Supporting Information

for

Biosynthesis of a Class III lantipeptide Catenulipeptin

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Materials

All oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases, DNA polymerases and T4 DNA ligase were purchased from New England Biolabs or Invitrogen. Media components for bacterial cultures were purchased from Difco laboratories. Chemicals were purchased from Fisher Scientific or from Aldrich unless noted otherwise. Endoproteinase GluC, and trypsin were purchased from Roche Biosciences. *E. coli* DH5α was used as host for cloning and plasmid propagation, and *E. coli* BL21 (DE3) was used as a host for expression of proteins and peptides.

General methods

All polymerase chain reactions (PCR) were carried out on a C1000™ thermal cycler (Bio-Rad). DNA sequencing was performed by the Biotechnology Center at the University of Illinois at Urbana-Champaign, using appropriate primers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager-DE-STR (Applied Biosystems) or a Bruker UltraFlextreme. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters).

Construction of His_6-K_3-AciA mutants. Site-directed mutagenesis of *aciA* was performed by multistep PCR. First, the amplification of *aciA* was carried out by thirty cycles of denaturing (94 °C for 20 s), annealing (58 °C for 30 s), and extending (72 °C for 20 s) using the AciA-FP and an appropriate mutant reverse primer to yield the 5' fragment of the mutant *aciA* gene (FP reaction). The PCR mixtures included 1×FailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Phusion DNA polymerase (Finnzymes) (0.04 U/μL), dNTP (2 mM), and primers (1 μM each). In parallel, a PCR reaction using an appropriate mutant forward primer and the AciA-RP primer was also conducted to produce 3' fragments of the mutated *aciA* gene using the same PCR conditions as FP reaction (RP reaction). The overlapping products from the FP and RP reactions were combined in equal amounts and extended by seven cycles of denaturing, annealing, and extending using the same PCR conditions. Following the extension, the AciA-FP and AciA-RP primers were added (final concentration, 2 μM) and the mixture

was incubated for another 25 cycles of denaturing, annealing, and extending. After amplification of the final PCR product, it was purified by 2% agarose gel electrophoresis. The resulting DNA inserts and empty pET15 vector were digested with NdeI and XhoI at 37 °C for 2 h. The digested inserts and vector products were purified by agarose gel electrophoresis and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA products were ligated with the digested pET15 vector at 24 °C for 5 h using T4 DNA ligase. *E. coli* DH5α cells were transformed with 5 µL of the ligation product by heat shock. Cells were plated on LB-ampicillin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL of LB-ampicillin medium. The cultures were grown at 37 °C for 15 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.

Expression and purification of His_6-K_3-AciA precursor peptides. *E. coli* BL21 (DE3) cells were transformed via electroporation with a pET15b AciA construct. A single colony transformant was used to inoculate 30 mL of LB medium supplemented with 100 µg/mL ampicillin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of LB containing 100 µg/mL ampicillin and cells were grown at 37 °C to $OD_{600} \approx 0.8$-1.0. IPTG was added to a final concentration of 1 mM and the culture was incubated at 37 °C for an additional 1 h. AciA peptides were expressed as insoluble peptides under such conditions. Cells were harvested by centrifugation at 12,000 ×*g* for 25 min at 4 °C, and the pellet was resuspended in 30 mL of start buffer (20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and stored at −80 °C. The cell paste was suspended in start buffer and the suspension was sonicated on ice for 20 min to lyse the cells. Cell debris was removed by centrifugation at 23,700 ×*g* for 30 min at 4 °C. The supernatant was discarded and the pellet containing the insoluble peptide was resuspended in 30 mL of start buffer. The sonication and centrifugation steps were repeated. Again the supernatant was discarded and the pellet was resuspended in 30 mL of buffer 1 (6 M guanidine HCl, 20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 0.5 mM imidazole). The sample was sonicated and insoluble material was removed by centrifugation at 23,700 ×*g* for 30 min at 4 °C, followed by filtration of the supernatant through a 0.45 µm filter. The filtered sample was applied to a 5 mL HisTrap HP (GE Healthcare Life Sciences) immobilized metal affinity chromatography (IMAC) column previously charged with $NiSO_4$ and equilibrated in buffer 1. The column was washed with two column volumes of buffer 1, followed by two column volumes of buffer 2 (4 M guanidine HCl, 20 mM NaH_2PO_4 , pH 7.5, 500 mM NaCl, 30 mM imidazole). The peptide was eluted with 1-2 column volumes of elution buffer (4 M guanidine HCl, 20 mM NaH_2PO_4 (pH 7.5), 500 mM NaCl, 1 M imidazole). The fractions were desalted using a ZipTipC18 and analyzed by MALDI-TOF MS. The fractions containing the desired peptide were pooled and purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) using a Waters Delta-pak™ C4 15 µm 300 Å 25 × 100 mm PrepPac® Cartridge. Solvents for the RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile / 20% water). A gradient of 2-100% of solvent B was executed over 45 min at a flow rate of 8 mL/min, and peptides were detected by absorbance at 220 nm. The fractions were analyzed by MALDI-TOF

MS. All the fractions containing the desired product were combined and the organic solvents were removed by rotary evaporation, followed by lyophilization to remove water. The product was kept at −20 °C for short-term storage and −80 °C for long-term storage. Typical yields from 1 L culture were 1-3 mg of His_6-K_3-AciA precursor peptide.

Expression and purification of His_6-AciKC and His_6-AciKC-ΔC. *E. coli* BL21 (DE3) cells were transformed with the pET28b AciKC or AciKC-ΔC construct via electroporation. A single colony transformant was used to inoculate 30 mL of LB medium supplemented with 50 μg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of LB containing 50 μg/mL kanamycin, and cells were grown at 37 °C to $OD_{600} \approx 0.6$. The culture was incubated at 4 °C on ice for 10 min, then IPTG was added to a final concentration of 0.1 mM, and the culture was incubated at 18 °C for an additional 16-20 h. Cells were harvested by centrifugation at 12,000 ×*g* for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 10% glycerol) and stored at −80 °C. All protein purification steps were performed at 4 °C. The cell paste was suspended in start buffer and the cells were lysed using a high pressure homogenizer (Avestin, Inc.). Cell debris was pelleted via centrifugation at 23,700 ×*g* for 20 min at 4 °C. The supernatant was injected via a superloop onto a fast protein liquid chromatography (FPLC) system (ÄKTA, GE Heathcare Life Sciences) equipped with a 5 mL HisTrap HP IMAC column previously charged with Ni^{2+} and equilibrated in start buffer. The column was washed with 50 mL of buffer A (30 mM imidazole, 20 mM Tris, pH 7.5, 200 mM NaCl) and the protein was eluted using a linear gradient of 0-100% B (buffer B = 200 mM imidazole, 20 mM Tris, pH 7.5, 200 mM NaCl) over 40 min at a 2 mL/min flow rate. UV (280 nm) was monitored and fractions were collected and analyzed by SDS-PAGE (4-20% Tris-glycine READY gel, BioRAD). The fractions containing AciKC were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (10 kDa MWCO, Millipore). Gel filtration purification was used to further purify AciKC. The concentrated protein sample was injected onto an FPLC system (ÄKTA) equipped with an XK16 16/60 (GE Healthcare Life Sciences) column packed with SuperDex 75 resin previously equilibrated in 20 mM HEPES (pH 7.5), 200 mM KCl. The protein was eluted with a flow rate of 0.9 mL/min. Both UV (280 nm) and conductance were monitored and fractions were collected. Misfolded/aggregated protein was efficiently separated from soluble, correctly folded protein and the desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit. The resulting protein sample was stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce) and typically yields were 15-30 mg of His_6-AciKC from 3 L of cell culture.

Table S1. Primer sequences for cloning and mutagenesis of AciA peptides

Primer name	sequence (5' ⟶ 3')
AciA-FP	ATCA CATATG AAAAAAAAA ATGACCGAGG AGATGACT
AciA-RP	ACTACTCGAGTCAGAGGTCGCAGAGCAG
AciA-S8A FP	GGC GGC GGC GAC GCG GGC CTG AGC GTC
AciA-S8A RP	GAC GCT CAG GCC CGC GTC GCC GCC GCC
AciA-S11A FP	TCG GGC CTG GCG GTC ACC GGC TGC AAC
AciA-S11A RP	GTT GCA GCC GGT GAC CGC CAG GCC CGA
AciA-C15A FP	GTC ACC GGC GCG AAC GGC CAC AGC
AciA-C15A RP	GCT GTG GCC GTT CGC GCC GGT GAC
AciA-C25A FP	GGCCACAGCGGCATCAGCCTGCTCGCGGACCTCTGA
AciA-C25A RP	TCAGAGGTCCGCGAGCAGGCTGATGCCGCTGTGGCC

Table S2. Parameters for GC/MS analysis of labionin.

Column	Model number: Phenomen ZB-1MS; Max temperature: 360 °C Nominal length: 30.0 m; Nominal diameter: 320.00 µm Nominal film thickness: 0.25 µm
Temperature program	Initial temp: 100 °C; Final temp: 320 °C Rate: 20 °C/min; Initial flow: 3.0 mL/min
Injection	Volume: 5 µL Pulsed split less
Initial pressure	11.92 psi
PCI-Mode	MS source: 250 °C, MS Quad: 150 °C

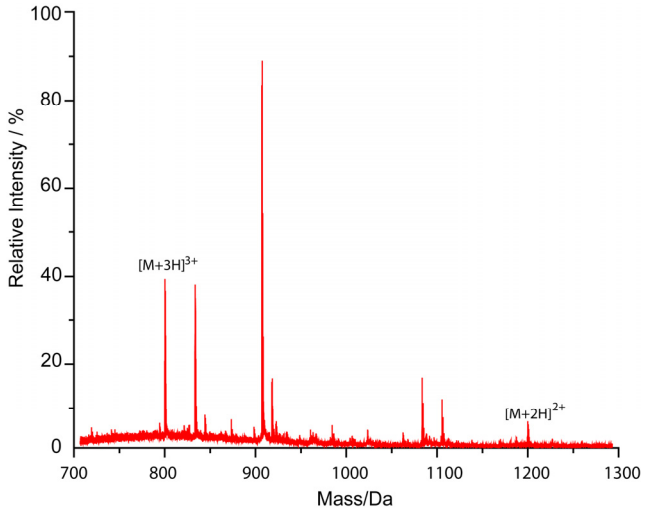


Figure S1a. ESI-MS spectrum of culture extract of *C. acidiphila* DSM 44928. The theoretical mass of triply charged catenulipeptin is 800.0277 Da. The observed triply charged putative catenulipeptin is 800.0283 Da. The theoretical mass of doubly charged catenulipeptin is 1199.5375 Da. Putative doubly charged catenulipeptin is observed with a mass of 1199.5452 Da.

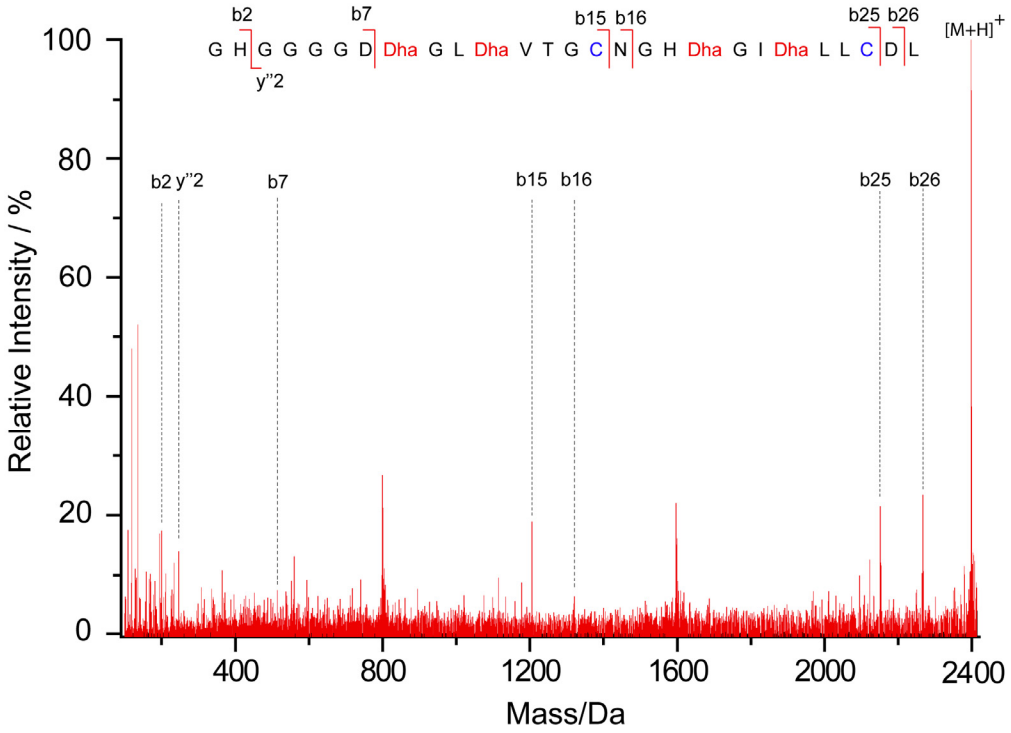


Figure S1b. Tandem MS spectrum of catenulipeptin from culture extract of *C. acidiphila* DSM 44928. Peaks that match the b ions and y'' ions generated from the fragmentation of catenulipeptin are labeled. The theoretical mass of catenulipeptin parent ion is 2398.0671 Da. The observed mass of the parent ion is 2398.0599 Da.

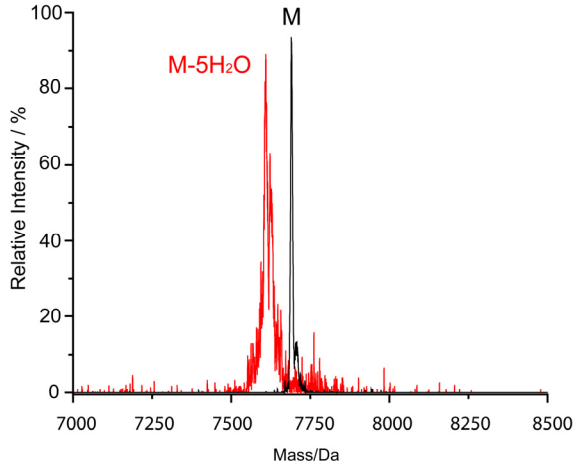


Figure S2a. Modification of AciA by AciKC with TTP. His_6-AciA was modified with AciKC in the presence of 5 mM TTP, 10 mM $MgCl_2$, and 0.1 mM TCEP in Tris (pH 8.0) buffer. The reaction was analyzed by MALDI-TOF MS. Black line indicates AciA peptide before assay; red line indicates modified AciA peptide after assay.

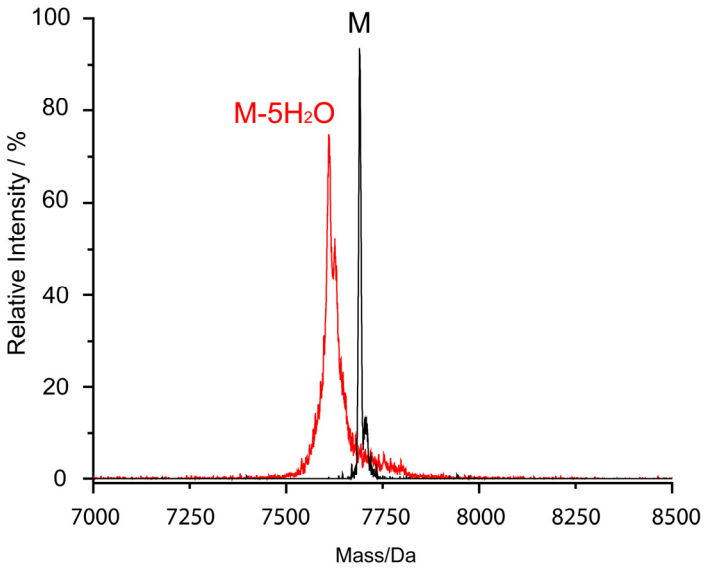


Figure S2b. Modification of AciA by AciKC with GTP. His_6-AciA was modified with AciKC in the presence of 5 mM GTP, 10 mM $MgCl_2$, and 0.1 mM TCEP in Tris (pH 8.0) buffer. The reaction was analyzed by MALDI-TOF MS. Black line indicates AciA peptide before assay; red line indicates modified AciA peptide after assay.

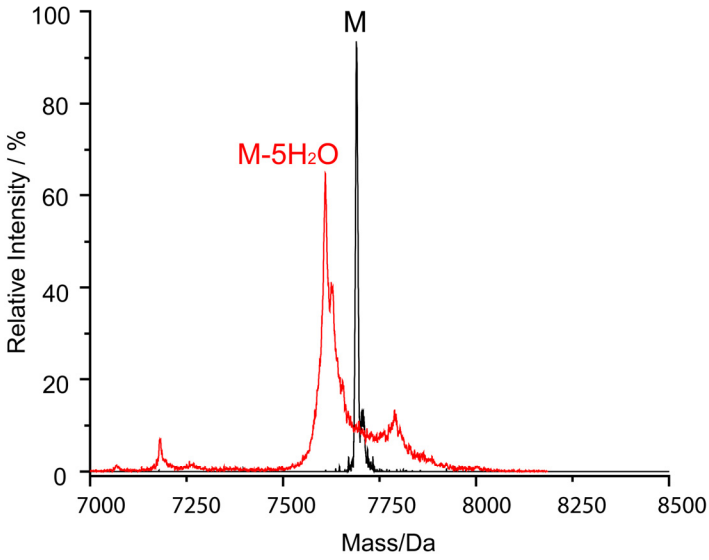


Figure S2c. Modification of AciA by AciKC with ATP. His_6-AciA was modified with AciKC in the presence of 5 mM ATP, 10 mM $MgCl_2$, and 0.1 mM TCEP in Tris (pH 8.0) buffer. The reaction was analyzed by MALDI-TOF MS. Black line indicates AciA peptide before assay; red line indicates modified AciA peptide after assay.

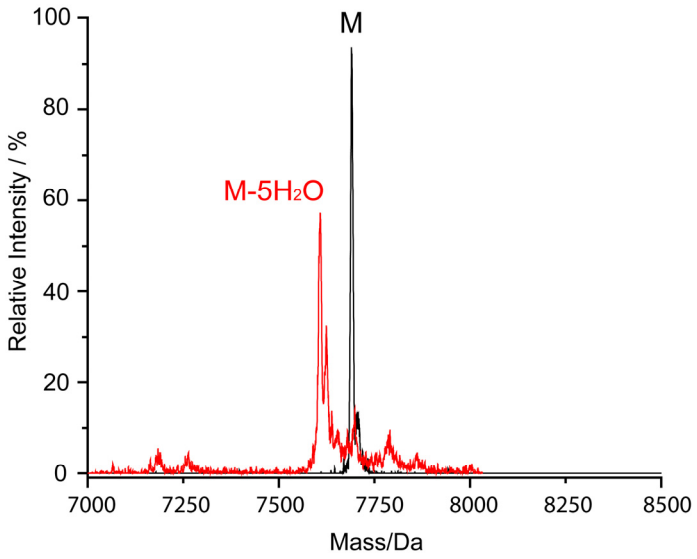


Figure S2d. Modification of AciA by AciKC with CTP. His_6-AciA was modified with AciKC in the presence of 5 mM CTP, 10 mM $MgCl_2$, and 0.1 mM TCEP in Tris (pH 8.0) buffer. The reaction was analyzed by MALDI-TOF MS. Black line indicates AciA peptide before assay; red line indicates modified AciA peptide after assay.

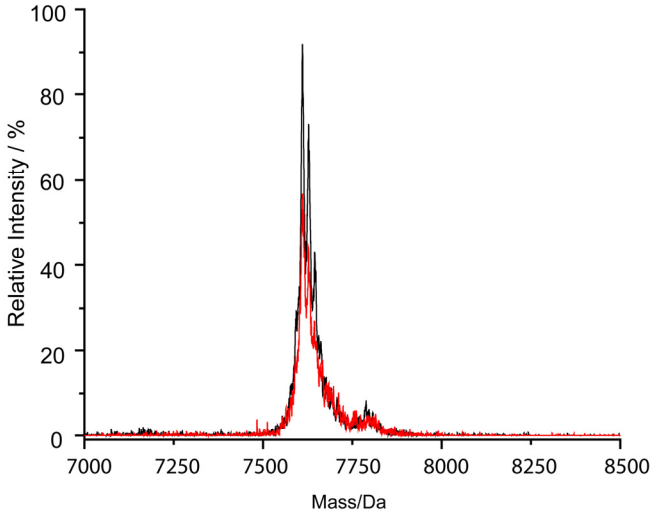


Figure S3. IAA assay to detect free cysteine thiol in modified AciA. After incubation of His_6-AciA with AciKC in the presence of 5 mM CTP, 10 mM $MgCl_2$, and 0.1 mM TCEP in Tris (pH 8.0) buffer, 5 mM IAA was added into the sample and the mixture was incubated for 2 h. The reaction was analyzed by MALDI-TOF MS. Black line indicates AciKC-trated AciA peptide before IAA addition; red line indicates AciKC-modified AciA peptide after IAA addition.

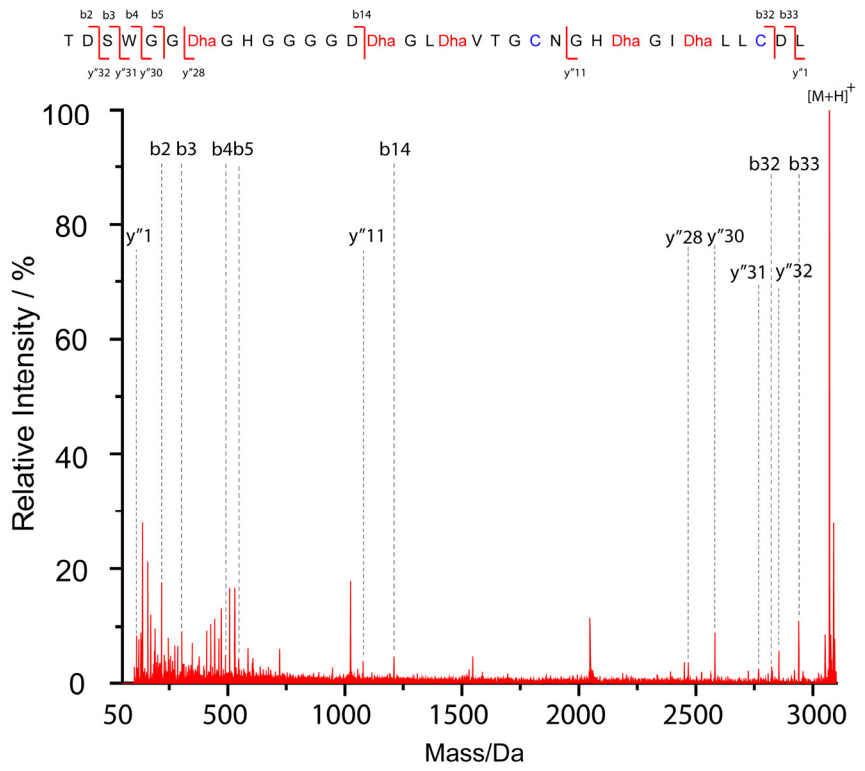


Figure S4a. ESI-MSMS analysis of the peptide spanning residues Thr(–7)-Leu27 of AciKC-modified AciA. AciA peptide (10 µM) was incubated with AciKC (2 µM) in the presence of NTP mixture (5 mM), Mg^{2+} (10 mM), and TCEP (0.1 mM) for 4 h at room temperature and then treated with Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

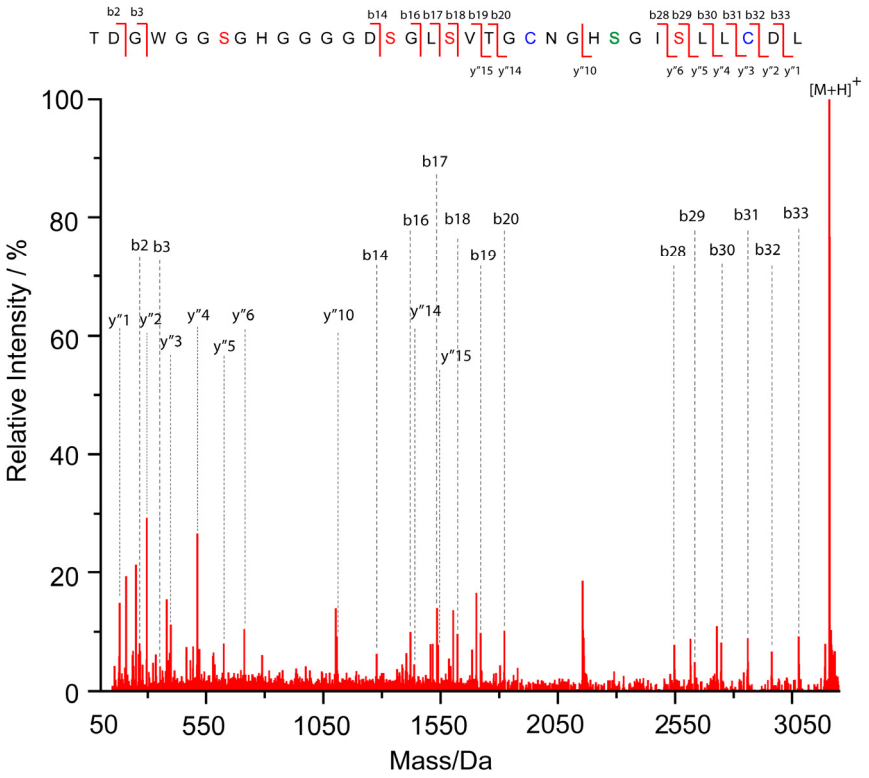


Figure S4b. ESI-MSMS analysis of the peptide spanning residues Thr(–7)-Leu27 of AciA peptide. AciA peptide (10 μM) was digested by endoprotease Glu-C. The resulting core peptide was analyzed by LC-ESI-MSMS.

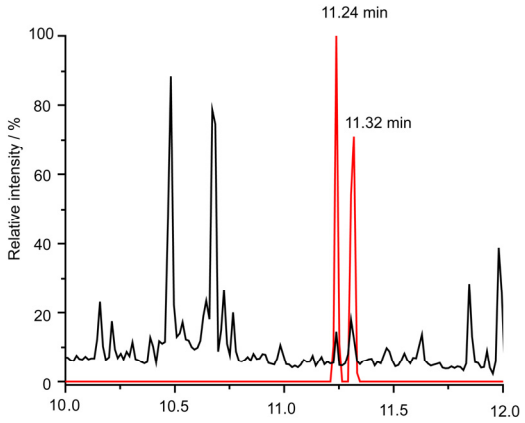


Figure S5a. GC-MS detection of labionin in AciKC-modified AciA. Shown is the gas chromatogram of hydrolyzed and derivatized AciKC-modified AciA. Normalized total ion chromatogram is in black, normalized Selected Ion Monitoring at m/z 668 Da (parent ion) is in red.

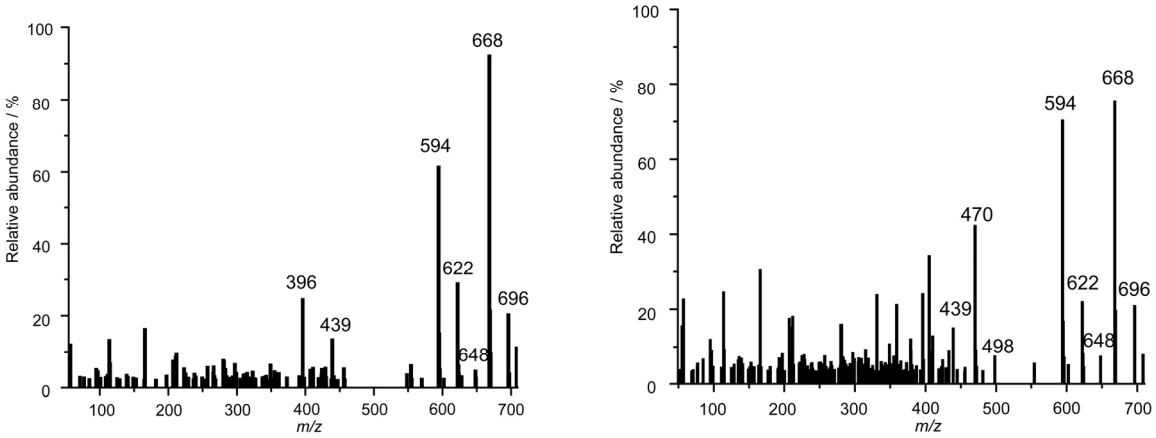


Figure S5b. GC-MS detection of labionin in modified AciA. **Left**. Mass spectrum of the SIM-identified peak at 11.24 min; **Right**. Mass spectrum of the SIM-identified peak at 11.32 min.

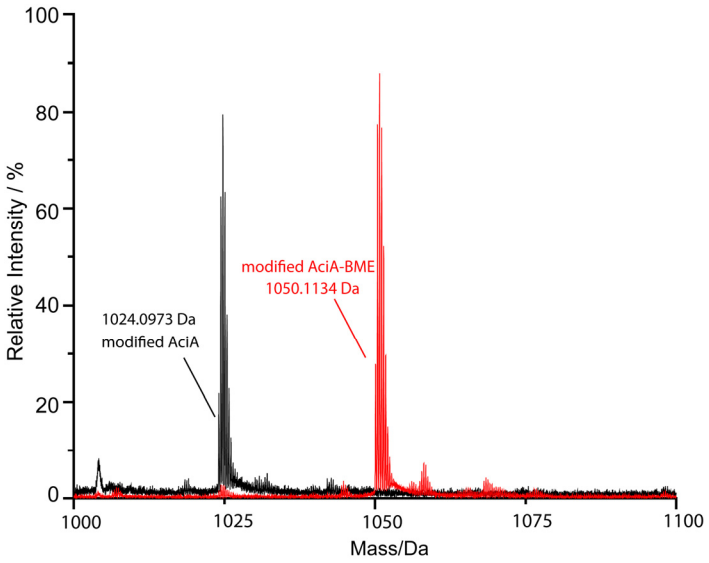


Figure S6. ESI-MS spectrum of βME addition to AciKC-modified AciA under alkaline condition. The purified AciKC-modified AciA peptide (10 µM) was incubated with 5 mM βME in Tris buffer (pH 8.5) for 2 h at 30 °C. The reaction mixture was subjected to LC-ESI-MS analysis. The triply charged AciKC-modified AciA after (red) and before (black) βME treatment shows a mass increase of 26.0161 Da, which represents one βME addition (78.0139 Da). No double addition is observed.

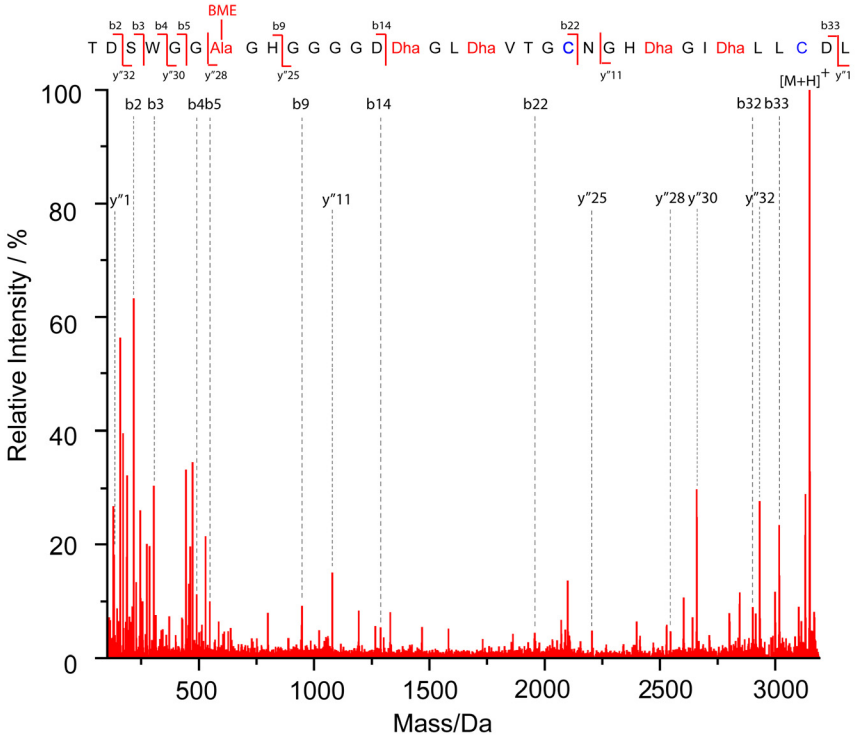


Figure S7. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of AciKC-modified AciA-βME adduct.

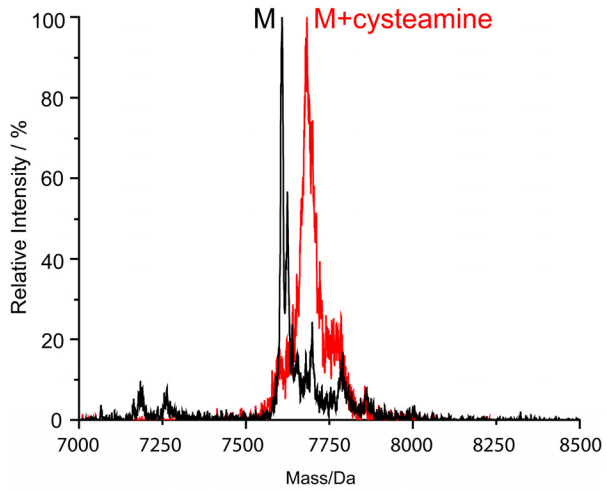


Figure S8a. Formation of the cysteamine adduct of AciKC-modified AciA peptide. AciKC-modified AciA peptide (30 µM) was incubated with cysteamine (5 mM) in 50 mM Tris buffer, pH 8.2 overnight at room temperature. The reaction was analyzed by MALDI-TOF-MS. The black line indicates the MALDI-TOF spectrum of AciKC-modified AciA peptide; the red line indicates the MALDI-TOF spectrum of the peptide cysteamine adduct. Theoretical mass of AciKC-modified AciA peptide cysteamine adduct is 7677.4 Da, observed AciKC-modified AciA peptide is 7678.2 Da.

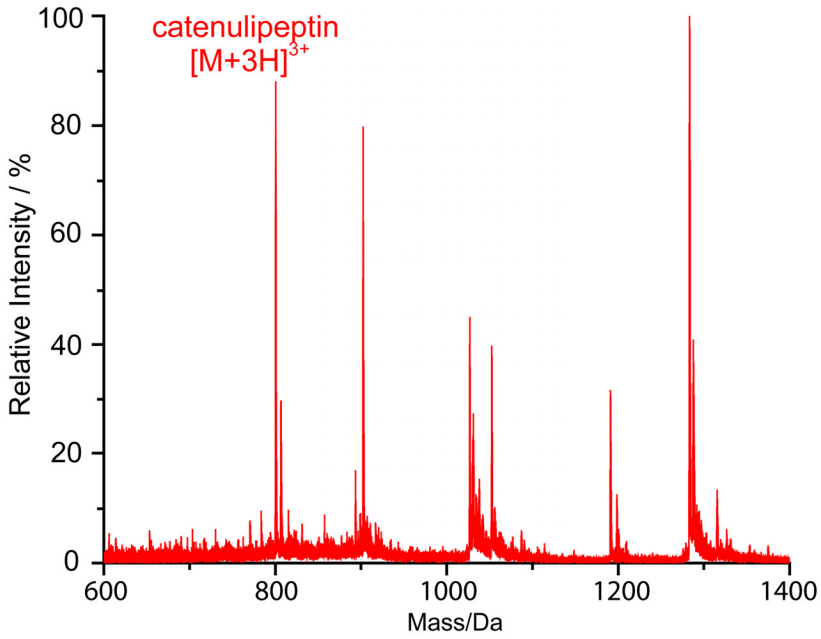
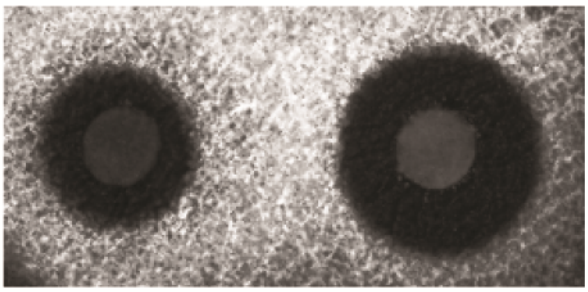


Figure S8b. Generation of catenulipeptin *in vitro*. The cysteamine adduct of AciKC-modified AciA peptide (30 µM) was subjected to trypsin (10 µg/mL) digestion in 50 mM Tris buffer, pH 8.0 for 3 h at room temperature. The reaction was then analyzed by ESI-MS. The triply charged catenulipeptin peak was observed at 800.0288 Da. The theoretical mass of triply charged catenulipeptin is 800.0285 Da.

A



B

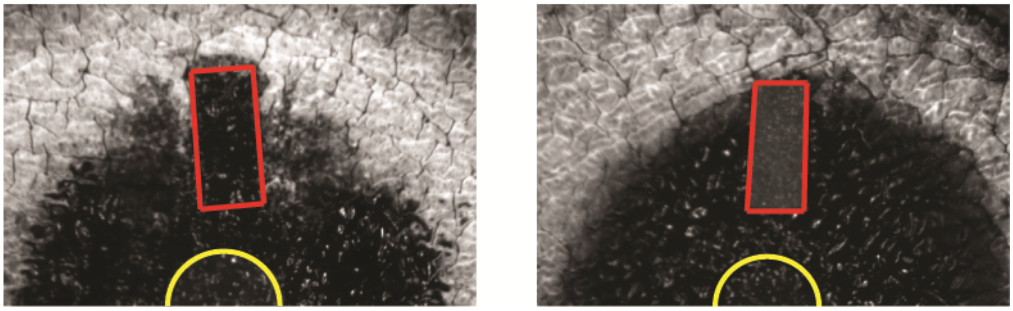


Figure S9. Catenulipeptin counteracts the effect of surfactin and partially restores aerial hyphae formation in *S. coelicolor* A3(2) (NRRL 16638). Spore suspensions of *S. coelicolor* A3 (2) (NRRL 16638) were generated using sterile glass beads to collect spores from an agar surface. Spores were washed from the surface of glass beads in 1 mL sterile water, vortexed, and dissociated in a sonicating water bath for 10 s to produce a uniform suspension. An aliquot of 200 µL of spore suspension was spread onto a YEME agar plate and allowed to grow for 2 h at 30 °C. Then a filter paper disk containing only surfactin (50 µg, 10 µL of a 5 µg/µL solution) (panel A, right) or a filter paper disk containing surfactin (50 µg, 10 µL of a 5 µg/µL solution) and catenulipeptin (25 µg, 10 µL of a 2.5 µg/µL solution) (panel A, left) was placed on the plate. The plate was incubated for 4 days at 30 °C until aerial hyphae were visible as a hairy, white surface on the agar plate. (B) A plate seeded with spores was prepared as described above. Then a filter paper disk containing surfactin (50 µg, 10 µL of a 5 µg/µL solution) was placed in the center of the agar plate. A filter paper strip containing 25 µg catenulipeptin was placed 0.3 cm from the edge of the surfactin-containing filter paper disk (left). On a second plate, a filter paper strip containing 5 µL water was placed 0.3 cm from the edge of the surfactin-containing filter paper disk as a control (right). The plate was incubated at 30 °C for 4 days until aerial hyphal development was visible. The edge of the filter paper strip is indicated by a red rectangle and the edge of the filter paper disk is indicated

by a yellow semicircle. The size of the filter paper disks used in these assays was 0.8 cm in diameter and the size of the paper strips was 0.8 cm (length) × 0.3 cm (width). Surfactin was dissolved in 50% ethanol/water at a concentration of 5 µg/µL. Catenulipeptin was dissolved in 50% acetonitrile/water at a concentration of 2.5 µg/µL. The solutions of compounds were first applied to filter papers and the filter papers were allowed to dry in air to remove the organic solvent before being placed onto the agar plates.

In panel A, the zone of inhibition of aerial hyphae formation is considerably smaller when catenulipeptin was applied together with surfactin. In panel B, hyphae formation is starting to be visible along the top sides of the strip containing catenulipeptin although not as robust as shown for SapB in reference 21. In that study, a filter paper disc containing 500 µg surfactin (50 µL of 10 µg/µL) was placed in the center of a YEME agar plate with wild type *S. coelicolor* spore lawn. A nylon strip soaked with 1 µg/µL SapB in DMSO was placed on the plate, 1 cm from the edge of the surfactin-containing filter disk. Much stronger hyphae formation was observed along the SapB containing strip (see Figure 3 in reference 21) as observed here with catenulipeptin.

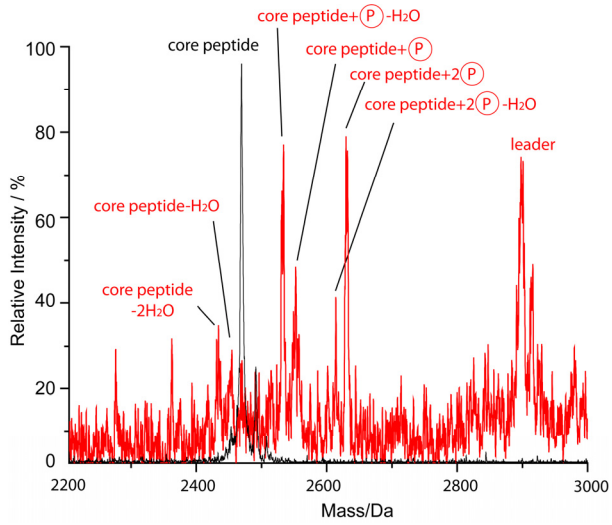


Figure S10. Modification of AciA core peptide in the presence of leader peptide supplied *in trans*. AciA core peptide (1 µM) was incubated with AciKC protein (2 µM) in the presence of ATP, Mg^{2+} and AciA leader peptide (2 µM). The reaction was analyzed by MALDI-TOF-MS. The black line indicates the MALDI-TOF spectrum of AciA core peptide; the red line indicates the MALDI-TOF spectrum of AciKC-modified AciA core peptide when leader peptide was supplied *in trans*.

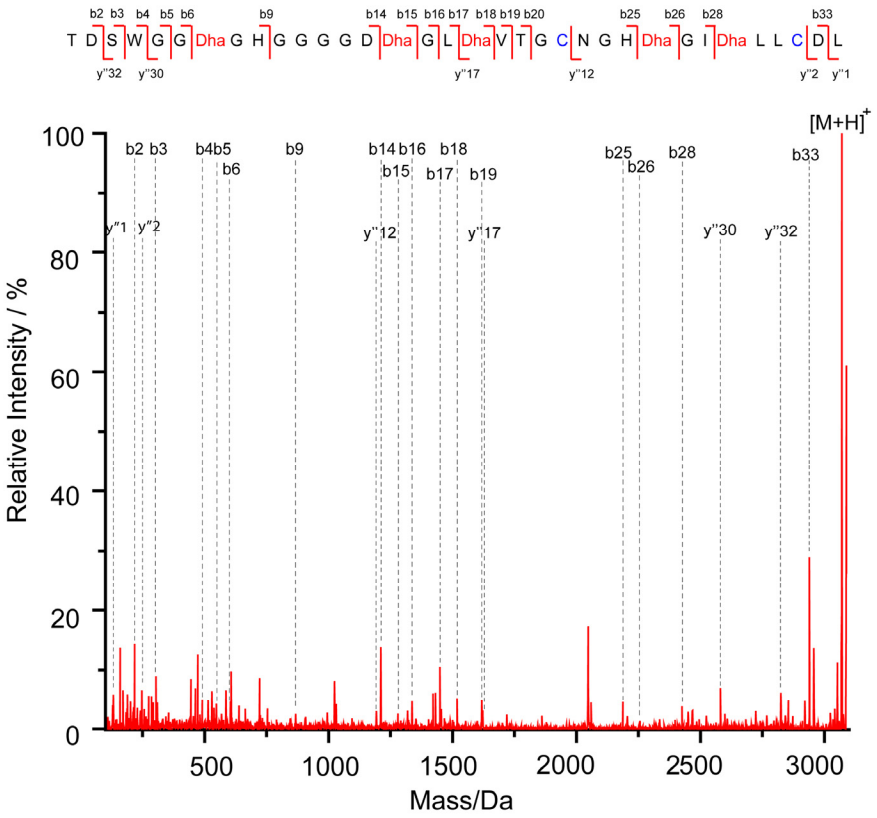


Figure S11. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of AciA modified by AciKC-ΔC. AciA peptide (10 μM) was incubated with AciKC-ΔC (2 μM) in the presence of NTP mixture (5 mM), Mg^{2+} (10 mM), and TCEP (0.1 mM) for 4 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

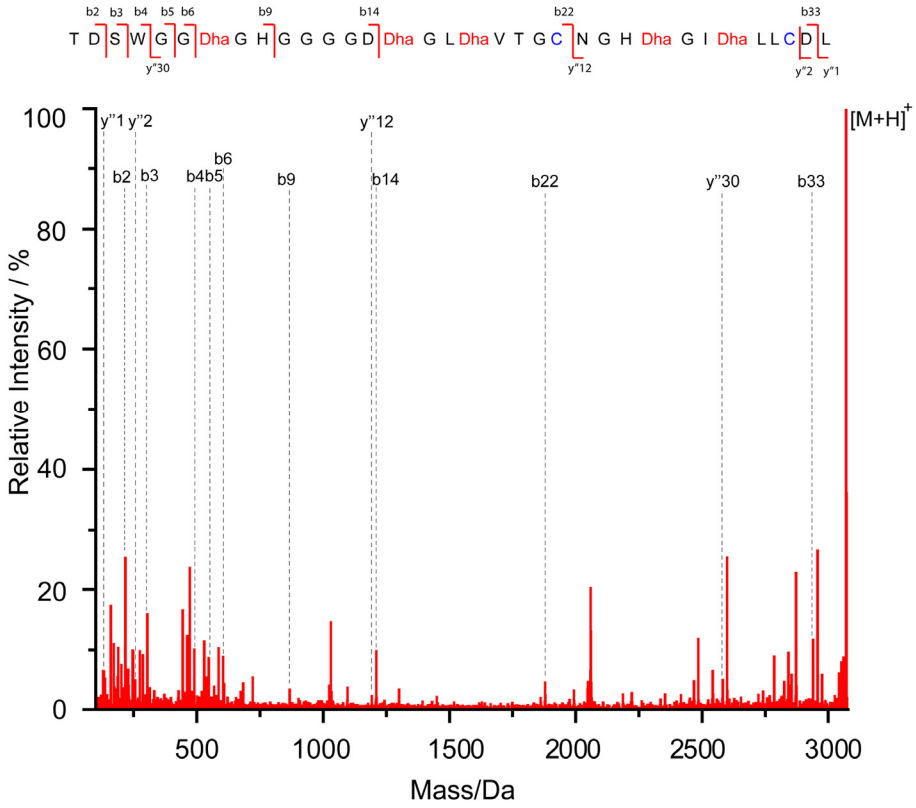


Figure S12a. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of dehydrated-AciA containing an intramolecular disulfide treated with AciKC and TCEP. Dehydrated-AciA peptide containing the disulfide (5 μM) was incubated with AciKC (5 μM), and TCEP (0.1 mM) for 1 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

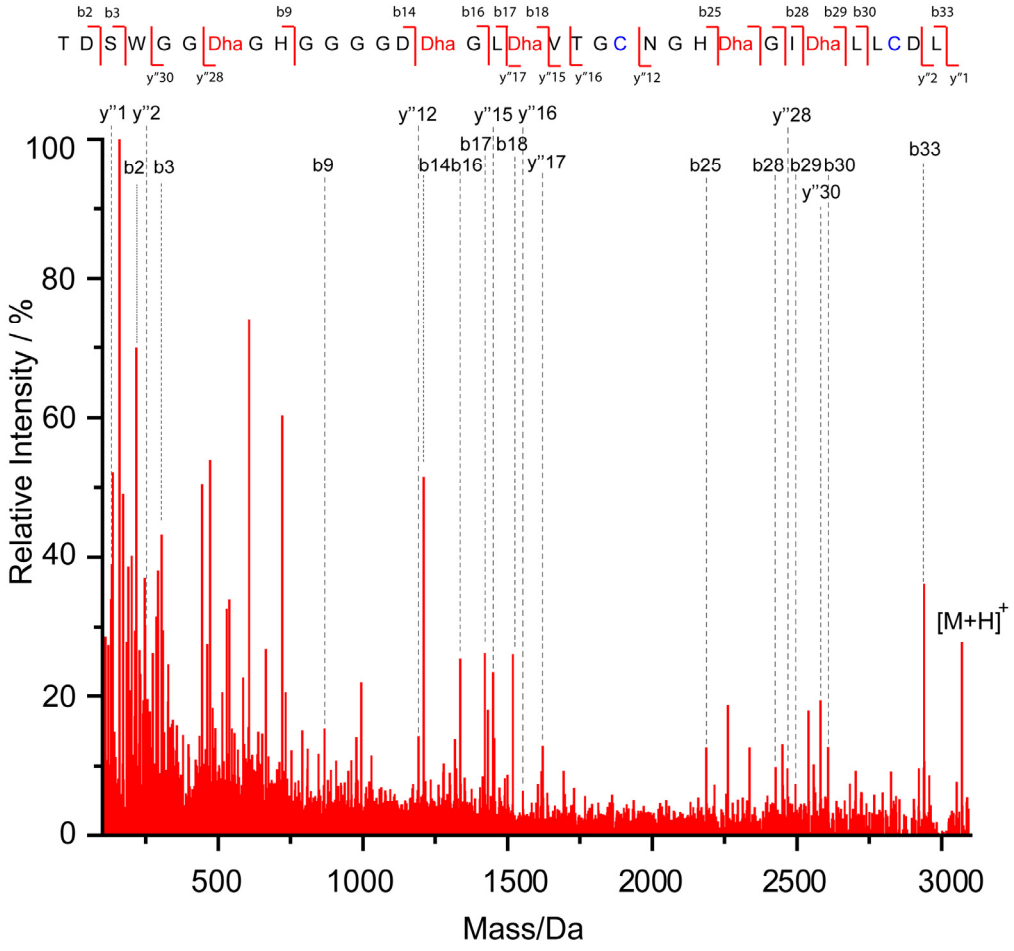


Figure S12b. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of dehydrated-AciA containing an intramolecular disulfide treated with TCEP. Dehydrated-AciA peptide containing the disulfide (5 µM) was incubated with TCEP (0.1 mM) for 1 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

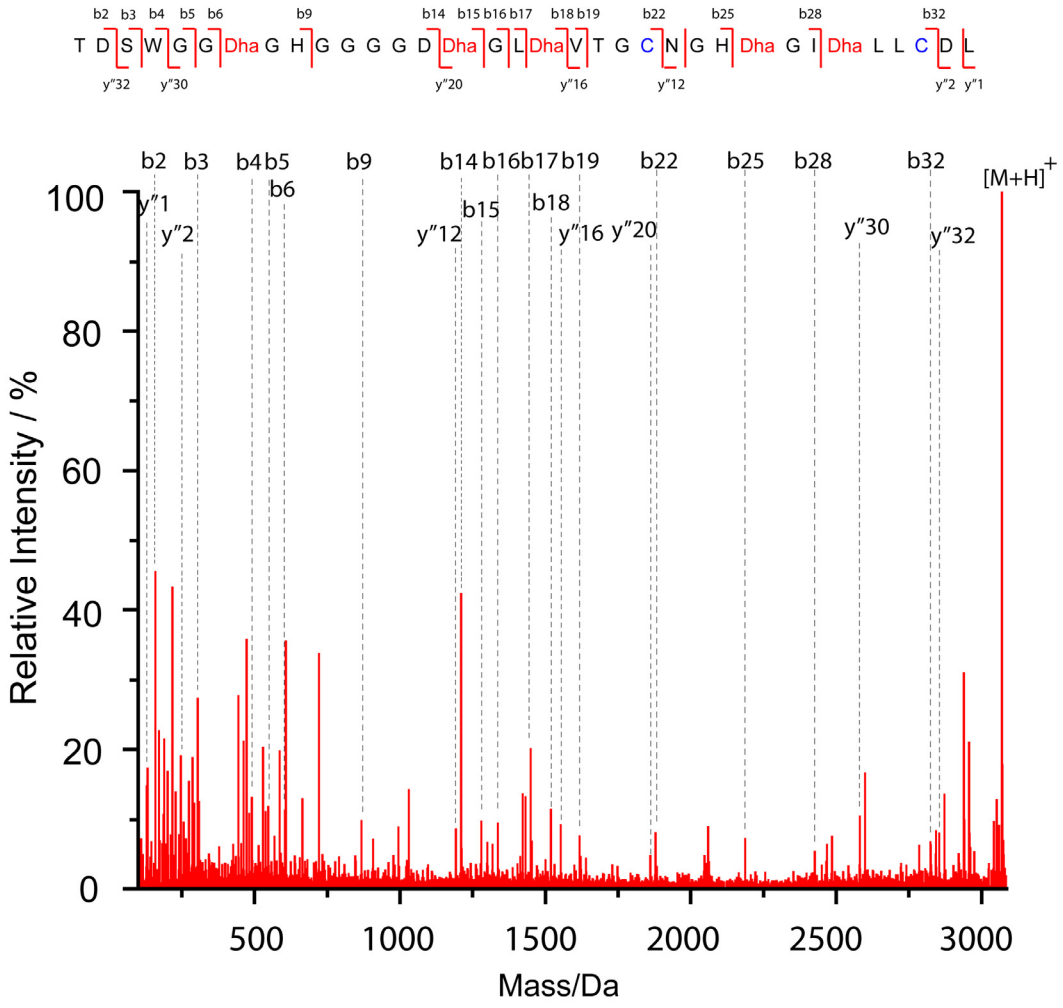


Figure S13. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of truncated dehydrated-AciA containing a disulfide treated with TCEP and AciKC. Dehydrated-AciA peptide containing the disulfide (5 µM) was first digested by endoprotease Glu-C and then incubated with AciKC (5 µM) and TCEP (0.1 mM) for 1 h at room temperature. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

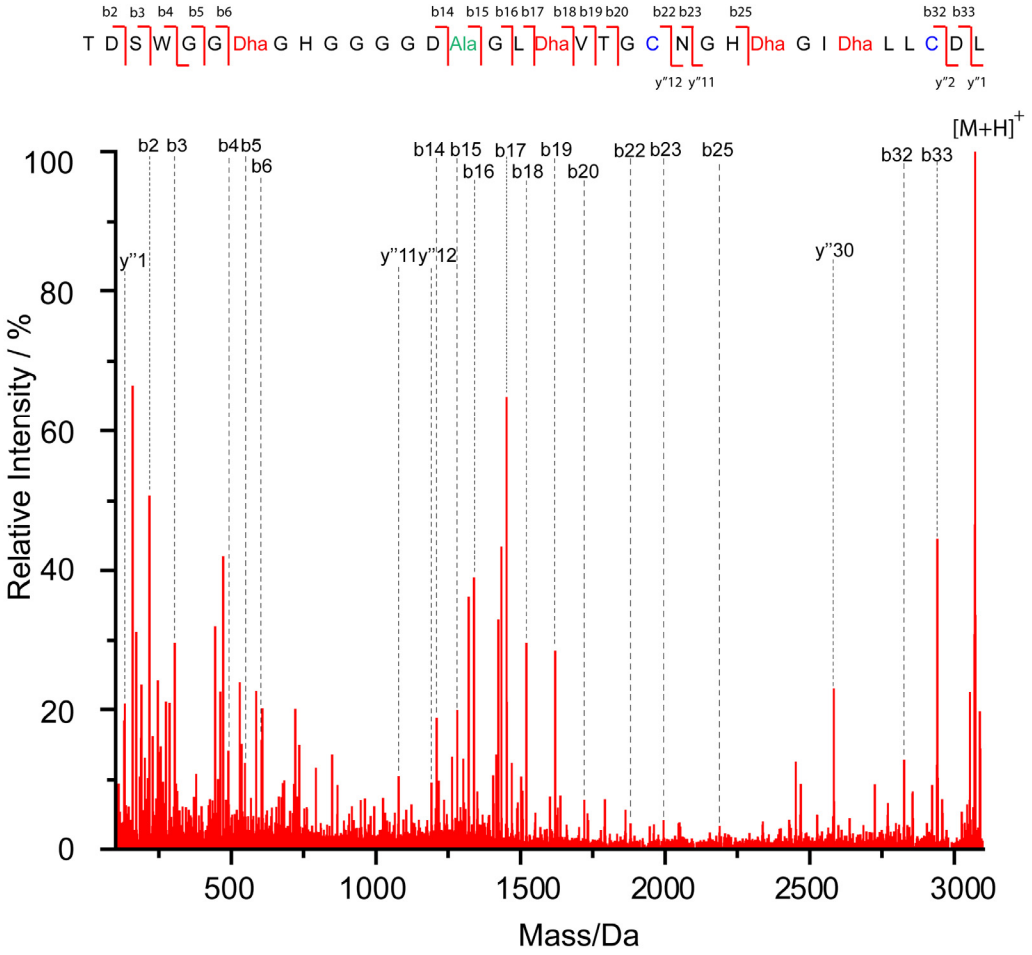


Figure S14a. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of AciKC-modified AciA-S8A with NTP. AciA-S8A peptide (10 µM) was incubated with AciKC (2 µM) in the presence of NTP mixture (5 mM), Mg^{2+} (10 mM), and TCEP (0.1 mM) for 4 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

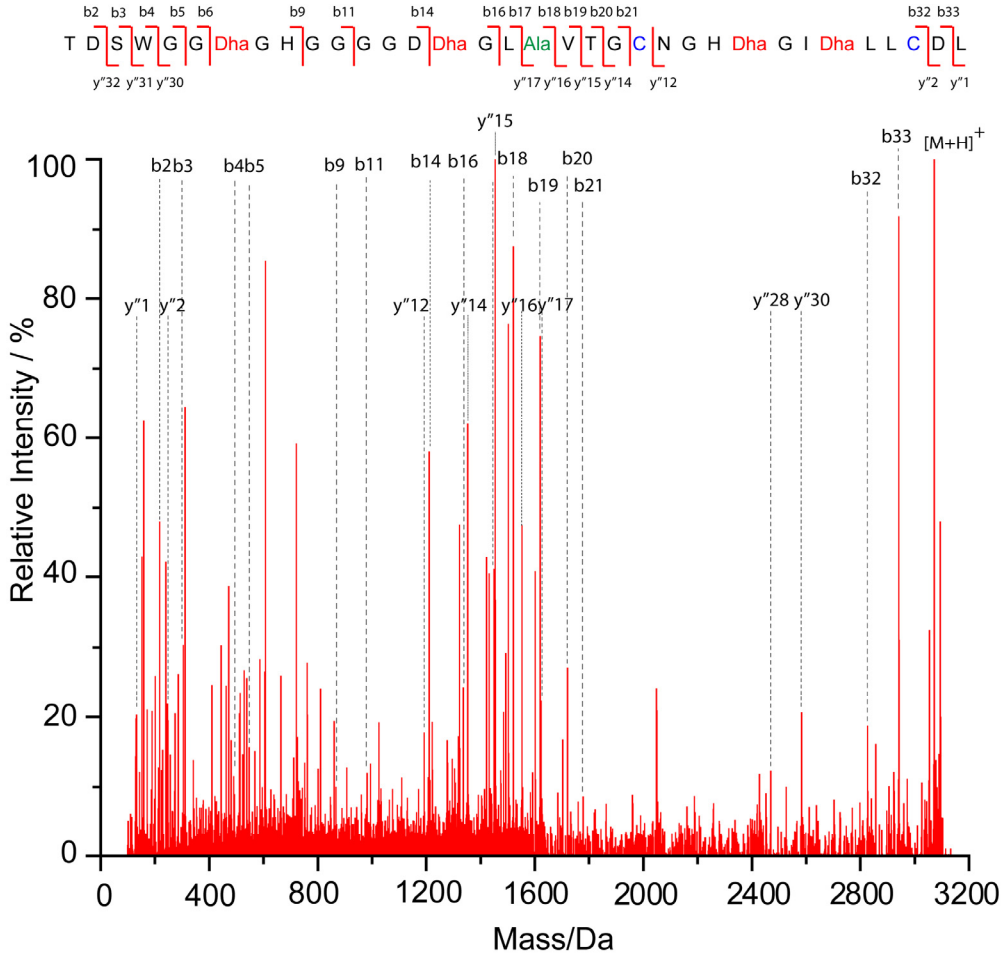


Figure S14b. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of AciKC-modified AciA-S11A with NTP. AciA-S11A peptide (10 μM) was incubated with AciKC (2 μM) in the presence of NTP mixture (5 mM), Mg^{2+} (10 mM), and TCEP (0.1 mM) for 4 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

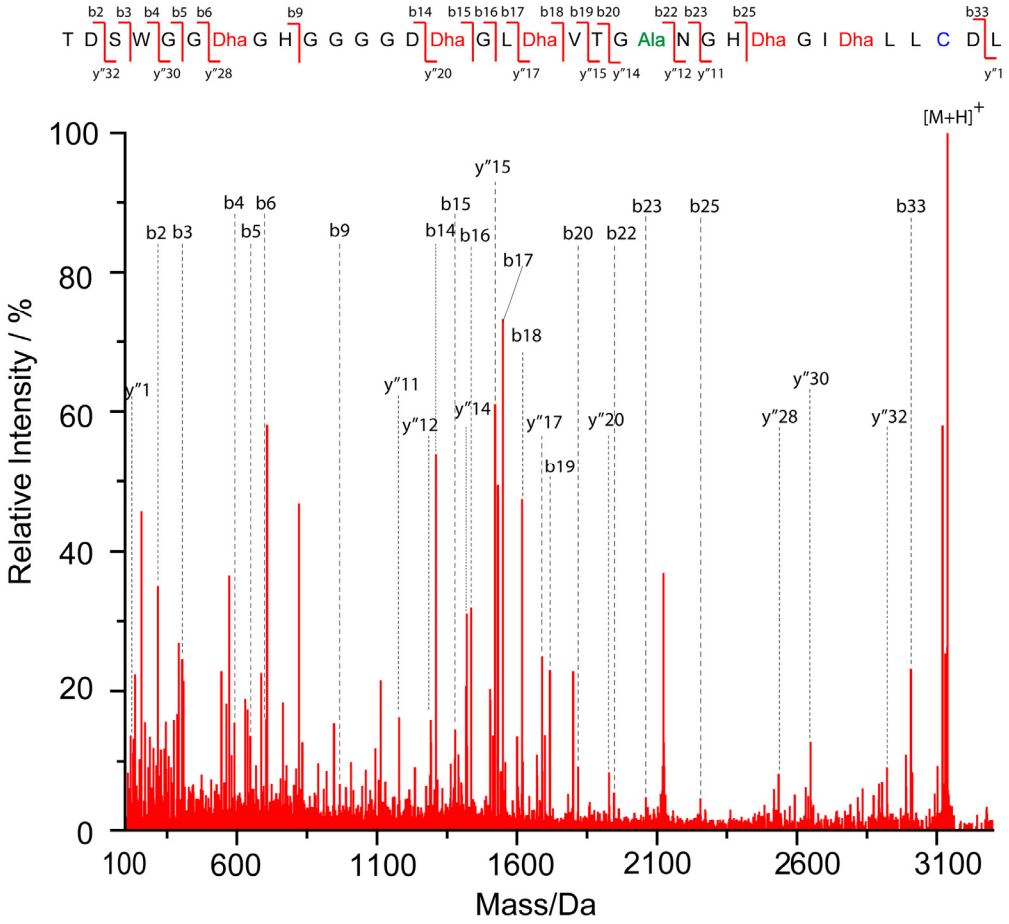


Figure S14c. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of AciKC-modified AciA-C15A with NTP. AciA-C15A peptide (10 μM) was incubated with AciKC (2 μM) in the presence of NTP mixture (5 mM), Mg^{2+} (10 mM), and TCEP (0.1 mM) for 4 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.

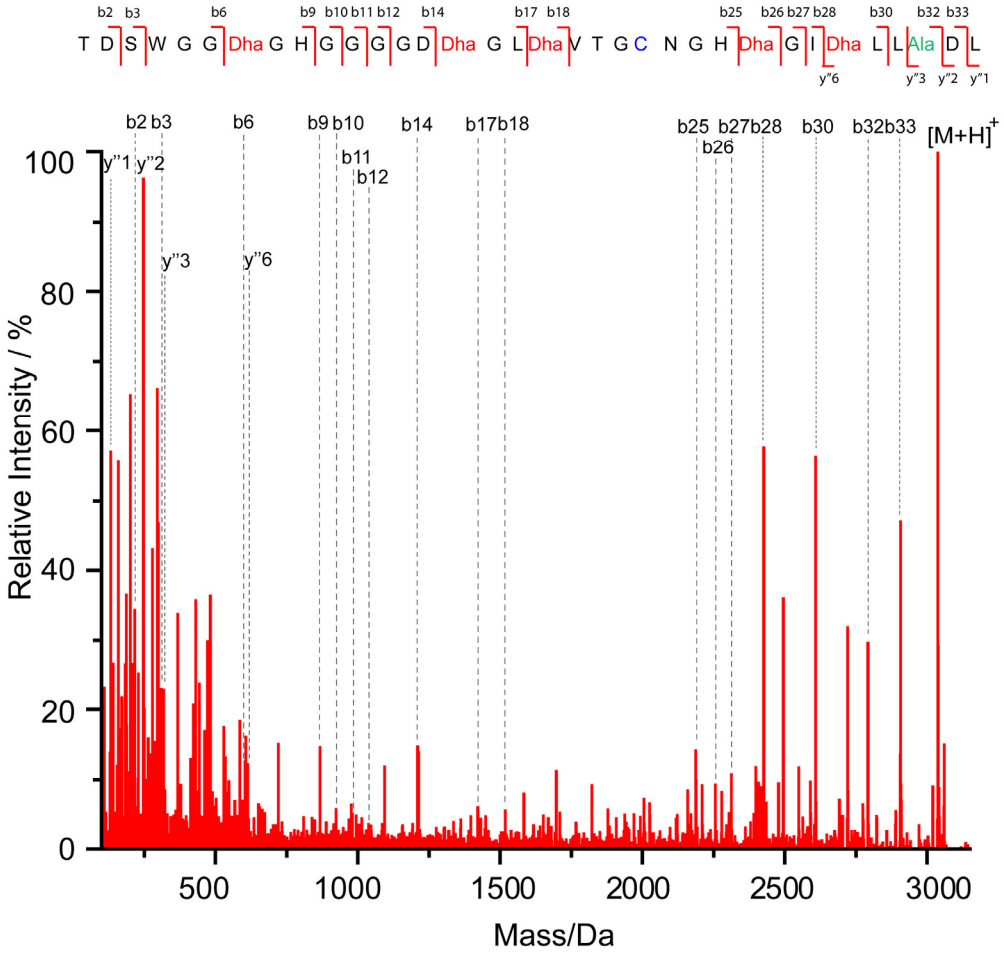


Figure S14d. ESI-MSMS spectrum of the peptide spanning residues Thr(–7)-Leu27 of AciKC-modified AciA-C25A with NTP. AciA-C25A peptide (10 µM) was incubated with AciKC (2 µM) in the presence of NTP mixture (5 mM), Mg^{2+} (10 mM), and TCEP (0.1 mM) for 4 h at room temperature and then cleaved by Glu-C endoprotease. The resulting modified core peptide was analyzed by LC-ESI-MSMS.