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Supporting Information

Enzyme-Primed Native Chemical Ligation Produces Autoinducing Cyclopeptides in Clostridia

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Experimental Procedures

Media components were purchased from Sigma, Roth and Difco. All chemicals were purchased from commercial suppliers (Sigma, Roth, *etc.*) without further purification. Oligonucleotide primer synthesis was performed by Eurofins Genomics. Restriction endonucleases were purchased from New England Biolabs. Standard PCRs and colony PCRs were performed using Phusion HF DNA polymerase and OneTaq Quick-Load 2 × Master Mix (New England Biolabs), respectively. All plasmids were constructed using either NEBuilder HiFi DNA Assembly Master Mix or T4 DNA ligase. For final verification, plasmids and PCR products were sequenced with GENEWIZ, Germany. All strains, plasmids and oligonucleotide primers used in this study are listed in Table S2, Table S3, and Table S4, respectively.

Bacterial strains and culturing conditions – *Escherichia coli* strains were grown in lysogeny broth (LB) shaken at 160 rpm or on LB agar plates at 37 °C with appropriate antibiotic selection (gentamicin, 200 µg · mL^{-1} and ampicillin 100 µg · mL^{-1}). All plasmid construction and storage was performed with *E. coli* TOP10.
Clostridia strains were cultivated under an anaerobic atmosphere (N_2:H_2:CO_2, 85:5:10 *v/v/v*) in a Whitley A35 anaerobic work station (Don Whitley Scientific) operating at 37 °C. To allow for gas exchange, cultures were grown in unsealed test tubes or glass bottles with a loosened lid. Routine cultivation of *Ruminiclostridium cellulolyticum* DSM 5812 strains (wild type and Δ*agrD1*) was performed in CM3 medium (modified).[1] For AIP production, *R. cellulolyticum* strains were cultivated in a modified version of VM medium[2] at pH 7.1. Medium components for 1 L VM (modified): 1 g KH_2PO_4, 3.4 g K_2HPO_4, 2.1 g urea, 10 g 3-(*N*-morpholino)propanesulfonic acid, 2 g yeast extract, 1 g $MgCl_2{\cdot}6H_2O$, 0.15 g $CaCl_2{\cdot}2H_2O$, 1.25 mg $FeSO_4{\cdot}7H_2O$, 1 g cysteine-HCl, 5.1 g cellobiose, 10 mL vitamin solution (250 mg · L^{-1} *p*-aminobenzoic acid, 250 mg · L^{-1} thiamine, 100 mg · L^{-1} D-biotin, 150 mg · L^{-1} nicotinic acid, 250 mg · L^{-1} riboflavin, 100 mg · L^{-1} cyanocobalamine, 270 mg · L^{-1} calcium pantothenate), 1 mL trace element solution (5 % (*v/v*) 10 M HCl, 5 g · L^{-1} $FeSO_4{\cdot}7H_2O$, 1.44 g · L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.12 g · L^{-1} $MnSO_4{\cdot}7H_2O$, 30 mg · L^{-1} H_3BO_3, 20 mg · L^{-1} $CoCl_2{\cdot}6H_2O$, 40 mg · L^{-1} $NiCl_2{\cdot}6H_2O$, 20 mg · L^{-1} Na_2SeO_4, 200 mg · L^{-1} $Na_2B_4O_7{\cdot}10H_2O$, 1 g · L^{-1} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$). Where appropriate, *R. cellulolyticum* cutures were supplemented with erythromycin (10 µg · mL^{-1} for agar and 10 µg · mL^{-1} for broth). *Ruminiclostridium josui* DSM 25723 and *Clostridium acetobutylicum* DSM 792 were grown in M122 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) and P2 medium,[3] respectively. *Clostridium cellulovorans* DSM 3052 and *Ruminiclostridium papyrosolvens* DSM 2782 were grown in CM3 medium (modified).

Generation of bacterial metabolite profiles – Clostridia cultures were grown until stationary phase, at which time each culture was extracted with 1 volume of ethyl acetate. The extract was dried over Na_2SO_4 and the ethyl acetate was removed using a rotary-evaporator. The resultant solid was dissolved in methanol to give a 500 × concentrate of the extract, then analyzed by HPLC-HRMS.

Plasmid construction – In order to generate an in-frame nonsense mutation in the genome of *R. cellulolyticum*, a knockout plasmid was generated as previously described with minor changes.[4] Briefly, the webtool CRISPy-web[5] was used to find a suitable target site containing the necessary PAM (NGG) sequence for knockout of the gene encoding the AIP precursor (Ccel_2126) via the CRISPR-nCas9 system.[6] A cassette containing the P4 promoter regulated sgRNA as well as the homologous arms (approx. 300 bp) surrounding the mutated N20 sequence was obtained by gene synthesis (Genewiz, Germany) and can be found in Table S5. This cassette was cut from the supplied vector with *Bsa*I and ligated with *Bsa*I-digested pCasC to give pCasC-Ccel2126-KO.
For genetic complementation, a plasmid expressing Ccel_2126 under the control of a constitutive promoter (P*thl*) and the corresponding empty vector control were constructed as follows. The shuttle plasmid pSOS95-oriT (unpublished data), that originates from pSOS95 and additionally contains the origin of transfer (*oriT*/*traJ* region) for conjugational plasmid transfer, was *Bam*HI/*Nar*I-digested. The gene Ccel_2126 was amplified from genomic DNA of *R. cellulolyticum* using primers JK744/JK745. The *thl* promoter together with a cloning site was generated by annealing of primers JK742/JK743. Both inserts were individually assembled with the plasmid backbone and resulted in pCPthl-*agrD1* and pCPthl-empty, respectively.
After isolation, all plasmids were verified by sequencing and prepared for electroporation into *R. cellulolyticum* or *R. cellulolyticum* Δ*agrD1* by methylation using the *Msp*I methyl transferase.[7]

Generation of *R. cellulolyticum* Δ*agrD1* and its genetic complementation – *R. cellulolyticum* Δ*agrD1*, which contains an in-frame nonsense mutation in Ccel_2126, was generated using pCasC-Ccel2126-KO as previously described.[4] The design of pCasC-Ccel2126-KO was such that successful editing of the target gene would introduce the desired mutation (an in-frame stop codon, TAA), as well as an *Spe*I restriction endonuclease site (ACTAGT) to facilitate screening of transformants. After electroporation, individual potential *R. cellulolyticum* Δ*agrD1* colonies were randomly picked and subjected to colony PCR using OneTaq DNA Polymerase with primers JK683 and JK684. The presence or absence of the restriction site (corresponding to edited or unedited Ccel2126) was established by digestion of the generated PCR products (1,026 bp) with restriction endonuclease *Spe*I, followed by agarose gel electrophoresis (1.5 % agarose gel) of the fragments. Pure mutant colonies were identified by the presence of the expected fragments of (580 bp and 446 bp) and lack of full-length PCR product (1,026 bp), while the PCR product remained undigested in the case of the wild-type control. In order to ensure the chosen mutant was free of wild-type contamination, a PCR using primers JK683 and JK685 was done. Primer JK685 binds in the unmutated N20 sequence of Ccel_2126 and thus only gives a product (460 bp) if wild-type cells remain. The purification of the undigested ΔCcel_2126 PCR product was performed using a Monarch PCR and DNA Cleanup Kit (New England Biolabs) and DNA sequencing with primer JK683 was used to confirm the presence of the desired mutation (Figure S12). The strain was subcultured in the absence of antibiotic selection until erythromycin resistance was lost, then lack of the KO plasmid was confirmed by PCR. For genetic complementation, the cured mutant strain *R. cellulolyticum* Δ*agrD1* was transformed with pCPthl-*agrD1* and pCPthl-empty following the same electroporation procedure as described elsewhere.[4] Transformants were selected on erythromycin-containing plates and verified by colony PCR.

Endospore formation assay – *R. cellulolyticum* strains (wild type and Δ*agrD1*) were grown in 5 mL of CM3 broth to early exponential phase (OD_{600nm} of 0.25–0.35). From these, 2.5 % inocula were made in three 1.5 mL volumes of VM broth supplemented with 0.5 % $CaCO_3$ (VM-Ca) to give three technical replicates for each assay condition. For *R. cellulolyticum* Δ*agrD1*, synthetic peptides were freshly dissolved in dimethyl sulfoxide (DMSO) and individually added to the VM-Ca cultures to give the desired final test concentration of **2**, **3**, **4** or **5** (0.5 % DMSO final concentration). Each time the assay was performed, untreated *R. cellulolyticum* wild type and *R. cellulolyticum* Δ*agrD1* cultures (containing 0.5 % DMSO final concentration) were included as positive and negative controls, respectively.
After 3 days incubation to enable sporulation, a 200 µL sample of each culture was heated to 80 °C for 15 min to kill vegetative cells. Serial dilutions (10^0–10^{-2}) of the heat-treated cell suspensions were performed in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7) and 20 µL aliquots were spotted onto VM agar.[8] Heat-resistant colony-forming units (CFU) that appeared after 5 days of incubation were counted. For each assay condition, the mean of the three technical replicates was calculated and the value noted as a biological replicate; the entire procedure was repeated on at least three separate occasions to give a minimum of three biological replicates. Statistical analysis was conducted using the unpaired t-test with Welch's correction in Graph Pad Prism 8.4.3.
Directly after addition to the cultures, the remainder of the stock solutions in DMSO were lyophilized and subjected to HPLC-HRMS to ensure the purity and condition of the compounds at the point of addition. In the case of **4**, an additional experiment was performed to check its stability under conditions mimicking those of the culture medium (at 37 °C in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.2) (Figure S29).

Bioinformatic analyses – Given the high level of sequence divergence in both the AgrB and AgrD protein families, putative AIP biosynthetic gene clusters in the Clostridia genomes were identified using PSI-BLAST with a concatenated query sequence comprised of previously reported AgrB/AgrD proteins. For this analysis, sequences from the following bacteria were used: *Staphylococcus aureus*, *Clostridioides difficile*, *C. acetobutylicum*, *Clostridium perfringens*, and *Clostridium botulinum*.
The enzyme responsible for leader peptide removal has not been identified in the Clostridia, but based on staphylococcal AIP biosynthesis, it is likely a general protease. We used 16S phylogeny for strain selection with the assumption that closely related strains would be more likely to have leader peptidases with identical patterns of specificity, while more distantly strains would be less likely. In this way, we aimed to test whether cAIP biosynthesis from an NTE-free precursor is a general feature of the Clostridia. For the 16s rRNA phylogenetic tree, sequences were aligned with MAFFT 7[9] and the alignment was manually adjusted. The phylogenetic tree was reconstructed using the neighbor-joining method with the maximum composite likelihood model and 1,000 bootstrap replicates in MEGA6.[10] The AgrB and AgrD multiple sequence alignments were performed with Clustal Omega using the default parameters.[11] The sequence logo diagram was made using the WebLogo[12] webtool and the AgrD sequence alignments using the default parameters.

Thin Layer Chromatography (TLC) – Merck precoated silica gel plates (60F-254) were used. Compounds were visualized using ultraviolet light irradiation at 254 nm and 366 nm or by using the following staining agents (dip, dry and heat development).
Staining solution A: $KMnO_4$ (1.00 g), K_2CO_3 (6.60 g), 5 % NaOH (1.70 mL) in H_2O (90 mL).
Staining solution B: Ninhydrin (1.00 g) in EtOH/HOAc (97:3, 100 mL).

Silica Gel Flash Chromatography – Purifications were performed using silica gel from Macherey & Nagel (particle size 40–60 µm) under approximately 0.3 bar pressure.

NMR – All NMR spectra (1D (^{1}H, ^{13}C and DEPT-135) and 2D (COSY, HSQC, HMBC)) were recorded on a Bruker Avance I 250, Fourier 300, Avance III 400, Avance III HD 500 or Avance III 600 system (600 MHz with Bruker cryo platform). The solvents used are deuterated (Deutero GmbH). Chemical shifts are given in ppm relative to the external standard Me_4Si. Residual peaks of the respective solvent were used as an internal standard (DMSO-d_6: δ_H = 2.50 ppm, δ_C = 39.52 ppm; MeOD-d_4: δ_H = 3.31 ppm, δ_C = 49.00 ppm, MeCN-d_3: δ_H = 1.94 ppm, δ_C = 118.26 ppm). The abbreviations used to describe the NMR spectra are as follows: s = singlet, d = doublet, t = triplet, q = quartet, qt = quintet, br = broad.

HPLC-HRMS and HPLC-HRMS/MS method A – This HPLC/HRMS analysis was performed on a Thermo Accela HPLC-system coupled to a QExactive Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) mass spectrometer equipped with an electrospray ion source. Separation was performed with an Accucore C18 column (2.1 × 100 mm, 2.6 µm, Thermo Fisher) operating at a flow rate of 200 µL · min^{-1}, with water + 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: starting 5 % solvent B to 98 % solvent B in 10 min, hold 98 % solvent B for 4 min.

HPLC-HRMS method B – This HPLC/HRMS analysis was performed on a Thermo Accela HPLC-system coupled to a Exactive Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) mass spectrometer equipped with an electrospray ion source. Separation was performed with an Betasil C18 column (2.1 × 150 mm, 3 µm, Thermo Fisher) operating at a flow rate of 200 µL · min^{-1}, with water + 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: starting 5 % solvent B for 1 min, then 5 % solvent B to 98 % solvent B in 15 min, then hold 98 % solvent B for 15 min.

HPLC-HRMS and HPLC-HRMS/MS method C – This HPLC/HRMS analysis was performed on a Thermo Ultimate3000 UHPLC-system coupled to a QExactive Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) mass spectrometer equipped with an electrospray ion source. The separation was performed on a Accucore C18 column (2.1 × 100 mm, 2.6 µm, Thermo Fisher) operating at a flow rate of 200 µL · min^{-1}, with water + 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: starting 5 % solvent B to 98 % solvent B in 10 min, hold 98 % solvent B for 4 min.

HPLC-HRMS and HPLC-HRMS/MS method D – This HPLC/HRMS analysis was performed on a Thermo Ultimate3000 UHPLC-system coupled to a QExactive Hybrid-Quadrupole-Orbitrap (Thermo Fischer Scientific) mass spectrometer equipped with an electrospray ion source. The separation was performed on a Accucore C18 column (2.1 × 100 mm, 2.6 µm, Thermo Fisher) operating at a flow rate of 200 µL · min^{-1}, with water + 0.1 % formic acid (solvent A) and acetonitrile + 0.1 % formic acid (solvent B) and the following gradient: starting 5 % solvent B to 98 % solvent B in 7 min, hold 98 % solvent B for 3 min.

HPLC-HRMS method E – High-resolution mass spectrometry (HR-MS) measurements for sum formula confirmation were performed on a LC-coupled MAXIS Impact ESI-TOF spectrometer (Bruker Daltronics, Bremen, Germany). Calculated masses were obtained using the software *ChemDraw Ultra* (CambridgeSoft Corporation) or *Xcalibur*. (Thermo Fisher Scientific)

HPLC- MS method A – Analyses were performed on a Shimadzu system with electrospray ion source consisting of a system controller (SLC-10AVP), a column oven (CTO-10ACVP), an auto-injector (SIL-10ADVP), a degasser (DGU-14A), two pumps (LC-10ATVP), a UV-vis-detector (SPD-10AVP), and a splitter (ICP 04-20). Eluents used: CH_3CN (A), water (B) containing 0.1 vol % HCOOH. Mass spectra were recorded by an ESI ion trap mass spectrometer (Finnigan Mat LCQ). The column used was a NUCLEODUR C18 Gravity 5 µm and the following gradient was applied: 10 min 10–95 % acetonitrile, 5 min 95 % acetonitrile, 3 min 10 % acetonitrile. Flow rate: 1 mL · min^{-1}.

HPLC- MS method B – Analyses were performed on a Shimadzu system with electrospray ion source consisting of a system controller (SLC-10AVP), a column oven (CTO-10ACVP), an auto-injector (SIL-10ADVP), a degasser (DGU-14A), two pumps (LC-10ATVP), a UV-vis-detector (SPD-10AVP), and a splitter (ICP 04-20). Eluents used: CH_3CN (A), water (B) containing 0.1 vol % HCOOH. Mass spectra were recorded by an ESI ion trap mass spectrometer (Finnigan Mat LCQ). The column used was a NUCLEODUR C18 Gravity 5 µm and the following gradient was applied: 10 min 30–95 % acetonitrile, 5 min 95 % acetonitrile, 3 min 30 % acetonitrile. Flow rate: 1 mL · min^{-1}.

HPLC-MS method C – LTQ Velos Ion Trap Benchtop LC-MS with electrospray ion source and Surveyor HPLC system (Thermo Fisher Scientific, Bremen). HPLC conditions using LTQ: C18 column (Phenomenex Kinetex XB-C18, 2.6 µm, 100 × 3 mm) and gradient elution (MeCN (0.1 % (*v/v*) HCOOH) / H_2O (0.1 % (*v/v*) HCOOH)) 10/90 for 1 min, going up to 100/0 in 8 min, then 100/0 for 4 min; flow rate 0.6 mL · min^{-1}; injection volume: 5 µL).

Preparative HPLC method A – This purification was performed on a Shimadzu model (LC-8A pump system) equipped with a diode array detector (type: SPD-M20A) and a Phenomenex Luna 10 u C18(2) 100 A AXIA (250 × 21.2 mm, 2 µm). The mobile phase was composed of H_2O + 0.1 % TFA and acetonitrile (ACN). The mobile phase was changing as follows: 0.01–4 min isocratic 25 % ACN, 4–24 min gradient 25 % to 100 % ACN. The flow rate was 18 mL · min^{-1}.

Preparative HPLC method B – This purification was performed on a Shimadzu model (LC-8A pump system) equipped with a diode array detector (type: SPD-M20A) and a Nucleodur C18 HTec VP250/10. The mobile phase was composed of H_2O + 0.1 % TFA and ACN. The mobile phase was changing as follows: 0.01−4 min isocratic 25 % ACN, 4–24 min gradient 25 % to 100 % ACN. The flow rate was 5 mL · min^{-1}.

Preparative HPLC method C – Separations were performed on a Shimadzu system consisting of a controller (SLC-10AVP), two pumps (LC-8A), and a UV-vis-detector (SPD-10AVP). Eluents used: CH_3CN (A), water (B) containing 0.1 vol % TFA. The column used was a NUCLEODUR C18 Gravity 5 µm VP 250/16 and the following gradient was applied: 30 min 30–95 % acetonitrile, 20 min 95 % acetonitrile, 10 min 30 % acetonitrile. 0.1 % TFA. Flow rate: 10 mL · min^{-1}. Detection at 220 nm.

Amide-coupling on solid support method A – A mixture of ethyl cyano(hydroxyimino)acetate (Oxyma Pure), 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy) dimethylaminomorpholino)] uronium hexafluorophosphate (COMU), diisopropyl ethyl amine (DIPEA) and the respective amino acid in 10 mL dimethylformamide (DMF) was added to the resin. The reaction was incubated rotating for the indicated amount of time until complete coupling was verified with the Kaiser test.

Amide-coupling on solid support method B – A solution of the respective (amino) acid, HBTU, HOBt and Et*N*iPr_2 in DMF (3 mL) was added to the resin and the mixture was shaken at room temperature for 2–3 h. The solvent was removed and the resin was washed with DMF (3 × 3 mL) and CH_2Cl_2 (3 × 3 mL). After evaporation of the solvents, the resin was stored at −25 °C under N_2-atmosphere.

Kaiser test. Three solutions were prepared: 1.) 1.7 mg KCN in 2.5 mL distilled water, 100 µL of this solution were mixed with 5 mL pyridine. 2.) 250 mg ninhydrin in 5 mL *n*-BuOH. 3.) 10 g Phenol in 5 mL *n*-BuOH. Some beats were removed after each coupling step and washed with DCM (2 × 1 mL). Two drops of each solution were added, and everything was heated to 110 °C. The color was compared with a negative and positive control after 5 minutes.

Fmoc-Deprotection on solid support method A – The resin was washed with DMF (2 × 10 min) and treated twice with piperidine (20 vol % in DMF, 3 × 3 min). The resin was washed three times with alternating DMF (10 mL) and isopropanol (10 mL).

Fmoc-Deprotection on solid support method B – The resin was swollen in DMF for 10 min and treated twice with piperidine (20 vol % in DMF, 5 min and 15 min). The resin was washed with DMF.

Cleavage of the peptide from the solid-support method A – The swollen resin was treated with a solution of hexafluoroisopropanol (10 vol % in CH_2Cl_2, 4 × 10 min). After each cleavage, the resin was washed with CH_2Cl_2

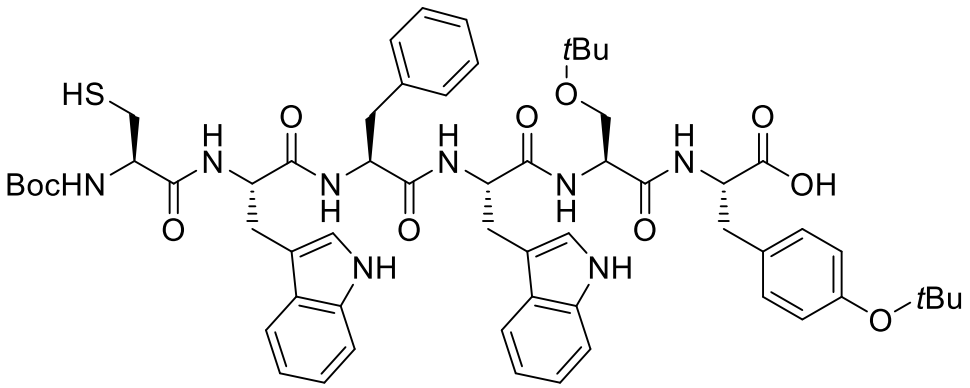
(4 × 5 min). The solvent was removed and the crude product was treated with water and saturated NaCl solution. After addition of CH_2Cl_2, the phases were separated and the organic layer was dried down. The crude was then purified using preparative HPLC (method A) to result in Boc- and *t*Bu-protected linear product.

Cleavage of the peptide from the solid-support method B – The swollen resin was treated with a solution of hexafluoroisopropanol (30 vol % in CH_2Cl_2, 3 × 30 min). After each cleavage, the resin was washed with CH_2Cl_2 (3 × 30 min). Toluene was added to the combined filtrates and the solvents were evaporated under reduced pressure to yield the corresponding peptide.

Determination of the half-life of thiolactone 1 using Ellman's reagent (ER) – The protected thiolactone **1b** (0.3 mg) was dissolved in TFA (300 µL) to give a 1 mg · mL^{-1} solution. The solution was aliquoted (100 µL) to wells of a 96-well plate to yield replicates and incubated for 30 minutes at room temperature. The samples were then dried under a stream of nitrogen and stored at 1 mbar at room temperature for 20 minutes to yield the crude thiolactone **1**. In the meantime, fresh 0.1 mM ER stock solution was prepared in 0.1 M phosphate buffer, pH 8 with 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 % DMSO. The plate was then placed into a Varioscan plate reader (Thermo Fisher) and the reaction was initiated by adding 50 mM MOPS buffer (pH 7.11, 100 µL) and ER (100 µL) to the wells using the auto injector system. After shaking, the absorption at 412 nm was measured approximately 10 sec after the addition of ER over the time course of 30 minutes at 37 °C. The experiment was performed on three different days as described (to give three experiments), with the samples aliquoted to give three replicates in two cases and two replicates in one case (Figure S11). Along with the reaction measurements (a) and (b), the following controls were measured (Figure S30): (c) 0.5 mM cysteine with ER; (d) ER; (e) ER in 50 mM MOPS buffer, pH 7.11; (f) thiolactone **1** in 50 mM MOPS buffer (without ER), pH 7.11; (g) water; (h) 50 mM MOPS buffer, pH 7.11.

General procedure for chemical probes – The crude extracts were dissolved in MeOH (~50 µL). For the NaOH treatment, an equal volume of NaOH (1 M) was added. After 1 h at room temperature the solution was filtered and measured by HPLC-HRMS. For the maleimide treatment *N*-benzylmaleimide (10 % in MeCN, 1 µL) was added. After 1 h at room temperature the solution was filtered and measured by HPLC-HRMS.

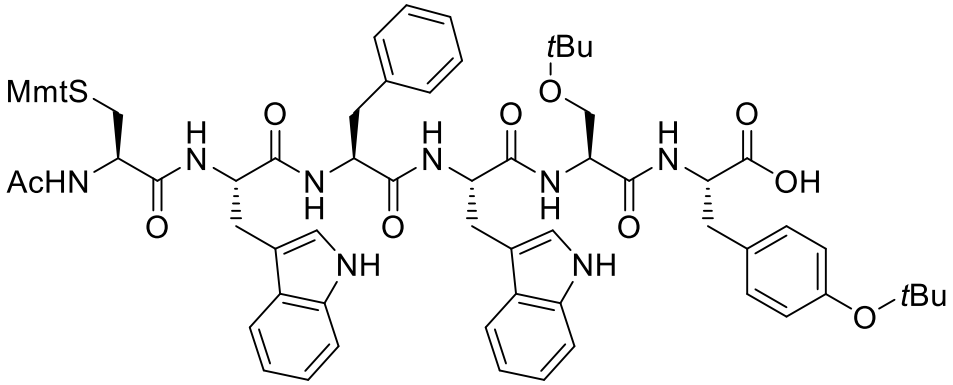
Solid phase peptide synthesis of Boc- and tBu-protected linear peptide 1a:



1a

Solid phase peptide synthesis was used to synthesise the linear protected peptide. The resin (1 g) loaded with protected tyrosine (0.78 mmol) was incubated in 17 mL DMF for 15 hours in a syringe. For the coupling, the solvent was removed and a mixture of ethyl cyano(hydroxyimino)acetate (Oxyma Pure), COMU, DIPEA and the respective amino acid in 10 mL DMF was added to the resin. The reaction was incubated rotating for the indicated time (see Table S7) until complete coupling was verified with the kaiser test. After each coupling and Fmoc deprotection step some beats were removed and washed with DMF (2 × 1 mL) and DCM (2 × 1 mL). They were treated with 20 % HFIP in isopropanol (1 mL, 15 min) to cleave the peptide from the resin and the solution was analyzed by HPLC-MS method C. The Fmoc protecting group was fully removed and the correct amino acid sequence could be validated. For Fmoc deprotection and peptide cleavage methods A were used with the respective amounts and times for every coupling step. The combined fractions gave 1.3 g of crude peptide, from which 605.6 mg were then treated with 10 % TFA (2 mL) and 2 % triethylsilane in DCM for 5 min before addition of 10 % pyridine in MeOH (2 mL) to quench the reaction. The solvent was removed and the crude product was taken up in DCM and washed with water and saturated NaCl solution. The organic layer was evaporated to give 441 mg crude product. The crude was then purified using preparative HPLC (method A) to result in Boc- and *t*Bu-protected linear peptide **1a** (240 mg, 0.22 mmol, 28 %). ^{1}H-NMR (500 MHz, DMSO-d_6): δ [ppm] = 12.73 (br, 1H), 10.75 (s, 1H), 10.70 (s, 1H), 8.14 (d, *J* = 7.7 Hz, 1H), 8.02 (d, *J* = 7.9 Hz, 1H), 7.99 (d, *J* = 7.9 Hz, 1H), 7.92–7.88 (m, 2H), 7.59 (d, *J* = 7.7 Hz, 1H) , 7.53 (d, *J* = 7.7 Hz, 1H), 7.30 (t, *J* = 9.5 Hz, 2H), 7.20–7.10 (m, 8H), 7.06–7.00 (m, 3H), 6.96 (t, *J* = 7.3 Hz, 1H), 6.91 (t, *J* = 7.5 Hz, 1H), 6.83 (d, *J* = 8.3 Hz, 3H), 4.65 (dt, *J* = 10.5 Hz, *J* = 5.6 Hz, 1H), 4.56–4.52 (m, 2H), 4.47 (dt, *J* = 10.5 Hz, *J* = 6.2 Hz, 1H), 4.37 (dt, *J* = 9.6 Hz, *J* = 7.1 Hz, 1H), 4.00 (dt, *J* = 4.5 Hz, 10.1 Hz, 1H), 3.44–3.41 (m, 1H), 3.37 (dt, *J* = 7.7 Hz, *J* = 2.1 Hz, 1H), 3.14 (dt, *J* = 5.2 Hz, *J* = 14.9 Hz, 1H), 3.06–2.98 (m, 4H), 2.92–2.86 (m, 2H), 2.77 (dt, *J* = 11.5 Hz, *J* = 4,8 Hz, 1H), 2.66–2.60 (m, 1H), 2.54 (t, *J* = 8,3 Hz, 1H), 2.09 (t, *J* = 8.5 Hz, 1H), 1.36 (s, 9H), 1.22 (s, 9H), 1.07 (s, 9H), ^{13}C-NMR (75 MHz, DMSO-d_6): δ [ppm] = 172.9, 171.5, 171.2, 170.4, 169.8, 155.7, 154.1, 138.0, 136.5, 132.2, 130.1, 129.6, 128.4, 127.9, 127.8, 126.6, 124.0, 123.9, 123.8, 121.3, 119.0, 118.9, 118.7, 118.6, 111.7 111.7, 110.3, 110.3, 78.9, 78.1, 73.4, 62.1, 57.2, 54.2, 53.9, 53.7, 53.6, 38.1, 36.7, 29.0, 28.6, 27.6, 26.9. HRMS (ESI$^+$-Orbitrap) *m/z* calculated for $C_{59}H_{74}N_8O_{11}S^+$: 1,103.5271 $[M+H]^+$; found: 1,103.5263 $[M+H]^+$.

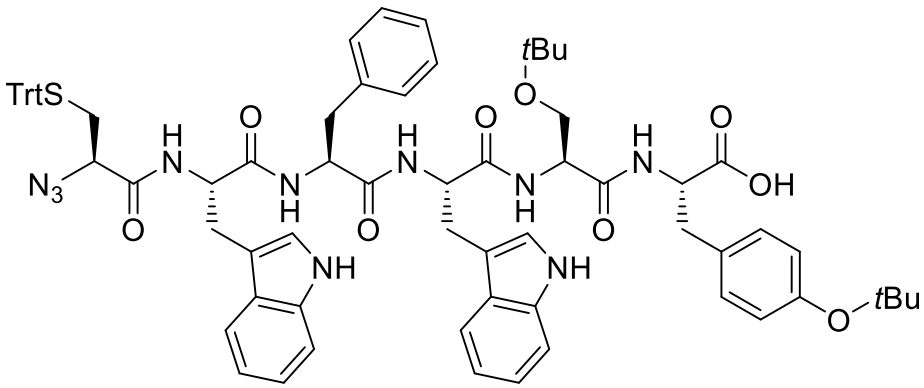
Solid phase peptide synthesis of Mmt-, Ac- and *t*Bu-protected linear peptide 4a:



4a

2-Chlorotrityl chloride resin (1.00 g, 0.8 mmol, 0.80 mmol · g^{-1}) was washed with CH_2Cl_2 (2 · 10 mL), swollen in CH_2Cl_2 (10 mL) and then shaken with Fmoc-Tyr(*t*Bu)-OH (368 mg, 800 µmol) and EtN*i*Pr_2 (685 µL, 4.00 mmol) in CH_2Cl_2/DMF (10 mL, 1:1 *v/v*) for 3 h. The resin was washed with CH_2Cl_2 (3 × 10 mL) and then treated with a solution of CH_2Cl_2/MeOH/EtN*i*Pr_2 (10 mL, 17:2:1 *v/v/v*, 3 × 1 min). The solvent was removed and the resin was washed with CH_2Cl_2 (4 × 10 mL), DMF (4 × 10 mL), and MeOH (4 × 10 mL) and then dried under reduced pressure. The loading was determined to be 0.68 mmol · g^{-1} by a test cleavage and measurement of the absorption of the formed piperidine-fulvene adduct.[2] The first four amino acids were connected using amide coupling and Fmoc deprotection methods B and the respective amounts and times. For the preparation of Ac protected peptide **4a**, a part of the resin (after step A: 460 mg, 0.48 mmol · g^{-1}, 221 µmol) was used. The resin was swollen and the Fmoc-group was cleaved as described in Fmoc-deprotection method B. Then, the resin was shaken with Fmoc-Cys(Mmt)-OH (544 mg, 880 µmol), HBTU (335 mg, 880 µmol) and HOBt (135 mg, 880 µmol) and EtN*i*Pr_2 (302 µL, 1.77 mmol) in DMF (4 mL) for 16 h. The resin was washed with DMF (4 × 4 mL) and MeOH (4 mL) and dried under reduced pressure. The such obtained resin (527 mg, 0.42 mmol · g^{-1}) was split up (2 × 260 mg, 2 × 109 µmol). For both fractions, the *N*-terminal Fmoc group was cleaved as described in Fmoc-deprotection method B. Both batches of resin were washed with DMF (4 × 4 mL) and MeOH (4 mL) and then dried under reduced pressure. For Ac protection of the *N*-terminal amino acid, the resin (260 mg, 0.42 mmol · g^{-1}, 109 µmol) was shaken with Ac_2O/DMF/pyridine (1.8 mL, 1:3:2 *v/v/v*) for 2 h. The peptide was cleaved from the resin as described in GP3 to afford peptide **4a** as a colorless solid which was used in the next step without further purification or characterization (123 mg, 93.3 µmol, 86 %). HPLC-MS method B (ESI^+): *m/z* = 1,318.1 $[M+H]^+$, 1,334.6 $[M+NH_4]^+$.

Solid phase peptide synthesis of Mmt- and tBu-protected linear peptide 5a:

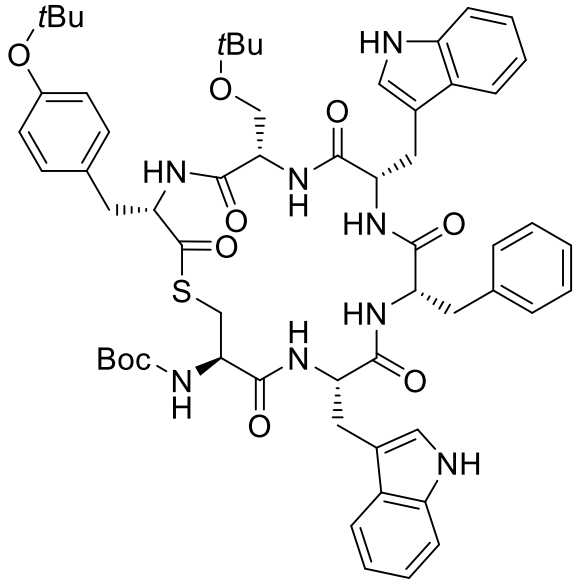


5a

2-Chlorotrityl chloride resin (1.00 g, 0.8 mmol, 0.80 mmol · g^{-1}) was washed with CH_2Cl_2 (2 × 10 mL), swollen in CH_2Cl_2 (10 mL) and then shaken with Fmoc-Tyr(*t*Bu)-OH (368 mg, 800 µmol) and EtN*i*Pr_2 (685 µL, 4.00 mmol) in CH_2Cl_2/DMF (10 mL, 1:1 *v/v*) for 3 h. The resin was washed with CH_2Cl_2 (3 × 10 mL) and then treated with a solution of CH_2Cl_2/MeOH/EtN*i*Pr_2 (10 mL, 17:2:1 *v/v/v*, 3 × 1 min). The solvent was removed and the resin was washed with CH_2Cl_2 (4 × 10 mL), DMF (4 × 10 mL), and MeOH (4 × 10 mL) and then dried under reduced pressure. The loading was determined to be 0.68 mmol · g^{-1} by a test cleavage and measurement of the absorption of the formed piperidine-fulvene adduct.[2] The first four amino acids were connected using amide coupling and Fmoc deprotection methods B and the respective amounts and times. For the preparation of peptide **5a** the resin (after step A: 250 mg, 0.48 mmol · g^{-1}, 120 µmol) was shaken with (*R*)-2-azido-3-(tritylthio)propanoic acid (187 mg, 480 µmol), HBTU (182 mg, 480 µmol) and HOBt (73.5 mg, 480 µmol) and EtN*i*Pr_2 (164 µL, 960 µmol) in DMF (4 mL) for 16 h. The resin was washed with DMF (4 × 4 mL) and CH_2Cl_2 (4 × 4 mL). The peptide was then cleaved from the resin as described in cleavage method B. Flash chromatography (25 g silica, petrol ether/acetone/HCOOH 1:1:1 *v/v/v*) afforded peptide **5a** as a colorless solid (113 mg, 88.9 µmol, 74 %, **d.r.* = 3:2). ^{1}H-NMR (methanol-d_4, 400 MHz): δ [ppm] = 7.52 (d, *J* = 8.3 Hz, 1H), 7.48 (d, *J* = 5.6 Hz, 1H), 7.37–7.32 (m, 6H), 7.29 (d, *J* = 10.7 Hz, 2H), 7.26–7.20 (m, 6H), 7.20–7.15 (m, 3H), 7.12–7.03 (m, 8H),

7.01–6.91 (m, 5H), 6.86 (d, *J* = 8.4 Hz, 2H), 4.66 (m, 2H), 4.53 (m, 2H), 4.38 (m, 1H), 3.52 (td, *J* = 9.1, 4.6 Hz, 1H), 3.29–2.97 (m, 8H), 2.90 (m, 1H), 2.70 (m, 1H), 2.55 (m, 1H), 2.44 (dd, *J* = 13.2, 8.4 Hz, 0.4H), 2.33 (dd, *J* = 13.2, 8.0 Hz, 0.6H), 1.25 (s, 9H), 1.08 (s, 5.3H), 1.07 (s, 3.6H) ppm. ^{13}C-NMR (methanol-d_4, 101 MHz): δ =174.1, 173.54, 173.45, 173.2, 173.11, 173.08, 171.7, 170.2, 170.1, 155.3, 145.7, 138.01, 137.97, 133.2, 131.0, 130.7, 130.32, 130.28, 129.5, 129.4, 129.1, 128.8, 128.8, 128.0, 127.7, 125.2, 124.7, 122.6, 122.5, 120.0, 119.9, 119.5, 119.4, 112.4, 110.8, 110.6, 110.32, 110.25, 79.5, 74.9, 68.3, 68.2, 63.2, 63.2, 62.5, 62.5, 56.0, 55.7, 55.6, 55.5, 55.4, 55.2, 55.0, 54.9, 38.4, 37.8, 35.1, 29.2, 28.6, 27.7 ppm. HRMS (ESI$^+$-TOF): *m/z* calculated for $C_{73}H_{79}N_{10}O_9S^+$: 1,271.5747 $[M+H]^+$; found: 1,271.5746 $[M+H]^+$.

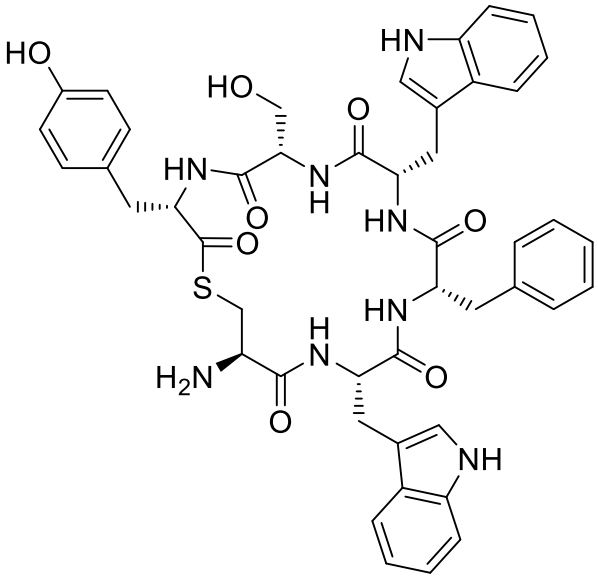
Synthesis of cyclic peptide 1b:



1b

The thiolactone formation was performed using the purified compound **1a** (135.6 mg; 0,123 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 63,5 mg; 0.415 mmol; 3.4 eq.), *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 129.4 mg; 0.675 mmol; 5.5 eq.), DIPEA (127 µL, 1.324 mmol; 10.8 eq.) and *N*,*N*-dimethylpyridine-4-amine (DMAP, 92.6 mg, 0.76 mmol, 6.2 eq.) and dissolving all in 140 mL DMF. The reaction was stirred at 20 °C until no starting material could be detected using HPLC-MS method C (~ 21 h). The thiolactone was then purified using preparative HPLC (method B) to yield compound **1b** (43.8 mg, 32 % yield). ^{1}H-NMR (500 MHz, DMSO-d_6): δ [ppm] = 10.91 (s, 1H), 10.75 (s, 1H), 9.08 (d, *J* = 4.1 Hz, 1H), 8.50 (dt, *J* = 10.9 Hz, *J* = 4.2 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 1H), 7.44 (d, *J* = 7.9 Hz, 1H), 7.35 (d, *J* = 7.9 Hz, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 7.27 (d, J = 8.3 Hz, 2H), 7.19–7.09 (m, 7H), 7.03 (q, *J* = 7.4 Hz, 2H), 6.97 (2d, *J* = 7.4 Hz, *J* = 7.1 Hz, 2H), 6.90 (d, *J* = 8.3 Hz, 2H), 4.73 (q, *J* = 7.6 Hz, 1H), 4.51–4.43 (m, 2H), 4.10 (s, 1H), 4.06–4.00 (m, 2H), 3.69 (dt, *J* = 6.8 Hz, *J* = 3.3 Hz, 1H), 3.33–3.20 (m, 3H), 3.14 (dd, *J* = 14.1 Hz, *J* = 2.9 Hz, 1H), 2.99–2.83 (m, 6H), 2.62 (dt, ,*J* = 7.2 Hz, *J* = 5.1 Hz, 1H), 1.40 (s, 9H), 1.28 (s, 9H), 0.86 (s, 9H), ^{13}C-NMR (125 MHz, DMSO-d_6): δ [ppm] = 202.3, 171.2, 171.2, 171.1, 170.5, 169.6, 155.1, 154.2, 137.3, 136.8, 136.6, 132.6, 130.1, 129.9, 128.4, 127.6, 127.5, 126.9, 123.7, 123.4, 121.7, 121.4, 118.9, 118.7, 118.5, 118.3, 112.0, 111.8, 110.4, 110.2, 78.9, 78.0, 72.8, 61.8, 60.4, 55.1, 54.6, 53.9, 53.4, 39.1, 36.6, 29.1, 28.8, 28.6, 27.4, 25.9. HRMS (ESI$^+$-Orbitrap) *m/z* calculated for $C_{59}H_{73}N_8O_{10}S^+$: 1,085.5165 $[M+H]^+$; found: 1,085.5155 $[M+H]^+$.

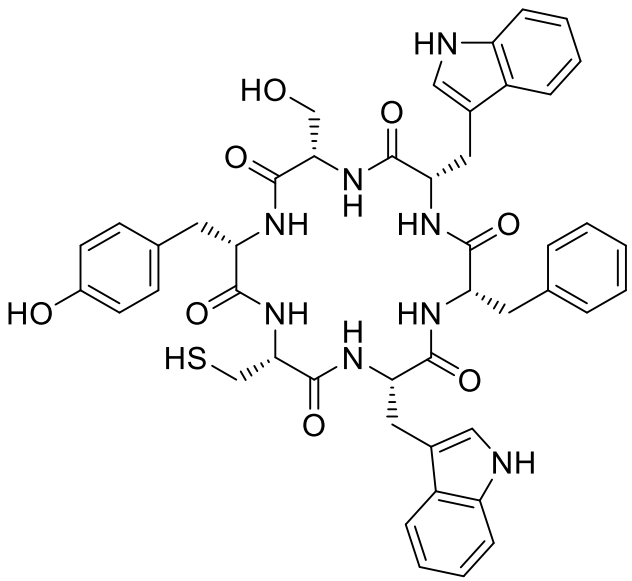
Synthesis of fully unprotected thiolactone 1:



1

The thiolactone **1b** (19.8 mg, 0,018 mmol) was treated with 95 % TFA in DCM (1 mL) for 1 h for deprotection of the hydroxy groups and the amine. The solvent was removed under a stream of nitrogen and the crude product was obtained as a yellow oil. It was handled under acidic conditions only, to sustain the thioester bond. The crude product was then purified using preparative HPLC (method B, but with 0.5 % TFA in solvent A and B. Additionally, TFA (4 mL) was added to each fraction.) to yield peptide **1** (6 mg, 38 % yield). (^{1}H-NMR (500 MHz, MeOD-d_4 + 1 % TFA-d): δ [ppm] = 8.34 (br, 0.2H), 8.12−8.06 (m, 1H), 7.52 (t, *J* = 8.3 Hz, 2H), 7.33 (dt, *J* = 9.3 Hz, *J* = 2.9 Hz, 2H), 7.19–7.06 (m, 8H), 7.02–6.99 (m, 4H), 6.93 (s, 1H), 6.76 (d, *J* = 8.5 Hz, 2H), 4.83–4.79 (m, 1H), 4.43 (br, 1H), 4.33 (t, *J* = 5.6 Hz, *J* = 4.3 Hz, 1H), 4.18–4.13 (m, 2H), 4.05 (t, *J* = 7.0 Hz, 1H), 3.80 (d, *J* = 13.8 Hz, 1H), 3.68 (dd, *J* = 11.8 Hz, *J* = 4.3 Hz, 1H), 3.63 (dd, *J* = 11.2 Hz, *J* = 6.8 Hz, 1H), 3.47 (d, *J* = 3.5 Hz, 2H), 3.35 (dd, *J* = 13.8 Hz, *J* = 3.9 Hz, 1H), 3.23 (dt, *J* = 13.62 Hz, *J* = 4.3 Hz, 1H), 3.15 (dt, *J* = 12.1 Hz, *J* = 3.1 Hz, 1H), 3.06−3.00 (m, 3H), 2.87 (dt, *J* = 11.4 Hz, *J* = 4.8 Hz, 1H), ^{13}C-NMR (125 MHz, MeOH-d_4 + 1 % TFA-d): δ [ppm] = 201.0, 175.7, 174.1, 173.7, 168.6, 157.9, 139.8, 138.7, 138.7, 132.1, 130.8, 130.1, 129.6, 128.98, 128.95, 128.23, 125.4, 125.1, 123.3, 123.1, 120.6, 120.4, 119.8, 119.6, 116.9 113.1, 113.0, 111.8, 110.5, 62.8, 62.5, 59.9, 58.3, 58.2, 56.9, 53.9, 38.1, 37.4, 29.8, 29.4, 27.2. HRMS (ESI$^+$-Orbitrap) *m/z* calculated for $C_{46}H_{49}N_8O_8S^+$: 873.3389 $[M+H]^+$; found: 873.3378 $[M+H]^+$.

Synthesis of cyclopeptide 2:

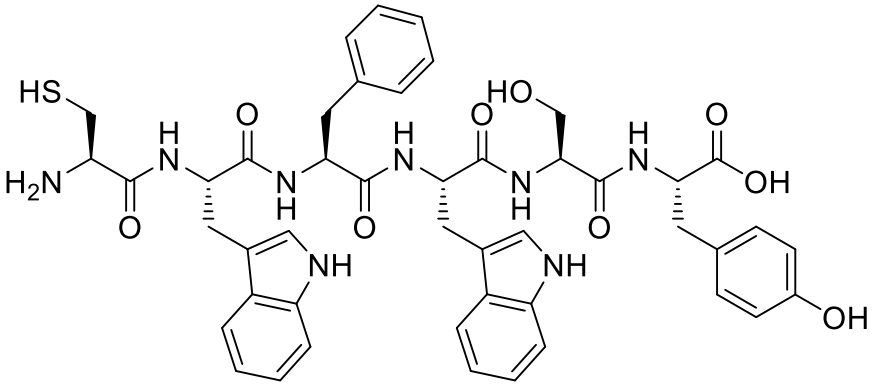


2

Peptide **1** (6 mg) was left in 50 mM MOPS, pH 7 for 2 h to fully rearrange into the cyclopeptide **2**. The pure compound was obtained using preparative HPLC (method B) in yield of 23 % as a colorless powder. ^{1}H-NMR (500 MHz, DMSO-d_6): δ [ppm] = 10.85 (s, 1H), 10.83 (s, 1H), 8.12 (q, *J* = 7.4 Hz, 2H), 8.03–7.94 (m, 4H), 7.61 (d, *J* = 7.9 Hz, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.34 (dd, *J* = 8.3 Hz, *J* = 1.7 Hz, 2H), 7.19 (s, 1H), 7.14–7.11 (m, 3H), 7.09–7.06 (m, 3H), 7.03–6.99 (m, 4H), 6.97–6.95 (m, 2H), 6.66 (d, *J* = 8.0 Hz, 2H), 4.38 (dt, *J* = 10.2 Hz, *J* = 6.2 Hz, 1H), 4.25 (q, *J* = 7.1 Hz, 1H), 3.13–

4.04 (m, 4H), 3.61 (s, 2H), 3.18 (d, *J* = 9.5 Hz, 1H), 3.15 (d, *J* = 8.8 Hz, 1H), 3.03–2.89 (m, 5H), 2.85–2.75 (m, 3H), 2.02 (t, *J* = 8.5 Hz, 1H), ^{13}C-NMR (125 MHz, DMSO-d_6): *δ* [ppm] = 171.6, 171.5, 171.1, 171.0, 170.5, 169.7, 156.3, 138.3, 136.6, 130.5, 129.4, 128.6, 128.4, 127.9, 127.6, 126.7, 124.1, 124.0, 121.5, 121.4, 118.8, 118.7, 118.6, 115.5, 111.9, 110.7, 110.5, 110.4, 61.3, 56.6, 56.4, 55.4, 54.9, 40.6, 40.5, 40.4, 40.4, 40.3, 40.2, 40.1, 40.0, 39.9, 39.8, 39.74, 39.67, 39.51, 36.7, 35.6, 27.2, 25.7. HRMS (ESI$^+$-Orbitrap) *m/z* calculated for $C_{46}H_{49}N_8O_8S^+$: 873.3389 $[M+H]^+$; found: 873.3398 $[M+H]^+$.

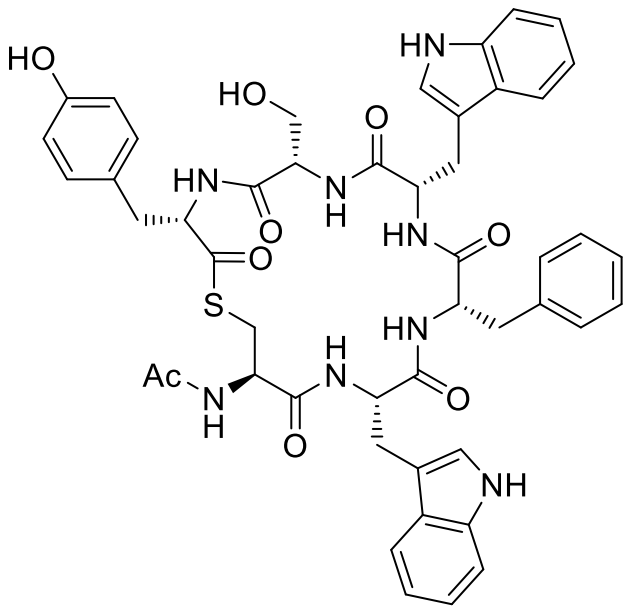
Solid phase peptide synthesis of unprotected linear peptide 3:



3

The protected thiolactone **1b** (9 mg, 0.0083 mmol) was dissolved in MeOH (1 mL), NaOH (50 µL, 1 M) was added, and the solution was stirred at 50 °C for 120 min. The solvent was then evaporated and the crude was dissolved in TFA (1 mL) for 60 min and the reaction was again stirred for 60 min at 50 °C before subjecting it to the preparative HPLC using method B. The title compound was obtained in a yield of 15 %. ^{1}H NMR (500 MHz, DMSO-d_6): *δ* [ppm] = 10.76 (s, 1H), 10.75 (s, 1H), 9.19 (s, 1H), 8.43 (s, 1H), 8.24 (d, *J* = 8.2 Hz, 1H), 8.17 (d, *J* = 7.8 Hz, 1H), 8.05 (d, *J* = 7.8 Hz, 1H), 7.94 (d, *J* = 7.6 Hz, 1H), 7.62 (d, *J* = 7.9 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 2H), 7.19 (s, 3H), 7.15 (s, 1H), 7.09 (s, 1H), 7.04 (t, *J* = 7.6 Hz, 2H), 7.00 (d, *J* = 8.3 Hz, 2H), 6.94 (td, *J* = 7.4 Hz, 4.8, 2H), 6.65 (d, *J* = 8.4 Hz, 2H), 4.64 (td, *J* = 8.0 Hz, 5.0, 1H), 4.57 (td, *J* = 8.5 Hz, 4.2, 2H), 4.38 (tq, *J* = 10.3 Hz, 5.8, 2H), 3.77 (d, *J* = 6.5 Hz, 1H), 3.56 (dt, *J* = 7.9 Hz, 4.0, 2H), 3.16 (dd, *J* = 14.9 Hz, 5.0, 1H), 3.08 (dd, *J* = 15.0 Hz, *J* = 4.5 Hz, 1H), 3.05–2.96 (m, 2H), 2.92 (dd, *J* = 13.9 Hz, *J* = 5.7 Hz, 1H), 2.86 (dd, *J* = 19.3 Hz, 6.5, 1H), 2.83–2.72 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) *δ* [ppm] = 172.7, 171.3, 170.9, 170.8, 169.7, 155.9, 137.7, 136.1, 136.0, 130.1, 129.2, 128.0, 127.4, 127.2, 126.1, 123.68, 123.63, 120.9, 120.8, 118.5, 118.2, 115.0, 111.23, 111.18, 109.9, 109.7, 61.7, 55.0, 53.9, 53.8, 53.5, 53.3, 37.5, 36.1, 27.8, 27.7, 26.0. HRMS (ESI$^+$-Orbitrap) *m/z* calculated for $C_{46}H_{50}N_8O_9S^+$: 891.3494 $[M+H]^+$; found: 891.3493 $[M+H]^+$.

Synthesis of cyclic peptide 4:

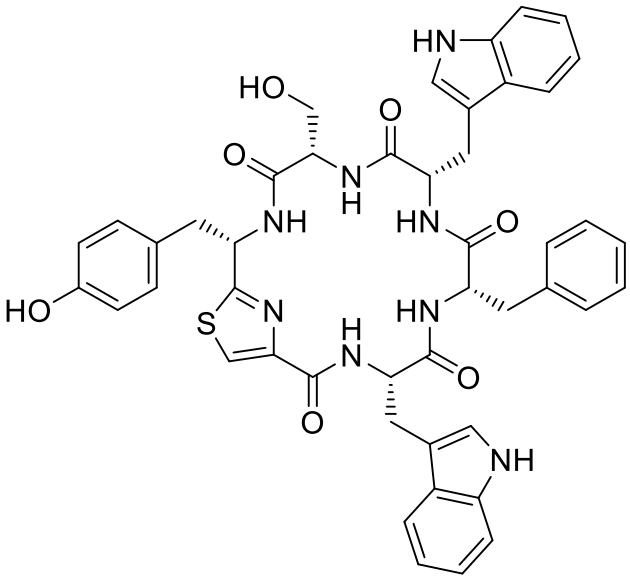


4

TFA (150 µL) and Et_3SiH (225 µL) were added to a solution of carboxylic acid **4a** (123 mg, 93.1 µmol) in CH_2Cl_2 (5 mL) and the solution was stirred 2 h at room temperature. Toluene (5 mL) was added and all volatile components were removed under reduced pressure to afford the *ω*-mercapto carboxylic acid. Solutions of PyBOP (58.2 mg, 112 µmol) and the *ω*-mercapto carboxylic acid in CH_2Cl_2/DMF (each 19 mL, 4.4:1 *v/v*) were given to a solution of Et*N*iPr_2 (31.9 µL,

186 µmol) in CH_2Cl_2 (20 mL) over 4 h via a syringe pump. The solution was stirred 24 h at room temperature. Toluene (10 mL) was added and the solvents were removed under reduced pressure. TFA/Et_3SiH/H_2O (3 mL, 95:2.5:2.5 *v/v/v*) were added to the crude peptide and the mixture was stirred 3 h at room temperature. The solvents were removed under reduced pressure. Preparative HPLC of the residue (method C) afforded cyclopeptide **4** as a colorless solid (8.37 mg, 9.15 µmol, 9.8 %). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ [ppm] = 10.88 (s, 1H), 10.78 (s, 1H), 9.21 (s, 1H), 9.08 (d, *J* = 5.8 Hz, 1H), 8.57 (dd, *J* = 11.2 Hz, *J* = 8.8 Hz, 2H), 8.28 (d, *J* = 8.7 Hz, 1H), 7.94 (d, *J* = 6.8 Hz, 1H), 7.55 (d, *J* = 7.7 Hz, 1H), 7.39−7.33 (m, 2H), 7.27 (t, *J* = 9.3 Hz, 2H), 7.25–7.05 (m, 10H), 7.05–7.00 (m, 2H), 6.97–6.91 (m, 2H), 6.69 (t, *J* = 10.3 Hz, 2H), 4.75 (dd, *J* = 14.3 Hz, *J* = 8.5 Hz, 1H), 4.48–4.39 (m, 1H), 4.38–4.31 (m, 1H), 4.31–4.24 (m, 1H), 4.08 (s, 1H), 4.00 (dt, *J* = 11.0 Hz, *J* = 5.5 Hz, 1H), 3.75 (dd, *J* = 11.1 Hz, *J* = 6.8 Hz, 1H), 3.61 (dd, *J* = 10.9 Hz, *J* = 3.8 Hz, 1H), 3.23–3.16 (m, 1H), 3.12–2.98 (m, 3H), 2.92 (m, 2H), 2.78 (m, 2H), 2.67 (m, 2H), 1.79 (s, 3H) ppm. ^{13}C-NMR (DMSO-d_6, 150 MHz, 297 K): δ [ppm] = 202.4, 171.5, 171.4, 170.9, 170.3, 170.1, 169.0, 156.4, 137.3, 136.7, 136.4, 130.7, 130.0, 128.4, 128.1, 127.7, 127.3, 126.8, 123.8, 123.8, 121.5, 121.3, 118.8, 118.8, 118.6, 118.2, 115.5, 111.9, 111.7, 110.5, 110.4, 62.2, 60.3, 56.7, 55.7, 55.0, 53.1, 52.2, 36.8, 28.8, 28.4, 26.4, 25.7, 22.7 ppm. HRMS (ESI$^+$-TOF) *m/z* calculated for $C_{48}H_{51}N_8O_9S^+$: 915.3494 $[M+H]^+$; found 915.3498 $[M+H]^+$.

Synthesis of cyclic peptide 5:



5

TFA (50 µL) and Et_3SiH (125 µL) were added to a solution of carboxylic acid **5a** (46.0 mg, 36.2 µmol) in CH_2Cl_2 (5 mL) and the solution was stirred 2 h at room temperature. Toluene (5 mL) was added and all volatile components were removed under reduced pressure to afford the *ω*-mercapto carboxylic acid. Solutions of PyBOP (28.2 mg, 54.3 µmol) and the *ω*-mercapto carboxylic acid in CH_2Cl_2/DMF (each 7.4 mL, 4.4:1 *v/v*) were given to a solution of Et*N*iPr_2 (12.4 µL, 72.4 µmol) in CH_2Cl_2 (7.8 mL) over 4 h via a syringe pump. The solution was stirred 24 h at room temperature. Toluene (10 mL) was added and the solvents were removed under reduced pressure. Flash chromatography (25 g silica, CH_2Cl_2/*i*PrOH 9:1) of the residue afforded the crude thiolactone that was used in the following step without any further characterization. The residue was solved in 2,6-lutidine (1 mL) and PPh_3 (14.2 mg, 54.1 µmol) was added. The solution was stirred at 60 °C for 6 h. The solvent was removed under reduced pressure and the residue was solved in CH_2Cl_2 (1 mL). At −10 °C, DBU (26.9 µL, 180 µmol) and $BrCCl_3$ (17.8 µL, 180 µmol) were added and the solution was stirred 4 h to reach room temperature. The volatile components were removed under reduced pressure and the residue was stirred in TFA/Et_3SiH/H_2O (1 mL, 95:2.5:2.5 *v/v/v*) for 2 h. The solvent was removed under reduced pressure and the residue was purified by preparative HPLC (method C) to afford thiazole **5** as a colorless solid (7.40 mg, 7.67 µmol, 21 %). ^{1}H-NMR (MeCN-d_3, 600 MHz): δ [ppm] = 9.20 (d, *J* = 9.7 Hz, 2H), 8.02 (d, *J* = 8.1 Hz, 1H), 7.88 (s, 1H), 7.70 (d, *J* = 7.7 Hz, 1H), 7.62 (d, *J* = 8.3 Hz, 1H), 7.40 (dd, *J* = 8.1, 3.9 Hz, 3H), 7.35 (s, 1H), 7.16–7.10 (m, 7H), 7.06 (m, 3H), 6.90 (d, *J* = 7.6 Hz, 3H), 6.86 (d, *J* = 5.8 Hz, 2H), 6.69 (d, *J* = 8.4 Hz, 2H), 5.24 (d, *J* = 6.2 Hz, 1H), 4.79 (d, *J* = 8.8 Hz, 1H), 4.25 (d, *J* = 6.1 Hz, 2H), 4.06–4.01 (m, 1H), 3.68 (s, 1H), 3.54–3.44 (m, 2H), 3.32 (dd, *J* = 14.7 Hz, *J* = 6.3 Hz, 1H), 3.28–3.17 (m, 4H), 3.05 (dd, *J* = 13.2 Hz, *J* = 3.8 Hz, 1H), 2.99 (dd, *J* = 13.1 Hz, *J* = 5.5 Hz, 1H), 2.94–2.84 (m, 2H) ppm. ^{13}C-NMR (MeCN-d_3, 150 MHz): δ [ppm] = 172.3, 172.2, 172.0, 170.7, 169.5, 161.8, 156.7, 150.4, 138.2, 137.5, 132.7, 131.5, 130.4, 129.7, 129.6, 129.4, 129.2, 128.5, 127.6, 124.9, 124.6, 124.4, 122.7, 122.6, 122.5, 120.1, 119.7, 119.5, 116.1, 112.4, 111.2, 110.8, 62.1, 57.2, 56.6, 56.1, 55.7, 53.7, 40.8, 37.6, 28.4, 27.6 ppm. HRMS (ESI$^+$-TOF) *m/z* calculated for $C_{46}H_{45}N_8O_7S^+$: 853.3126 $[M+H]^+$; found 853.3150 $[M+H]^+$.

Figure S1. AIP biosynthetic loci identified in the *R. cellulolyticum* genome. Representation of the four putative AIP biosynthetic gene clusters identified in the *R. cellulolyticum* genome (see Table S1). The key (below) indicates the encoded protein products: AgrD, precursor peptide; AgrB, macrocyclase; AgrC, sensor kinase; AgrA; response regulator. No gene encoding a leader peptidase is depicted since the enzyme responsible for leader peptide removal in clostridial AIP biosynthesis remains unknown. The locus tags for the genes encoding the putative AgrD precursor peptides (*Rc*-AIP1–4) are displayed for each cluster. The predicted core peptide sequences (see Figure S2) are surrounded by brown boxes, with the cysteine residues at the putative cyclization positions underlined.

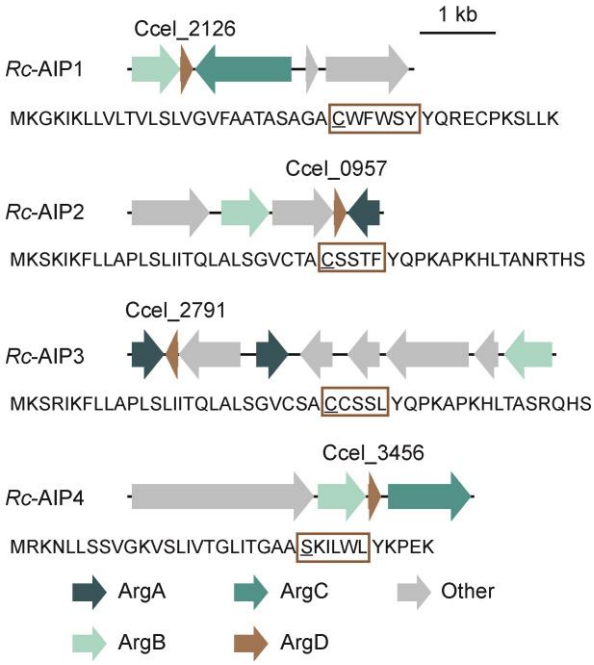
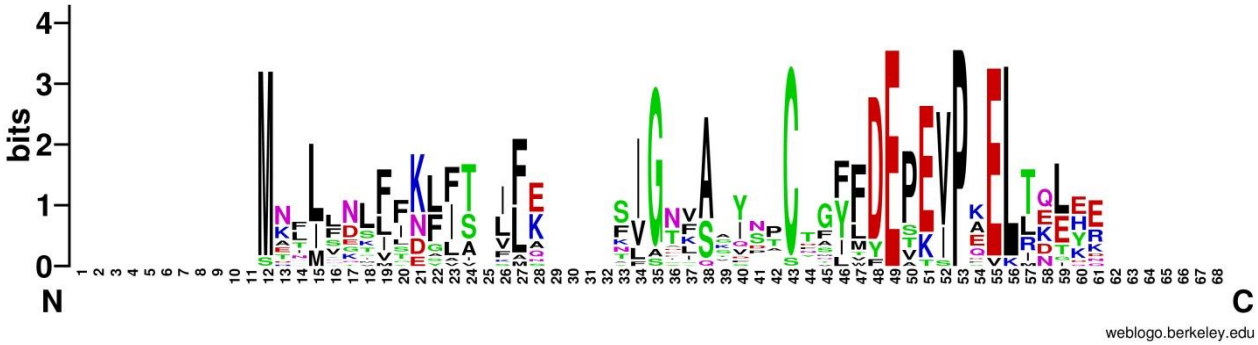


Figure S2. Prediction of the core peptide sequences for *R. cellulolyticum* AgrD1–4. A) WebLogo of structurally characterized AIPs. Although the sequences of the precursor peptides are highly divergent, two features are universally conserved: an ExxxP ArgB recognition motif in the follower peptide and a cyclizable residue (Cys or Ser) ten amino acids upstream of the conserved proline. The (thio)lactone macrocycle is formed between residues 43 and 47. See Table S6 for the identity of the sequences used to generate the WebLogo diagram. **B)** Alignment of *Rc*-AgrD1–4 to the structurally characterized AIPs from *Staphylococcus aureus*. The conserved proline in the AgrB recognition sequence and the (thio)lactone-forming residues are colored orange and blue, respectively. Amino acids comprising the *N*-terminal extensions on the thiolactone-containing macrocycles of structurally verified AIPs are underlined.

A



B

```
Saur_AgrD_I          MNTLFNLFFDFITGILKNIGNIAAYST C-DFIM DEVEVPKELTQLHE--      46
Saur_AgrD_II         MNTLVNMFFDFIIKLAKAIGIVSGVNA C-SSLF DEPKVPAELTNLYDK-      47
Saur_AgrD_III        MKKLLNKVIELLVDFFNSIGYKAAYIN C-DFLL DEAEVPKELTQLHE--      46
Saur_AgrD_IV         MNTLLNIFFDFITGVLKNIGNVASYST C-YFIM DEVEVPKELTQLHE--      46
Ccel_0957            MKSKIKFLLAPLSLIITQLALSGVCTA C-SSTF YQPKAPKHLTANRTHS      48
Ccel_2791            MKSRIKFLLAPLSLIITQLALSGVCSA C-CSSL YQPKAPKHLTASRQHS      48
Ccel_2126            MKGKIKLLVLTVLSLVGVFAATASAGA CWFWSY YQRECPKSLLK-----      44
Ccel_3456            MRKNLLSSVGKVSLIVTGLIT-GAA-- SKILWL YKPEK-----------      35
                     *.  .   .  :  .  :  .          .
```

Figure S3. HPLC-HRMS detection of *Rc*-AIP1, an AIP produced by *R. cellulolyticum*. Extracted ion chromatograms (EICs) (HPLC-HRMS method B) showing the detection of the mass consistent with the prediction for mature *Rc*-AIP1 lacking an *N*-terminal extension (NTE) (calc. *m/z* 873.3389 $[M+H]^+$; found *m/z* 873.3398) $[M+H]^+$) in the metabolite profile of *R. cellulolyticum*. No signals were observed corresponding to the predicted exact masses of *Rc*-AIP1 bearing NTEs of 1–4 residues. The core peptide sequence for *Rc*-AIP1 is depicted surrounded by a brown box, with the cysteine residue at the putative cyclization position underlined. Residues shown outside the box form the hypothetical NTEs.

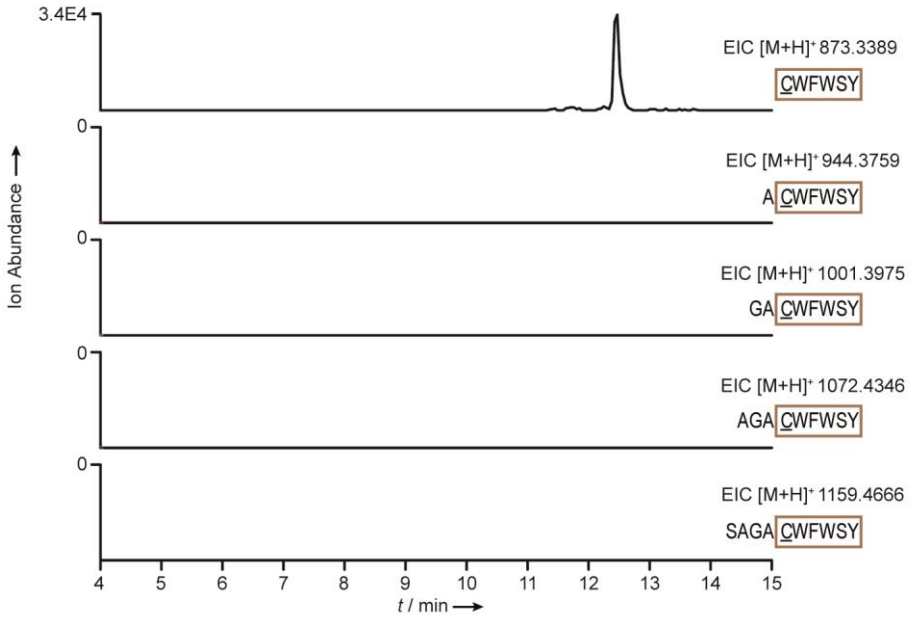
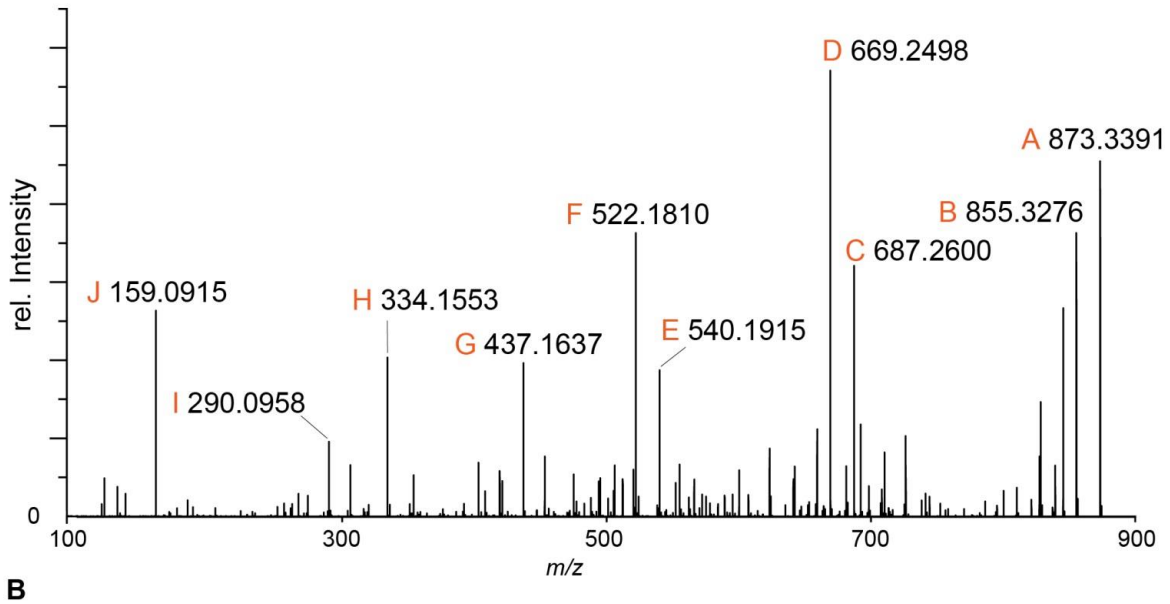
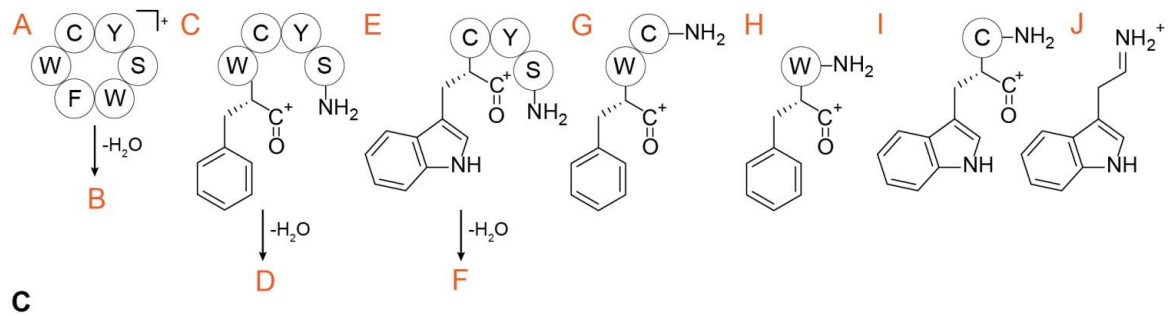


Figure S4. HRMS²-based fragmentation of candidate AIPs and the synthetic reference cyclopeptide 2. A) HRMS²-based fragmentation (HPLC-HRMS method A) of *Rp*-AIP1 (18 NCE, *m/z* 873.3389 $[M+H]^+$) detected in a crude extract from *R. papyrosolvens*. The fragmentation pattern is representative of those also observed for the synthetic reference cyclopeptide **2**, as well as *Rc*-AIP1 and *Rj*-AIP1 detected in crude extracts from *R. cellulolyticum* and *R. josui*, respectively (Figure S14). All observed fragment masses were in agreement with a cyclopeptide of amino acid sequence CWFWSY (see Panel B and C). **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.

A



B



C

fragment	sum formula	$[M+H]^+$
A	$C_{46}H_{49}N_8O_8S^+$	873.3389
B	$C_{46}H_{47}N_8O_7S^+$	855.3283
C	$C_{35}H_{39}N_6O_7S^+$	687.2595
D	$C_{35}H_{37}N_6O_6S^+$	669.2490
E	$C_{26}H_{30}N_5O_6S^+$	540.1911
F	$C_{26}H_{28}N_5O_5S^+$	522.1806
G	$C_{23}H_{25}N_4O_3S^+$	437.1642
H	$C_{20}H_{20}N_3O_2S^+$	334.1550
I	$C_{14}H_{16}N_3O_2S^+$	290.0958
J	$C_{10}H_{11}N_2^+$	159.0917

Figure S5. Overview of synthetic strategies. The synthetic strategies towards compounds **1−5** with precursors are depicted.

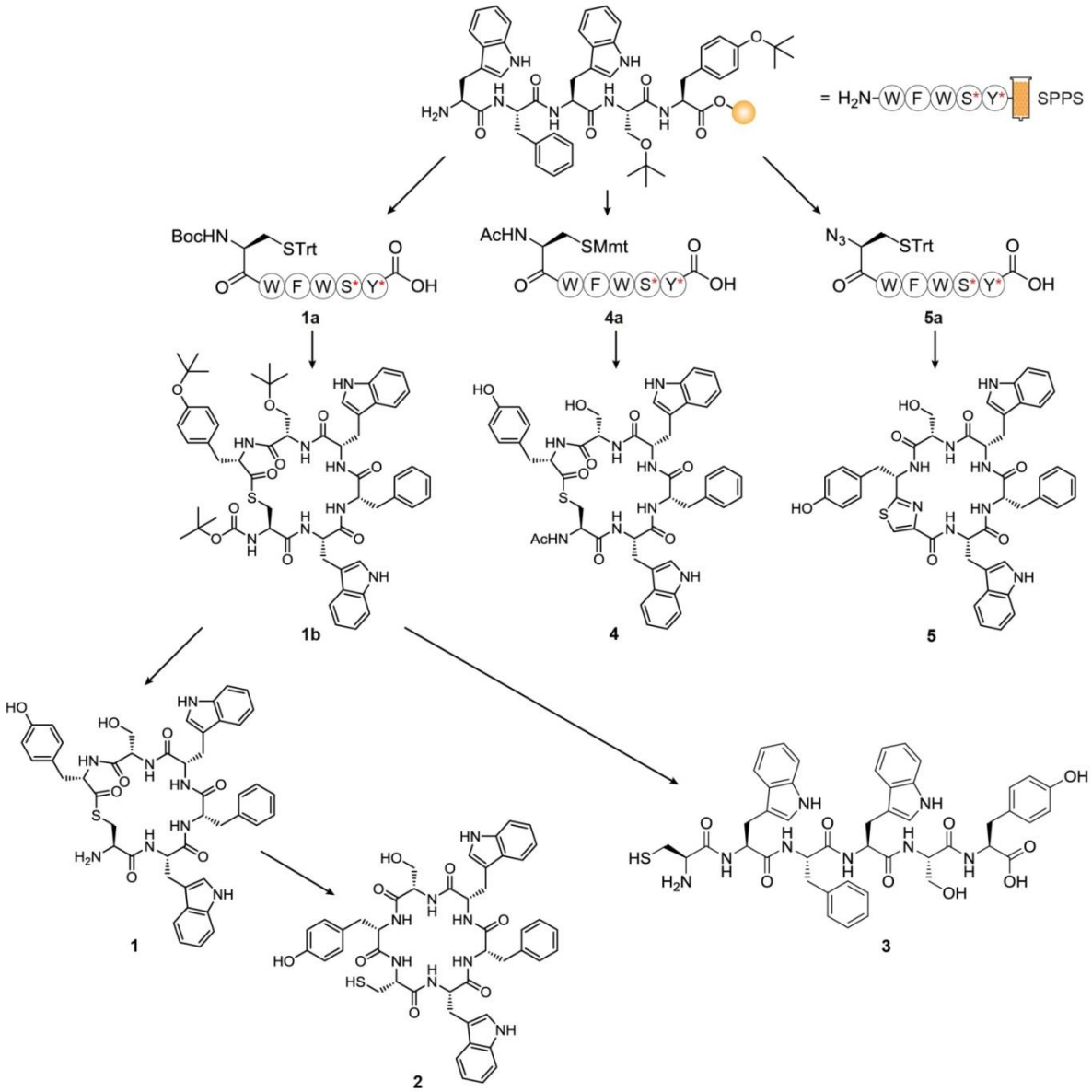


Figure S6. ^{13}C-NMR spectrum of synthetic reference thiolactone 1. Confirmation of the formation of the synthetic reference thiolactone **1** by ^{13}C-NMR.

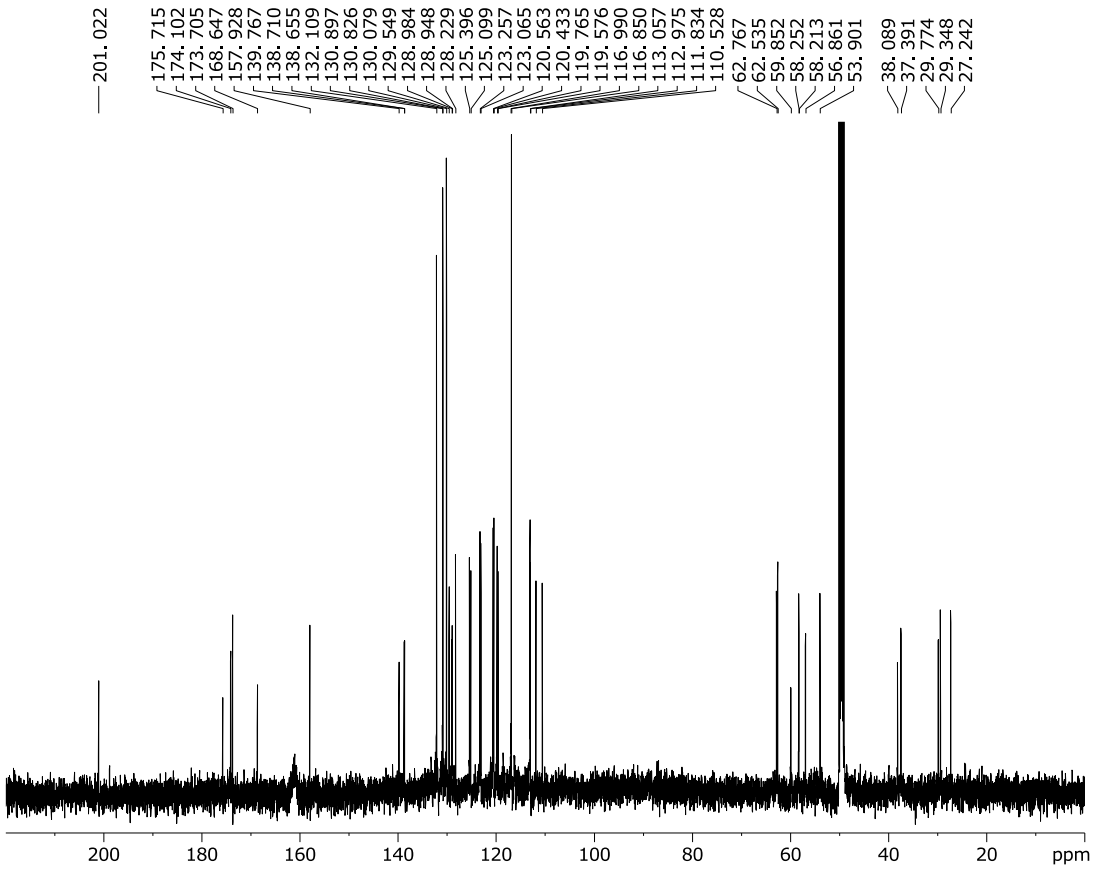


Figure S7. Comparison by HPLC-HRMS of retention time and accurate mass of *Rc*-AIP1 with references. Extracted ion chromatogram (EIC) and mass spectrum (HPLC-HRMS method A) showing the detection of the mass consistent with the prediction for mature *Rc*-AIP1 lacking an *N*-terminal extension (calc. *m/z* 873.3389 $[M+H]^+$; found *m/z* 873.3398) $[M+H]^+$) in the metabolite profile of *R. cellulolyticum*. Upon comparison to the synthetic references thiolactone **1** and cyclopeptide **2,** the retention time of *Rc*-AIP1 aligned with that of **2**, proving that the AIP of *R. cellulolyticum* is a homodetic cyclopeptide (cAIP). The mass spectra shown for thiolactone **1** and cyclopeptide **2** originate from the major EIC signal in each case. SSPS, solid-phase peptide synthesis.

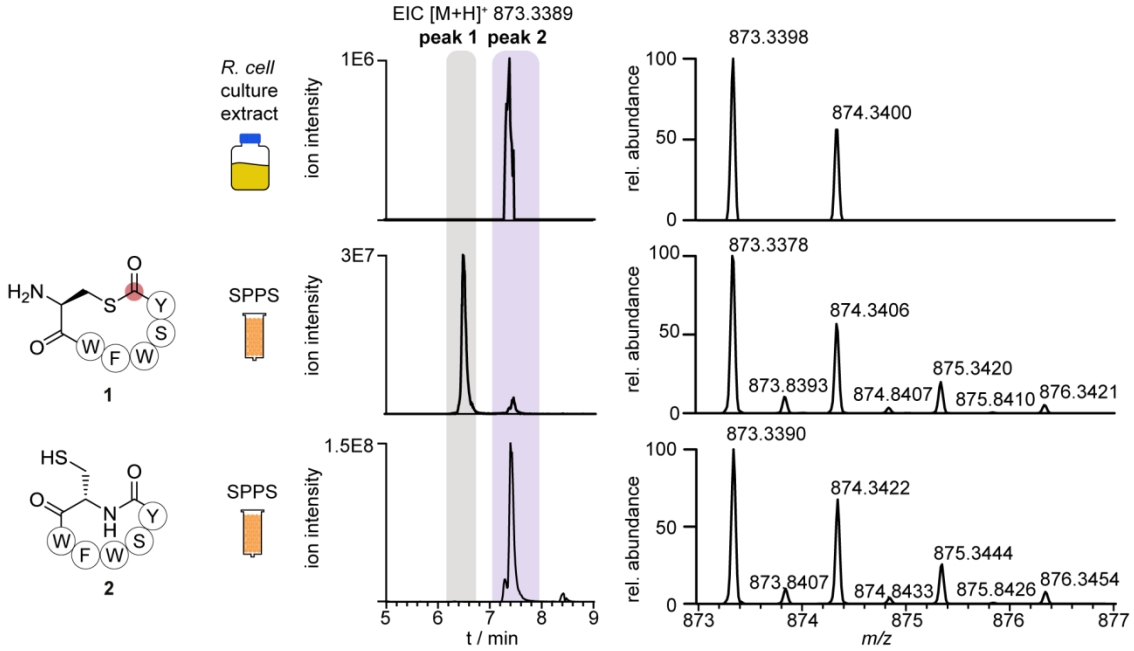


Figure S8. HRMS^2 spectra of the synthetic thiolactone 1. Comparison of HRMS^2 spectra (HPLC-HRMS method A) of the molecular ion masses (*m/z* 873.3389 $[M+H]^+$) corresponding to peak 1 and peak 2 of the synthetic thiolactone **1** (see Figure 2B and Figure S7), with 15 and 22 normalized collision energy (NCE).

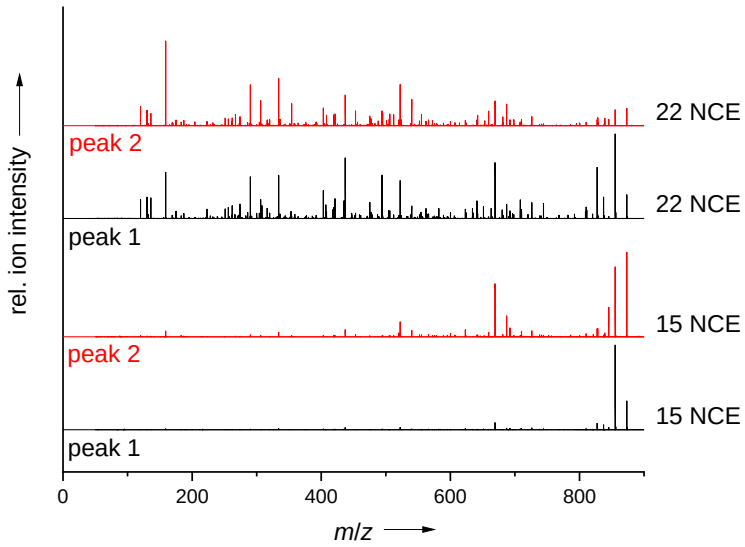


Figure S9. Thiolactone 1 spontaneously converts into lactam 2. Extracted ion chromatograms (EICs) (HPLC-HRMS method A) of *m*/*z* 873.3389 $[M+H]^+$ (5 ppm window) shows the conversion of thiolactone **1** into lactam **2** over time (d, days).

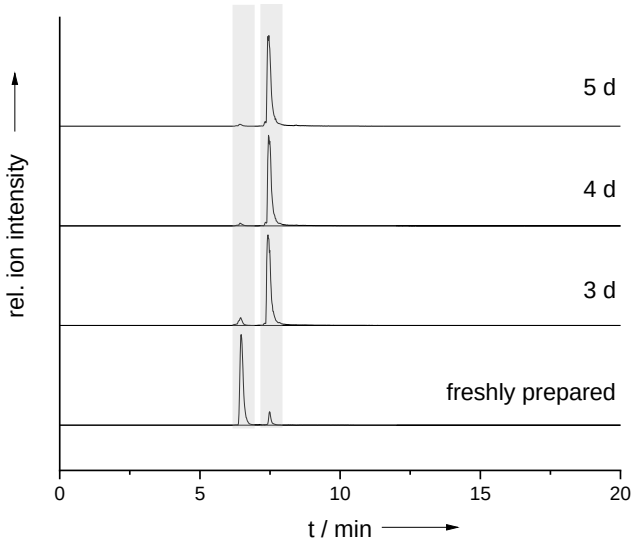


Figure S10. ^{13}C-NMR spectrum of synthetic reference cyclopeptide 2. Confirmation of the formation of the cyclopeptide **2** by ^{13}C-NMR.

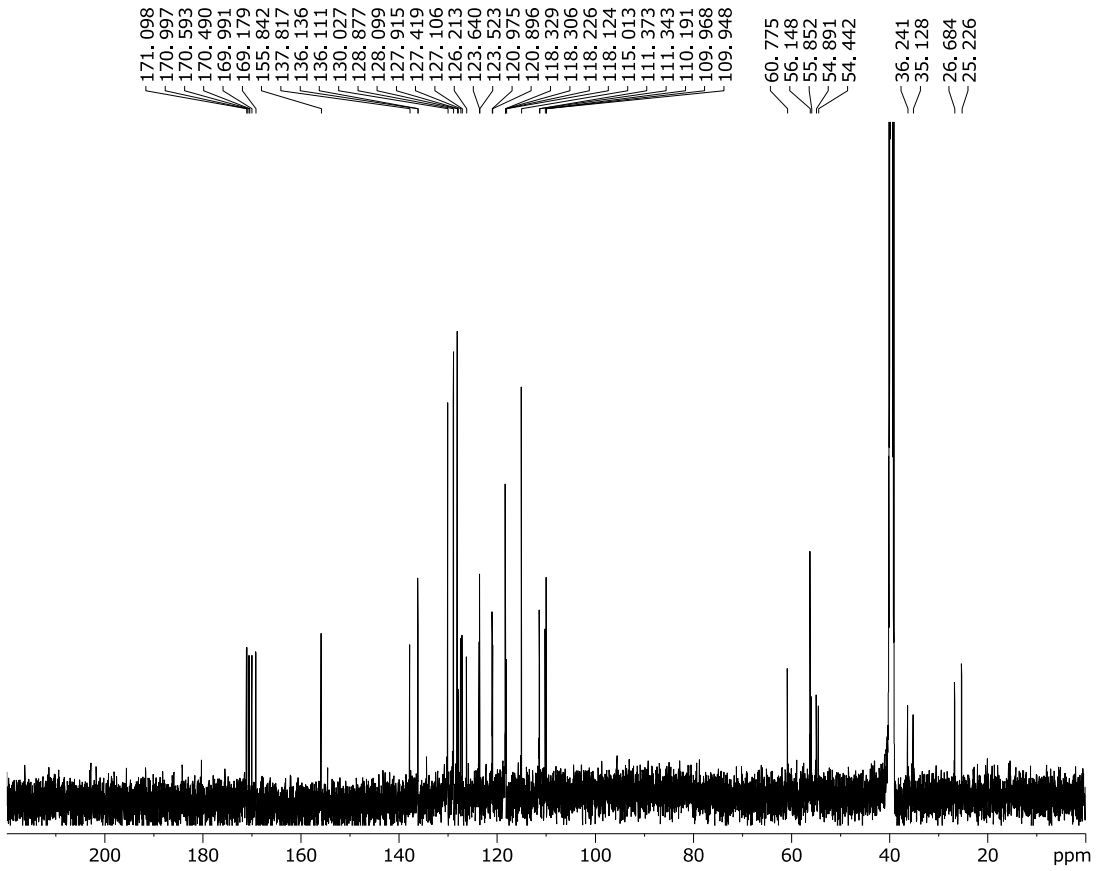


Figure S11. Rearrangement kinetics of thiolactone 1 measured with Ellman's reagent. The stability of **1** was measured by reaction of the spontaneously formed free thiol in **2** with Ellman's reagent. The normalized absorbance at 412 nm is shown over the time course of 2000 sec. As a non-linear regression, the data points were fitted with a one-phase-association equation. The time points at which half of the molecules (half-life, $t_{1/2}$) had rearranged from thiolactone **1** to cyclopeptide **2**, *i. e.* free thiol, is shown in each case. A control experiment is shown in Figure S30.

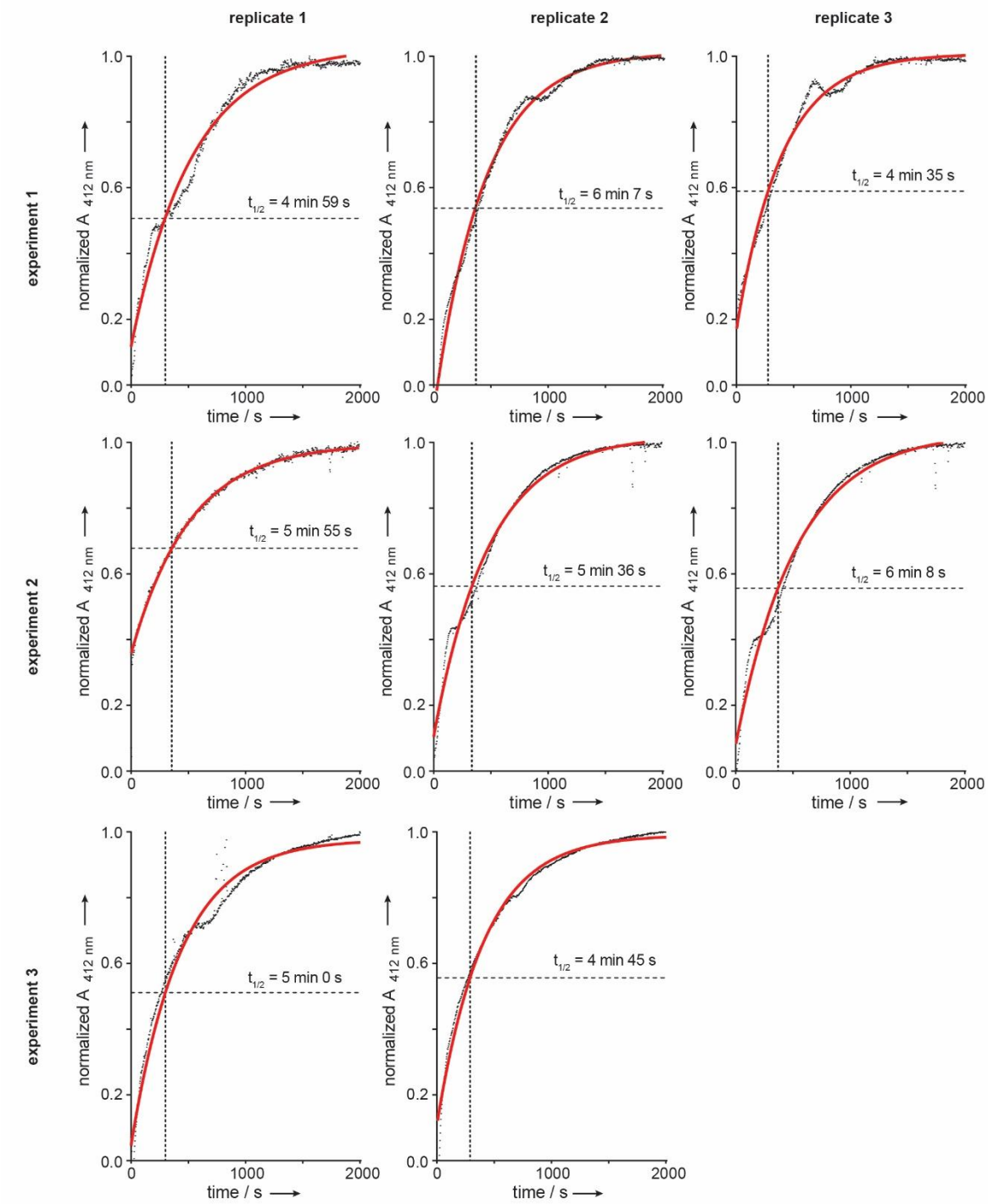


Figure S12. Generation of *R. cellulolyticum* Δ*agrD1* by CRISPR-nCas9 genome editing. A) A portion of the Ccel_2126 sequence is shown (*agrD1*) with the region of the targeted mutation displayed beneath (Δ*agrD1*, colored red). The location of the stop codon is indicated by an asterisk. **B)** Agarose gel (1.5 %) showing *Spe*I-digested Ccel2126-specific PCR products amplified from single colonies of the indicated strains. The PCR amplicon from the wild-type (WT) control (1026 bp) does not contain a *Spe*I recognition site and is not digested by *Spe*I. The PCR amplicon (1026 bp) from the Δ*agrD1* strain contains one *Spe*I recognition site and is digested to the expected 580 bp and 446 bp products by *Spe*I. L, GeneRuler DNA ladder Mix (Thermo Scientific). **C)** DNA sequence chromatogram verifying the precise insertion of the mutated sequence (highlighted) in *R. cellulolyticum* Δ*agrD1.*

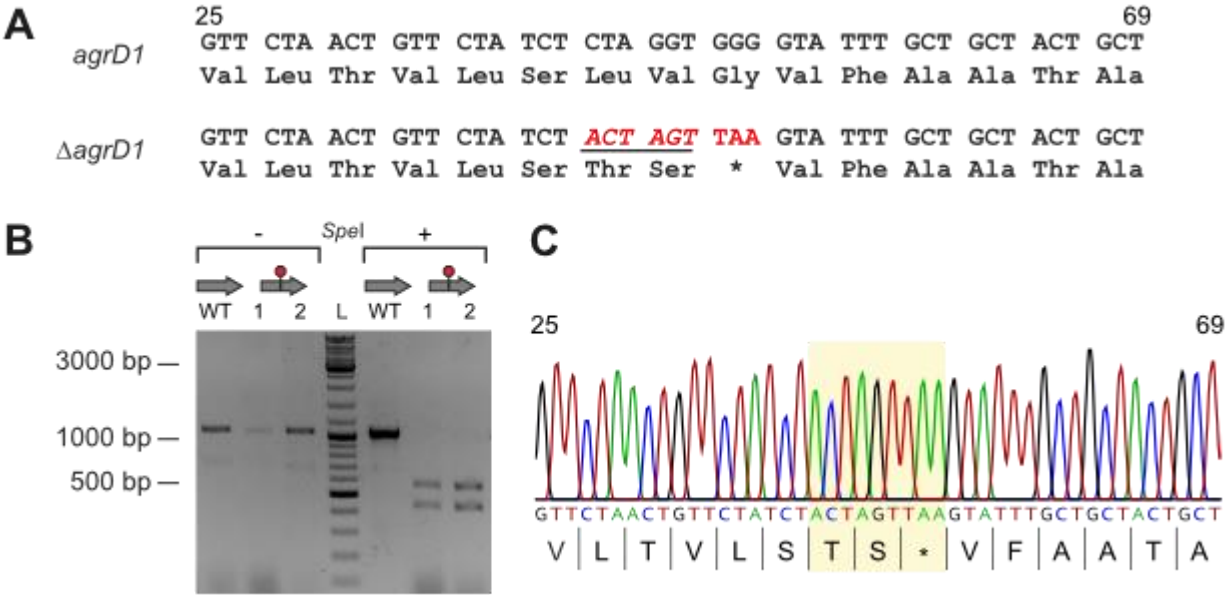


Figure S13. Bioinformatic screen for AIP biosynthetic gene clusters in diverse Clostridia. A) Maximum-likelihood phylogenetic tree of 16s rRNA sequences from the diverse Clostridia subjected to the bioinformatic screen with 1000 bootstrap replicates. *Staphylococcus aureus* was selected as the outgroup. The number in parenthesis indicates the numbers of AIP biosynthetic loci identified in each genome (see Table S1 for additional information). Extracts from the strains colored red were analyzed by HPLC-HRMS, resulting in the detection of several candidate AIPs, which were ultimately shown to be cAIPs. **B)** WebLogo of all AgrD precursor peptides identified in this bioinformatic screen. Despite their divergence, the presence in the predicted core peptide (underlined) of a cyclizable residue (red asterisk) 10–11 residues upstream of the conserved proline in the follower peptide is universally conserved.

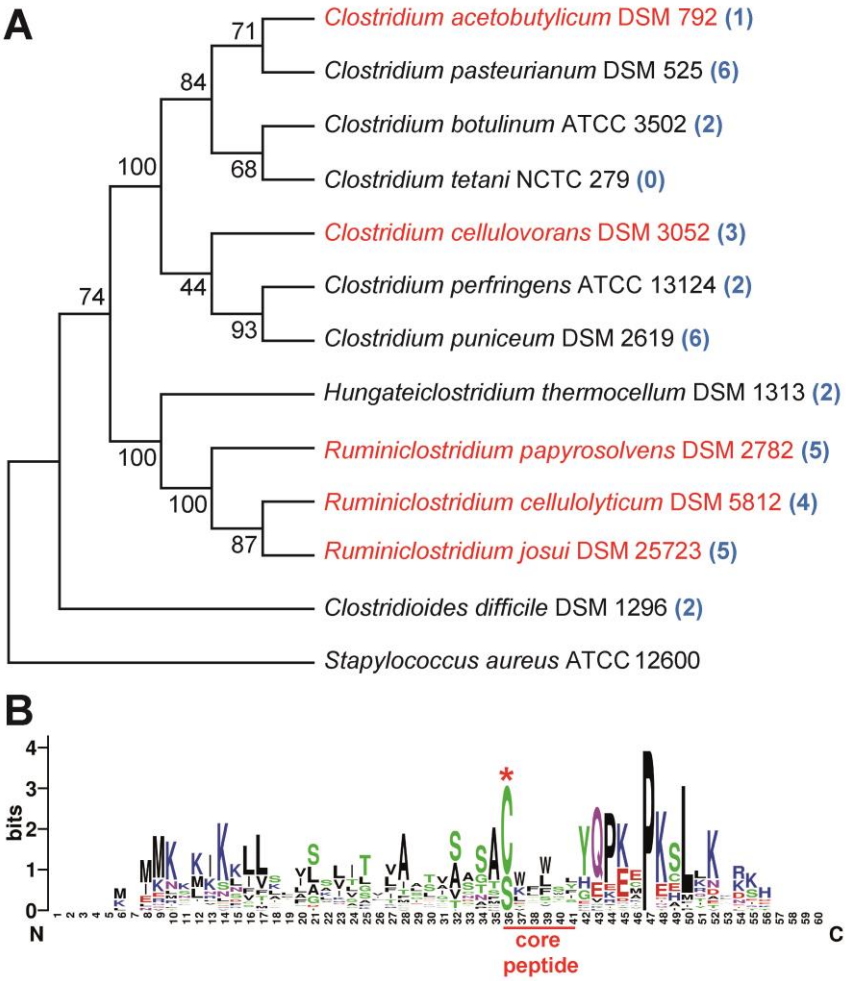


Figure S14. Three *Ruminiclostridium* species produce AIPs with identical fragmentation pattern. Comparison of HRMS^2 spectra (HPLC-HRMS method A) of the molecular ion masses (*m/z* 873.3389 $[M+H]^+$) corresponding to compounds eluting at around 7.4 min from the crude extracts of *R. cellulolyticum* (22 NCE), *R. josui* (18 NCE), *R. papyrosolvens* (18 NCE), and the synthetic reference cyclopeptide **2** (18 and 22 NCE).

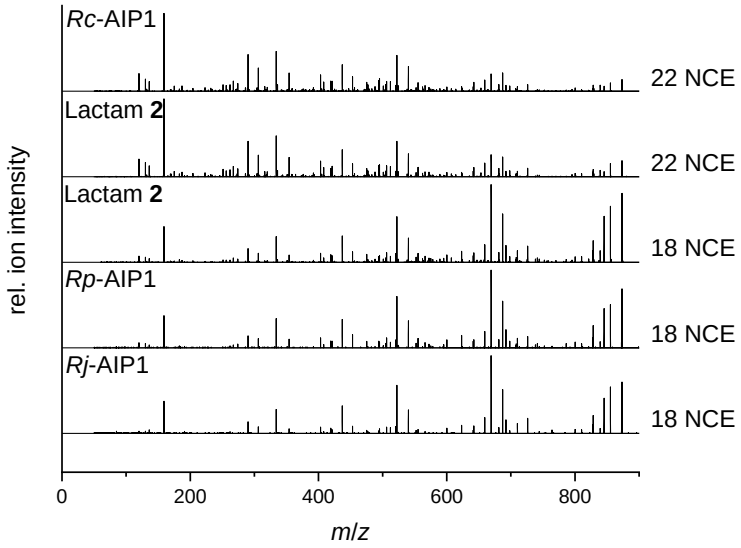
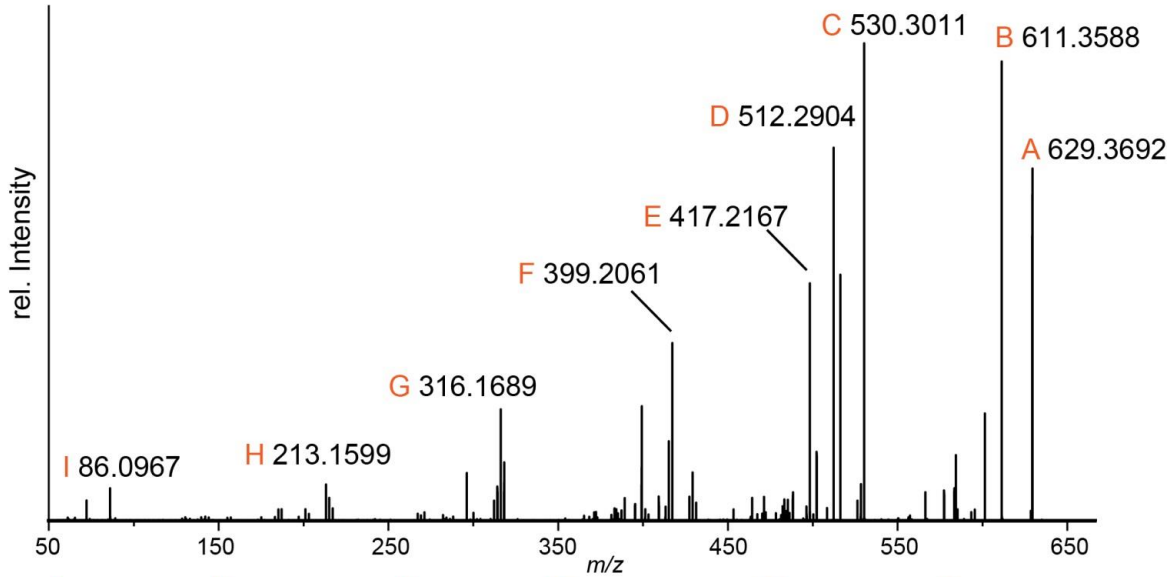
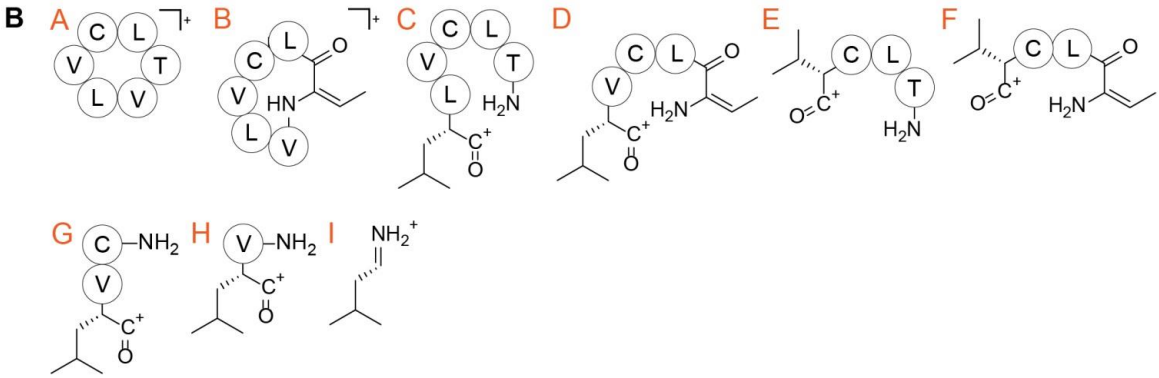


Figure S15. HRMS²-based fragmentation of *Ca*-AIP1 from *C. acetobutylicum*. A) Representative fragmentation pattern (HPLC-HRMS method A) of *Ca*-AIP1 detected in a crude extract from *C acetobutylicum*. All observed fragment masses were in agreement with a cyclopeptide of amino acid sequence CVLVTL (see Panel B and C). **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.

A



B

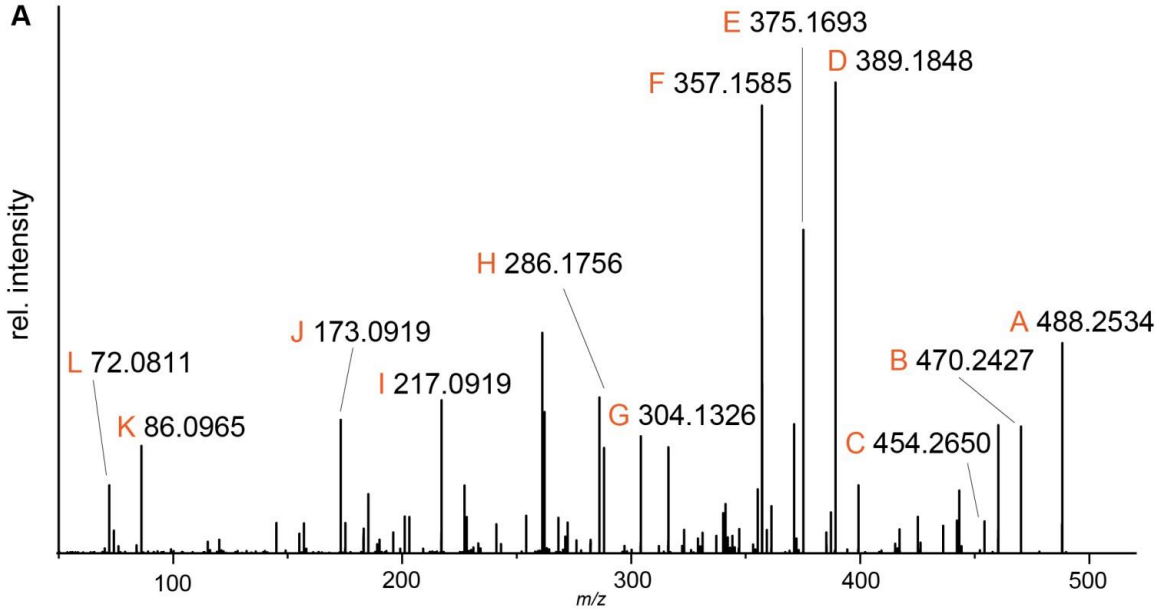


C

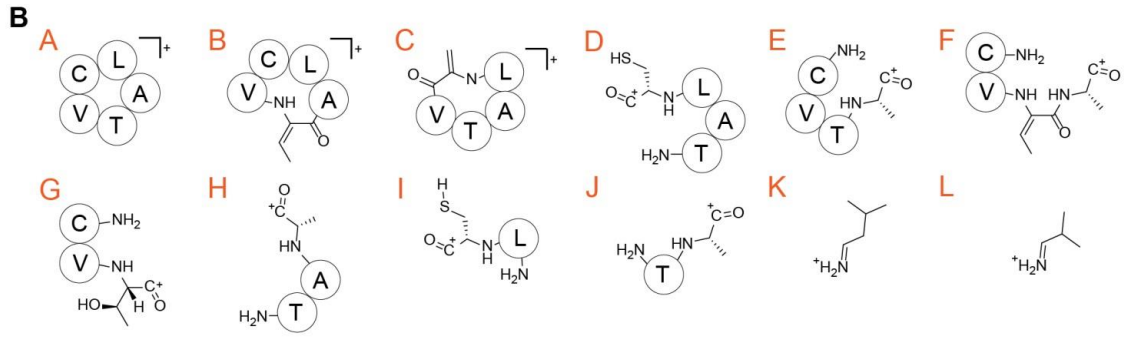
fragment	sum formula	$[M+H]^+$
A	$C_{29}H_{53}N_6O_7S^+$	629.3691
B	$C_{29}H_{51}N_6O_6S^+$	611.3585
C	$C_{24}H_{44}N_5O_6S^+$	530.3007
D	$C_{24}H_{42}N_5O_5S^+$	512.2901
E	$C_{18}H_{33}N_4O_5S^+$	417.2166
F	$C_{18}H_{31}N_4O_4S^+$	399.2061
G	$C_{14}H_{26}N_3O_3S^+$	316.1689
H	$C_{11}H_{21}N_2O_2^+$	213.1598
I	$C_5H_{12}N^+$	86.0864

Figure S16. HRMS²-based fragmentation of *Cc*-AIP1 from *C. cellulovorans*. A) Representative fragmentation pattern (HPLC-HRMS method A) of *Cc*-AIP1 detected in a crude extract from *C. cellulovorans*. All observed fragment masses were in agreement with a cyclopeptide of amino acid sequence CVTAL (see Panel B and C). **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.

A



B

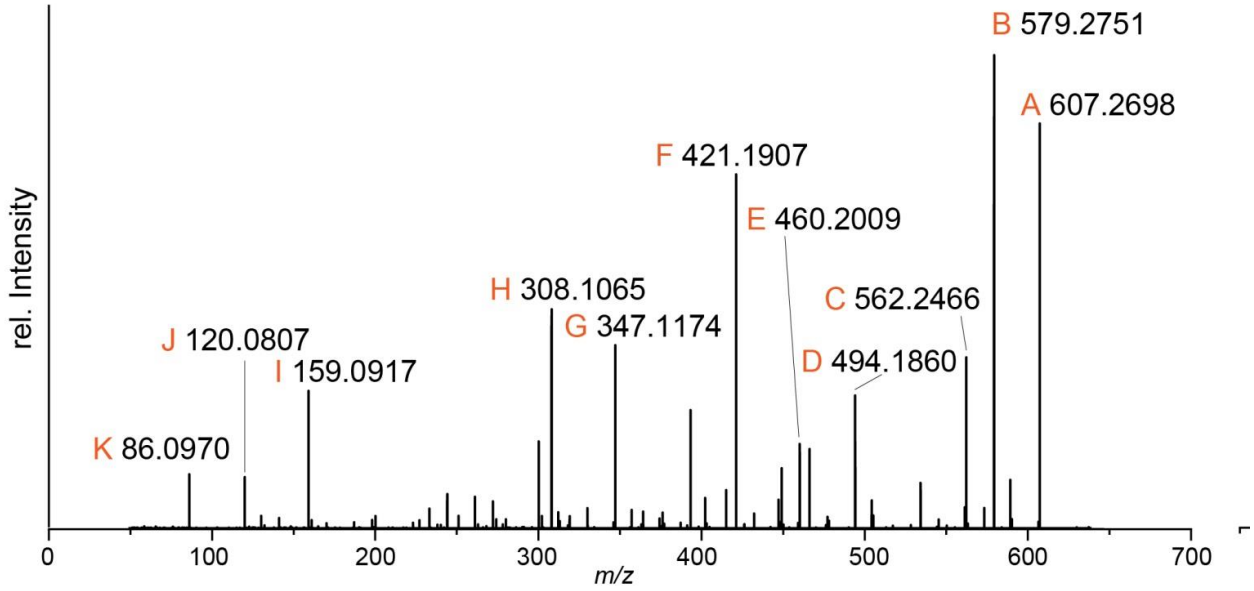


C

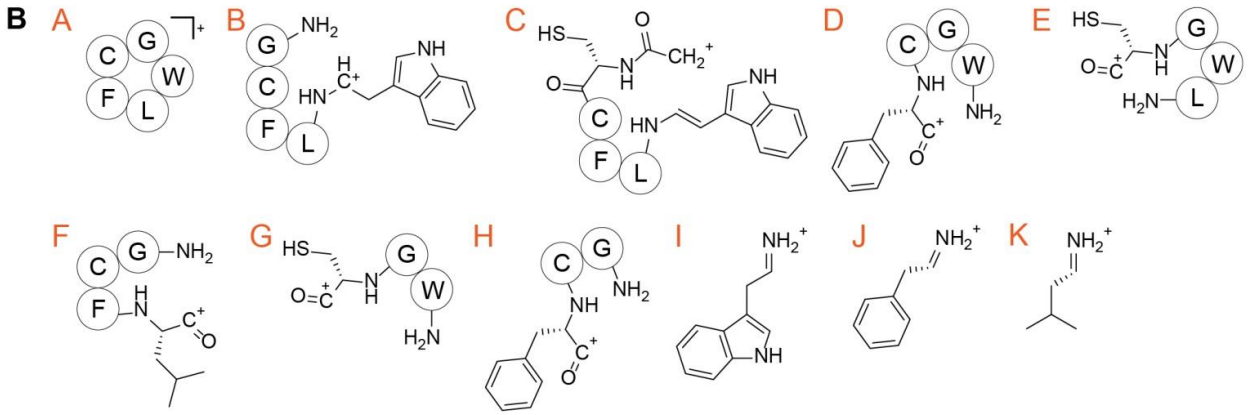
fragment	sum formula	[M+H]⁺
A	$C_{21}H_{36}N_5O_6S^+$	488.2537
B	$C_{21}H_{36}N_5O_5S^+$	470.2432
C	$C_{21}H_{36}N_6O_6^+$	455.2660
D	$C_{16}H_{29}N_4O_5S^+$	389.1853
E	$C_{15}H_{27}N_4O_5S^+$	375.1697
F	$C_{15}H_{25}N_4O_4S^+$	357.1591
G	$C_{12}H_{22}N_3O_4S^+$	304.1326
H	$C_{13}H_{24}N_3O_4^+$	286.1761
I	$C_9H_{17}N_2O_2S^+$	217.1005
J	$C_7H_{13}N_2O_3^+$	173.0921
K	$C_5H_{12}N^+$	86.0964
L	$C_4H_{10}N^+$	72.0808

Figure S17. HRMS^2-based fragmentation of *Rp*-AIP2 from *R. papyrosolvens*. A) Representative fragmentation pattern (HPLC-HRMS method A) of *Rp*-AIP2 detected in a crude extract from *R. papyrosolvens*. All observed fragment masses were in agreement with a cyclopeptide of amino acid sequence CFLWG (see Panel B and C). **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.

A



B

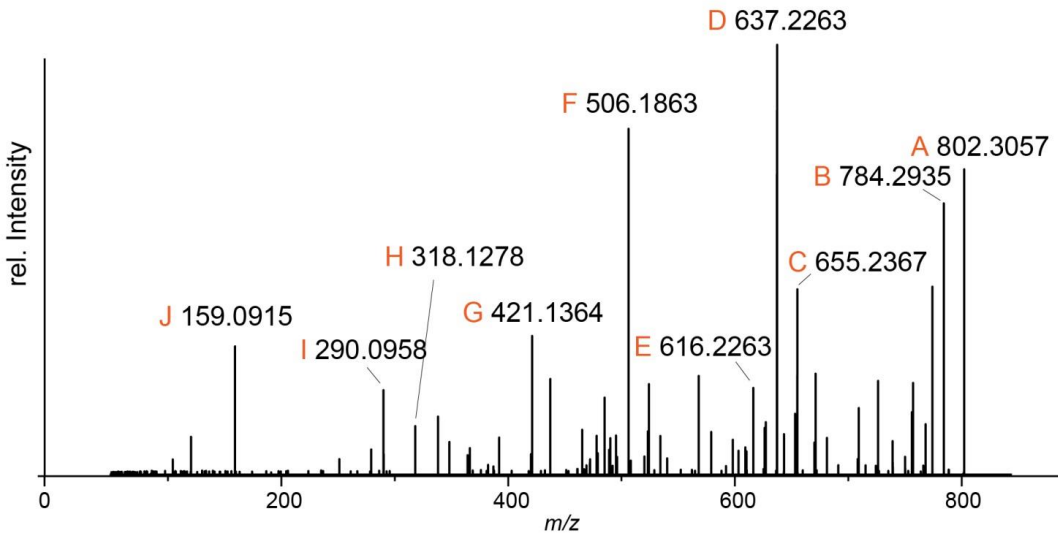


C

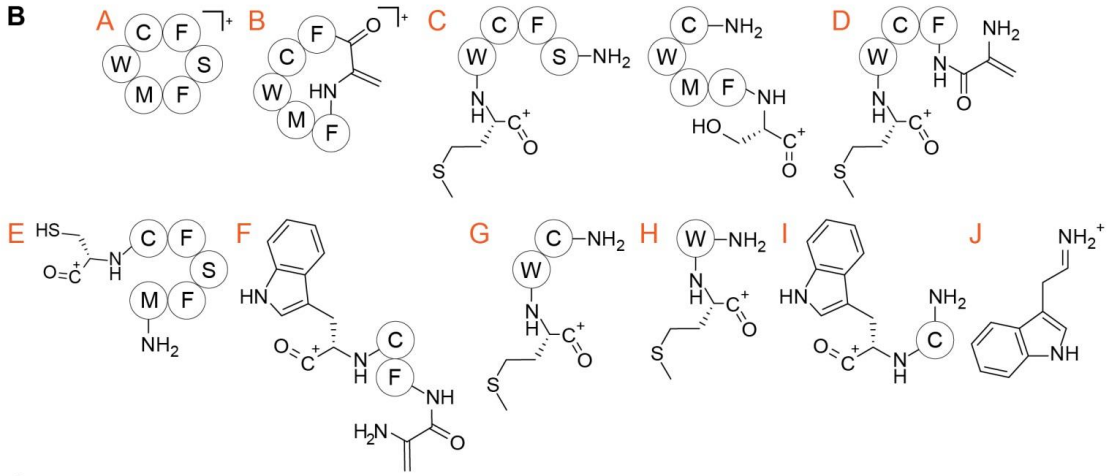
fragment	sum formula	$[M+H]^+$
A	$C_{31}H_{39}N_6O_5S^+$	607.2697
B	$C_{30}H_{39}N_6O_4S^+$	579.2748
C	$C_{30}H_{36}N_5O_4S^+$	562.2483
D	$C_{25}H_{28}N_5O_4S^+$	494.1857
E	$C_{22}H_{30}N_5O_4S^+$	460.2013
F	$C_{20}H_{29}N_4O_4S^+$	421.1904
G	$C_{16}H_{19}N_4O_3S^+$	347.1172
H	$C_{14}H_{18}N_3O_3S^+$	308.1063
I	$C_{10}H_{11}N_2^+$	159.0917
J	$C_8H_{10}N^+$	120.0808
K	$C_5H_{12}N^+$	86.0964

Figure S18. HRMS²-based fragmentation of *Rj*-AIP2 from *R. josui*. A) Representative fragmentation pattern (HPLC-HRMS method A) of *Rj*-AIP2 detected in a crude extract from *R. josui*. All observed fragment masses were in agreement with a cyclopeptide of amino acid sequence CWMFSF (see Panel B and C). **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.

A



B



C

fragment	sum formula	$[M+H]^+$
A	$C_{40}H_{48}N_7O_7S_2^+$	802.3051
B	$C_{40}H_{46}N_7O_6S_2^+$	784.2946
C	$C_{31}H_{39}N_6O_6S_2^+$	655.2367
D	$C_{31}H_{37}N_6O_5S_2^+$	637.2261
E	$C_{29}H_{38}N_5O_6S_2^+$	616.2258
F	$C_{26}H_{28}N_5O_4S^+$	506.1857
G	$C_{19}H_{25}N_4O_3S_2^+$	421.1363
H	$C_{16}H_{20}N_3O_2S^+$	318.1271
I	$C_{14}H_{16}N_3O_2S^+$	290.0958
J	$C_{10}H_{11}N_2^+$	159.0917

Figure S19. Overview of method to distinguish thiolactone or cyclopeptide form of AIPs. General scheme depicting the reaction of a cAIP (homodetic peptide) with *N*-benzylmaleimide resulting in the formation of a thiol adduct (mass increase of 187.0633 Da, red) and the absence of hydrolysis by NaOH (blue). In contrast, an AIP would not react with *N*-benzylmaleimide but the presence of a thiolactone moiety would render it susceptible to basic hydrolysis (mass increase of 18.0106 Da, blue).

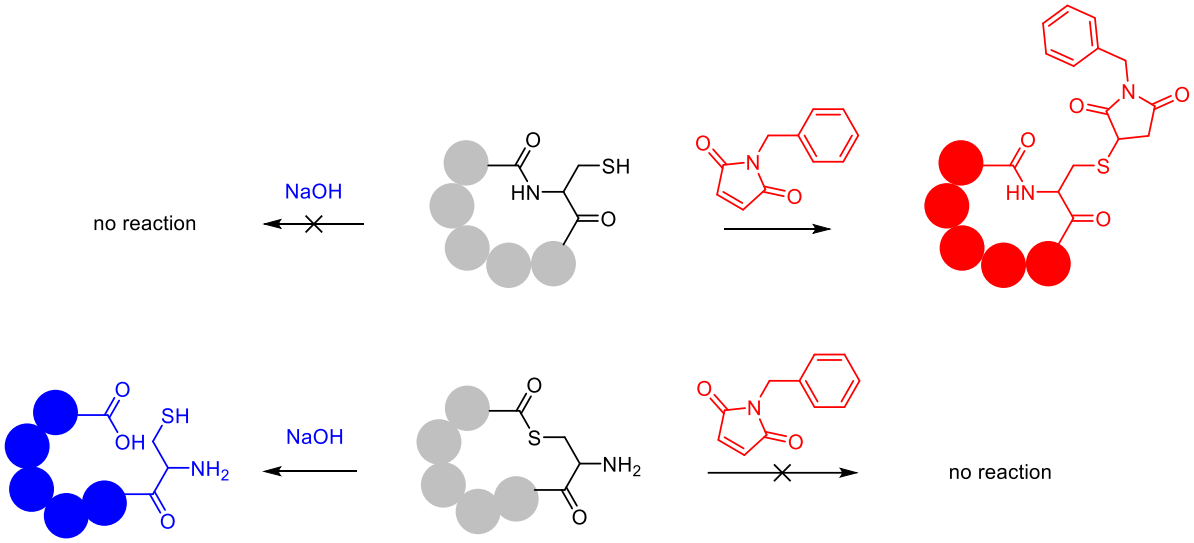
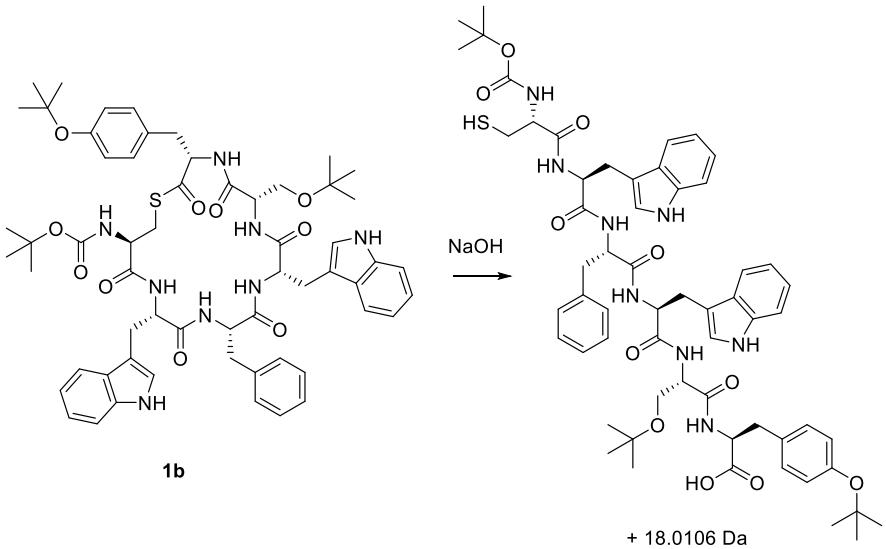


Figure S20. Thiolactone susceptibility to NaOH hydrolysis. Reaction scheme (above) and HPLC-HRMS chromatograms (below) (HPLC-HRMS method C) depicting the sensitivity of the protected thiolactone **1b** to NaOH treatment. 1 M NaOH (*v*:*v* = 1:1) was added to a solution of **1b** in MeOH at 20 °C. The extracted ion chromatograms of the mass corresponding to the fully protected thiolactone **1b** (*m/z* 1,085.5165 $[M+H]^+$) and the mass of the linear form (mass increase of 18.0106 Da, *m/z* 1,103.5271 $[M+H]^+$) are shown over a period of 16 hours. Due to the instability of thiolactone **1**, **1b** was used for method validation. These data show a rapid ring-opening occurred.



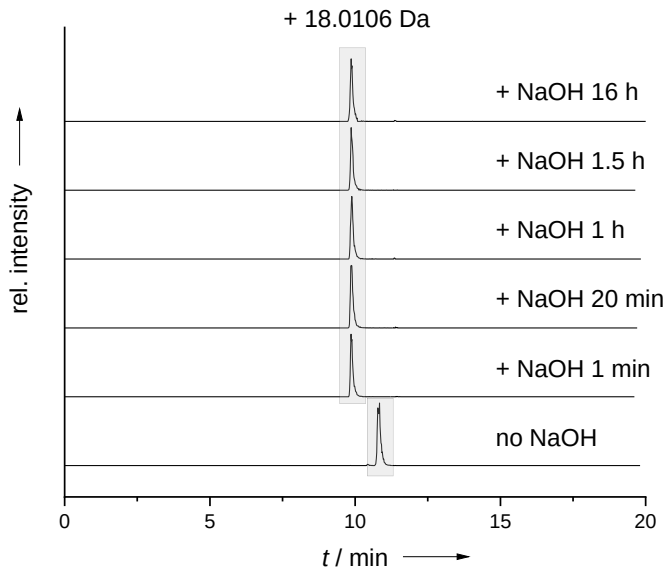
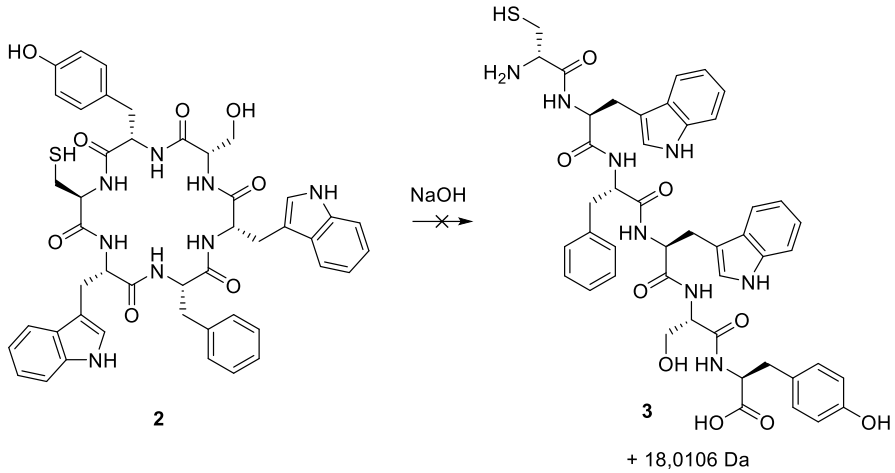


Figure S21. Lactam resistance to NaOH hydrolysis. Reaction scheme (above) and HPLC-HRMS chromatograms (below) (HPLC-HRMS method C) depicting the stability of lactam **2** to NaOH treatment. 1 M NaOH (*v*:*v* = 1:1) was added to a solution of **2** in MeOH at 20 °C. The extracted ion chromatograms of the mass corresponding to the lactam **2** (*m/z* 873.3389 $[M+H]^+$) and the mass of the linear form (mass increase of 18.0106 Da, *m/z* 891.3494 $[M+H]^+$) are shown over a period of 15 h. The HPLC-HRMS chromatogram of the linear peptide **3** is shown for reference. At 15 h, a trace amount of hydrolysis was detected; $HRMS^2$ measurements of the linear form showed that the cleavage occurred randomly at different amide bonds. No ring-opening was observed at the chosen reaction time of 1 h for NaOH treatment of crude extracts.



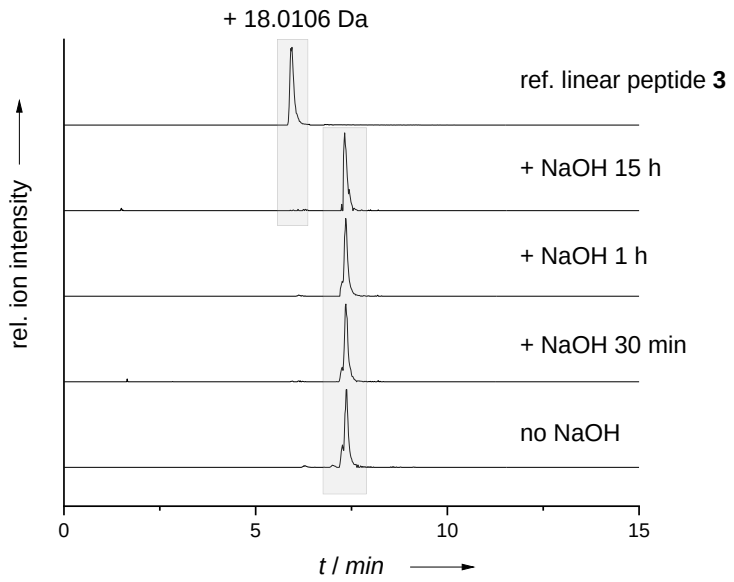
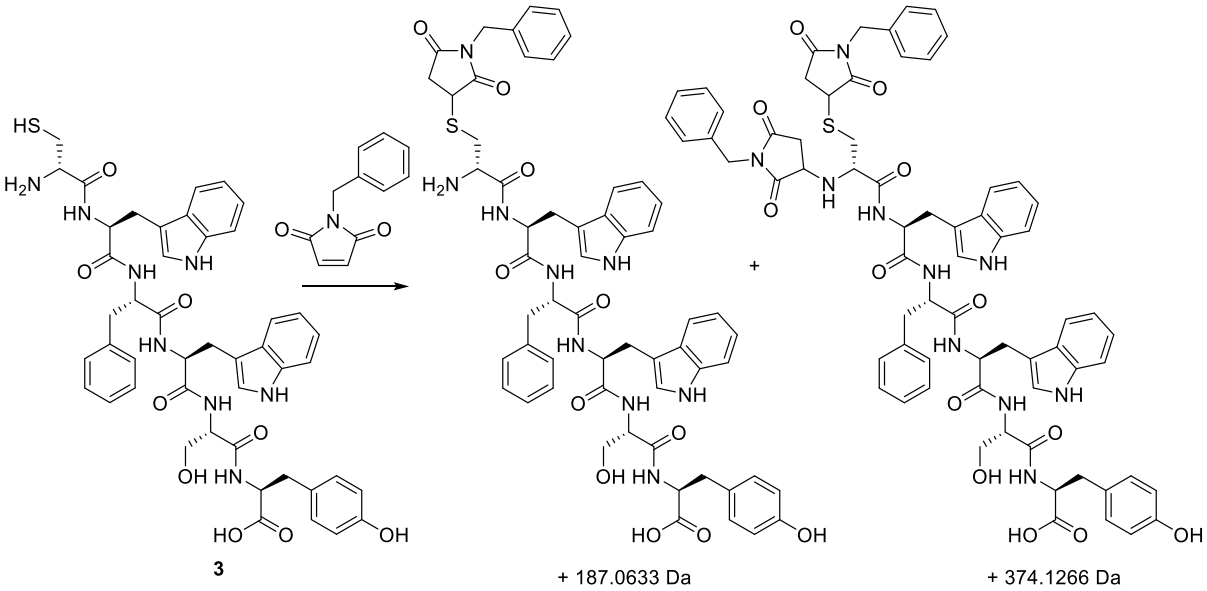


Figure S22. Chemoselective labeling of free thiols by *N*-benzylmaleimide. Reaction scheme (above) and HPLC-HRMS chromatograms (below) (HPLC-HRMS method C) depicting the reaction of the linear peptide **3** with *N*-benzylmaleimide. A solution of *N*-benzylmaleimide (10 mg in 100 µL MeCN, 1 µL) was added to a solution of the linear peptide **3** in MeOH (*v*:*v* = 1:1) at 20 °C. The extracted ion chromatograms of the mass corresponding to **3** (*m/z* 891.3494 $[M+H]^+$), the mass of **3** labeled once with *N*-benzylmaleimide (mass increase of 187.0633 Da, *m/z* 1,078.4128 $[M+H]^+$), and the mass of **3** labeled twice with *N*-benzylmaleimide (mass increase of 374.1266 Da, *m/z* 1,265.4761 $[M+H]^+$) are shown over a period of 18 hours. Due to the instability of thiolactone **1**, **3** was used for method validation to test whether off-target reaction with the amine occurs. After 1 h, most of **3** (*m/z* 891.3494) had reacted with *N*-benzylmaleimide to form the once-labeled adduct. Only after prolonged reaction times was the mass of twice-labeled **3** detected.



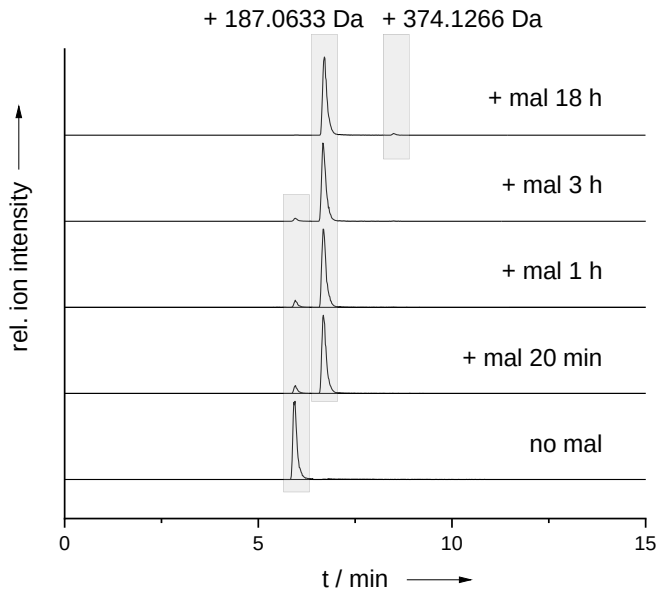


Figure S23. Retention time of controls for method to distinguish thiolactone or cyclopeptide form of AIPs. Extracted ion chromatograms (EICs) (HPLC-HRMS method A) of synthetic standards with increasing retention time: *m/z* 891.3494 $[M+H]^+$ corresponding to linear peptide **3** at 5.93 min (blue); *m/z* 873.3389 $[M+H]^+$ corresponding to thiolactone **1** at 6.47 min, with traces of the cyclopeptide **2** (green); *m/z* 873.3389 $[M+H]^+$ corresponding to cyclopeptide **2** at 7.49 min (black); *m/z* 1,060.4022 $[M+H]^+$ corresponding to *N*-benzylmaleimide adduct of cyclopeptide **2** at 8.27 min (red).

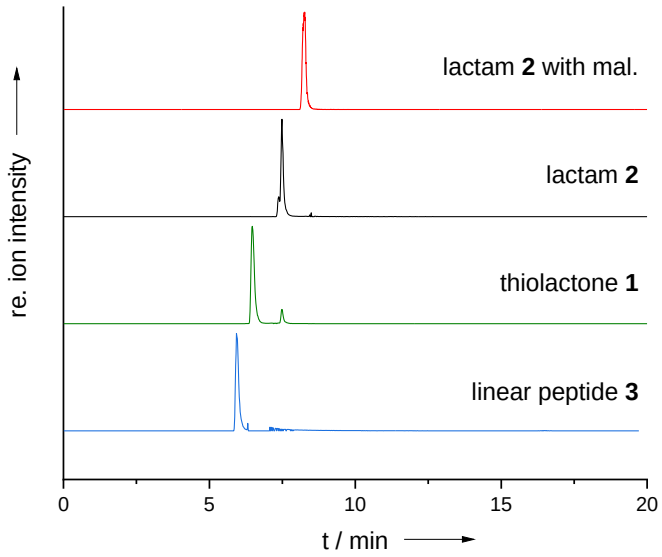


Figure S24. Demonstration of expected cAIP behavior of *Rc*-AIP1 in crude extract. Extracted ion chromatograms (EICs) (HPLC-HRMS method D) showing the detection of masses (1.5 ppm window) in a *R. cellulolyticum* Δ*agrD1* pCPthl-*agrD1* crude extract consistent with the unreacted cAIP (*m/z* 873.3389 $[M+H]^+$, black), the expected mal-adduct (*m/z* 1060.4022 $[M+H]^+$, red) after *N*-benzylmaleimide treatment (+ mal.), and no ring opening (*m/z* 891.3494 $[M+H]^+$, blue) after NaOH treatment (+ NaOH). Reference compounds are shown for comparison.

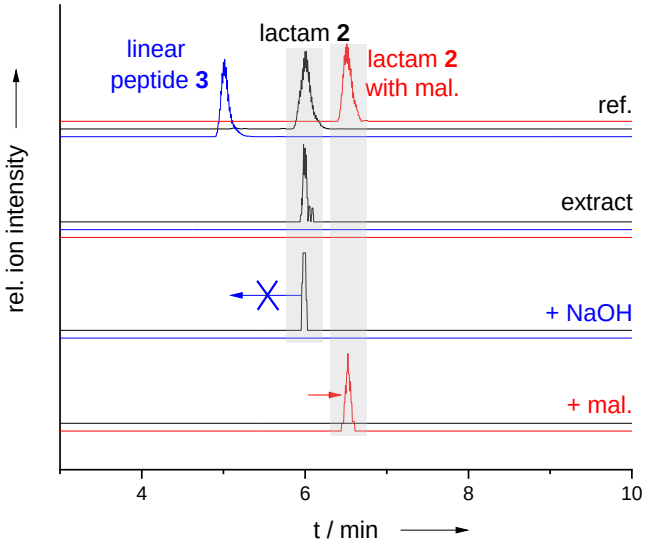
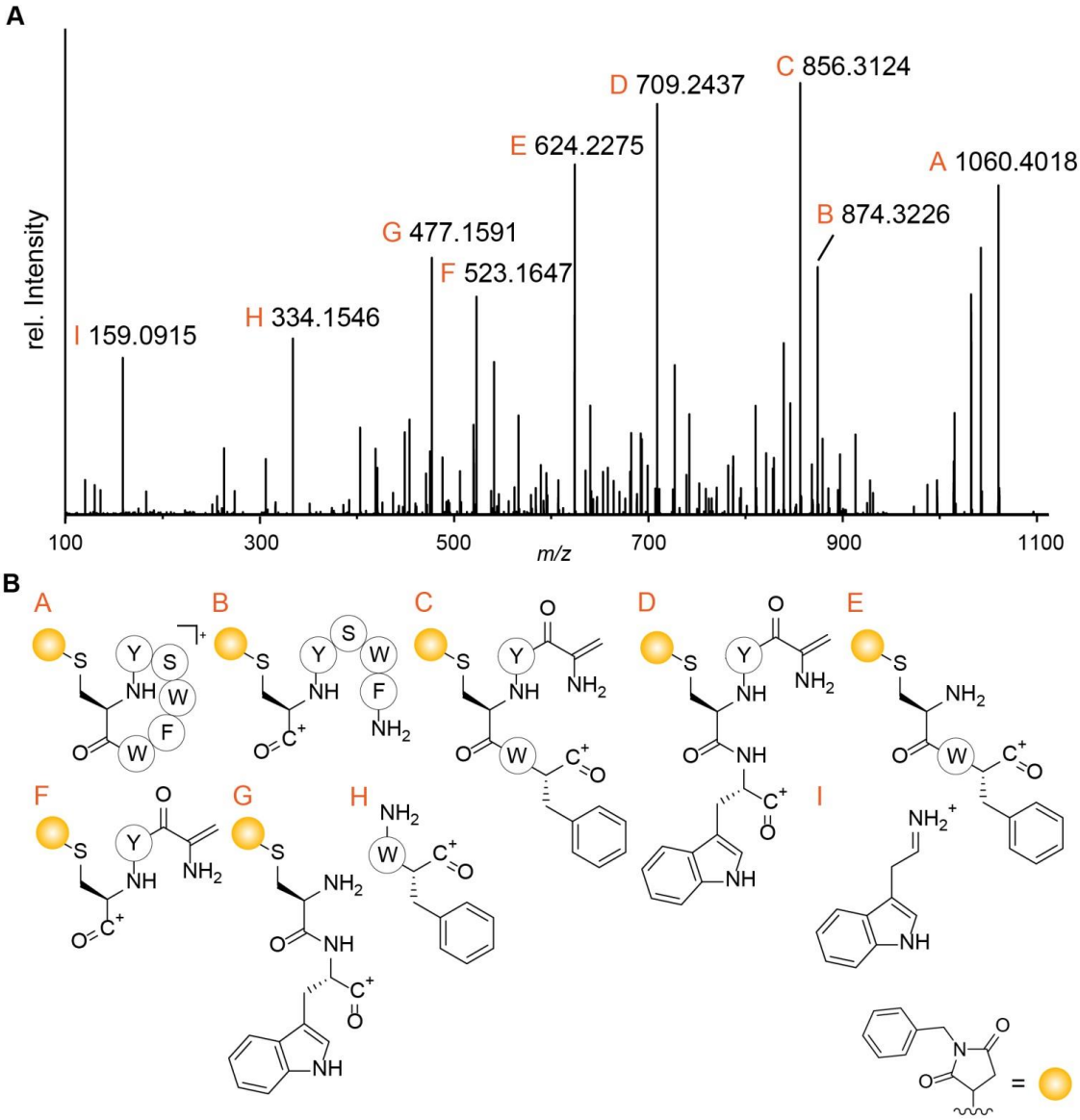


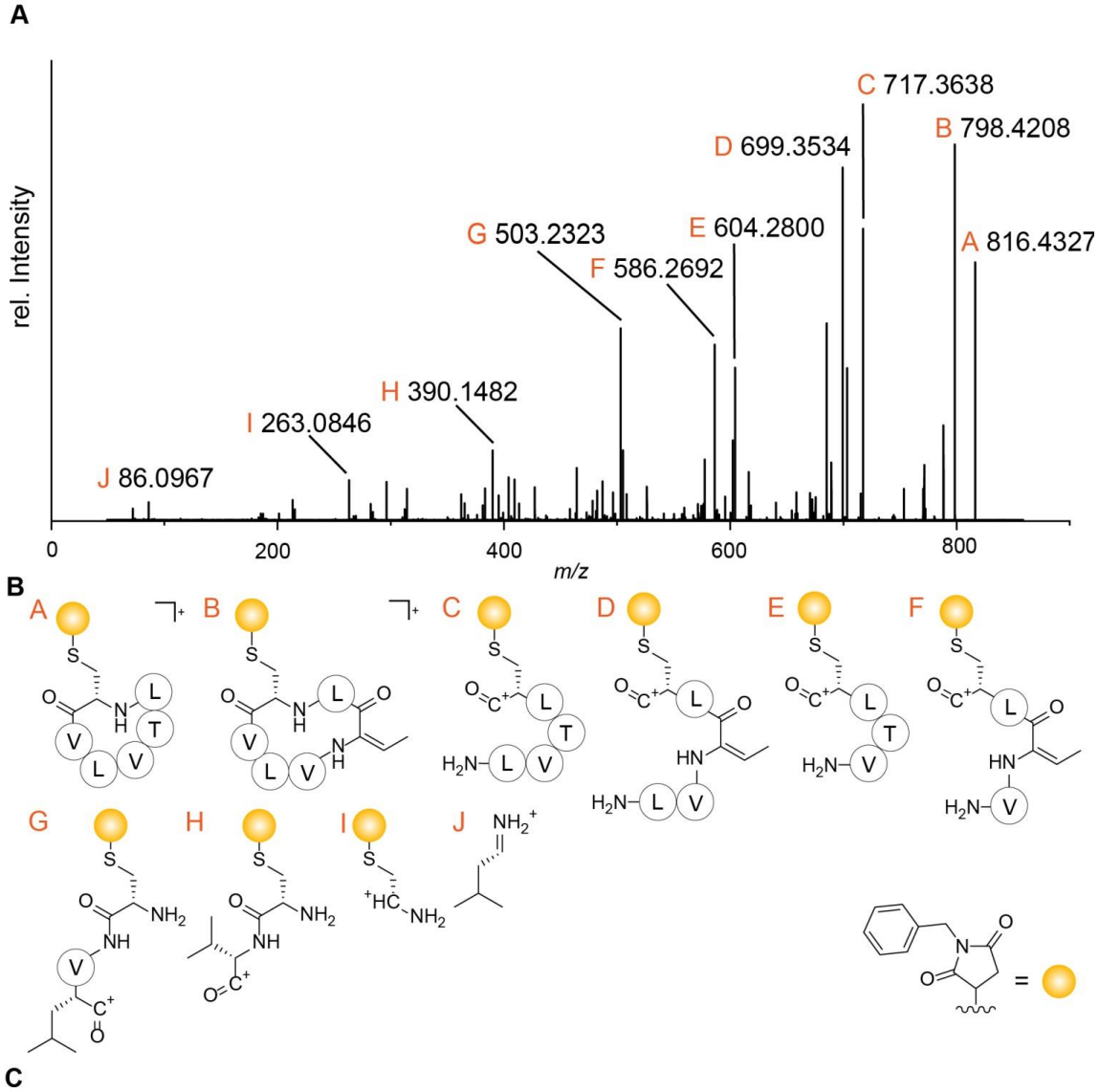
Figure S25. Fragmentation pattern of maleimide adducts of *Rc*-AIP1, *Rp*-AIP1, *Rj*-AIP1 and cyclopeptide 2. A) Representative fragmentation pattern (HPLC-HRMS method A) is shown for maleimide-labeled *Rc*-AIP1, *Rp*-AIP1, *Rj*-AIP1 from crude extracts of *R. cellulolyticum*, *R. papyrosolvens* and *R. josui*, respectively, as well as maleimide-labeled synthetic reference cyclopeptide **2**. **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.



C

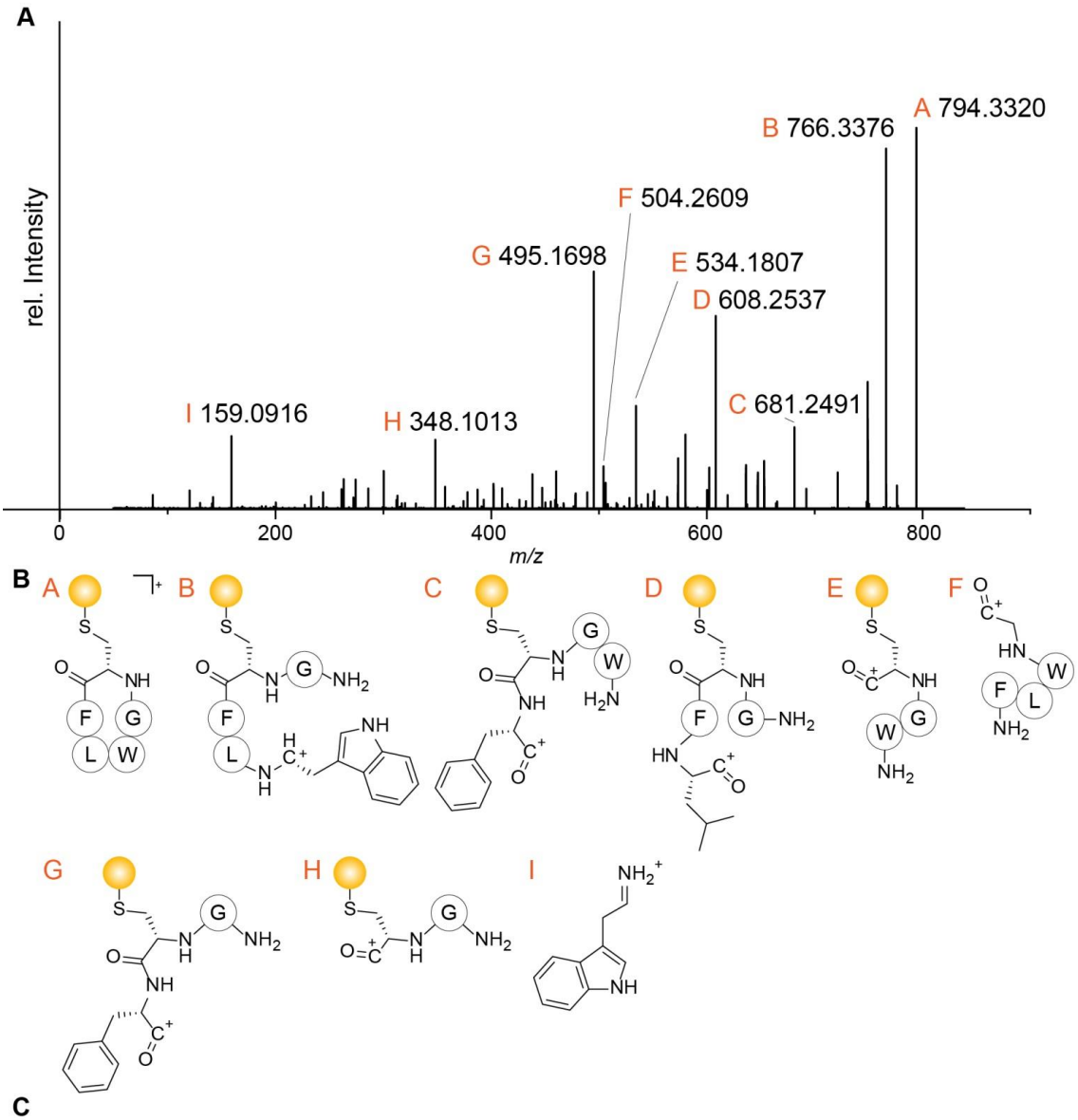
fragment	sum formula	[M+H]$^+$
A	$C_{57}H_{58}N_9O_{10}S^+$	1,060.4022
B	$C_{46}H_{48}N_7O_9S^+$	874.3229
C	$C_{46}H_{46}N_7O_8S^+$	856.3123
D	$C_{37}H_{37}N_6O_7S^+$	709.2439
E	$C_{34}H_{34}N_5O_5S^+$	624.2275
F	$C_{26}H_{27}N_4O_6S^+$	523.1646
G	$C_{25}H_{25}N_4O_4S^+$	477.1591
H	$C_{20}H_{20}N_3O_2^+$	334.1550
I	$C_{10}H_{11}N_2^+$	159.0917

Figure S26. Fragmentation pattern of the maleimide adduct of *Ca*-AIP1 from *C. acetobutylicum*. A) Representative fragmentation pattern (HPLC-HRMS method A) is shown for maleimide-labeled *Ca*-AIP1 detected from a crude extract of *C. acetobutylicum*. **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.



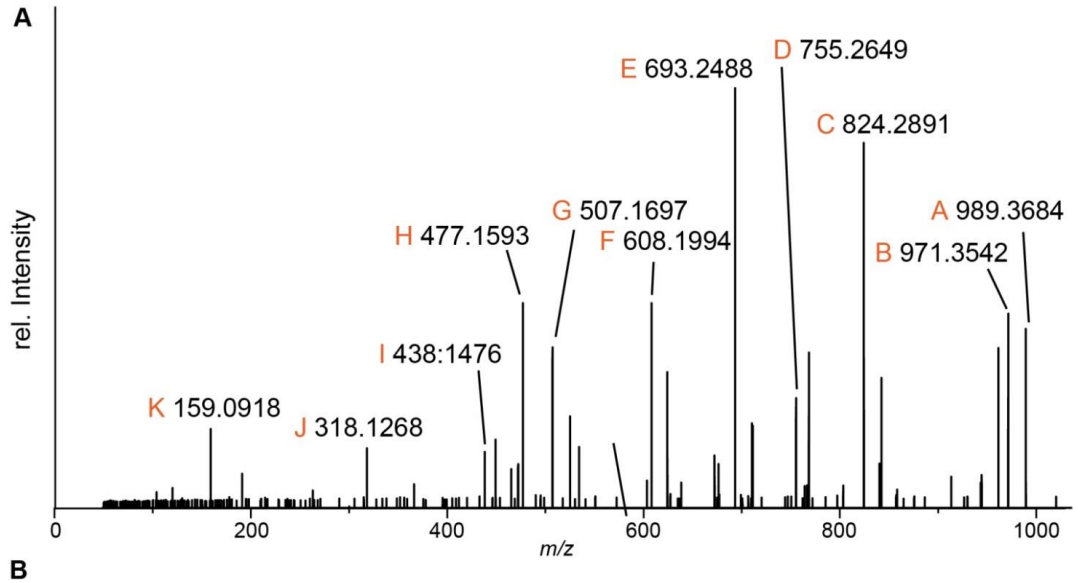
fragment	sum formula	$[M+H]^+$
A	$C_{40}H_{62}N_7O_9S^+$	816.4324
B	$C_{40}H_{60}N_7O_8S^+$	798.4219
C	$C_{35}H_{53}N_6O_8S^+$	717.3640
D	$C_{35}H_{51}N_6O_7S^+$	699.3534
E	$C_{29}H_{42}N_5O_7S^+$	604.2799
F	$C_{29}H_{40}N_5O_6S^+$	586.2694
G	$C_{25}H_{35}N_4O_5S^+$	503.2323
H	$C_{19}H_{24}N_3O_4S^+$	390.1482
I	$C_{13}H_{15}N_2O_2S^+$	263.0849
J	$C_5H_{12}N^+$	86.0864

Figure S27. Fragmentation pattern of the maleimide adduct of *Rp*-AIP2 from *R. papyrosolvens*. A) Representative fragmentation pattern (HPLC-HRMS method A) is shown for maleimide-labeled *Rp*-AIP2 detected from a crude extract of *R. papyrosolvens*. **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.

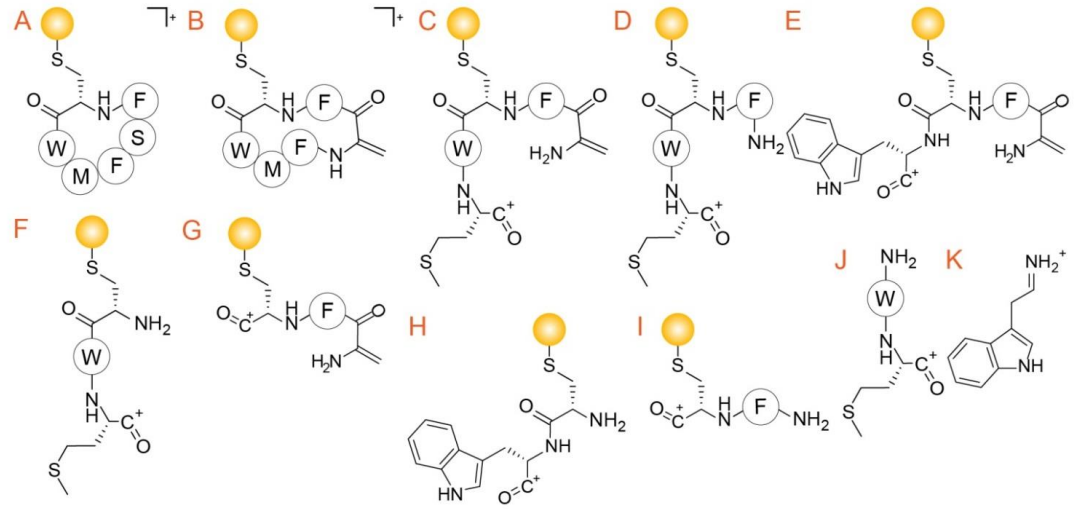


fragment	sum formula	$[M+H]^+$
A	$C_{42}H_{48}N_7O_7S^+$	794.3330
B	$C_{41}H_{48}N_7O_6S^+$	766.3381
C	$C_{36}H_{37}N_6O_6S^+$	681.2490
D	$C_{31}H_{38}N_5O_6S^+$	608.2637
E	$C_{27}H_{28}N_5O_5S^+$	534.1806
F	$C_{28}H_{34}N_5O_4S^+$	504.2605
G	$C_{25}H_{27}N_4O_5S^+$	495.1697
H	$C_{16}H_{18}N_3O_4S^+$	348.1013
I	$C_{10}H_{11}N_2^+$	159.0917

Figure S28. Fragmentation pattern of the maleimide adduct of *Rj*-AIP2 from *R. josui*. A) Representative fragmentation pattern (HPLC-HRMS method A) is shown for maleimide-labeled *Rj*-AIP2 detected from a crude extract of *R. josui*. **B)** Shown are possible ions. **C)** Listed are exact masses and respective sum formulas.



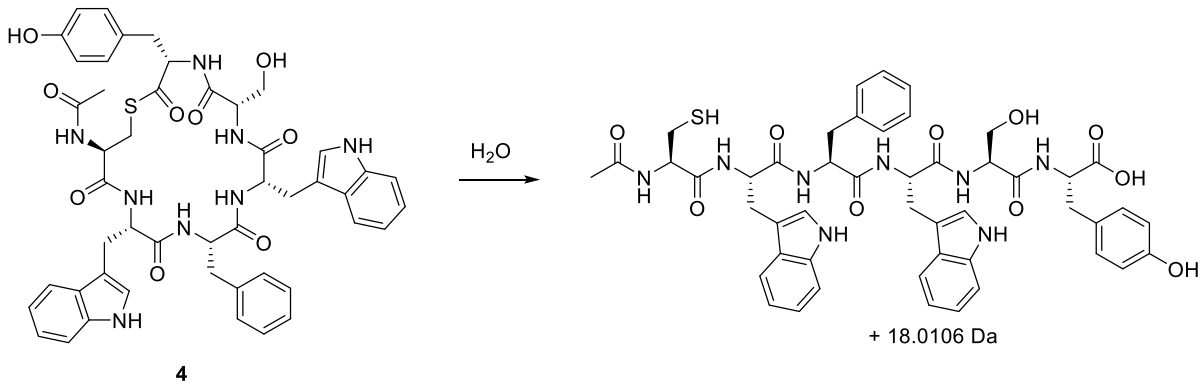
B



C

fragment	sum formula	$[M+H]^+$
A	$C_{51}H_{57}N_8O_9S_2^+$	989.3684
B	$C_{51}H_{55}N_8O_8S_2^+$	971.3579
C	$C_{42}H_{46}N_7O_7S_2^+$	824.2895
D	$C_{39}H_{43}N_6O_6S_2^+$	755.2680
E	$C_{37}H_{37}N_6O_6S^+$	693.2490
F	$C_{30}H_{34}N_5O_5S_2^+$	608.1996
G	$C_{26}H_{27}N_4O_5S^+$	507.1697
H	$C_{25}H_{25}N_4O_4S^+$	477.1591
I	$C_{23}H_{24}N_3O_4S^+$	438.1482
J	$C_{16}H_{20}N_3O_2S^+$	318.1271
K	$C_{10}H_{11}N_2^+$	159.0917

Figure S29. Investigation of the stability of analogue 4. Reaction scheme (above) and HPLC-HRMS chromatograms (below) (HPLC-HRMS method D) depicting the hydrolysis of derivative **4**. The chromatograms (absorbance at λ=280 nm) show *N*-terminally acetylated derivative **4** after incubation at 37 °C in 50 mM MOPS (pH 7.2) for the indicated time (d, days). For comparison, the extracted ion chromatograms (EICs) corresponding to **4** (*m/z* 915.3494 $[M+H]^+$) and hydrolyzed **4** (*m/z* 933.3600 $[M+H]^+$) are shown for the 3-day sample (the hydrolysis reaction is depicted below). After 3 days (the time-scale of the endospore formation assay), some of the compound was hydrolyzed but the majority remained unchanged.



4

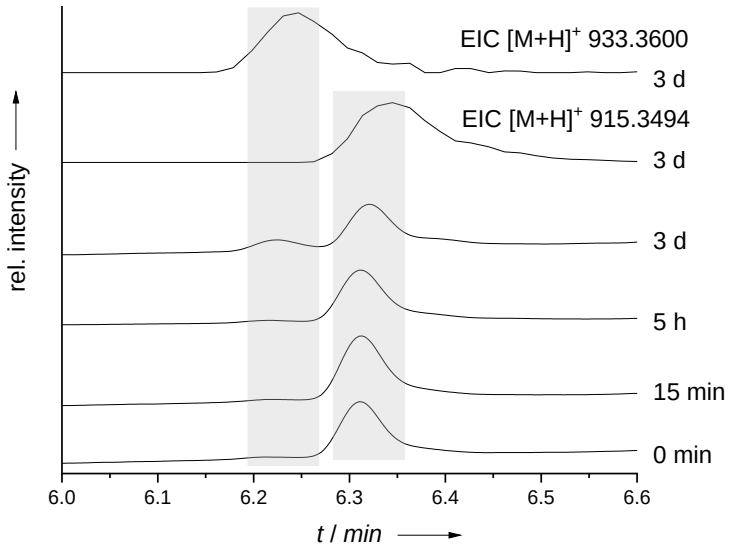


Figure S30. Controls for determination of rearrangement kinetics using Ellman's reagent. Shown is the increase of absorbance at 412 nm for the controls to determine the kinetics of rearrangement of the thiolactone moiety of **1** to the lactam, giving the cyclopeptide **2**. As a baseline, the values for Ellman's Reagent (ER) in 50 mM MOPS were subtracted from the measured values of **1** in 50 mM MOPS with ER.

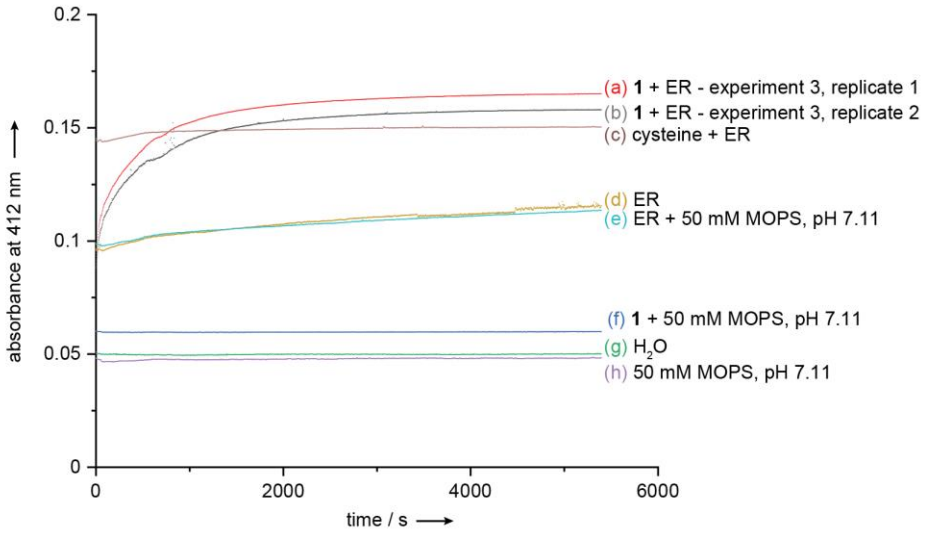


Figure S31. ^{1}H-NMR spectra of peptide 1a.

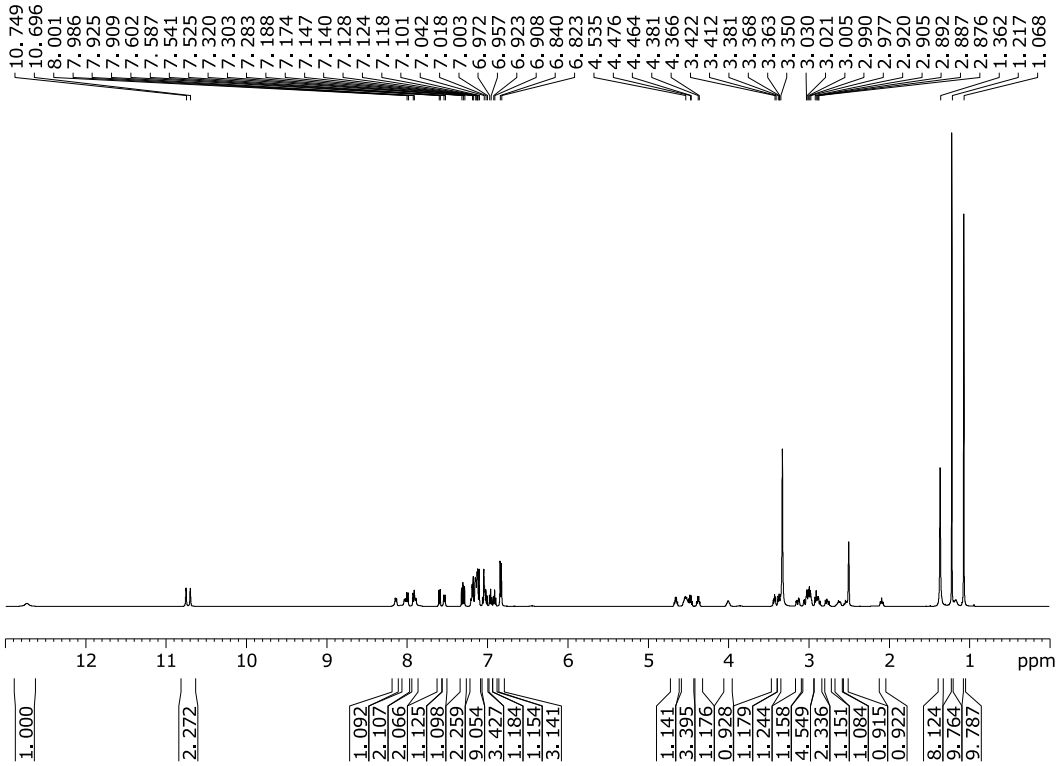


Figure S32. ^{13}C-NMR spectrum of peptide 1a.

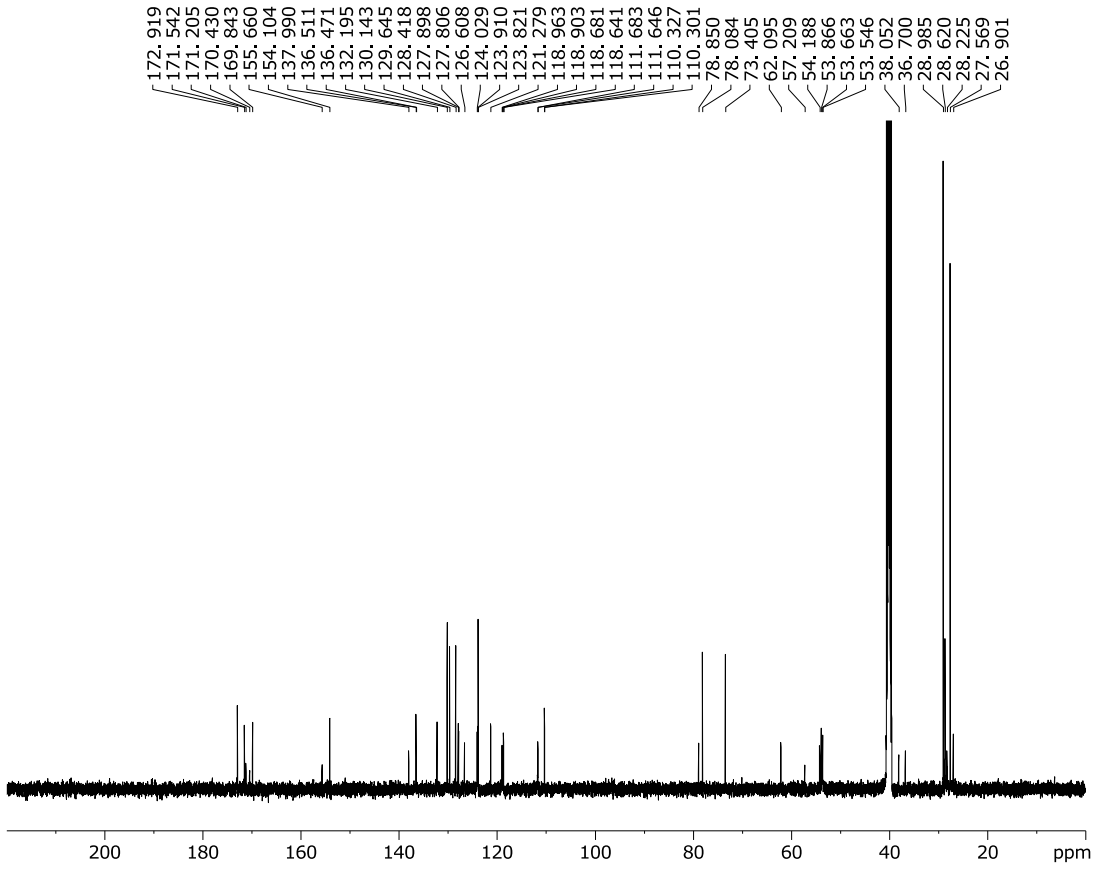


Figure S33. ^{1}H-NMR spectrum of linear peptide 3.

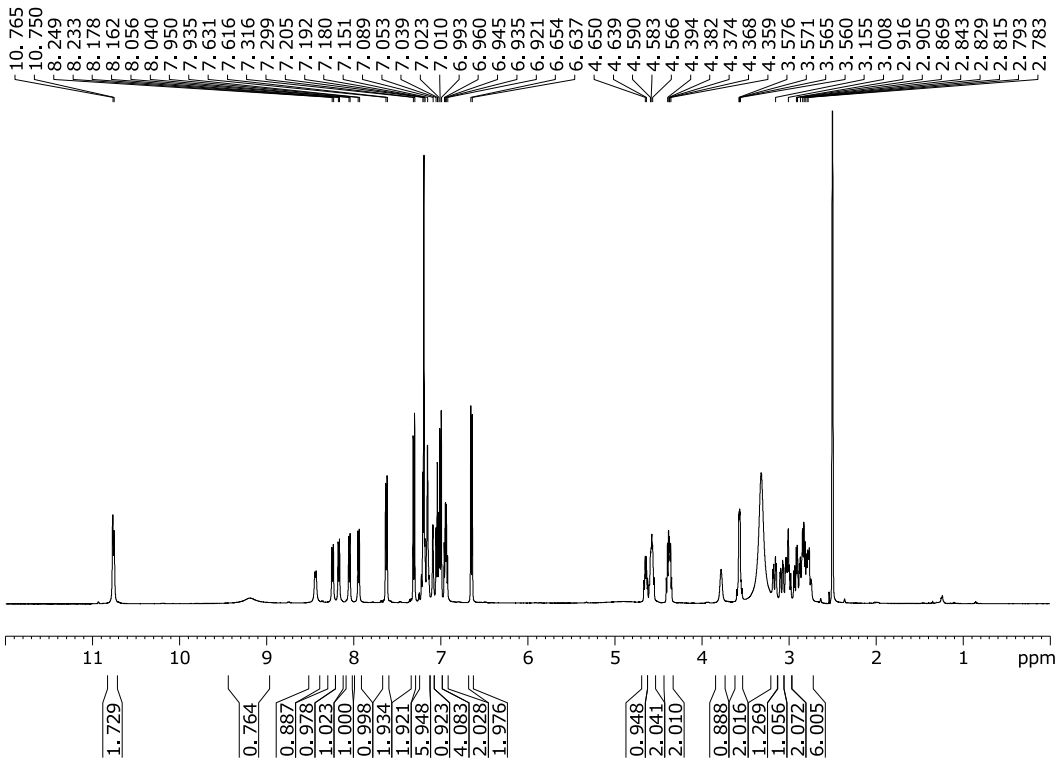


Figure S34. ^{13}C-NMR spectrum of linear peptide 3.

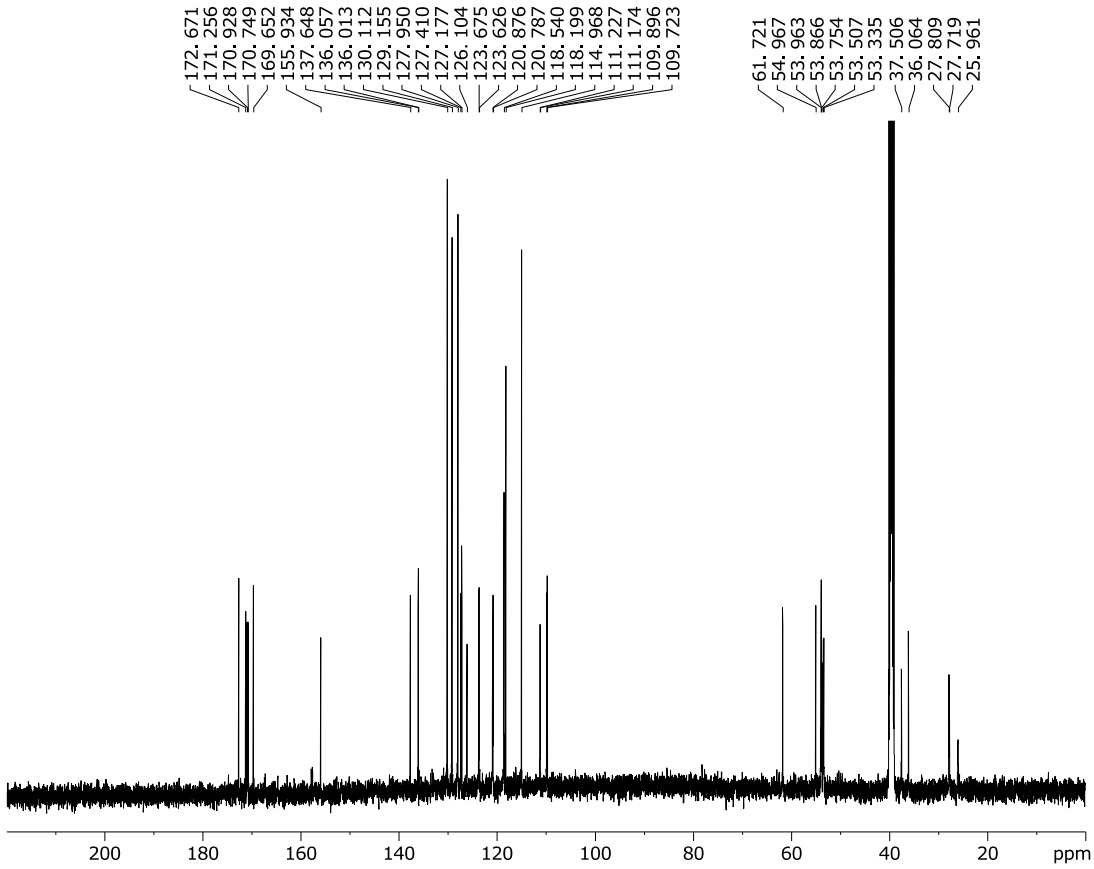


Figure S35. ^{1}H-NMR spectrum of peptide 5a.

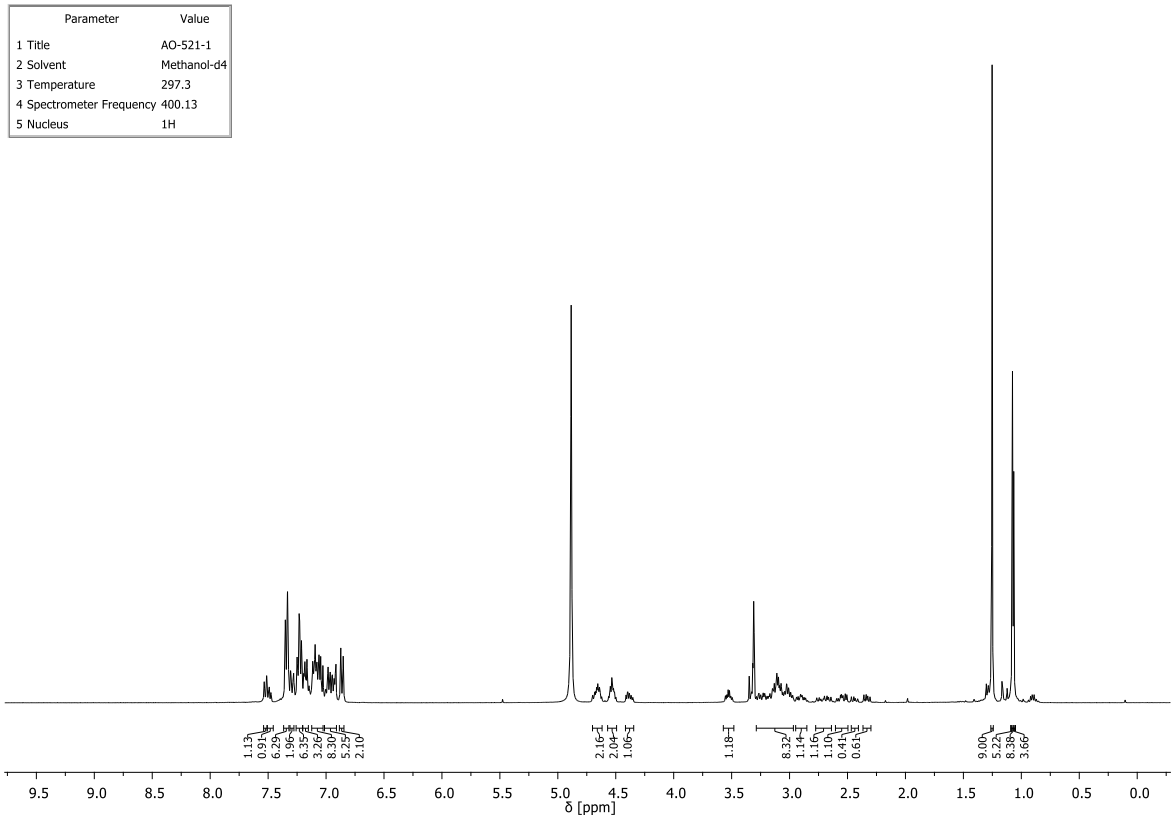


Figure S36. ^{13}C-NMR spectrum of peptide 5a.

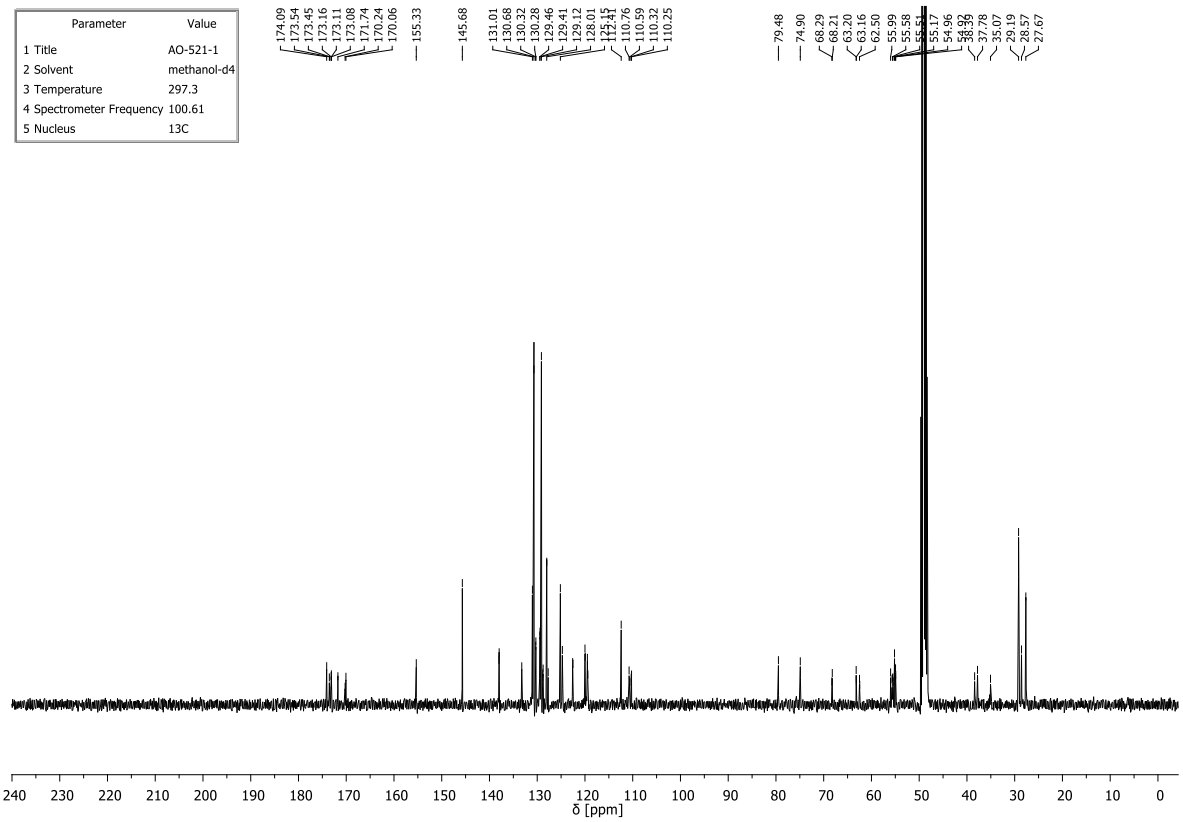


Figure S37. ^{1}H-NMR spectrum of peptide 1b.

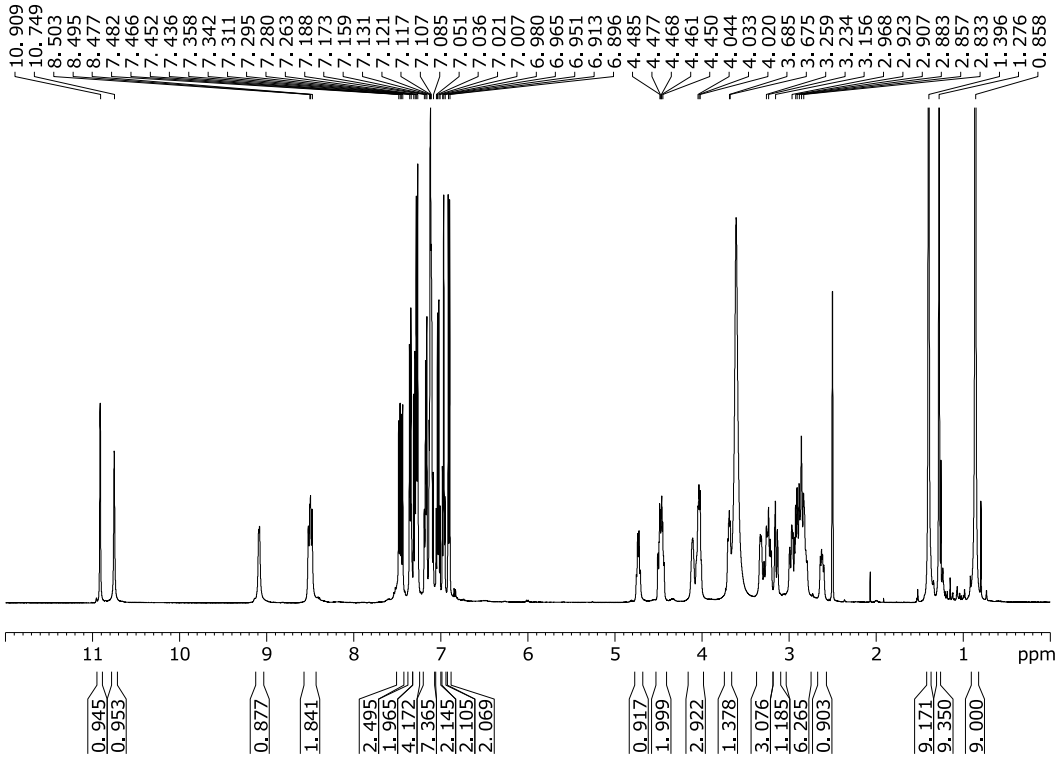


Figure S38. ^{13}C-NMR spectrum of peptide 1b.

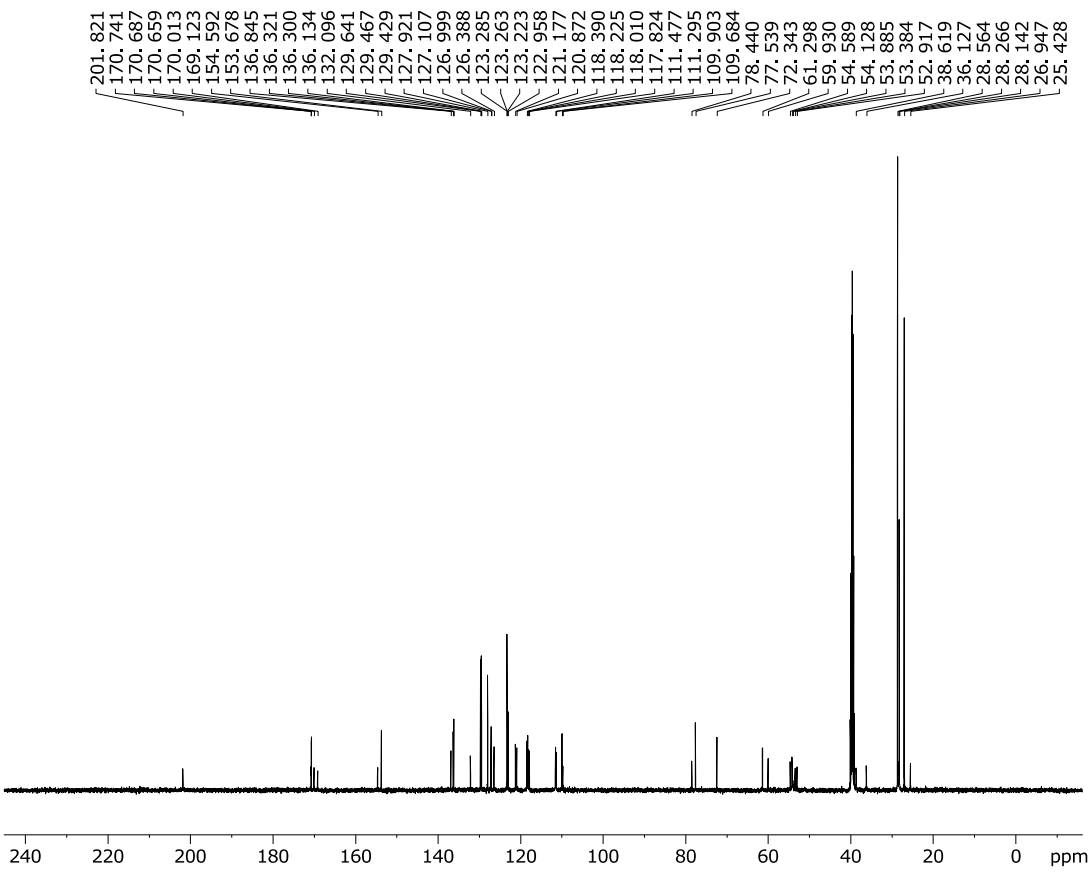


Figure S39. ^{1}H-NMR spectrum of thiolactone reference 1.

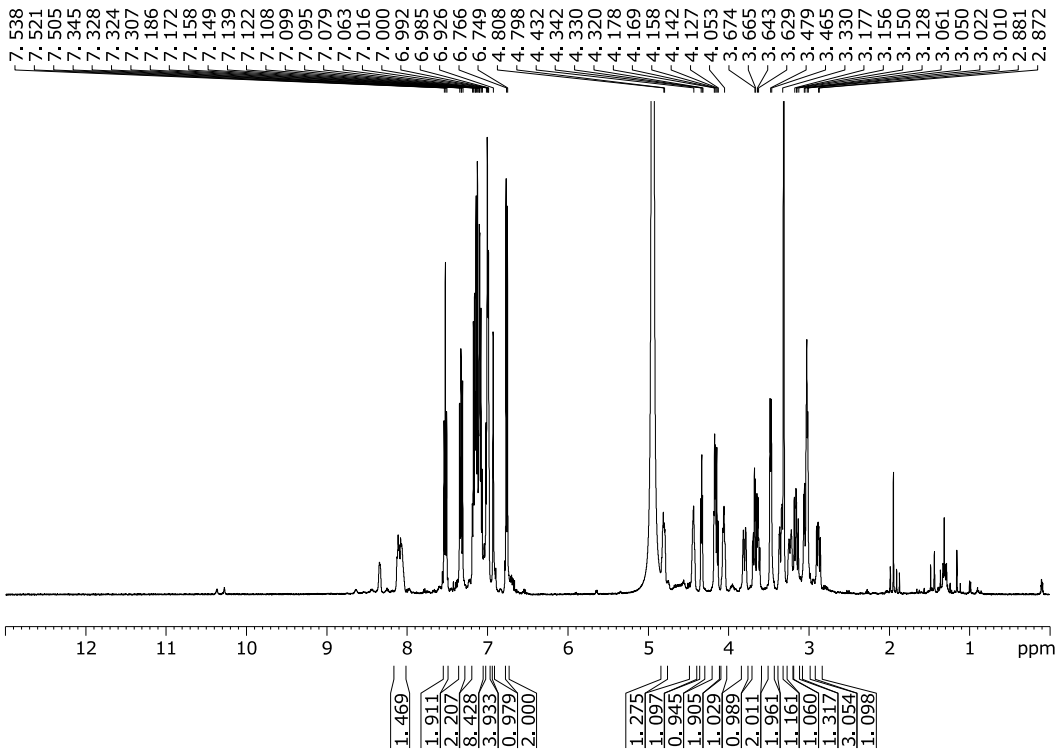


Figure S40. ^{13}C-DEPT-NMR spectrum of thiolactone reference 1.

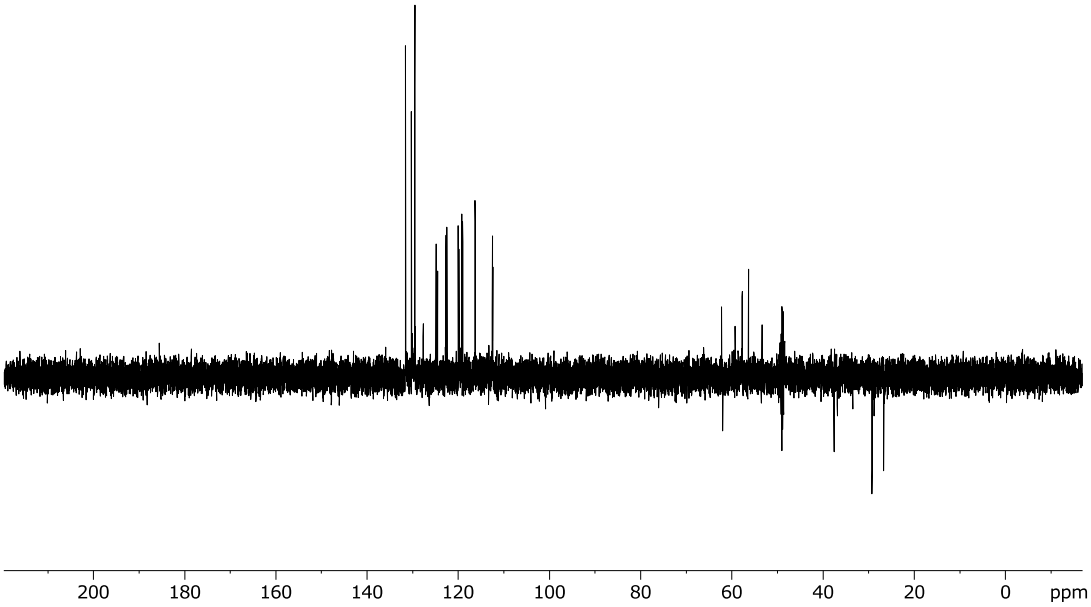


Figure S41. ^{1}H,^{1}H-COSY NMR spectrum of thiolactone reference 1.

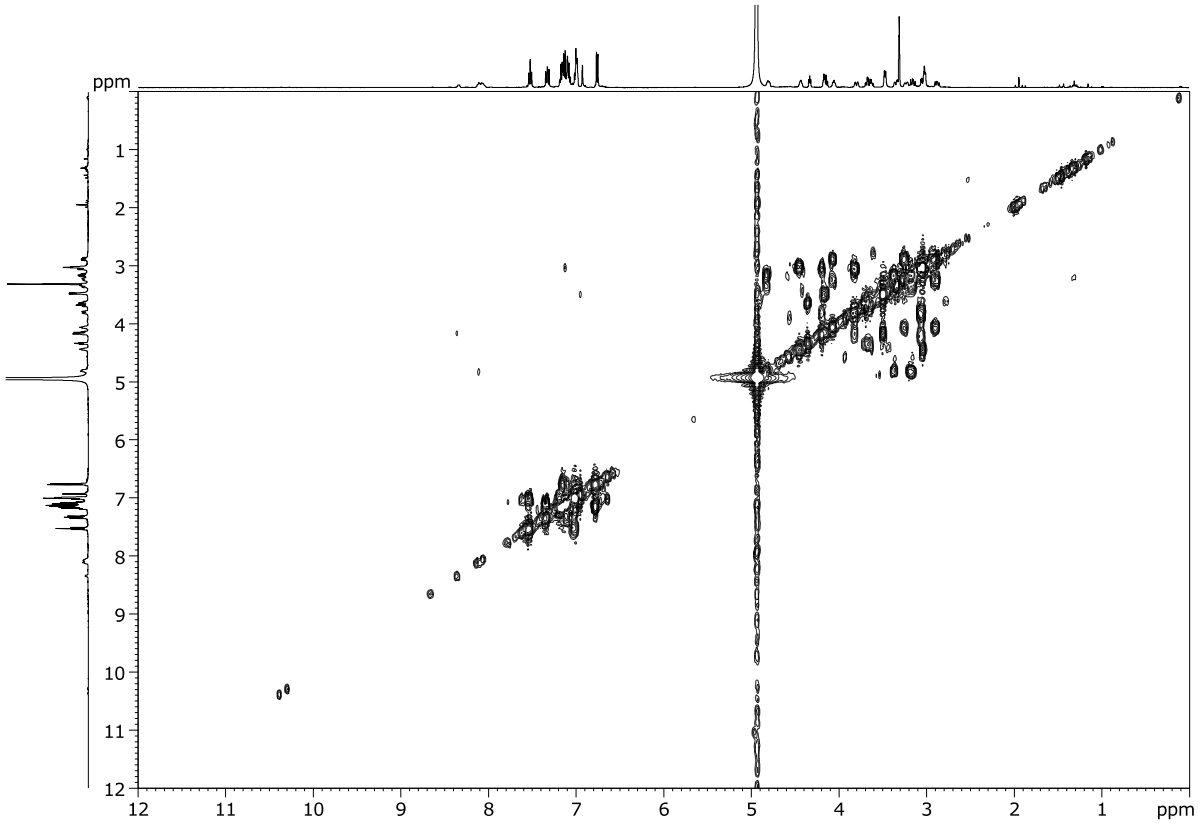


Figure S42. $^{1}H,^{13}C$-HSQC NMR spectrum of thiolactone reference 1.

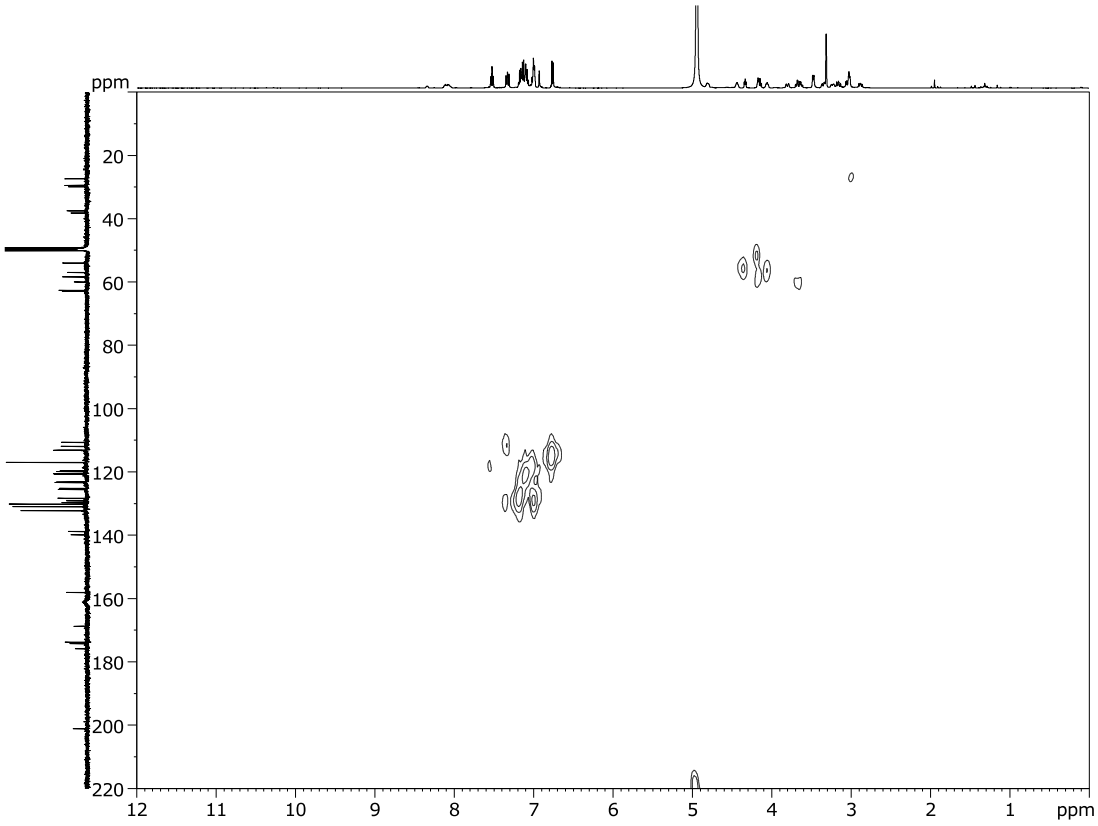


Figure S43. $^{1}H,^{13}C$-HMBC NMR spectrum of thiolactone reference 1.

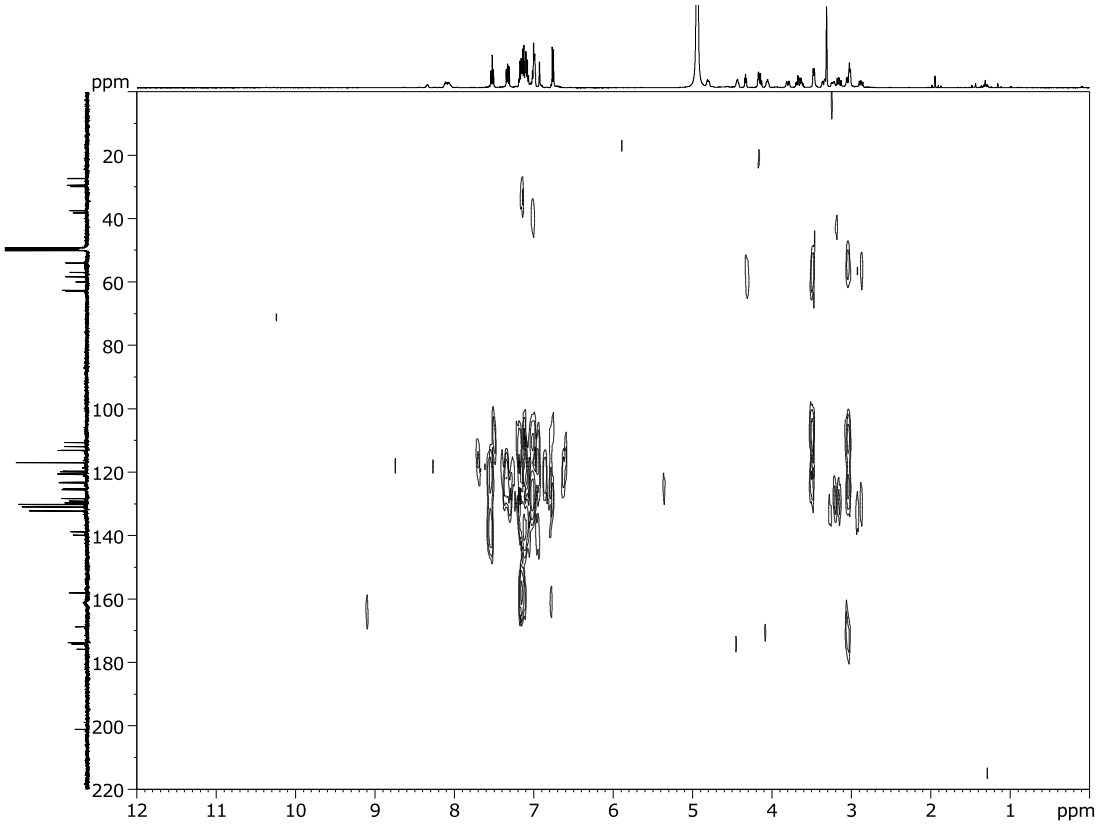


Figure S44. ^{1}H-NMR spectrum of cyclopeptide reference 2.

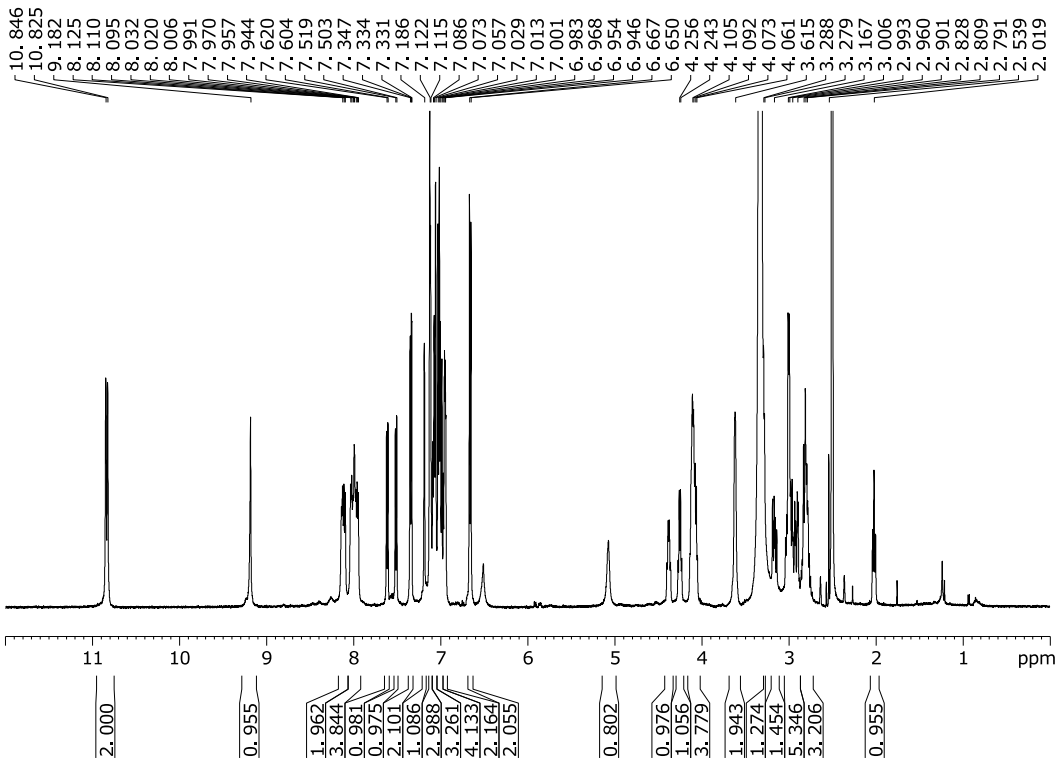


Figure S45. ^{13}C-DEPT-NMR spectrum of cyclopeptide reference 2.

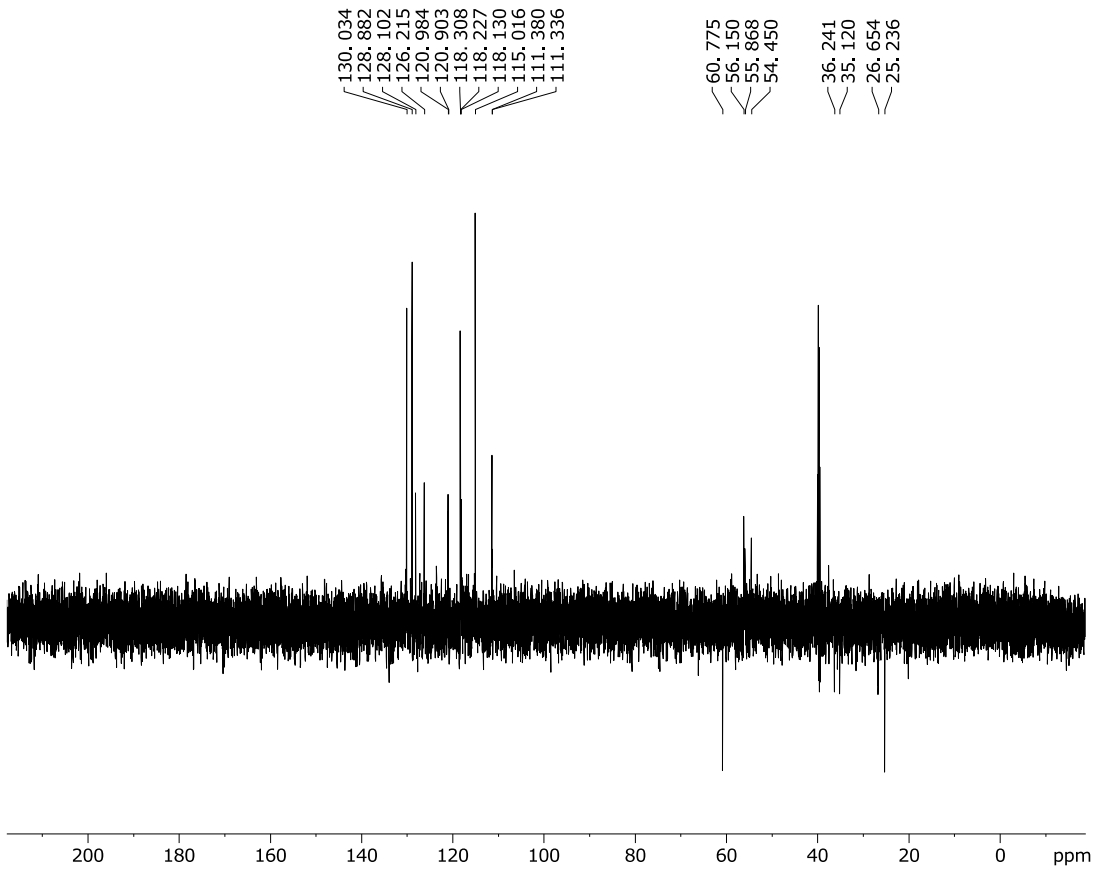


Figure S46. $^{1}H,^{1}H$-COSY NMR spectrum of cyclopeptide reference 2.

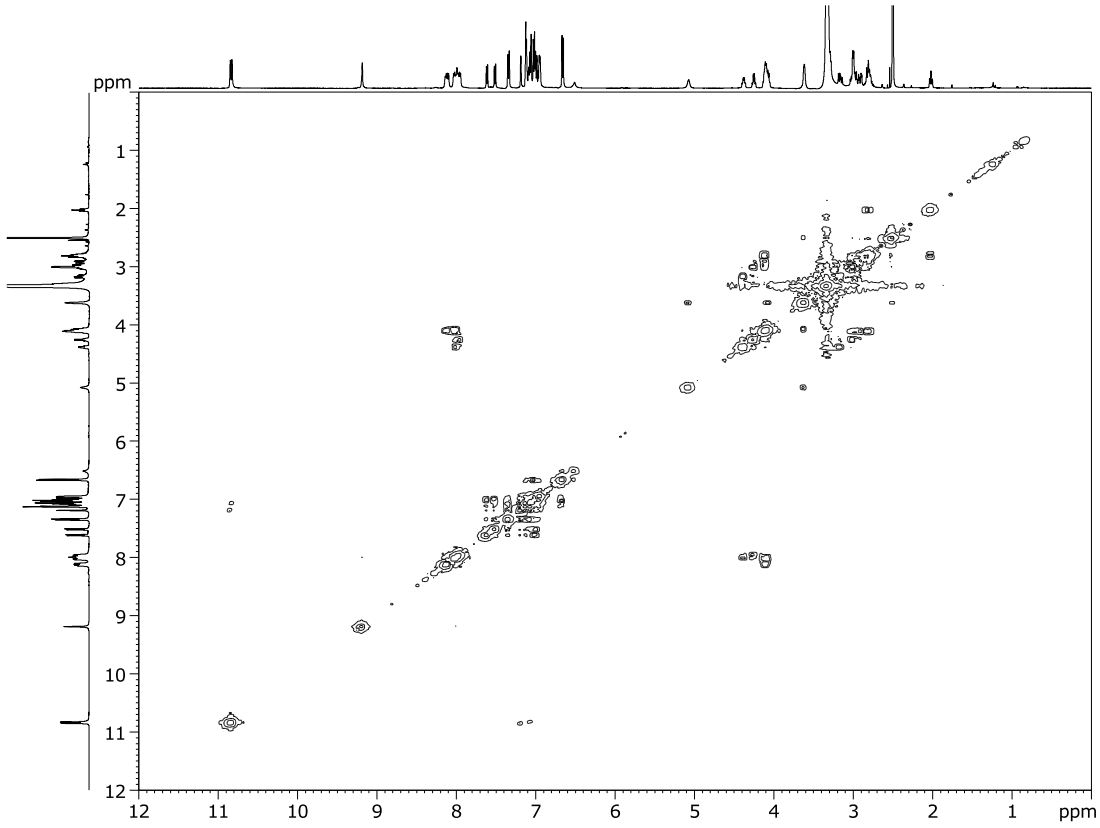


Figure S47. $^{1}H,^{13}C$-HSQC NMR spectrum of cyclopeptide reference 2.

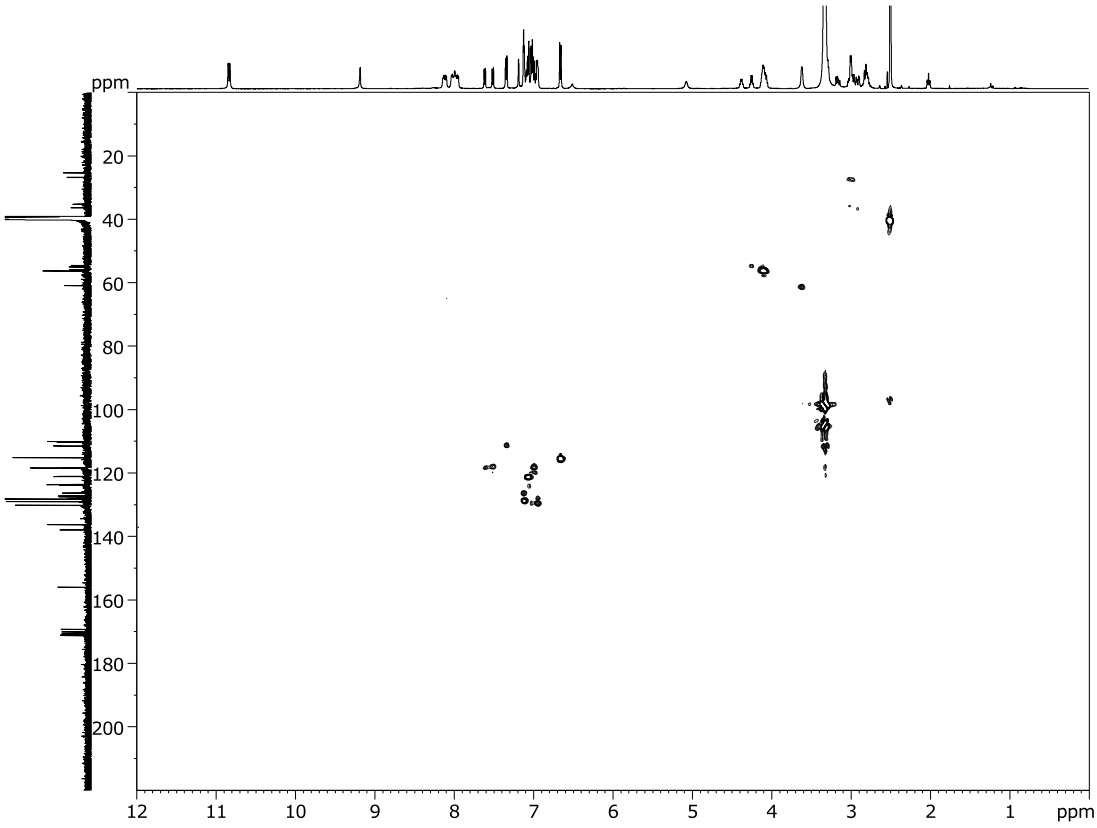


Figure S48. $^{1}H,^{13}C$-HMBC NMR spectrum of cyclopeptide reference 2.

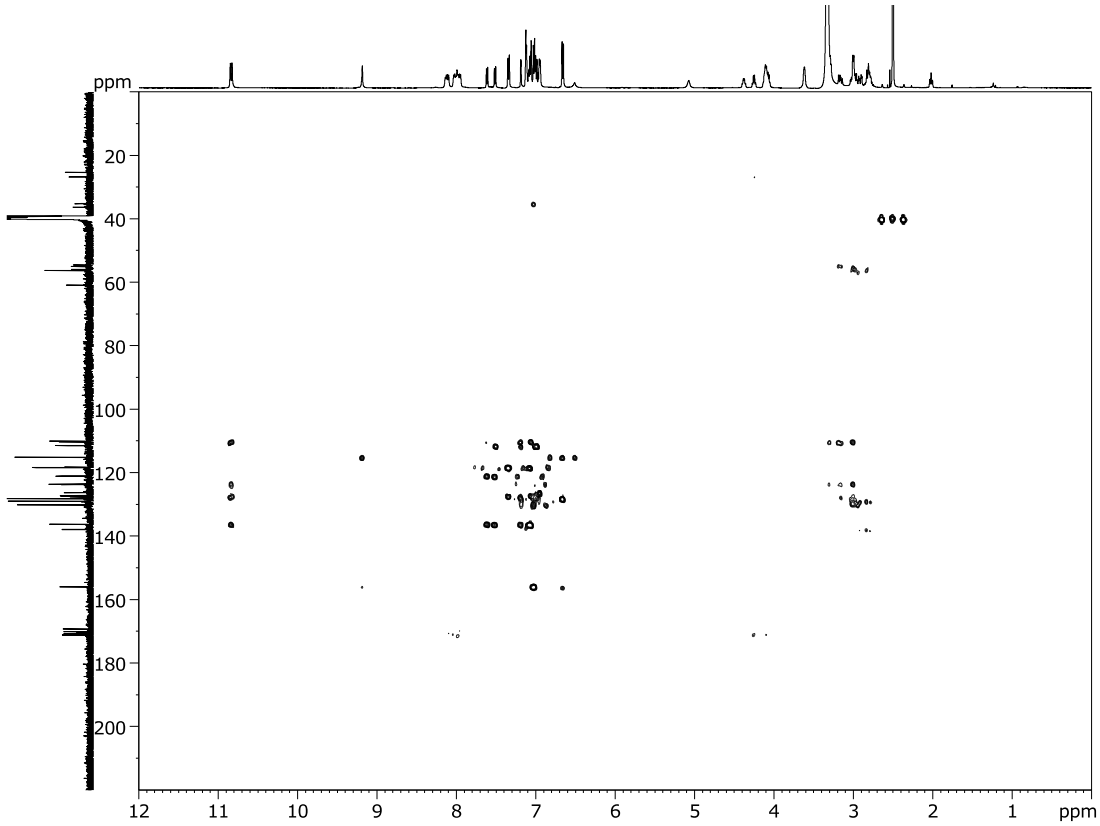


Figure S49. ^{1}H-NMR spectrum of *N*-acetylated analogue 4.

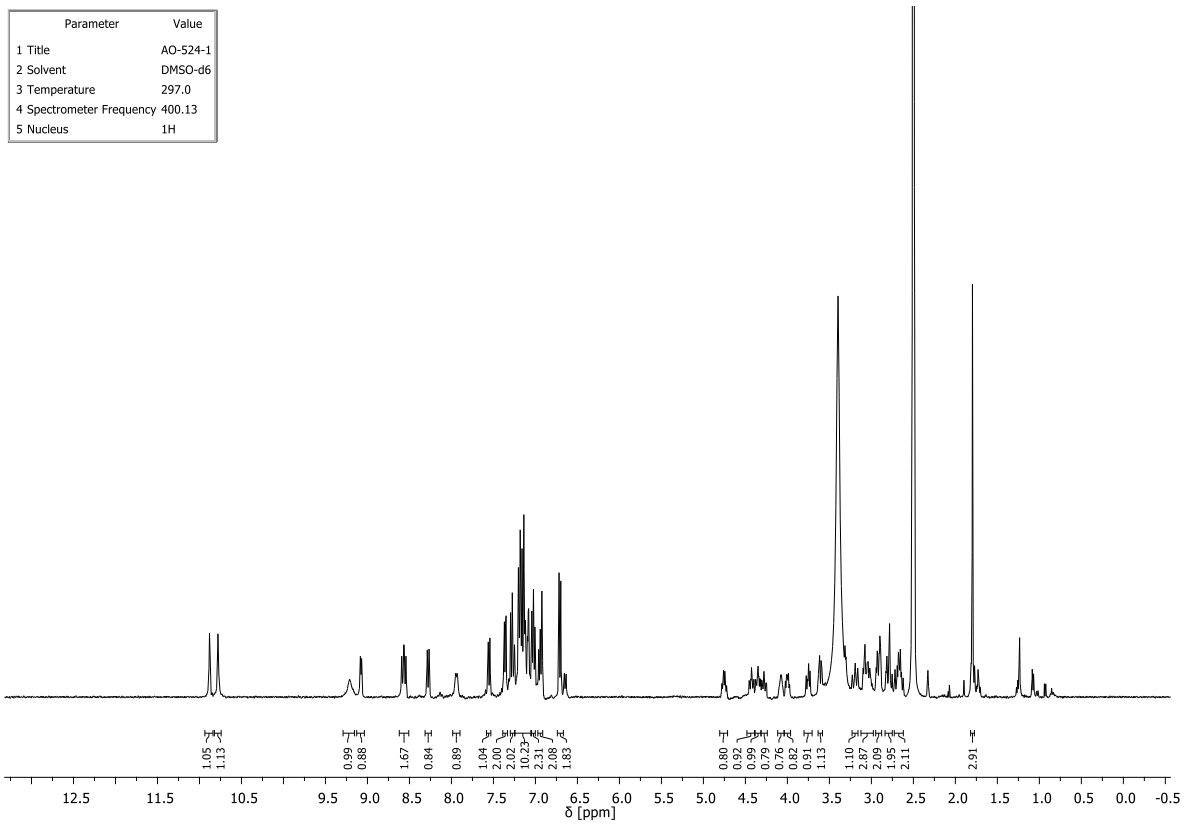


Figure S50.^{13}C-NMR spectrum of *N*-acetylated analogue 4.

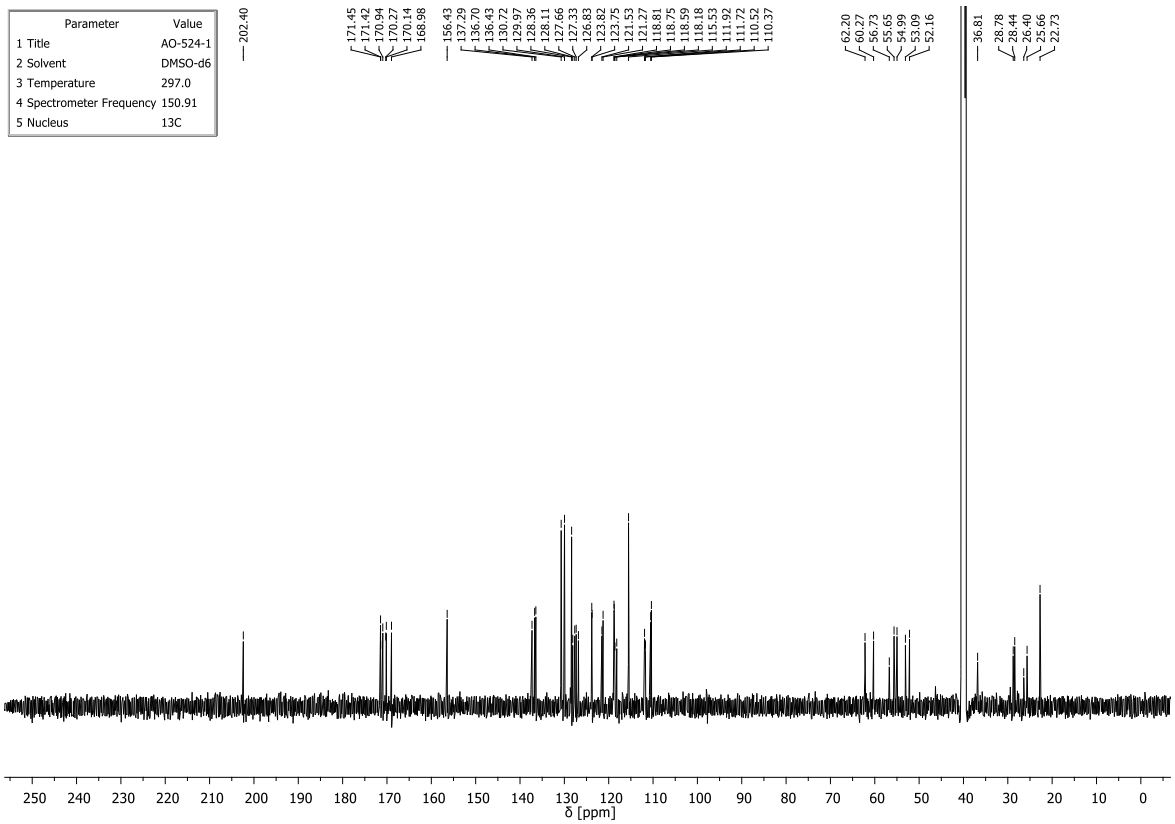


Figure S51. ^{1}H-NMR spectrum of thiazole-containing analogue 5.

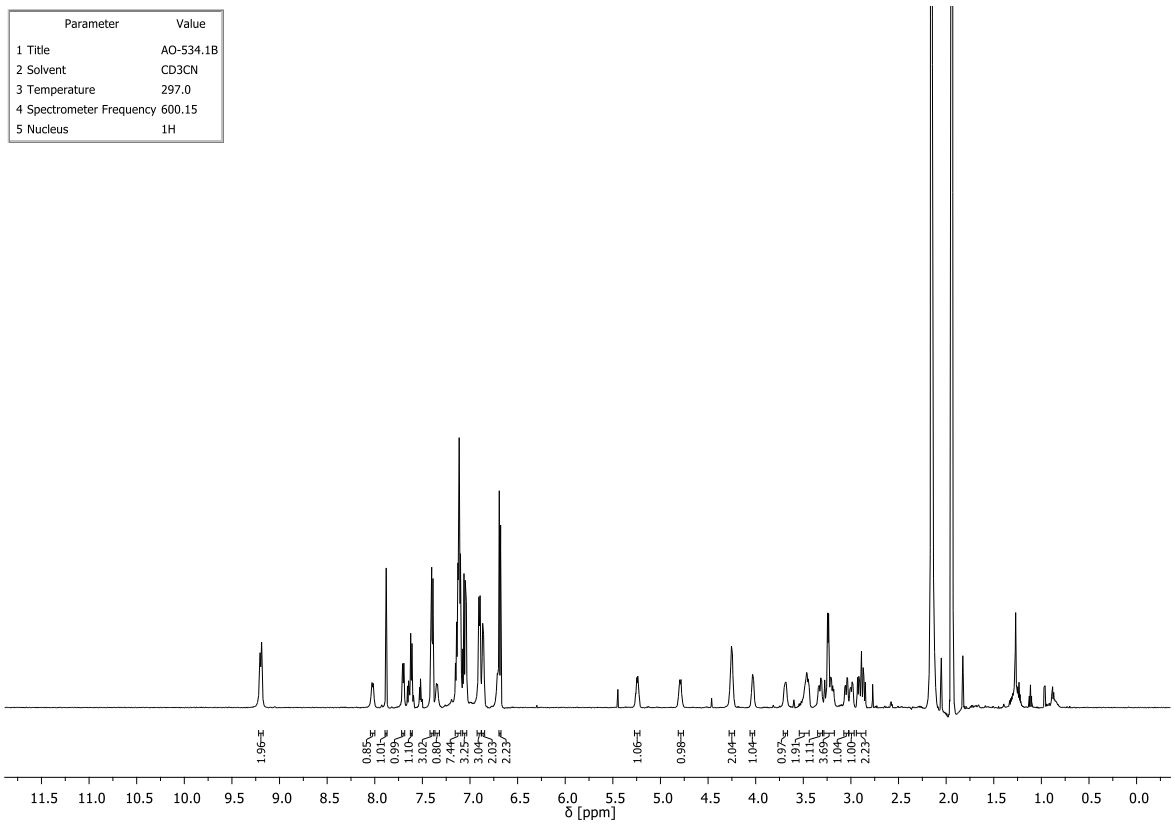


Figure S52. ^{13}C-NMR spectrum of thiazole-containing analogue 5.

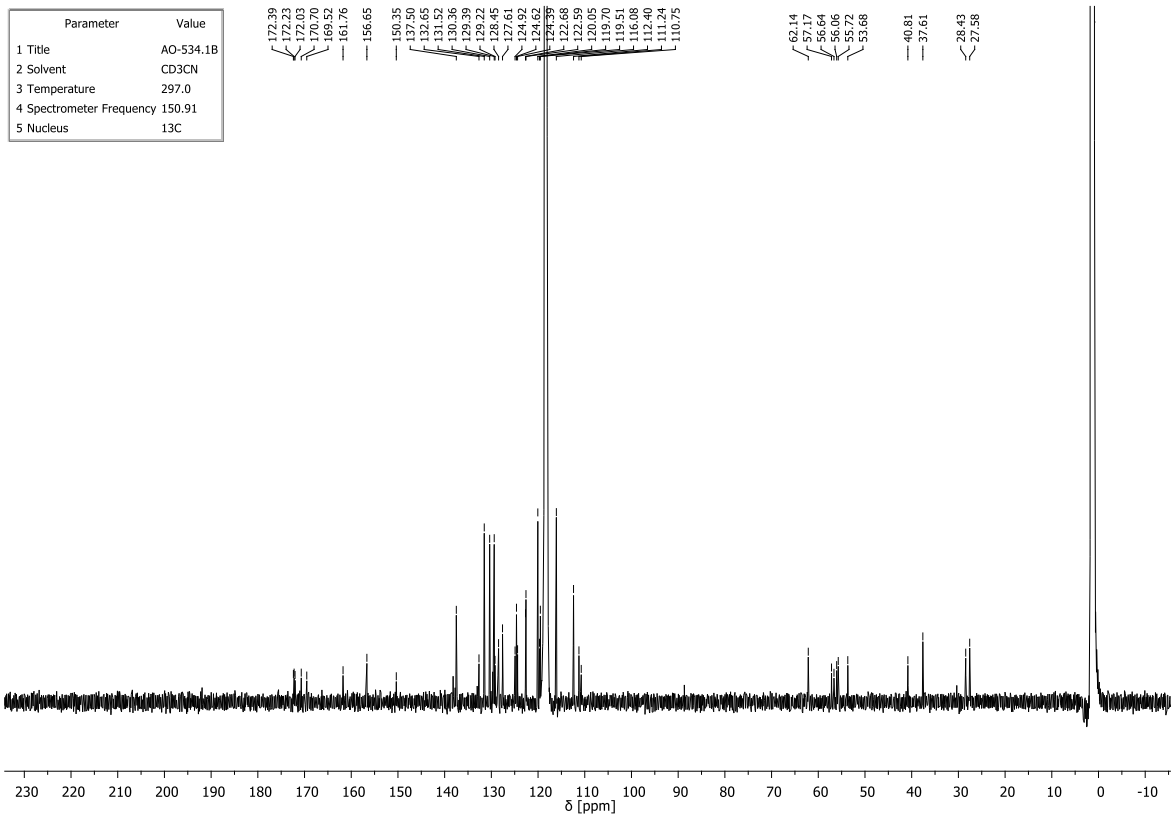


Figure S53. Structure elucidation of thiolactone 1 and cyclopeptide 2.

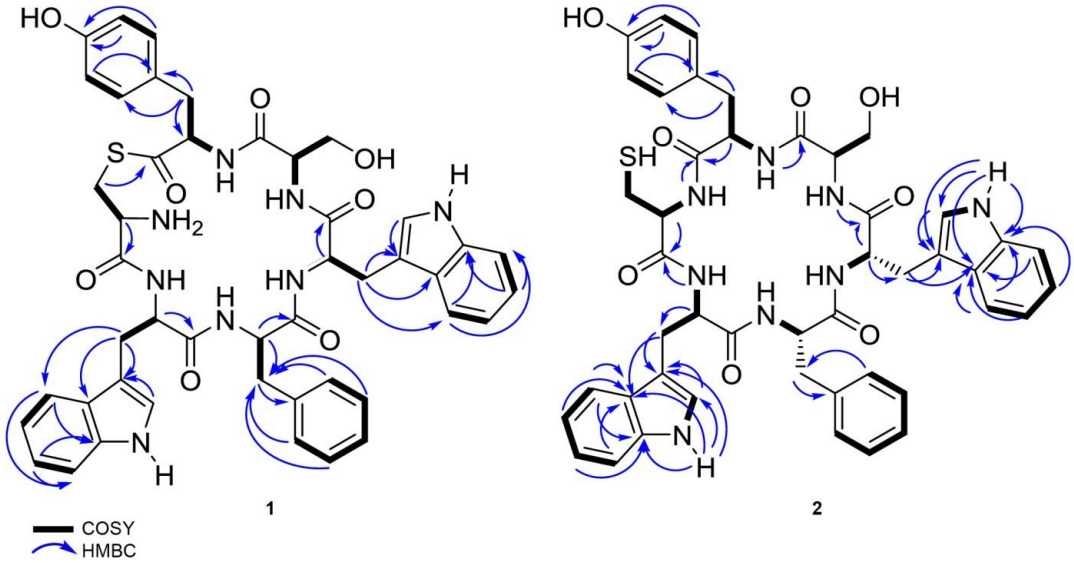


Table S1. Putative AIP biosynthetic gene clusters identified in diverse Clostridia. Strains that were screened in this study are highlighted in yellow. Clusters from which a compound was detected are colored red. The predicted core peptide sequence for each AgrD precursor peptide is underlined.

Strain	AgrB	AgrD	AgrA	AgrC	Sequence of AgrD	Mature cAIP sequence
***Ruminiclostridium cellulolyticum* DSM 5812**	Ccel_0955	Ccel_0957	Ccel_0958	-	MKSKIKFLLAPLSLIITQLALSGVCTA**CSSTF**YQP KAPKHLTANRTHS	-
	Ccel_2798	Ccel_2791	Ccel_2790, Ccel_2793	-	MKSRIKFLLAPLSLIITQLALSGVCSA**CCSSL**YQP KAPKHLTASRQHS	-
	Ccel_2127	Ccel_2126	-	Ccel_2125	MKGKIKLLVLTVLSLVGVFAATASAGA**CWFWSY** YQRECPKSLLK	**CWFWSY**
	Ccel_3455	Ccel_3456	-	Ccel_3457	MRKNLLSSVGKVSLIVTGLITGAA**SKILWL**YKPE K	-
***Ruminiclostridium josui* DSM 25723**	-	K412_RS21970	K412_RS016960	K412_RS0116955	MKNKLKLIVLMALSVIGITAATASAGA**CWMFSF**Y QKECPTSLLK	**CWMFSF**
	K412_RS0102150	K412_RS21625	K412_RS0102135	K412_RS0102140	MKIRPHKIKLFVLLGITMISLFFSTASANG**CWLW SM**HQRKCPESLIK	-
	K412_RS0111890	K412_RS21865	-	-	MKSKIKLLLAPLSLLITFLALSGVSTA**CCSSY**YQP KAPKHLTTRKSH	-
	K412_RS0114620	K412_RS21915	-	-	MNKKSKLLSIIDSNVTAIAEKKAENF**SFLWG**HQP RVPKSLKTIKK	-
	K412_RS0101445	K412_RS21605	-	K412_RS0101435	MKGKIKLLVLTVLSLIGVFAATASAGA**CWFWSY** YQKECPKSLLK	**CWFWSY**
	K412_RS0108210	Unannotated	-	K412_RS0108220	MKKNLLKTVGKVSLIVTGLITGAA**SKILWL**YQPE K	-
***Ruminiclostridium papyrosolvens* DSM 2782**	Cpap_1414	Cpap_1415	-	Cpap_1416	MRKNLLKSVGKVSLIVTGLITGAA**SKILWL**YQPE K	-
	Cpap_0110	Cpap_0111	-	Cpap_0112	MKGKIKLLVLTVLSLIGVFAATASAGA**CWFWSY** YQKDCPKSLLK	**CWFWSY**
	Cpap_0129	Cpap_0138	-	-	MKSKIKFLLTPLSIIITLLALSGVCSA**CMSSY**YQP KAPKHLKKH	-
	Cpap_3744	Cpap_3749	-	-	MKAKIKLLFVPLSLLITLLALSGVCSA**CYGYY**YQ PKAPKHLTVRRSH	-
	Cpap_1450	Cpap_1451	-	-	MDNTKKILSLIDSGVTKVAETKADNF**CFLWG**YQ PKTPKALKKIKK	**CFLWG**
***Clostridium acetobutylicum* DSM 792**	CA_C0078	CA_C0079	CA_C0081	CA_C0080	MNLKEQLNKVNDKFIKGLGKASMKIGEQANGK**C VLVTL**YEPKMPEELLKENIDK	**CVLVTL**
***Clostridium cellulovorans* DSM 3052**	CLOCEL_RS06570	CLOCEL_RS06575	CLOCEL_RS06550	CLOCEL_RS06555	MRKTKSKVLSLFAVLTTVIASMVASSA**CFWYY**Y QPEEPEILRDK	-
	CLOCEL_RS06160	CLOCEL_RS22225	-	-	MKNSNKKVLSLISAITTKAAEDLSNST**CVTAL**YQ PKEPKSLQKK	**CVTAL**
	CLOCEL_RS21235	Clocel_4254	CLOCEL_RS21225	CLOCEL_RS21230	MRNMREELIKIGDMVIGEIGNEAVKLGDRARGL **CLFLNV**YEPVIPIELLDDIE	-
***Hungateiclostridium thermocellum* DSM 1313**	Clo1313_0947	Clo1313_0946	-	Clo1313_0945	MLRALKALKRSDLLALLLGWLSVIAIFIAQASSNS **CTSWAI**EQPKIPKSLIKQD	-
	Clo1313_3021	-			-	-
	-	Clo1313_2818			MKNKVLSAISAVLFVIASMVASSA**SFFIF**YQPKTP KALKK	-
***Clostridium botulinum* A ATCC 3502**	CBO_RS01850	CBO_RS01855	-	CBO_RS01840	MKKQLKEKCTKVTAKLLKSVAYSTADSA**CHLGI** YQPKEPKSLRK	-
	CBO_RS01815	CBO_RS01820	-	-	MKKLNKKVLMLVATFTTLLASIVASSA**CYWCV**Y QPKEPKCLREE	-
***Clostridioidies difficile* DSM 1296**	CDIF1296T_02890	Unannotated	-	-	MKKFIVRFMKFASSLALSTAILSANST**CPWII**HQP KVPKEISNLKKTN	-
	CDIF1296T_03445	CDIF1296T_03446	CDIF1296T_03448	CDIF1296T_03447	MKKIALNLLKNISALSFGIAVLSANSA**SSWVA**HQ AKEPQALQKLKK	-

Table S1 (continued). Putative AIP biosynthetic gene clusters identified in diverse Clostridia. The predicted core peptide sequence for each AgrD precursor peptide is underlined.

Strain	AgrB	AgrD	AgrA	AgrC	Sequence of AgrD	Mature cAIP sequence
***Clostridium pasteurianum*DSM 525**	F502_RS 14735	F502_RS 20285	-	F502_RS14740	MKNKLKNTLKKNISVIFKRTAFSTSASA**CTFSF**YQ PKEPKCLQKK	-
	F502_RS 05975	F502_RS 05970	-	-	MKKKLLIVVAAVTTVVASVIASSA**CVFFF**YQPEEP KCLQKR	-
	F502_RS 04745	F502_RS 20410	F502_RS 04750	F502_RS04780	MRTLKIKKCIAIALCTVSMIIAETSSSM**CLFFLA**KE TKMPKSLYKVD	-
	F502_RS 13030	F502_RS 21025	-	-	MRNMKAKISDFTEKFADVIGEVSIKISDKTCEK**CF ILGI**YEPNIPIEILKENARK	-
	F502_RS 17030	F502_RS 21050	F502_RS 17020	F502_RS17025	MINKKLHKSILNKAGFCLIAVASFITVSS**SLFLL**GE PTPPKSLYND	-
	F502_RS 04435	F502_RS 20040	F502_RS 04450	F502_RS04440	MKNIKNLLVSRSMKIAGFVALFLGTIVITPA**STLGS** HQPKCPDEFLK	-
Clostridium perfringens ATCC 13124	CPF_RS0 8805	CPF_RS0 8800	-	-	MKKLNKNLLTLFAALTTVVATTVATSA**CLWFT**HQ PEEPKSLRDE	-
	CPF_RS0 4265		-	-	MRKSILSMLALLTTLTINASTT**SFATI**GLEEMPES MKKSR	-
***Clostridium puniceum* DSM 2619**	CLPUN_5 2280	CLPUN_5 2290	-	-	MENKIKKSIVVLVSNLCTKMALSVSASA**CLWNS**Y QPKEPKCLEKWNSKEL	-
	-	CLPUN_4 4270	-	-	MVNYLKKSLTSFISAICTQMAFTVSASA**CNWSS**F QPEEPKCLRNKE	-
	CLPUN 5 3090	CLPUN 5 3100	CLPUN 5 3130	CLPUN 53110, CLPUN_53120	MQKSISFFSVMLLNLFKLVSKT**SCPLTW**GEPDYP KELLK	-
	CLPUN_1 5330	CLPUN_1 5270	CLPUN_1 5320	CLPUN_15310	MKITKIKSFLALSLSAVAMLVASVSSTM**CCTHF**EE PKMPKSLYKVD	-
	CLPUN_1 9800	CLPUN_1 9810	-	CLPUN_19820	MIKKILGEFLKKLSDVMITEATA**SLVGS**GVEEMPE SMKKLR	-
	CLPUN 1 3950	CLPUN 1 3960	-	CLPUN_13970	MIKKIFGEILKKISDSMITLAPA**SYWGS**GVEEMPK SLKNLR	-
***Clostridium tetani* ATCC 13124**	-	-	-	-	-	-

Table S2. Strains used in this study.

Strain	Relevant genotype and descriptions	Source
Ruminiclostridium cellulolyticum	Wild type: DSM 5812	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH
R. cellulolyticum ΔagrD1	CRISPR-nCas9-inactivated Ccel_2126	This study
R. cellulolyticum Δ*agrD1* pCPthl-empty	*R. cellulolyticum* Δ*agrD1* with empty expression plasmid	This study
R. cellulolyticum Δ*agrD1* pCPthl-*agrD1*	*R. cellulolyticum* Δ*agrD1* with Ccel_2126 expression plasmid	This study
Escherichia coli TOP10	General cloning strain	Laboratory strain

Table S3. Plasmids used in this study.

Plasmid	Description	Source
pCasC	Expresses *cas9* nickase (D10A). Amp^R, Gm^R, Ery^R. 11.2 kb	[4]
pCasC-Ccel2126-KO	1-step CRISPR-nCas9 knockout vector for Ccel_2126. Gm^R, Ery^R. 10.7 kb	This study
pSOS95	Expression plasmid for *Clostridia* spp. Amp^R, Ery^R. 7.8 kb	[13]
pCPthl-empty	Empty expression plasmid for *Clostridia* spp., contains *oriT*. Amp^R, Ery^R. 5.8 kb	This study
pCPthl-*agrD1*	pCPthl-empty containing Ccel_2126. Amp^R, Ery^R. 5.9 kb	This study

Markers: Amp^R, ampicillin resistance; Gm^R, gentamicin resistance; Ery^R, erythromycin resistance

Table S4. Oligonucleotide primers used in this study.

Name	Sequence (5' → 3')	Use
JK683	CTATTGGAGAAGTTGATTC	Screening for Ccel_2126 mutants, amplification of Ccel_2126 for mutant verification
JK684	GTTCCGGGAATTACTGAAG	Screening for Ccel_2126 mutants, amplification of Ccel_2126 for mutant verification
JK685	GTTCTATCTCTAGTTGGG	Screening for Ccel_2126 mutants for wild-type contamination
JK742	GTTTATCTGTTACCCCGTAGGATCCAGAATTTAAA AGGAGGGATTCATATGGATTTATGAATTGGCGCC ACTTAATGATTTGCCAG	Construction of pCPthl-empty
JK743	CTGGCAAATCATTAAGTGGCGCCAATTCATAAAT CCATATGAATCCCTCCTTTTAAATTCTGGATCCTA CGGGGTAACAGATAAAC	Construction of pCPthl-empty
JK744	GTTTATCTGTTACCCCGTAGGATCCAGAATTTAAA AGGAGGGATTCATATGAAAGGTAAAATTAAATTG	Construction of pCPthl-*agrD1*
JK745	GCAAATCATTAAGTGGCGCCTTACTTGAGTAATG ATTTTG	Construction of pCPthl-*agrD1*

Table S5. Sequence of the synthesized cassette for gene knockout via CRISPR-nCas9.

Target	Sequence of gene synthesis cassette
Ccel_2126	TACCGC*GAGACC*TTGACAAATTTATTTTTTAAAGTTAAAATTAAGTTG**AACTGTTCTATCTCTAGTTG**GT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTC GGTGCTTTTTTTGAATTCTCTAGAGTCTTACAATCAGGATAGAAAACGAATGTATTAAAGTACCGGATAT TAAAAACTCATCAAAGGGTGCAGATCGAGGAAACGGATTAATAATTGCAAATAAAATAATCGCTTCATA TAAGAATATTTTAAGTTCAACTACCTTCGATGAAAACAGAATGGTATTTTTACAGGTTCTAAGAATAATA GCAAAAGAGGCTTAATAGCCTCTTTTTGCTTACTTGAGTAATGATTTTGGACATTCCCTTTGATAATAAG ACCAAAACCAGCATGCACCTGCAGAAGCAGTAGCAGCAAATA**C<u>TTA</u>*ACTAGT*AGATAGAACAGTT**AGA ACCAGCAATTTAATTTTACCTTTCATGTAATTATCCCACCCCTCTTATTTTTTGTTAATCTATACACTATT GGTGTAATGTTAACCGAAGATATTATTGTAATCACTGAAATGATATTTGAGTAAATATTAGGAACTATTA ATGTAACTGTAAAGCATATAACTAATAATATACTACCCTTTATCCTTAACTCCCGTTTTCTTTTTTCAGTA ACAATAGGTTTTGACACCAGATCTGCAGGTGCGTATAGAAATACAAAAATTCCGGATATAGCAAATAAA ACTATATTTAAGTGCGTG*GAGACC*

*Bsa*I restriction site, sgRNA cassette with P4 promoter and **target specific N20 sequence**, homologous arms surrounding a **mutated N20-PAM sequence containing a <u>stop codon</u> and a *Spe*I RES**, *Bsa*I restriction site

Table S6. Sequences of structurally verified AIPs used for the WebLogo diagram. Amino acid sequences are listed for the structurally verified AIPs used for the WebLogo shown in Figure S2A. The sequences of the core peptides are indicated in bold.

ID	GenBank ID	Sequence	Organism
Lplan AgrD	WP_011102233.1	MKQKMYEAIAHLFKYVGAKQLVMC**CVGIW**FETKIPDELRK	*Lactobacillus plantarum*
Lmon AgrD	EAA0099852.1	MKNMNKSVGKFLSRKLEEQSMKVADSSMSKA**CFMFV**YEPKSPFVKMQEK	*Listeria monocytogenes*
Saur AgrD I	ABB29295.1	MNTLFNLFFDFITGILKNIGNIAAYST**CDFIM**DEVEVPKELTQLHE	*Staphylococcus aureus*
Saur AgrD II	WP_072416134.1	MNTLVNMFFDFIIKLAKAIGIVSGVNA**CSSLF**DEPKVPAELTNLYDK	*Staphylococcus aureus*
Saur AgrD III	WP_115288203.1	MKKLLNKVIELLVDFFNSIGYKAAYIN**CDFLL**DEAEVPKELTQLHE	*Staphylococcus aureus*
Saur AgrD IV	WP_089535699.1	MNTLLNIFFDFITGVLKNIGNVASYST**CYFIM**DEVEVPKELTQLHE	*Staphylococcus aureus*
Schr AgrD	WP_107381457	MAIFETLFNLFTTLFKTLGNLASINP**CTGFF**DEPEVPQELRETE	*Staphylococcus chromogenes*
Sepi AgrD II	AAO05235.1	MENIFNLFIKFFTTILEFIGTVAGDSV**CASYF**DEPEVPEELTKLYE	*Staphylococcus epidermidis*
Shae AgrD	RIO86649.1	MTVLVDLIIKLFTFLLQSIGTVASFTP**CTTYF**DEPEVPEELTNTKN	*Staphylococcus haemolyticus*
Shom AgrD	NAM95812.1	MTFITDLFIKLFSLILETVGTLATYST**CYGYF**DESEVPEELTNLER	*Staphylococcus hominis*
Shyi AgrD	NJH67186.1	MAFFDTLLNLVTISFKSLGNFAKINP**CTVFF**DEPEVPEELRQGE	*Staphylococcus hyicus*
Sint AgrD	AAL65836.1	MRILEVLFNLITNLFQSIGTFARIPT**STGFF**DEPEIPAELLEEEK	*Staphylococcus intermedius*
Slug AgrD	AAD51710.1	MNLLSGLFTKGISAIFEFIGNFSAQDI**CNAYF**DEPEVPQELIDLQRKQLIESV	*Staphylococcus lugdunensis*
Slug AgrD II	EFU83103.1	MNLLSGLFTKGISVIFEFIGNFSVQDM**CNGYF**DEPEVPQELIDLHRN	*Staphylococcus lugdunensis*
Ssap AgrD	WP_069838087.1	MNVIKSISKSISKSISKSISNYFAKVFASIGSISTINP**CFGYT**DESEIPKELTDLYE	*Staphylococcus saprophyticus*
Ssch AgrD	WP_050345386.1	MKFLDSLLKLLTSVFASISTYAKYPF**CIGYF**YEPEIPAELLEEE	*Staphylococcus schleiferi*
Ssim AgrD	ERS92975.1	MDLLNGIFKLFAFIFEKIGNLAKYNP**CLGFL**DEPTVPKELLEEDK	*Staphylococcus simulans*
Svit AgrD	WP_016912307.1	MNLLATLFSKSASNFLSSFGEKAVIRG**CTAFL**DETEVPAELLKEKQ	*Staphylococcus vitulinus*
Swar AgrD	AGC90169.1	MEFLVNLFFKFFTSIMEFVGFVAGYSP**CTNFF**DEPEVPSELTKIYE	*Staphylococcus warneri*

Table S7. Amounts of reagents used in every step of the SPPS of coupling in method A.

amino acid	Oxyma Pure	COMU	DIPEA	time of reaction
Fmoc-Ser-(OtBu) 794.7 mg; 2.07 mmol; 2.66 eq.	313.4 mg; 2.2 mmol; 2.8 eq.	857.3 mg; 2.0 mmol; 2.5 eq.	680 µL; 4.0 mol; 5.1 eq.	1 h 11 min
Fmoc-Trp 838.1 mg; 2.0 mmol; 2.6 eq.	279.7 mg; 2.0 mmol; 2.5 eq.	838.6 mg; 1.96 mmol; 2.5 eq.	680 µL; 4.0 mol; 5.1 eq.	42 min
Fmoc-Phe 788.3 mg; 2.0 mmol; 2.6 eq.	316.6 mg; 2.2 mmol; 2.9 eq.	974 mg; 2.3 mmol; 2.9 eq.	680 µL; 4.0 mol; 5.1 eq.	35 min
Fmoc-Trp 1037 mg; 2.4 mmol; 3.1 eq.	289 mg; 2.0 mmol; 2.6 eq.	936.5 mg; 2.2 mmol; 2.8 eq.	680 µL; 4.0 mol; 5.1 eq.	1 h 40 min
Trt-S-Cys-NH-Boc 970.2 mg; 2.1 mmol; 2.7 eq.	281.6 mg; 2.0 mmol; 2.5 eq.	1.209 g; 2.4 mmol; 3.1 eq.	collidine instead of DIPEA was used here first: 517 µL; 4.7 mmol; 6.0 eq. Later 340 µL (2 mmol; 2.6 eq.) DIPEA was added.	(1) 1 h 40 min (2) 27 min

Table S8. Amounts of reagents used in every step of the SPPS of coupling in method B.

amino acid	HBTU	HOBt	EtN*i*Pr$_2$	time of reaction
Fmoc-Ser(*t*Bu)-OH 522 mg, 1.36 mmol, 4 eq.	516 mg, 1.36 mmol, 4 eq.	208 mg, 1.36 mmol, 4 eq.	466 µL, 2.72 mmol, 8 eq.	3 h
Fmoc-Trp-OH 580 mg, 1.36 mmol, 4 eq.	516 mg, 1.36 mmol, 4 eq.	208 mg, 1.36 mmol, 4 eq.	466 µL, 2.72 mmol, 8 eq.	3 h
Fmoc-Phe-OH 527 mg, 1.36 mmol, 4 eq.	516 mg, 1.36 mmol, 4 eq.	208 mg, 1.36 mmol, 4 eq.	466 µL, 2.72 mmol, 8 eq.	3 h
Fmoc-Trp-OH 580 mg, 1.36 mmol, 4 eq.	516 mg, 1.36 mmol, 4 eq.	208 mg, 1.36 mmol, 4 eq.	466 µL, 2.72 mmol, 8 eq.	3 h
At that point, the resin (calc. loading 0.48 mmol) was split up for different couplings				

References

[1] T. Lincke, S. Behnken, K. Ishida, M. Roth, C. Hertweck, *Angew. Chem., Int. Ed.* **2010**, *49*, 2011-2013.
[2] W. Higashide, Y. Li, Y. Yang, J. C. Liao, *Appl. Environ. Microbiol.* **2011**, *77*, 2727-2733.
[3] S. H. Baer, H. P. Blaschek, T. L. Smith, *Appl. Environ. Microbiol.* **1987**, *53*, 2854-2861.
[4] K. L. Dunbar, H. Büttner, E. M. Molloy, M. Dell, J. Kumpfmüller, C. Hertweck, *Angew. Chem., Int. Ed.* **2018**, *57*, 14080-14084.
[5] K. Blin, L. E. Pedersen, T. Weber, S. Y. Lee, *Synth. Syst. Biotechnol.* **2016**, *1*, 118-121.
[6] aI. Fedorova, A. Arseniev, P. Selkova, G. Pobegalov, I. Goryanin, A. Vasileva, O. Musharova, M. Abramova, M. Kazalov, T. Zyubko, T. Artamonova, D. Artamonova, S. Shmakov, M. Khodorkovskii, K. Severinov, *Nucleic Acids Res.* **2020**, *48*, 2026-2034; bT. Xu, Y. Li, Z. Shi, C. L. Hemme, Y. Li, Y. Zhu, J. D. Van Nostrand, Z. He, J. Zhou, *Appl. Environ. Microbiol.* **2015**, *81*, 4423-4431.
[7] G.-Z. Cui, W. Hong, J. Zhang, W.-L. Li, Y. Feng, Y.-J. Liu, Q. Cui, *J. Microbiol. Methods* **2012**, *89*, 201-208.
[8] P. Thomas, A. C. Sekhar, R. Upreti, M. M. Mujawar, S. S. Pasha, *Biotechnol. Rep.* **2015**, *8*, 45-55.
[9] K. Katoh, J. Rozewicki, K. D. Yamada, *Brief. Bioinform.* **2019**, *20*, 1160-1166.
[10] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, *Mol. Biol. Evol.* **2013**, *30*, 2725-2729.
[11] F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson, D. G. Higgins, *Mol. Syst. Biol.* **2011**, *7*, 539.
[12] G. E. Crooks, G. Hon, J. M. Chandonia, S. E. Brenner, *Genome Res.* **2004**, *14*, 1188-1190.
[13] S. B. Tummala, N. E. Welker, E. T. Papoutsakis, *Appl. Environ. Microbiol.* **1999**, *65*, 3793-3799.