Biosynthesis of Translation Inhibitor Klebsazolicin Proceeds through Heterocyclisation and N-terminal Amidine Formation Catalysed by a Single YcaO Enzyme

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SUPPORTING INFORMATION
(Figures S1-S7, Tables S1 and S2, Materials and Methods)

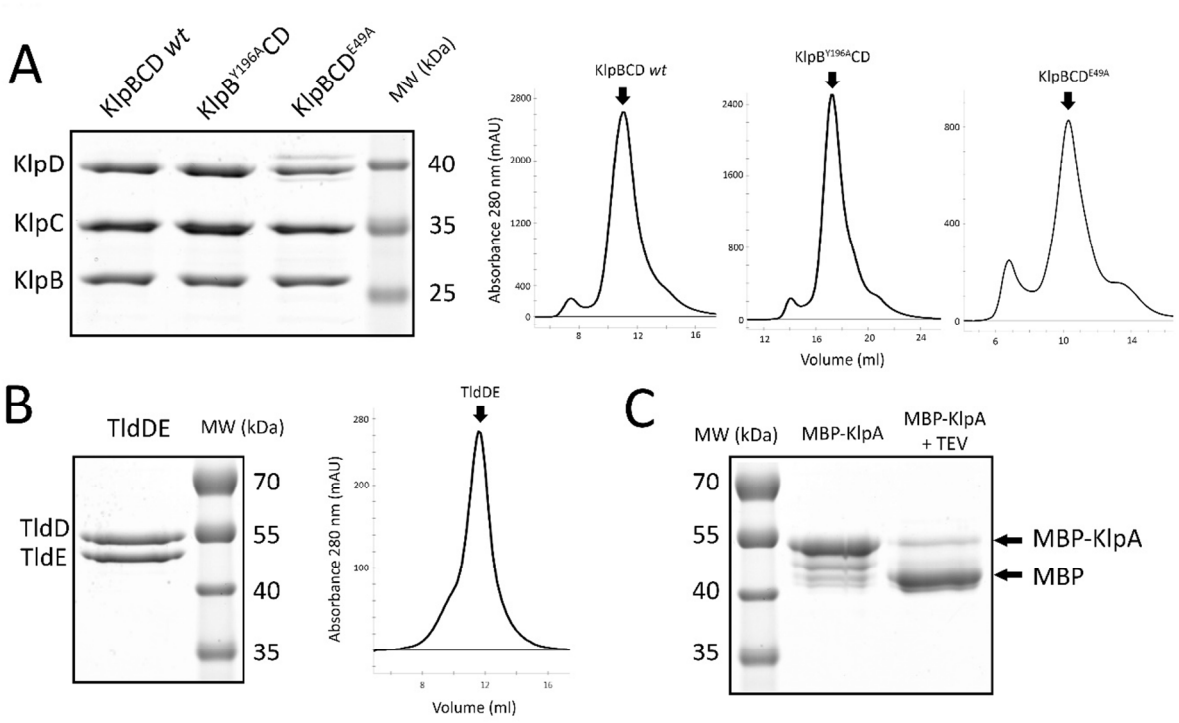

Figure S1.
Purification of the proteins used for *in vitro* reactions of KLB precursor peptide modification. **A.** Gel-filtration traces (**A**, *right*) of affinity purified synthetase KlpBCD complexes (*wt* and two mutant variants), top fractions loaded at denaturing gel with the protein marker (right lane). **B.** Purification of TldD/E complex: gel-filtration trace (**B**, *right*) and SDS-PAGE analysis of the top fraction (**B**, *left*). **C.** SDS-PAGE analysis of KlpA precursor peptide purification: first lane – protein marker with molecular masses (in kDa), second lane – elution after amylose affinity chromatography of MBP-KlpA, third lane – after the cleavage of the MBP-tag with TEV protease.

Figure S2.

Conserved positions in YcaO-domain containing dehydratases and azoline dehydrogenases. A. Fragments of multiple sequence alignment of KlpD cyclodehydratase with YcaO-containing enzymes from TOMM biosynthetic gene clusters (SagD – streptolysin, McbD – microcin B17, BmbE – bottromycin, GodD – goadsporin, PznD – plantazolicin) and *E. coli* standalone YcaO (Ec-YcaO). Conserved amino acid residues taking part in the ATP-binding site formation are colored in red. Black arrow indicated the residue substituted for alanine in KlpBCD^{E49A} mutant complex. **B.** Fragment of multiple sequence alignment of KlpB dehydrogenase with homologs from other TOMM biosynthetic gene clusters (McbC – microcin B17, SagB – streptolysin, GodE – goadsporin. PznB – plantazolicin). Conserved pair of residues Lys-Tyr participating in catalysis is colored in red. Black arrow indicates the residues substituted for alanine in KlpB^{Y196A}CD mutant complex.

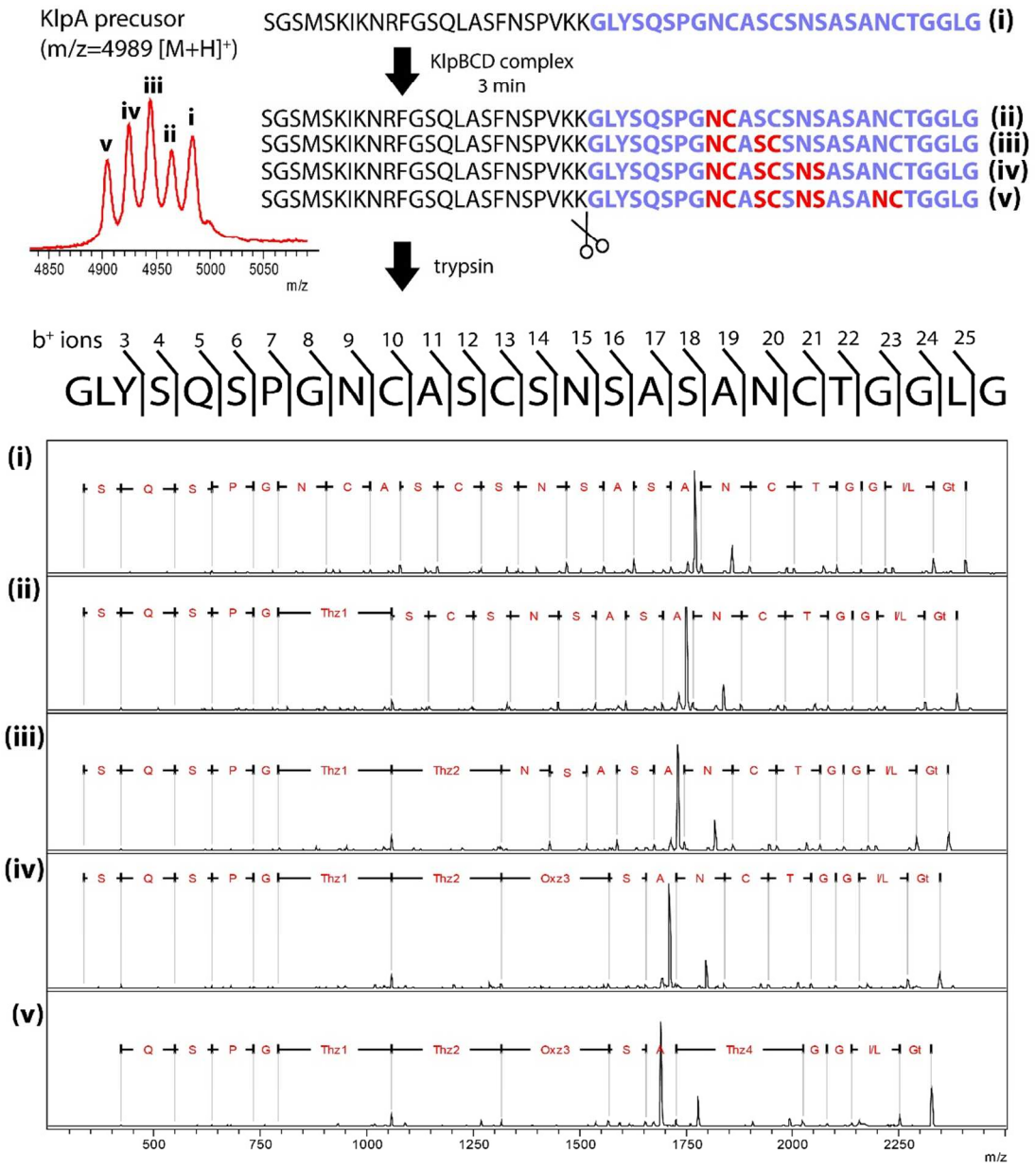

Figure S3.

An N- to C-terminal directionality of KlpA precursor peptide processing by KlpBCD modification complex. KlpA modification intermediates accumulated after 3 minutes of *in vitro* reaction with KlpBCD were digested with trypsin. MALDI-TOF-MS-MS spectra of five resulting peaks are presented, peaks corresponding to b^+-ions are indicated.

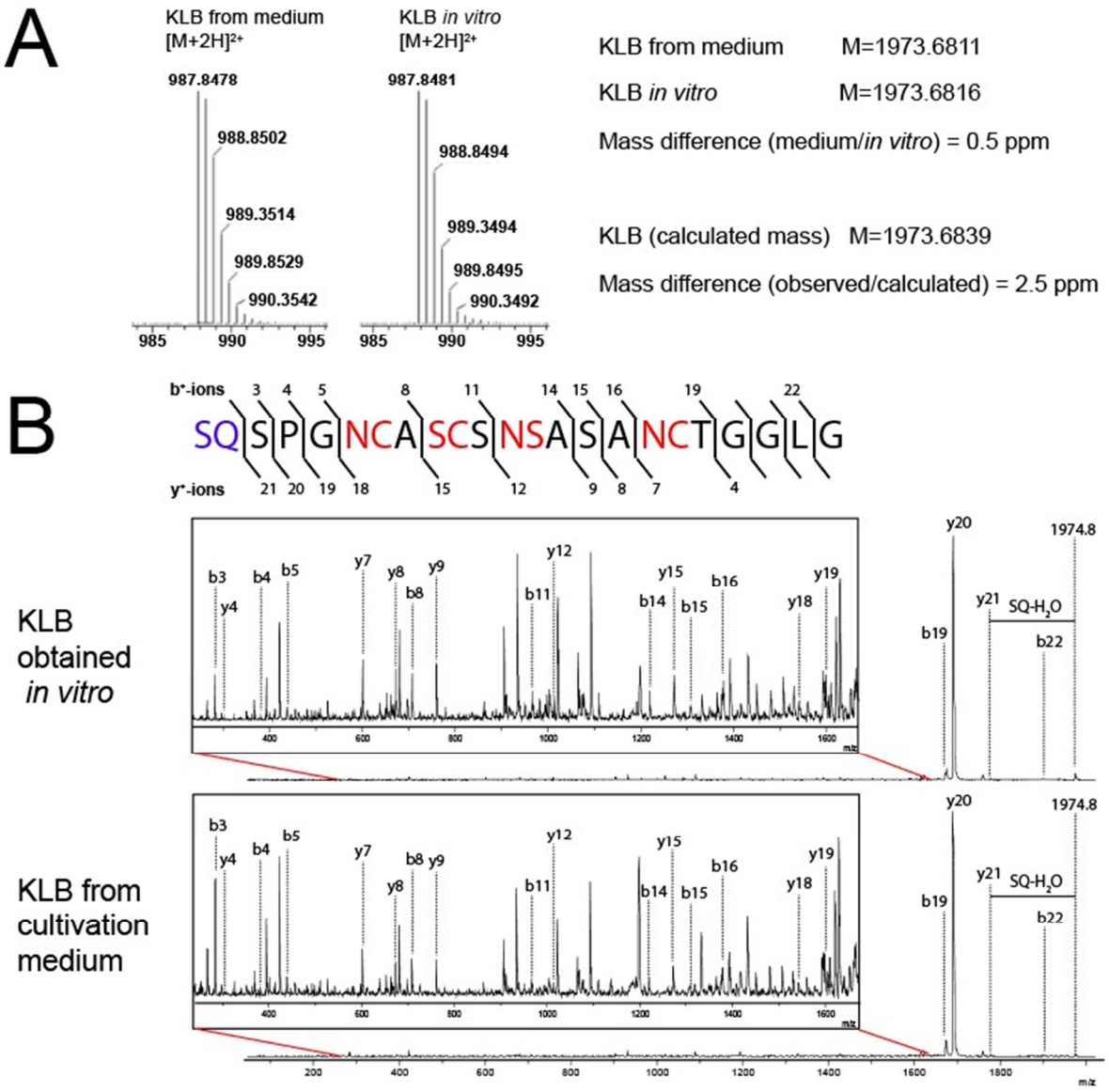

Figure S4. A. High resolution mass-spectra of KLB obtained in *in vitro* reaction and KLB purified from the cultivation medium. Monoisotopic m/z values are consistent with $C_{76}H_{107}N_{27}O_{30}S_3$ brutto formula (m/z_{calc}=1974.6839). **B.** Comparison of MS-MS spectra of KLB obtained *in vitro* and purified from the cultivation medium. Diagram at the top shows KLB with the observed b^+- and y^+-ions labeled in the structure. The same ions are labeled in the spectra. Amino acid residues taking part in posttranslational modifications are shown in color (red – core azole cycles, blue – N-terminal amidine cycle).

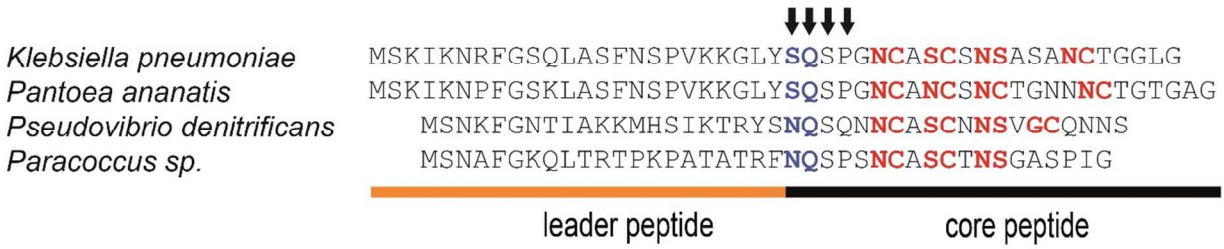

Figure S5.
Sequences of the predicted precursor peptides – homologs of KlpA. Predicted leader and core parts are shown in orange and black respectively, amino acid residues predicted to take part in the formation of posttranslational modifications are shown in red (azoles) and blue (amidine). Black arrows mark residues, subjected to site-specific mutagenesis in KlpA precursor peptide.

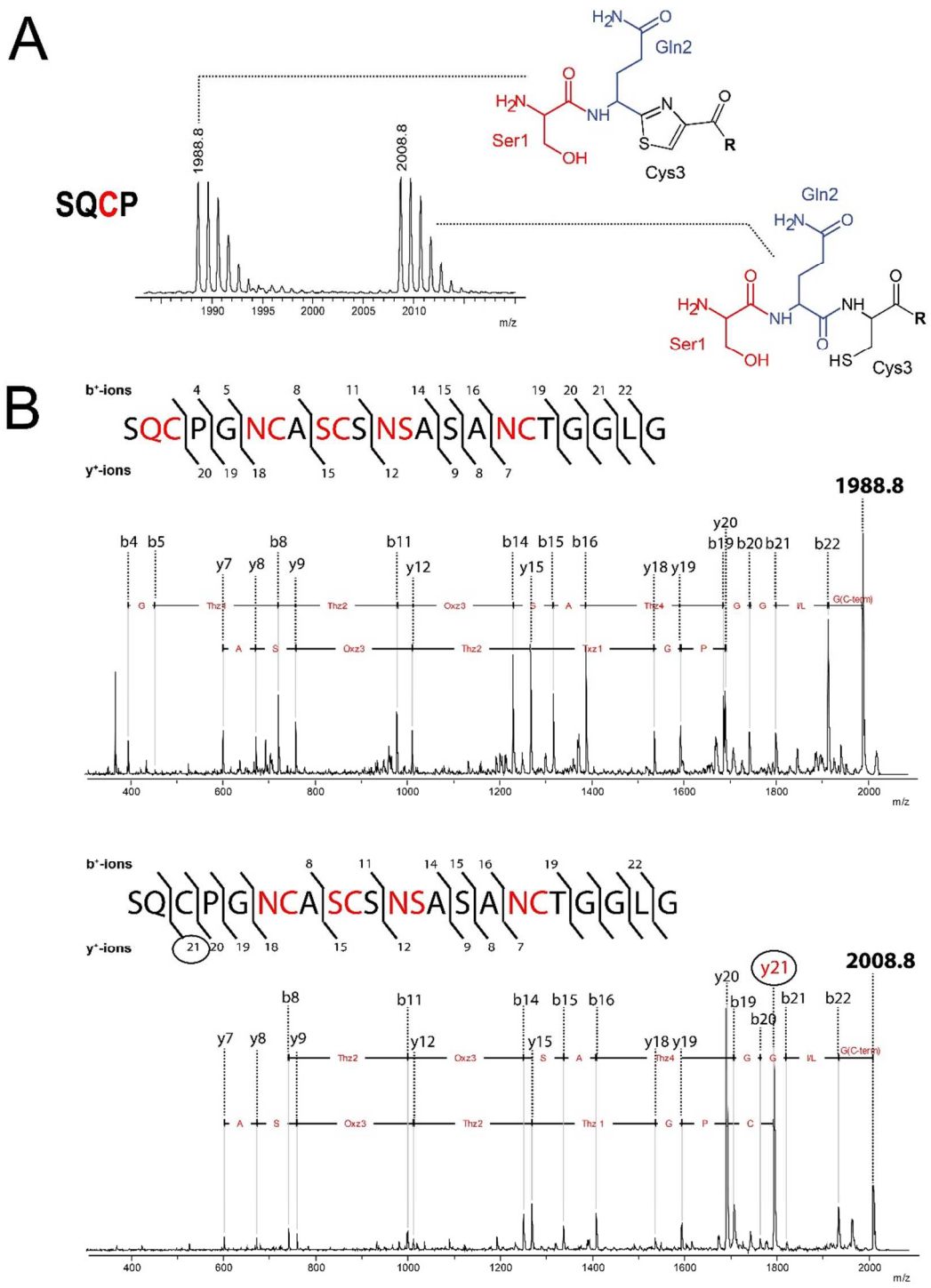

Figure S6.

A. MALDI-MS analysis of *in vitro* modification reaction containing $KlpA^{S3C}$ precursor peptide and proposed structure of the N-terminal region of the two obtained compounds (m/z = 1988.8 and 2008.8 $[M+H]^+$). **B.** Comparison of MS-MS spectra for compounds **1988.8** and **2008.8** shows a similar set of fragment ions except for y_{21}^+-ion (shown in the circle), which is detected only in the spectrum of **2008.8**. This is consistent with the presence of uncyclized cysteine residue, while in **1988.8** this residue is a part of a thiazole cycle. Diagrams for two compounds show the detected ions and amino acid residues involved in the formation of azole cycles (labeled in red).

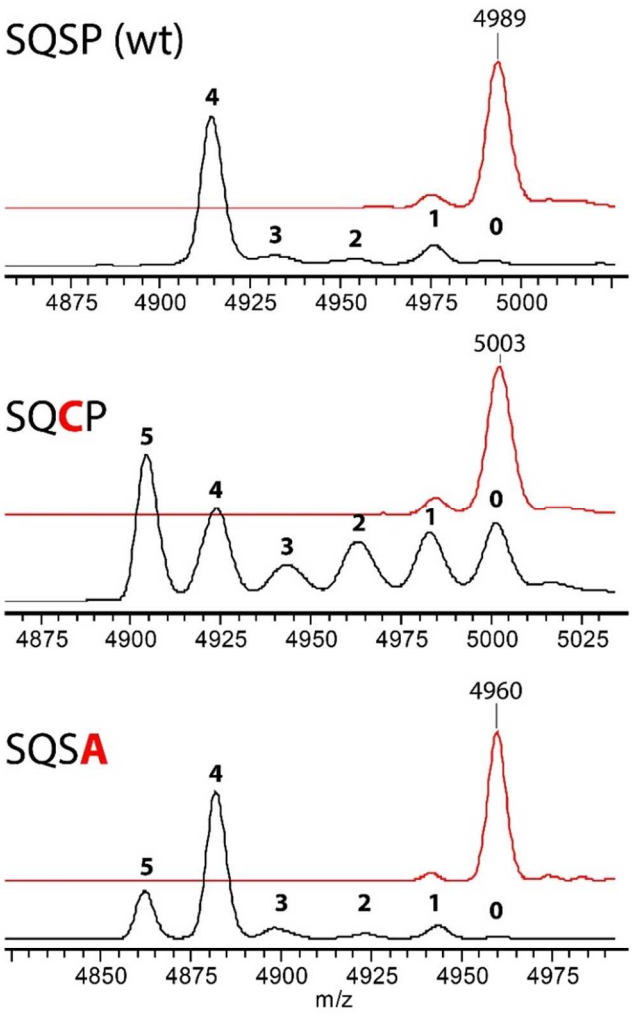

Figure S7.
MALDI-MS analysis of products of *in vitro* modification of wild type KlpA and two mutant precursor peptides. The substitutions S3C and P4A enable the formation of the fifth core cycle after KlpBCD treatment, which is not observed for the wild type peptide. The spectrum before modification is shown in red, after the reaction – in black. Three pairs of spectra are aligned according to the number of cycles present.

Table S1.
Nucleotide sequences of primers used for molecular cloning

Primer name	Primer sequence (5'-3')	Purpose
klpcF	TATTAGAGCTCCATTTATTCGAGAGCCCCAATCACG	Molecular cloning of *klpBCD* wild type and mutant variants
klpdR	AATTAGGTACCTCATGCGAATGGTATATGCCCTTTG	
klpb196F	GGATAAACATATCTGTAAAGCTGGATACAGAGGATTTCGG	Site mutagenesis in klpB gene (Tyr196Ala)
klpb196R	CCTCTGTATCCAGCTTTACAGATATGTTTATCCAACAATG	
klpd49F	CATGAAAAAGTCTTTAGGTGCAGCACTAGAAAG	Site mutagenesis in klpD gene (Glu49Ala)
klpd49R	CTTTCTAGTGCTGCACCTAAAGACTTTTTCATG	
klpa-MBPF	ATTATAGGATCCATGTCTAAAATCAAGAATCGTTTTGG	Molecular cloning of *klpA* gene to pet28MBP_TEV vector
klpa-MBPR	TATTCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACT GGC	
klpaF	ATAATCCATGGCTAAAATCAAGAATCGTTTTGG	Molecular cloning of *klpA* gene
klpaR	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTA	
klpaS1N	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACTGGCAC TATTTGAACAACTTGCACAATTACCCGGTGACTGATTATAGAGTCC CTTCTT	Molecular cloning for preparation of KlpA mutants, primers for cloning of other mutants can be found in Metelev and Osterman *et al*., *Nat. Chem. Biol.* 2017, ref (3) in the main text.
klpaQ2A	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACTGGCA CTATTTGAACAACTTGCACAATTACCCGGTGACGCACTATAGAGT CCCTTCTT	
klpaS3C	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACTGGCA CTATTTGAACAACTTGCACAATTACCCGGACACTGACTATAGAGT CCCTTCTTC	
klpaS3T	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACTGGCA CTATTTGAACAACTTGCACAATTACCCGGGGTCTGACTATAGAGT CCCTTCTTC	
klpaS3X	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACTGGCA CTATTTGAACAACTTGCACAATTACCCGGNNNCTGACTATAGAGT CCCTTCTTC	
klpaP4A	ATTATCTCGAGTTAACCTAAGCCACCTGTACAATTAGCACTGGCA CTATTTGAACAACTTGCACAATTACCCGCTGACTGACTATAGAGT CCCTTCTTC	

Table S2.
Results of MALDI-MS analysis of the mutant KLB variants from the cell cultivation medium.
Modified residues are labeled in red (thiazole and oxazole cycles) and in blue (N-terminal amidine cycle). *X=F, Y, N, L, I, V, P, G, K, A, M

Mutated position	Sequence of products detected	N-terminal modification
wt	**SQ**SPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring
Ser1	**AQ**SPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring
Ser1	**NQ**SPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring
Gln2	**SA**SPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring
Gln2	**SN**SPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring
Ser3	S**QC**PG**NC**A**SC**S**NS**ASA**NC**TGGLG **SQ**CPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Thiazole (major) +Amidine ring (minor)
Ser3	**SQ**TPG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring
Ser3*	SQ**X**PG**NC**A**SC**S**NS**ASA**NC**TGGLG	**No modification**
Pro4	**SQ**SAG**NC**A**SC**S**NS**ASA**NC**TGGLG S**QS**AG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring (~70%) + Oxazole (~30%)
Pro4	**SQ**SQG**NC**A**SC**S**NS**ASA**NC**TGGLG S**QS**QG**NC**A**SC**S**NS**ASA**NC**TGGLG	Amidine ring (~95%) + Oxazole (~5%)

MATERIALS AND METHODS

Bacterial strains and growth conditions

The following bacterial strains were used in the study: *Escherichia coli* BL21(DE3) Gold; *E. coli* BW25113, BW25113*ΔtldD* and BW25113*ΔpmbA(ΔtldE)* (JW3212 and JW4194 from the Keio collection [24] respectively). Cultures of bacterial cells were grown in LB medium (per 1L: 5 g of NaCl, 10 g of tryptone, and 5 g of yeast extract), 2xYT medium (per 1L: 5 g of NaCl, 16 g of tryptone, and 10 g of yeast extract) or M9 minimal medium (per 1L: 7.25 g $Na_2HPO_4 \cdot 2H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mM $MgSO_4$, 100 μM $CaCl_2$, and 10 μg/ml thiamine) supplemented with 1% (v/v) glycerol. When necessary, antibiotics were added to the medium at following concentrations: 100 μg/ml for ampicillin and 50 μg/ml for kanamycin.

Cloning and mutagenesis

klpCBD fragment was amplified from pBAD-*klpBCDE* plasmid (Metelev and Osterman *et al., Nat. Chem. Biol.* 2017, ref (3) in the main text) with oligonucleotides klpcF and klpdR **(Table S1).** The amplified product was digested with SacI and KpnI and cloned into pBAD His/B vector treated with the same enzymes. Site-directed PCR mutagenesis with oligonucleotide pairs klpb196F-klpb196R and klpd49F-klpd49R **(Table S1)** was used to create constructions for the production of $KlpB^{Y196A}CD$ and $KlpBCD^{E49A}$ mutant variants of the synthetase complex respectively.

klpA gene was amplified from plasmid pET28a-*klpA* (Metelev and Osterman *et al*., *Nat. Chem. Biol.* 2017, ref (3) in the main text) with klpa-MBPF and klpa-MBPR **(Table S1)** and cloned into pet28MBP-TEV vector using BamHI and NotI restriction sites. This resulted in the plasmid pet28MBP-TEV-*klpA* further used for KLB precursor peptide MBP-fusion expression.

Mutated variants of KLB precursor peptide were generated by amplifying *klpA* gene using the following pairs of oligonucleotides: klpaF–klpaS1A, klpaF–klpaS1N, klpaF–klpaQ2A, klpaF–klpaQ2N, klpaF–klpaS3C, klpaF–klpaS3T, klpaF–klpaS3X and klpaF–klpaP4A **(Table S1)**. Amplified fragments were cloned into pET28a vector using NcoI and XhoI sites, resulting in pET28a-$klpA^{S1A}$, pET28a-$klpA^{S1A}$, pET28a-$klpA^{Q2A}$, pET28a-$klpA^{G2A}$, pET28a-$klpA^{S3C}$, pET28a-$klpA^{S3T}$ and pET28a-$klpA^{P4A}$ plasmids and the library of mutant plasmids pET28a-$klpA^{S3X}$ (where X=Phe, Tyr, Asn, Leu, Val, Pro, Gly, Lys, Ala or Met). These plasmids were then used as templates for *klpA* gene variants amplification and cloning in pET28-MBP-TEV vector as described above for wild type *klpA*.

Purification of KlpBCD enzyme complexes

For purification of His-tagged BCD protein complexes, 1.5 L of 2xYT medium containing 100 μg/mL ampicillin was inoculated with 20 mL of overnight culture of *E.coli* BW25113 cells, transformed with a relevant plasmid (pBAD-*klpCBD,* pBAD-*klpCB*^{Y196A}*D* or pBAD-*klpCBD*^{E49A}*)*. Culture was grown at 37°C to an optical density OD_{600} of 0.6, induced with 1 mM arabinose and allowed to grow for 16-18 hours at 22°C. Cells were harvested by centrifugation at 5,000 rpm for 10 min, resuspended in Lysis Buffer [20 mM Tris·HCl pH 8.0, 150 mM NaCl, 10% glycerol (v/v), 5 mM imidazole, 2 mg/ml lysozyme] supplemented with protease inhibitor cocktail (Thermo) and lysed by sonication. Cell debris was removed by centrifugation at 14,000 rpm for 30 min, lysate was filtered through 0.22 μm nitrocellulose filter (Millipore) and applied to pre-equilibrated 1 mL HiTrap Chelating HP column charged with Ni^{2+} (GE Healthcare). The column was washed with 5 volumes of Lysis Buffer followed by 25 volumes of Wash Buffer [20 mM Tris·HCl pH 8.0, 150 mM NaCl, 10% glycerol (v/v), 10 mM imidazole]. His-tagged protein complexes were eluted with 5 mL of Elution Buffer [20 mM Tris·HCl pH 8.0, 150 mM NaCl, 10% glycerol (v/v), 250 mM imidazole]. Fractions containing highest protein concentrations (according to Bradford colorimetric assay (Bio-Rad)) were immediately loaded onto Superdex 200 10/300 GL gel-filtration column (GE Healthcare) equilibrated with Protein Storage Buffer [10 mM Tris·HCl pH 8.0, 50 mM NaCl, 5% glycerol (v/v)]. Peak fractions were collected, aliquoted, frozen in liquid nitrogen and stored at -80°C. Protein concentration for each of three complexes (KlpBCD, KlpB^{Y196A}CD and KlpBCD^{E49A}) was measured with DirectDetect IR-spectrometer (Millipore).

Purification of MBP-tagged KlpA peptides

For purification of MBP-tagged peptides (KlpA and its variants with single amino acid substitutions), 0.75 L of 2xYT medium containing 50 μg/mL kanamycin was inoculated with 20 mL of overnight culture of *E.coli* BL21(DE3) Gold, transformed with relevant plasmid (e.g. pET28MBP-TEV-*klpA*). Culture was grown at 37°C to the OD_{600} of 0.6, induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and allowed to grow for 3.5 hours at 37°C. Cells were harvested by centrifugation at 5,000 rpm for 10 min, resuspended in MBP Lysis Buffer [50 mM Tris·HCl pH 7.5, 500 mM NaCl, 2.5% glycerol (v/v), 0.1% Triton X-100, 2 mg/mL lysozyme] supplemented with protease inhibitor cocktail (Thermo) and lysed by sonication. Cell debris was removed by centrifugation at 14,000 rpm for 30 min, lysate was filtered through 0.22 μm nitrocellulose filter (Millipore) and applied to pre-equilibrated 1 mL MBPTrap column (GE

Healthcare). The column was washed with 25 volumes of MBP Wash Buffer [50 mM Tris·HCl pH 7.5, 400 mM NaCl, 2.5% glycerol (v/v)]. MBP-tagged peptides were eluted from the column with 5 mL of phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4] supplemented with 10 mM maltose. Fractions with highest protein concentration were determined using Bradford colorimetric assay (Bio-Rad), aliquoted, frozen in liquid nitrogen and stored at -80°C. Protein concentrations in purified MBP-fusion samples were measured with DirectDetect IR-spectrometer (Millipore).

***In vitro* synthetase assay**

MBP tag was removed from the fused peptide substrates by 16 hours of incubation with TEV protease (30°C, 1:100 by weight, supplemented with 10 mM DTT). The modification of KlpA precursor peptide was carried out at 37°C for 60 minutes in Reaction Buffer [50 mM Tris·HCl pH 7.5, 125 mM NaCl, 20 mM $MgCl_2$, 2.5% glycerol (v/v), 10 mM DTT] supplemented with 2 mM ATP when needed. Reaction mixture (total volume 50 µL) contained 10 µM of precursor peptide and 1 µM of KlpBCD modification complex. When needed, the reaction was also supplemented with 0.5 µM of TldD/E protease. Reaction was stopped by the addition of the equal volume of 1% trifluoroacetic acid (TFA). Before MALDI-MS analysis, the reactions were desalted using ZipTip C18 tips (Millipore).

Purification of *in vitro* synthesized compounds

To purify compounds **1, 3** and **4** from *in vitro* reactions, reaction mixtures (without the addition of 0.1% TFA) were supplemented with fresh DTT to 10 mM, incubated for 15 minutes at 37°C, and loaded onto Agilent HF Bond Elut LRC-C18 Cartridge (500 mg). Cartridge was washed extensively with water and 5% aqueous MeCN. Compounds were eluted with three bed volumes of 50% MeCN. Eluates were subjected to HPLC purification on Agilent ZORBAX Eclipse Plus C18 HPLC column; (4.6x150 mm, 5µm particle size). The column was equilibrated with 50 mM ammonium acetate (pH 7.5) and developed in linear gradient of MeCN in 50 mM ammonium acetate (10% to 20% MeCN in 10 min for **1** and **4**; 10% to 30% MeCN in 15 min for **3**). Fractions containing the compounds of interest were dried under vacuum, dissolved in 100% DMSO, aliquoted, and stored at -20°C.

LC-MS, MALDI-ToF-MS and MS-MS analysis

The samples were analysed by LC-MS on a Synapt G2-Si mass spectrometer coupled to an Acquity UPLC system (Waters, Manchester, UK). Aliquots of the samples were injected onto an Acquity UPLC® BEH C18 column, 1.7 μm, 1x100 mm (Waters) and eluted with a gradient of 1-60% acetonitrile in 0.1% formic acid in 11 min at a flow rate of 0.08 ml min^{-1}. The mass spectrometer was controlled by the Masslynx 4.1 software (Waters) and operated in positive MS-TOF and resolution mode with a capillary voltage of 3 kV and a cone voltage of 40 V in the m/z range of 50-2000. Leu-enkephalin peptide (0.25 μM, Waters) was infused at 10 μl min-1 as a lock mass and measured every 20 s. Spectra were generated using the Masslynx 4.1 software and by combining a number of scans followed by automatic peak detection including lock mass re-calibration.

MALDI-MS mass spectra were recorded on an Ultraflextreme (Bruker Daltonics, Germany) MALDI-ToF-ToF-MS instrument in a positive ion measurement mode. The spectra of unprocessed precursors (**2** and **3**) were obtained in a linear mode with an accuracy of measuring the average m/z up to 1 Da. The spectra of compounds with removed leader peptide (**1** and **4**) were obtained in reflecto-mode with an accuracy of measuring the monoisotopic m/z up to 0.1 Da. The fragmentation spectra were obtained using the Lift mode, the accuracy of measuring the daughter ions was within 0.2 Da. Sample aliquots were mixed on a steel target with a 30 mg/ml 2,5-dihydroxybenzoic acid in 0.5% trifluoroacetic acid and 30% acetonitrile water solution (Aldrich).

Bioactivity evaluation

For *in vivo* bioactivity test the reporter strain BW25113-pDualrep2 was used as previously described.[25] Briefly, 2 μl of tested solutions of compounds were applied to agar plate that already contained a lawn of the reporter strain. 10 mM KLB (purified from groth medium/obtained *in vitro*), 20 μM erythromycin and levofloxacin solutions were used. After overnight incubation of the plate at 37°C, it was scanned by ChemiDoc (Bio-Rad): “Cy3-blot” for RFP and “Cy5-blot” for Katushka2S.

For *in vitro* translation inhibition assay the in vitro transcribed firefly luciferase mRNA (final concentration 0.02 μM) was translated in the *E. coli* S30 cell-free system for linear templates (Promega). Reactions were carried out in 10 μL aliquots at 37°C for 30 minutes in presence of 0.1 mM D-luciferin with simultaneous detection of the signal by Victor X5 reader (Perkin Elmer).