# SUPPORTING INFORMATION
# Facile Removal of Leader Peptides from Lanthipeptides by Incorporation of a Hydroxy Acid

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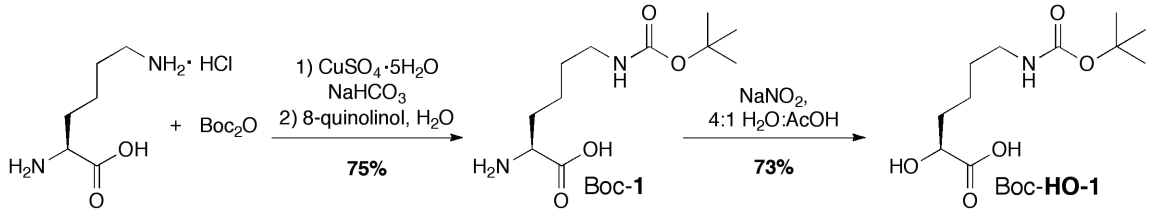
## Materials

All compounds used in the synthetic schemes were purchased from Sigma Aldrich with the exception of L-lysine monohydrochloride (Eastman Chemical Company), 8-quinolinol (Fisher Scientific), di-tert-butyl dicarbonate ($Boc_2O$, Fluka Analytical), propargyl bromide (TCI America), and 3-bromo-L-phenylalanine (H-Phe(3Br)-OH, Chem Impex Int.). 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. Oligonucleotide primers used for molecular cloning were purchased from Integrated DNA Technologies. Phusion High-Fidelity DNA polymerase, T4 DNA ligase, Gibson Assembly master mix, and all restriction endonucleases were purchased from New England Biolabs. Gel extraction, plasmid miniprep, and PCR purification kits were purchased from QIAGEN. Endoproteinases LysC and GluC were purchased from Roche Applied Science and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) was purchased from Sigma Aldrich. Other items procured for cell culture and peptide work included isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology), kanamycin monosulfate (IBI Scientific), chloramphenicol (IBI Scientific), and L(+)-arabinose (Fisher Scientific). MALDI-TOF MS analyses were conducted at the Mass Spectrometry Facility (UIUC) using an UltrafleXtreme TOF/TOF (Bruker Daltonics). Amberlite XAD-16 was purchased from Alfa Aesar. SP-Sepharose Fast Flow resin was purchased from GE Healthcare Life Sciences.

## Synthesis of amino and hydroxy acids Boc-1 and Boc-HO-1




### H-Lys(Boc)-OH (Boc-1)

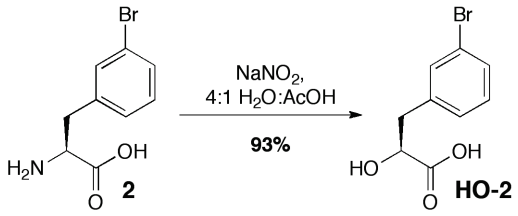
Into a 250 mL round-bottomed flask was added L-lysine monohydrochloride (3.74 g, 20.5 mmol, 1 equiv) in 1 M aqueous $NaHCO_3$ (41 mL). To this mixture was slowly added $CuSO_4$-5 $H_2O$ (2.56 g, 10.25 mmol, 0.5 equiv) followed by solid $NaHCO_3$ (1.72 g, 20.5 mmol). Finally, $Boc_2O$ (5.82 g, 26.7 mmol, 1.3 equiv) in acetone (24.6 mL) was added and the reaction was stirred overnight at room temperature. The reaction was then quenched with MeOH (6.0 mL) and was stirred for 2 h followed by the addition of $H_2O$ (20 mL) and EtOAc (20 mL). The blue solid was then filtered, washed with $H_2O$, dried and used immediately in the subsequent reaction.

To a vigorously stirred suspension of the intermediate just generated in $H_2O$ (200 mL) was added 8-quinolinol (3.88 g, 26.7 mmol). The reaction was stirred overnight at which time the green suspension was filtered and the solid was washed with $H_2O$ (100 mL) and discarded. The filtrate was washed with DCM (2 × 100 mL) and the aqueous layer was concentrated to a smaller

volume by rotary evaporation and lyophilized yielding compound Boc-**1** (3.74 g, 75%) as a fluffy white solid.[1]
$^{1}$H NMR: (499.695 MHz, 1:1 $D_2O$:$CD_3OD$) δ=1.40 (m, 11H), 1.49 (m, 2H, $CH_2$) 1.76 (m, 2H, $CH_2$), 3.04 (t, J = 7 Hz, 2H, $CH_2$) 3.52 (t, J = J Hz, 1H, CH).
$^{13}$C NMR: (499.434 MHz, 1:1 $D_2O$:$CD_3OD$) δ = 22.4, 27.9, 29.3, 31.8, 40.0, 55.4, 80.1, 158.1, 176.8.
HRMS $[M+Na]^+$ $C_{11}H_{23}N_2O_4^+$ calc'd = 266.9632, found = 266.9642.

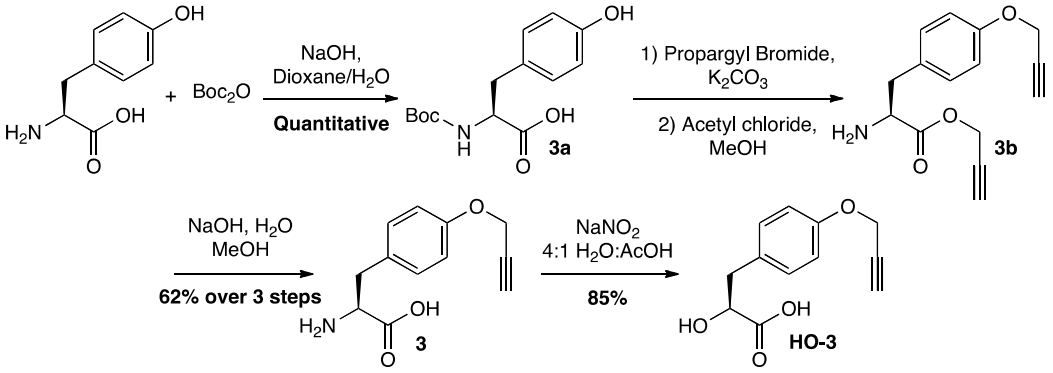
**HO-Lys(Boc)-OH (Boc-HO-1)**
Into a 250 mL round-bottomed flask was placed Boc-**1** (1.4 g, 5.69 mmol) dissolved in 57 mL of 4:1 $H_2O$:AcOH and the flask was placed in a 0 ºC ice bath. To this mixture was slowly added aqueous sodium nitrite (12 mL, 1 M) via a syringe pump over 30 min. The reaction was stirred for 4 h at room temperature at which point methylamine (40% in $H_2O$, 0.9 mL) was added and the reaction was stirred for 10 min. The reaction was then acidified with aqueous 1 M HCl (until the pH reached 2.5) and product was extracted with EtOAc (3 × 50 mL). The organic layer was dried with $MgSO_4$, filtered, and the filtrate was concentrated via rotary evaporation. To the yellow liquid was added 60% aqueous MeCN/0.1% TFA (2 mL) and this solution was lyophilized to yield Boc-**HO-1** (1.02 g, 73% yield) as a yellow oil.[2] Characterization data matched that previously reported.[3]

**Synthesis of HO-Phe(3Br)-OH (HO-2)**




**HO-Phe(3Br)-OH (HO-2)**
Into a 250 mL round-bottomed flask was placed **2** (0.8 g, 3.4 mmol) dissolved in 32 mL of 4:1 $H_2O$:AcOH and the flask was placed in a 0 ºC ice bath. To this mixture was slowly added aqueous sodium nitrite (6.6 mL, 1 M) via a syringe pump over 30 min. The reaction was stirred for 4 h at room temperature at which point methylamine (40% in $H_2O$, 0.5 mL) was added and the reaction was stirred for 10 min. The reaction was then acidified with aqueous 1 M HCl (until the pH reached 2.5) and product was extracted with EtOAc (3 × 50 mL). The organic layer was dried with $MgSO_4$, filtered, and the filtrate was concentrated via rotary evaporation. To the orange liquid was added 60% aqueous MeCN/0.1% TFA (2 mL), which was lyophilized to yield **HO-2** (750 mg, 93% yield) as an orange oil.[2]
$^{1}$H NMR: (499.434 MHz, $CD_3OD$) δ=2.90 (dd, J = 14.0, 8.0 Hz, 1H, $CH_2$), 3.09 (dd, J = 14.0, 4.0 Hz, 1H, $CH_2$) 4.35 (m, 1H, CH), 7.16 (m, 1H, $CH_{phenyl}$) 7.22 (m, 1H, $CH_{phenyl}$), 7.33 (dt, J = 10.0, 1.8 Hz, 1H, $CH_{phenyl}$), 7.43 (t, J = 2.5 Hz, 1H, $CH_{phenyl}$).
$^{13}$C NMR: (499.434 MHz, $CD_3OD$) δ = 39.9, 71.2, 122.0, 128.3, 129.5, 129.8, 132.5, 140.4, 175.7.
HRMS $[M+Na]^+$ $C_9H_9BrNaO_3^+$ calc'd = 266.9632, found = 266.9642.

**Synthesis of amino and hydroxy acids 3 and HO-3**




**Synthetic scheme of HO-Tyr(propargyl)-OH (HO-3)**

**Boc-Tyr-OH (3a)**

Into a 500 mL round-bottomed flask was placed L-tyrosine (4.0 g, 22.0 mmol, 1 equiv) dissolved in dioxane:$H_2O$ (2:1, 150 mL). To this solution was added aqueous NaOH (1 M, 50 mL) and $Boc_2O$ (5.28 g, 24.2 mmol, 1.1 equiv) sequentially. The reaction was stirred at room temperature for 3 h, acidified to pH 2.3 with 5 M HCl, and product was extracted with EtOAc (3 × 50 mL). The organic layer was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and concentrated via rotary evaporation yielding **3a** (6.18 g, quantitative) as a crystalline white solid. Characterization data matched that previously reported.[4]

**H-Tyr(propargyl)-O-propargyl (3b)**

Into a 250 mL round-bottomed flask was placed **3a** (6.2 g, 22.1 mmol, 1 equiv) and $K_2CO_3$ (9.16 g, 66.3 mmol, 3.0 equiv) suspended in anhydrous DMF (37 mL). The flask was cooled in a 0 ºC ice bath and propargyl bromide (7.89 g, 5.03 mL, 66.3 mmol, 3 equiv) was added dropwise. The reaction was warmed to room temperature and stirred for 20 h. At this time, $H_2O$ (150 mL) was added and the aqueous layer was extracted with $Et_2O$ (3 × 100 mL). The organic layer was washed with $H_2O$ (100 mL) and sat. aqueous NaCl (2 × 100 mL), dried with $MgSO_4$, and concentrated by rotary evaporation yielding a yellow-orange oil. Characterization data matched that previously reported.[5]

Into a separate 250 mL round-bottomed flask containing anhydrous MeOH (74.6 mL) at 0 ºC was slowly added acetyl chloride (9.54 g, 8.64 mL, 121.6 mmol). This solution was added to the oil isolated above, the resulting solution was warmed to room temperature and stirred for 16 h. All volatile components were removed on a vacuum line yielding **3b**, which was immediately used in the subsequent step.

**H-Tyr(propargyl)-OH (3)**

Into a 250 mL round-bottomed flask was placed intermediate **3b** dissolved in MeOH (22 mL) followed by aqueous NaOH (2 M, 32 mL). The reaction was stirred at room temperature for 20 h, acidified to pH 3.0 with concentrated HCl, and cooled at 4 ºC overnight. The following morning a tan precipitate had formed, which was filtered, washed with acidified cold $H_2O$ (pH 3.0), and dried yielding **3** (3.0 g, 62% over 3 steps) as a tan solid. Characterization data matched that previously reported.[5]

**HO-Tyr(propargyl)-OH (HO-3)**
Into a 250 mL round-bottomed flask was placed **3** (2.55 g, 10 mmol) dissolved in 100 mL of 4:1 $H_2O$:AcOH and the flask was placed in a 0 ºC ice bath. To this mixture was slowly added aqueous sodium nitrite (20 mL, 1 M) via a syringe pump over 30 min. The reaction was stirred for 4 h at room temperature at which point methylamine (40% in $H_2O$, 1.56 mL) was added and the reaction was stirred for 10 min. The reaction was then acidified with aqueous 1 M HCl (until the pH reached 2.5) and product was extracted with EtOAc (3 × 50 mL). The organic layer was dried with $MgSO_4$, filtered, and the filtrate was concentrated via rotary evaporation. To the orange liquid was added 60% aqueous MeCN/0.1% TFA (2 mL), which was lyophilized to yield **HO-3** (2.2 g, 85% yield) as an orange oil. Characterization data matched that previously reported.[5]

**Construction of plasmids for coexpression**
DNA constructs were assembled by overlap extension PCR, followed either by digestion and ligation or Gibson Assembly[6] of the PCR product into the appropriate plasmid.

**Overlap extension PCR**
A 5' gene fragment was amplified by PCR using Phusion High-Fidelity DNA polymerase from a template containing the sequence of interest using a forward primer with the desired restriction site and a reverse primer either incorporating the internal mutation or insertion or beginning at the first nucleotide immediately after the internal mutation or insertion. An overlapping 3' gene fragment was amplified by PCR from the same template using a reverse primer with the desired restriction site and a forward primer incorporating the internal mutation. For primers used, see Table S1. Then, a PCR with the overlapping gene fragments and the primers with the desired restriction sites was used to generate the full-length mutants; the overlapping gene fragments were first added to the PCR mixture and after 8 rounds of amplification the primers with the desired restriction sites were added. After digestion of the mutant gene fragment and the appropriate vector with the desired restriction enzymes, the PCR products containing point mutations, insertions, or deletions were ligated into the plasmid using T4 DNA ligase. Incorporation of the correct mutation into the plasmid DNA was confirmed by sequencing at ACGT, Inc.

**General protocol for PCR**
To a 0.2 mL Eppendorf tube was added $H_2O$ (34 µL), buffer HF (10 µL, 5X stock solution, provided with Phusion High-Fidelity DNA polymerase), template (0.5 µL, 100 ng/µL), primers (1 µL each, 100 µM), DMSO (1 µL), dNTPs (1 µL, 10 mM), and Phusion High-Fidelity DNA polymerase (0.5 µL, 2,000 U/mL, NEB). The DNA template was amplified using the following protocol. The reaction was 1) heated to 95 ºC for 2 min; 2) heated at 95 ºC for 30 s; 3) heated at annealing temperature (see individual cases below) for 30 s; 4) heated at 72 ºC for the elongation time (see below); 5) steps 2-4 were repeated for 29 cycles; 6) heated at 72 ºC for 10 min.

**Construction of inserts for pRSFDuet*(lctA(A–1I/K1X)/lctM/pylT*)**
The *lctA* mutant was generated in MCS1 of a pRSFDuet vector with *lctM* in MCS2 using pRSFDuet(*lctAwt/lctM*)[7] as template by overlap extension PCR. The primers *lctA EcoRI FMP 5'* and *lctA(A–1I/K1X) FMP 3'* were used to make the 5' overlapping fragment and the primers *lctA(A–1I/K1X) RMP 5'* and *lctA NotI RMP 3'* were used to make the 3' overlapping fragment (Table S1). The restriction sites were EcoRI and NotI. The annealing temperature was 60 $^{o}$C with

an elongation time of 20 s. After overlap extension PCR, the inserts were ligated into MCS1 of a pRSFDuet vector as described below.
*pylT* was amplified by PCR from pEVOL*(pylRS/pylT)* using *pylT AgeI 5'* and *pylT DrdI 3'* as primers (Table S1) and the product was digested using AgeI and DrdI. The gene fragment was then ligated into the *lctA* plasmids constructed as described above that had been treated with the same restriction enzymes generating pRSFDuet*(lctA(A–1I/K1X)/lctM/pylT)*.

**Construction of inserts for plasmids pRSFDuet*(nukA)/nukM/pylT)* and pRSFDuet*(nukA(A–1I/K1X)/nukM/pylT)***
*nukA* was amplified by PCR from pET15b*(nukA)*[8] using *nukA Gibson 5'* and *nukA Gibson 3'* as primers (Table S1). The annealing temperature was 58 $^{o}$C with an elongation time of 20 s. The gene fragment was then inserted by Gibson Assembly into MCS1 of pRSFDuet-*(lctA(K1X)/lctM/pylT)*, which had been treated with EcoRI and NotI, producing pRSFDuet*(nukA)/lctM/pylT)*.
*nukM* was amplified by PCR from genomic DNA extracted from *Staphylococcus warneri* JCM 11004 using *nukM Gibson 5'* and *nukM Gibson 3'* as primers (Table S1). The annealing temperature was 55 $^{o}$C with an elongation time of 150 s. The gene fragment was then inserted by Gibson Assembly into MCS2 of pRSFDuet*(nukA/lctM/pylT)*, which had been treated with BglII and XhoI, producing pRSFDuet*(nukA/nukM/pylT)*.
The *nukA(A–1I/K1X)* mutant was generated using pET15b*(nukA)* as template by overlap extension PCR and ligated into MCS1 of pRSFDuet*(nukA)/nukM/pylT)* by Gibson Assembly, producing pRSFDuet*(nukA(A–1I/K1X)/nukM/pylT)*. The primers *nukA Gibson 5'* and *nukA(A–1I/K1X) FMP 3'* were used to make the 5' overlapping fragment and the primers *nukA(A–1I/K1X) RMP 5'* and *nukA Gibson 3'* were used to make the 3' overlapping fragment. The parent plasmid was treated with EcoRI and NotI. The annealing temperature was 58 $^{o}$C with an elongation time of 20 s.

**Digestion of plasmids and DNA inserts**
To a 1.7 mL Eppendorf tube with insert or plasmid DNA (40 μL, 100-250 ng/μL) was added digestion buffer (5 μL; NEB, provided with the restriction enzymes), digestion enzyme(s) (1.5 μL, concentrations are below), BSA (if necessary, 0.5 μL, 100X, provided with digestion enzyme), and $H_2O$ (to 50 μL). The components were incubated at 37 $^{o}$C for 2-4 h. At this time calf intestine phosphatase (CIP) (1 μL) was added to plasmid DNA only and the sample was incubated for an additional 1 h. The samples were then analyzed by DNA gel electrophoresis and the desired band was purified by a QIAGEN Gel Extraction kit eluting in buffer EB (30 μL).
*Note:* Both digestion enzymes were added in one single digest if the buffer was compatible. If buffer was not compatible, two successive digests were performed.

NotI = 10,000 U/mL, EcoRI = 20,000 U/mL, AgeI = 5000 U/mL, DrdI = 5000 U/mL, CIP = 10,000 U/mL.

**Ligation of digested plasmid and DNA insert**
To three 0.6 mL Eppendorf tubes was added plasmid DNA (1 μL, 100 ng/μL). Insert DNA (1 μL of a solution of different concentrations to give a ratio of plasmid to insert of 1:1, 1:10 and 1:100) was then added as well as T4 DNA ligase reaction buffer (0.7 μL, provided with the ligation enzyme), T4 DNA ligase (0.8 μL, 400,000 cohesive end U/mL), and $H_2O$ (to 5 μL). The

samples were incubated at 18 °C for 16-20 h at which time the entire reactions were used to transform *E. coli* DH5α cells.

**Gibson Assembly**[6]

To a 0.6 mL Eppendorf tube was added plasmid DNA (1 μL, 25 ng/μL), insert DNA (equal mol amount), Gibson Assembly Mastermix (2.5 μL, NEB), and $H_2O$ (to 5 μL). The samples were incubated at 50 ºC for 15 min at which time the entire reaction was used to transform *E. coli* DH5α cells.

**Table S1**. Primers used in molecular cloning.

| | |
|---|---|
| ***lctA EcoRI FMP 5':*** | CCT CTG GCG GAT CCG AAT TCG ATG AAA TGA AAG AAC AAA AC |
| ***lctA(A–1I/K1X) FMP 3':*** | TCC ACT GCC GCC CTA AAT ACC TAA AAT AAG G |
| ***lctA(A–1I/K1X) RMP 5':*** | CCT TAT TTT AGG TAT TTA GGG CGG CAG TGG A |
| ***lctA NotI RMP 3':*** | CGG TTA AAG CGG CCG CTT AAG AGC AGC AAG TAA ATA C |
| | |
| ***pylT AgeI 5':*** | GAG TAT GAA CCG GTT GTG CTT CTC AAA TGC CTG A |
| ***pylT DrdI 3':*** | GTA CTG AGA GAC AGT CTA GTC CAT GCA AAA AAG CCT GCT C |
| | |
| ***nukA Gibson 5'*** | CAC AGC CAG GAT CCG ATG GAA AAT TCT AAA GTT ATG AAG G |
| ***nukA Gibson 3'*** | CGA CTT AAG CAT TAT GCT TAT GAA CAA CAA GTA AAT ACA AAT TG |
| ***nukA(A–1I/K1X) FMP 3'*** | GAT TAC TCC TGA CTT TTT CTA AAT TCC TAA GAC TTC ATT C |
| ***nukA(A–1I/K1X) RMP 5'*** | GAA TGA AGT CTT AGG AAT TTA GAA AAA GTC AGG AGT AAT C |
| | |
| ***nukM Gibson 5'*** | GGA GAT ATA CAT ATG GCA GAT CTC ATG AAC AAC ATT AAA GTT GAA CAA TTT AG |
| ***nukM Gibson 3'*** | CGG TTT CTT TAC CAG ACT CGA GTT AAA TGT TTG GTA ACA ATA TAC CTA ATT C |

**Heterologous production, purification, and characterization of LanA analogues**

The lanthipeptides were expressed using *E. coli* BL21 (DE3) cells transformed with a pRSFDuet plasmid containing the *lanA*, *lanM,* and *pylT* genes and a pEVOL plasmid containing the *pylRS* (2 copies of either wt or N346A/C348A mutant) and *pylT* genes (1 copy). The later plasmid was reported previously.[9] For production of Δ1-lacticin 481 and Δ1-3-nukacin ISK-1 only the pRSFDuet plasmid was used because non-canonical amino or hydroxy acid was not incorporated. All coexpression cultures were incubated at 18 ºC overnight after induction with IPTG except for the coexpression cultures in which Boc-**1**, **2**, or **3** or Boc-**HO-1**, **HO-2**, or **HO-3** were incorporated into LctA, which were incubated at 37 ºC for 4 h after induction. Composition of 1 L enriched LB: 35 g tryptone, 20 g yeast extract, and 5 g NaCl.

**Overexpression and purification of $His_6$-tagged modified peptide mutants**

An overnight culture of *E. coli* BL21 (DE3) cells was added to a culture flask containing enriched LB (1:100; volume overnight culture: volume overexpression culture) and appropriate antibiotics depending on the plasmids used (1:1000 v:v; kanamycin (50 mg/mL), chloramphenicol (25 mg/mL)). The culture was then incubated in a 37 ºC shaker (220 rpm) until the optical density reached 1.0. For LctA(K1X) and LctA(A–1I/K1X), overexpression was immediately 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

amino or hydroxy acid (dissolved in 1 M NaOH; 2 mM final concentration; pH of culture solution was adjusted back to neutral with 5 M HCl) and the flask was incubated for 4 h in a shaker at 37 ºC and 220 rpm. Conversely, for NukA(A–1I/K1X), wt NukA, and wt LctA, the culture was first placed on ice (5 min) to cool to around 18 ºC. Overexpression was then induced by the addition of just IPTG for wt NukA and wt LctA and by arabinose and IPTG in the presence of non-proteinogenic amino or hydroxy acid for NukA(A–1/K1X). The flasks were then incubated overnight 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 $^{o}$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 $^{o}$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 non-specific 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 preparative reversed phase high-performance liquid chromatography (RP-HPLC) (solvent A = 0.1% TFA in $H_2O$; solvent B = 80% acetonitrile in $H_2O$, 0.086% TFA) using a gradient from 2-80% solvent B in 45 min and lyophilized (column used and retention times are described below). The yield after desalting was between 2.1-5.0 mg/L culture for **X**-mLctA and 0.8 mg/L culture for **HO-1**-mNukA.

**GluC cleavage of X-mLctA incorporating HO-1, HO-2, or HO-3**
To RP-HPLC purified and lyophilized peptide was added HEPES buffer (500 µL, pH 7.5, 50 mM final concentration) and GluC (1 µL, 2 mg/mL). The reaction was incubated for 2 h at 37 ºC. The reaction was analyzed by MALDI-TOF MS.

**LysC cleavage of $His_6$-mLctA or $His_6$-mNukA**
To RP-HPLC purified and lyophilized peptide was added HEPES buffer (500 µL, pH 7.5, 50 mM final concentration) and LysC (3 U/100 µL). The reaction was incubated at 37 ºC until complete as determined by MALDI-TOF MS analysis.

**Boc removal of HO-1-mLctA and HO-1-mNukA**
To RP-HPLC purified and lyophilized peptide was added aqueous 5% TFA (2 mL). The reaction was incubated for 4 h at 50 ºC. The peptidic solution was then concentrated by purging with a nitrogen stream and used in the alkaline hydrolysis reaction without further purification.

**Alkaline hydrolysis of HO-1-mLctA, HO-2-mLctA, HO-3-mLctA, and HO-1-mNukA**
To the concentrated peptide solution from the Boc-removal reaction (for HO-**1**) or RP-HPLC purified and lyophilized peptide (for HO-**2** and HO-**3**), was added CAPS buffer (4 mL, 250 mM, pH 10.5) and 1 M NaOH (until pH reached 10.5). The reaction was incubated for 2 h at 37 ºC and purified by preparative RP-HPLC on a C18 column (solvent A = 0.1% TFA in $H_2O$; solvent B = 80% acetonitrile in $H_2O$, 0.086% TFA) using a gradient from 2-80% solvent B in 45 min

(column used and retention times are described below). Fractions containing product were collected and lyophilized.

**N-ethylmaleimide assay of Δ1-3 nukacin ISK-1**

To Δ1-3 nukacin ISK-1 (2 μL, 80 μM) in HEPES buffer (15 μL, 50 mM, pH 6.3) was added TCEP (2 μL, 2 mM). The solution was incubated at room temperature for 30 min at which time N-ethylmaleimide (2 μL, 10 mM) was added. The solution was incubated for an additional 90 min at 37 ºC.

**Isolation of authentic lacticin 481**

*Lactococcus lactis* subsp. *lactis* CNRZ 481 was grown for 9 h at 30 ºC without agitation in EG'P media (1.74 L, Elliker broth medium without gelatin) supplemented with sodium β-glycerophosphate (26.1 g, (15 g/L)) maintaining the culture pH at 5.5 by adding aliquots of 3 M ammonium hydroxide every hour. After 9 h the pH was again adjusted to 5.5 and cells were removed by centrifugation (11,900 × g, 20 min). The culture supernatant was heat-treated (80 ºC, 1 h) to deactivate proteases, cooled to 4 ºC, and the lantibiotic was concentrated with an ammonium sulfate precipitation step, saturating the solution at 60% at 4 °C (649.4 g of ammonium sulfate into 1.74 L culture supernatant). The sample was vigorously stirred for 4 h and then centrifuged (17,100 × g, 45 min, 4 ºC). After resuspension of the pellet in Sorensen buffer (20 mL, 176 mM $NaH_2PO_4$, 24 mM $Na_2HPO_4$, pH 6.0), lacticin 481 was further purified by solid phase extraction using a Vydac 214SPE3000 C4 column, with 20 mM ammonium acetate and MeCN as eluting solvents. After addition of the lantibiotic solution, the column was washed with 20 mM ammonium acetate and 20 mM ammonium acetate with 30% MeCN. Peptide was then eluted with 20 mM ammonium acetate with 40% MeCN. The fraction containing product was concentrated by lyophilization and further purified by analytical RP-HPLC (Grace Vydac C18 column 214TP54) using a gradient from 30-40% MeCN in 20 mM ammonium acetate, pH 5.5, over 30 min. The combined fractions were lyophilized yielding 6 mg of partially purified lacticin 481. In previous isolations of authentic lacticin 481 in our laboratory no further purification was performed. However, these older batches of lacticin 481 were likely contaminated with ammonium acetate as the $IC_{50}$ value experimentally determined in this manuscript after an additional HPLC step was > 3-fold lower.

**RP-HPLC purification of lacticin 481 isolated from *L. lactis* subsp. *lactis* 481**

To the lyophilized partially purified lacticin 481 (2 mg) from above was added $H_2O$ (4 mL) and the peptide was purified by preparative RP-HPLC on a C18 column (solvent A = 0.1% TFA in $H_2O$; solvent B = 80% acetonitrile in $H_2O$, 0.086% TFA). Product eluted from 26.5-27.0 min yielding 300 μg of pure lacticin 481. This peptide was then used for MIC studies.

**Isolation of nukacin ISK-1 from *Staphylococcus warneri*[10]**

A 100 mL overnight culture of *S. warneri* (JCM 11004) was used to inoculate 8 L of MRS media and the cells were incubated for 12 h in a 37 ºC shaker (220 rpm). The cells were removed by centrifugation (8,000 ×*g*, 4 ºC, 20 min) and the culture supernatant was incubated with 10 g of Amberlite XAD-16 per liter of culture for 3 h in a 14 ºC shaker (220 rpm). The resin was washed with $H_2O$ (800 mL) and EtOH (800 mL) and the lantibiotic was eluted with 70% aqueous iPrOH (800 mL). The elution solvent was removed by rotary evaporation and the brown residue was dissolved in 20 mM phosphate buffer (pH 5.7) and passed through an SP-Sepharose (Sigma Chemical) cation exchange column (0.5 mL resin per liter of culture). The resin was washed with

20 mM phosphate buffer (100 mL, pH 5.7) and the lantibiotic was eluted with 0.5 M NaCl (200 mL). The solution was concentrated by rotary evaporation and purified by preparative RP-HPLC (Phenomenex C18 column (00G-4253-N0)) (solvent A = 0.1% TFA in $H_2O$; solvent B = 80% acetonitrile in $H_2O$, 0.086% TFA) using a gradient from 2%-38% in 25 min and 38%-60% in 10 min. Fractions containing partially purified nukacin ISK-1, as analyzed by MALDI-TOF MS, were collected and lyophilized. The combined fractions were further purified by analytical RP-HPLC (Phenomenex C18 column (00G-4253-E0)) using a gradient from 2%-35% solvent B in 5 min and 35%-45% solvent B in 20 min. Product eluted between 17.6-18.2 min. Combined fractions containing nukacin ISK-1, as judged by MALDI-TOF MS, were lyophilized to afford 6.0 mg of pure nukacin ISK-1.

**Agar diffusion growth inhibition assay of lacticin 481, nukacin ISK-1, and analogues**

HPLC-purified peptides were dissolved in water, lyophilized and redissolved in PBS to remove traces of TFA remaining from the HPLC solvents. Overnight cultures of *L. lactis* HP were diluted in M-17 agar containing 0.5 % glucose to an $OD_{600}$ of 0.05. For lacticin 481 and analogues a total of 10 µL of 12.5 µM solution was spotted. For nukacin ISK-1 and analogues a total of 10 µL of the following peptide concentrations were spotted: authentic nukacin ISK-1 from producer strain *S. warneri* (17.5 µM), Δ1-3 nukacin ISK-1 (40 and 20 µM), and **HO-1**-nukacin ISK-1 (40, 20, 10, and 5 µM). The plates were incubated at 30 ºC for 18 h.

**Purification of LanA analogues and final yields of lantibiotics**

$His_6$-mLctA. RP-HPLC: $R_t$ = 29.0-34.0 min (C4, Waters Delta-pak™).
Boc-**HO-1**-mLctA. RP-HPLC: $R_t$ = 32.7-35.7 min (C4, Waters Delta-pak™).
**HO-2**-mLctA. RP-HPLC: $R_t$ = 34.0-37.0 min (C4, Waters Delta-pak™).
**HO-3**-mLctA. RP-HPLC: $R_t$ = 31.0-36.0 min (C4, Waters Delta-pak™).
$His_6$-mNukA. RP-HPLC: $R_t$ = 25.5-26.5 min (C5, Phenomenex (00G-4092-N0)).
Boc-**HO-1**-mNukA. RP-HPLC: $R_t$ = 27.5-28.5 min (C5, Phenomenex (00G-4092-N0)).
Δ1-lacticin 481. Yield: 0.16 mg/L culture; RP-HPLC: $R_t$ = 27.9-28.3 min (C18, Phenomenex (00G-4253-N0)).
**HO-1**-lacticin 481. Yield: 0.10 mg/L culture; RP-HPLC: $R_t$ = 27.2-28.2 min (C18, Phenomenex).
**HO-2**-lacticin 481. Yield: 0.04 mg/L; RP-HPLC: $R_t$ = 31.8-32.3 min (C18, Phenomenex).
**HO-3**-lacticin 481. Yield: 0.045 mg/L; RP-HPLC: $R_t$ = 30.4-31.0 min (C18, Phenomenex).
Δ1-3-nukacin ISK-1. Yield: 0.5 mg/L RP-HPLC: $R_t$ = 29.0-30.0 min (C18, Phenomenex).
**HO-1**-nukacin ISK-1. Yield: 0.05 mg/L; RP-HPLC: $R_t$ = 23.2-23.8 min (C18, Phenomenex).

**Liquid media growth assays to determine antibiotic $IC_{50}$ and MIC values**

Ninety six-well microtiter plates (Corning Costar) were utilized for anaerobic cultures. Each well contained 90 µL of an overnight culture containing *L. lactis* HP (approximately $1\times10^8$ CFU $mL^{-1}$) and a 10x stock of peptide (10 µL) of the desired concentration. In addition, each plate contained several blank (growth media with no bacteria) and control (sterile deionized water in place of peptide) wells. The plates were incubated at 30 ºC without agitation. The optical density at 600 nm ($OD_{600}$) was recorded after 16 h for lacticin 481 and analogues and 18 h for nukacin ISK-1 and analogues. The MIC was determined as the lowest concentration at which no cell growth was observed after 16-18 h.

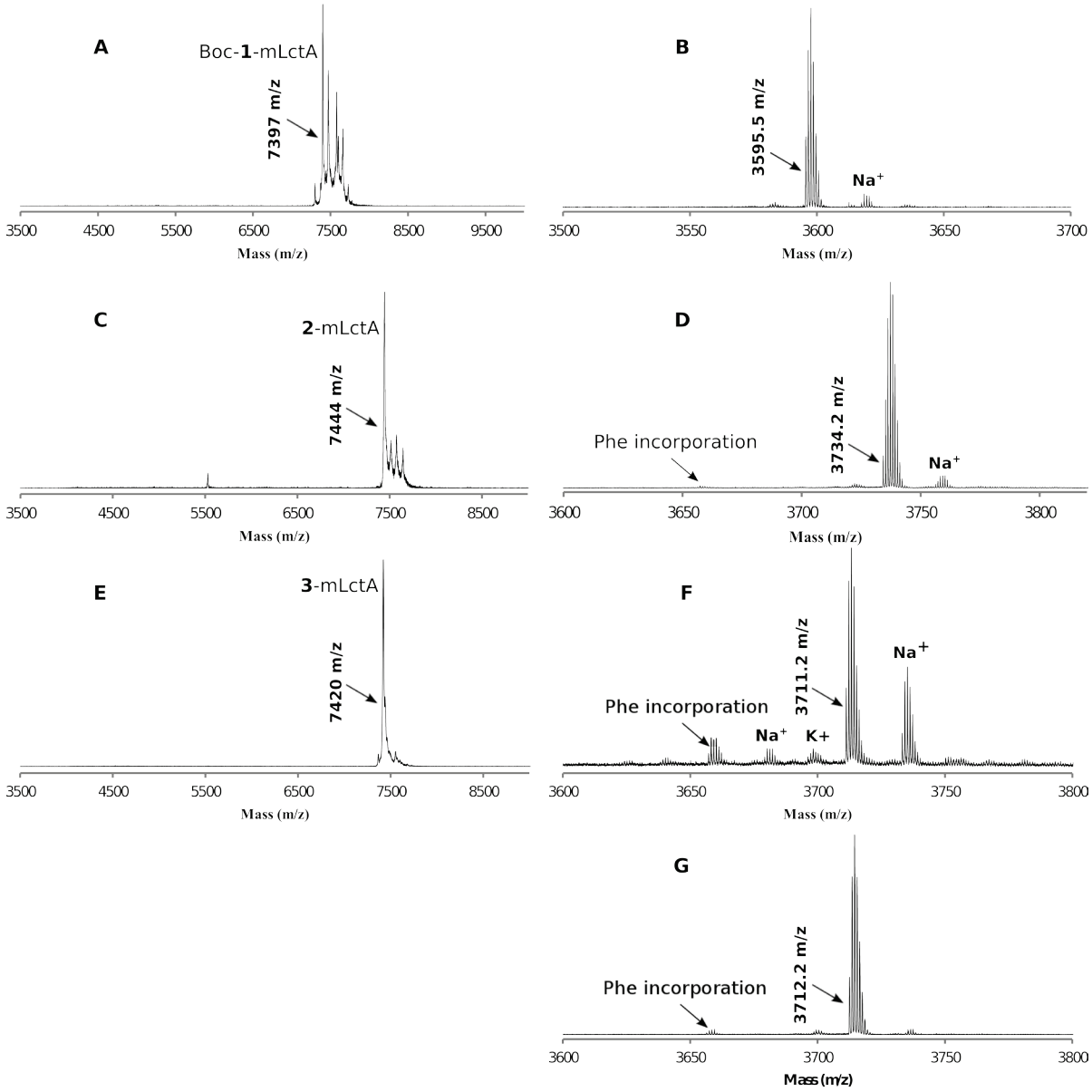



**Figure S1**. MALDI-TOF MS spectra of full-length (left panels) and GluC-cleaved $His_6$-LctA(K1X) (right panels). The protease GluC hydrolyzes the amide bond immediately C-terminal to Glu–8 yielding **X**-mLctA(–7-27). The modification of the LctA core region can be analyzed more accurately in this shorter peptide than in the full-length precursor peptide because gluconylation and phosphogluconylation adducts to the N-terminal His-tag are removed and monoisotopic masses can be observed.[11] No proteolysis occurs after Glu13 in the LctA core peptide because the lanthionine rings protect the residue. A) Boc-**1**-mLctA ($[M+H]^+$ calc'd = 7389 Da). B) GluC-cleaved Boc-**1**-mLctA ($[M+H]^+$ calc'd = 3595.7-*0* Da). C) **2**-mLctA ($[M+H]^+$ calc'd = 7428 Da). D) GluC-cleaved **2**-mLctA ($[M+H]^+$ calc'd = 3734.7-*0* Da). E) **3**-mLctA ($[M+H]^+$ calc'd = 7403 Da). F) GluC-cleaved **3**-mLctA ($[M+H]^+$ calc'd = 3710.7-*0* Da). G) GluC-cleaved **HO-3**-mLctA ($[M+H]^+$ calc'd = 3711.7-*0* Da). The spectra in panels A, C, and E present average masses whereas the spectra in panels B, D, F, and G present monoisotopic masses (*0*). The isotope pattern of panel D also clearly shows the presence of a bromine atom. The peaks labeled $Na^+$ and $K^+$ represent a sodium and potassium adduct respectively. The mass difference between calculated and observed in C and E is likely due to oxidation of the His-tag or leader peptide. This mass difference disappears after leader peptide removal.

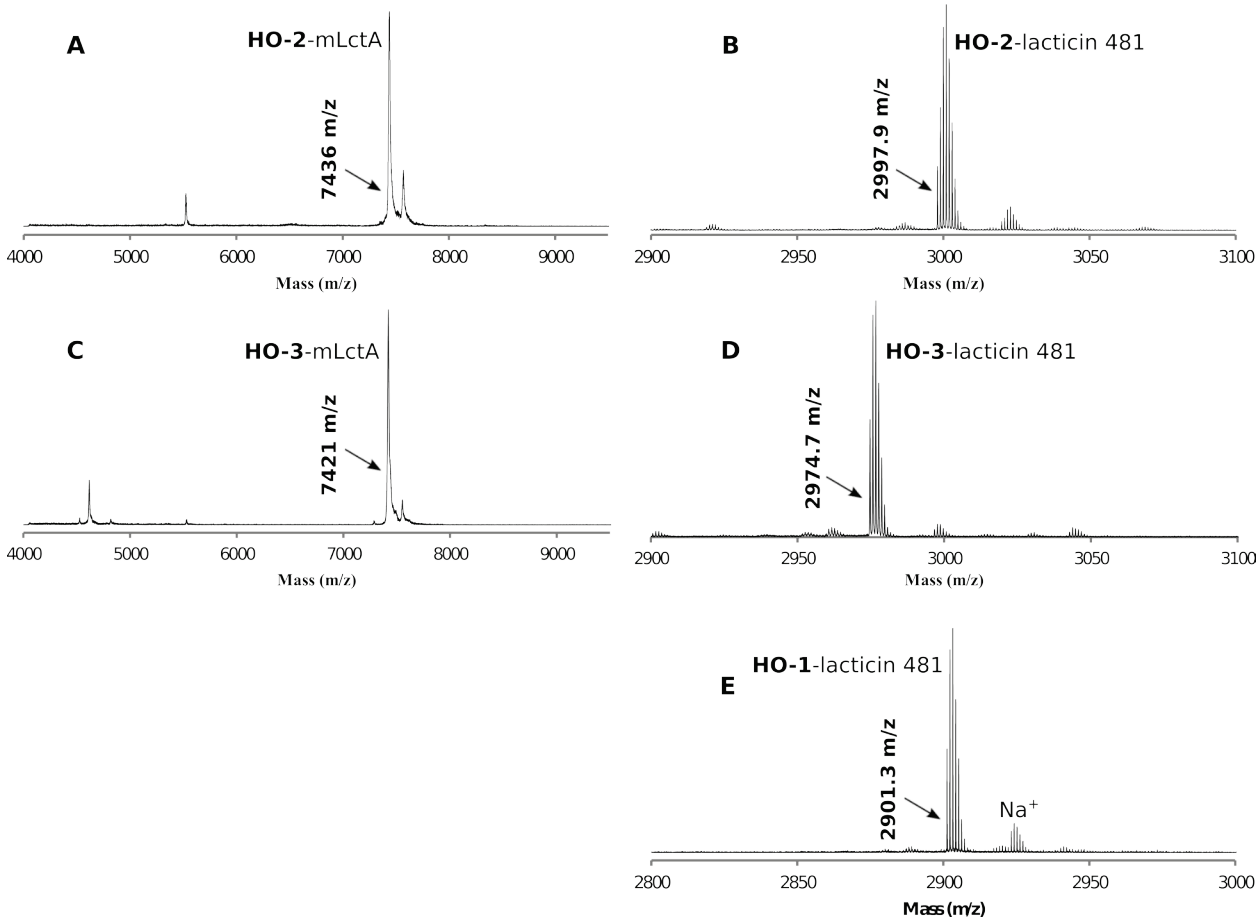
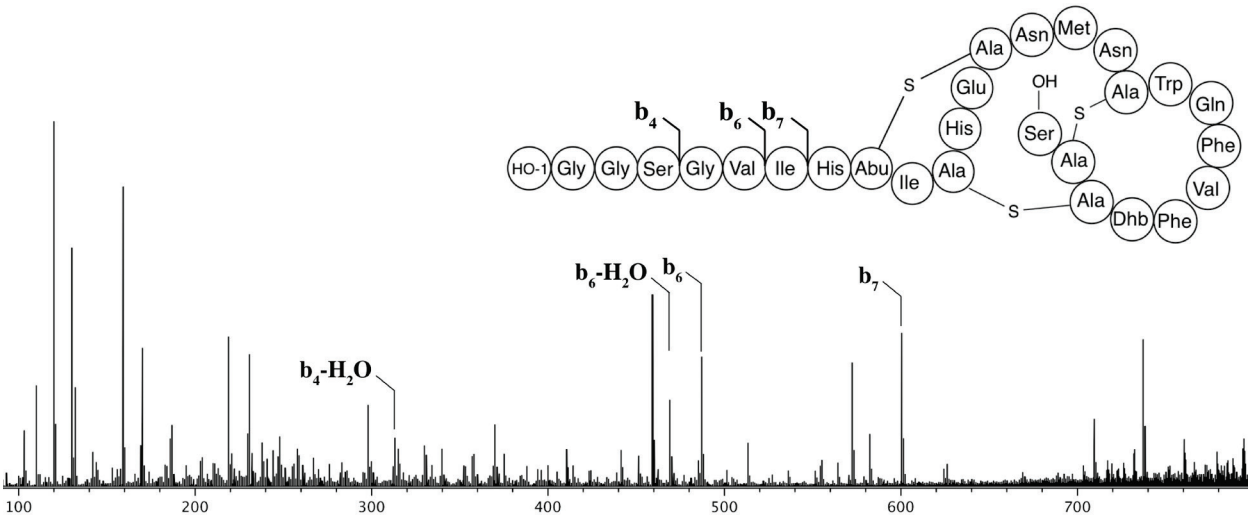
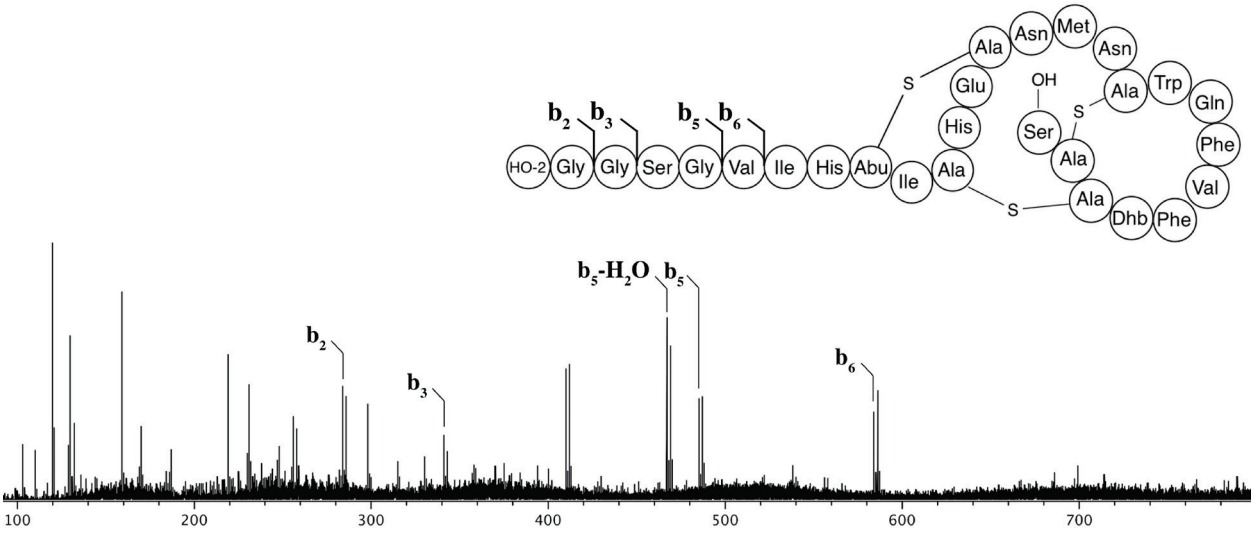



**Figure S2**. MALDI-TOF MS spectra of full-length (left panels) and base-hydrolyzed (right panels) LctM-modified $His_6$-LctA(A–1I/K1X) incorporating **HO-2** or **HO-3**. A) **HO-2**-mLctA ($[M+H]^+$ calc'd = 7429 Da). B) Base-hydrolyzed **HO-2**-mLctA ($[M+H]^+$ calc'd = 2998.1-*0* Da). C) **HO-3**-mLctA ($[M+H]^+$ calc'd = 7404 Da). D) Base-hydrolyzed **HO-3**-mLctA ($[M+H]^+$ calc'd = 2974.2-*0* Da). E) Base-hydrolyzed **HO-1**-mLctA ($[M+H]^+$ calc'd = 2901.3-*0* Da). The spectra in panels A and C present average masses whereas the spectra in panels B, D, and E present monoisotopic masses. The latter are inconsistent with an N-terminal amine but fully consistent with an N-terminal hydroxyl group. The isotope pattern of **HO-2-**lacticin 481 also clearly demonstrates the presence of a bromine atom. The peak labeled $Na^+$ represents a sodium adduct.

**A**


HO-1 Gly Gly Ser Gly Val Ile His Abu Ile Ala Ala Dhb Phe Val Phe Gln Trp Ala Asn Met Asn Ala Glu His Ser Ala S OH
b4 b6 b7
b6-H2O b6 b7
b4-H2O
100 200 300 400 500 600 700

**B**


HO-2 Gly Gly Ser Gly Val Ile His Abu Ile Ala Ala Dhb Phe Val Phe Gln Trp Ala Asn Met Asn Ala Glu His Ser Ala S OH
b2 b3 b5 b6
b5-H2O b5
b2
b3
b6
100 200 300 400 500 600 700

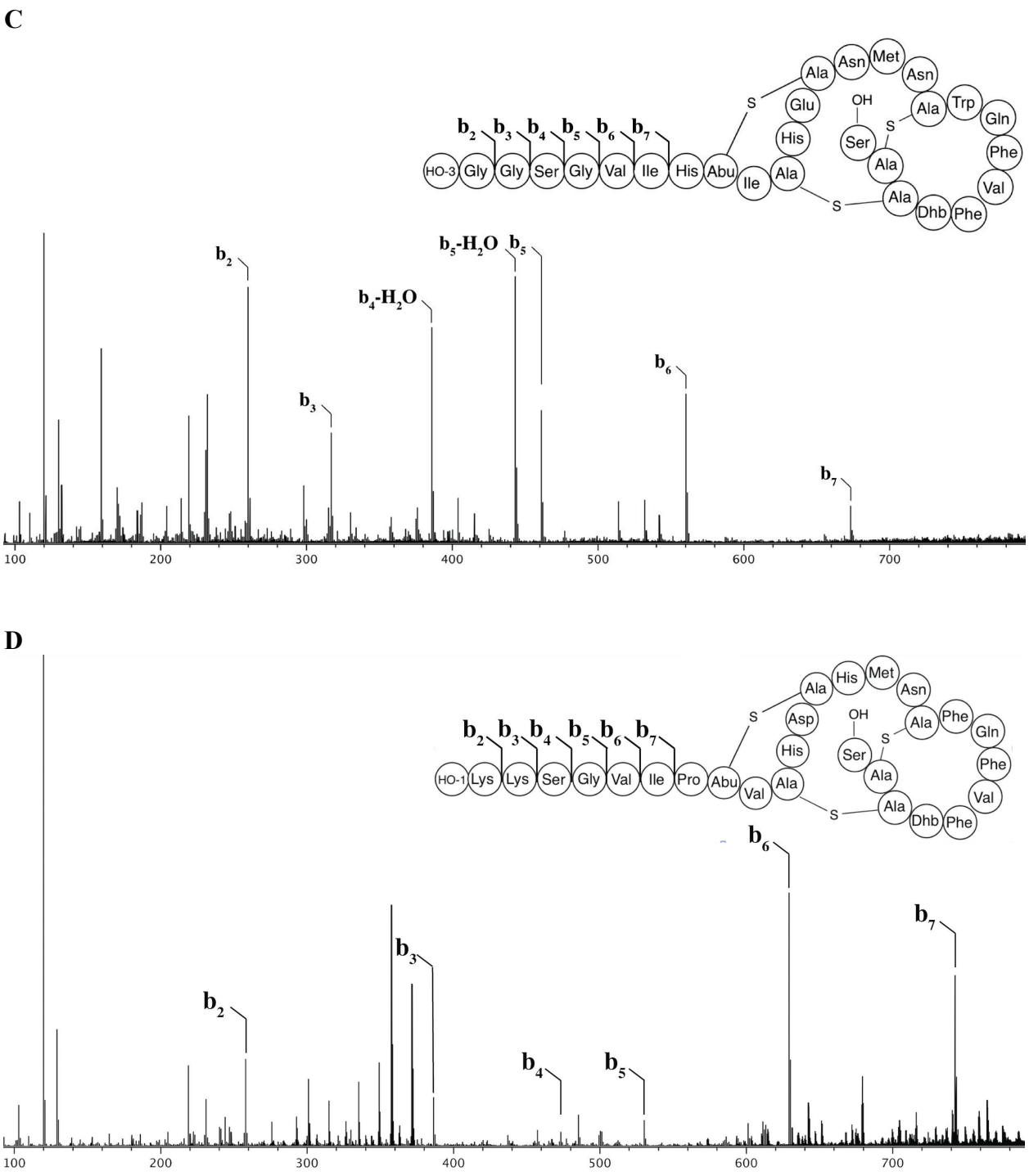
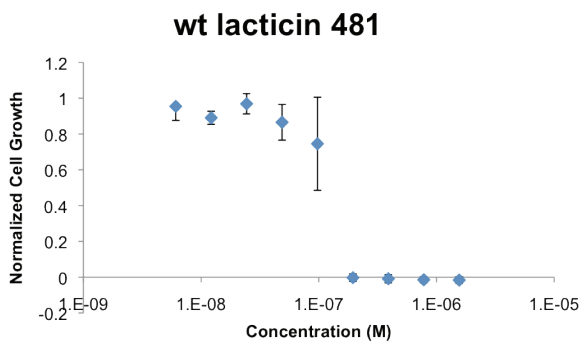
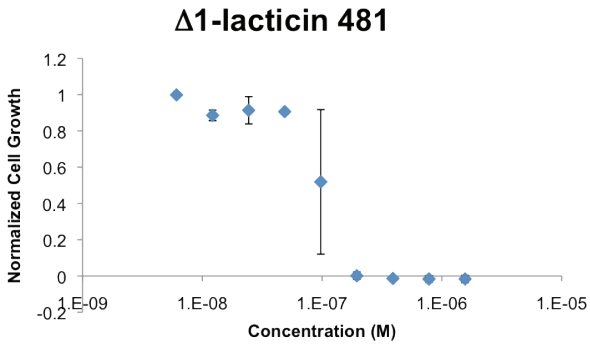


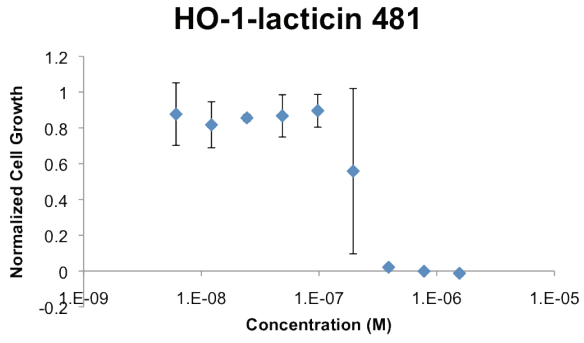

**Figure S3.** Tandem MS fragmentation data of A) **HO-1**-lacticin 481, B) **HO-2**-lacticin 481, C) **HO-3**-lacticin 481, and D) **HO-1**-nukacin ISK-1. Each annotated ion is within 10 ppm of the theoretical mass. The isotope pattern of the peaks of **HO-2-**lacticin 481 also clearly demonstrates the presence of a bromine atom.



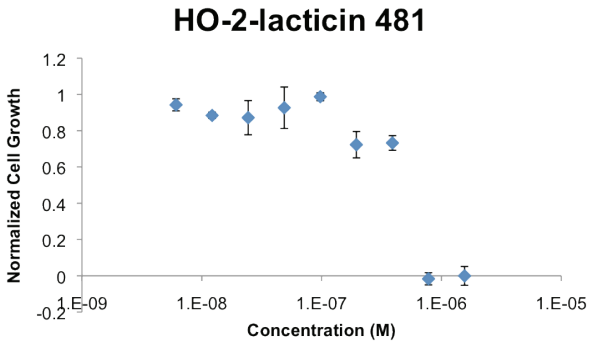

**wt lacticin 481**
$IC_{50}$ = 100 ± 25 nM
MIC = 195 nM



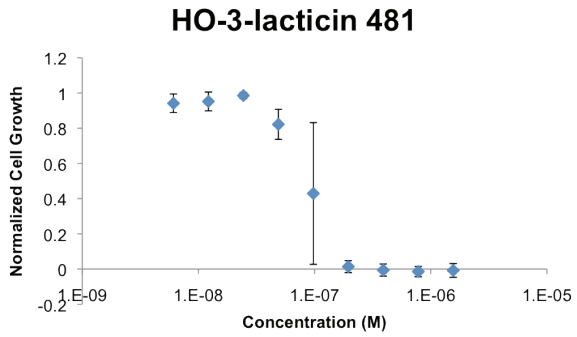

**Δ1-lacticin 481**
$IC_{50}$ = 90 ± 20 nM
MIC = 195 nM



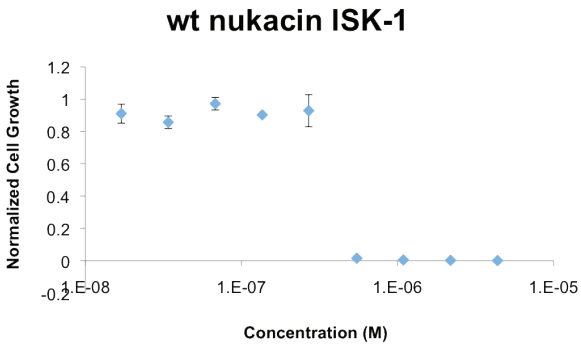

**HO-1-lacticin 481**
$IC_{50}$ = 200 ± 70 nM
MIC = 390 nM



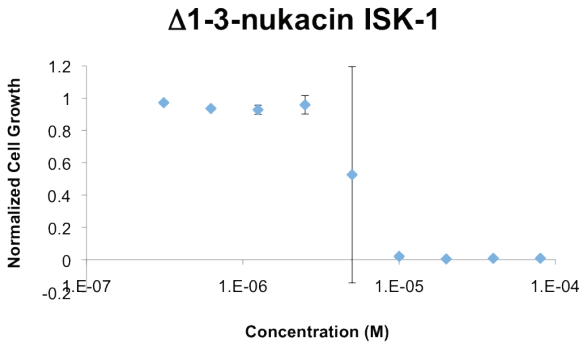

**HO-2-lacticin 481**
$IC_{50}$ = 3.50 ± 0.150 μM
MIC = 6250 nM



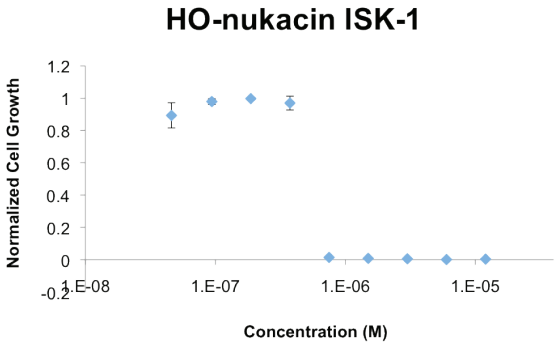

**HO-3-lacticin 481**
$IC_{50}$ = 700 ± 280 nM
MIC = 1560 nM




**wt nukacin ISK-1**
$IC_{50}$ = 400 ± 80 nM
MIC = 550 nM




**Δ1-3-nukacin ISK-1**
$IC_{50}$ = 5900 nM ± 2500 nM
MIC = 10000 nM




**HO-nukacin ISK-1**
$IC_{50}$ = 560 nM ± 100 nM
MIC = 750 nM

**Figure S4.** Liquid media growth assays to determine the antimicrobial activities of wt lacticin 481, nukacin ISK-1, and mutant peptides against *L. lactis* HP. Each data point represents the average of multiple assays. The IC50 value for each growth assay was computed using the dose response calculation on OriginPro. Each reported $IC_{50}$ value is the average of the triplicate runs for lacticin and duplicate runs for nukacin ISK-1 with the standard deviation provided as the error. The $IC_{50}$ value for Δ1-lacticin 481 in this manuscript is lower than previously reported.[12] However, in that manuscript Δ1-lacticin 481 was synthesized as an N15R/F21H mutant, rather than the wt sequence 481 that was used here.

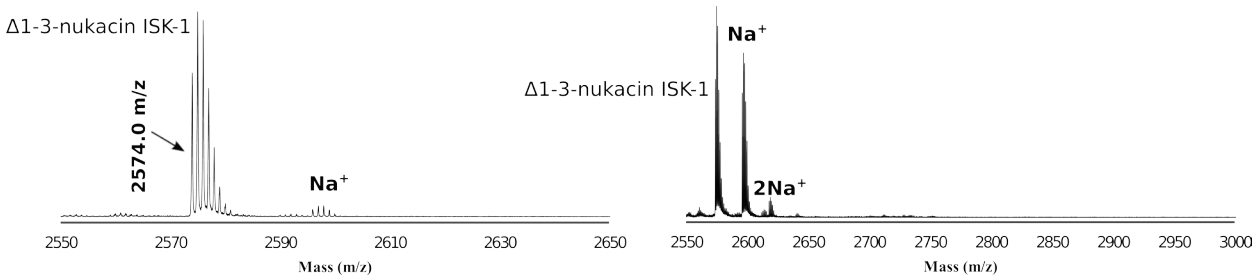



**Figure S5.** Biosynthesis of Δ1-3-nukacin ISK-1 in *E. coli*. Left, Δ1-3-nukacin ISK-1 $[M+H]^+$ calc'd = 2574.1-*0* Da (monoisotopic mass). Coexpression cultures containing a pRSFDuet plasmid encoding NukA and NukM were grown at 18 ºC after IPTG induction. The peak labeled $Na^+$ represents a sodium adduct. Right, N-ethylmaleimide (NEM) assay to probe for free cysteines on biosynthesized Δ1-3-nukacin ISK-1. NEM adduct = +125 Da. No NEM adducts were observed by MALDI-TOF MS signifying three successful cyclizations. Both panels represent monoisotopic masses.

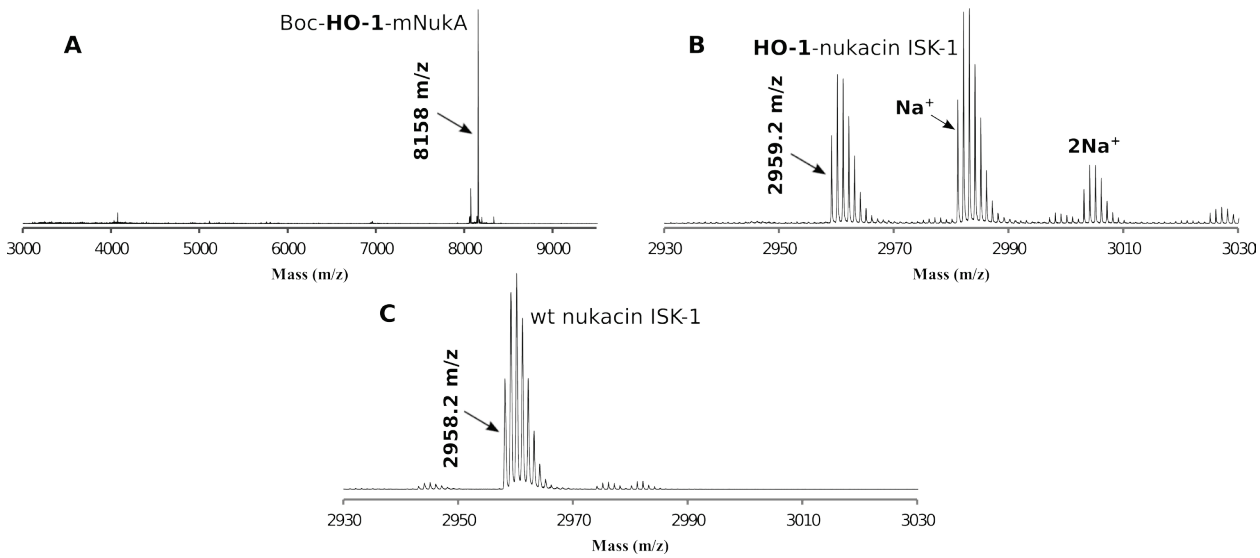



**Figure S6.** Incorporation of **HO-1** into $His_6$-NukA(A–1I/K1X). A) Boc-**HO-1**-mNukA(A–1I/K1X) ($[M+H]^+$ calc'd = 8157 Da; average mass), B) Boc-deprotected and hydrolyzed **HO-1**-mNukA(A–1I/K1X) producing **HO-1**-nukacin ISK-1 ($[M+H]^+$ calc'd = 2959.3 Da, monoisotopic mass). $Na^+$ represents a sodium adduct. C) Authentic nukacin ISK-1 ($[M+H]^+$ calc'd = 2958.3-*0* Da, monoisotopic mass).

**References for Supporting Information**

(1) Weijak, S.; Masiukiewicz, E.; Rzeszotarska, B. *Chem. Pharm. Bull.* **1999**, *47*, 1489.
(2) Deechongkit, S.; You, S. L.; Kelly, J. W. *Org. Lett.* **2004**, *6*, 497.
(3) Li, Y. M.; Yang, M. Y.; Huang, Y. C.; Li, Y. T.; Chen, P. R.; Liu, L. *ACS Chem. Biol.* **2012**, *7*, 1015.
(4) Zakharova, V. M.; Serpi, M.; Krylov, I. S.; Peterson, L. W.; Breitenbach, J. M.; Borysko, K. Z.; Drach, J. C.; Collins, M.; Hilfinger, J. M.; Kashemirov, B. A.; McKenna, C. E. *J. Med. Chem.* **2011**, *54*, 5680.
(5) Zhang, Z.; Yin, L.; Xu, Y.; Tong, R.; Lu, Y.; Ren, J.; Cheng, J. *Biomacromolecules* **2012**, *13*, 3456.
(6) Gibson, D. G.; Young, L.; Chuang, R. Y.; Venter, J. C.; Hutchison, C. A., 3rd; Smith, H. O. *Nat Methods* **2009**, *6*, 343.
(7) Oman, T. J.; Knerr, P. J.; Bindman, N. A.; Velasquez, J. E.; van der Donk, W. A. *J. Am. Chem. Soc.* **2012**, *134*, 6952.
(8) Patton, G. C.; Paul, M.; Cooper, L. E.; Chatterjee, C.; van der Donk, W. A. *Biochemistry* **2008**, *47*, 7342.
(9) Wang, Y. S.; Fang, X.; Wallace, A. L.; Wu, B.; Liu, W. R. *J. Am. Chem. Soc.* **2012**, *134*, 2950.
(10) Aso, Y.; Okuda, K.; Nagao, J.; Kanemasa, Y.; Thi Bich Phuong, N.; Koga, H.; Shioya, K.; Sashihara, T.; Nakayama, J.; Sonomoto, K. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1403.
(11) Geoghegan, K. F.; Dixon, H. B.; Rosner, P. J.; Hoth, L. R.; Lanzetti, A. J.; Borzilleri, K. A.; Marr, E. S.; Pezzullo, L. H.; Martin, L. B.; LeMotte, P. K.; McColl, A. S.; Kamath, A. V.; Stroh, J. G. *Anal. Biochem.* **1999**, *267*, 169.
(12) Knerr, P. J.; Oman, T. J.; Garcia De Gonzalo, C. V.; Lupoli, T. J.; Walker, S.; van der Donk, W. A. *ACS Chem. Biol.* **2012**, *7*, 1791.