Supporting Information

Substrate Tolerance of the Biosynthetic Enzymes of Glycosylated Lanthipeptide NAI-112

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General methods and materials

All oligonucleotides were purchased from Genscript Biotech (Nanjing, China). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Phanta® Max Master Mix were purchased from Vazyme Biotech (Nanjing, China). Media components for bacterial cultures were purchased from Thermo Fisher (Waltham, MA, USA). Chemicals were purchased from Aladdin Reagent (Shanghai, China) or from Sigma-Aldrich (Schnelldorf, Germany) unless noted otherwise. Endoproteinase GluC was purchased from Roche Biosciences (Basel, Switzerland). *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.

All polymerase chain reactions (PCR) were carried out on a C1000 Touch™ thermal cycler (Bio-Rad). DNA sequencing was performed by the Genscript Biotech, using appropriate primers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out on Bruker UltraFlextreme. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Triple TOF 4600 System (AB Sciex) equipped with a Prominence Ultra Fast Liquid Chromatography (UFLC) system (Shimadzu). UV-Vis spectrometry was conducted using Cary 300 (Agilent Technologies).

Conditions for all ESI-MS and MS/MS were set as follows: nebulizer gas: 55 psi; heater gas : 55 psi; curtain gas: 35 psi; drying temperature: 550 °C; ion spray voltage: 5500 V; declustering potential: 100 V; collision energy: 35 V (positive); collision energy spread: 10 V. The mass range and accumulation time are 400-4000 m/z, 250 ms for ESI-MS and 100-2000 m/z, 100 ms for MS/MS, respectively. Collision-induced dissociation (CID) was performed for fragmentation of the respective peptide ions. Calibration solutions purchased from AB SCIEX were used for instrument calibration, and high resolution was chosen in the ESI+ mode.

General nomenclature

In this paper, we will use the standardized nomenclature recommended by the lanthipeptide community in 2013. This nomenclature numbers residues in the precursor peptides starting with the first residue of the core peptide. Residues in the leader peptide are indicated with negative numbers counting back from the junction between the leader peptide and core peptide.

Primers used in this study

Primer name	Sequence (5'-3')
AplA BamHI F	TGCTGGATCCAGTCCAGGAAATTCTGGAACTGCAAGAAC TGCC
AplA HindIII R	TGCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCG GCCAAC
$AplA_{P11insW}$ HindIII R	GCTAAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCG GCCAACCCCACGGGCAC
$AplA_{P11insAAA}$ HindIII R	GCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCGG CCAACCCGCGGCCGCCGGGCACGGGCTGC
$AplA_{W13insAAA}$ HindIII R	AGCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCG GCGCCGCCGCCCA

$AplA_{P11W-W13A}$ HindIII R	GCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCGG CGCACCCCAGCACGGGCTGCTAACAGAC
$AplA_{W13C}$ HindIII R	GCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCGG GCAACCCGGGCACGGGCTGC
$AplA_{W13S}$ HindIII R	GCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCGG GCTACCCGGGCACGGGCTGC
$AplA_{W13R}$ HindIII R	GCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCGG ACGACCCGGGCACGGGCTGC
$AplA_{W13E}$ HindIII R	AAAAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCGG TTCACCCGGGCACGGGCTGC
$AplA_{T3A-S16A}$ BamHI F	ACTCGGCCAACCCGGGCACGGGCTGCTAACAGACAGCGC GCTAACCAG
$AplA_{T3A-S16A}$ HindIII R	ATCGAAGCTTTTAACAGTTGGACCAGGTAAACGCACTCG GCCAACCCGGGCAC
$LabA_{T18A}$ HindIII R	GCTAAGCTTTTAACAGTTGGACCACGCAAAGGAACTCGG CCAACCCG
$LabA_{T18S}$ HindIII R	AGCTAAGCTTTTAACAGTTGGACCAGCTAAAGGAACTCG GCCAACCCG
$AplA_{W19insA-S20insA}$ HindIII R	AAAAAGCTTTTAACAGTTCGCGGAGGCCCAGGTAAAGGA ACTCGGCCAACCCGGGCACG
$AplA_{S20insA}$ HindIII R	AAAAAGCTTTTAACAGTTGGCGGACCAGGTAAAGGAACT CGGCCAACCCGG
$AplA_{C10A}$ HindIII R	TTAACAGTTGGACCAGGTAAAGGAACTCGGCCAACCCGG CGCCGGGCTGCTAACAGACA
$AplA_{C22A}$ HindIII R	AGCTAAGCTTTTACGCGTTGGACCAGGTAAAGGAACTCG GCCAAC
$AplA_{P11A}$ HindIII R	TTAACAGTTGGACCAGGTAAAGGAACTCGGCCAACCCGC GCACGGGCTGCTAACAGACA
$AplA_{P14A}$ HindIII R	AGCTAAGCTTTTAACAGTTGGACCAGGTAAAGGAACTCG CCCAACCCGGGCACGGGCTG
$AplA_{P11A-P14A}$ HindIII R	TTAACAGTTGGACCAGGTAAAGGAACTCGCCCAACCCGC GCACGGGCTGCTAACAGACA
AplG NdeI F	GCTCATATGCGCGTGCTGCTGTATTGCTACG
AplG XhoI R	GCTCTCGAGTTAGCGATGGACCAGACGTTCCAGAACT
AplKC NdeI F	AGCTCATATGGTTCTGGATACCCGTTATATTCGCTTTTGCC GTC
AplKC XhoI R	AGCTCTCGAGTTATTCTTCAGAATCGGCACCCGCGGAG
$AplKC_{K63A}$ F	CCGGAACAAGGTTGGGCAGTTCATCTGGCGTCC
$AplKC_{K63A}$ R	GGACGCCAGATGAACTGCCCAACCTTGTTCCGG
$AplKC_{H65A}$ F	CAAGGTTGGAAAGTTGCTCTGGCGTCCACCCTG
$AplKC_{H65A}$ R	CAGGGTGGACGCCAGAGCAACTTTCCAACCTTG
$AplKC_{Y120A}$ F	GGTAAATTTATTACCATCGCCCCGCTGGACGAAGCC
$AplKC_{Y120A}$ R	GGCTTCGTCCAGCGGGGCGATGGTAATAAATTTACC

$AplKC_{K249A}$ F	GAACGTGTTGTCCTGGCAGAAGCGCGTCCG
$AplKC_{K249A}$ F	CGGACGCGCTTCTGCCAGGACAACACGTTC
$AplKC_{D359A}$ F	GTCGTGCTGGGTGCGGTTCATCCGAAAAACATT
$AplKC_{D359A}$ R	AATGTTTTTCGGATGAACCGCACCCAGCACGAC
$AplKC_{N364A}$ F	GATGTTCATCCGAAAGCCATTCTGCTGCGCGAT
$AplKC_{N364A}$ R	ATCGCGCAGCAGAATGGCTTTCGGATGAACATC
$AplKC_{D376A}$ F	CAGCCGGTGTTCATTGCGTTCGAATTTTCGGGT
$AplKC_{D376A}$ R	ACCCGAAAATTCGAACGCAATGAACACCGGCTG
$AplG_{S10A-D13A}$ F	TATTGCTACGGCGCCCGTGGTGCTGTTCAGCCG
$AplG_{S10A-D13A}$ R	CGGCTGAACAGCACCACGGGCGCCGTAGCAATA
$AplG_{E358A}$ F	TGTCCGTTCCATCGTGCACAGGCCCAATGGT
$AplG_{E358A}$ R	ACCATTGGGCCTGTGCACGATGGAACGGACA

Molecular cloning of the *aplA*, *aplKC* and *aplG* genes

Plasmids containing target genes were synthesized by Genscript Biotech and PCR amplified by 30 cycles of denaturing (95 °C for 30 s), annealing (65 °C for 30 s), and extending (72 °C, 1 min/kb) using high fidelity Phanta® DNA Polymerase. Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using an Omega Biotech Cycle Pure Kit. The target DNA fragment and pRSFDuet-1 vector were digested in separate reactions containing 1X NEB buffer (New England Biolabs) with selected pair of restriction enzymes for 2 h at 37 °C. The digested products were purified by agarose gel electrophoresis, and the DNA fragments were extracted from the gel using an Omega Biotech Gel Extraction Kit. The resulting DNA products were ligated at 16 °C for 12 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/ μL). *E. coli* DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using an Omega Biotech Plasmid Mini Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

Construction of plasmids for coexpression of His_6-AplA, AplKC and AplG.

Amplification of the two genes was performed using high fidelity Phanta® DNA Polymerase following the protocol described above. Restriction digestions were performed with NdeI and XhoI endonucleases following standard protocol. The digested products of *aplA* and *aplKC* genes were purified by agarose gel electrophoresis and ligated with pRSFDuet-1 and pACYCDuet-1 vectors, respectively. The resulting DNA products were ligated at 16 °C for 12 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/ μL). *E. coli* DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-kanamycin and LB-chloramphenicol agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin and LB-chloramphenicol medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using an Omega Biotech Plasmid Mini Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

Mutagenesis of AplKC, AplG and AplA

Mutagenesis of AplKC was carried out by two-stage protocol, starting with the generation of

primers using two parallel asymmetric PCRs, followed by an exponential whole-plasmid amplification. PCR amplified by 30 cycles of denaturing (95 °C for 30 s), annealing (65 °C for 30 s), and extending (72 °C, 1 min/kb) using high fidelity Phanta® DNA Polymerase. Amplifications were confirmed by 1% agarose gel electrophoresis, and the PCR products were purified using an Omega Biotech Cycle Pure Kit. The target DNA fragment was digested in reaction containing 1X NEB buffer (New England Biolabs) with DpnI for 3 h at 37 °C. *E. coli* DH5α cells were transformed with 2.5 μL of the digested product by heat shock, and cells were plated on LB- agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-chloramphenicol medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using an Omega Biotech Plasmid Mini Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

Co-expression of AplKC with His_6-AplA.

E. coli BL21(DE3) cells were transformed with pRSFDuet-1-His_6-ApLA and pACYCDuet-1-AplKC plated on a Luria broth (LB) agar plate containing 50 mg/L of kanamycin and 35 mg/mL of chloramphenicol. A single colony was used to inoculate a 5 mL culture of LB supplemented with 50 mg/L of kanamycin and 35 mg/mL of chloramphenicol. The culture was grown at 37 °C for 12 h and was used to inoculate 4 L of LB containing 50 mg/L of kanamycin and 35 mg/mL of chloramphenicol. Cells were grown at 37 °C to OD_{600}~0.6-0.8 before IPTG was added to a final concentration of 0.2 mM and the culture was incubated at 18 °C for additional 16 h before harvesting.

Overexpression and purification of AplKC and AplG modified AplA peptide.

E. coli BL21(DE3) cells were transformed with pRSFDuet-1-His_6-AplA-AplG and pACYCDuet-1-AplKC and plated on a Luria broth (LB) agar plate containing 50 mg/L of kanamycin and 35 mg/L of chloramphenicol. A single colony was used to inoculate a 5 mL culture of LB supplemented with 50 mg/L of kanamycin and 35 mg/L of chloramphenicol at 37 °C for 12 h. The culture was used to inoculate 4 L of LB containing 50 mg/L of kanamycin and 35 mg/L of chloramphenicol. Cells were grown at 37 °C to OD_{600}~0.6-0.8 before IPTG was added to a final concentration of 0.2 mM and the culture was incubated at 18 °C for another 16 h before harvesting.

Purification of AplA peptides produced by co-expression

Cell 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

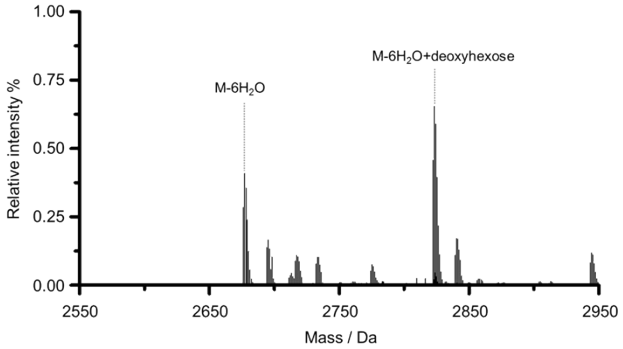
volumes of elution buffer (4 M guanidine HCl, 20 mM NaH_2PO_4 , pH 7.5, 500 mM NaCl, 1 M imidazole). The fractions were desalted using a ZipTipC_{18} and analyzed by MALDI-TOF MS. The fractions containing the desired peptide were pooled and purified by preparative reverse-phase high-performance liquid 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 210 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. The product was kept at −80 °C for long-term storage.

Strain	Genome	Glycosyltransferase
Streptomyces albus subsp. albus strain NRRL F-4371	NZ_LMZE01000299.1	WP_060733177.1
Streptomyces sp. NRRL F-2664	NZ_JOFX01000007.1	WP_030764500.1
Kitasatospora aureofaciens strain NRRL B-2658	NZ_LGUY01000246.1	WP_003981554.1
Streptomyces peucetius strain NRRL WC-3868	NZ_JOCK01000055.1	WP_032916214.1
Pseudonocardia sp. Ae168_Ps1	NZ_MCIK01000001.1	WP_075297995.1
Streptomyces rimosus subsp. rimosus strain NRRL WC-3927	NZ_JOBO01000038.1	WP_033015994.1
Streptomyces rimosus subsp. rimosus strain NRRL WC-3904	NZ_JOCQ01000054.1	WP_030673038.1
Streptomyces rimosus subsp. rimosus ATCC 10970	NZ_ANSJ01000034.1	WP_003981554.1
Streptomyces rimosus subsp. pseudoverticillatus strain NRRL WC-3896	NZ_LGCV01000431.1	WP_053804725.1
Amycolatopsis albispora strain WP1	NZ_CP015163.1	WP_162788447.1
		WP_113692533.1
Actinosynnema mirum DSM 43827	NC_013093.1	WP_015803558.1
		WP_015803555.1
Actinosynnema pretiosum subsp. pretiosum strain ATCC 31280	NZ_CP029607.1	WP_015803558.1
		WP_118947401.1
Streptomyces ruber NRRL ISP-5378	NZ_JOAQ01000001	WP_051910126.1
		WP_030363693.1
Microbacterium sp. SLBN-146	VFMR01000001.1	TQJ31151.1

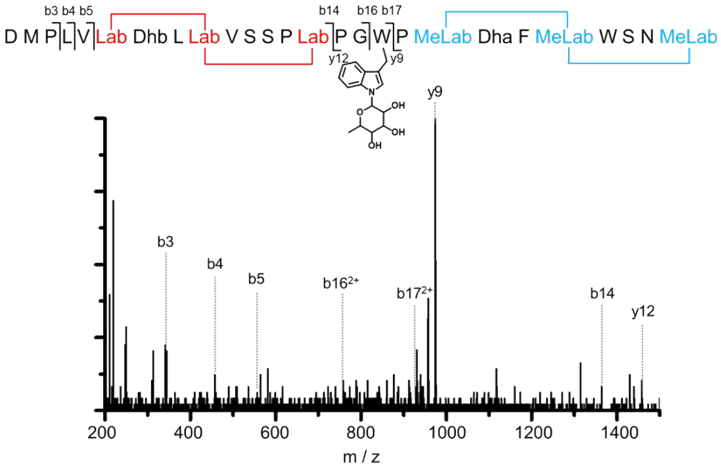
Table S1. Putative lanthipeptide biosynthetic gene clusters with glycosyltransferases. The contig numbers of the gene clusters and the accession numbers of putative glycosyltransferases are provided.

A) Co-expression of AplA with AplKC and AplG in *E. coli*

B) LC-MS analysis of the digestion products



C) LC-MS/MS analysis of the [M-6H_2O+deoxyhexose] segment



Species	Predicted mass / Da	Observed mass / Da	Error / ppm	Species	Predicted mass / Da	Observed mass / Da	Error / ppm
b3	344.12751	344.12665	2.5	y9	974.38257	974.38662	4.2
b4	457.21158	457.20990	3.7	y12	1460.5940	1460.5897	2.9
b5	556.27999	556.27803	3.5	$b16^{2+}$	759.36004	759.36306	4.0
b14	1363.6385	1363.6399	1.0	$b17^{2+}$	925.42865	925.42034	9.0

Figure S1. Co-expression of AplA with AplKC and AplG in *E. coli*. A) His_6-tagged AplA was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA after GluC digestion. Deconvoluted mass of two derivatives: [M-6H_2O], calc. 2676.1599, obs. 2676.1693; [M-6H_2O+deoxyhexose], calc. 2822.2179, obs. 2822.2306. C) LC-MS/MS analysis of the [M-6H_2O+deoxyhexose] segment. The b and y ions are listed in the table and marked in the spectrum. The amino acid of this peptide is shown on top.

Figure S2. Sequence alignment of AplKC with Protein Kinase A and known class III lanthipeptide synthetases. The blue star indicates the amino acid that may interact with the α and β phosphates of ATP. The red star indicates the amino acid that may direct the hydroxyl for attack on the γ phosphate of ATP. Cyan stars indicate amino acids that may chelate the Mg^{2+} in the active site of enzymes.

The accession numbers of listed proteins in GenBank are listed as follows: PKA (NP_032880.1), AplKC (AHB63593.1), AciKC (WP_015792832.1), AviKC (WP_010988898.1), CruKC (WP_012853928.1), EryKC (WP_009949110.1), FlaKC (WP_012920096.1), GriKC (WP_012380882.1), LabKC (CAX48971.1), RamKC (NP_630756.1), StaKC (WP_013015817.1).

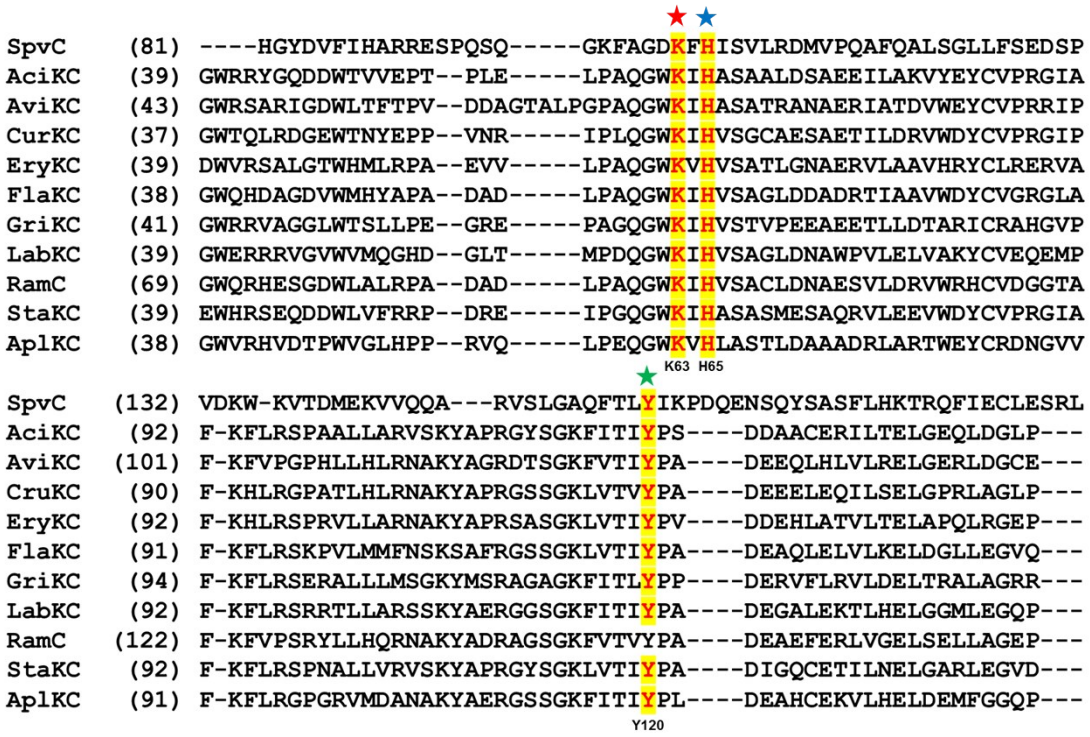


Figure S3. Sequence alignment of AplKC with phosphothreonine lyase SpvC and known class III lanthipeptide synthetases. The red star indicates the amino acid that may act as a general base to abstract the α proton. The blue star indicates the amino acid may act as a general acid to facilitate cleavage of the Cβ-OP bond. The cyan star indicates the amino acid that may act as a hydrogen bond acceptor.

The accession numbers of protein in GenBank are listed as follows: SpvC (WP_001122242.1), AplKC (AHB63593.1), AciKC (WP_015792832.1), AviKC (WP_010988898.1), CruKC (WP_012853928.1), EryKC (WP_009949110.1), FlaKC (WP_012920096.1), GriKC (WP_012380882.1), LabKC (CAX48971.1), RamKC (NP_630756.1), StaKC (WP_013015817.1).

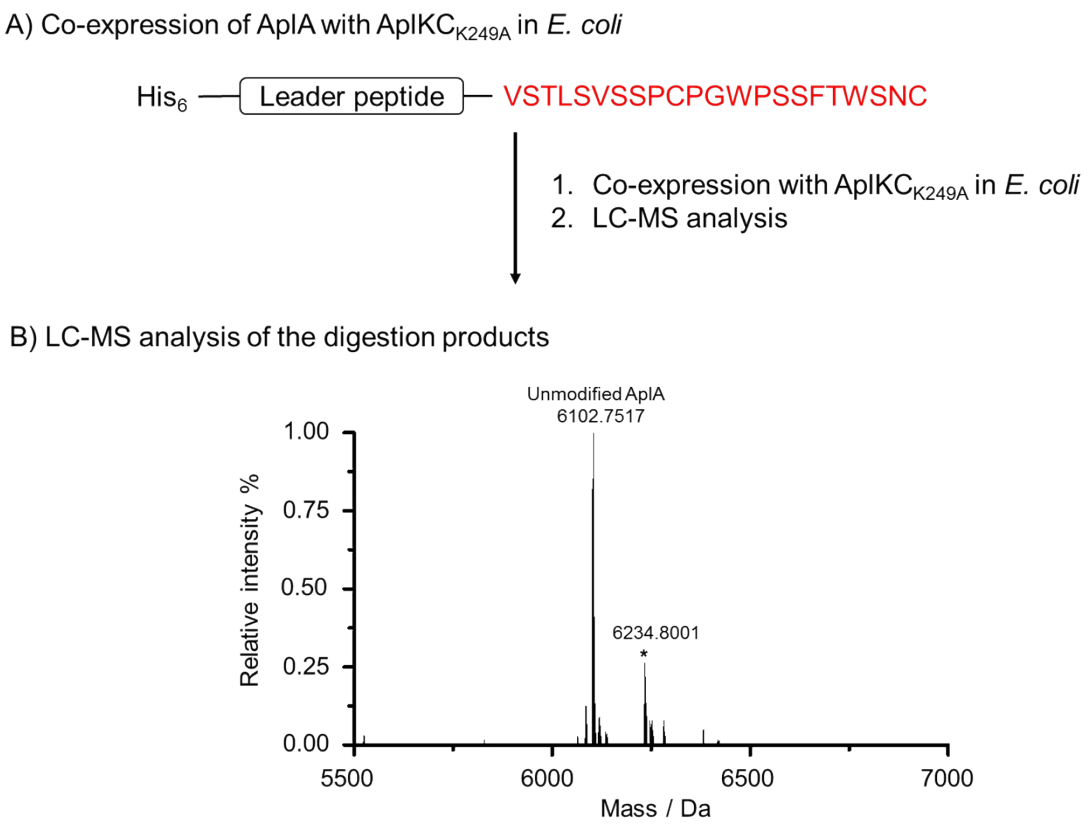


Figure S4. Co-expression of AplA with AplKC$_{K249A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed With AplKC$_{K249A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of the AplA product. Deconvoluted mass of one derivative: unmodified AplA, calc. 6102.7635, obs. 6102.7517. The peak marked with star does not match with any possible AplA product and may be produced by *E. coli*.

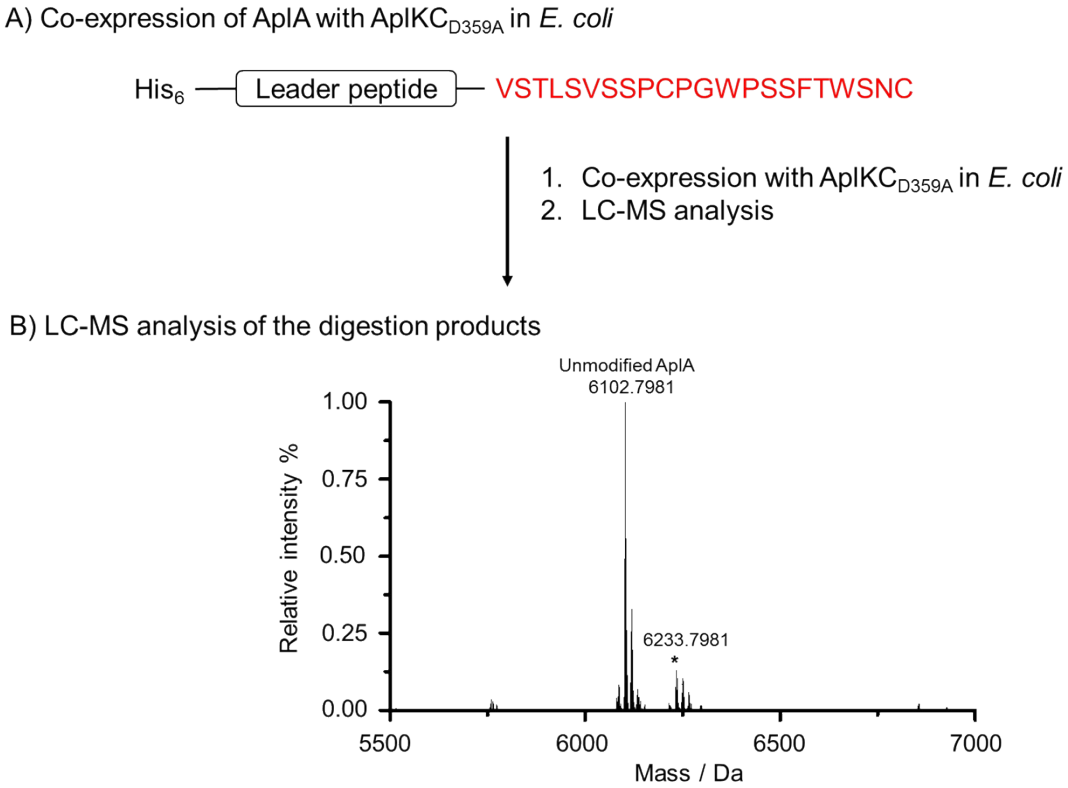


Figure S5. Co-expression of AplA with AplKC$_{D359A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed With AplKC$_{D359A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of AplA product. Deconvoluted mass of one derivative: unmodified AplA, calc. 6102.7635, obs. 6102.7981. The peak marked with star does not match with any possible AplA product and may be produced by *E. coli*.

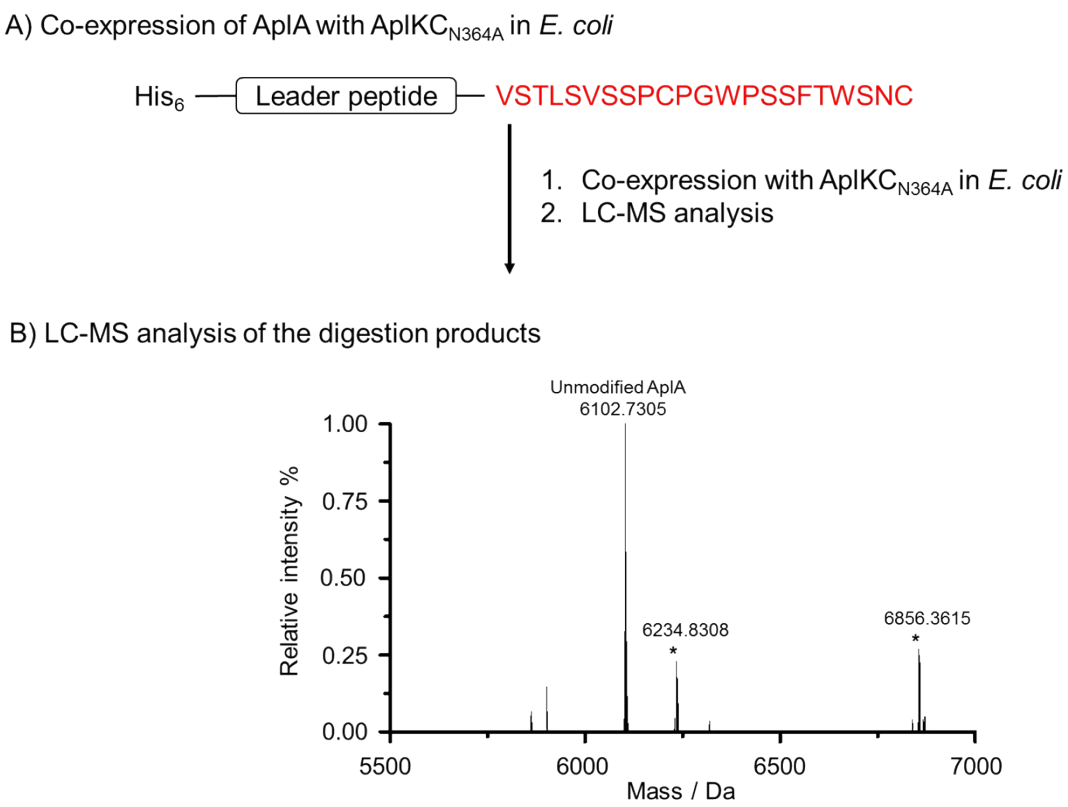


Figure S6. Co-expression of AplA with AplKC$_{N364A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed With AplKC$_{N364A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of AplA. Deconvoluted mass of one derivative: unmodified AplA, calc. 6102.7635, obs. 6102.7305. The peak marked with star does not match with any possible AplA product and may be produced by *E. coli*.

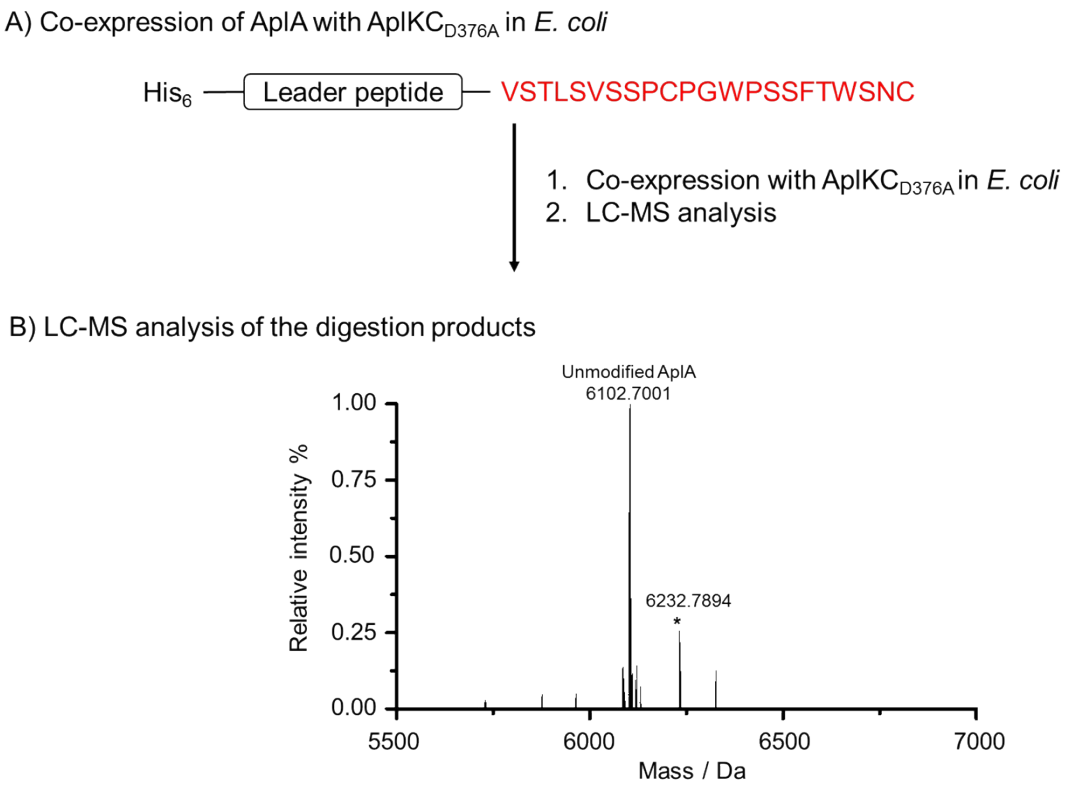


Figure S7. Co-expression of AplA with AplKC$_{D376A}$ in *E. coli*. A) His6-tagged AplA was co-expressed With AplKC$_{D376A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of AplA product. Deconvoluted mass of one derivative: unmodified AplA, calc. 6102.7635, obs. 6102.7001. The peak marked with star does not match with any possible AplA product and may be produced by *E. coli*.

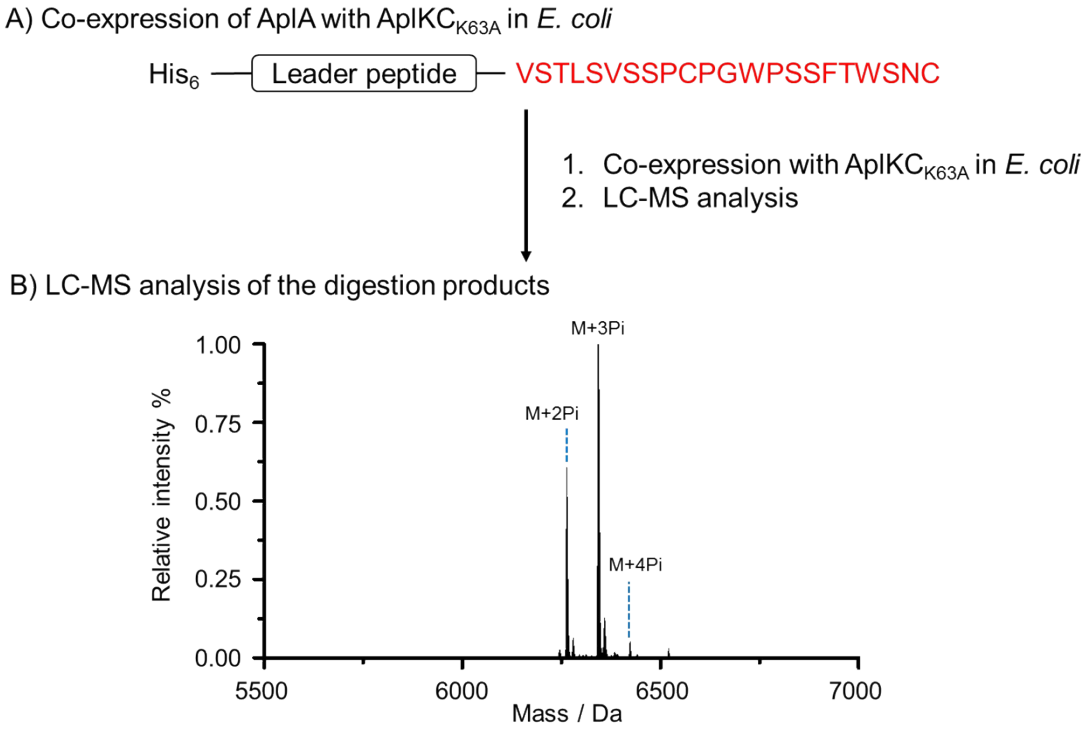


Figure S8. Co-expression of AplA with AplKC$_{K63A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed With AplKC$_{K63A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of AplA products. Deconvoluted mass of three derivatives: [M+2Pi], calc. 6262.6962, obs.6262.6092; [M+3Pi], calc. 6342.6625, obs. 6342.7368; [M+4Pi], calc. 6422.6288, obs. 6422.5968.

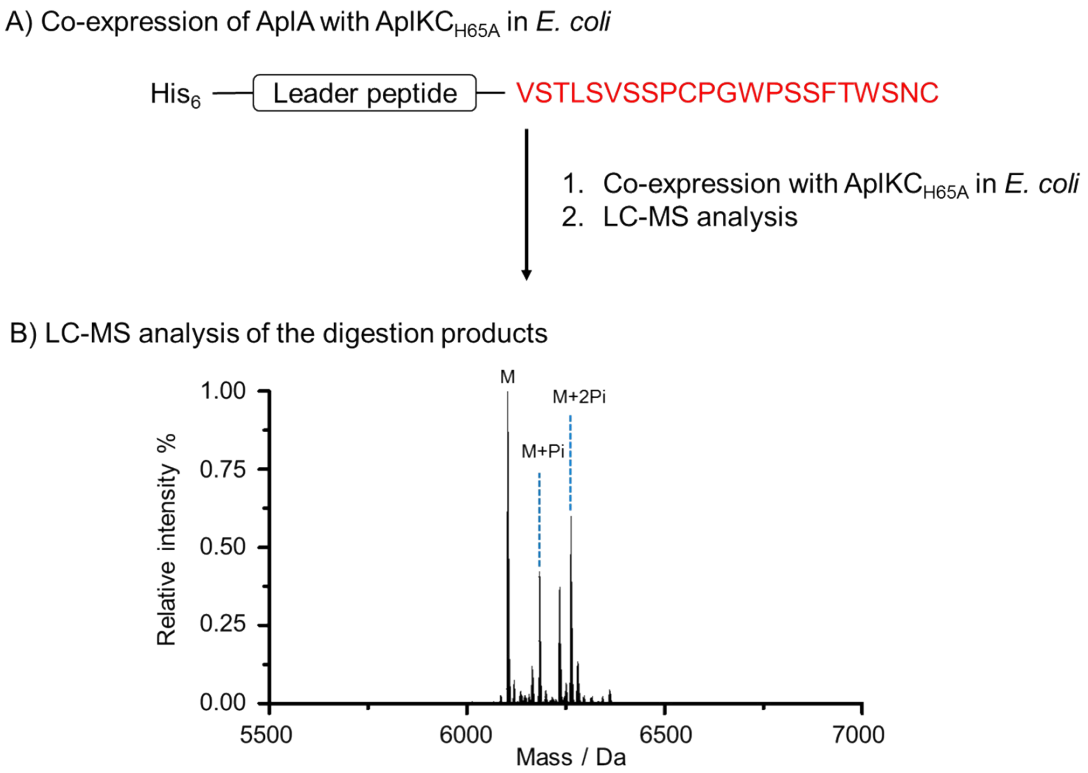


Figure S9. Co-expression of AplA with AplKC$_{H65A}$ in *E. coli.* A) His$_6$-tagged AplA was co-expressed With AplKC$_{H65A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of AplA products. Deconvoluted mass of three derivatives: [M], calc. 6102.7635, obs. 6102.6738; [M+Pi], calc. 6182.7298, obs. 6182.6158; [M+2Pi], calc. 6262.6962, obs. 6262.5759.

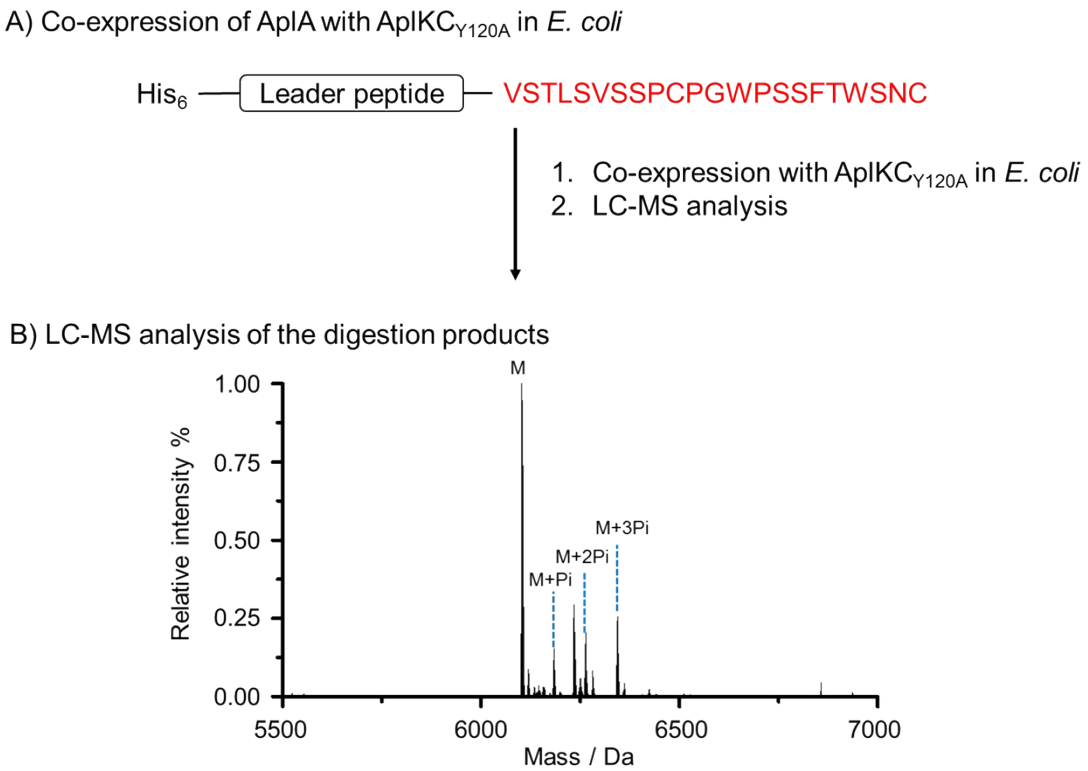
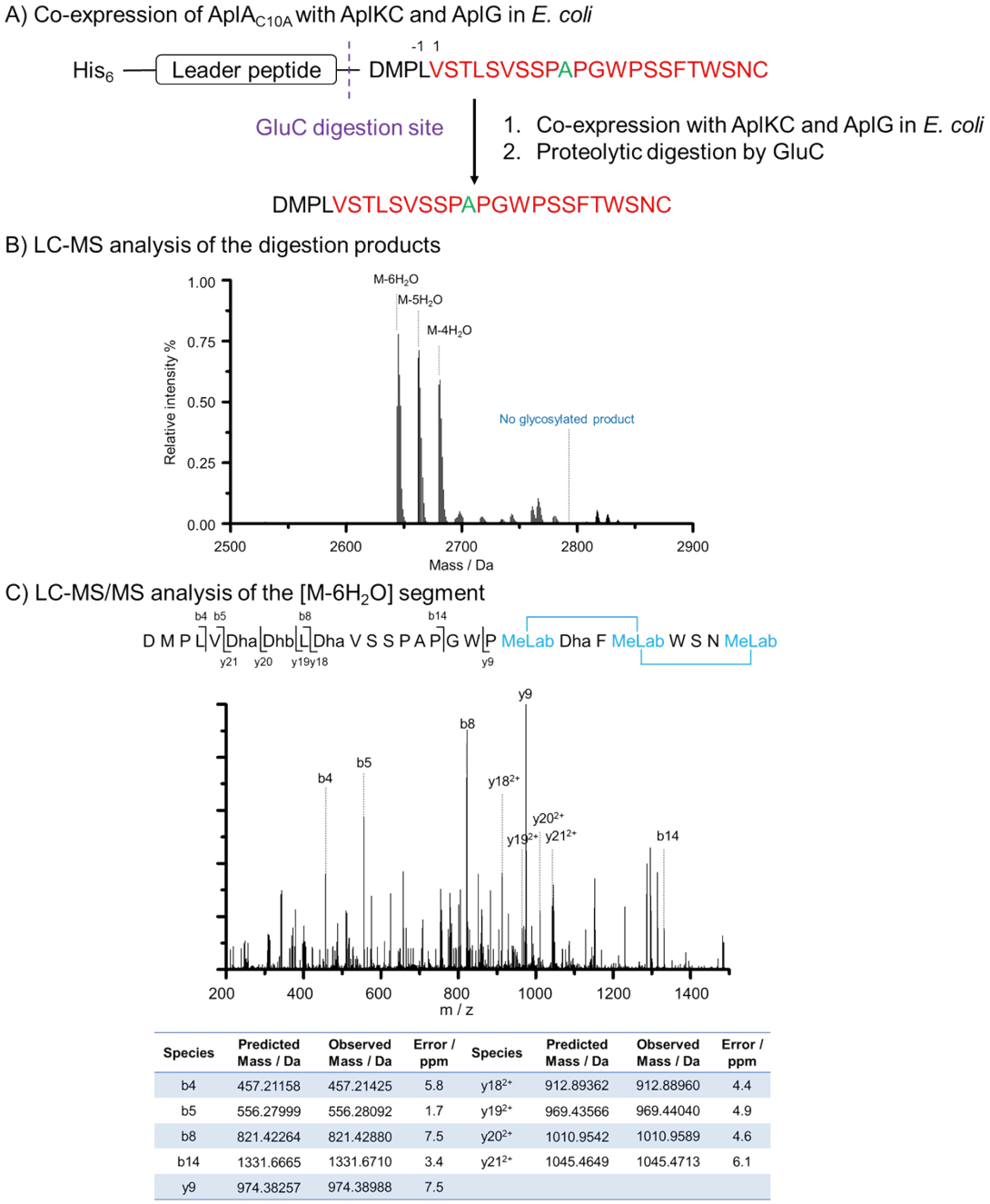


Figure S10. Co-expression of AplA with AplKC$_{Y120A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed With AplKC$_{Y120A}$ in *E. coli*. After purified the product was subjected to LC-MS analysis. B) HRMS analysis of AplA products. Deconvoluted mass of four derivatives: [M], calc. 6102.7635, obs. 6102.7096; [M+Pi], calc. 6182.7298, obs. 6182.7156; [M+2Pi], calc. 6262.6962, obs. 6262.6399; [M+3Pi], calc. 6342.6625, obs. 6342.6231.

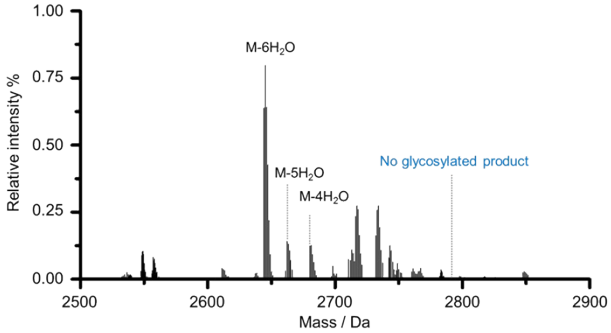


Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b4	457.21158	457.21425	5.8	$y18^{2+}$	912.89362	912.88960	4.4
b5	556.27999	556.28092	1.7	$y19^{2+}$	969.43566	969.44040	4.9
b8	821.42264	821.42880	7.5	$y20^{2+}$	1010.9542	1010.9589	4.6
b14	1331.6665	1331.6710	3.4	$y21^{2+}$	1045.4649	1045.4713	6.1
y9	974.38257	974.38988	7.5				

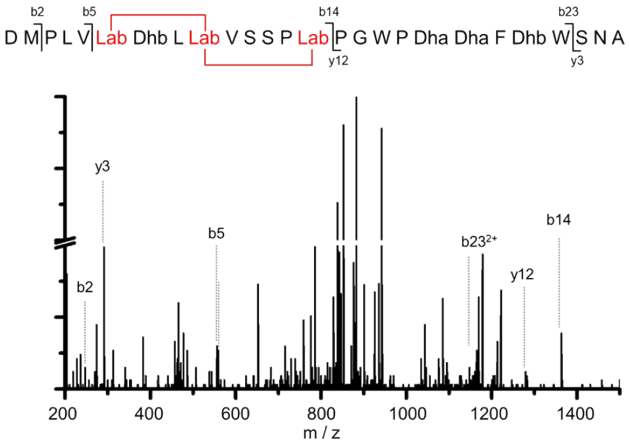
Figure S11. Co-expression of AplA$_{C10A}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{C10A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA$_{C10A}$ and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC-AplG modified AplA$_{C10A}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O], calc. 2644.1879, obs. 2644.2020; [M-5H$_2$O], calc. 2662.1985, obs. 2662.2143; [M-4H$_2$O], calc. 2680.2091, obs. 2680.2277. C) LC-MS/MS analysis of the [M-4H$_2$O] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the modified peptide is shown on top.

A) Co-expression of $AplA_{C22A}$ with AplKC and AplG in *E. coli*

B) LC-MS analysis of the digestion products

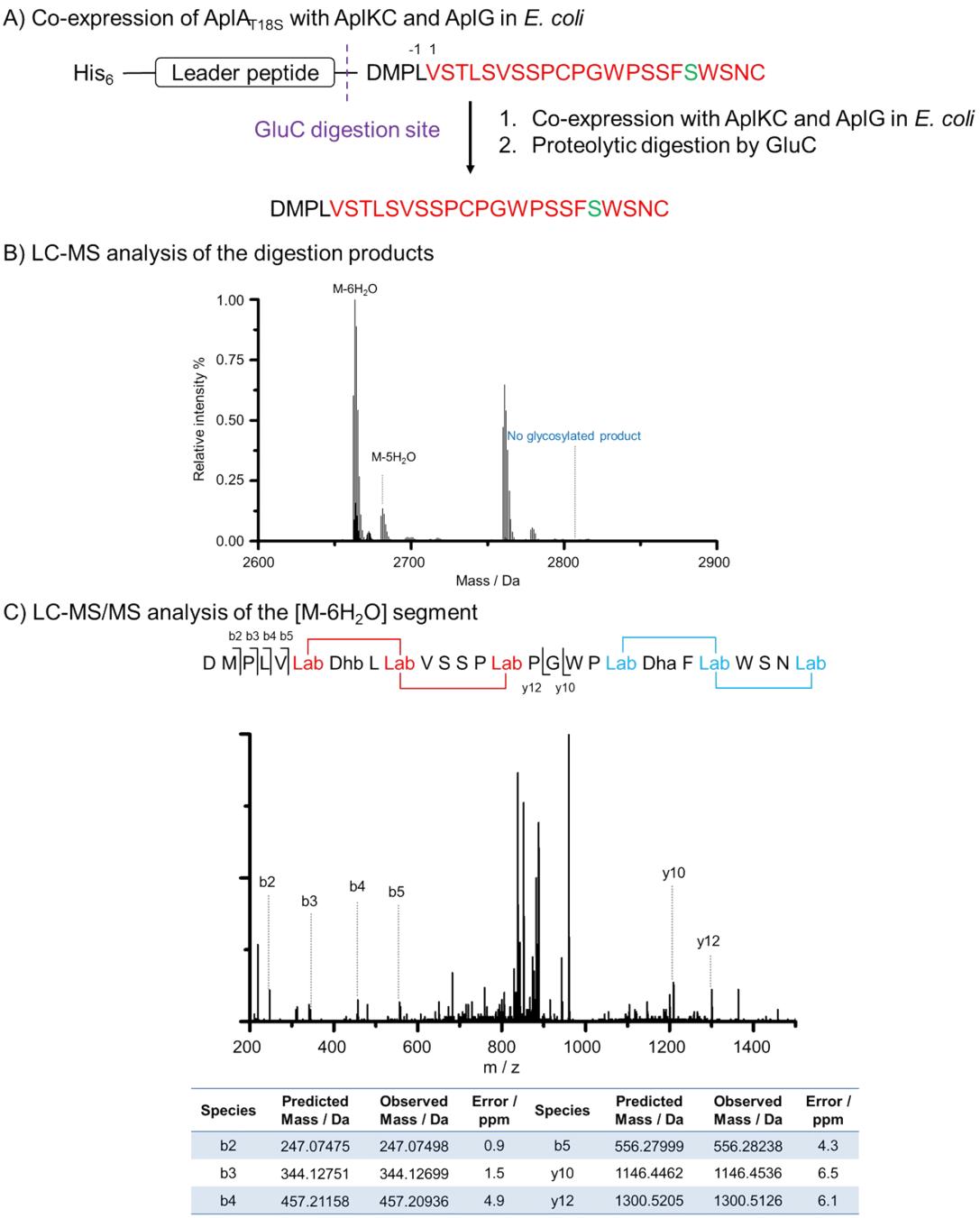


C) LC-MS/MS analysis of the [M-$6H_2O$] segment



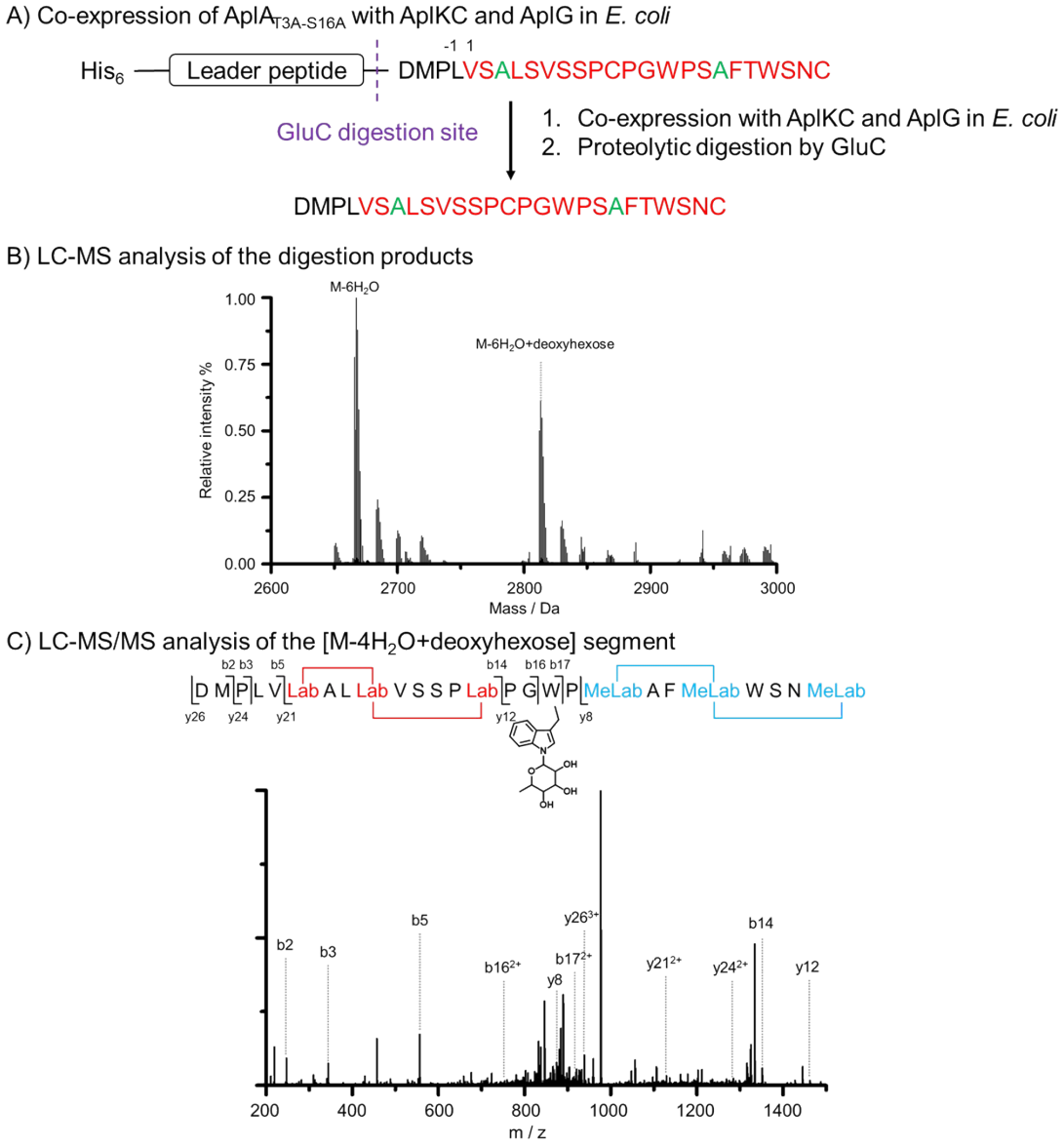
Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07619	5.8	y3	291.12993	291.13023	1.0
b5	556.27999	556.28304	5.5	y12	1282.5640	1282.5655	1.2
b14	1363.6385	1363.6424	2.9	$b23^{2+}$	1178.0400	1178.0488	7.7

Figure S12. Co-expression of $AplA_{C22A}$ with AplKC and AplG in *E. coli*. A) His_6-tagged $AplA_{C22A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM $AplA_{C22A}$ and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified $AplA_{C22A}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-$6H_2O$], calc. 2644.1879, obs. 2644.1799; [M-$5H_2O$], calc. 2662.1985, obs. 2662.1914; [M-$4H_2O$], calc. 2680.2091, obs. 2680.1841. C) LC-MS/MS analysis of the [M-$6H_2O$] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the modified peptide is shown on top.



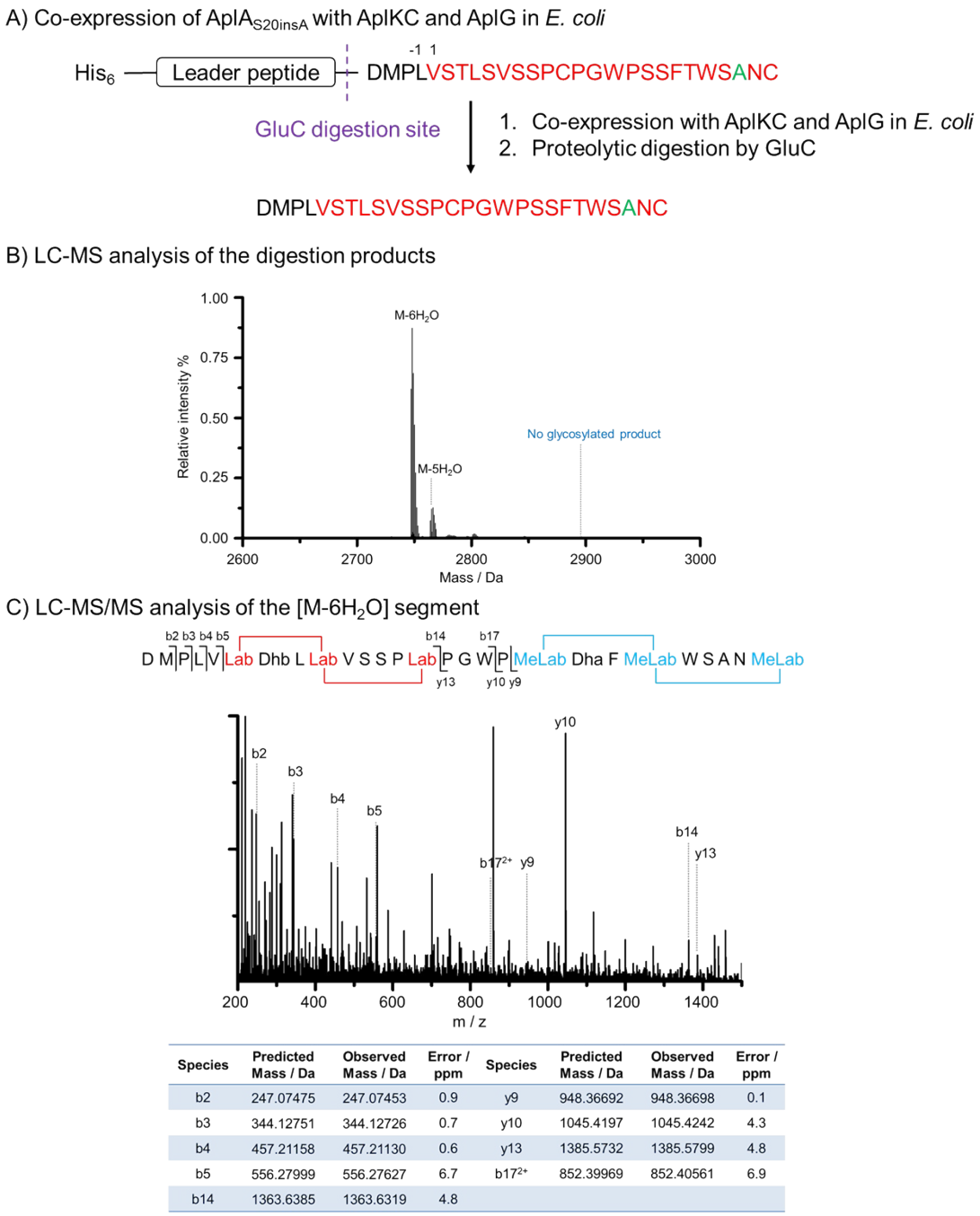
Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07498	0.9	b5	556.27999	556.28238	4.3
b3	344.12751	344.12699	1.5	y10	1146.4462	1146.4536	6.5
b4	457.21158	457.20936	4.9	y12	1300.5205	1300.5126	6.1

Figure S13. Co-expression of AplA$_{T18S}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{T18S}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA$_{T18S}$ and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{T18S}$ after GluC digestion. Deconvoluted mass of two derivatives: [M-6H$_2$O], calc. 2662.1443, obs. 2662.1815; [M-5H$_2$O], calc. 2680.1549, obs. 2680.1942. C) LC-MS/MS analysis of the [M-6H$_2$O] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the modified peptide is shown on top.



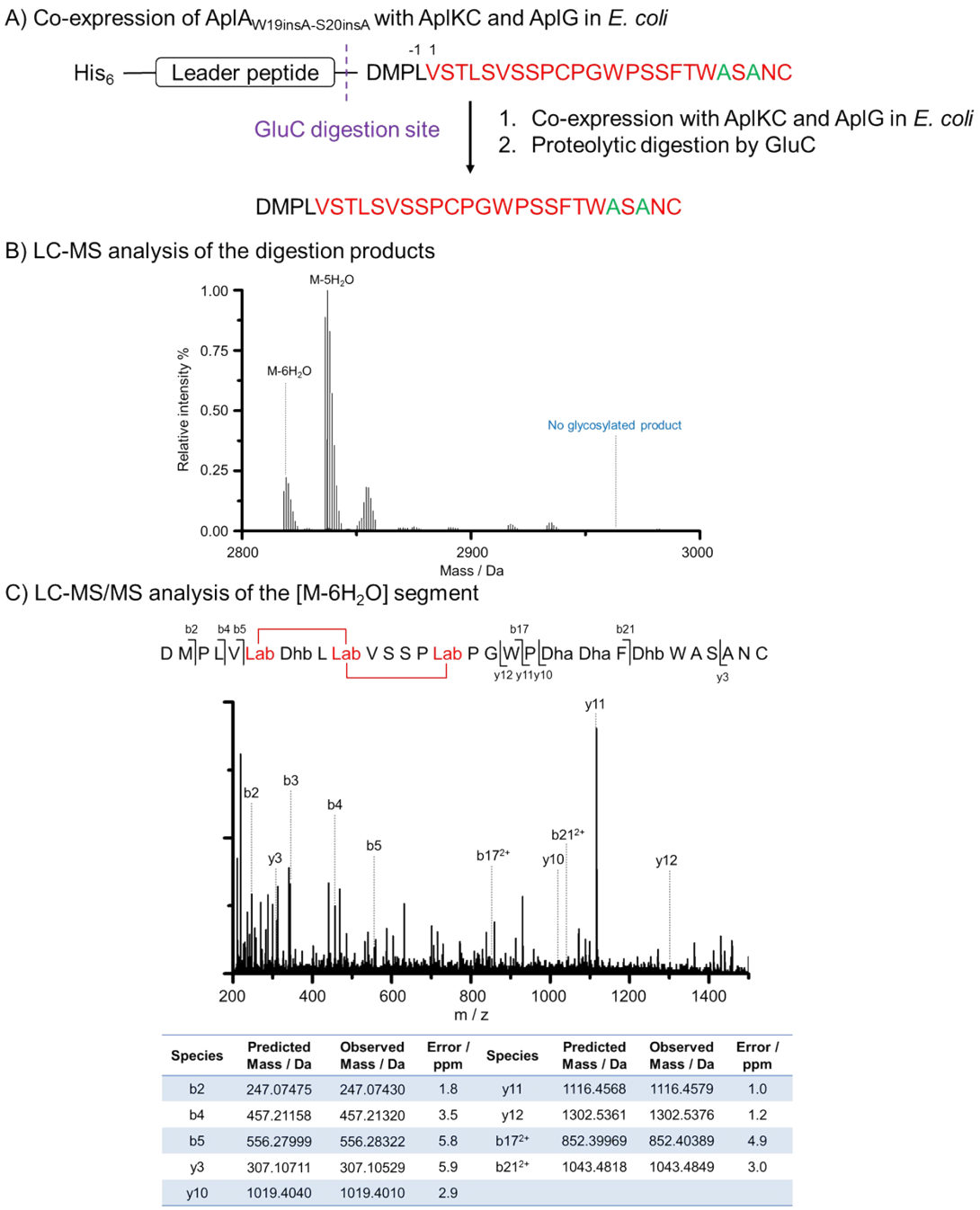
Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07544	2.8	$b16^{2+}$	753.36004	753.36186	2.4
b3	344.12751	344.12784	1.0	$b17^{2+}$	919.42865	919.43208	3.7
b5	556.27999	556.28101	1.8	$y21^{2+}$	1129.4878	1129.4885	0.6
b14	1351.6385	1351.6401	1.2	$y24^{2+}$	1284.0904	1284.0876	2.2
y8	879.34545	879.34946	4.6	$y26^{3+}$	938.41850	938.41750	1.0
y12	1462.6097	1462.6143	3.1				

Figure S14. Co-expression of AplA$_{T3A-S16A}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{T3A-S16A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA$_{T3A-S16A}$ and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC AplG modified AplA$_{T3A-S16A}$ after GluC digestion. Deconvoluted mass of two derivatives: [M-4H$_2$O], calc. 2666.1755, obs. 2666.1873; [M-4H$_2$O+deoxyhexose], calc. 2812.2335, obs. 2812.2443. C) LC-MS/MS analysis of the [M-4H$_2$O+deoxyhexose] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the modified peptide is shown on top.



Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07453	0.9	y9	948.36692	948.36698	0.1
b3	344.12751	344.12726	0.7	y10	1045.4197	1045.4242	4.3
b4	457.21158	457.21130	0.6	y13	1385.5732	1385.5799	4.8
b5	556.27999	556.27627	6.7	b17^{2+}	852.39969	852.40561	6.9
b14	1363.6385	1363.6319	4.8				

Figure S15. Co-expression of AplA$_{S20insA}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{S20insA}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{S20insA}$ and 1 µM GluC at 37 oC for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{S20insA}$ after GluC digestion. Deconvoluted mass of two derivatives: [M-6H$_2$O], calc. 2747.1970, obs. 2747.2007; [M-5H$_2$O], calc. 2765.2076, obs. 2765.2071. C) LC-MS/MS analysis of the [M-6H$_2$O] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the modified peptide is shown on top.



Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07430	1.8	y11	1116.4568	1116.4579	1.0
b4	457.21158	457.21320	3.5	y12	1302.5361	1302.5376	1.2
b5	556.27999	556.28322	5.8	b17^{2+}	852.39969	852.40389	4.9
y3	307.10711	307.10529	5.9	b21^{2+}	1043.4818	1043.4849	3.0
y10	1019.4040	1019.4010	2.9				

Figure S16. Co-expression of AplA$_{W19insA-S20insA}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{W19insA-/S20insA}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{W19insA-S20insA}$ and 1 µM GluC at 37 oC for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{W19insA-S20insA}$ after GluC digestion. Deconvoluted mass of two derivatives: [M-6H$_2$O], calc. 2818.2342, obs. 2818.2564; [M-5H$_2$O], calc. 2836.2448, obs. 2836.2694. C) LC-MS/MS analysis of the [M-6H$_2$O] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the modified peptide is shown on top.

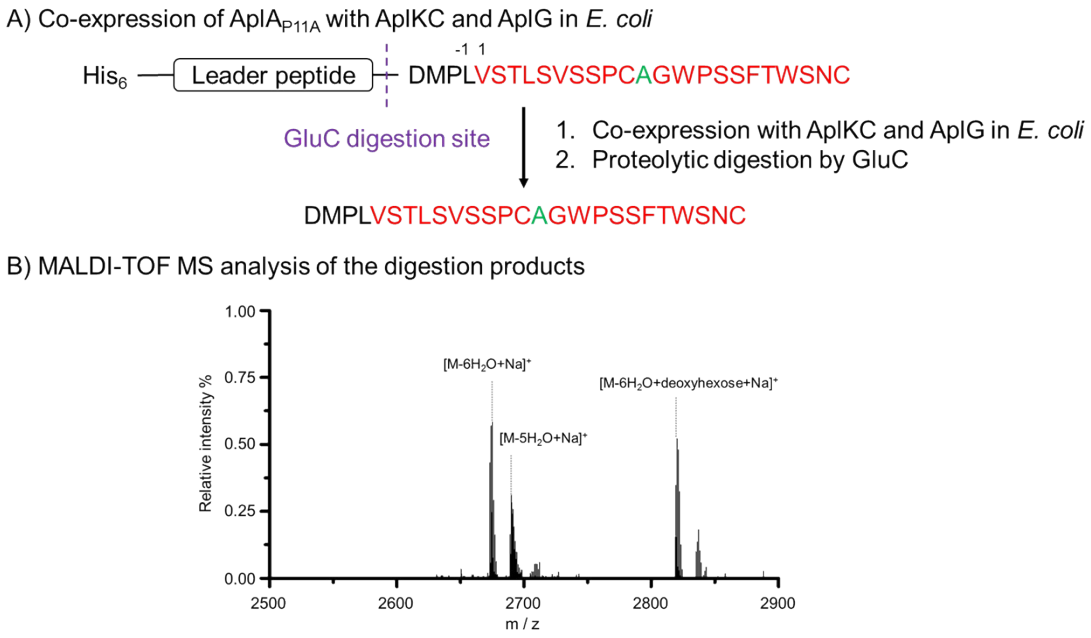


Figure S17. Co-expression of AplA$_{P11A}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{P11A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{P11A}$ and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{P11A}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+Na]$^+$, calc. 2673.1243, obs. 2673.1880; [M-5H$_2$O+Na]$^+$, calc. 2691.1349, obs. 2691.2000. [M-6H$_2$O+deoxyhexose+Na]$^+$, calc. 2819.1825, obs. 2819.2420.

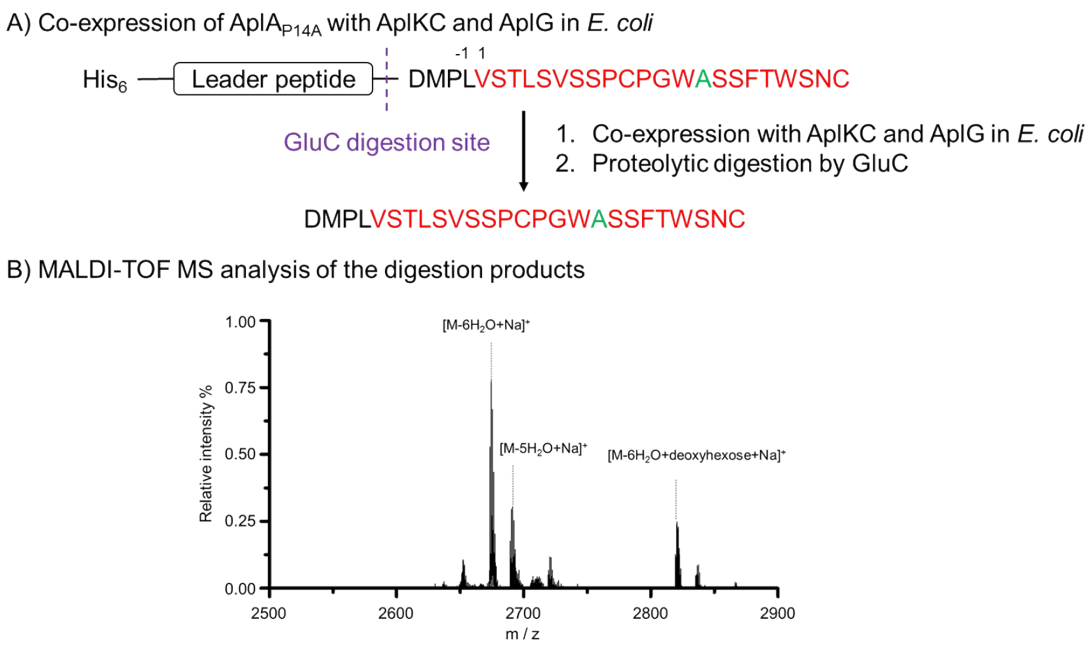


Figure S18. Co-expression of AplA$_{P14A}$, AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{P14A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{P14A}$ and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{P14A}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+Na]$^+$, calc. 2673.1243, obs. 2673.4000; [M-5H$_2$O+Na]$^+$, calc. 2691.1349, obs. 2691.4020. [M-6H$_2$O+deoxyhexose+Na]$^+$, calc. 2819.1825, obs. 2819.4970.

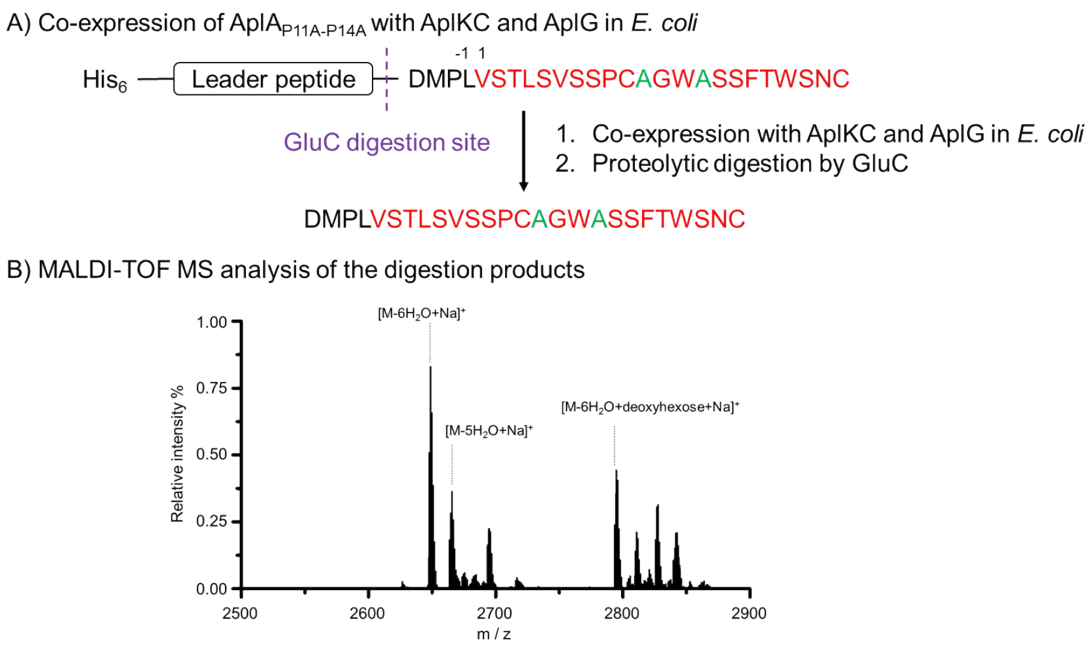


Figure S19. Co-expression of AplA$_{P11A-P14A}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{P11A-P14A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{P11A-P14A}$ and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{P11A-P14A}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+Na]$^+$, calc. 2647.1086, obs. 2647.7176; [M-5H$_2$O+Na]$^+$, calc. 2665.1192, obs. 2665.6950. [M-6H$_2$O+deoxyhexose+Na]$^+$, calc. 2793.1668, obs. 2693.8400.

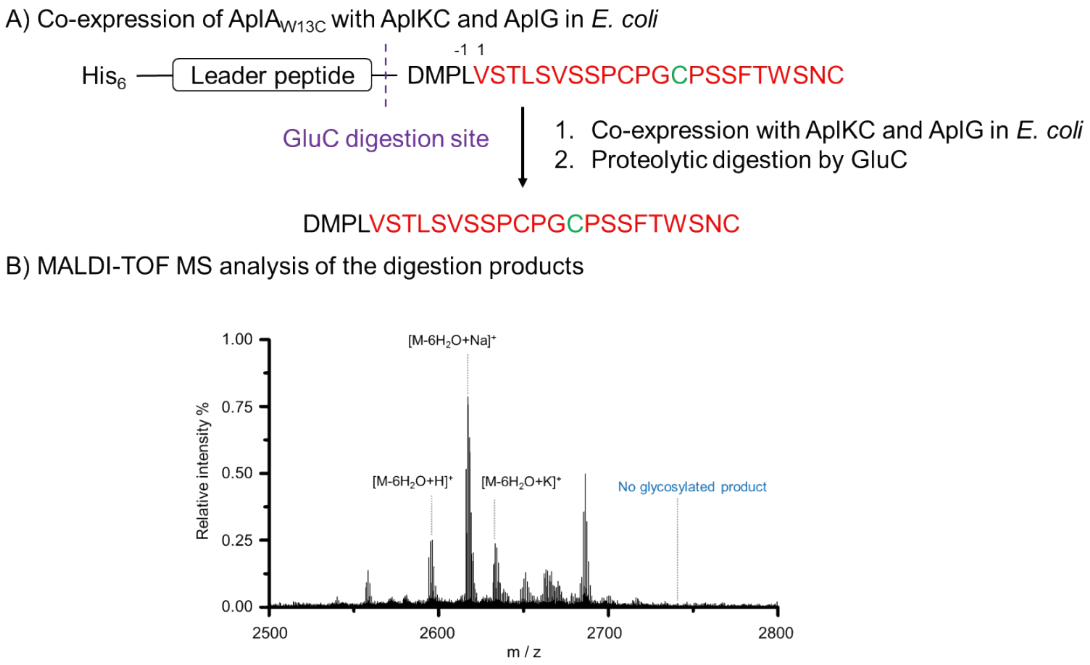


Figure S20. Co-expression of AplA$_{W13C}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{W13C}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA$_{W13C}$ and 1 μM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{W13C}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+H]$^+$, calc. 2594.0898, obs. 2594.2400; [M-6H$_2$O+Na]$^+$, calc. 2616.0796, obs. 2616.2440. [M-6H$_2$O+K]$^+$, calc. 2632.0536, obs. 2632.2130.

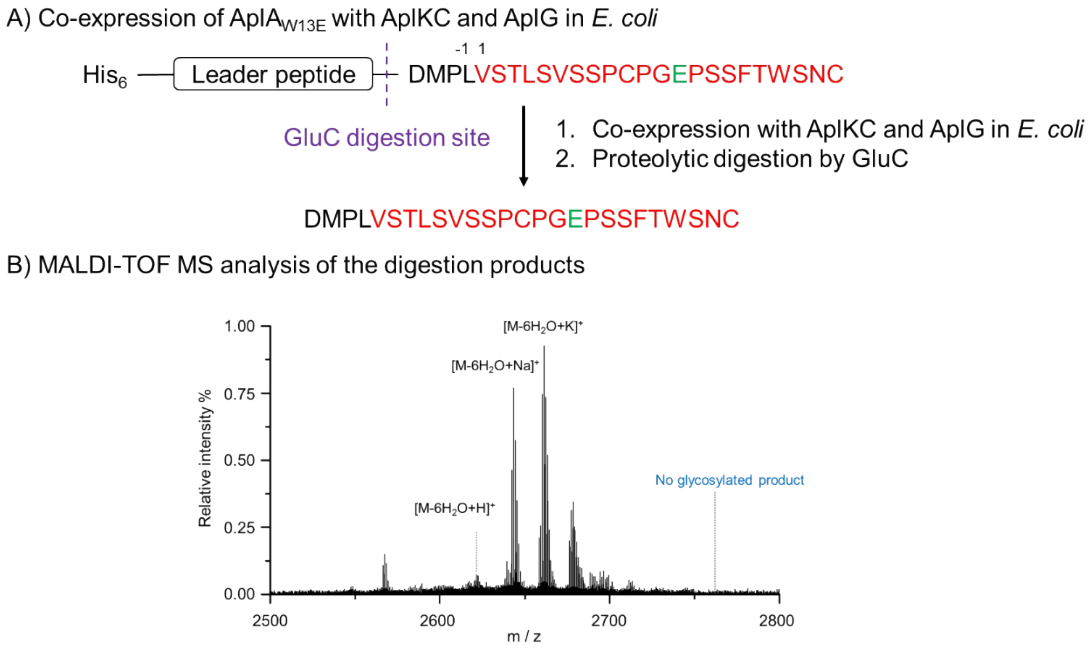


Figure S21. Co-expression of AplA$_{W13E}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{W13E}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{W13E}$ and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{W13E}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+H]$^+$, calc. 2620.1232, obs. 2620.4950; [M-6H$_2$O+Na]$^+$, calc. 2642.1130, obs. 2642.1080. [M-6H$_2$O+K]$^+$, calc. 2658.1130, obs. 2658.3410.

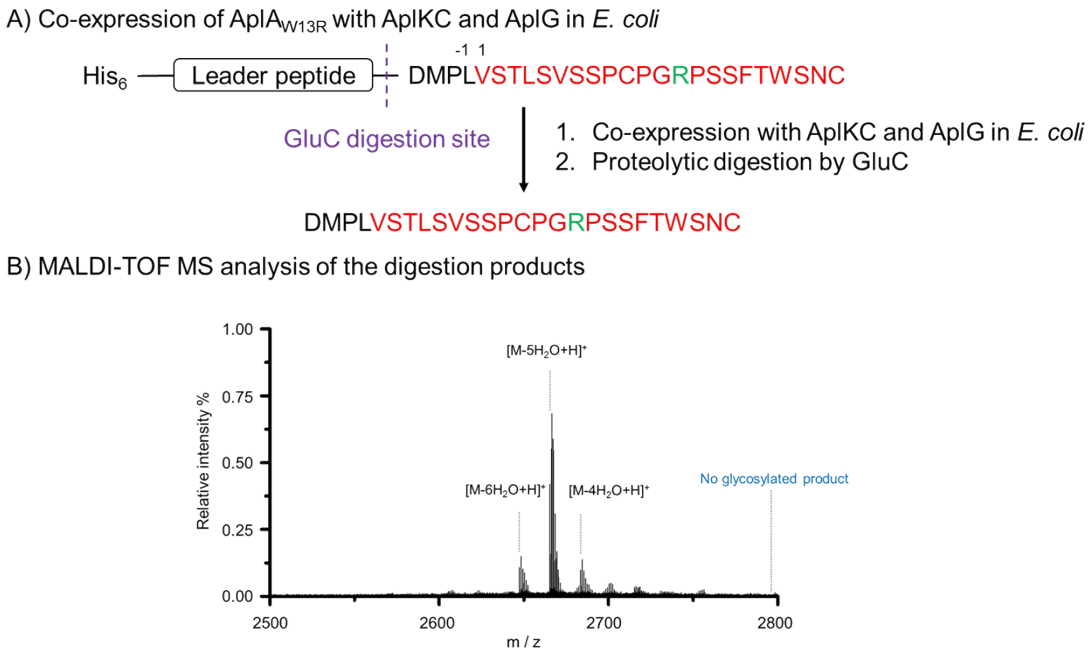


Figure S22. Co-expression of AplA$_{W13R}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{W13R}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{W13R}$ and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{W13R}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+H]$^+$, calc. 2647.1817, obs. 2647.4100; [M-5H$_2$O+H]$^+$, calc. 2665.1923, obs. 2665.4180. [M-4H$_2$O+H]$^+$, calc. 2683.2080, obs. 2683.4280.

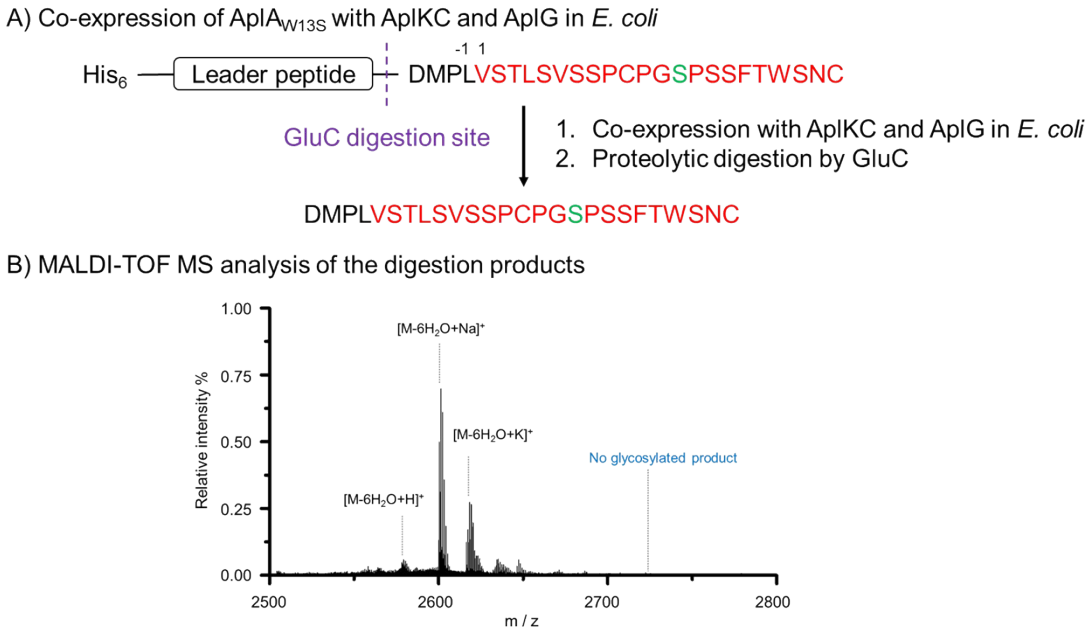
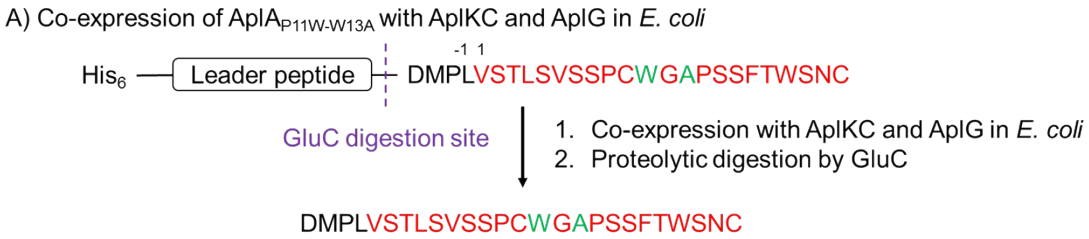
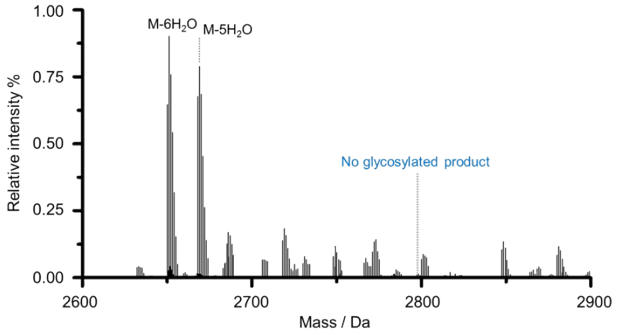


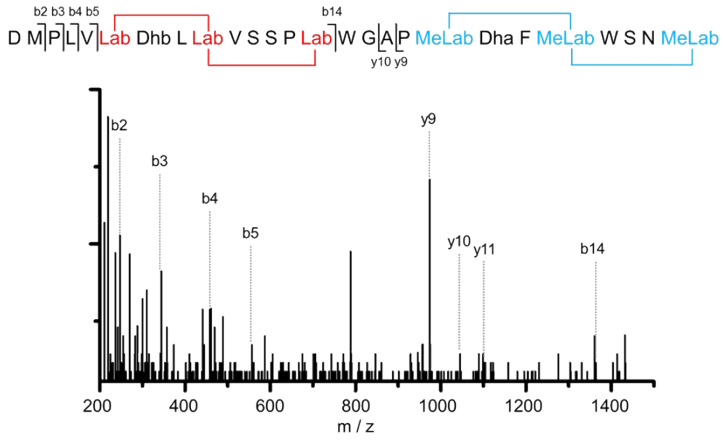
Figure S23. Co-expression of AplA$_{W13S}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{W13S}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{W13S}$ and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA$_{W13S}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O+H]$^+$, calc. 2578.1127, obs. 2578.2940; [M-6H$_2$O+Na]$^+$, calc. 2600.1025, obs. 2600.3460. [M-6H$_2$O+K]$^+$, calc. 2616.1025, obs. 2616.2910.



B) LC-MS analysis of the digestion products



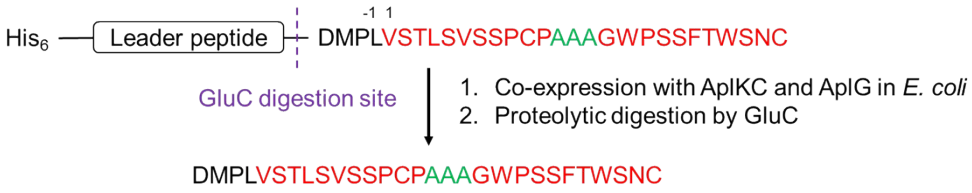
C) LC-MS/MS analysis of the [M-6H$_2$O] segment



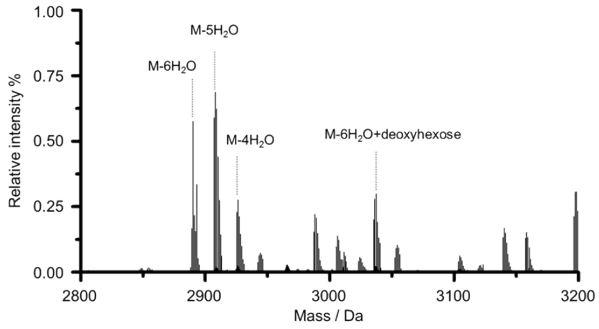
Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07497	0.9	b14	1363.6385	1363.6410	1.8
b3	344.12751	344.12780	0.8	y9	974.38257	974.37663	6.1
b4	457.21158	457.21326	3.7	y10	1045.4197	1045.4237	3.8
b5	556.27999	556.28217	3.9				

Figure S24. Co-expression of AplA$_{P11W-W13A}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{P11W-W13A}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{P11W-W13A}$ and 1 µM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{P11W-W13A}$ after GluC digestion. Deconvoluted mass of two derivatives: [M-6H$_2$O], calc. 2650.1443, obs. 2650.1402; [M-5H$_2$O], calc. 2668.1549, obs. 2668.1508. C) LC-MS/MS analysis of the [M-6H$_2$O] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the peptide is shown on top.

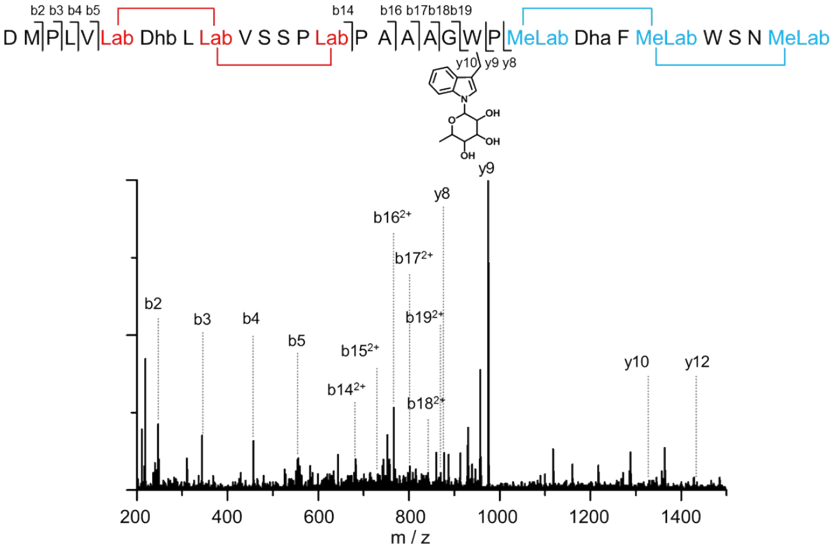
A) Co-expression of AplA$_{P11insAAA}$ with AplKC and AplG in *E. coli*



B) LC-MS analysis of the digestion products

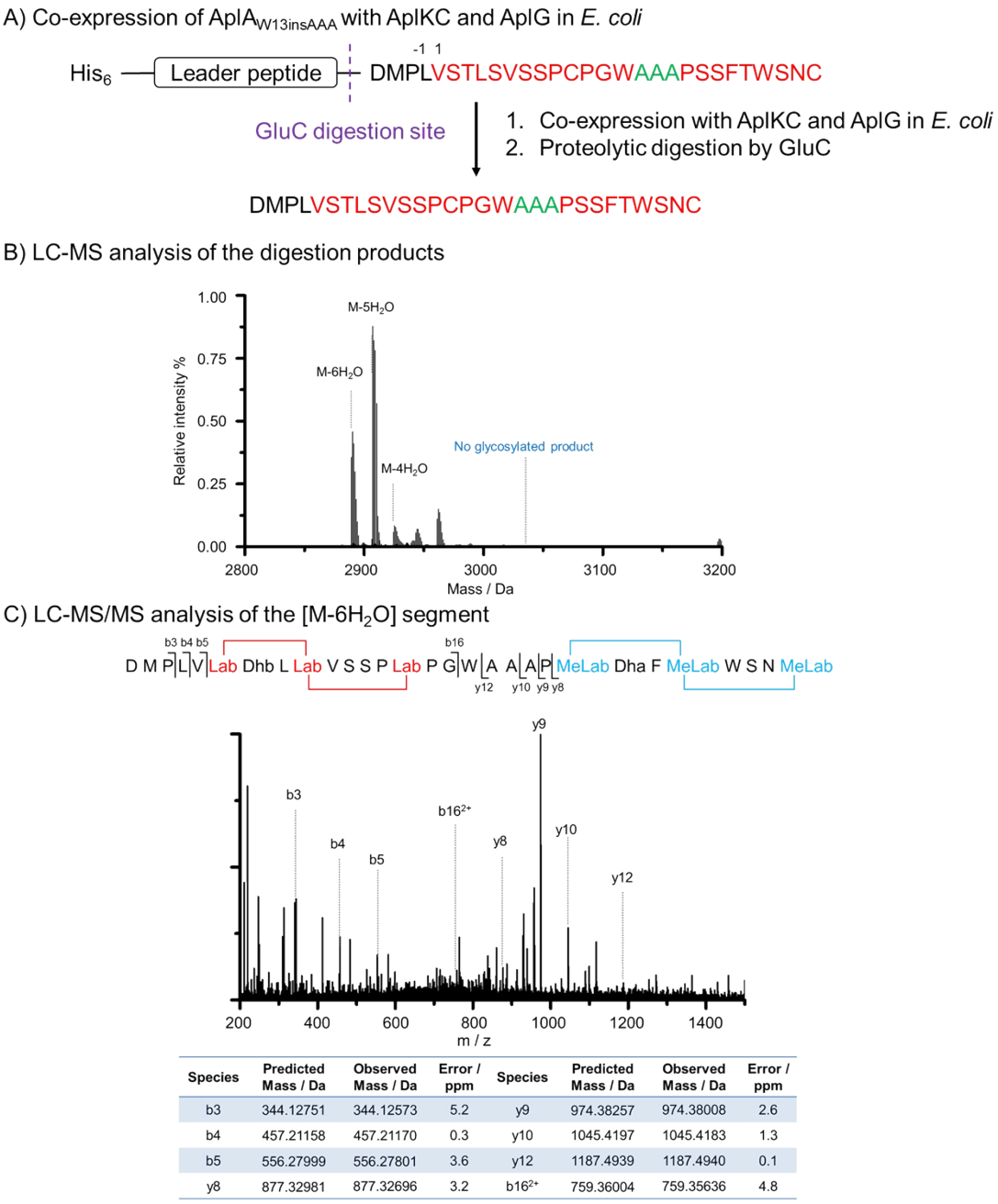


C) LC-MS/MS analysis of the [M-6H$_2$O+deoxyhexose] segment



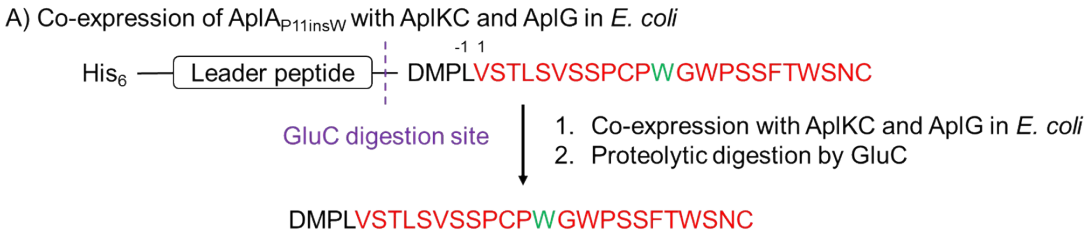
Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07450	1.0	b14^{2+}	682.32292	682.32268	0.4
b3	344.12751	344.12757	0.2	b16^{2+}	766.36786	766.37090	4.0
b5	556.27999	556.27865	2.4	b17^{2+}	801.88642	801.88732	1.1
y8	877.32981	877.33054	0.8	b18^{2+}	837.40498	837.40511	0.2
y9	974.38257	974.38820	5.8	b19^{2+}	865.91571	865.91067	5.8
y10	1306.5198	1306.5170	2.1				

Figure S25. Co-expression of AplA$_{P11insAAA}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{P11insAAA}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA$_{P11insAAA}$ and 1 µM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{P11insAAA}$ after GluC digestion. Deconvoluted mass of four derivatives: [M-6H$_2$O], calc. 2889.2713, obs. 2889.2878; [M-5H$_2$O], calc. 2907.2819, obs. 2907.3003; [M-4H$_2$O], calc. 2925.2925, obs. 2925.3103; [M-6H$_2$O+deoxyhexose], calc. 3035.3293, obs. 3035.3465. C) LC-MS/MS analysis of the [M-6H$_2$O+deoxyhexose] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the peptide is shown on top.

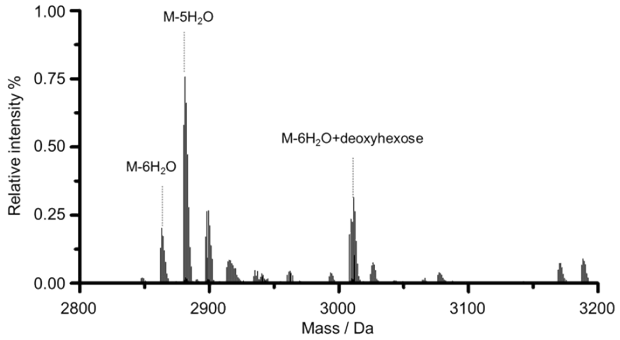


Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b3	344.12751	344.12573	5.2	y9	974.38257	974.38008	2.6
b4	457.21158	457.21170	0.3	y10	1045.4197	1045.4183	1.3
b5	556.27999	556.27801	3.6	y12	1187.4939	1187.4940	0.1
y8	877.32981	877.32696	3.2	b16^{2+}	759.36004	759.35636	4.8

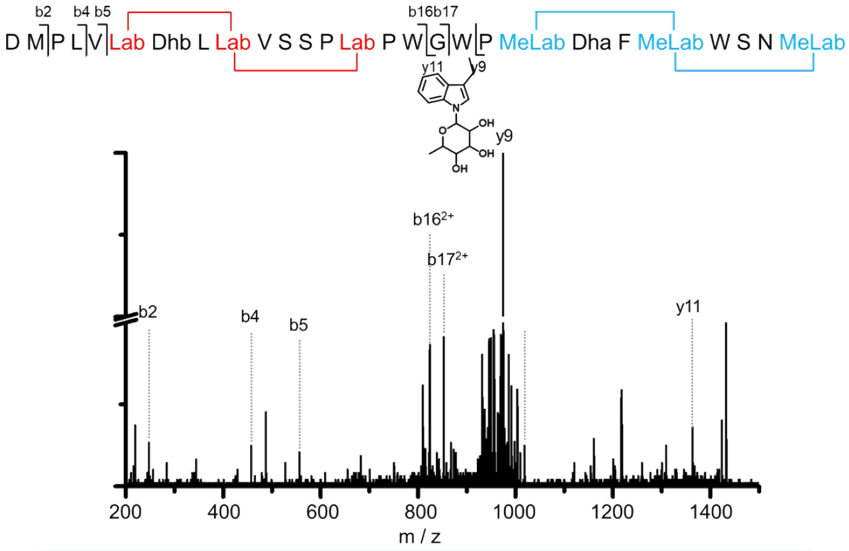
Figure S26. Co-expression of AplA$_{W13insAAA}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{W13insAAA}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA$_{W13insAAA}$ and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{W13insAAA}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O], calc. 2889.2713, obs. 2889.2824; [M-5H$_2$O], calc. 2907.2819, obs. 2907.3031; [M-4H$_2$O], calc. 2925.2925, obs. 2925.3146. C) LC-MS/MS analysis of the [M-6H$_2$O] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the peptide is shown on top.



B) LC-MS analysis of the digestion products



C) LC-MS/MS analysis of the [M-6H$_2$O+deoxyhexose] segment



Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm	Species	Predicted Mass / Da	Observed Mass / Da	Error / ppm
b2	247.07475	247.07451	1.0	y11	1363.5413	1363.5415	0.1
b4	457.21158	457.21232	1.6	b16^{2+}	823.88896	823.89054	1.9
b5	556.27999	556.28235	4.2	b17^{2+}	852.39969	852.40423	5.3
y9	974.38257	974.38663	4.2				

Figure S27. Co-expression of AplA$_{P11insW}$ with AplKC and AplG in *E. coli*. A) His$_6$-tagged AplA$_{P11insW}$ was co-expressed with AplKC and AplG in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to LC-MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 μM AplA$_{P11insW}$ and 1 μM GluC at 37 °C for 12 h. B) LC-MS analysis of the AplKC, AplG modified AplA$_{P11insW}$ after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O], calc. 2862.2392, obs. 2862.2451; [M-5H$_2$O], calc. 2880.2498, obs. 2880.2545; [M-6H$_2$O+deoxyhexose], calc. 3008.2972, obs. 3008.3038. C) LC-MS/MS analysis of the [M-6H$_2$O+deoxyhexose] segment. The *b* and *y* ions are listed in the table and marked in the spectrum. The amino acid sequence of the peptide is shown on top.

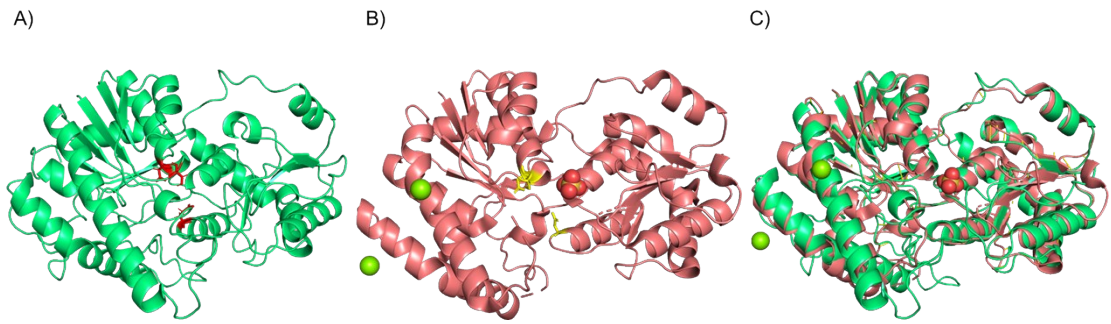


Figure S28. Predicted structure of AplG, crystal structure of GtfB and overlay of these two structures. A) Predicted structure of AplG using I-TASSER. Residues S10, D13 and E358 were marked in red. B) Crystal structure of GtfB (PDB:1IIR). Active site residues S10, D13 and D332 were marked in yellow. Green spheres are magnesium ion. Sulfate ion is in the center of the protein. C) Structural overlay of AplG and GtfB.

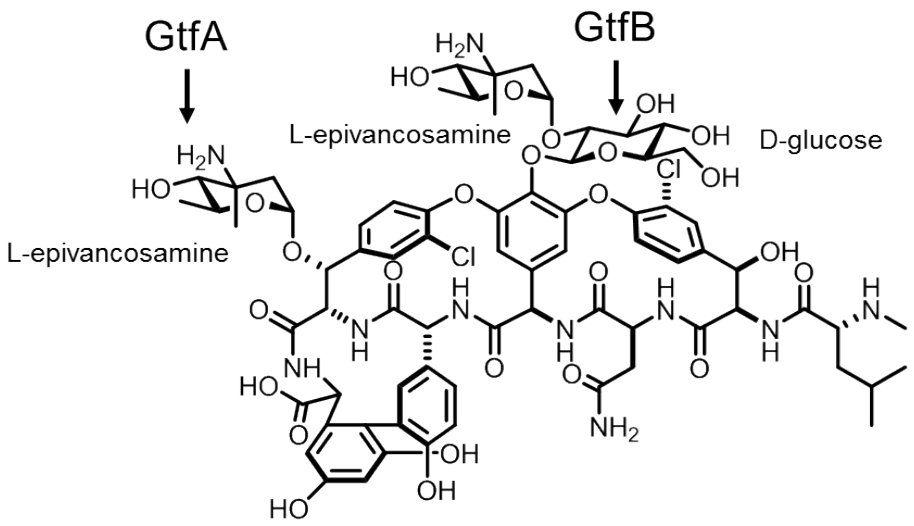


Figure S29. Structure of chloroeremomycin and the roles of GtfA and GtfB. GtfA catalyzes the addition of 4-*epi*-vancosamine to the desvancosaminyl vancomycin substrate. GtfB transfers the glucose moiety to the vancomycin aglycone acceptor.

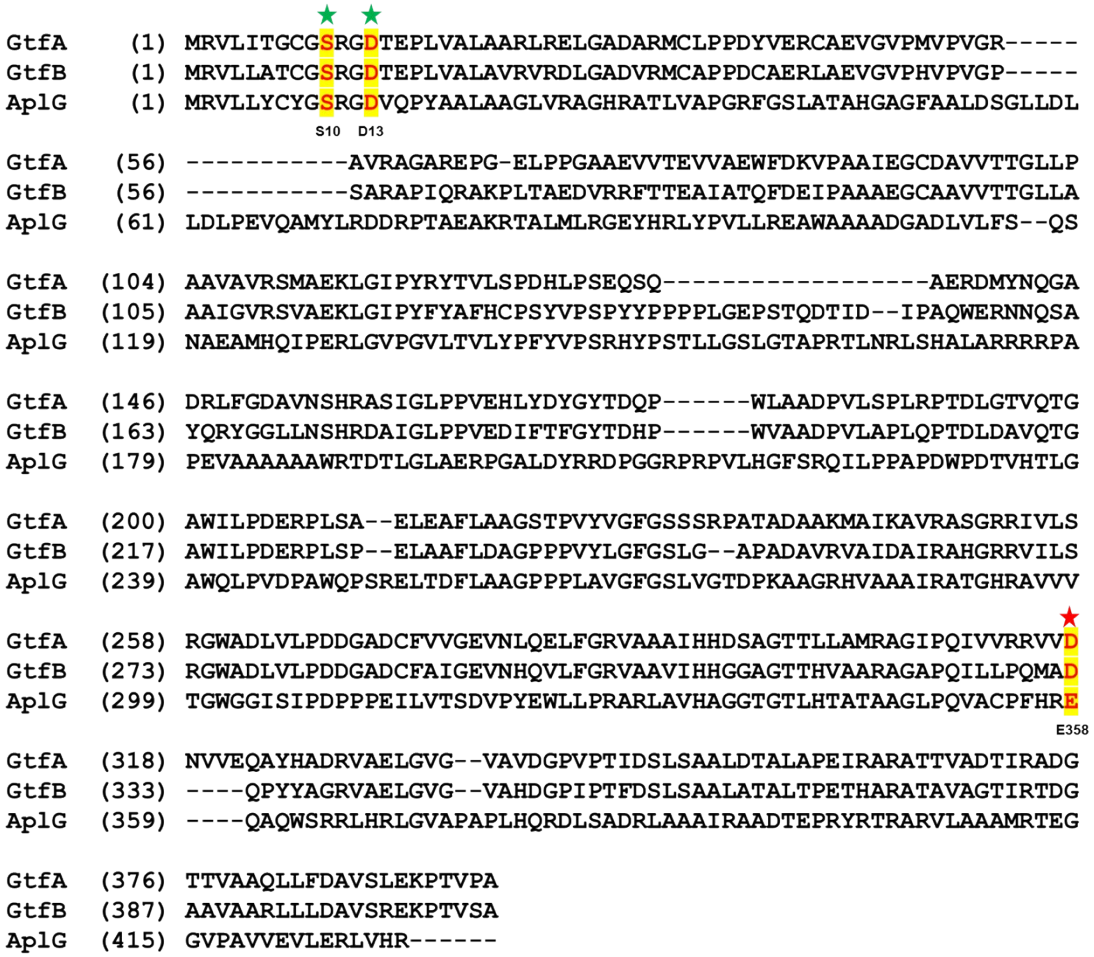


Figure S30. Sequence alignment of AplG with GtfA and GtfB. The accession numbers of protein in GenBank are listed as follows: AplG (AHB63595.1), GtfA (AAB49292.1), GtfB (AAB49293.1). Cyan star indicates the amino acid that may bind the reactive hydroxyl group of vancomycin aglycon substrate. Red star indicates the amino acids that may as a catalytic base.

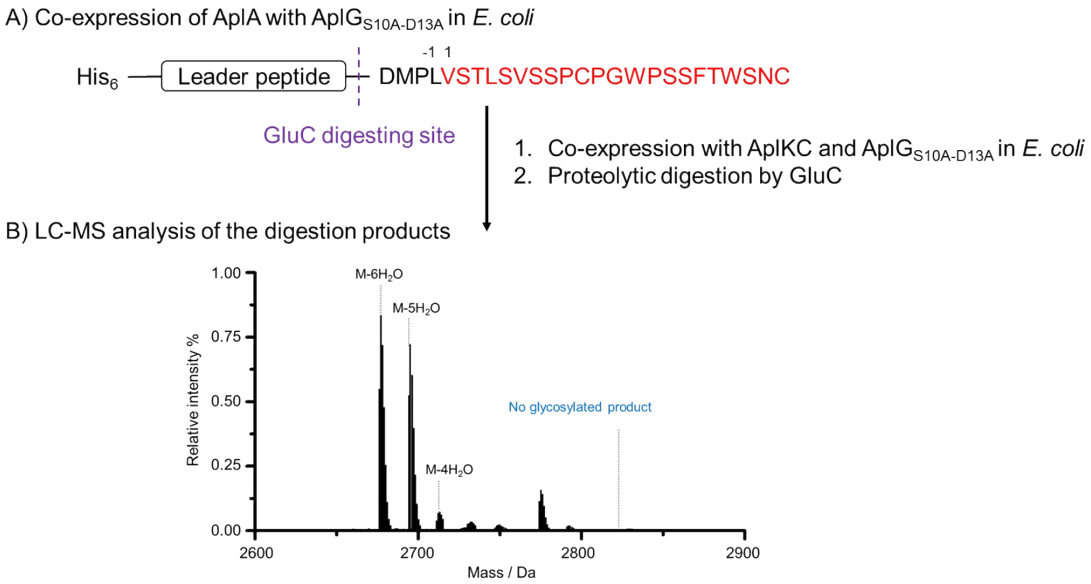


Figure S31. Co-expression of AplA with AplKC and AplG$_{S10A-D13A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed with AplKC and AplG$_{S10A-D13A}$ in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA and 1 µM GluC at 37 ºC for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O], calc. 2676.1672, obs. 2676.1530; [M-5H$_2$O], calc. 2694.1778, obs. 2694.1628. [M-4H$_2$O], calc. 2712.1884, obs. 2712.1594.

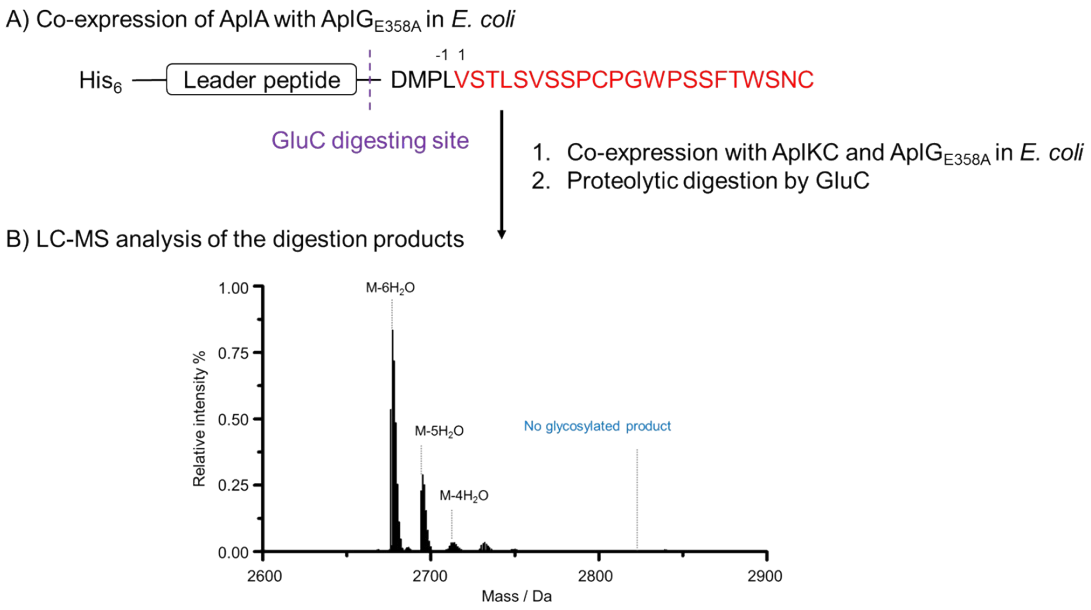


Figure S32. Co-expression of AplA with AplKC and AplG$_{E358A}$ in *E. coli*. A) His$_6$-tagged AplA was co-expressed with AplKC and AplG$_{E358A}$ in *E. coli*, purified and digested by GluC. The reaction mixture was subjected to MALDI-TOF MS analysis. Reaction conditions: 50 mM Tris HCl, pH 8.0, 100 µM AplA and 1 µM GluC at 37 °C for 12 h. B) MALDI-TOF MS analysis of the AplKC modified AplA after GluC digestion. Deconvoluted mass of three derivatives: [M-6H$_2$O], calc. 2676.1672, obs. 2676.1655; [M-5H$_2$O], calc. 2694.1778, obs. 2694.1726. [M-4H$_2$O], calc. 2712.1884, obs. 2712.1658.