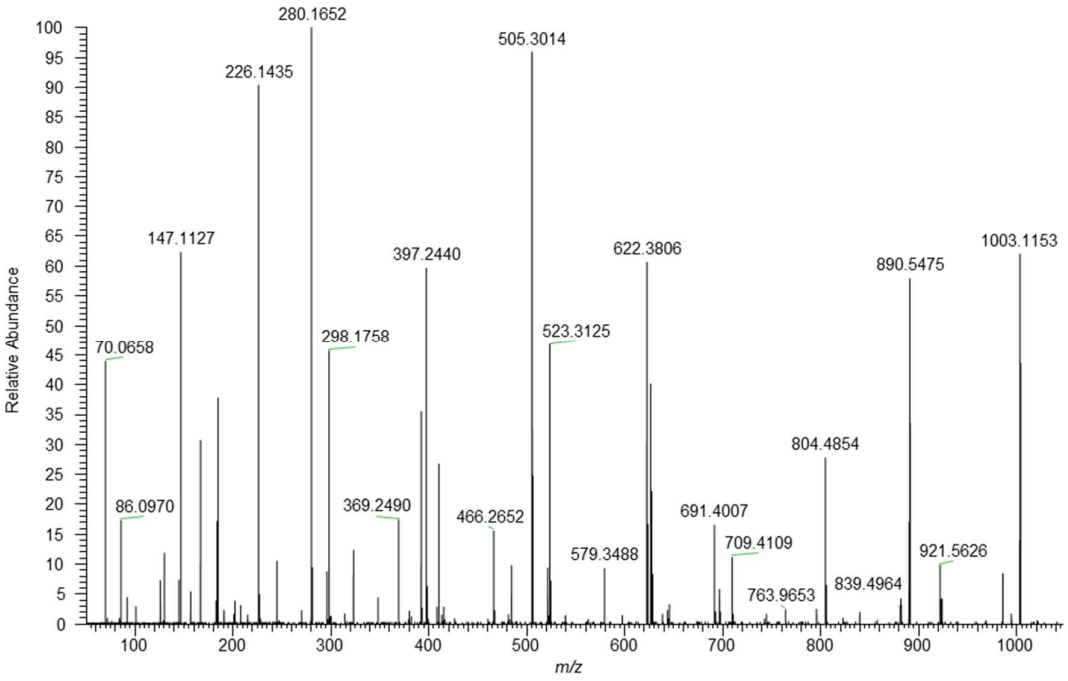


Fig. S10. HRMSMS spectrum of tolaasin C (**2**). *m/z* 1003.10 $[M + 2H]^{2+}$ was used as parent ion.

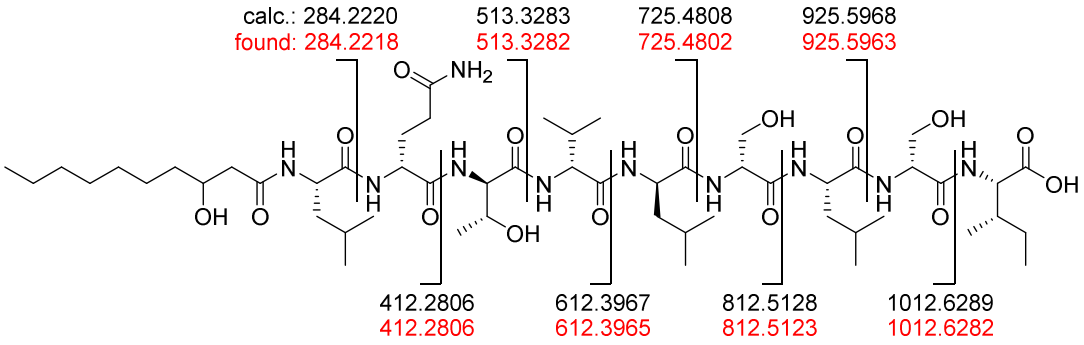


Fig. S11. MSMS analysis of pseudodesmin C (**5**) detected in bacterial co-cultures. Values represent calculated (black) and found (red) *m/z* values.

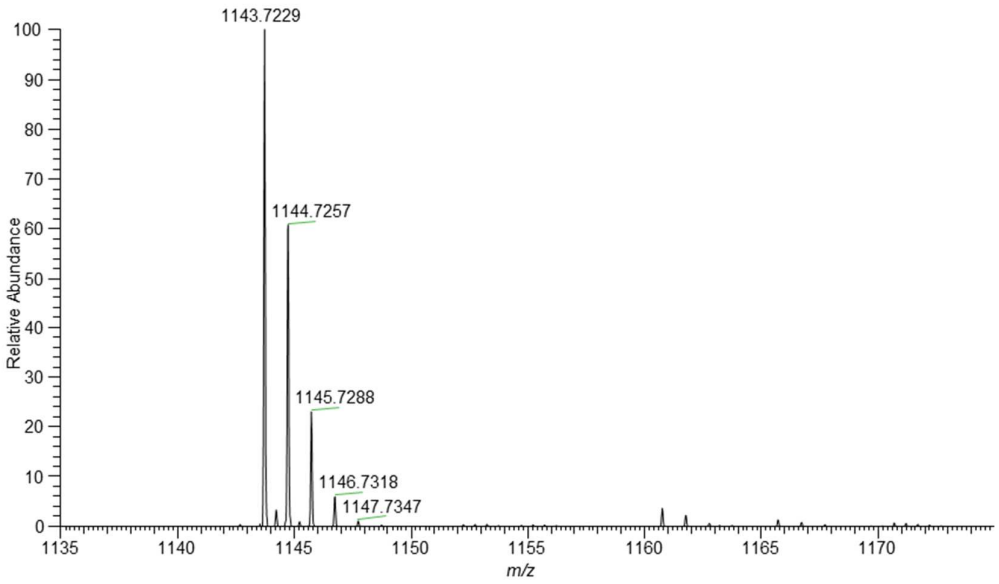


Fig. S12. Isotope pattern of pseudodesmin C (**5**, $[M + H]^+$), detected in bacterial co-cultures.

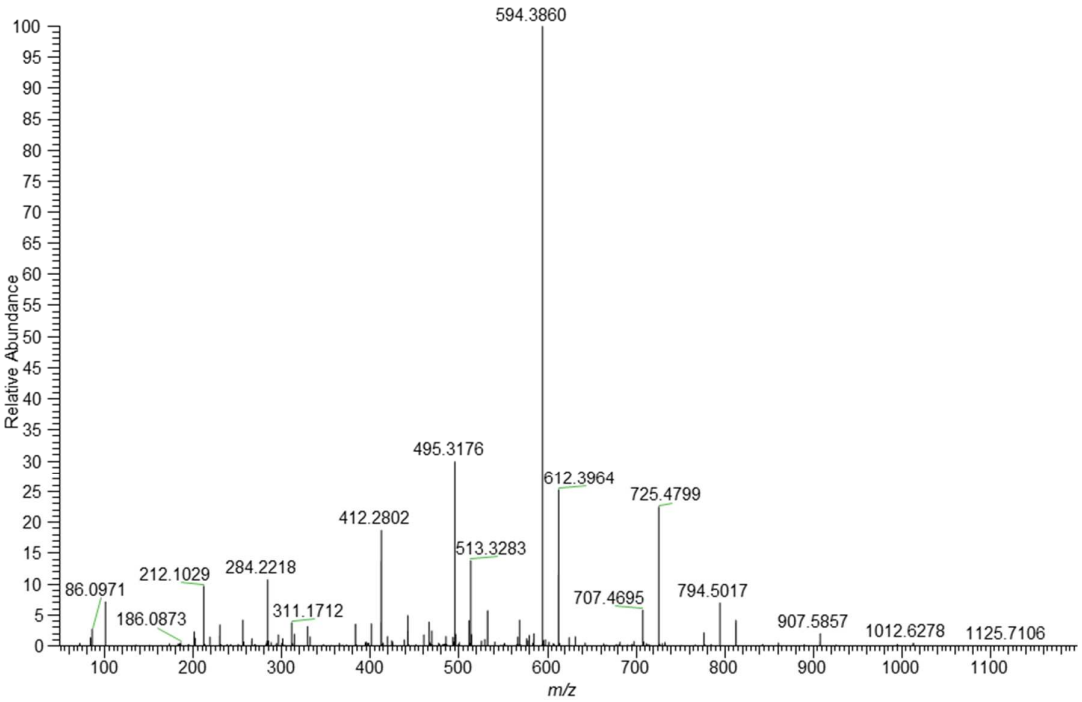


Fig. S13. HRMSMS spectrum of pseudodesmin C (**5**).

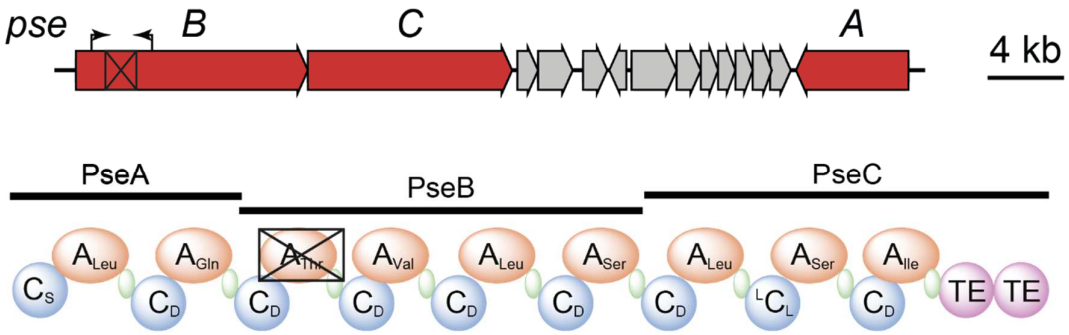


Fig. S14. Pseudodesmin biosynthetic gene cluster with predicted NRPS domain structure and substrate specificities of adenylation domains. Genes predicted to code for NRPS are highlighted in red, primer binding sites used for confirmation of successful deletion are indicated with arrows, crossed box marks deleted area/encoded domains. C: condensation domain; C_S: starter condensation domain; C_D: dual E/C domain (E: epimerase) performing condensation and epimerization; $^{L}C_L$: condensation domain linking an L-amino acid with an L-amino acid; A: adenylation domain; TE: thioesterase domain; green: thiolation domain.

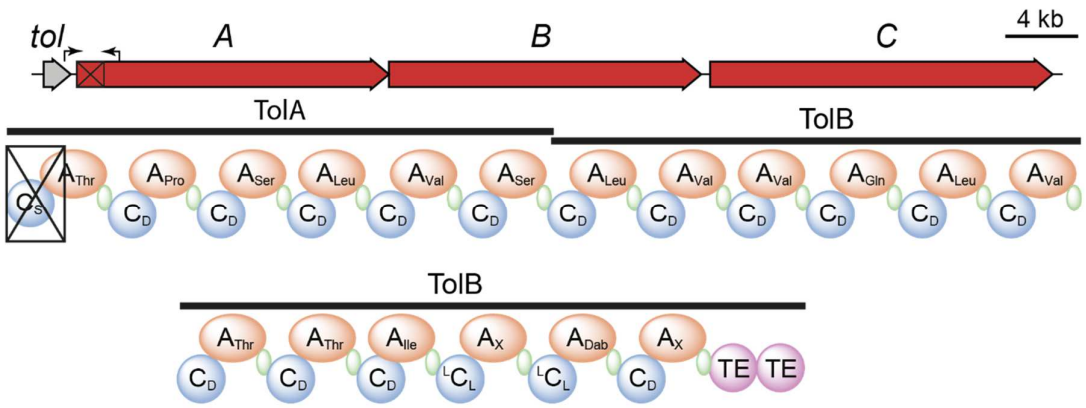


Fig. S15. Tolaasin biosynthesis gene cluster with predicted NRPS domain structure, and substrate specificities of adenylation domains. Genes predicted to code for NRPS are highlighted in red, primer binding sites used for confirmation of successful deletion are indicated with arrows, crossed box marks deleted areas/encoded domains. C: condensation domain; C_S: starter condensation domain; C_D: dual E/C domain (E: epimerase) performing condensation and epimerization; $^{L}C_L$: condensation domain linking an L-amino acid with an L-amino acid; A: adenylation domain; TE: thioesterase domain; green: thiolation domain.

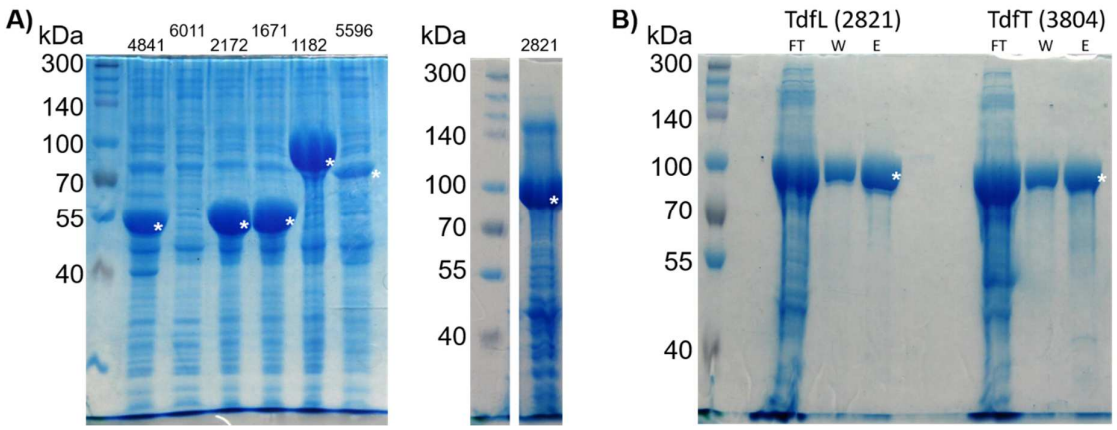


Fig. S16. SDS PAGE of heterologously produced proteins. **A)** Lysates obtained from *E. coli* BL21 (DE3) pET-28-xxxx (xxxx: 4841, 6011, 2172, 1671, 1182, 5596, or 2821). cultures that were used for activity assays. Asterisks indicate proteins of interest. Calculated molecular weights of proteins: 51.9 kDa (4841); 95.4 kDa (6011); 52.6 kDa (2172); 52.1 kDa (1671); 95.3 kDa (1182); 73.5 kDa (5596); 83.6 kDa (2821/TdfL). **B)** SDS-PAGE of fractions obtained during purification of TdfL and TdfT. FT: flow through; W: wash; E: elution. Asterisks mark fractions used for further enzyme assays. Calculated molecular weights of proteins: 83.6 kDa (TdfL); 82.5 kDa (TdfT).

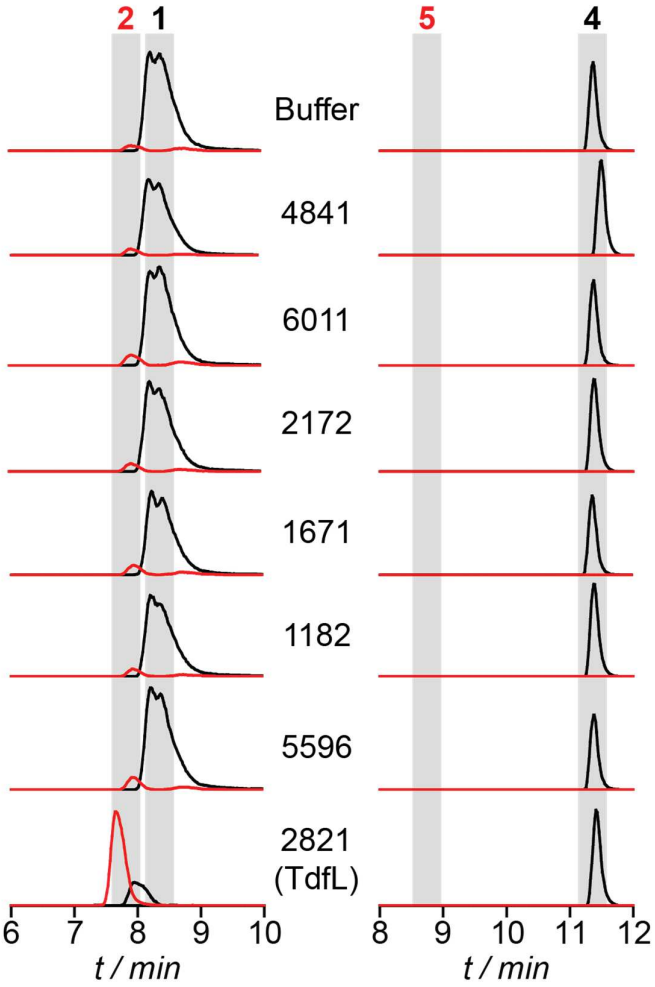


Fig. S17. Bioactivity assays of heterologously produced proteins. For tags see Table S4. Only protein 2821 (TdfL) converted tolaasin I (**1**) into tolaasin C (**2**). None of the proteins showed activity towards pseudodesmin A (**4**) since **5** could not be detected. EICs of tolaasin I (**1** left) and pseudodesmin A(**4**, right) in black and EICs of tolaasin C (**2**, left) and pseudodesmin C (**5**, right) highlighted in red.

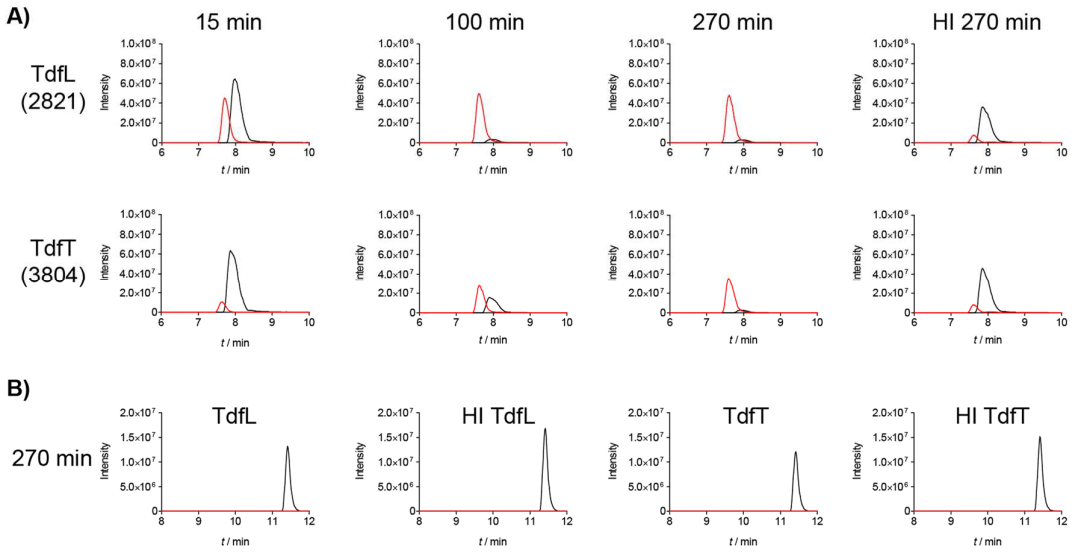


Fig. S18. Bioactivity assays using TdfL and TdfT. **A)** Tolaasin I (**1**, EIC in black) used as a substrate and conversion into tolaasin C (**2**, EIC in red) was monitored over time at an enzyme to substrate ratio of 1:400. Heat inactivated (HI) enzymes were used as a negative control. **B)** Pseudodesmin A (**4**, EIC in black) used as a substrate. No conversion into pseudodesmin C (**5**, EIC in red) could be observed after 270 min at an enzyme to substrate ratio of 1:80. Heat inactivated (HI) enzymes were used as a negative control.

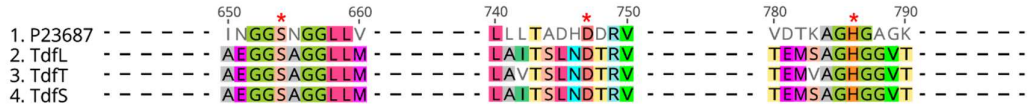


Fig. S19. Active sites in TdfL (2821), TdfT (3804), and TdfS. Alignment of P23687 (prolyl endopeptidase) from *Sus scrofa* with TdfL, TdfT, and TdfS, active site residues based on annotation of P23687 highlighted with asterisks.

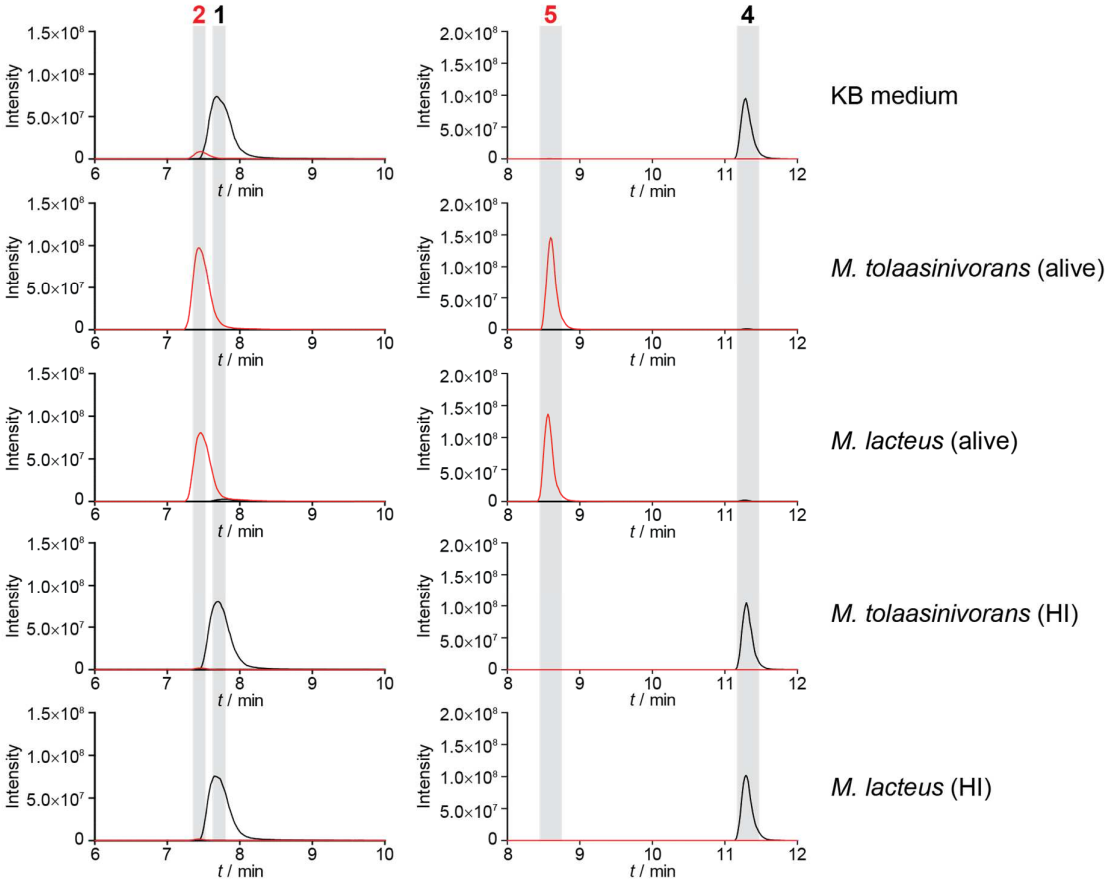


Fig. S20. LC profiles of hydrolysis assays using living and autoclaved (HI) cultures of *M. tolaasinivorans* and *M. lacteus*. Red: EIC traces of linearized peptides **2** and **5**; black: EIC traces of cyclic peptides **1** and **4**.

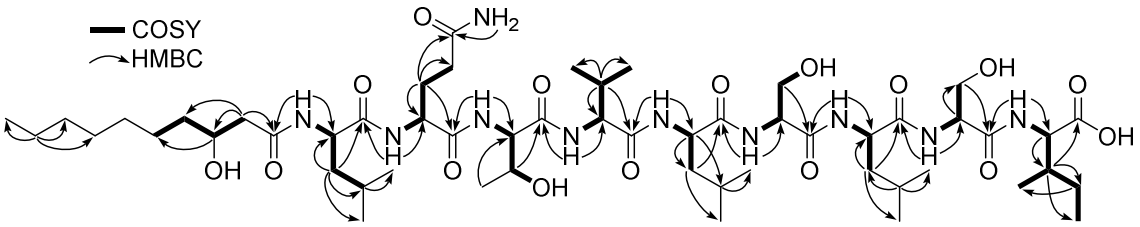


Fig. S21. Key COSY and HMBC correlations used for structure elucidation of pseudodesmin C (**5**). Assigned signals can be found in Table S5

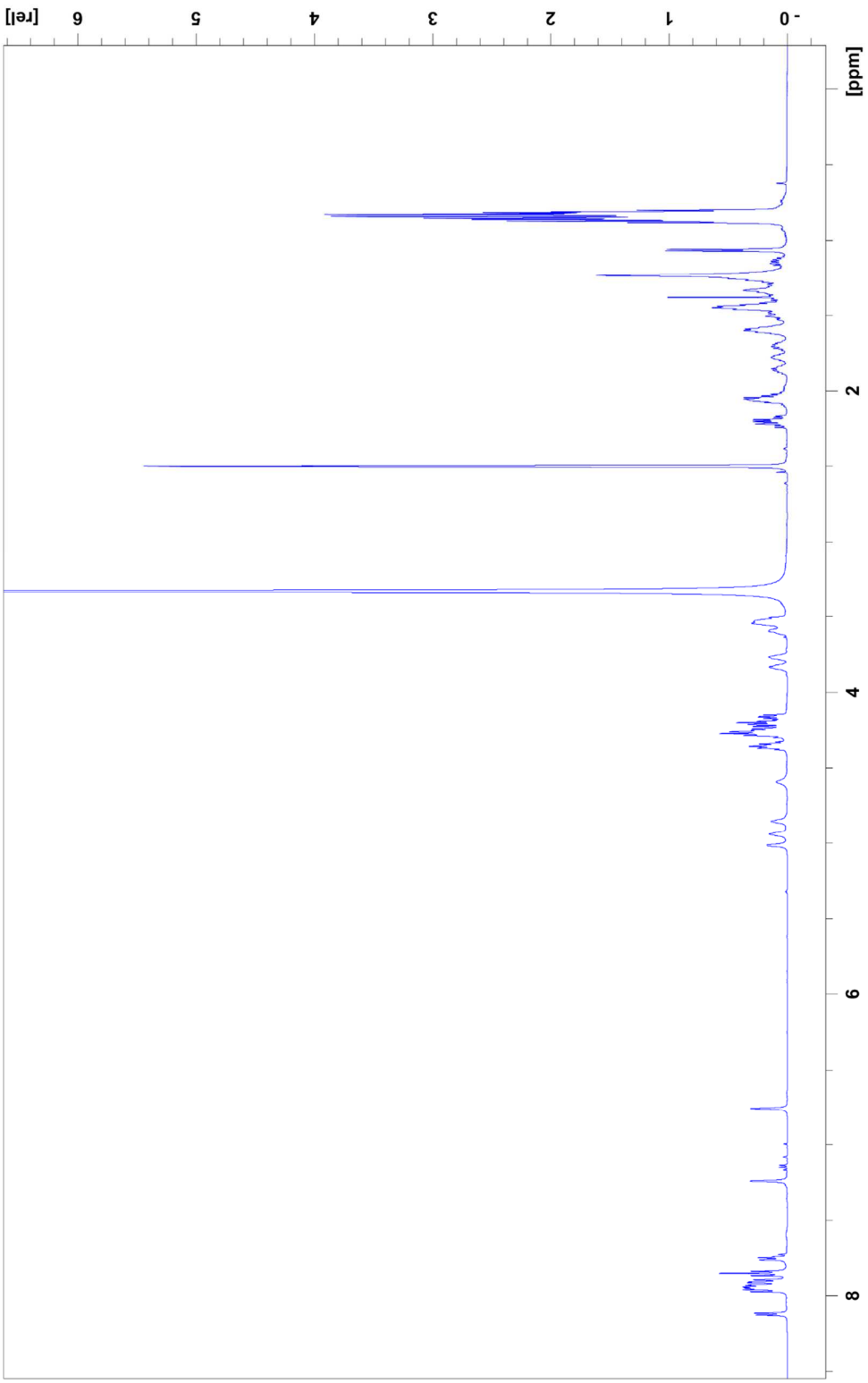


Fig. S22. ^{1}H NMR spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 600 MHz).

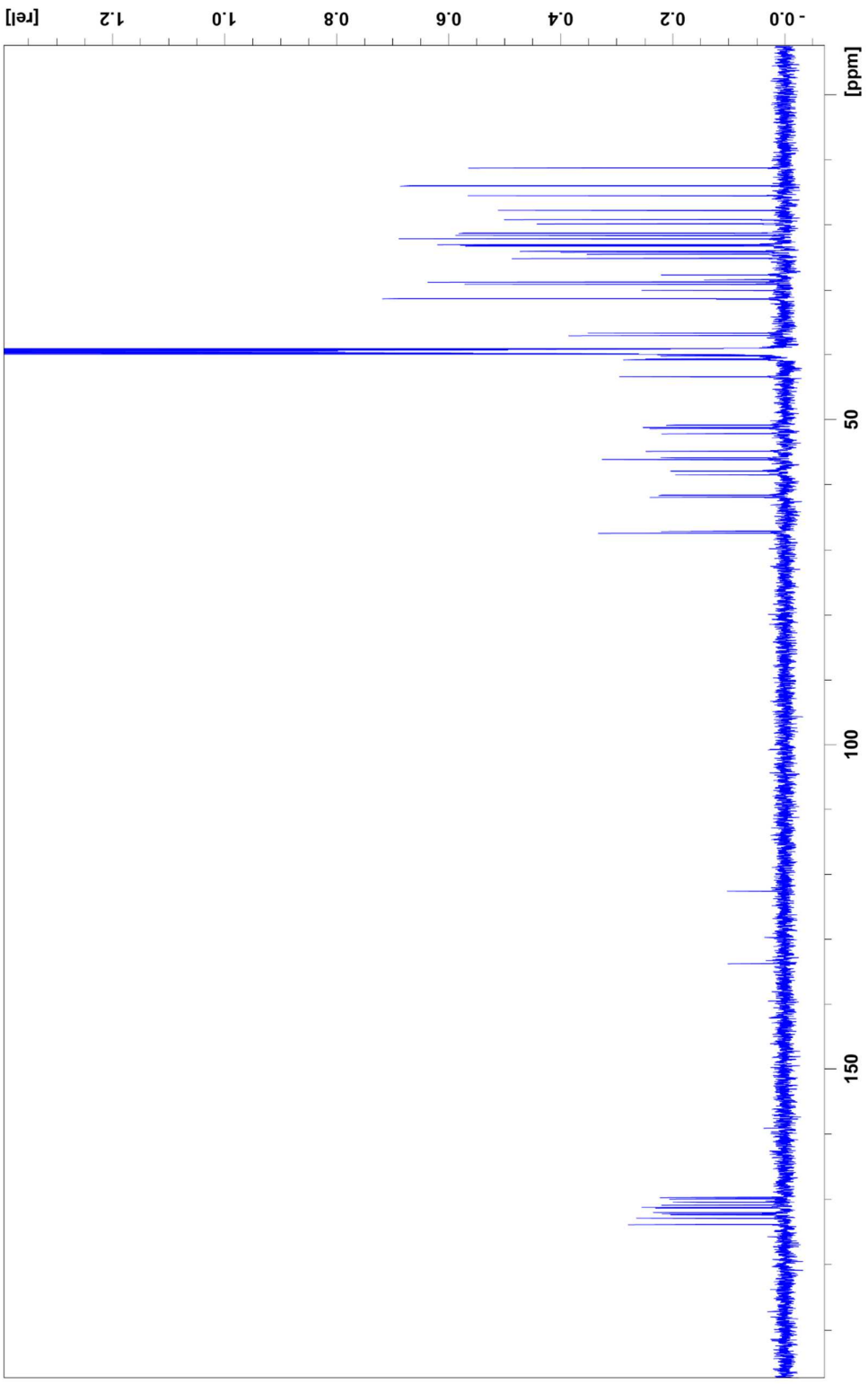


Fig. S23. ^{13}C NMR spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 150 MHz).

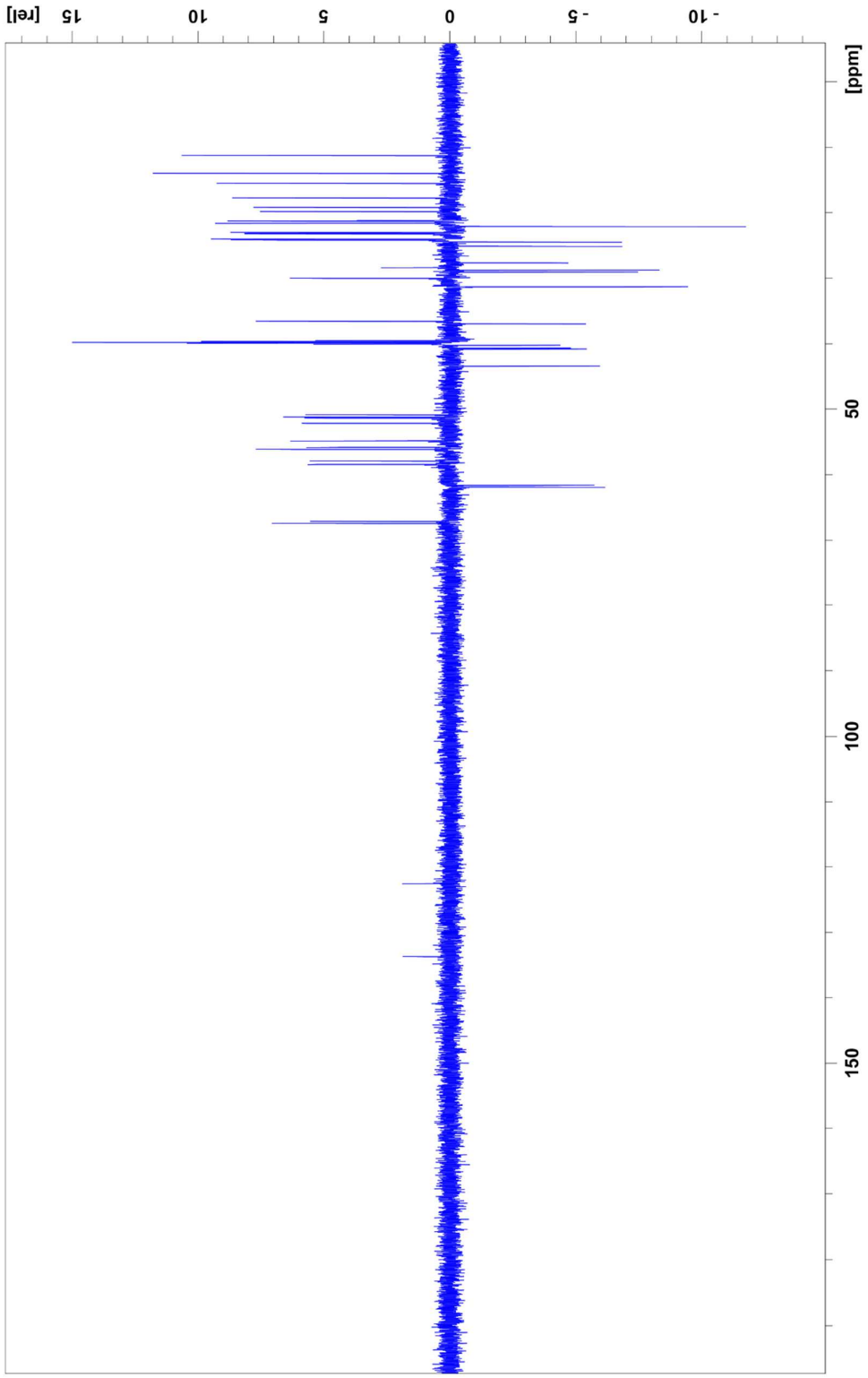


Fig. S24. Dept-135 spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 150 MHz).

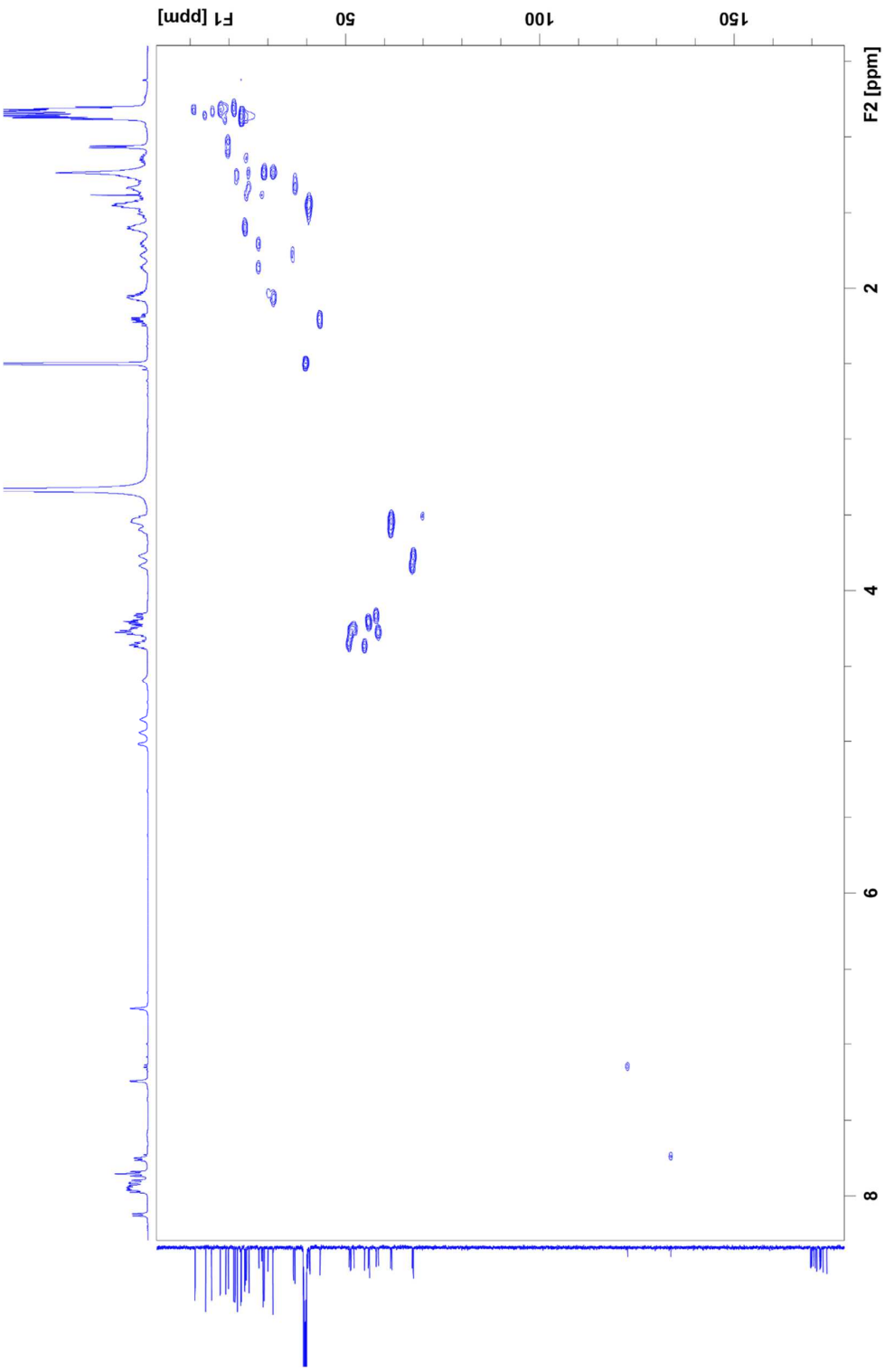


Fig. S25. HSQC spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 600 MHz).

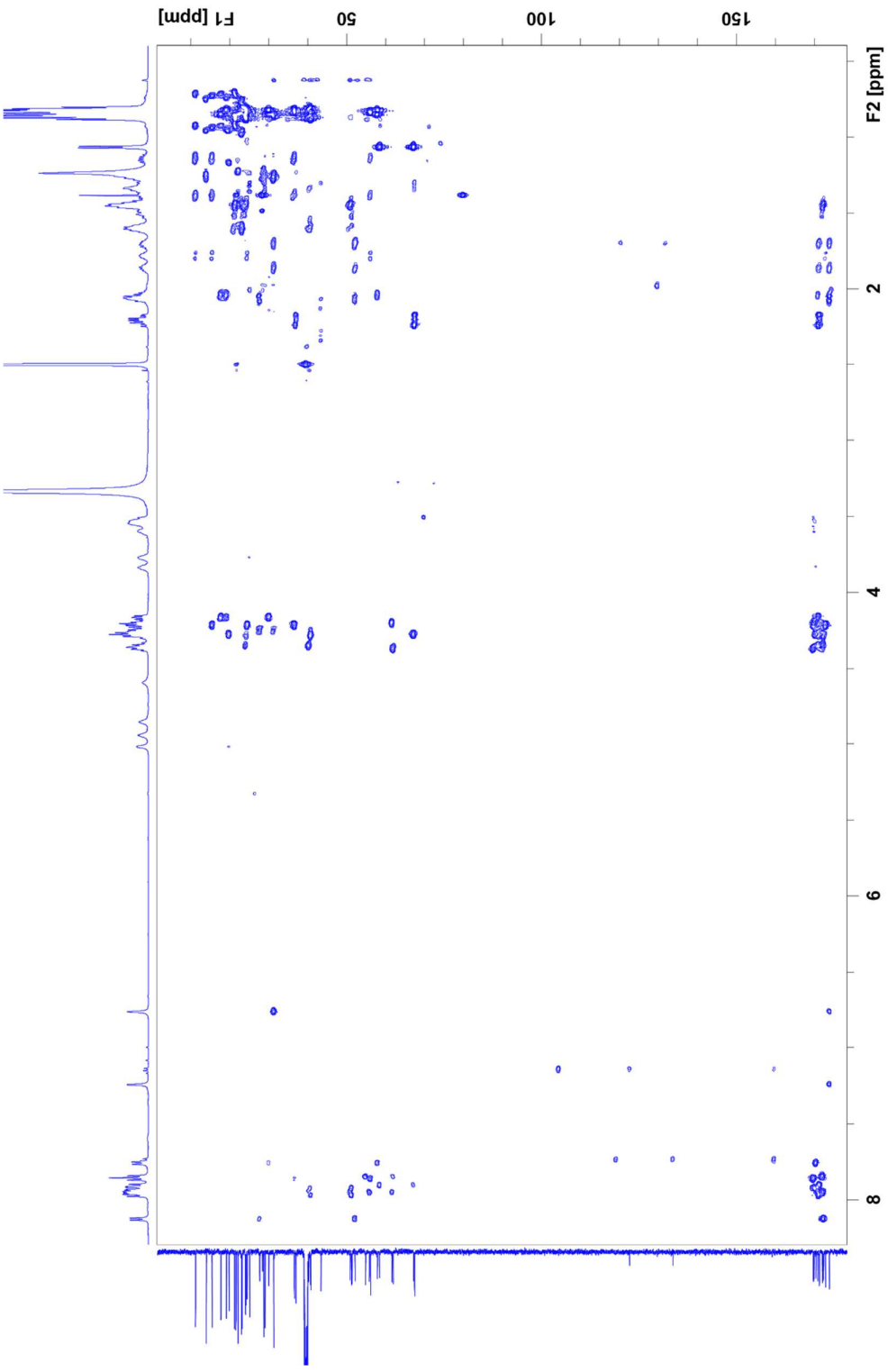


Fig. S26. HMBC spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 600 MHz).

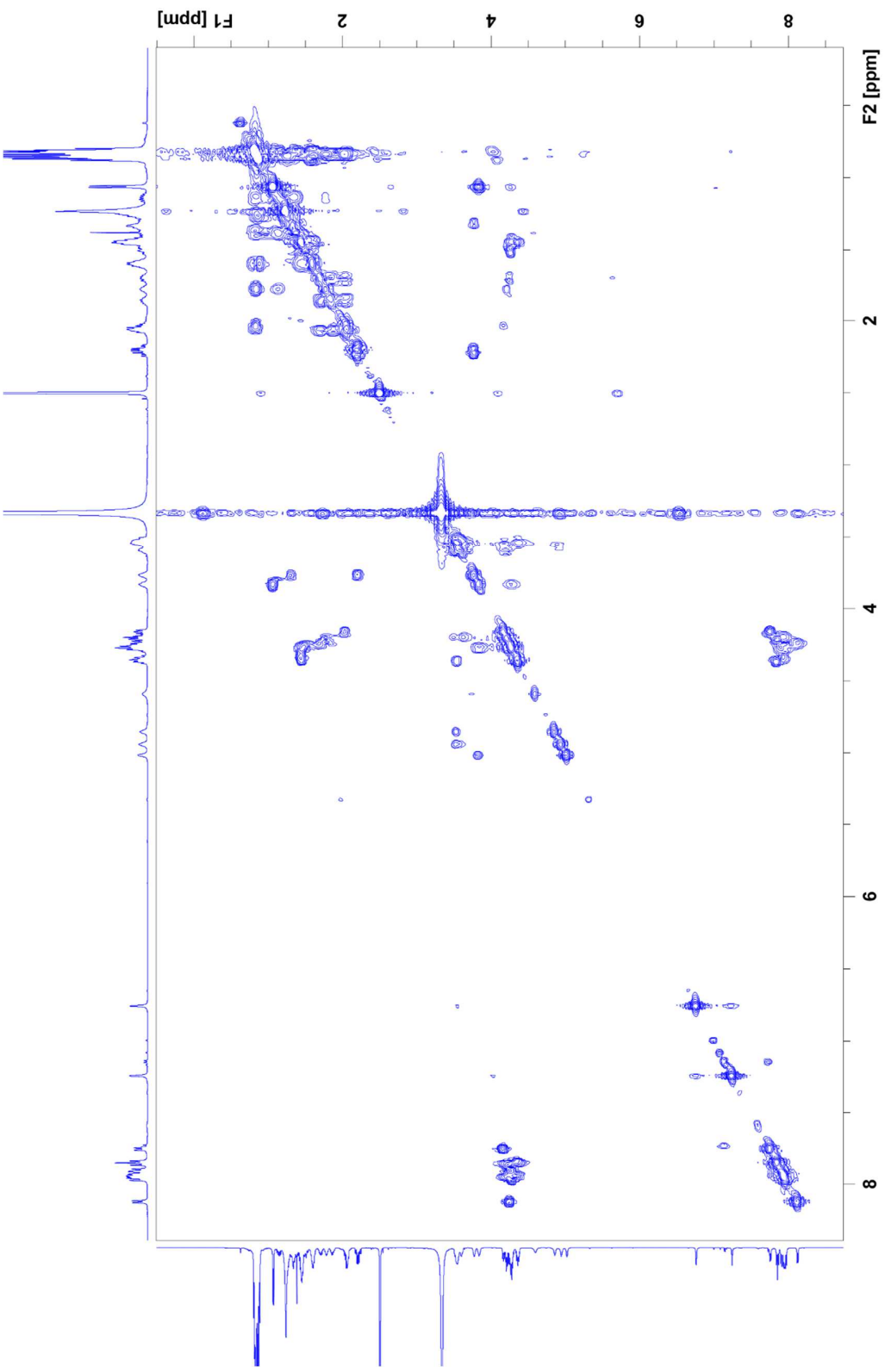


Fig. S27. ^{1}H-^{1}H COSY spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 600 MHz).

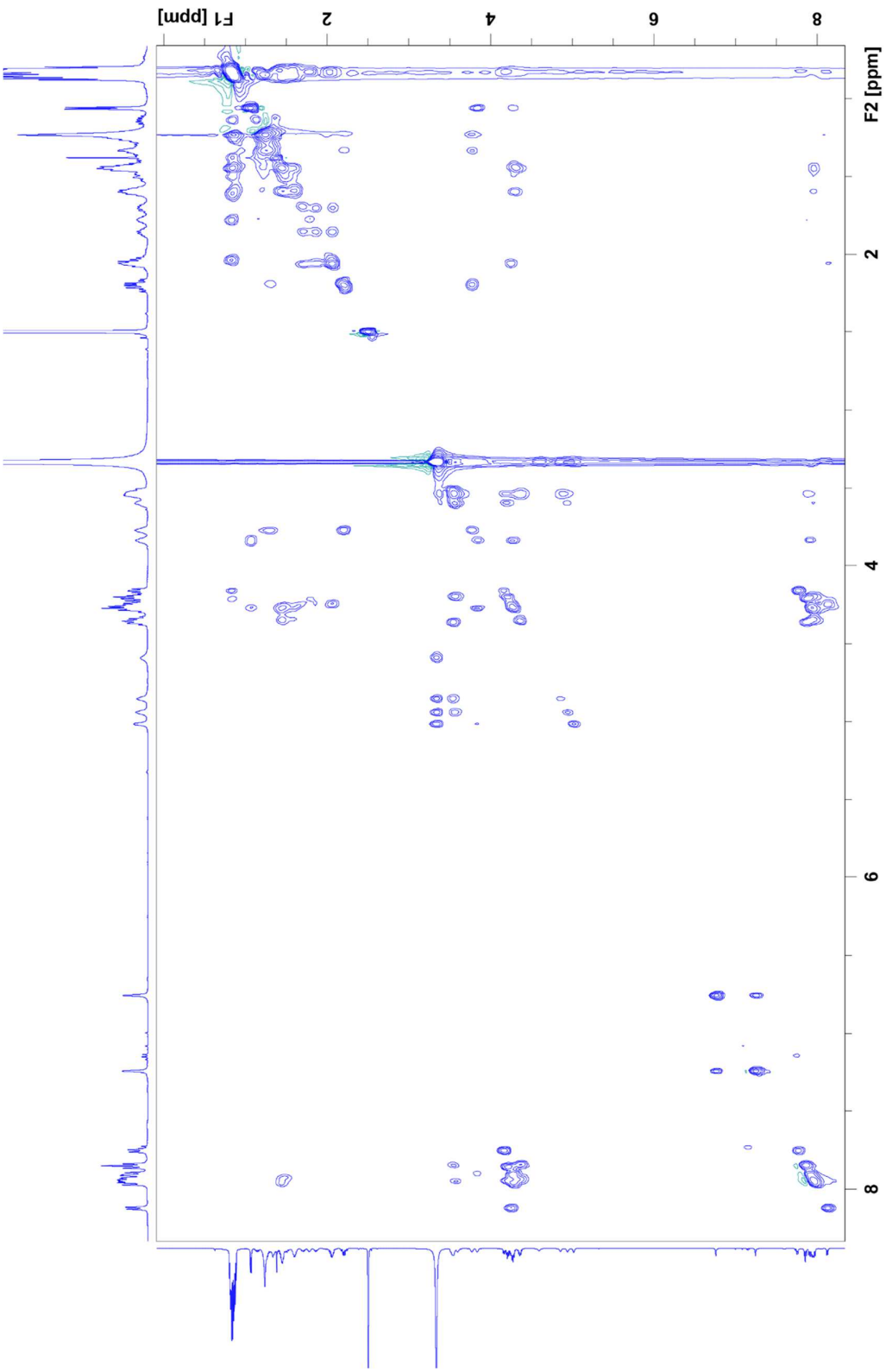


Fig. S28. ^{1}H-^{1}H TOCSY spectrum of pseudodesmin C (**5**) (DMSO-d_6, 300 K, 600 MHz).

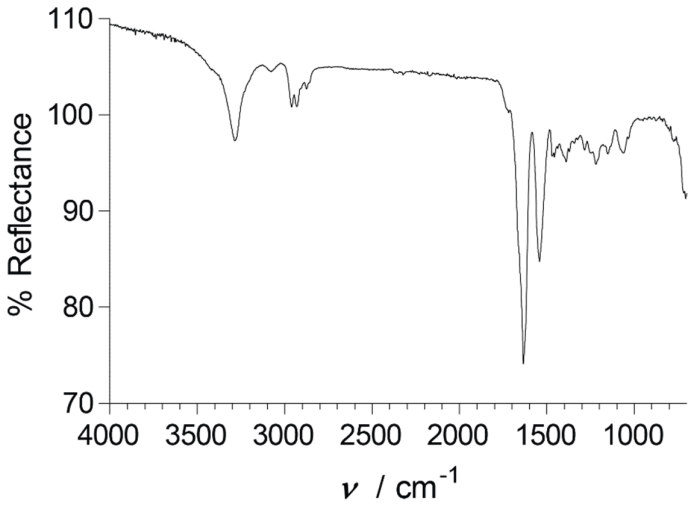


Fig. S29. IR spectrum of pseudodesmin C (**5**) recorded on a Jasco Fourier Transform Infrared Spectrometer 4100 using the ATR technique.

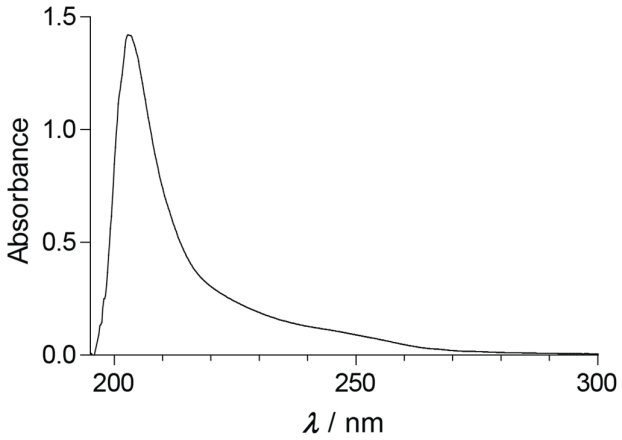


Fig. S30. UV absorption spectrum of pseudodesmin C (**5**) recorded on a Shimadzu UV-1800 spectrometer (dissolved in methanol).

Table S1. Strains used in this study.

Species	Strain	Relevant characteristics	Source or reference
E. coli	TOP10	General cloning host strain	Invitrogen
	BL21 (DE3)	General expression strain	Agilent
	S17-1 λpir	Conjugation strain	
P. tolaasii	DSM19342	Wild type, environmental isolate	DSMZ
	Δ*tolA*	1.7 kb deleted in *tolA*	This study
	Δ*pseB*	1.7 kb deleted in *pseB*	This study
M. tolaasinivorans	DSM15179	Wild type, environmental isolate	(6)
M. lacteus	DSM15177	Wild type, environmental isolate	(6)

Table S2. Primers used in this study. Capital letters indicate annealing basepairs; underlines mark restriction sites

Primer	Nucleotide sequence (5' to 3')	Restriction site
Tol-LA-fw	ggaagcataaatgtaaagcaAGCAAAAACGCCTGGCCC	-
Tol-LA-rev	cgtccagatcGAAGGTTTGGGGCTTCATACG	-
Tol-RA-fw	ccaaaccttcGATCTGGACGCAACGAGC	-
Tol-RA-rev	ggaaattaattaaggtaccgAAAGCGTTCGGCGTTGAG	-
Pse-LA-fw	ggaagcataaatgtaaagcaCTGCGCCACCTGGCGCGG	-
Pse-LA-rev	cttgctgcccCAGGCGCTGGTTGTCGAAGCGC	-
Pse-RA-fw	ccagcgcctgGGGCAGCAAGAGGTGCAG	-
Pse-RA-rev	ggaaattaattaaggtaccgCTGCAAGCCGAAGGCCAG	-
4841-fw	cgacatatGATGATTCCCCCGAGAGC	NdeI
4841-rev	cgaaagctttcaTCATGATCGTTTCCCTTC	HindIII
6011M-fw	cgacatatgCCCGGAGAAAACCTGACC	NdeI
6011M-rev	ccaggatccttATTACGCCCGGGCGTCG	BamHI
2172-fw	cgacaTATGAACGCCTCGAACCACACC	NdeI
2172-rev	ccaggatccttATTATTTGGTCTGGCCGTT	BamHI
1671-fw	cgacaTATGAACGCCTCGAACCACACC	NdeI
1671-rev	ccaggatccttATTATGCGCCGTGTCGGT	BamHI
1182L-fw	gcacatatgcTGCCCGGAGAAAACCTG	NdeI
1182L-rev	ccaggatcccTACTAGCGCTGCGCGTC	BamHI
5596-fw	cgacatATGACGTCTCCCAACTCGACC	NdeI
5596-rev	ccaggatcctcaTCACCAGATGGTGACG	BamHI
2821-fw	cgacaTATGGTTTCGACATCCGCATCC	NdeI
2821-rev	cgaaagcttctaCTAGTTGGTGGGCTCG	HindIII
3804-fw	cgacatATGGCTTCAGCATCCGCACC	NdeI
3804-rev	cgaaagcttctaCTAGTGCGCGGCTCCGTC	HindIII

Table S3. Plasmids used in this study.

Plasmid	Relevant characteristics	Source or reference
pEXG2	Conjugation plasmid, *sacB*, Gm^R	(7)
pEXG2-Tol	pEXG2 with homologues regions targeting *tolA*	This study
pEXG2-Pse	pEXG2 with homologues regions targeting *pseB*	This study
pJET1.2/blunt	Cloning vector	Thermo Scientific
pET-28c(+)	Expression vector, *lacI*, Kan^R	Merck KGaA
pET-28-4841	pET-28 containing gene for 4841	This study
pET-28-6011	pET-28 containing gene for 6011	This study
pET-28-2172	pET-28 containing gene for 2172	This study
pET-28-1671	pET-28 containing gene for 1671	This study
pET-28-1182	pET-28 containing gene for 1182	This study
pET-28-5596	pET-28 containing gene for 5596	This study
pET-28-2821	pET-28 containing *tdfL* (gene for 2821)	This study
pET-28-3804	pET-28 containing *tdfT* (gene for 3804)	This study

Table S4. Candidate proteins identified by bioactivity guided fractionation.

Protein	NCBI accession #	Strain	Size (aa)	Blast annotation
1182	WP_121688897.1	*M. lacteus*	849	aminopeptidase N
6011	WP_121648965.1	*M. tolaasinivorans*	849	aminopeptidase N
2172	WP_121687967.1	*M. lacteus*	473	dipeptidase
1671	WP_121647172.1	*M. tolaasinivorans*	473	dipeptidase
4841	WP_121688271.1	*M. lacteus*	460	insulinase family protein
5596	WP_121689693.1	*M. lacteus*	658	peptidase M13
2821 (TdfL)	WP_121688317.1	*M. lacteus*	738	S9 family peptidase
3804 (TdfT)	WP_121649136.1	*M. tolaasinivorans*	731	S9 family peptidase

Table S5. ^{1}H and ^{13}C assignments of pseudodesmin C **(5)** (DMSO-d_6, 300 K, 600 MHz) **.**

		^{13}C δ[ppm]	^{1}H δ[ppm]
HDA	CO	171.17	-
	$^{\alpha}CH_2$	43.4	2.21
	$^{\beta}CH$	67.43	3.77
	$^{\gamma}CH_2$	36.96	1.32
	$^{\delta}CH_2$	25.12	1.34 & 1.23
	$^{\varepsilon}CH_2$	29.05	1.24
	$^{\xi}CH_2$	28.74	1.24
	$^{\eta}CH_2$	31.27	1.24
	$^{\theta}CH_2$	22.1	1.26
	$^{\iota}CH_3$	13.97	0.86
	OH	-	4.59
Leu1	NH	-	7.96
	CO	172.33	-
	$^{\alpha}CH$	51.35	4.28
	$^{\beta}CH_2$	40.8	1.45
	$^{\gamma}CH$	24.17	1.6
	$^{\delta}CH_3$	23.2	0.86
	$^{\delta}CH_3$	21.59	0.81
Gln2	NH	-	8.12
	CO	171.28	-
	$^{\alpha}CH$	52.15	4.25
	$^{\beta}CH_2$	27.64	1.86 & 1.70
	$^{\gamma}CH2$	31.32	2.06
	$^{\delta}CO$	173.83	-
	NH_2	-	7.24 & 6.76
Thr3	NH	-	7.9
	CO	170.39	-
	$^{\alpha}CH$	58.43	4.27
	$^{\beta}CH$	67.13	3.84
	$^{\gamma}CH_3$	19.81	1.07
	OH	5.01	-
Val4	NH	-	7.75
	CO	170.86	-
	$^{\alpha}CH$	57.87	4.16
	$^{\beta}CH$	30	2.04
	$^{\gamma}CH_3$	19.16	0.86
	$^{\gamma}CH_3$	17.73	0.82

		^{13}C δ[ppm]	^{1}H δ[ppm]
Leu5	NH	-	7.93
	CO	172.19	-
	$^{\alpha}CH$	50.83	4.35
	$^{\beta}CH_2$	40.23	1.45
	$^{\gamma}CH$	24.01	1.6
	$^{\delta}CH_3$	23.12	0.86
	$^{\delta}CH_3$	21.28	0.81
Ser6	NH	-	7.94
	CO	169.91	-
	$^{\alpha}CH$	55.83	4.2
	$^{\beta}CH_2$	61.58	3.57
	OH	-	4.94
Leu7	NH	-	7.92
	CO	172	-
	$^{\alpha}CH$	51.19	4.28
	$^{\beta}CH_2$	40.66	1.45
	$^{\gamma}CH$	24	1.6
	$^{\delta}CH_3$	23	0.86
	$^{\delta}CH_3$	21.22	0.81
Ser8	NH	-	7.84
	CO	169.69	-
	$^{\alpha}CH$	54.84	4.37
	$^{\beta}CH_2$	61.9	3.53
	OH	-	4.85
Ile9	NH	-	7.86
	CO	172.86	-
	$^{\alpha}CH$	56.11	4.22
	$^{\beta}CH$	36.55	1.78
	$^{\gamma}CH_3$	13.97	0.86
	$^{\gamma}CH_2$	24.47	1.14 & 1.38
	$^{\delta}CH_3$	11.22	0.82

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