Incorporation of non-proteinogenic amino acid incorporation in class I and II lantibiotics

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Supporting Information

Materials

All oligonucleotides, restriction endonucleases, and DNA polymerases were purchased from Integrated DNA Technologies, 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. Non-canonical amino acids were purchased from Chem Impex International. Solvents commonly used in peptide purification, including trifluoroacetic acid (TFA) and acetonitrile (MeCN), were obtained in RP-HPLC grade or better and used directly without further purification. Endoproteinase LysN and trypsin were purchased from Roche Biosciences or Worthington Biosciences. *E. coli* DH5α was used as host for cloning and plasmid isolation, and *E. coli* BL21 (DE3) or genomically recoded organism (GRO) C321.ΔprfA-T7RNAPΔrneΔompTΔlon[1] were used as a host for co-expression.

General methods

All polymerase chain reactions (PCR) were carried out on a C1000™ thermal cycler (Bio-Rad). DNA sequencing was carried out by ACGT Inc. using appropriate primers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics) at Mass Spectrometry Facility (UIUC).

Construction of pRSFDuet-1/*nisA mutants*+*nisB* and pCDFDuet-1/*nisC/pylT*

The amber codon TAG was introduced into nisA/nisB/pRSFDuet-1 at different positions of *nisA*, generating co-expression vectors encoding NisA-T2B, NisA-I4V/S5B/L6G, and NisA-M21B, where B stands for the amber stop codon. The mutants were generated using QuikChange PCR with the *nisA/nisB/pRSFDuet-1* vector as the template.[2] The primer sequences are listed in Table S1. The PCR mixture included 1× Phusion HF buffer (New England BioLabs), dNTP mixture (2 mM each), primers (stock solution 100 μM each, final concentration 1 μM each), DMSO (2%, v/v), template plasmid *nisA/nisB/pRSFDuet-1* (~ 40 ng) and Phusion High-Fidelity DNA Polymerase (0.02 U/μL). The PCR amplification was performed by twenty five cycles of denaturing (98 °C for 10 s), annealing (57.5 °C for 30 s), and extending (72 °C for 150 s). The product pNisA1.x (Table S3) was purified by QIA quick PCR Purification Kit (QIAGEN). The purified PCR product was mixed with 2 μL of 10x NEBuffer 4

and 2 μL of DpnI (stock solution 20,000 U/mL, New England Biolabs), followed by incubation at 37 °C for 1 h. DpnI was heat-inactivated by incubation at 80 °C for 20 min. A 5 μL aliquot of the resulting solution was used for heat shock transformation of *E. coli* DH5α.

One copy of *nisC* was inserted between *Bgl*II and *Xho*I restriction sites into the MCS2 of *pCDFDuet-1* via PCR amplification, digestion, and ligation using *nisC/pACYCDuet-1*[2] as the PCR template. In order to raise the copy number of MmPylT expressed in the host cells, one copy of Mmpyrrolysyl-tRNA (pylT) was introduced between *Bam*HI and *Not*I restriction sites into the MCS1 of the constructed *nisC*/*pCDFDuet-1* using the the *pEVOL-pylT-mmPylRS(2X)-N346A/C348A* vector as the PCR template, resulting in *pNisTC* (Table S3). The primer sequences are listed in Table S1.

A copy of *nisB* with *E. coli*-optimized codons was inserted into MCS2 of *pNisTC* after the *nisC* gene separated by ribosome binding site (rbs) via PCR amplification using appropriate primers and then Gibson ligated using the vendor's protocol to arrive at *pNisTCB* plasmid. An additional copy of *nisB* was inserted in a similar fashion after the first copy of *nisB* gene separated by a rbs to produce *pNisTCBB*.

Table S1. Sequences of oligonucleotide primers used to prepare analogs of nisin.

Primer Name	Primer Sequence (5'-3')
proK_BamHI_FP	ATA ATA TGG GAT CCG TGT GCT TCT CAA ATG CCT GAG GCC
tRNA_NotI_RP	ATA ATA TGG CGG CCG CCA TGC AAA AAA GCC TGC TCG TTG
NisA_T2B_FP	CAG GTG CAT CAC CAC GCA TTT AGA GTA TTT CGC TAT G
NisA_T2B_QRP	GCG TGG TGA TGC ACC TGA ATC TTT CTT CG
NisA_M21B_FP	GAG CTC TGA TGG GTT GTA ACT AGA AAA CAG CAA CTT G
NisA_M21B_QRP	GTT ACA ACC CAT CAG AGC TCC TGT TTT ACA ACC
NisC_T7_PacI_FP	TCC AGG CAT TAA TTA ACG AAA TTA ATA CGA CTC ACT ATA GGG G
NisC_AvrII_RP	ATA ATA TGC CTA GGT CAT TTC CTC TTC CCT CCT TTC
NisC/RBS/optNisB/Fgib	GAT TTT TTG AAA GGA GGG AAG AGG AAA TGA AGG AGA TAT ACC ATG ATC AAA AGC TCC TTT AAA GCA CAG
optNisB/PacI/pCDFDuet/Rgib	GCG GTG GCA GCA GCC TAG GTT AAT TAA TTA TTT CAT ATA TTC TTC GCT CAC AAA CAG ACG

NisBend/RBS/NisB/Fgib	TTC GGA AGA ATA CAT GAA ATG AAG GAG ATA TAC CAT GAT AAA AAG TTC ATT TAA AGC TCA ACC G
pCDF/PacI/NisB/Rgib	GCG GTG GCA GCA GCC TAG GTT AAT TAA TCA TTT CAT GTA TTC TTC CGA AAC AAA C

Construction of pRSFDuet-1/*lctA mutants+lctM+pylT*

The amber codon TAG was introduced into *histagged-lctA(wt)/lctM/pylT/*pRSFDuet-1 at different positions of *lctA*, generating co-expression vectors encoding LctA-W19B, LctA-F21B, and LctA-F23B, where B stands for the amino acid to be introduced via the amber stop codon. The mutants were generated using QuikChange PCR with the *histagged-lctA(wt)/lctM/pylT/*pRSFDuet-1[3] vector as the template. The primer sequences are listed in Table S2. To improve solubility of these peptides,[4] the N15R mutation was introduced via QuikChange PCR.

Table S2. Oligonucleotide sequences of primers used for preparing analogs of lacticin 481.

Primer Name	Primer Sequence (5'-3')
LctA_Trp19Stop_N15R_FP	CA ATT TCT CAT GAA TGT CGC ATG AAT AGC TAG CAA TTT G
LctA_Trp19Stop_N15R_RP	CAA ATT GCT AGC TAT TCA TGC GAC ATT CAT GAG AAA TTG
LctA_F21Stop_FP*	ATG AAT AGC TGG CAA TAG GTA TTT ACT TGC TGC
LctA_F21Stop_RP*	GCA GCA AGT AAA TAC CTA TTG CCA GCT ATT C
LctA_Phe23Stop_FP*	AGC TGG CAA TTT GTA TAG ACT TGC TGC TC
LctA_Phe23Stop_RP*	GAG CAG CAA GTC TAT ACA AAT TGC CAG C
LctA_N15R_FP_new	CA ATT TCT CAT GAA TGT CGC ATG AAT AGC TGG C
LctA_N15R_RP_new	GCC AGC TAT TCA TGC GAC ATT CAT GAG AAA TTG

Heterologous production, purification, and characterization of lacticin 481 analogs

The lacticin 481 analogs were expressed using *E. coli* BL21 (DE3) cells transformed with pLctAMx, and pMmPyl (Table S3). An overnight culture of these cells was added to a culture flask containing synthetic media[5] lacking Phe, and containing kanamycin (25 mg/mL) and chloramphenicol (12.5 mg/mL). The culture was then incubated in a 37 ºC shaker (220 rpm) until the optical density reached 1.0. After cooling at 4 ºC for 15 min, overexpression was induced with arabinose (0.2% final concentration) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.25 mM final concentration) in the

presence of non-proteinogenic acid (2 mM final concentration; dissolved using appropriate amount of 1 M NaOH right before use), and the flask was incubated for 12 h in a shaker at 37 ºC and 220 rpm. The following morning the cells were harvested by centrifugation (11,867 ×*g*, 4 ºC, 20 min), the supernatant was discarded and the cells were lysed using a cell homogenizer (Avestin Emulsiflex-C3; 5,000 PSI) in LanA start buffer (2 mL per 100 mL culture; 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol, pH 7.5). The soluble and insoluble fractions were then separated by centrifugation (22,789 ×*g*, 4 °C, 20 min) and the soluble fraction was saved for purification. The insoluble fraction was suspended in LanA lysis buffer (1 mL per 100 mL culture; 6 M guanidine-HCl, 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, pH 7.5) and the suspension was sonicated. The soluble and insoluble fractions were again separated by centrifugation (22,789 ×*g*, 4 °C, 20 min) and the modified His_6-LanA peptide was purified from the combined soluble layers using a Ni HisTrap HP column (5 mL, GE Healthcare). The column was washed with LanA wash buffer (4 M guanidine-HCl, 20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole, pH 7.5) to remove nonspecific binding proteins. The His_6-LanA peptide was eluted with LanA elute buffer (3 × 5 mL, 4 M guanidine-HCl, 20 mM NaH_2PO_4, 100 mM NaCl, 500 mM imidazole, pH 7.5). Finally, the peptide solution was desalted by C4 SPE column (Grace Vydac).

Proteolysis of full-length His-tagged modified LctA mutants, and purification of lacticin 481 analogs After solid phase extraction, the full-length peptides were purified further on a C5 Phenomenex column in a Shimadzu Prep-HPLC Instrument and were observed to elute in a time range of 22-25 min when the gradient was set from 2% solvent B to 100%A (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water) over a time period of 45.0 min at a flow rate of 7.0 mL /min. Lacticin 481 analogues were obtained via the proteolytic removal of the leader peptide using the endoproteinase LysN. The full-length peptides were dissolved in water, and incubated in 50 mM Tris buffer (pH 8.0), followed by the addition of LysN at a concentration of 1:100 (w/w) with respect to the peptide and incubated overnight at room temperature. Extent of cleavage was monitored using MALDI-TOF MS. The peptides were then purified using a C18-Phenomenex column in a Agilent Analytical HPLC Instrument and were observed to elute in a time range of 23-26 min when the gradient was set from 2% solvent B to 100%A (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water) over a time period of 45.0 min at a flow rate of 1.0 mL /min.

Heterologous production, purification, and characterization of nisin analogues using *E. coli* BL21 (DE3) The nisin analogs were expressed using *E. coli* BL21 (DE3) cells transformed with pNisABx, pMmPyl, and either pNisTC or pNisTCB or pNisTCBB (Table S3).

An overnight culture of *E. coli* BL21 (DE3) cells was added to a culture flask containing synthetic media[5] lacking Phe, and containing kanamycin (17 mg/mL), chloramphenicol (10 mg/mL) and

spectinomycin (17 mg/mL). The culture was then incubated in a 37 ºC shaker (220 rpm) until the optical density reached 1.0. After cooling at 4 ºC for 15 min, overexpression was induced with arabinose (0.2% final concentration) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.25 mM final concentration) and the flask was incubated for 16 h in a shaker at 18 ºC and 220 rpm. The following morning the cells were harvested by centrifugation (11,867 ×*g*, 4 ºC, 20 min), the supernatant was discarded, and the cells were lysed using a cell homogenizer (Avestin Emulsiflex-C3; 5,000 PSI) in LanA start buffer (2 mL per 100 mL culture; 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol, pH 7.5). The soluble and insoluble layers were then separated by centrifugation (22,789 ×*g*, 4 °C, 20 min) and the soluble layer was saved for purification. The insoluble layer was suspended in LanA lysis buffer (1 mL per 100 mL culture; 6 M guanidine-HCl, 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, pH 7.5) and the suspension was sonicated. The soluble and insoluble layers were again separated by centrifugation (22,789 ×*g*, 4 °C, 20 min) and the modified His_6-LanA peptide was purified from the combined soluble layers using a Ni HisTrap HP column (5 mL, GE Healthcare). The column was washed with LanA wash buffer (4 M guanidine-HCl, 20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole, pH 7.5) to remove nonspecific binding proteins. The His_6-LanA peptide was eluted with LanA elute buffer (3 × 5 mL, 4 M guanidine-HCl, 20 mM NaH_2PO_4, 100 mM NaCl, 500 mM imidazole, pH 7.5). Finally, the peptide solution was desalted with a C4 SPE column (Grace Vydac).

Table S3. List of plasmids used.

Plasmid	**Marker**	**Insert/Mutation**	**Site**	**Reference**
phistagged-nisA/nisB/pRSFDuet-1	Kanamycin	His-tagged-*nisA (wt)* and *nisB* genes	*nisA* inserted between *Bam*HI and *Hind*III; *nisB* inserted between *Nde*I and *Xho*I	[2]
pNisAB1 derivative of *phistagged-nisA/nisB/pRSFDuet-1*	Kanamycin	*His-tagged-nisA-T2B* (B = amber stop codon) and *nisB* genes		This work
pNisAB2 derivative of *phistagged-nisA/nisB/pRSFDuet-1*	Kanamycin	*His-tagged-nisA*-I4V/S5B/L6G (B = amber stop codon) and *nisB* genes		This work
pNisAB3	Kanamycin	*Histagged-nisA*-M21B (B = amber		This work

derivative of *phistagged-nisA/nisB/pRSFDuet-1*		stop codon) and *nisB* genes		
pNisC/pCDFDuet-1	Spectinomycin	*nisC*	*Bgl*II and *Xho*I (MCS2)	This work
pNisTC	Spectinomycin	*nisC* and *mmPylT tRNA* in pCDFDuet-1	*Bam*HI and *Not*I (MCS1) in *pNisC/pCDFDuet-1*	This work
pNisTCB	Spectinomycin	*nisB, nisC* and *mmPylT tRNA* in pCDFDuet-1	*rbs-nisB* gene inserted right after the *nisC* gene in *pNisTC*	This work
pNisTCBB	Spectinomycin	*nisB (2X), nisC* and *mmPylT tRNA* in pCDFDuet-1	Additional copy of *rbs-nisB* inserted right after the *nisB* gene in *pNisTCB*	This work
pMmPyl	Chloramphenicol	*pylT-mmPylRS(2X)-N346A/C348A* in pEVOL	Sequential insertion of two copies of N346A/C348A *pylT* between *Spe*I and *Sal*I; *pylT* gene inserted between *Apa*LI and *Xho*I	[6]
phistagged-lctA(wt)/lctM/pylT/pRSFDuet-1	Kanamycin	*Histagged-lctA(wt), lctM and pylT*	*lctA(wt)* inserted between *Eco*RI and *Not*I*; lctM* inserted between *Bgl*II *and Xho*I*; pylT* inserted between *Age*I and *Drd*I	[3]

pLctAM1 derivative of *phistagged-lctA(wt)/lctM/pylT/*pRSFDuet-1	Kanamycin	*Histagged-lctA-N15R/W19B* (B = amber stop codon), *lctM* and *pylT*		This work
pLctAM2 derivative of *phistagged-lctA(wt)/lctM/pylT/*pRSFDuet-1	Kanamycin	*Histagged-lctA-N15R/F21B* (B = amber stop codon), *lctM* and *pylT*		This work
pLctAM3 derivative of *phistagged-lctA(wt)/lctM/pylT/*pRSFDuet-1	Kanamycin	*Histagged-lctA-N15R/F23B* (B = amber stop codon), *lctM* and *pylT*		This work

Heterologous production, purification, and characterization of nisin analogues using *GRO*

The nisin analogues were expressed using GRO transformed with with pNisABx, pMmPyl and pNisTC (Table S3). This strain was always maintained at temperatures below 32 ºC (mostly 30 ºC) to avoid triggering lambda-red mediated mutagenesis.[1, 7]

An overnight culture of *E. coli* BL21 (DE3) cells was added to a culture flask containing LB media containing kanamycin (17 mg/mL), chloramphenicol (10 mg/mL) and spectinomycin (17 mg/mL). The culture was then incubated in a 30 ºC shaker (220 rpm) until the optical density reached 0.8. After cooling at 4 ºC for 15 min, overexpression was induced with arabinose (0.2% final concentration) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM final concentration) in the presence of non-proteinogenic acid (4 mM final concentration; dissolved using an appropriate amount of 1 M NaOH right before use) and the flask was incubated for 20 h in a shaker at 18 ºC and 220 rpm. The following morning the cells were harvested by centrifugation (11,867 ×*g*, 4 ºC, 20 min), the supernatant was discarded and the cells were lysed using a cell homogenizer (Avestin Emulsiflex-C3; 5,000 PSI) in LanA start buffer (2 mL per 100 mL culture; 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol, pH 7.5). The soluble and insoluble fractions were then separated by centrifugation (22,789 ×*g*, 4 °C, 20 min) and the soluble layer was saved for purification. The insoluble fraction was suspended in LanA lysis buffer (1 mL per 100 mL culture; 6 M guanidine-HCl, 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, pH 7.5) and the suspension was sonicated. The soluble and insoluble layers were again separated by centrifugation (22,789 ×*g*, 4 °C, 20 min) and the modified His_6-LanA peptide was purified from the combined soluble layers using a Ni HisTrap HP column (5 mL, GE Healthcare). The

column was washed with LanA wash buffer (4 M guanidine-HCl, 20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole, pH 7.5) to remove nonspecific binding proteins. The His_6-LanA peptide was eluted with LanA elute buffer (3 × 5 mL, 4 M guanidine-HCl, 20 mM NaH2PO4, 100 mM NaCl, 500 mM imidazole, pH 7.5). Finally, the peptide solution was desalted by using a C4 SPE column (Grace Vydac).

Proteolysis of full-length His-tagged NisA mutants, and purification of nisin analogues

After solid phase extraction, the full-length peptides were purified further on a C5-Phenomenex column using a Shimadzu Prep-HPLC Instrument and were observed to elute in a time range of 23-25 min when the gradient was set from 2% solvent B to 100%A (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water) over a time period of 45.0 min at a flow rate of 7.0 mL /min. Nisin analogues were obtained via the proteolytic removal of the leader peptide using the endoproteinase trypsin. The full-length peptides were dissolved in water, and incubated in 50 mM Tris buffer (pH 8.0), followed by the addition of trypsin at a concentration of 1:100 (w/w) with respect to the peptide and incubated for 3 h at room temperature. The extent of cleavage was monitored using MALDI-TOF MS. The peptides were then purified using a C18-Phenomenex column in a Agilent Analytical HPLC Instrument and were observed to elute in a time range of 22-24 min when the gradient was set from 2% solvent B to 100%A (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water) over a time period of 45.0 min at a flow rate of 1.0 mL /min.

Agar diffusion growth inhibition assay of lacticin 481, nisin and analogs

HPLC-purified peptides were dissolved in water, lyophilized and redissolved in water. Isolation of authentic lacticin 481 has been described previously.[3] For lacticin 481 bioactivity assays, overnight cultures of *L. lactis* HP were diluted in M-17 agar containing 0.5 % glucose to an OD_{600} of 0.05. A total of 10 μL of 12.5 μM solution was spotted. The plates were incubated at 30 ºC for 20 h.

For nisin bioactivity assays, overnight cultures of *L. lactis* HP were diluted in M-17 agar containing 0.5% glucose to an OD_{600} of 0.05. A total of 10 μL of 5 μM commercial, HPLC-purified nisin and analogues was spotted. The plates were incubated at 30 ºC for 18 h.

Determination of MIC and IC_{50} values against liquid cultures of *L. lactis* HP

Ninety-six well microtiter plates were used to determine MIC and IC_{50} values. A total volume of culture in each well was 100 μL; the experimental wells contained 15 μL of diluted peptide at defined concentrations and 85 μL of a culture of *L. lactis* HP indicator strain (OD_{600} = 0.9 – 1.0) in GM17 medium (M17 media with 2% glucose) diluted 1-to-10 in fresh liquid GM17 medium. The peptide concentrations ranged from 25 μM to 11.9 nM. Each plate contained two lanes for controls: 85 μL fresh GM17 medium and 15 μL sterile Millipore water, and 85 μL of untreated 1-in-10 diluted culture and 15 μL sterile Millipore water. The optical density at 600 nm (OD_{600}) was recorded at hourly intervals

from 0 to 5 h with an additional measurement at 18 h using a BioTek Synergy 2 plate reader. Plates were incubated at 30 ºC between readings. The MIC values reported are the concentrations where no growth was observed after 18 h.

Blanks (growth medium and sterile Millipore water only) were averaged and then subtracted from the averages of triplicate experimental readings. Growth curves vs. peptide concentration were developed, and curve fits for IC_{50} determination were produced by fitting data with OriginPro 8 software using the dose-response curve. IC_{50} values were calculated from this fit. The fitted curves are shown in Figure S12.

SUPPLEMENTARY FIGURES:

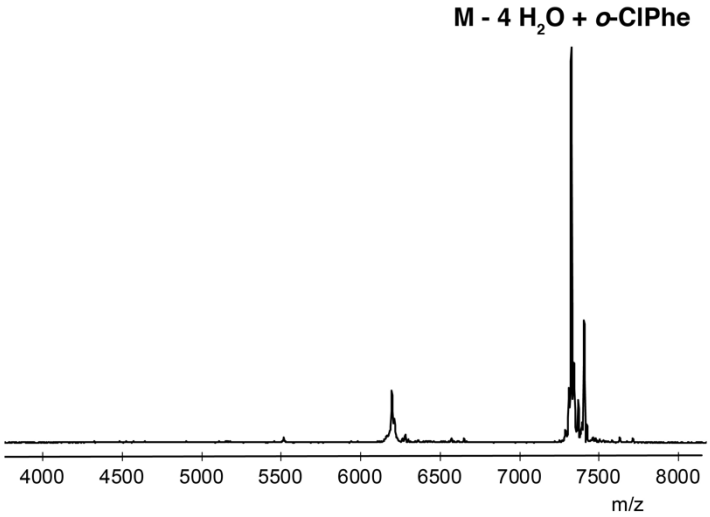


Figure S1. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/W19*o*-ClPhe modified by LctM in *E. coli* BL21(DE3).

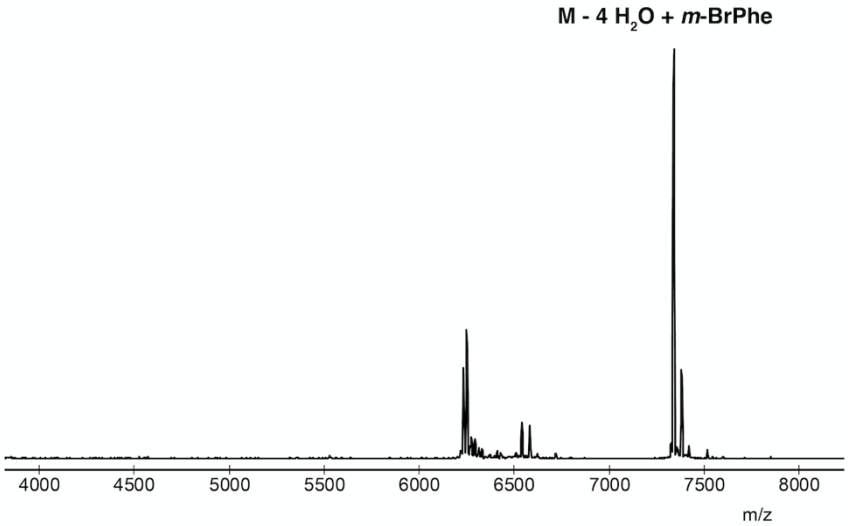


Figure S2. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/W19*m*-BrPhe, modified by LctM in *E. coli* BL21(DE3).

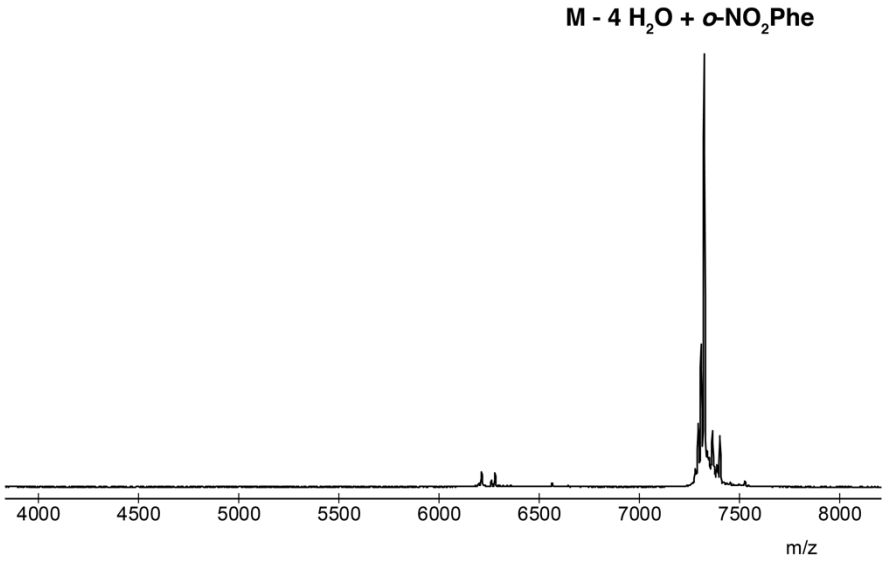


Figure S3. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/W19*o*-NO_2Phe, modified by LctM in *E. coli* BL21(DE3).

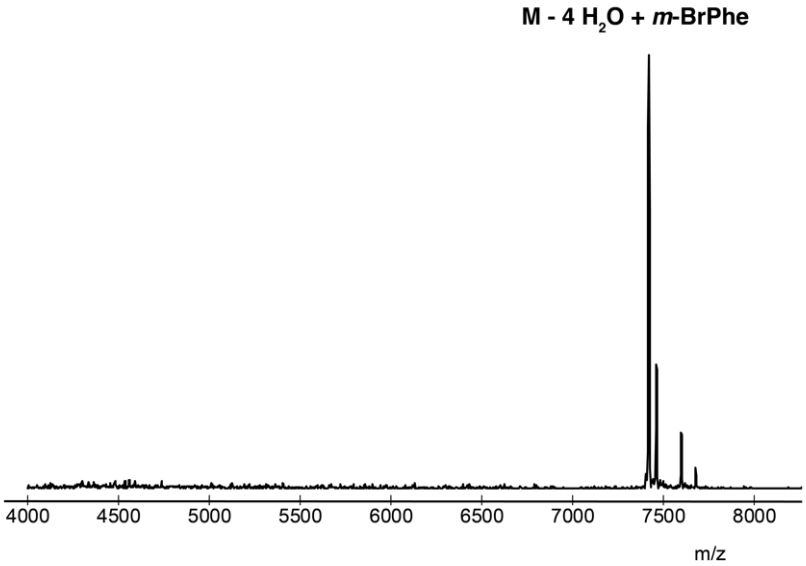


Figure S4. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F21*m*-BrPhe, modified by LctM in *E. coli* BL21(DE3).

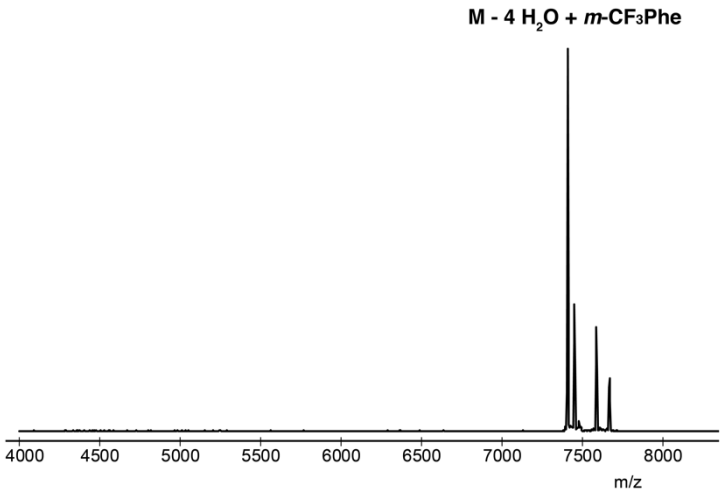


Figure S5. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F21*m*-CF_3Phe, modified by LctM in *E. coli* BL21(DE3).

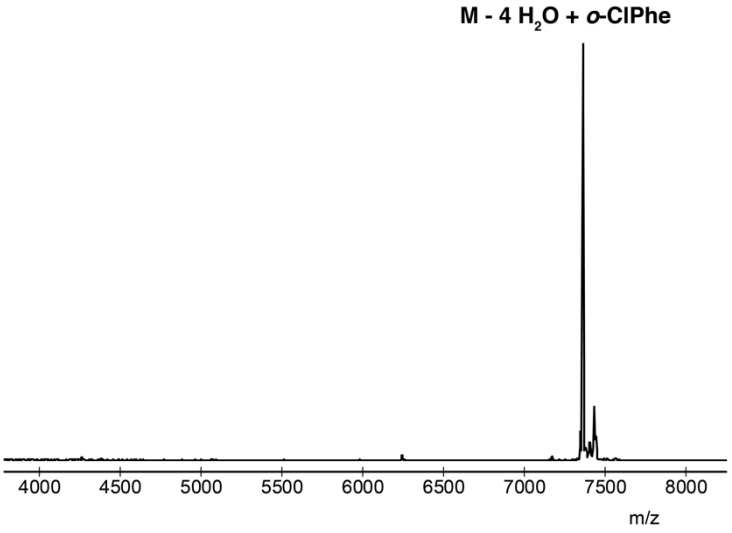


Figure S6. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F21*o*-ClPhe, modified by LctM in *E. coli* BL21(DE3).

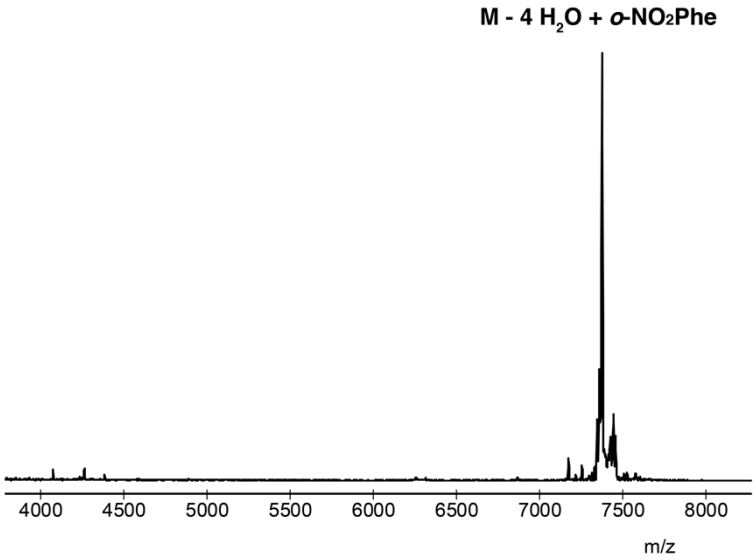


Figure S7. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F21*o*-NO_2Phe, modified by LctM in *E. coli* BL21(DE3).

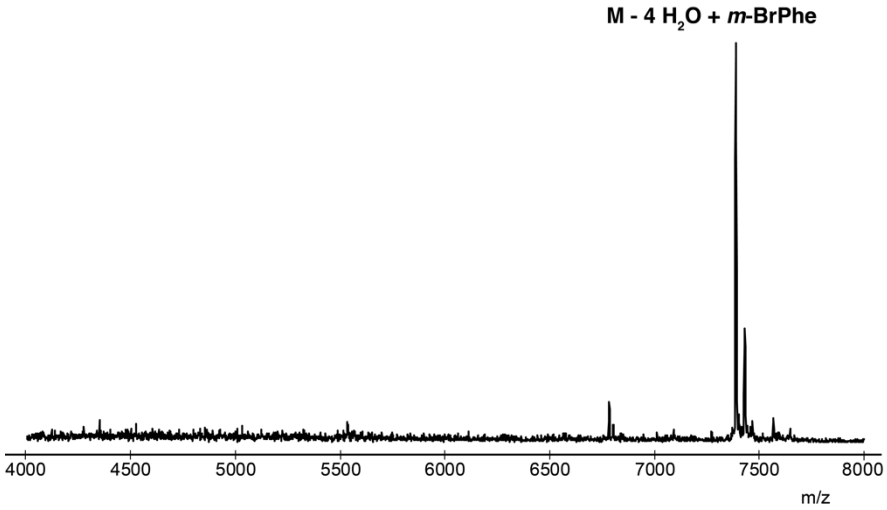


Figure S8. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F23*m*-BrPhe, modified by LctM in *E. coli* BL21(DE3).

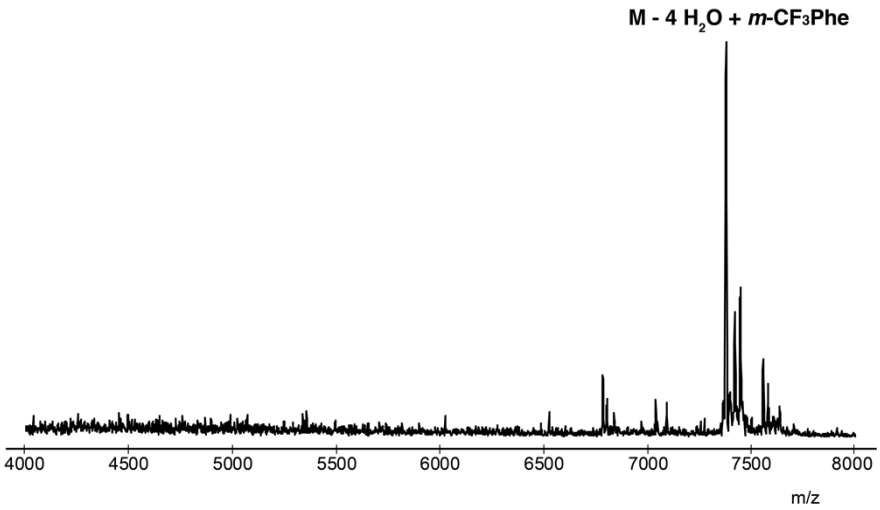


Figure S9. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F23*m*-CF_3Phe, modified by LctM in *E. coli* BL21(DE3).

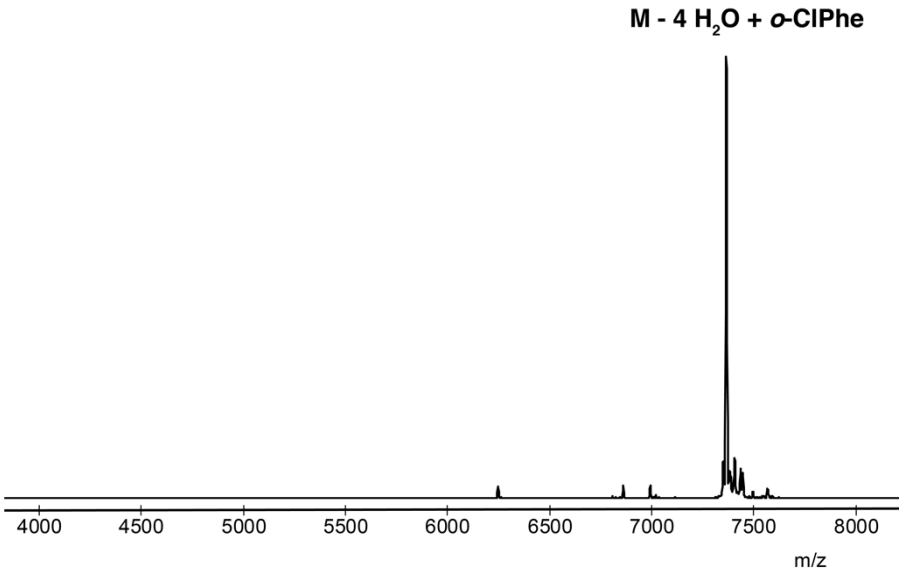


Figure S10. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F23*o*-ClPhe, modified by LctM in *E. coli* BL21(DE3).

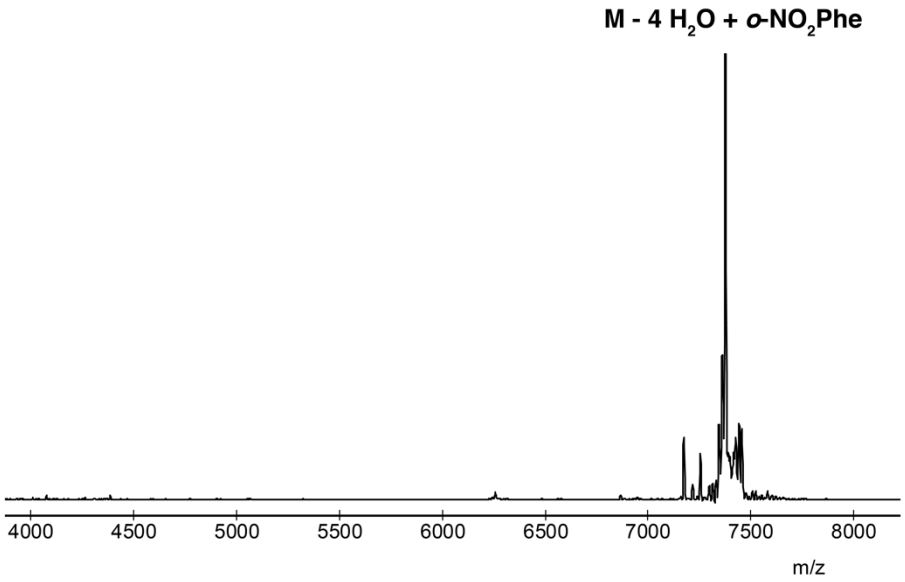
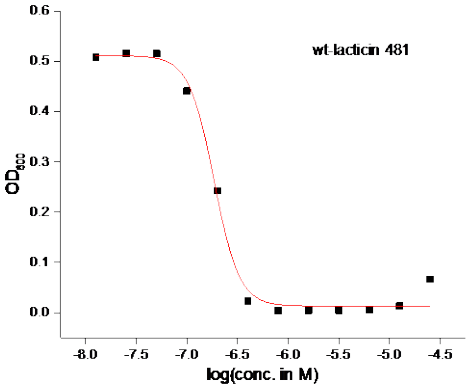
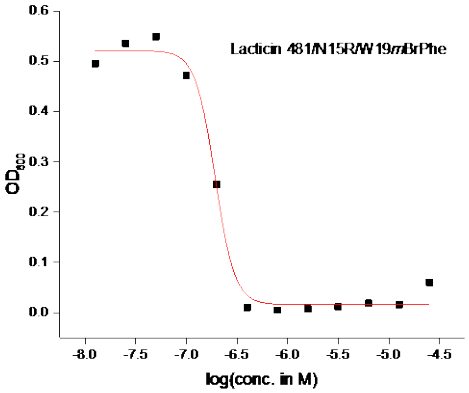


Figure S11. MALDI-TOF mass spectrum of His-tagged-LctA-N15R/F23*o*-NO_2Phe, modified by LctM in *E. coli* BL21(DE3).

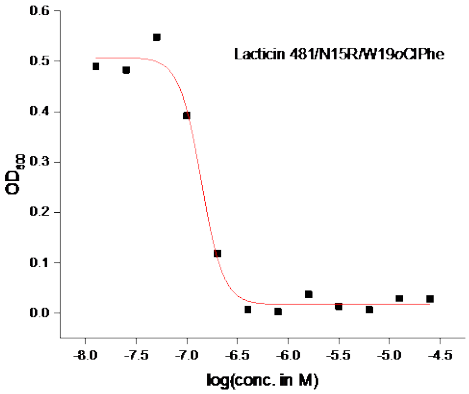
Figure S12. IC_{50} values determined by fitting growth inhibition data wt-lacticin 481 and five analogs against *L. lactis* HP grown in liquid medium.



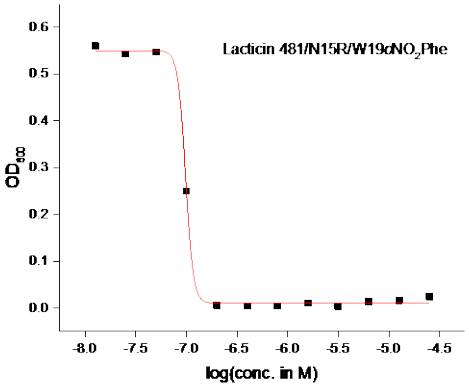
MIC = 390 nM
IC_{50} = 185 ± 10 nM.



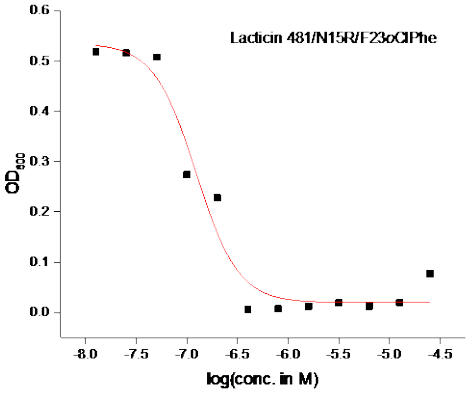
MIC = 390 nM
IC_{50} = 192 ± 9 nM



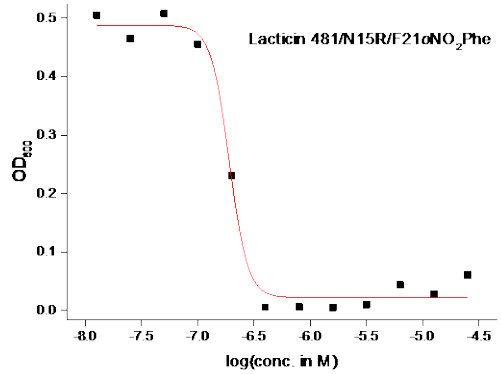
MIC = 390 nM
IC_{50} = 138 ± 8 nM.



MIC = 195 nM
IC_{50} = 98 ± 8 nM



MIC = 390 nM
IC_{50} = 123 ± 21 nM.



MIC = 390 nM
IC_{50} = 190 ± 9 nM

Table S4. Masses observed in MALDI-TOF mass spectra of LctA mutants after purification of full-length LctM-modified peptides. Masses shown are for 4-fold dehydrated products.

Mutant	Mass calculated for $[M+H]^+$ peak	Mass observed for $[M+H]^+$ peak
His-tagged-LctA-N15R/W19*o*-ClPhe (Fig. S1)	7326.53	7326
His-tagged-LctA-N15R/W19*m*-BrPhe (Fig. S2)	7370.99	7371
His-tagged-LctA-N15R/W19*m*-CF_3Phe (Fig. 3 main text)	7360.09	7360
His-tagged-LctA-N15R/W19*o*-NO_2Phe (Fig. S3)	7337.07	7337
His-tagged-LctA-N15R/F21*m*-BrPhe (Fig. S4)	7410.03	7411
His-tagged-LctA-N15R/F21*m*-CF_3Phe (Fig. S5)	7399.13	7399
His-tagged-LctA-N15R/F21*o*-ClPhe (Fig. S6)	7365.57	7366
His-tagged-LctA-N15R/F21*o*-NO_2Phe (Fig. S7)	7376.13	7375
His-tagged-LctA-N15R/F23*m*-BrPhe (Fig. S8)	7410.03	7411
His-tagged-LctA-N15R/F23*m*-CF_3Phe (Fig. S9)	7399.13	7400
His-tagged-LctA-N15R/F23*o*-ClPhe (Fig. S10)	7365.57	7366
His-tagged-LctA-N15R/F23*o*-NO_2Phe (Fig. S11)	7376.13	7376

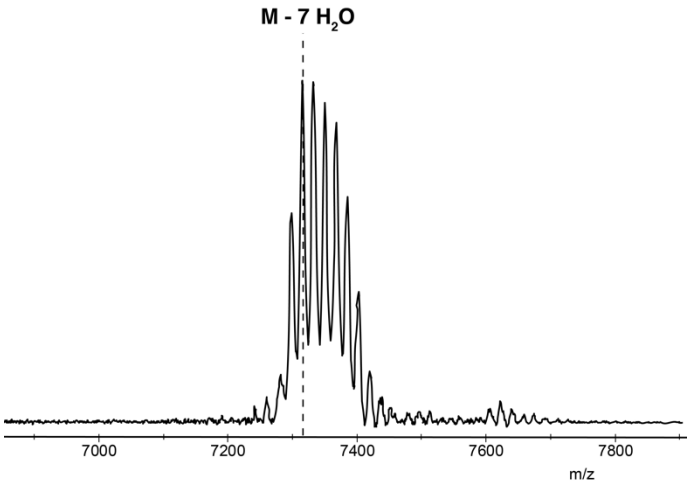


Figure S13. MALDI-TOF mass spectrum of wild type NisA modified by NisB and NisC in *E. coli* BL21(DE3) in synthetic media at 18 °C. Although the desired 7- and 8-fold dehydrated peptides are present, peptides that are dehydrated to a lesser extent are prevalent. m/z calculated for $[M+H]^+$ peak for 7-fold dehydrated His-tagged-NisA = 7319.45, m/z observed for $[M+H]^+$ peak for the labeled 7-fold dehydrated His-tagged-NisA = 7319.

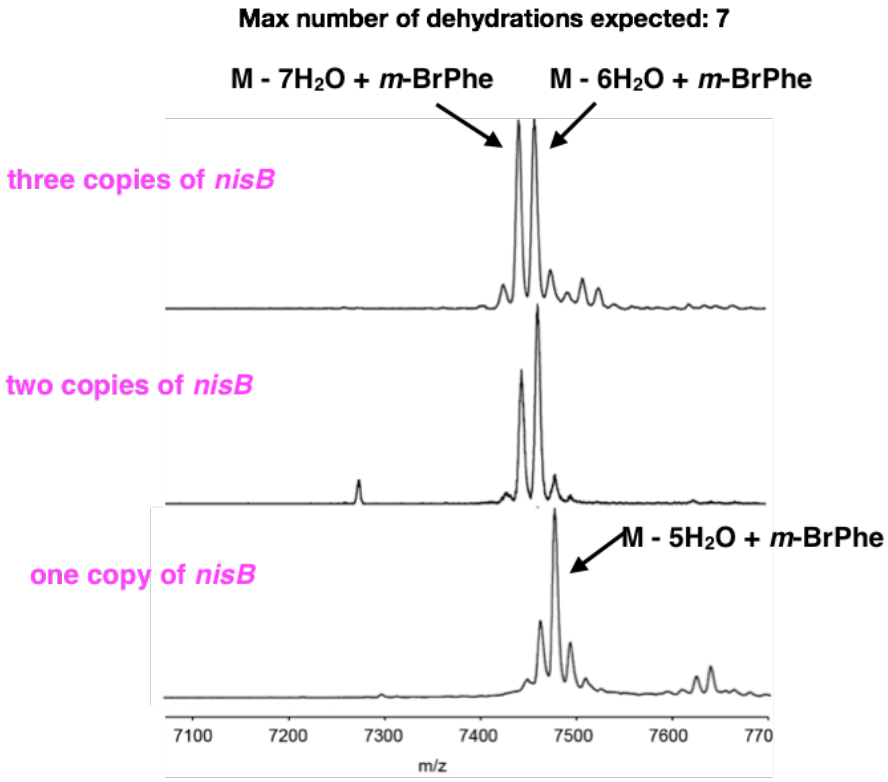


Figure S14. MALDI-TOF mass spectra of NisA-T2*m*-BrPhe modified by NisB and NisC in *E. coli* BL21(DE3) in synthetic media with multiple copies of *nisB* gene expressed.

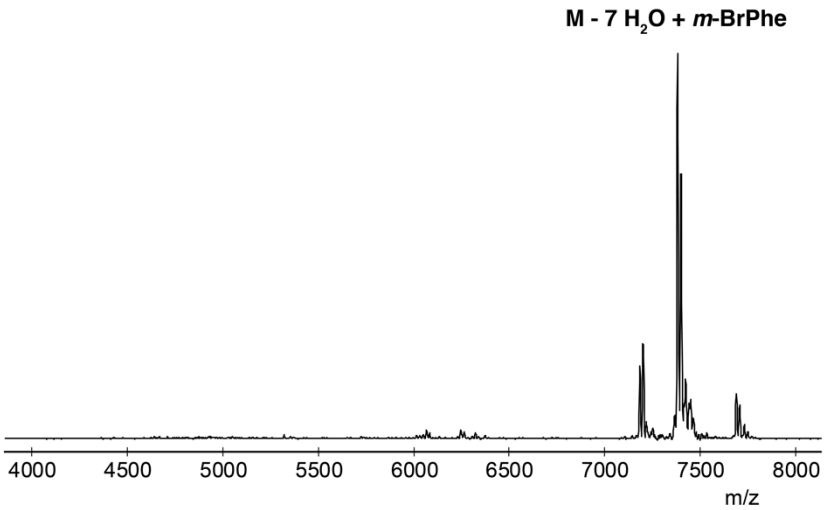


Figure S15. MALDI-TOF mass spectrum of NisA-I4V/S5B/L6G with S5 substituted by *m*-BrPhe, modified by NisB and NisC in *E. coli* (C321.ΔprfA-T7RNAPΔrneΔompTΔlon).

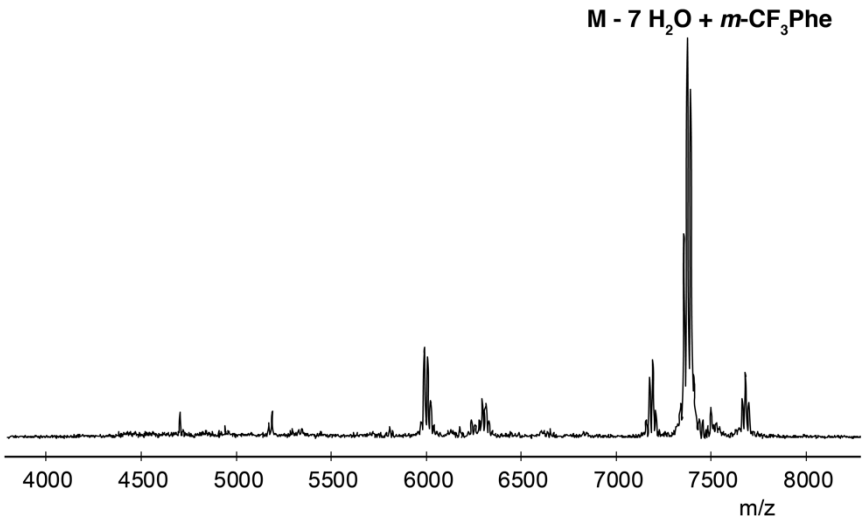


Figure S16. MALDI-TOF mass spectrum of NisA-I4V/S5B/L6G with S5 substituted by *m*-CF_3Phe, modified by NisB and NisC in in *E. coli* (C321.ΔprfA-T7RNAPΔrneΔompTΔlon).

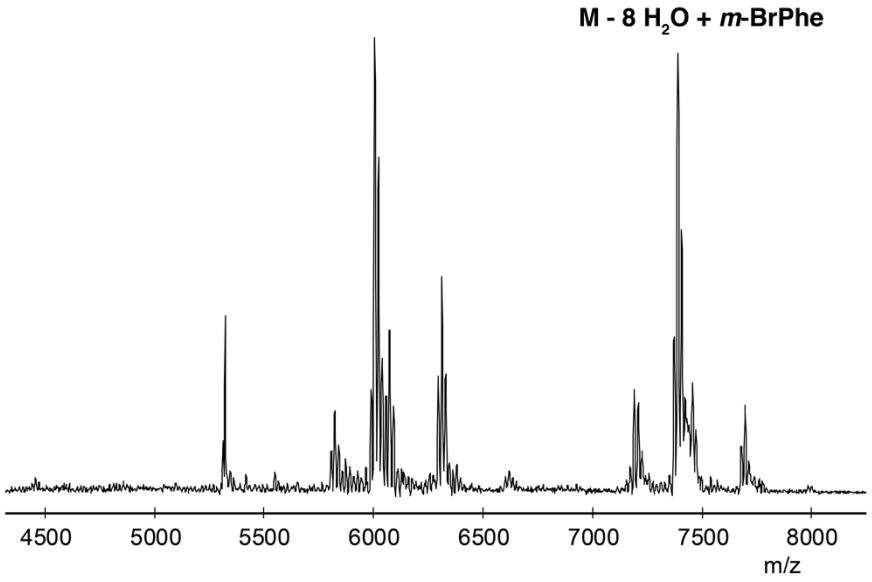


Figure S17. MALDI-TOF mass spectrum of NisA-M21*m*-BrPhe, modified by NisB and NisC in in *E. coli* (C321.ΔprfA-T7RNAPΔrneΔompTΔlon). The peptide was expresed in very low yields.

Table S5. Masses observed in MALDI-TOF mass spectra of NisA mutants after purification of full-length NisB/C-modified peptides expressed in in *E. coli* (C321.ΔprfA-T7RNAPΔrneΔompTΔlon). Masses shown are for 7-fold dehydrated products for mutations at Thr2 and Ser5, and 8-fold dehydrated peptides for mutation at Met21.

Mutant	Mass calculated for the major peak, $[M+H]^+$	Mass observed for the major peak, $[M+H]^+$
His-tagged-NisA-T2*m*-BrPhe (Fig. in the main text)	7444.42	7445
His-tagged-NisA-I4V/S5*m*-BrPhe/L6G	7388.31	7389
His-tagged-NisA-I4V/S5*m*-CF_3Phe/L6G	7377.41	7378
His-tagged-NisA-M21*m*-BrPhe	7396.32	7396

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