Supporting Information

In Vitro Mutasynthesis of Lantibiotic Analogues Containing Nonproteinogenic Amino Acids

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Materials. Fmoc amino acids and resins were purchased from Advanced ChemTech, Novabiochem, or Chem-Impex, with the exception of Fmoc-*N*-butyl glycine, which was synthesized according to published procedures. The nonproteinogenic amino acids used in this report are shown in Figure S1 below. HOBt (1-hydroxybenzotriazole), DIC (N,N' diisopropylcarbodiimide) and HBTU (O-(1H-benzotriazole-1-yl)-*N*,*N*,*N'*,*N'*-tetramethylammoniumhexafluorophosphate) were purchased from Advanced ChemTech. Solvents commonly used in peptide synthesis and purification, including dimethylformamide (DMF), trifluoroacetic acid (TFA), and acetonitrile (MeCN) were obtained in HPLC grade or better and used directly without further purification. Piperidine, pyridine, morpholine, and diisopropylethylamine (DIPEA) were purchased from Acros. Endoproteinase LysC was obtained from Roche Biosciences. 2-Mercaptoethanol (BME) was obtained from Aldrich and tris-(2-carboxyethyl)phosphine (TCEP) was obtained from Molecular Probes as the TCEP-HCl salt. The ligand *tris*-(benzyltriazolylmethyl)amine (TBTA)[1] and 6-azido hexanoic acid[2] were synthesized according to published procedures. His_6-LctA Asn15Arg/Phe21His, required for IVM to provide **4**, was overexpressed in *E. coli* and purified as previously reported.[3]

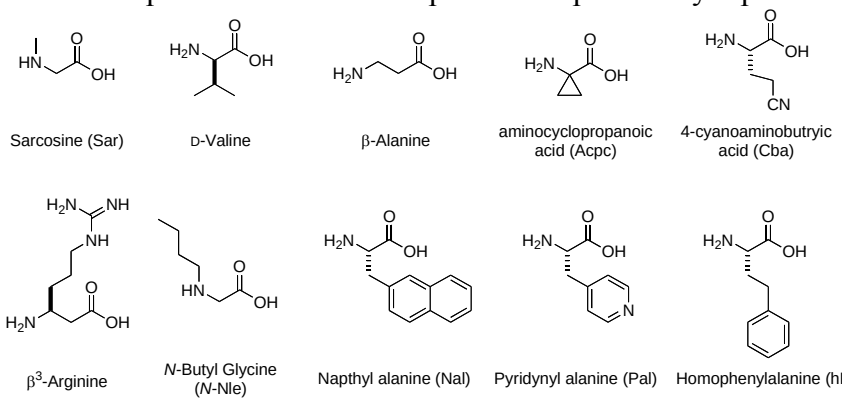

Figure S1. Structures and nomenclature of the nonproteinogenic amino acids utilized in this report.

Solid-phase peptide synthesis. Peptides were synthesized using standard Fmoc solid phase chemistry using an automated peptide synthesizer (either Aapptec 396 or Rainin PS3 systems). Pre-loaded Wang resin (0.1 mmol) and Fmoc-amino acids (0.4 mmol) were used. Resins were first swollen in DMF (8 mL) for 30 min. Fmoc-amino acids were coupled using *O*-benzotriazole-*N,N,N',N'*,-tetramethyl-uroniumhexafluoro-phosphate (HBTU, 0.4 mmol) as coupling reagent with *N*-methylmorpholine (NMM,

4.4% in DMF, 8 mL) as activating reagent. Fmoc deprotections were performed using piperidine (4 x 3 min, 20% in DMF, 8 mL). Couplings were performed from 45 min to 3 h and coupling efficiency was monitored by the Kaiser test.[4] To minimize racemization during couplings, Fmoc-Cys derivatives (0.4 mmol) were coupled using 1-hydroxybenzotriazole (HOBT, 0.4 mmol) and *N,N'*-diisopropylcarbodiimide (DIC, 0.4 mmol) in DMF (8 mL).[5] For the synthesis of N-terminally azide-functionalized peptides, 6-azido hexanoic acid (0.4 mmol) was coupled using HOBT (0.4 mmol) and DIC (0.4 mmol). Unless stated otherwise, the terminal Fmoc group was deprotected in the last step of the synthesis followed by washing of the resin with DMF (5 x 10 mL), ethanol (5 x 10 mL), and CH_2Cl_2 (5 x 10 mL) and drying for 4-6 h in a vacuum dessicator. Peptide cleavage from the resin was achieved by stirring the resin in a mixture of TFA (10 mL), water (150 μL), ethanedithiol (150 μL), and triisopropylsilane (150 μL) for 1.5 h at room temperature. The resin was then filtered and the filtrate concentrated under reduced pressure. The concentrated crude peptide was washed with cold diethyl ether (3 x 10 mL) and lyophilized from 10% aqueous acetic acid following filtration through a 0.45 μm filter. The lyophilized crude peptides were purified by preparative RP-HPLC on a Waters Delta-Pak™ C18 column (2.5 cm x 10.0 cm) employing a water-acetonitrile solvent system. Analytical RP-HPLC separations were conducted on a Beckman System Gold instrument with a Vydac C4 or C18 analytical column. The standard HPLC separation conditions employed a gradient from 2-100% of solvent B over 45 min (A: H_2O, 0.1% TFA; B: 80% CH_3CN in H_2O v/v, 0.086% TFA). Peptides were detected by their absorbance at 220 nm. Fractions were lyophilized and those containing product analyzed by either ESI-TOF or MALDI-TOF MS in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois at Urbana-Champaign.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Met16βAla** Cys14, Cys25, Cys26 (StBu)*. R_t = 32.3-34.3 min. LRMS (ESI): calcd $C_{142}H_{223}N_{43}O_{39}S_6$ [M+H] 3347.5, observed 3347.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Glu13Cba** Cys14, Cys25, Cys26 (StBu)*. R_t = 34.4-35.6 min. LRMS (MALDI): calcd $C_{144}H_{226}N_{44}O_{37}S_7$ [M+H] 3388.52, observed 3393.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Gly5Sar** Cys14, Cys25, Cys26 (StBu)*. R_t = 33.4-34.3 min. LRMS (MALDI): calcd $C_{145}H_{229}N_{43}O_{39}S_7$ [M+H] 3421.53, observed 3426.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/**Phe21Pal** Cys14, Cys25, Cys26 (StBu)*. R_t = 32.8-34.1 min. LRMS (MALDI): calcd $C_{146}H_{228}N_{42}O_{39}S_7$ [M+H] 3418.52, observed 3423.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Gly5Acpc** Cys14, Cys25, Cys26 (StBu)*. R_t = 33.1-34.3 min. LRMS (MALDI): calcd $C_{146}H_{229}N_{43}O_{39}S_7$ [M+H] 3433.53, observed 3437.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Val6D-Val** Cys14, Cys25, Cys26 (StBu)*. R_t = 32.9-34.6 min. LRMS (MALDI): calcd $C_{144}H_{227}N_{43}O_{39}S_7$ [M+H] 3407.51, observed 3412.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Phe23homoPhe** Cys14, Cys25, Cys26 (StBu)*. R_t = 33.8-35.0 min. LRMS (MALDI): calcd $C_{145}H_{229}N_{43}O_{39}S_7$ [M+H] 3421.53, observed 3422.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Met16N-Nle** Cys14, Cys25, Cys26 (StBu)*. R_t = 33.3-34.8 min. LRMS (MALDI): calcd $C_{145}H_{229}N_{43}O_{39}S_6$ [M+H] 3389.56, observed 3392.

*6-Azido hexanoic acid-modified LctA(1-27) **Asn15β³-Arg**/Phe21His Cys14, Cys25, Cys26 (StBu)*. R_t = 32.7-33.8 min. LRMS (MALDI): calcd $C_{145}H_{229}N_{43}O_{39}S_7$ [M+H] 3421.53, observed 3421.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Trp19Nal** Cys14, Cys25, Cys26 (StBu*). R_t = 34.3-35.8 min. LRMS (MALDI): calcd $C_{146}H_{228}N_{42}O_{39}S_7$ [M+H] 3418.52, observed 3418.

*6-Azido hexanoic acid-modified LctA(1-27) Asn15Arg/Phe21His/**Gly2β-Ala/Gly3β-Ala/Gly5β-Ala** Cys14, Cys25, Cys26 (StBu)*. R_t = 32.4 min. LRMS (MALDI): calcd $C_{147}H_{233}N_{43}O_{39}S_7$ [M+H] 3449.56, observed 3451.

$^{-23}$ $^{-1}$

Synthesis of *1*. LctA leader peptide (sequence: KEQNSFNLLQEVTESELDLILGG) was synthesized on pre-loaded H-Gly-2'-chlorotrityl resin by traditional Fmoc-based solid-phase peptide synthesis on an automated peptide synthesizer, as described above. The N-terminal Fmoc-protecting group was not removed prior to cleavage from the resin. Cleavage of the fully-protected peptide from the resin was accomplished by stirring the resin in an 8:1:1 mixture of CH_2Cl_2:trifluoroethanol:acetic acid for 45 min. The solvents were removed via rotary evaporation. Acetic acid was removed by repeated rotary evaporation with hexanes (5 x 5 mL). The crude product was suspended in a 1:1 mixture of DMF and THF (10 mL total volume) and to this was added a mixture of 3-butyn-1-amine hydrochloride (4 equiv., AB Chem Inc.), diisopropylethylamine (DIPEA, 4 equiv.), HOBT (4 equiv.), and DIC (4 equiv.), with stirring continuing for 4-6 h at room temperature. Solvent was then removed by rotary evaporation and a 20% piperidine/DMF mixture was added to remove the terminal Fmoc protecting group. Stirring was continued for 30 min, followed by removal of piperidine and DMF under reduced pressure. Side-chain protecting groups were then removed by stirring the crude product in a 87.5:5:5:2.5 mixture of TFA:water:ethanedithiol:TIPS for 1.5-2 h. Solvent was removed by rotary evaporation, which was followed by trituration of the peptide with cold diethyl ether. The peptide was redissolved in 0.5% aqueous TFA/MeCN and purified by preparative C18 RP-HPLC and the mass verified by MALDI-TOF MS analysis. LRMS (MALDI): calcd $C_{116}H_{187}N_{29}O_{40}$ [M+H] 2627.35; observed 2629.

Preparation of triazole-linked substrates (*3*). An equimolar ratio of azide- and alkyne-modified HPLC-purified peptides (0.2-6.0 mg each) were dissolved in 0.5-2.0 mL of a 50% mixture of dioxane and 5 mM TRIS buffer, pH 7.0 and sparged with N_2. In a separate flask, 0.1-0.5 mg $CuSO_4$ and 0.1-0.5 mg of *tris*-(benzyltriazolylmethyl)amine (TBTA) were dissolved in 0.50 mL of a 50% mixture of dioxane and 5 mM TRIS, pH 7.0 and sparged with N_2. To the cupric solution was added 10-20 μL of anhydrous hydrazine by syringe to form the active Cu(I) species. This resulted in a color change from light blue to light brown. The cuprous solution was cannulated into the peptide solution and the mixture stirred for 45 min to 2 h. The crude reaction mixture was acidified to pH 1-2 with 1 M HCl prior to preparative C18 RP-HPLC purification.

*Triazole-linked LctA(−23-27)Asn15Arg/**Phe21Pal** Cys14, Cys25, Cys26 (StBu)*. R_t = 33.5-35.2 min. LRMS (MALDI): calcd $C_{262}H_{415}N_{71}O_{79}S_7$ [M+H] 6044.82, observed 6049.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Gly5Acpc** Cys14, Cys25, Cys26 (StBu)*. R_t = 34.9-36.2 min. LRMS (MALDI): calcd $C_{262}H_{416}N_{72}O_{79}S_7$ [M+H] 6059.88, observed 6064.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Glu13Cba** Cys14, Cys25, Cys26 (StBu)*. R_t = 35.5-36.7 min. LRMS (MALDI): calcd $C_{260}H_{413}N_{73}O_{77}S_7$ [M+H] 6014.87, observed 6019.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Met16β-Ala** Cys14, Cys25, Cys26 (StBu)*. R_t = 33.6-35.0 min. LRMS (MALDI): calcd $C_{258}H_{410}N_{72}O_{79}S_6$ [M+H] 5973.85, observed 5978.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Gly5Sar** Cys14, Cys25, Cys26 (StBu)*. R_t = 35.1-36.6 min. LRMS (MALDI): calcd $C_{261}H_{416}N_{72}O_{79}S_7$ [M+H] 6047.88, observed 6050.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Val6D-Val** Cys14, Cys25, Cys26 (StBu)*. R_t = 34.5-35.5 min. LRMS (MALDI): calcd $C_{260}H_{414}N_{72}O_{79}S_7$ [M+H] 6033.86, observed 6034.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Met16N-Nle** Cys14, Cys25, Cys26 (StBu)*. R_t = 34.4-36.1 min. LRMS (MALDI): calcd $C_{261}H_{416}N_{72}O_{79}S_6$ [M+H] 6015.91, observed 6021.

*Triazole-linked LctA(−23-27)**Asn15β³-Arg**/Phe21His Cys14, Cys25, Cys26 (StBu)*. R_t = 34.3-35.9 min. LRMS (MALDI): calcd $C_{261}H_{416}N_{72}O_{79}S_7$ [M+H] 6047.88, observed 6051.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Trp19Nal** Cys14, Cys25, Cys26 (StBu)*. R_t = 35.1-36.2 min. LRMS (MALDI): calcd $C_{262}H_{415}N_{71}O_{79}S_7$ [M+H] 6044.87 observed 6048.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Gly2β-Ala/Gly3β-Ala/Gly5β-Ala** Cys14, Cys25, Cys26 (StBu)*. R_t = 34.7-36.3 min. LRMS (MALDI): calcd $C_{263}H_{420}N_{72}O_{79}S_7$ [M+H] 6075.91, observed 6079.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Phe23hPhe** Cys14, Cys25, Cys26 (StBu)*. R_t = 35.6-37.3 min. LRMS (MALDI): calcd $C_{261}H_{416}N_{72}O_{79}S_7$ [M+H] 6047.88 observed 6048.

Deprotection of –StBu protected triazole-linked substrates. HPLC-purified triazole-linked LctA substrates were resuspended in 2-2.5 mL of 100 mM TRIS buffer, pH 8.0 containing 50-100 mM 2-mercaptoethanol and 10 mM TCEP. The mixtures were heated to 60 ^oC for 30 min at 150 W using a CEM Discover microwave reactor. The crude samples were acidified to pH 1-2 with 1 M HCl and purified by preparative C18 or analytical C4 RP-HPLC. The fractions containing product were analyzed by MALDI-TOF MS.

*Triazole-linked LctA(−23-27)Asn15Arg/**Phe21Pal***. R_t = 29.8-31.5 min. LRMS (MALDI): calcd $C_{250}H_{391}N_{71}O_{79}S_4$ [M+H] 5780.77, observed 5784.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Gly5Acpc***. R_t = 29.7-31.3 min. LRMS (MALDI): calcd $C_{250}H_{393}N_{72}O_{79}S_4$ [M+H] 5795.78, observed 5798.

*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Glu13Cba***. R_t = 30.3-31.9 min. LRMS (MALDI): calcd $C_{248}H_{390}N_{73}O_{77}S_4$ [M+H] 5750.77, observed 5755.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Met16β-Ala***. R_t = 29.2-30.8 min. LRMS (MALDI): calcd $C_{246}H_{391}N_{72}O_{79}S_3$ [M+H] 5709.76, observed 5712.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Gly5Sar***. R_t = 29.6-30.5 min. LRMS (MALDI): calcd $C_{249}H_{392}N_{72}O_{79}S_4$ [M+H] 5783.78, observed 5787.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Val6D-Val***. R_t = 29.3-30.9 min. LRMS (MALDI): calcd $C_{248}H_{390}N_{72}O_{79}S_4$ [M+H] 5769.76, observed 5774.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Met16N-Nle***. R_t = 29.8-31.8 min. LRMS (MALDI): calcd $C_{249}H_{392}N_{72}O_{79}S_3$ [M+H] 5751.80, observed 5754.
*Triazole-linked LctA(−23-27)**Asn15β³-Arg**/Phe21His*. R_t = 28.8-30.5 min. LRMS (MALDI): calcd $C_{249}H_{392}N_{72}O_{79}S_4$ [M+H] 5783.78, observed 5784.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Trp19Nal***. R_t = 29.8-31.4 min. LRMS (MALDI): calcd $C_{250}H_{391}N_{71}O_{79}S_4$ [M+H] 5780.77, observed 5783.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Gly2β-Ala/Gly3β-Ala/Gly5β-Ala***. R_t = 29.1-30.7 min. LRMS (MALDI): calcd $C_{251}H_{396}N_{72}O_{79}S_4$ [M+H] 5811.81, observed 5814.
*Triazole-linked LctA(−23-27)Asn15Arg/Phe21His/**Phe23hPhe***. R_t = 31.0-32.1 min. LRMS (MALDI): calcd $C_{249}H_{392}N_{72}O_{79}S_4$ [M+H] 5783.78, observed 5792.

General procedure for LctM Assays. His-LctM was overexpressed and purified as previously reported.[6] For 20 μL assays intended only for mass spectrometric analysis, a RP-HPLC fraction containing the desired synthetic peptide (Abs_{220} ~ 0.5-1.6, ca. 20-100 μg) was redissolved in 20 μL of sterile Millipore water. Typically, 2-5 μL (1-10 μg) of substrate solution was used for each assay. To the substrate was added 10-13 μL of a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 25 μg/mL BSA. His-LctM (5 μL of 0.5-2.0 μM stock solution) was added to the buffered peptide suspension and the assay was incubated for 10 min to 24 h at 25 °C. For large scale LctM assays intended for analytical HPLC purification, 0.5-1.5 mg of peptide was dissolved in 6.0 mL of a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, and 25 μg/mL BSA. His-LctM (0.1-0.6 μM final concentration) was added to the buffered solution and the assay was incubated for 1-6 h at 25 °C. Assays were analyzed by MALDI-TOF MS. The samples for MALDI-TOF MS were prepared as follows: 5 μL of assay was removed and quenched with 1.0 μL of 0.1% TFA, then loaded onto a C18 Zip-tip. The assay product was eluted using 4 μL of α–hydroxycinnamic acid matrix (prepared in 70% MeCN with 0.1% TFA). The sample was spotted on the MALDI target plate using 1.5 μL of eluent. Purification of the assay products was conducted on an analytical C4 RP-HPLC column, using the gradient elution conditions noted previously.
*LctM assay **3** containing a **Val6D-Val** mutation*. LRMS (MALDI-TOF): calcd 5770 (M), 5716 (M–54), 5698 (M–72). Observed 5699, 5718 (45%). MALDI-TOF MS analysis of this assay is depicted in Figure 2 of the main text.
*LctM assay **3** containing a **Asn15β³-Arg** mutation*. LRMS (MALDI-TOF): calcd 5784 (M), 5730 (M–54), 5712 (M–72). Observed 5716. MALDI-TOF MS analysis of this assay is depicted in Figure 2 of the main text.

LctM assay of ***3*** *containing a* ***Trp19Nal*** *mutation*. LRMS (MALDI-TOF): calcd 5781 (M), 5727 (M–54), 5709 (M–72). Observed 5710. MALDI-TOF MS analysis of this assay is depicted in Figure 2 of the main text.

LctM assay of ***3*** *containing a* ***Phe23hPhe*** *mutation.* LRMS (MALDI-TOF): calcd 5784 (M), 5730 (M–54), 5712 (M–72). Observed 5713, 5730 (40%). MALDI-TOF MS analysis of this assay is depicted in Figure 2 of the main text.

LctM assay of ***3*** *containing* ***Gly2β-Ala/Gly3β-Ala/Gly5β-Ala*** *mutations*. LRMS (MALDI-TOF): calcd 5812 (M), 5758 (M–54), 5740 (M–72). Observed 5744, 5762 (45%). MALDI-TOF MS analysis of this assay is depicted in Figure S2A.

LctM assay of ***3*** *containing a* ***Met16β-Ala*** *mutation*. LRMS (MALDI-TOF): calcd 5710 (M), 5666 (M–54), 5648 (M–72). Observed 5643, 5661 (30%). MALDI-TOF MS analysis of this assay is depicted in Figure S2B.

LctM assay of ***3*** *containing a* ***Met16N-Nle*** *mutation*. LRMS (MALDI-TOF): calcd 5752 (M), 5698 (M–54), 5680 (M–72). Observed 5685, 5702 (60%). MALDI-TOF MS analysis of this assay is depicted in Figure S2C.

LctM assay of ***3*** *containing a* ***Gly5Sar*** *mutation*. LRMS (MALDI-TOF): calcd 5784 (M), 5730 (M–54), 5712 (M–72). Observed 5712. MALDI-TOF MS analysis of this assay is depicted in Figure S2D.

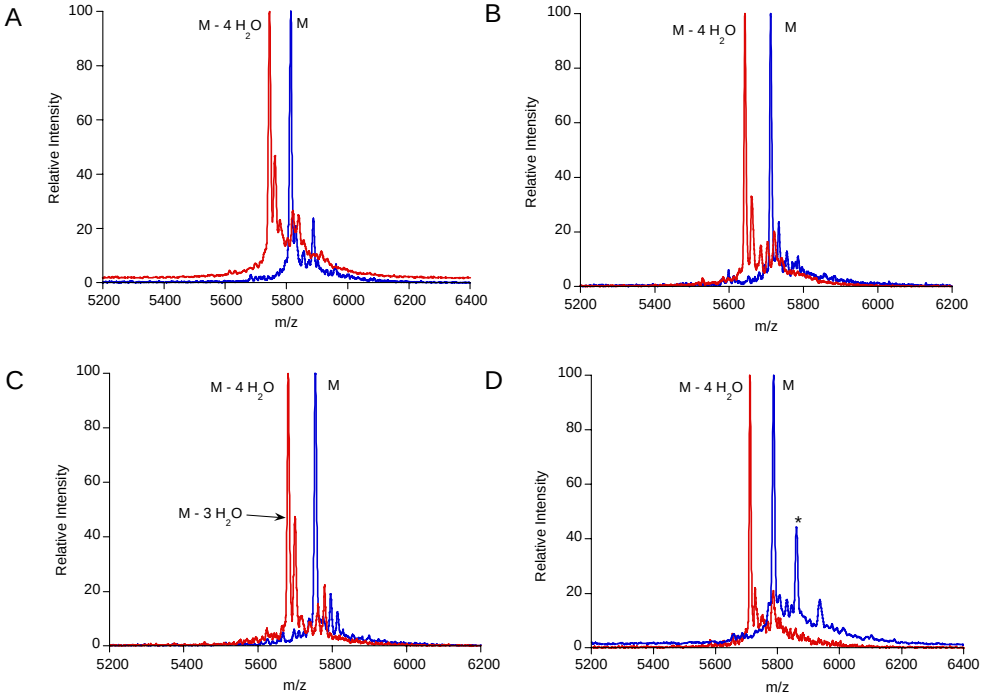

Figure S2. MALDI-TOF mass spectrum following incubation of **3** containing (A) Gly2/Gly3/Gly5β-Ala, (B) Met16β-Ala, (C) Met16*N*-Nle, and (D) Gly5Sar mutations with LctM in the presence of Mg^{2+} and ATP. For each assay, the starting materials are shown in blue and the products following incubation with LctM are depicted in red. In assay D, an asterisk marks an unknown impurity.

*LctM assay of **3** containing a **Glu13Cba** mutation*. LRMS (MALDI-TOF): calcd 5751 (M), 5697 (M–54), 5679 (M–72). Observed 5675, 5692 (40%). MALDI-TOF MS analysis of this assay is depicted in Figure S3A.
*LctM assay of **3** containing a **Gly5Acpc** mutation*. LRMS (MALDI-TOF): calcd 5796 (M), 5742 (M–54), 5724 (M–72). Observed 5725, 5741 (25%). MALDI-TOF MS analysis of this assay is depicted in Figure S3B.
*LctM assay of **3** containing a **Phe21Pal** mutation*. LRMS (MALDI-TOF): calcd 5781 (M), 5727 (M–54), 5709 (M–72). Observed 5709, 5727 (65%). MALDI-TOF MS analysis of this assay is depicted in Figure S3C.

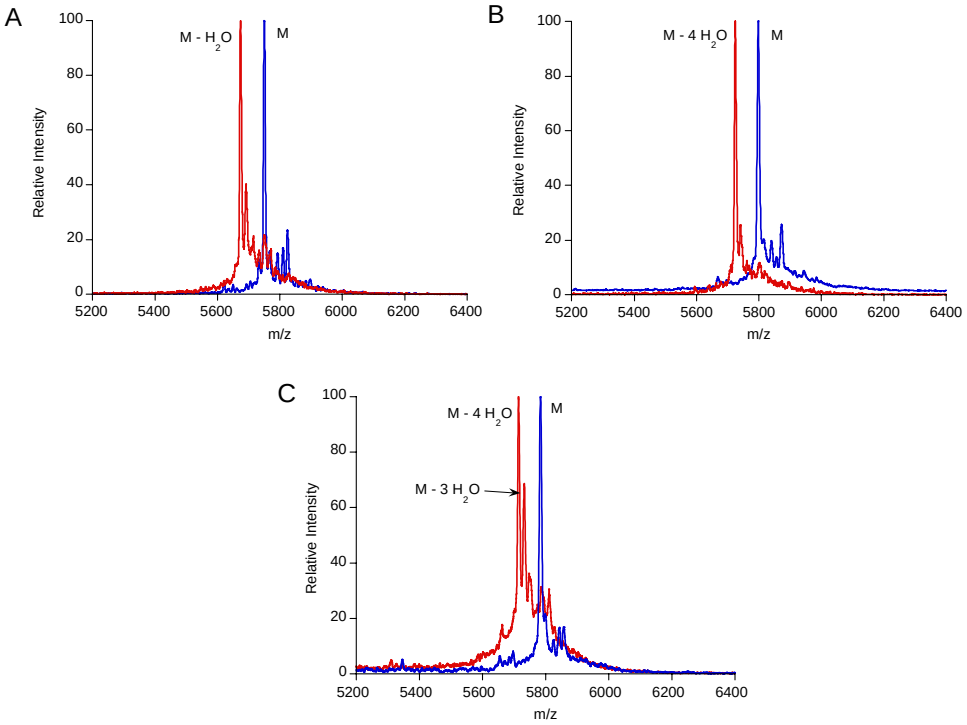

Figure S3. MALDI-TOF mass spectrum following incubation of **3** containing (A) Glu13Cba, (B) Gly5Acpc, and (C) Phe21Pal mutations with LctM in the presence of Mg^{2+} and ATP. For each assay, the starting materials are shown in blue and the products following incubation with LctM are depicted in red.

LysC cleavage of LctM-modified peptides. To HPLC-purified LctM-treated substrates in a 0.7 mL tube was added 250 µL of 100 mM TRIS buffer at pH 8.3 and 4-6 µL of a stock solution of endoproteinase LysC (Roche Applied Science, 3 U in 50 µL 100 mM TRIS buffer, pH 8.3). The mixtures were incubated at 37 ^{o}C for 1.5-2 h, then purified by analytical C4 RP-HPLC using a gradient of 2-100% B over 90 min, where A is 0.1% TFA and B is 80% MeCN/H_2O with 0.086% TFA. The collected fractions were analyzed by MALDI-TOF MS and resuspended in 75 µL water. Following HPLC purification, the concentration of each analogue was determined via spectrophotometric analysis at wavelengths of 260 and 280 nm. A plot of concentration versus absorbance for authentic lacticin 481 was used as a standard curve. The concentration of each analogue is provided below.
***Product 4** containing a **Trp19Nal** mutation.* LRMS (MALDI): calcd $C_{122}H_{175}N_{37}O_{33}S_4$ [M+H] 2815.20, observed 2815. Concentration = 127 µM.

Product 4 *containing* ***Gly2β-Ala/Gly3β-Ala/Gly5β-Ala*** *mutations*. LRMS (MALDI): calcd $C_{123}H_{180}N_{38}O_{33}S_4$ [M+H] 2846.25, observed 2846, 2703 (25%). Concentration = 127 μM.

Product 4 *containing a* ***Gly5Acpc*** *mutation*. LRMS (MALDI): calcd $C_{122}H_{176}N_{38}O_{33}S_4$ [M+H] 2830.21, observed 2830. Concentration = 172 μM.

Product 4 *containing a* ***Val6D-Val*** *mutation*. LRMS (MALDI): calcd $C_{120}H_{174}N_{38}O_{33}S_4$ [M+H] 2804.20, observed 2804. Concentration = 178 μM.

Product 4 *containing a* ***Phe21Pal*** *mutation*. LRMS (MALDI): calcd $C_{122}H_{175}N_{37}O_{33}S_4$ [M+H] 2815.20, observed 2815. Concentration = 136 μM.

Product 4 *containing a* ***Gly5Sar*** *mutation*. LRMS (MALDI): calcd $C_{121}H_{176}N_{38}O_{33}S_4$ [M+H] 2818.21, observed 2818. Concentration = 81 μM.

Product 4 *containing a* ***Phe23hPhe*** *mutation*. LRMS (MALDI): calcd $C_{121}H_{176}N_{38}O_{33}S_4$ [M+H] 2818.21, observed 2816. Concentration = 115 μM.

Product 4 *containing a* ***Asn15β^3-Arg*** *mutation*. LRMS (MALDI): calcd $C_{121}H_{176}N_{38}O_{33}S_4$ [M+H] 2818.21, observed 2822. Concentration = 147 μM.

Product 4 *containing a* ***Met16N-Nle*** *mutation*. LRMS (MALDI): calcd $C_{121}H_{176}N_{38}O_{33}S_3$ [M+H] 2786.24, observed 2790, 2808 (20%). Concentration = 308 μM.

Product 4 *containing a* ***Met16β-Ala*** *mutation*. LRMS (MALDI): calcd $C_{118}H_{170}N_{38}O_{33}S_3$ [M+H] 2744.20, observed 2749. Concentration = 109 μM.

Product 4 *containing a* ***Glu13Cba*** *mutation.* LRMS (MALDI): calcd $C_{120}H_{173}N_{39}O_{31}S_4$ [M+H] 2785.20, observed 2783. Concentration = 90 μM.

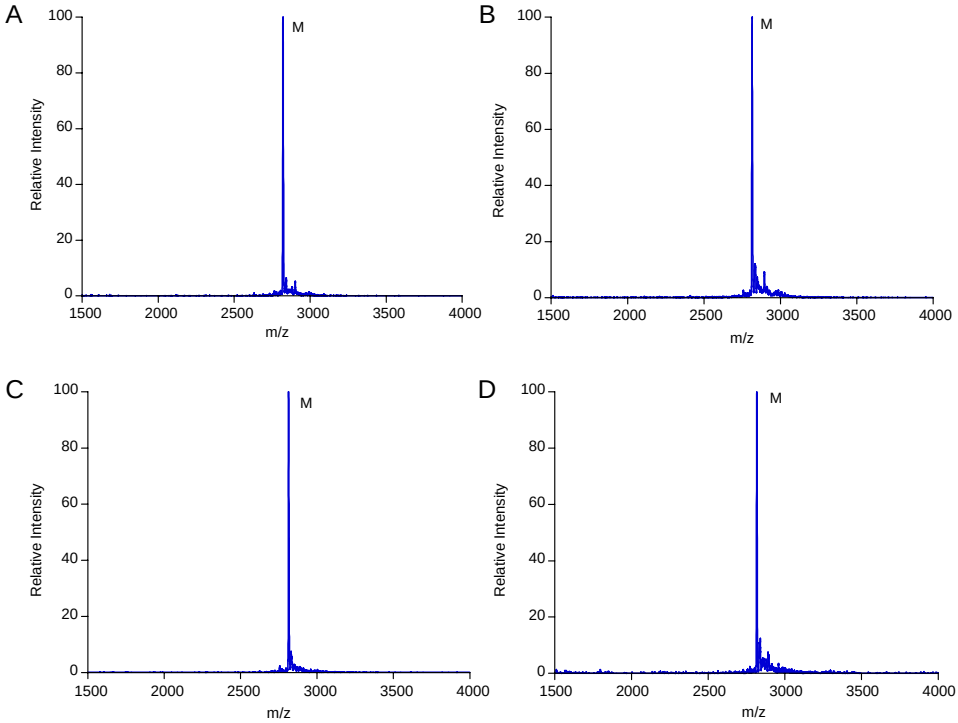

Figure S4. Representative MALDI-TOF mass spectra of **4** containing (A) Asn15β3-Arg, (B) Phe21Pal, (C) Trp19Nal, and (D) Gly5Sar mutations. The spectra were obtained following C4 RP-HPLC purification of endoproteinase LysC proteolysis reactions.

***p*-Hydroxymercuribenzoic acid (PMBA) cyclization analysis**. To test for the presence of free thiol groups, PMBA assays were performed as previously described.[7] To approximately 0.5 μL of each lacticin 481 analogue solution was added 5 μL of 10 mM TCEP and 4 M guanidine hydrochloride and the sample was incubated at 25 °C for 15 min. Subsequently, 5 μL of a 10 mM solution of PMBA was added and the sample incubated at 25 °C for 12-16 h. Thiol modification would be expected to result in a mass increase of 320 Da. Positive control PMBA assays were conducted using non-LctM treated triazole-linked peptides. PMBA adduct formation was not detected by MALDI MS for any of the lacticin 481 analogues, indicating that each was fully cyclized.

Agar well diffusion bioactivity assays. Inhibitory activity was measured using agar well diffusion assays. Molten agar GM17 medium (1.5%, 20 mL) or agar LB medium (1.5%, 20 mL) was seeded with *Lactococcus lactis* HP indicator strain or *Bacillus subtilis* ATCC 6633, respectively (75 μL of overnight culture at $O.D._{600}$ = 1.6-2.0), and dispensed into a sterile tray and allowed to solidify at 25 °C. An additional volume of 20 mL of seeded molten agar was poured over the solidified bottom layer and a sterile 96-well PCR tray was gently placed in the molten top layer. Upon solidification, the PCR tray was removed, yielding wells of roughly 10-12 μL volume. Known amounts of lacticin 481 analogues were added to each well (each added in 10 μL volumes). For larger plates with well volumes of approximately 40 μL, the second layer of molten agar had a volume of 40 mL (Figure S6) and the amount of compound added ranged from 25-40 μL in different experiments. The plates were then incubated overnight at 25 °C or 30 °C. Establishment of a zone of clearance indicated the presence of active peptides. The diameter of the zone was measured using analytical calipers (Mitutoyo) with an error range of ± 0.05 mm.

Evaluation of biological activity of lacticin 481 analogues:

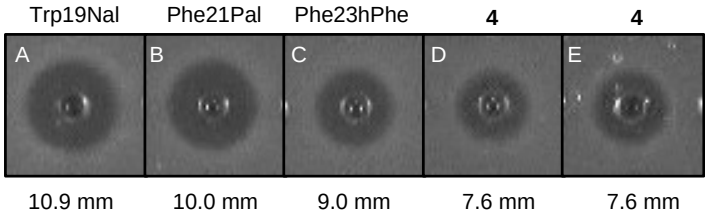

Figure S5. Evaluation of biological activity of lacticin 481 analogues against *L. lactis* HP. Samples containing approximately 21 μM of each lacticin 481 analogue were spotted on a 96-well GM17 media agar plate (10 μL total volume, ca. 580 ng) and incubated overnight at 25 °C. The size of the zone of inhibition following overnight incubation was measured using calipers and is indicated below each zone. Two samples of **4** were tested, from separate preparations. Wells A through D are the same as Figure 3 in the main text.

Agar diffusion assay for the evaluation of lacticin analogues with decreased activity:

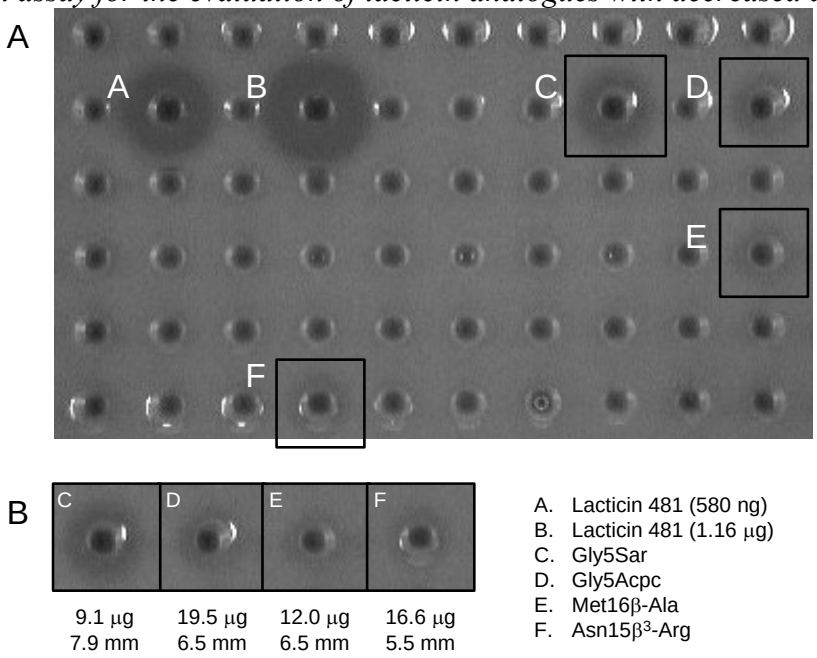

Figure S6. Evaluation of biological activity of lacticin 481 analogues against *L. lactis HP*. (A) 96-well agar diffusion plate containing unnatural lacticin 481 analogues. Due to the much lower activity of these analogues, all material that was purified was applied to the wells in 40 μL volumes. Even at these larger amounts, analogues containing Gly5Sar, Gly5Acpc, Met16β-Ala, and Asn15β^3-Arg mutations showed small zones of inhibition while compounds containing Glu13Cba, Met16*N*-Nle, Val6D-Val, and concomitant β-Ala mutations at Gly2, Gly3 and Gly5 did not demonstrate bioactivity within the limits of this assay. (B) Close-up of bioactive compounds, with the amount of each compound spotted and the diameter of the zone of inhibition depicted at bottom. The concentrations were Gly5Sar, 81 μM; Gly5Acpc, 172 μM; Met16β-Ala, 109 μM; Asn15β^3-Arg, 147 μM.

Summary of activity of lacticin 481 analogues against L. lactis HP: The diameter of the zone of inhibition against *L. lactis* HP was used to roughly compare the activity of the lacticin 481 analogues. For active analogues (Trp19Nal, Phe21Pal, Phe23hPhe, and **4**), where the same amount of each compound (21 μM) was spotted, the diameter was used to provide an estimate of relative activity compared to **4**. For less active mutants (Gly5Sar, Gly5Acpc, Asn15β^3-Arg, and Met16β-Ala), the amount of each compound needed to provide a zone of inhibition was used to estimate the decrease in activity compared to **4**.

Mutant	Active	Relative Activity**
Trp19Nal	++	2.1
Phe21Pal	++	1.7
Phe23hPhe	++	1.4
Asn315Arg/Phe21His	++	1.0
Gly5Sar	+	-16
Gly5Acpc	+	-34
Asn15β^3-Arg	+	-29
Met16β-Ala	+	-21
Val6D-Val	-	N/A
Glu13Cba	-	N/A
Met16*N*-Nle	-	N/A
Gly2,3,5β-Ala	-	N/A

Compared to **4

Table S1. Summary of biological activity (agar well diffusion assay) of lacticin 481 analogues against *L. lactis* HP. The relative activity of each analogue, compared to **4**, was determined using the diameter of the zone of inhibition (for compounds with ++ activity) or from the amount of compound needed to provide a zone of inhibition (for compounds marked with + activity).

Bioactivity assay against Bacillus subtilis ATCC 6633 *in Luria Bertani agar*:

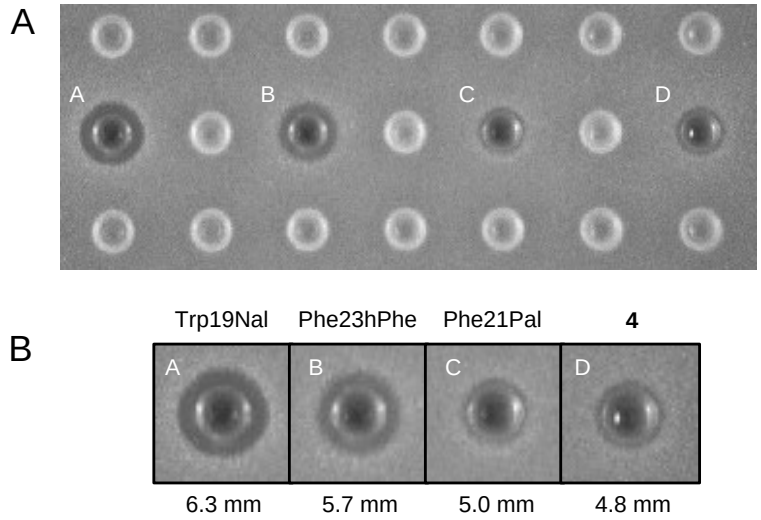

Figure S7. Evaluation of biological activity of lacticin 481 analogues against *Bacillus subtilis* ATCC 6633. (A) A portion of a 96-well agar diffusion plate containing one row of spotted compounds. A solution of approximately 123 μM of each compound was spotted in a total volume of 10 μL (3.48 μg). (B) Enlarged depiction of the wells containing each lacticin 481 analogue. The size of the zone of inhibition (diameter, in mm) following overnight incubation was measured using calipers and is indicated below each zone.

Validation of agar diffusion assay for comparative evaluation of bioactivity. To determine the extent to which agar diffusion bioactivity assays can be used for comparative analysis of bioactivity of lacticin 481 analogues, a range of amounts of authentic lacticin 481 (2.5 μM to 80.0 μM, 10 μL total volume) was spotted against *L. lactis* HP on M17 agar medium and the zones of inhibition measured using analytical calipers following overnight incubation at 25 ^{o}C. The results are depicted Figure S8.

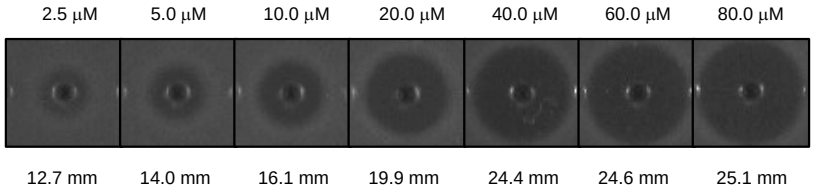

Figure S8. Zones of inhibition (in mm, bottom) of varying amounts of lacticin 481 (top) following overnight incubation against *L. lactis* HP at 25 °C on M17 agar medium.

At low concentrations of lacticin 481 added (2.5 μM → 40 μM), the size of the zone of inhibition appears to correlate linearly with the amount of compound used. However, at higher amounts of lacticin 481, this correlation no longer applies. The diameter of the zone of inhibition for each concentration of lacticin 481 was plotted to investigate the range of appropriate amounts of compound that would provide a linear relationship with activity. This plot is shown in Figure S9. A linear relationship between concentration of lacticin 481 spotted and the diameter of the zone of inhibition was noted at concentrations lower than 40 μM. The concentrations of lacticin 481 mutants used for bioactivity assays fall within this range (21 μM for Trp19Nal, Phe21Pal, Phe23hPhe, Figure 3 and S5). This concentration is depicted by an orange arrow in Figure S9.

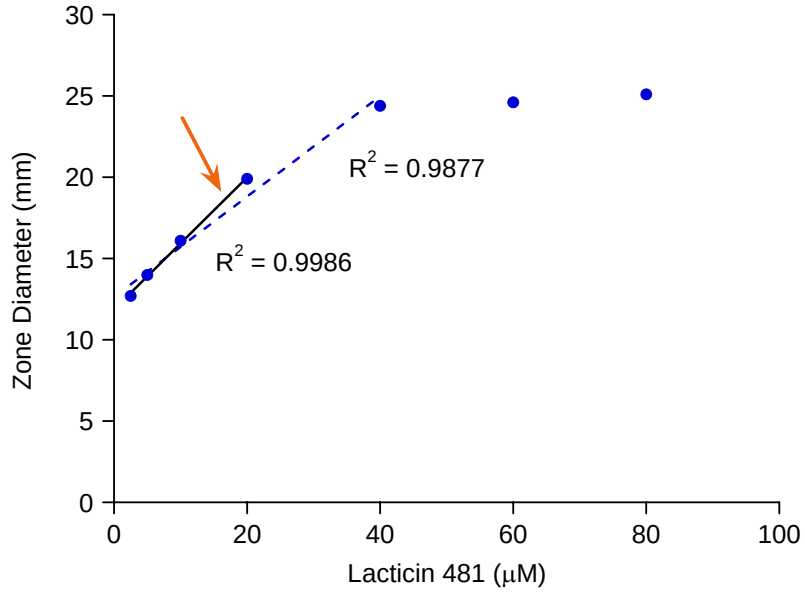

Figure S9. Plot of zone of inhibition (diameter, in mm) versus amount of lacticin 481 spotted (μM) for agar diffusion bioactivity assay against *L. lactis* HP on GM17 agar. Linear regression using the lowest four (solid line) or five (dashed line) amounts of lacticin 481 spotted provided R^2 values of 0.9986 and 0.9877, respectively. The amount of lacticin 481 mutants spotted for quantitative evaluation of bioactivity (21 μM, see Figures 3 or S5) is denoted by an orange arrow.

Determination of MIC values against liquid cultures of *L. lactis* HP

Ninety-six well microtiter plates were used to determine MIC and IC_{50} values of indicator strains. A total volume of culture in each well was 200 μL; the experimental wells contained 50 μL of diluted peptide at defined concentrations and 150 μL of a culture of *L. lactis* HP indicator strain (OD_{600} = 0.9 – 1.0) in GM17 medium diluted 1-to-10 in fresh liquid GM17 medium. Working peptide concentrations ranged from 25 μM to 24.4 nM. Each plate contained several control (150 μL fresh GM17 medium and 50 μL sterile Millipore water) and blank wells (150 μL of untreated 1-in-10 diluted culture and 50 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 24 h using a BioTek Synergy 2 plate reader. Plates were incubated at 30 ^{o}C between readings. Blanks (growth medium and sterile Millipore water only) were subtracted from experimental readings. Growth curves vs. peptide concentration were developed, and curve fits for IC_{50} and MIC determination were produced by fitting data with OriginPro 8 software using the dose-response curve with the equation: $y = A1 + (A2 - A1) / (1 + 10^{(Logx0 - x)p})$, with p = variable Hill slope. IC_{50} and MIC values were calculated from this fit, and triplicate readings were averaged.

Lacticin 481 analogue	MIC	IC_{50}
Compound **4**	6.25 μM	2.29 ± 0.09
Trp19Nal	3.12 μM	1.45 ± 0.08
Phe23hPhe	3.12 μM	1.54 ± 0.16
Phe21Pal	12.5 μM	5.18 ± 0.18

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