Lacticin 481: in vitro reconstitution of lantibiotic synthetase activity

Lili Xie, Leah M. Miller, Olga Averin, Champak Chatterjee, Neil L. Kelleher,* and Wilfred A. van der Donk*  

Supporting Information

Materials and Methods

E. coli DH5α and BL21(DE3) were used as hosts for cloning and expression, respectively. Transformants were selected by plating on LB agar plates containing 100 µg/mL ampicillin (FisherBiotech) and/or 50 µg/mL kanamycin (Sigma). pET-15b and pET-28b were obtained from Novagen. pTXB1 was purchased from NEB. E. coli strains were grown in LB or TB (terrific broth) medium. Lacticin 481 producing strain Lactococcus lactis CNRZ 481 was obtained from the CNRZ culture collection (INRA, Jouy-en-Josas, France) and was cultured in GM17 broth (4 % M17 and 0.5 % glucose) at 30 °C without aeration for isolating chromosomal DNA.

Restriction enzymes were obtained from either GibcoBRL or NEB. Calf intestinal alkaline phosphatase (CIP) was purchased from Promega. IPTG was purchased from CalBiochem. T4 DNA ligase was obtained from NEB. Primers were purchased from the UIUC Biotechnology Center. dNTPs were obtained from GibcoBRL. DNA polymerases were purchased from GibcoBRL or Stratagene. Chelating Sepharose Fast Flow resin and SP Sepharose Fast Flow resin were purchased from Amersham Pharmacia Biotech. Chitin resin and amylose resin were purchased from NEB. The metal chelating column for the BioCad FPLC system was self-packed using POROS 20 MC resin (metal chelate affinity packing) obtained from PerSeptive Biosystem. RP-HPLC was performed on either a Rainin system (Dynamax model SD-200 pump and model UV-1 detector), a Beckman Gold system (Model 125 solvent module and model 166 detector), or a Waters system (Model 600 controller, Delta 600 pump, and 2487 dual wavelength absorbance detector) with a Vydac C4 analytical (0.46 cm x 25 cm), semi-preparative (1.0 cm x 25 cm), or preparative column (2.2 cm x 25 cm), or a Waters PrepLC™ 25 mm Module preparative C18 column, monitoring at 220 nm. Solvents for RP-HPLC were solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in acetonitrile). MALDI-TOF mass spectrometry was carried out on a Voyager-DE-STR (Applied Biosystem) in the Mass Spectrometry Laboratory, School of Chemical Sciences, UIUC. The instrument has an accuracy of 0.05% for small to medium sized peptides and proteins when external calibration is used.

Genomic DNA from L. lactis CNRZ 481 strain was isolated by the DNAzol method (Molecular Research Center, Inc). Plasmid DNA was isolated using QIAGEN Plasmid Purification Kits (QIAGEN). DNA purification after restriction digestions was performed using QIAquick PCR or gel purification kit (QIAGEN). DNA sequencing was performed using the appropriate primers on an ABI PRISM automated sequence model vision 2.1.1 at the Biotechnology Center of UIUC.

Cloning of LctM

The lctM gene was PCR amplified using the primers 5’-CGACTAGCTAGC-ATGAAAAAAAGACTTAC-3’ and 5’-CCGCTCGAGTTAATCAAACATATGGCAT-3’. The PCR product was digested with NheI and XhoI restriction enzymes and ligated.
into pET28b to generate a His<sub>6</sub>-tagged LctM construct (pET28b-LctM(NB)H2). The sequence of LctM insert was checked by DNA sequencing.

**Expression and Purification of His<sub>6</sub>-LctM**

BL21(DE3) cells transformed with pET28b-LctM(NB)H2 were grown in TB medium at 37 °C and induced with 0.5 mM IPTG when OD<sub>600nm</sub> reached 0.8-0.9. The cells were then continually shaken at 18 °C for 22 h and then harvested. The cell paste was resuspended in start buffer (20 mM Tris, pH 8.3, 1 M NaCl, 10 % glycerol) and stored at –80 °C until use. All purification steps were performed at 4 °C. Cell paste (11 g) in 50 mL of start buffer was sonicated on ice for 20 min. After centrifugation, the supernatant (48 mL) was loaded onto a POROS<sup>®</sup>20 MC 7.8 mL column at 2 mL/min. The column was washed with 150 mL of start buffer at 2 mL/min. Then the flow rate was increased to 4 mL/min and the column was washed with an additional 100 mL of start buffer, followed by 40 mL of 30 mM imidazole buffer (20 mM Tris, pH 7.2, 100 mM NaCl, 30 mM imidazole), and eluted with 100 mL of a 30 mM to 500 mM imidazole gradient in 20 mM MOPS, pH 7.2, 100 mM NaCl buffer. The His<sub>6</sub>-LctM containing fractions were combined and directly loaded onto a SP cation exchange column (27 mL, 15 mm diameter) pre-equilibrated with MOPS buffer (20 mM MOPS, pH 7.1, 100 mM NaCl). About 20 mg of His<sub>6</sub>-LctM was obtained (1.16 mg/mL, 19 mL) based on Bradford assay. Mass spectrometric analysis (MALDI) revealed a mass of 109,094 ± 120 (3 determinations) (calcld 109,161).

**Cloning of His<sub>6</sub>-LctA**

The lctA gene was amplified using the primers 5'-GGGAATTCCATATG-AAAGAACAAAACCTTTTTAA-3' and 5'-CGCGGATCCCTTAAAGGCAGCAAGTA. The PCR product was digested with NdeI and BamHI restriction enzymes and ligated into the pET15b vector. The resulting pET15b-LctA#4 plasmid was used for the overexpression of His<sub>6</sub>-LctA. DNA sequencing revealed a nucleotide change (A33G) leading to a silent mutation (Q11Q).

**Expression and Purification of His<sub>6</sub>-LctA**

BL21(DE3) cells carrying the plasmid pET15b-LctA were induced with 1 mM IPTG at 37 °C at OD<sub>600nm</sub> = 0.5-0.7, and grown for an additional 3 h. Cells were harvested by centrifugation, and the cell pellet (11 g) was resuspended in 40 mL of start buffer2 (20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.5, 500 mM NaCl, 0.5 mM imidazole) and lysed by sonication. After centrifugation, the supernatant was discarded and the pellet was washed twice with start buffer2 to remove trapped proteins. The resulting pellet was resuspended in 10 mL of denaturing buffer (start buffer2 containing 6 M guanidine hydrochloride). The mixture was incubated at rt for 1 h to completely redissolve the protein. The remaining insoluble material was removed by centrifugation and the supernatant was mixed with Ni<sup>2+</sup>-charged chelating resin (~10 mL) at rt for 30 min by rotation in a 50 mL centrifuge tube. The resin mixture was then transferred into a column. The column was drained, washed with denaturing buffer, and 30 mM imidazole buffer containing 6 M guanidine. His<sub>6</sub>-
LctA was eluted with denaturing buffer containing 100 mM EDTA. Fractions containing His$_6$-LctA were concentrated by Amicon ultrafiltration using a YM1 membrane (Millipore). His$_6$-LctA was further purified by RP-HPLC using a C18 preparative column (Waters). The retention time (t$_R$) was 15.5 min using a gradient of 2-100 % B over 20 min (B = 100 % MeCN/0.1% TFA) on a C4 Vydac analytical column. About 32 mg of HPLC purified peptide was obtained (~6.5 mg per liter of culture). MALDI-TOF MS for His$_6$-LctA calcd. 7710 (M+H), found 7710. ESI-FT-MS mass calcd. 7708.59-4, found 7708.60-4 (1 ppm error, external calibration with bovine ubiquitin, 8564.64-5). For all FT-ESI data reported herein, the mass difference (in units of 1.0024 Da) between the most abundant isotopic peak and the monoisotopic peak is denoted in italics after each M$_r$ value.

**Activity Assay**

The HPLC purified His$_6$-LctA peptide was redissolved in water and the concentration was determined by UV-vis according to the estimated extinction coefficient ($\varepsilon_{280nm} = 5690$) from the ExPASy ProtParam tool. About 10 µL of His$_6$-LctA was mixed with 1 µL of 10 x assay buffer (final concentration; 25 mM Tris, 5 mM MgCl$_2$, 5 mM DTT, 0.5 mM ATP, 12.5 µg/mL BSA, pH 7.5) and ~1 µL of ZnCl$_2$ (final conc. 0.36 µM). Peptide precipitation was observed upon addition of the buffer if the peptide concentration was too high. His$_6$-LctM was added (1 µM) and the reaction mixture was incubated at rt for 28 h. For MALDI MS analysis, 1 µL of the assay sample was mixed with 9 µL of sinapinic acid pre-dissolved in MeCN:H$_2$O (2:1) in 0.1 % TFA and 1 µL of the mixture was applied on the MALDI target. MALDI MS calcd. 7710 (M+H), 7638 (M-72+H), found 7638 (M-72+H) as the major peak. Small amounts of species corresponding to 7656 (M-54+H), 7674 (M-36+H), 7692 (M-18+H) and 7710 (M+H) were also observed in MALDI MS. ESI-FT-MS for the major product: mass calcd. 7636.55-4, found 7636.62-4 (9 ppm error, external calibration with bovine ubiquitin, 8564.64-5).

**Bioassay**

The lyophilized assay product (~ 0.1 µg) was redissolved in 6 µL of Millipore water. To the solution, 4 µL of a solution of LysC in 100 mM Tris, pH 8.5 (0.4 ng/µL) was added and the mixture was incubated at 37 °C for 3 h. The resulting mixture was checked by MALDI-TOF MS. Three fragments were expected and two were observed: calcd, 2310 ([1-21]), 2591 ([22-44]), 2773 ([45-70]); found, 2593 ([22-44]), 2775 ([45-70]). Inhibitory activity was assayed by the solid agar medium test. A GM17 agar plate was seeded with the test strain by mixing 100 mL of liquid GM17 agar at 50 °C with 1.5 mL of an overnight culture. After agar solidification, wells were created in the medium and the samples were added to the wells. The activity of the starting peptide, LctM-product, and the product and starting peptide treated with LysC were determined using a solid agar diffusion bioassay with indicator strain *L. lactis* CNRZ 117 (See Figure S1).

**FT-MS/MS conditions**

Samples for MS/MS analysis were resuspended in 78% acetonitrile, 20% water, 2% acetic acid and introduced via electrospray ionization into a custom built 8.5 Tesla Q-FTMS. Data acquisition was performed with the MIDAS datastation and then stored as 512 K data sets. Isopro v3.0 was used to generate theoretical isotopic distributions which
were fit to experimental data by least squares to assign the most abundant peak. Instrument specifics have been described previously (SI). For all FT-ESI data reported herein, the mass difference (in units of 1.0024 Da) between the most abundant isotopic peak and the monoisotopic peak is denoted in italics after each \( M_r \) value.

**IRMPD:** In the FTMS cell the ions of interest were isolated using a stored waveform inverse Fourier transform (SWIFT). After isolation, the selected ions were irradiated with a 75 W \( \text{CO}_2 \) laser which induced fragmentation along the peptide backbone.

**Multipole Dissociation:** For multipole dissociation, ions of interest were first selectively filtered in the quadrupole and then fragmented by lowering the axial offset on the accumulation multipole. The fragment ions were then directed down to the cell via multiple ion guides where they were excited and detected.

**Generation of LctA mutants**

**His\textsubscript{6}\text{LctA(5-51)}** The partial \( lctA \) gene (13-156) was amplified using the primers 5’-GGGAATTCCATATGAAACTCTTTAATCTTTC-3’ and 5’-CGCGGATCCTTTAAAGAGCAGCAAGTA-3’ with the plasmid pET15b-LctA as the template. The PCR product was gel purified, digested with \( NdeI \) and \( BamHI \), and ligated into a pET15b vector. The resulting pET15b-LctA-N5#1 plasmid was used for the overexpression of His\textsubscript{6}\text{LctA(5-51)}. DNA sequencing revealed a nucleotide change (A33G) leading to a silent mutation (Q11Q).

**His\textsubscript{6}\text{LctA(10-51)}** The partial \( lctA \) gene (28-156) was amplified using the primers 5’-GGGAATTCCATATGCTTTAAGAAGTGACA-3’ and 5’-CGCGGATCCTTTAAGAGCAGCAAGTA-3’ with genomic DNA as the template. The PCR product was digested with \( NdeI \) and \( BamHI \), and ligated into a pET15b vector. The resulting pET15b-LctA-L10#3 plasmid was used for the overexpression of His\textsubscript{6}\text{LctA(10-51)}. DNA sequencing confirmed the desired sequence.

**His\textsubscript{6}\text{LctA(25-51)}** The partial \( lctA \) gene (73-156) was amplified using the primers 5’-GGGAATTCCATATGAAAGCGGACGTGGA-3’ and 5’-CGCGGATCCTTTAAGAGCAGCAAGTA-3’ with the plasmid pTXB1-LctA as the template. The PCR product was digested with \( NdeI \) and \( BamHI \) restriction enzymes, and ligated into a pET15b vector. The resulting plasmid was called pET15b-LctA-K25-intein-CBD#2 which was used to obtain the truncated peptide His\textsubscript{6}\text{LctA(25-51)}. DNA sequencing confirmed the presence of the desired sequence.

**His\textsubscript{6}\text{LctA(1-37)}** The partial \( lctA \) gene (1-111) was amplified using the primers 5’-GGGAATTCCATATGAAAGCGGACGTGGA-3’ and 5’-ATATGCTCTTTACCGATCATGAGAGATTGT-3’ with genomic DNA as the template. The PCR product was digested with \( NdeI \) and \( SapI \) restriction enzymes, and ligated into a pTXB1 vector. The DNA fragment LctA-37E-intein-CBD was cut out of the resulting pTXB1-LctA-37E plasmid using \( NdeI \) and \( BamHI \), and this fragment was ligated into the pET-15b vector. This resulted in the plasmid pET15b-LctA-37E-intein-CBD#2 which was used to obtain truncated peptides His\textsubscript{6}\text{LctA(1-37)} and His\textsubscript{6}\text{LctA(1-38)C38U} by EPL. DNA sequencing revealed a nucleotide change (T48C) leading to a silent mutation (S16S).

**His\textsubscript{6}\text{LctA-T48S.}** The \( lctA \) gene was amplified using the primers 5’-GGGAATTCCATATGAAAGCGGACGTGGA-3’ and 5’-CGCGGATCCTTTAAGAGCAGCAAGTA-3’ with pET15b-LctA as the template. The PCR product was
digested with NdeI and BamHI restriction enzymes and ligated into a pET15b vector. The resulting pET15b-LctA-T48S#2 plasmid was used for the overexpression of His\textsubscript{6}-LctA-T48S. DNA sequencing revealed a nucleotide change (A33G) leading to a silent mutation (Q11Q) in addition to the desired mutation.  

**His\textsubscript{6}-LctA-T48A.** The lctA gene was amplified using the primers 5’-GGGAATTCCATATGAAAGAACAAAACCTCTTTTAA-3’ and 5’-CGCGGATCC-TTAAGAGGCAGCATGCA-3’ with pET15b-LctA as the template. The PCR product was digested with NdeI and BamHI restriction enzymes and ligated into a pET15b vector. The resulting pET15b-LctA-T48A#6 plasmid was used for the overexpression of His\textsubscript{6}-LctA-T48A. DNA sequencing revealed a nucleotide change (A33G) leading to a silent mutation (Q11Q) in addition to the desired mutation.  

**His\textsubscript{6}-LctA-C49A.** The lctA gene was amplified using the primers 5’-GGGAATTCCATATGAAAGAACAAAACCTCTTTTAA-3’ and 5’-CGCGGATCC-TTAAGAGGCAGCTAGTA-3’ with genomic DNA as the template. The PCR product was digested with NdeI and BamHI restriction enzymes and ligated into the pET15b vector. The resulting pET15b-LctA-C49A#1 plasmid was used for the overexpression of His\textsubscript{6}-LctA-C49A. DNA sequencing revealed a nucleotide change (A33G) leading to a silent mutation (Q11Q) in addition to the desired mutation.  

**His\textsubscript{6}-LctA-C49S.** The lctA gene was amplified using the primers 5’-GGGAATTCCATATGAAAGAACAAAACCTCTTTTAA-3’ and 5’-CGCGGATCC-TTAAGAGGCAGCTAGTA-3’ with genomic DNA as the template. The PCR product was digested with NdeI and BamHI restriction enzymes and ligated into the pET15b vector. The resulting pET15b-LctA-C49S#4 plasmid was used for the overexpression of His\textsubscript{6}-LctA-C49S. DNA sequencing matched the published sequence and showed the desired mutation.  

**General procedure for overexpression and purification of His\textsubscript{6}-LctA mutants.** BL21(DE3) cells carrying the corresponding plasmid were induced with 1 mM IPTG at 37 °C at OD\textsubscript{600nm} = 0.5-0.7, and grown for an additional 3 h. Cells were harvested by centrifugation, and the cell pellet (11 g) was resuspended in 40 mL of the start buffer\textsubscript{2} (20 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.5, 500 mM NaCl, 0.5 mM imidazole) and lysed by sonication. After centrifugation, the supernatant was discarded and the pellet was washed twice with the start buffer\textsubscript{2} to remove trapped proteins. The resulting pellet was resuspended in 10 mL of the denaturing buffer (the start buffer\textsubscript{2} containing 6 M guanidine hydrochloride). The mixture was incubated at 25 °C for 1 h to completely dissolve the protein. The remaining insoluble material was removed by centrifugation and the supernatant was mixed with Ni\textsuperscript{2+}-charged chelating resin at 25 °C for 30 min by rotation in a 50 mL centrifuge tube (Corning). The resin mixture was then transferred into a column. The column was drained, washed with the denaturing buffer, and 30 mM imidazole buffer containing 4 M guanidine. The peptide was eluted with the buffer containing 50 mM EDTA, 20 mM Tris, pH 6.5, 4 M guanidine. Fractions containing the peptide were collected and purified by RP-HPLC using a C18 preparative column (Waters).  

**General procedure for overexpression and purification of the truncated LctA from the intein system.** BL21(DE3) cells carrying the pET15b-LctA-37E-intein-CBD#2 plasmid were induced with 0.5 mM IPTG at 25 °C at OD\textsubscript{600nm} = 0.5-0.7, and grown for an additional 6 h. Cells were harvested by centrifugation, and the cell pellet was resuspended in cell lysis buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA) and
lysed by sonication. After centrifugation, the supernatant containing the truncated peptide was purified by chitin affinity chromatography and subsequent intein mediated cleavage with β-mercaptoethanol (100 mM) at 25 °C. The fractions containing His6-LctA(1-37) were combined, lyophilized, redissolved in minimum solvent and purified further by RP-HPLC. For expressed protein ligation (EPL), the peptide-intein-CBD containing resin was incubated with MESNA (50 mM) and Cys (1 mM) at 25 °C for 15 h before elution. Elution fractions containing the peptide were further purified by RP-HPLC using a C4 Vydac analytical column. The HPLC fractions were lyophilized and analyzed by MALDI-TOF MS.

**Overexpression, preparation and purification of LctA(1-38)C38U.** BL21(DE3) cells carrying the pET15b-LctA-37E-intein-CBD#2 plasmid were induced with 0.5 mM IPTG at 25 °C at OD600 = 0.6-0.7 and grown for an additional 6 h. Cells were harvested by centrifugation and the cell pellet was resuspended in cell lysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1mM EDTA, 0.1 % Tween-20) and lysed by sonication. After centrifugation, the supernatant containing the LctA(1-37)-intein-CBD fusion protein was bound to chitin affinity resin with gentle shaking at 4 °C for 2 h. The resin was then washed with wash buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA) until no absorbance was detected at 220 nm in the flow-through. Expressed protein ligation was performed by incubating the resin with bound peptide-intein-CBD with L-selenocysteine, generated in situ by the reduction of L-seleocystine (4 mM) with tris(2-carboxyethyl)phosphine (TCEP) (8 mM), and MESNA (100 mM) for 18 h at 25 °C under argon. Eluted fractions containing the desired peptide were further purified by RP-HPLC using a C4 Vydac analytical column and product fractions were lyophilized and analyzed by MALDI-TOF MS.

**Assays with mutant LctA peptides.** The assays with the mutant peptides were carried out essentially the same as described above for the wt His6-LctA peptide. Mass spectrometry data is listed below.

**His6-Lcm assay with His6-LctA(5-51).** MALDI-TOF MS calcd. 7324 (M), 7252 (M-72), found 7325 (M+H+), 7252 (M-72), 7270 (M-54), 7288 (M-36).

**His6-Lcm assay with His6-LctA(10-51).** MALDI-TOF MS calcd. 6749 (M), 6677 (M-72), found 6769 (M), 6677(M-72), 6695 (M-54), 6472 (M-36).

**His6-Lcm assay with His6-LctA(25-51).** MALDI-TOF MS calcd. 5137 (M), 5065 (M-72), found 5134 (M), 5116 (M-18), 5098 (M-36), 5080 (M-54).

**His6-Lcm assay with His6-LctA(1-38).** MALDI-TOF MS calcd. 6161 (M), 6125 (M-36), found 6125 (M-36). ESI-FTMS monoisotopic mass calcd. 6156.98-0 (M), 6120.94-0 (M-36).

**His6-Lcm assay with His6-LctA(1-37).** MALDI-TOF MS calcd. 6208 (M), 6172 (M-36), found 6210 (M), 6174 (M-36), 6192 (M-18).

**His6-Lcm assay with His6-LctA-T48S.** MALDI-TOF MS calcd. 7696 (M), 7624 (M-72), found 7624 (M-72), 7642 (M-54), 7660 (M-36). ESI-FTMS monoisotopic mass calcd. 7690.56-0 (M), 7618.56-0 (M+H), 7618.53-0 (M-72).

**His6-Lcm assay with His6-LctA-T48A.** MALDI-TOF MS calcd. 7680 (M), 7626 (M-54), 7624 (M-54), 7680 (M).

**His6-Lcm assay with His6-LctA-C49A.** MALDI-TOF MS calcd. 7678 (M), 7606 (M-72), found 7606 (M-72), 7624 (M-54), 7642 (M-36), 7760 (M-72+DTT). ESI-FTMS
monoisotopic mass calcd. 7672.61-0 (M), 7600.57-0 (M-72), found 7670.59-0 (M-2; disulfide between Cys38 & Cys50), 7600.59-0 (M-72).

**His6-LctM assay with His6-LctA-C49S.** MALDI-TOF MS calcd. 7694 (M), 7622 (M-72), found 7622 (M-72), 7640 (M-54), 7658 (M-36), 7604 (M-90), 7694 (M), 7758 (M-90+DTT), 7776 (M-72+DTT). ESI-FTMS monoisotopic mass calcd. 7688.60-0 (M), 7616.56-0 (M-72), found 7688.65-0 (M), 7616.50-0 (M-72), 7598.60-0 (M-90).

**His6-LctM assay with His6-LctA(1-38)C38U.** ESI-FTMS monoisotopic mass calcd. 6204.88-0 (M), 6168.86-0 (M-36), found 6204.91-0 (M), 6168.86-0 (M-36).
Fig. S1. Small aliquots of the following samples were added in the wells: (1) His$_6$-LctA, (2) His$_6$-LctA treated with LysC, (3) His$_6$-LctA modified by LctM, (4) His$_6$-LctA modified by LctM and treated with LysC, (5) cell free broth of L. lactis CNRZ 117, (6) cell free broth of L. lactis CNRZ 481 containing lacticin 481, (7) 50 mM Tris buffer, pH 8.5, (8) LysC in 50 mM Tris buffer, pH 8.5.
Fig. S2. Tandem MS on His-LctA(1-38) and the product obtained after LctM-catalyzed modification. Top panel: Sequence of His-LctA(1-38) and its product with key fragments highlighted. The fragmentation pattern of the starting peptide and product is almost identical with exception of the fragments in the C-terminus that are observed for the starting peptide but not in the product as a result of cyclization of the A-ring. The y-ion resulting from fragmentation between Ile31 and His32 localizes the dehydrations to the last 7 residues. Bottom panel (A) Key fragment ions. (B) Fragmentation spectra of His-LctA(1-38) starting material and enzymatic product (-36.02 Da). Formation of the b55 (blue) and b56 (red) ions should be inhibited in the product by the presence of a ring between Dhb33/Dha35 and Cys38. Both the b55 and b56 ions are present with > 20 S/N ratio in the starting material. (C) Both of these fragment ions are not observed in key portions of the CAD fragmentation spectrum for the product. If the b55 and b56 ions were observed in the product, they would appear with a -36.02 Da mass shift due to two dehydrations. Overall, panels B and C provide convincing evidence that a thioether ring has been formed. All masses are reported as monoisotopic masses.
Fig. S3. Tandem MS on His₆-LctA(1-38)C38U and the product after LctM-catalyzed modification. Top panel: The fragmentation pattern is similar for both the starting peptide and the product with the exception of fragmentation between Glu37 and Sec38 that is absent in the product. This is a result of cyclization by LctM catalyzed addition of the selenol side chain of Sec38 to a dehydrated residue. The two dehydrations observed in the product can be localized to the last 6 residues by the γ-ion arising from fragmentation between His32 and Thr33. Bottom panel: (A) Key fragment ion. (B) Fragmentation spectrum of the His₆- LctA(1-38)C38U starting material showing the b₅₆ ion fragment. (C) Formation of the b₅₆ ion would be inhibited in the product spectrum due to ring formation between Dhb33/Dha35 and Sec38. Indeed the b₅₆ ion fragment is absent in the spectrum of the enzymatic product (-36.02 Da). The red arrow indicates the expected position at Δm = -36.02 Da of the b₅₆ ion as a consequence of two dehydrations in the product. The absence of the b₅₆ ion is convincing evidence for thioether ring formation catalyzed by LctM. All masses are reported as monoisotopic masses. Ions corresponding to non-diagnostic fragments are labeled in black.
Major differences are seen in the fragmentation pattern of the starting peptide and product in the region of the A- and B-ring. That is fragmentations are observed between His32, Thr33, Ile34, Ser35, and His36 in the starting peptide but not the product as a result of formation of the A-ring. Similarly, fragmentations are observed between Ser42, Trp43, Gln44, Phe45, Val46, Phe47, and Thr48 in the starting peptide but not in the product as a result of formation of the C-ring. However, the fragmentations between Asn39 and Met40 and between Asn41 and Ser42 are observed in both peptides clearly showing the absence of the B-ring.

In addition to the M-72 product, a product was formed resulting of loss of 90 Da. MS/MS on this product showed that the newly introduced Ser in the mutant substrate is dehydrated in this peptide. Specifically, the b-ions resulting from fragmentation between Cys50 and residue 49 and between residues 49 and 48 showed unambiguously that residue 49 has lost 18 Da compared to the Ser precursor. Similar inspection of other b-ions in the C-terminal region once more verify dehydration of Thr33, Ser35, Ser42, and Thr48. Of note is that many fragments are generated in the C-terminal 10 amino acids in the product suggesting that the dehydration of Ser49 interferes with formation of the C-ring.

REFERENCES