Thesis Changes Log

Name of Candidate: Tatyana Zyubko

PhD Program: Life Sciences

Title of Thesis: efficient in vivo synthesis of lasso peptide pseudomycoidin proceeds in the absence of leader and leader peptidase

Supervisor: Prof. Konstantin Severinov

Chair of PhD defense Jury: Prof. Douglas A. Mitchell, Prof. Sylvie Rebuffat, Prof. Dmitry Ivankov, Prof. Konstantin Lukyanov, Prof. Timofei Zatsepin.

Email*:* ***pochta.tz@gmail.com***

Date of Thesis Defense: 19/12/19

The thesis document includes the following changes in answer to the external review process.

Dear jury members,

I am immensely grateful to all my reviewers for a careful reading of my manuscript as well as the critical remarks and numerous useful comments. All your suggestions have been addressed and the required information was added where necessary. Below are the answers to specific points you have raised.

Reviewer: Prof. Douglas A. Mitchell

- "*Page 9: there are font size inconsistencies in the abbreviations listing. MBP, "maltose" typo."*
  Answer: Thank you.The font size was made uniform, a typo corrected.

- "*Page 11: self-immunity is only needed if there is self-toxicity. Many metabolites could be toxic to other organisms but not the producer itself. Consider rephrasing."*
  Answer: Thank you for the suggestion, the sentence was rephrased. Now it reads (page 10): "Additionally, the clusters often encode an export system for the mature product and in some cases of self-toxic antimicrobial peptides (AMP), a self-immunity system."

- "*Page 11 (and throughout the thesis): abbreviations are defined and then not used. Numerous instances of BGC not being used after being defined. PTM on page 12, etc. etc."*
  Answer: A list of abbreviations has been updated and the use of abbreviations throughout the text was made consistent.

- "*Figure 1: need NH2 group for the N-terminus, not NH."*
  Answer: I have corrected the figure, thank you.

- "*Page 12: bottromycin has a follower, not a leader."*
  Answer: Thank you. The sentence has been corrected. Now it is (page 10):" Far less frequently (i.e., in bottromycins) the follower sequence is present at the C-terminus of the precursor peptide."

- "*Figure 2: Cyanobactin misspelled. Also, inconsistent hyphenation in "lasso peptide". Recommend not using hyphenation*"
  Answer: Thank you for the suggestion, I have corrected the figure.

- "*Figure 3: Low quality. Use standardized drawing settings so all chemical structures in the thesis are consistent."*
  Answer: I changed the figure for the one with a better quality.

- "*Page 13: Gram-negative is a phenotype and not useful for taxonomic classification. For instance, eukaryotes are "gram-negative" but microcin B17 does not kill them."*
  Answer: I agree with this comment, the sentence was ambiguous. Now the text reads as follows (page 12): "**Microcin B17** (MccB17) is produced by various *E. coli* strains [1] and inhibits DNA gyrase [2]–[5]."

- "*Page 14: TOMM is a term that is no longer in usage. Just use LAP."*
  Answer: TOMM abbreviation has been changed for LAP in the whole text.

- "*Figure 4 (and elsewhere): If figures are being reproduced from others' publications, it should be clearly stated as such in the caption."*
  Answer: All the figures have been carefully worked through and the original sources acknowledged in captions.

- "*Page 15: there is a square character in place of a the Greek symbol, I presume, in paragraph 2. The same is true for page 29."*
  Answer: It is, I added the symbol again.

- "*Page 16: by my count, there are 70 reported lasso peptides, so if table 1 is meant to be comprehensive, it is not. This page also has grammar issues in the first and last paragraphs. Lastly, to say lasso peptides "normally" are threaded is misleading. There is no reason to believe any are unthreaded. The lassomycin report really muddied the water on this topic. Regarding the top of page 18: the NMR solution structure of lassomycin was obviously incorrectly assigned by the authors. There is no reason to believe lassomycin unthreaded in that solvent. However, this is what was observed by Link (fuscanodin, 2019 JACS)."*
  Answer: Thank you for a through reading of my manuscript.
  (1)The missing peptides have been added to the table (subterisin, leepeptin, cattlecin, ubonodin, mycetohabin-15, -16 and pandonodin). Overall, the final number of peptides listed in the Table 1 now is 69. The final figure of 69 is different as compared to your list it may arise from the same peptide having multiple names (RP-71955 = siamycin III =

aborycin; fuscanodin = fusilassin; lariatin A without C-terminal GlyPro = lariatin B; etc.). (2) I may agree with your suggestion about lassomycin, but there is no reason to believe that all the undescribed lasso peptides will be found to have a threaded structure.

- "*Figure 5: not particularly pleasing on the eye. If you wish to show the topological chirality, there are better ways to depict these structures are non-superimposable mirror images of one another.*"
  Answer: I added a top view of the right- and left-handed peptides in Figure 5, hope it makes it better. Now it looks as follows:

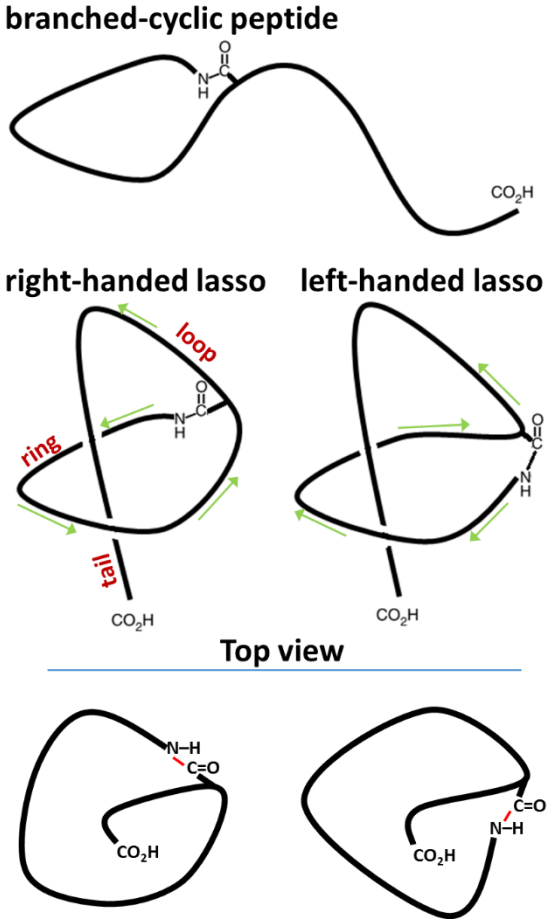

***Figure 5*. A scheme of an unthreaded branched-cyclic peptide in comparison with a threaded one**. Lasso peptides are chiral; only right-handed ones have been found.

- "*Page 18: what is so "unusual" about a macrolactam bond being formed? This type of PTM is very common.*"
  Answer: Thank you for this remark. The lasso-peptide PTMs involve not only the macrolactam ring formation but also an unusual folding of the precursor peptide (resulting in the C-tail trapped through the ring).

- "*Table 1: recommend Courier font for the core sequence column so the character spacing is consistent.*"
  Answer: The font was changed.

- "*Figure 8: I think some discussion of the pre-folded state is in order. As is, it would be hard for a non-expert to know what is happening here.*"
  Answer: To make the Figure clearer I modified it and added more detailed captions. Now it looks as follows"

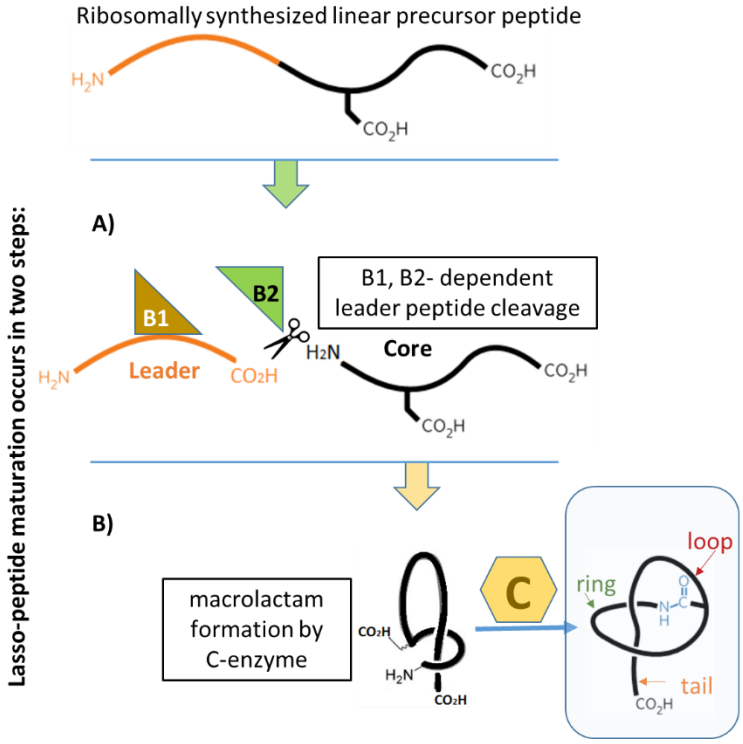

Figure 10. **A typical lasso peptide biosynthetic pathway by essential processing enzymes occurs in two steps:** A) leader peptide cleavage; B) catalysis of the isopeptide bond formation in the prefolded core peptide resulting in the N-terminal macrolactam ring trapped with the C-terminal tail.

- "*Page 27: I am fairly certain that StrA leader peptide binds to StrB as a beta strand, not alpha helix. Also, does it make physical sense that LarB1 could interact with the core of LarA?*"
  Answer:I am particularly grateful for this comment and agree with it. I was not entirely correct in this conclusion. To be more precise, the X-ray crystallography was performed on the SuiB, a close orthologue of StrB, rather than on StrB, so we cannot claim the StrB-StrA interaction with absolute certainty[1]. Indeed the leader peptide of SuiA during interaction with SuiB was shown to form an alpha-helix [1], but rather than binding to the RRE domain of SuiB , SuiA leader peptide interacts with the SuiB catalytic barrel. So, I'm going to excise the relevant passage from the thesis.

  Now the text reads as follows (page 30): "A number of co-crystallization and NMR experiments have clearly demonstrated that a wHTH motifs are strongly associated with their respective leader precursor peptides [6]–[10]. During this interaction the leader sequence can form a β-strand with the β-sheets of wHTH motifs of enzymes LynD [6], NisB [9], TfuB1 [10], PqqD [8], etc."

- "*Page 28: Most B2 proteins do not have a conserved Asp residue, so calling this a catalytic dyad would be more accurate.*"
  Answer: Thank you for pointing this out, the wording has been changed. Now it is (page 31):

"The B2 proteins contain conserved Cys-His-Asp/Cys-His residues forming the catalytic triad/dyad typical for transglutaminase peptide family."

- "*Page 29: Although it hasn't been definitely proven, the prevailing view is that the lasso cyclase would emulate the AsnB reaction. Thus, the N-terminus displaces AMP (not ADP) from the carboxylate sidechain."*
  Answer: Thank you for this comment. Yet I can't use this point in my literature review since it should be accompanied with references.

- "*Figure 9: ATP is not required for leader peptide cleavage, thus this figure should be corrected. The text on page 31 should be corrected to so as to not propagate incorrect information. Also, Figure 10. It's pretty clear that the ATPase activity is attributed to co-purification of an ATP-dependent chaperone."*

  Answer: The text and figures were corrected. Now it is:

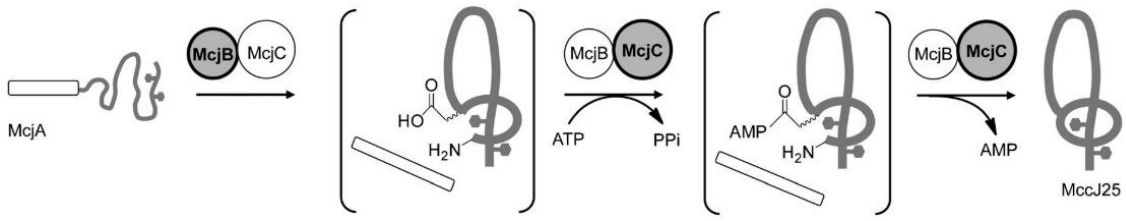

Figure 11. **Proposed two-step biosynthetic scheme of microcin J25 maturation in the presence of ATP and Mg^{2+} ions**. McjB and McjC presumably interact to form the synthetase. The functional enzyme at each step is indicated with bold outlines, the grey empty bar represents the leader peptide. *Adapted from* [11].

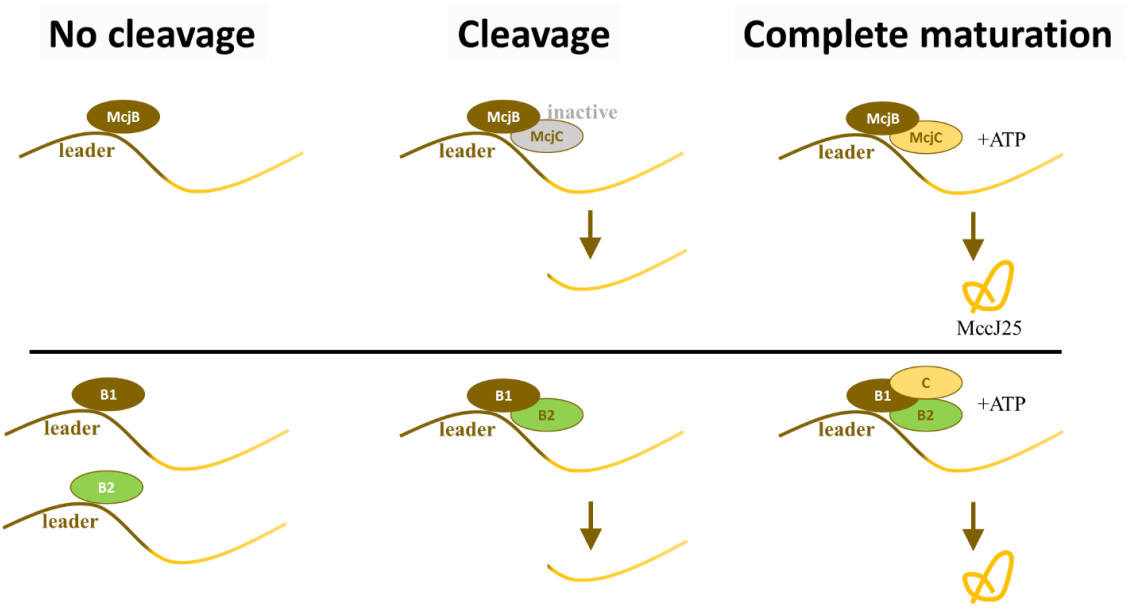

Figure 12. **A scheme of *in vitro* processing of lasso-precursor peptides with various combinations of precursor peptides, maturation enzymes and ATP**. The top panel represents data relevant to MccJ25 biosynthetic system with fused B-protein. The bottom panel shows the scheme for separated B1 and B2. *Adapted from refs*. [11], [12].

- "*Page 30: What is the direct evidence that McjC can recognize and cyclize a linear lasso peptide (to yield a branched cyclic structure, I presume)? How can you be sure it didn't recognize a pre-folded substrate, then it forms the macrolactam, which immediately unfolds after dissociation from the enzyme since the steric locks where insufficient? This seems more plausible to me, given what is known in the current literature."*
  Answer: Thank you for this comment. It might be a speculative suggestion, yet maturation of the modified mcj25 precursor peptide (involving the F10-V11 or G12-G14 deletions only with the lock residues F19-Y20 still present), results in the formation of branched mcj25. However, in case of these deletions in a prefolding state the lock residues could be placed above the ring. Yet, our substrate-mutation analysis has demonstrated the brunched cyclic topology for the cyclized H22F and H23F psm, while L21F was shown to be threaded (with the same ring size as well as the Phe "lock" residue). If the cyclization occurs only with a prefolded substrate, then we cannot explain the biosynthesis of these cyclized derivatives.

- "*Page 36: The first paper on RODEO found 1400. The more recent publication catalogued ~3000 lasso BGCs. Other corrections needed are: capistruin and mcj25 bind to enzymes while siamycin does not. It binds lipid II!"*
  Answer: **(1)** You are absolutely correct regarding the predicted with RODEO lasso BGCs of there are ~3000; and I mention it in the section "Lasso peptides: structure, classification". To improve the clarity of this passage I added the reference to the page 39 as well. Now it reads as follows: "Using the C-centric approach they found more than 1300 new clusters, while more recent paper reported about 3000 novel lasso BGCs [13], [14]."
  (2) You are also correct regarding the cellular targets of capistruin, mcj25 and siamycin. However, my text provides the same information and the corrections are not needed.

- "*Page 37: portions of the middle paragraph feel repetitive and thus unnecessary*"
  Answer: I have rephrased the paragraph. Now it is shorter and reads as follows: "As most lasso peptide clusters encode export pumps, they must be functioning outside the producer cells. Presumably, some lasso peptide can signal to other bacteria of the same or different species. Most effects associated with the non-bacterial targets were found via a systematic screening and might merely be coincidental, whereas the antibacterial activities are likely to be native functions."

- "*Page 38: several inaccuracies on this page. 1. The epitope that was grafted into the mcj25 loop was to engender integrin binding, not angiotensin. The reference to Bode here is inappropriate, as he didn't even make a "lasso peptide" in that study. Also, to my knowledge, there have not been any other chemical syntheses of lasso peptides except for the BI-32169 case. What are the other reports (reference 166 does not make a lasso peptide, as previously stated) The rest of the thesis (the actual science part, as opposed to background) is considerably stronger and much freer of errors."*
  Answer: Thank you for the comments, I agree with you and made the necessary corrections in the text and references. Now the passage reads as following (page 24): "The epitope molecular grafting approach has been effectively applied to create a microcin J25 derivative that was able to specifically bind and inhibit integrin [15] as well as D-enantiomer of BI-32169, which shows a strong glucagon receptor antagonist activity and much higher enzymatic stability compared to the L-lasso peptide [16], [17]."

- "*Figure 20: appears a bit fuzzy, and thus harder to interpret."*
  Answer: I replaced the figure with a bigger one. Hope it is clearer now (page 60):

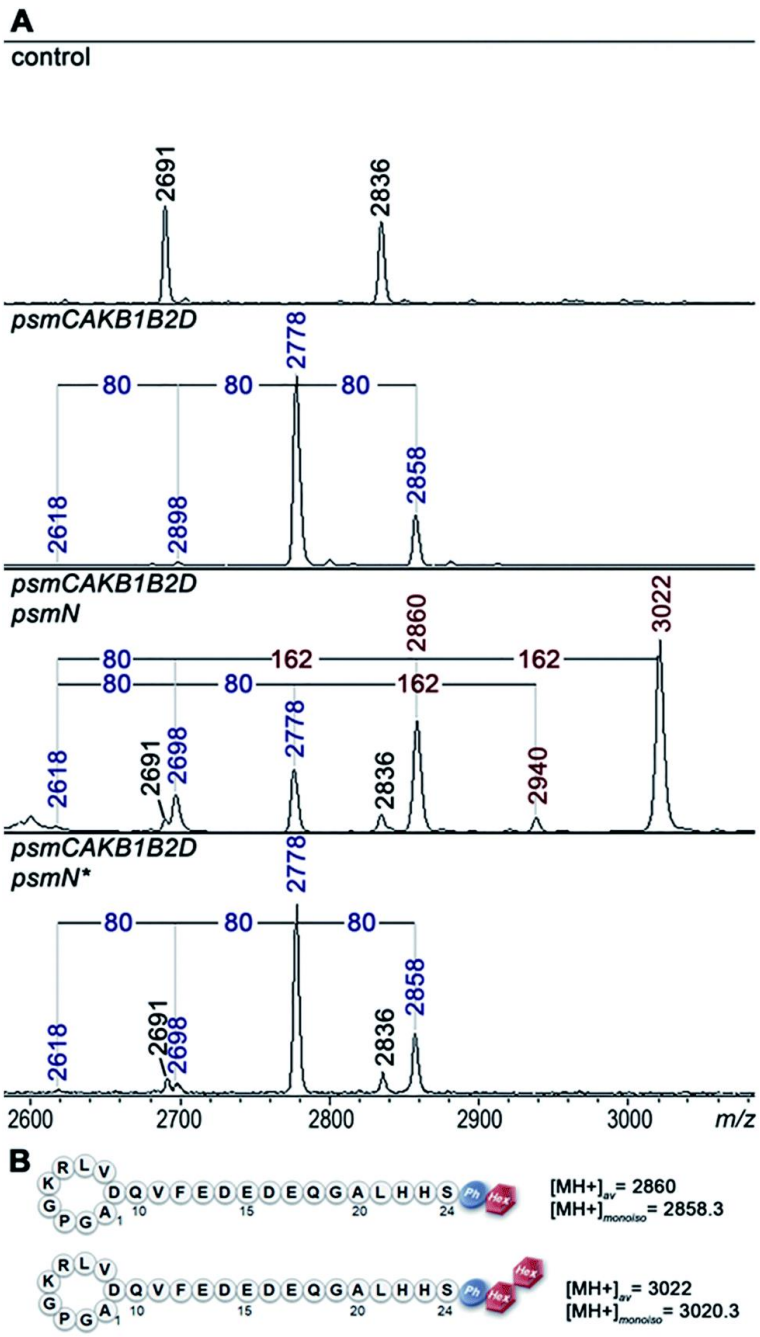

***Figure 21*. PsmN is required for an additional post-translational modification of phosphorylated pseudomycoidin.** (A) MALDI-MS analysis of *E. coli* BL21(DE3) cells containing control plasmids, or plasmids expressing the *psm* cluster with *psmN* deleted, with or without additional plasmid expressing wild-type, or mutant (*psmN**, encodes the D134A D136A double mutant) *psmN*. The average m/z values of mass-peaks specific for cells carrying the *psm* genes are marked with blue font and for *psmN*-dependent peaks, brown color font. (B) Schematic structures of fully modified pseudomycoidins.

- "*Figure 23: Polymyxa is misspelled. Also, the length of B1 should be shorter than B2. The ORF arrows should be drawn to scale."*

Answer: All the necessary corrections were meticulously made here and throughout the manuscript. The Figure 23 now looks as follows:

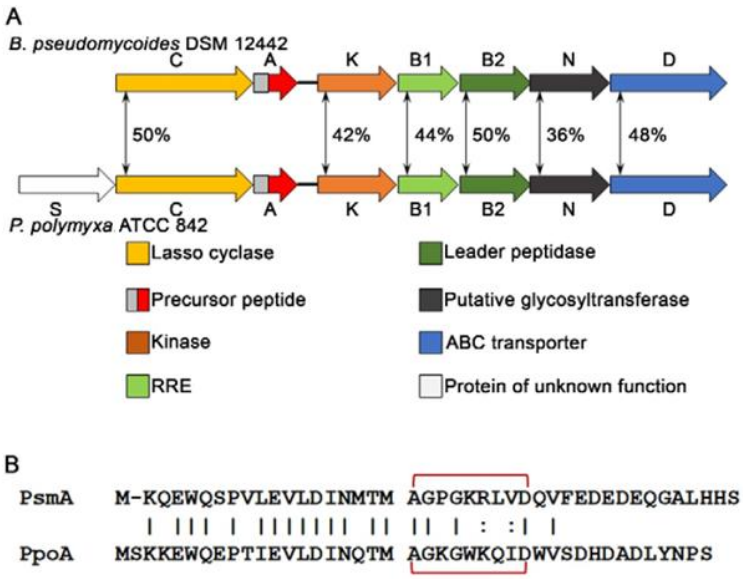

Figure 24. **Comparison of lasso peptide gene clusters from *B. pseudomycoides* DSM 12442 and *P. polymyxa* ATCC 842.** (A) A schematic representation of the gene arrangements in the clusters. Genes are indicated by arrows, and homologous genes are indicated by identical colors. Numbers indicate the percentage of identity between homologous protein sequences. Known or putative gene product functions are listed at the bottom. PpoS has homology to the sulfotransferase family of proteins. (B) An alignment of precursor peptides from *B. pseudomycoides* DSM 12442 and *P. polymyxa* ATCC 842. Amino acids forming the macrolactam ring are linked by red brackets.

- "*Figure 25 (and anywhere high-resolution MS data are shown): provide calc mass values and give error in ppm."*
  Answer: The missing numbers were added (pages 67-68, Figures 26-27 captions): "Calculated value for brutto formulae: 2616.2439; error: 0.03 ppm. Internal calibration (ACTH fragment18-39 (2465.1989Da) and Insulin oxidized B chain (3494.6513Da)."

- "*Figure 28: Was the Y-shaped (tryptic product) ever characterized by MS/MS to ensure the authors are making conclusions based on the right information?"*
  Answer: Yes, we analyzed the trypsin digested product with MS/MS and HPLC followed by MS (the data are not shown) prior performing the double digest analysis of psm.

- "*Page 73: The evidence that pseudomycoidin isolated from heterologous expression had undergone unthreading would be more compelling if H/D exchange was performed. If unthreaded, literally all amide NH's would exchange immediately. If threaded, several residues in the ring and tail would very slowly exchange. This can be done on very small amounts of material by using MS."*
  Answer: Thank you for the recommendation, that is great idea.

- "*Figure 33/35: Curious as to why H23F and L21F variants were prepared as opposed to Trp. Larger is better if one was suspecting unthreading."*
  Answer: In this regard, we just wanted to find the lock residue for this peptide and the Phe residue was the first randomly chosen candidate among bigger amino acids (Tyr, Trp). The first attempt was successful, so we decided not to make any further ones.

- "*Page 82: Bioinformatics surveys have been published using both the cyclase and the leader peptidase as the "foothold". They yield very similar BGCs thus it is highly improbable that "many" lasso BGCs were overlooked. Surely there are a few but the data would suggest they are rare overall."*
  Answer: Thank you for the remarks, all the necessary corrections were implemented.

Reviewer: Prof. Sylvie Rebuffat

- "*Figure 1: RiPP biosynthesis uses in many cases bifunctional ABC transporters called PCAT (Peptidase Containing ATP-binding Transporters) or AMS (ABC transporter Maturation Secretion), which ensure both proteolysis (protease) and export (ABC exporter) (see the review Beis et al 2019, Res Microbiol and reference herein). This has to be mentioned and the figure modified accordingly.*"
  Answer: I am particularly grateful for this information. I have used this reference and incorporated into my literature review (page 10): "Typically, the export system consists of the ABC-exporters belonging to one of the two major types: transporters that exclusively export AMPs or bifunctional transporters that ensure both the maturation of AMP by cleaving the leader region of precursor and secreting the mature peptide (Peptidase-Containing ATP-binding Transporters) [18]."

- "*Throughout the manuscript (p. 11, p. 15, p. 23, 24, etc) "small" is used to qualify peptides or peptide precursors of 20 to 50 amino acids, while a peptide considered as "small" contains less than 10 amino acids. This should be corrected. Similar page 16, the description of lasso peptides has not to insist on their "small" size (they contain 14 to 26 amino acids...).*"
  Answer: I have removed the word "small" in many.

- "*Throughout the manuscript change "express" to "produce" or "synthesized" when required: "expression" is incorrect for any compound — genes are expressed, whereas compounds are either modified from peptides or synthesized by enzymes and thus "produced" by bacteria and other organisms.*"
  Answer: Thank you for this suggestion, I have made the necessary changes throughout the text of my thesis.

- "*Table 1 page 19 is a very good point in the chapter as it aims at assembling all lasso peptides discovered and identified until now. To improve it, appropriate references for each peptide have to be included in the Table. In addition some recent lasso peptides, which have been evidenced to adopta lasso fold have not been included, for instance: subterisin (Kuroha 2017, Tetrahedron Lett 58, 3429-32); specialicin (Kaweewan 2019, Bioorg Med Chem, 26, 6050-55); leepeptin (Gomez-Erscribano 2019, Appl Environ Microbiol doi: 10.1128/AEM.01752-19). Litterature in the domain has to be carefully checked and the missing peptides have to be added.*"
  Answer: Many thanks for pointing this out, I have added seven novel lasso peptides into the table (subterisin, leepeptin, cattlecin, ubonodin, mycetohabin-15, -16 and pandonodin).The references for all listed peptides have been added as well.

- "*Figure 7 page 22 shows a very heterogeneous representation of lasso peptides (color/no color, amino acid numbering/no numbering; moreover the figure seems to show that some lasso peptides adopt a lasso topology (siamycin, paeninodin), while others would be in the branched-cyclic topology, while this has not been assessed (citrulassin which is known to be in the lasso fold and lassomycin which has been shown to adopt a non-lasso fold are represented similarly). This figure has to be modified and clarified and the lasso or cyclic-branched topology has to be specified for the different peptides.*"
Answer: I have modified figure 7 following your suggestions. Now it looks as follows (page 22):

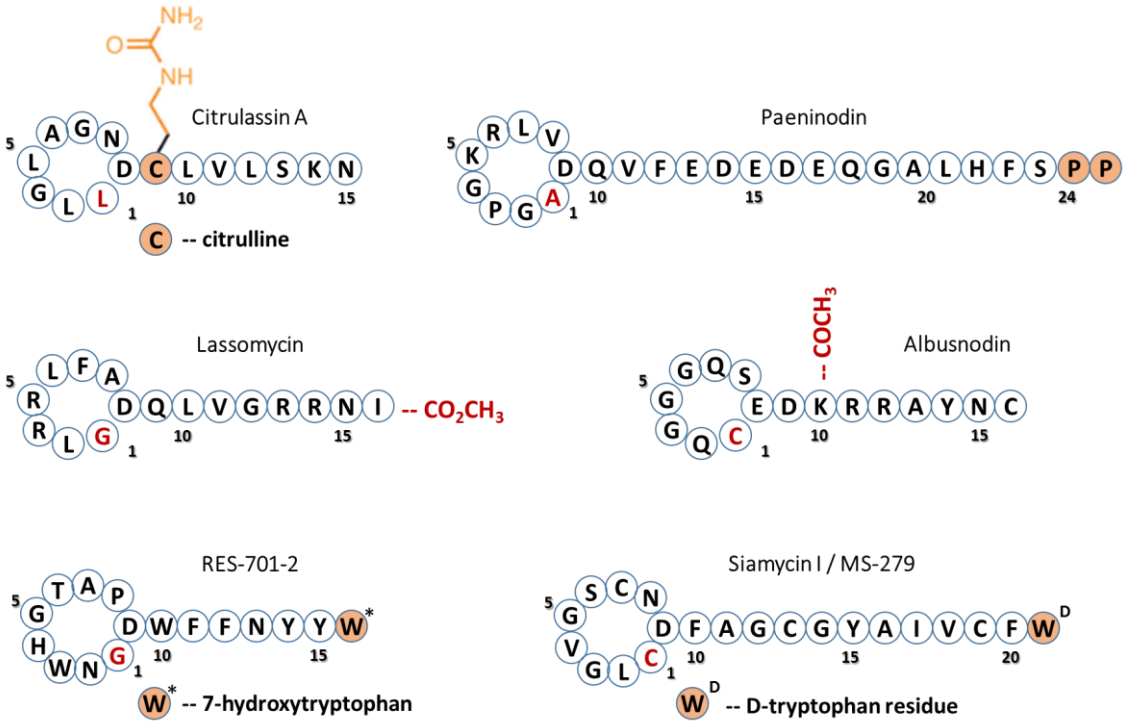

***Figure 8. Lasso* peptides with additional modifications**. Peptides carrying C-terminal tailoring modifications: lassomycin, [19] with a methyl group at C-terminal carboxyl; RES-701-2 hydroxylated at the side chain of C-terminal Trp [20]; siamycin I / MS-279 [21] with D-Trp residue at the C-terminus, albusnodin with acetyllysine post-translational modification, and paeninodin phosphorylated at the side chain of C-terminal serine [22]. A recently reported lasso peptide citrulassin A possesses Arg9 modified to citrulline [13], [23].

- "*The paragraph on lasso peptide chemical synthesis, which describes the chemistry of the tentative chemical synthesis of lasso peptides published, is included in the section describing the perspectives opened by using the lasso peptide scaffold for creating novel bioactive peptides (section 1.7 Lasso peptides as an efficient scaffold for molecular grafting). This chemical approach should be mentioned page 23 as a short section entitled "Chemical synthesis of lasso peptides" before section "1.3 Enzymatic biosynthesis of lasso peptides", and only a brief reminder included in section 1.7.*"
Answer: The paragraph has been moved as you suggest.

- "*Page 37 last line, legend to figure 29 page 71: change "rotaxane" to "{1Jrotaxane"; page 71 line 5, page _73 lines 5 and 9, specify which type of "rotaxane". [1]rotaxane or "[2]rotaxane", is concerned; (the lasso topology is a [1]rotaxane and the branched-cyclic non lasso topology where a tail fragment remains blocked inside without covalent bond is a [2]rotaxane); thus specifying which type of rotaxane is involved is essential.*"

Answer: Thank you for the explanation, all mentions of "rotaxanes" in the thesis were revised.

- "*Page 50 two last lines: m/z values (here MH*) should not be expressed in Da; this has to be corrected.*"
  Answer: The "Da" was excised, now it looks as following (page 53) : "The expected average [MH+] of the lasso peptide formed from PsmA, if one assumes processing at the leader-core junction based on similarity to paeninodin, is 2618 (Figure 14.B).".

- "*Page 51 third paragraph, the sentence describing the presence of m/z ions typical of phosphorylated pseudomycoidin species is unclear and has to be reworded.*"
  Answer: Thank you for pointing at this ambiguity, the sentence has been edited and now reads as following: "Three peaks, with the average m/z = 2698, 2778, and 2858 differed from the m/z = 2618 peak and from each other by 80 atomic unit increments, that is a value matching a phosphate group".

- "*Page 53 first line and legend to figure 16, the amino acid sequence AGPGTSTPD is not found in pseudomycoidin but in paeninodin. Change to the correct amino acid sequence.*"
  Answer: Thank you for this suggestion, the legend was revised (page 54): "***Figure 16.*** **MALDI MS-MS analysis of unphosphorylated and phosphorylated forms of pseudomycoidin**. Upper panel – the fragmentation spectrum of unphosphorylated pseudomycoidin (average m/z = 2618, monoisotopic m/z = 2616.2). The peaks corresponding to the C-terminal fragments of the lasso peptide are marked. Low-intensity daughter peaks are magnified in the insert. The daughter peak with m/z = 877 matches the macrocycle formed from the N-terminal AGPGKRLVD peptide. Middle and lower panels – fragmentation spectra of monophosphorylated (average m/z = 2698, monoisotopic m/z = 2696.2) and diphosphorylated (average m/z = 2778, monoisotopic m/z = 2776.2) pseudomycoidin. Fragment analysis shows the presence of phosphate group(s) attached to the C-terminal serine residue. Mass differences of 98 and 80 Da match H_3PO_4 and -HPO_3 groups, respectively."

- "*Pages 78-79: it is surprising that the brief NMR analysis of natural pseudomycoidin and its L21F variant does not describe the typical NOE correlations that should occur between amino acids from the ring and the loop that unambiguously assign a lasso topology. Even if a complete three-dimensional structure analysis of the two pseudomycoidins is not included in the scope of the PhD, this specific point should be added or discussed, as it is critical and the only information discussed in the manuscript only allows to confirm the presence of the macrolactam ring closed between A1 and D9 side-chain. In addition enlargements of the important regions in the TOCSY/NOESY 2D spectra should be added.*"
  Answer: We performed NMR analysis of a wild-type (branched cycle) pseudomucoidin and the L21F mutant that appeared to have the threaded lasso topology based on biochemical assays. The NMR data confirmed these conjectures. The NMR data were quite sound but we did not collect enough data needed to determine a three-dimensional structure of pseudomucoidin, a task which may not be possible at the moment. As it has been reported earlier by the Marahiel group for a very similar compound paeninodin [24]: "Therefore, we hypothesize that paeninodin may adopt more than one stable conformation in solution. In addition, we propose that these coexisting conformations

undergo rapid chemical exchange, which results in overlapping signals and hinders NMR structure determination".

- "*Although the list of references is appropriate, up to date references and particularly reviews published recently on lasso peptides and associated domains, and showing the field is highly competitive and raises high interest, have to be included (on lasso peptides: Hegemann J, ChemBiochem 2019, Tan S, Antibiotics 2019; Cheung-Lee WL, J Industrial Microbiol Biotechnol 2019; on transporters: Beis K, Res Microbiol 2019).*"
  Answer: Thank you for this recommendation, I have implemented the new information and the references to recent studies to my text.
  Page 10: "Typically, the export system consists of the ABC-exporters belonging to one of the two major types: transporters that exclusively export AMPs or bifunctional transporters that ensure both the maturation of AMP by cleaving the leader region of precursor and secreting the mature peptide (Peptidase-Containing ATP-binding Transporters) [18]."
  Page 28: "RREs consist of three N-terminal b-strands and three C-terminal a-helices. They typically bind the leader peptide in a cleft between the b3-strand and a3-helix [25]."
  Pages 16-17: "The heat stability of lasso peptides depends on many factors such as the size of the ring, the plug residues and the amino acid sequence forming the macrolactam ring [69]. In fact, the xanthomonins contain the smallest so far known macrolactam rings consisting of 7 residues only, which makes the WT xanthomonins and variants thereof extremely stable. Only variants of xanthomonin II with the plug smaller than Ser residue are thermally unstable fold [63]. For the heat-sensitive lasso peptides having 8aa rings, such as caulosegnin I, the thermal stability can be accomplished with a larger lower plug residue, e.g. with the E16W substitution [71]. The heat-sensitive astexin-1 involving 9aa macrolactam ring the same as caulosegnin I can be made heat-stable by the F15W substitution of the lower plug residue [57]. At the same time, the caulosegnin III, caulonodin VI and caulondin V containing the largest known 9aa-macrocycles do not possess thermal stability, even when the biggest amino acid, Trp, is used as a lower plug [65], [71]."

  Page 18: "The chromatographic behavior of the branched cyclic and threaded lasso peptides is usually distinct (different retention time), while their masses are identical (Figure 6) [62], [69], [76].

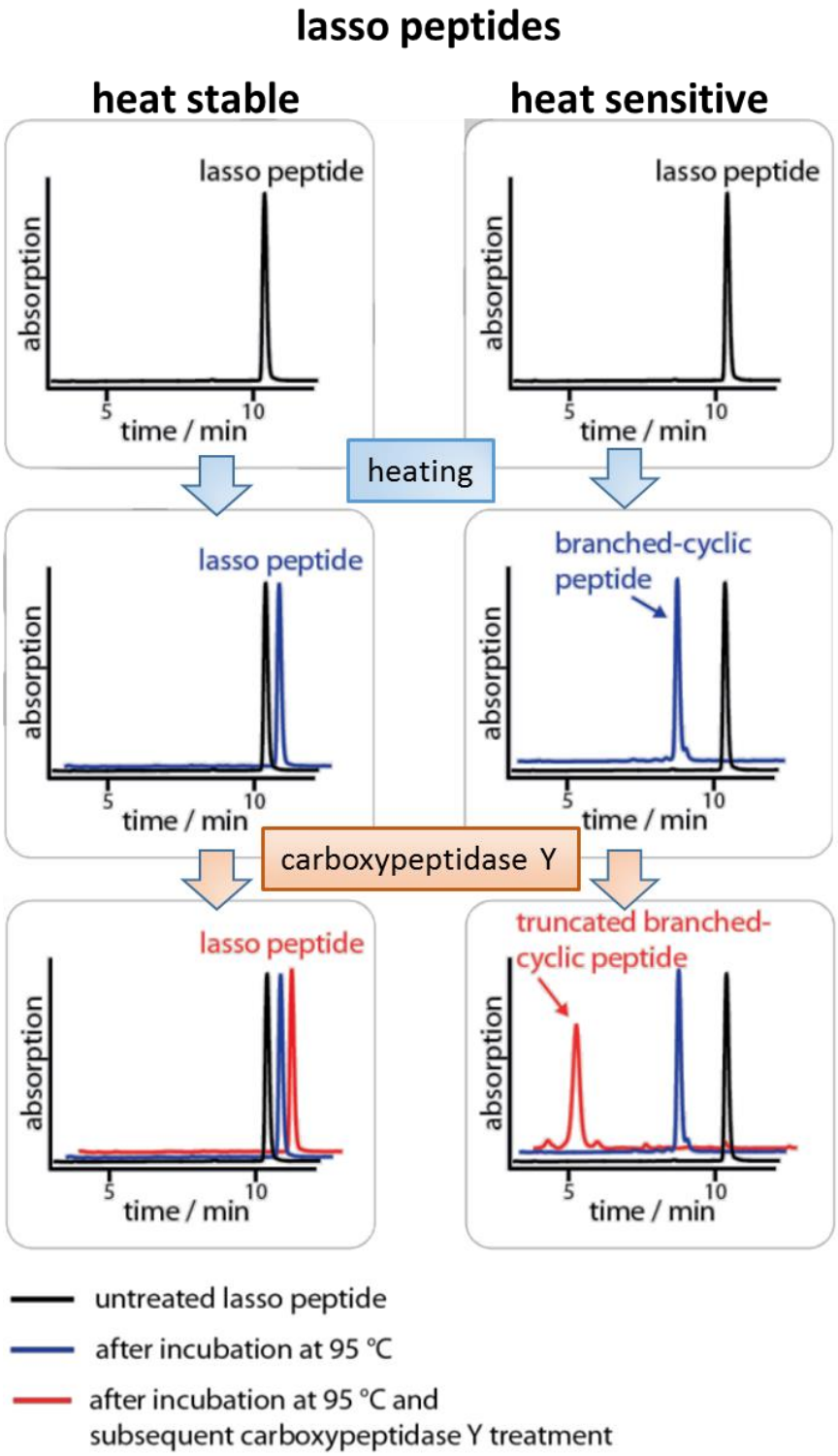

Figure 6. **Comparison of the LC traces of a heat stable and heat sensitive lasso peptide:** after thermal treatment (middle panel) and subsequent carboxypeptidase Y digestion (lower panel). *Adapted from* [69]."

Also, the paragraph "To identify the threaded lasso-structure, carboxypeptidase Y (CPDY) digestion is commonly used [64], [69]. CPDY is capable of nonspecific hydrolyzation of a peptide bond at the C-terminal end of the peptide. In the case of lasso peptides, the action is directed to the tail region. The tail trapped through the ring is assumed to be protected from degradation through the steric hindrance formed by the surrounding macrolactam ring: the enzyme would stop cleaving the tail close to the ring resulting in a ring-loop containing product. On the contrary, an unthreaded peptide will undergo further degradation to a stable macrocycle product (Figure 6, lower panel)" **has**

been moved from the results section to the section "1.2. Lasso peptides: structure, classification."

- "*The list of references is very badly presented. It has to be checked and corrected carefully as it contains a lot of mistakes: page numbering are lacking (for instance [56, 68, 97, 98, 104, 105, 112, 117... and many others]), or are wrong (for instance [72, 85,...]), author names (or part of author names) are lacking (for instance [2, 65, 116 and many others]), names of the journals are lacking (for instance [40, 47, 83, 97,... and many others]); in many references, genus and species names of bacteria and other organisms have to be in italics; papers cannot be "in press if published before 2019 (for instance [49]); some references are in full capitals ([150]. In addition, all author names or at least four or five and not only the first one have to be cited in some references (for instance [60]).*"
  Answer: The list of references was corrected.

- "*In supplementary materials, it is required to provide titles to the tables.*"
  Answer: The titles have been added: "Table S1. The nucleic acid sequences of the genes, consisting of the psm cluster; Table S2. The list of oligonucleotides used in the study."

- "*Quality of certain figures has to be improved, particularly Fig 16 and 19 where m/z values are difficult to read, or Fig 20B.*"
  Answer: I have replaced the figure 20 (page 60) with a bigger one, but the figures 16 and 19 were left as is, because it is hard to fit them into a single page given the amount of information on them.

- "*When abbreviations are used they should be defined the first time they appear and then used throughout the manuscript (ex: microcin J25 p. 25, which has been abbreviated since page 15).*"
  Answer: I've replaced most of the relevant phrases with their abbreviations. A few of them were left as is for the purpose of readability.

- "*Figure 2: change "cianobactin" to "cyanobactin".*"
  Answer: Thank you for the suggestion; I have revised the figure note.

- "*Throughout the manuscript, do not use capital letter for the names of peptides (such as p. 13: lantipeptides, page 19 all cited lasso peptides, page 25 microcin, etc.).*"
  Answer: I have corrected this throughout the text, thank you.

- "*Page 15 in the subtitle, change "MccC"" to "McC", as MccC is not the microcin C but one of the modification enzymes.*"
  Answer: Thank you for this point; I have made the corrections.

- "*Page 16, four lines before the end reword the sentence.*"
  Answer: I have rephrased these sentences. Now it reads as following: "Lasso peptides are compact molecules containing a 7–9-residue N-terminal macrolactam ring followed by a C-terminal peptide tail, which is normally threaded through the ring forming a

"lasso"- like topology [30] (Figure 5). Thus, the threaded lasso peptides have three distinct regions: the ring, the loop above it and the threaded tail below it."

- "*Page 29, add the Greek letter required.*"
  Answer: I have added the symbol, thank you for noticing this.

- "*Page 33 line 10 change "by an ATP-binding cassette (ABC) transporters" to "by ATP-binding cassette (ABC) transporters".*"
  Answer: I have made correction, thank you for this.

- "*Page 51 change "phosphate residue" to "phosphate group".*"
  Answer: The typo has been revised.

Reviewer: Prof. Dmitry Ivankov

- "*p. 69: I see no evidence that trypsin cleaves exclusively between Lys5 and Arg6. I would say that trypsin cleaves additionally after Arg6, giving a mixture of the differently cleaved peptides. This does not influence the following analysis, so no reason to make a stronger unjustified statement*."
  Answer: I agree with you, but in our case the product of this cleavage was not detected via MS or HPLC-MS analysis.

- "*There is little details about the bioinformatics analysis given in the paper. I am not sure that a reference to the protocol from some paper is enough for PhD thesis. At least, Tatyana could give the parameters of PSI-BLAST*."
  Answer: Thank you for the suggestion, I agree with you, so the description was expanded. Now the paragraph is longer and reads as follows: "The bioinformatic search for the new lasso-cluster in the *Firmicutes* (taxid:1239) genomes was carried out against non-redundant GenBank CDS translations (excluding models (XM/XP) and uncultured/environmental sample sequences). The web-based Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST) was applied using C protein from paeninodin cluster (PadeC) as a bite [24]. The number of target sequences (hitlist size) was set to 500; the matrix for scoring parameters was BLOSUM62; expected value threshold set up to 10. The number of identified C proteins derived from other than paeninodin origin Paenibacillaceae family were then checked manually to search the nearby presence of genes coding for B2 protein homologue as well as a leader peptide. As a result, the cluster from *Bacillus pseudomycoides* was chosen for future research".

- "*It is not explained why Tatyana chose E.coli BL21 strain, not E.coli K12 strain.*"
  Answer: The *E.coli BL21* cells are typically used in our laboratory for the inducible protein expression.

- "*P. 31: Are "Mccj25", "McJ25", and "MccJ25" the designations of the same stuff?"*
  Answer: The McJ25 and Mccj25 are typos of the MccJ25. It has been revised.

- "*Some typos, the annotated pdf was sent to Tatyana."*
  Answer: Thank you for all your work and time spared, I have implemented all the corrections suggested.

Reviewer: Prof. Konstantin Lukyanov

- "*Literature citation in the text could be simplified, e.g., "[1], [2], [3], [3][4], [5], [6], [7], [8], [9]" -> [1-9]; "[2], [4], [6], [11]" -> [2, 4, 6, 9]; etc.*"
  Answer: The list of citations has been corrected.

- "*Page 15, missed symbol: "attached to the -carboxyl group.*"
  Answer: I have added the symbol again.

- "*Page 51, misprint "The psmCA genes were cloned on the sane…" (-> same)."*
  Answer: A typo has been corrected.

- "*Figure 20B is of too low resolution (not all letters are clear)."*
  Answer: I have replaced the figure with a bigger one (see above).

- "*Page 78: "With the help of collaborators, we recorded NMR spectra…" I think it is worth mentioning their names and institution.*"
  Answer: Collaborators' name and the Institute have already been added (page 78): "With the help of the team of Dr. Guy Lippens from Toulouse Biotechnology Institute, France, we recorded NMR spectra of wild-type and L21F pseudomycoidins purified from cells harboring *psmCAB1B2D* and *psmCA^{C}D* gene sets, correspondingly, in an aqueous phosphate buffer."

Reviewer: Prof. Timofei Zatsepin

- "*I recommend intensive text proofreading to remove typos and improve the text – for example, slang like "collected elution fractions were confirmed by MALDI MS" (p. 46), "heightened stability" (p.12), "nanomolar affinity" is not true for KD 200 nM (p.27) should be corrected."*
  Answer: Many corrections including the ones you suggested have been made.

- "*Intensive proof-reading for the list of the cited literature is the must – now numerous references are filled only partially (for example, ref. 153, 156,158,161, 165, etc.)"*
  Answer: The list of citations was corrected.

- "*semi-preparative C18 column" (p.46) – semi-preparative starts from diameter = 10 mm, please remove."*
  Answer: Thank you for this remark, I have removed this word. Now the sentence look like this (page 46): "Secondly, reversed-phase high-performance liquid chromatography (RP-HPLC) on the C18 column (Jupiter, 5 µm particle size C18 300 Å, LC Column 250 x 4.6 mm) applying gradient elution from 19 to 25% aqueous ACN/ 0.1% TFA in 40 min using UV detection at 210 nm was performed."

[1] J. L. San Millan, R. Kolter, and F. Moreno, “Plasmid genes required for microcin B17 production,” *J. Bacteriol.*, vol. 163, no. 3, pp. 1016–1020, 1985.

[2] M. Lavina, A. P. Pugsley, and F. Moreno, “Identification, mapping, cloning and characterization of a gene (sbm A) required for microcin B17 action on Escherichia coli K12,” *J. Gen. Microbiol.*, vol. 132, no. 6, pp. 1685–1693, 1986.

[3] M. R. Baquero, M. Bouzon, J. Varea, and F. Moreno, “sbmC, a stationary-phase induced SOS Escherichia coli gene, whose product protects cells from the DNA replication inhibitor microcin B17,” *Mol. Microbiol.*, vol. 18, no. 2, pp. 301–311, 1995.

[4] J. L. Vizán, C. Hernández-Chico, I. del Castillo, and F. Moreno, “The peptide antibiotic microcin B17 induces double-strand cleavage of DNA mediated by E. coli DNA gyrase.,” *EMBO J.*, vol. 10, no. 2, pp. 467–476, 1991.

[5] W. M. Parks, A. R. Bottrill, O. A. Pierrat, M. C. Durrant, and A. Maxwell, “The action of the bacterial toxin, microcin B17, on DNA gyrase,” *Biochimie*, vol. 89, no. 4, pp. 500–507, 2007.

[6] J. Koehnke *et al.*, “Structural analysis of leader peptide binding enables leader-free cyanobactin processing,” *Nat. Chem. Biol.*, vol. 11, no. 8, pp. 558–563, 2015.

[7] C. A. Regni, R. F. Roush, D. J. Miller, A. Nourse, C. T. Walsh, and B. A. Schulman, “How the MccB bacterial ancestor of ubiquitin E1 initiates biosynthesis of the microcin C7 antibiotic,” *EMBO J.*, vol. 28, no. 13, pp. 1953–1964, 2009.

[8] R. L. Evans, J. A. Latham, Y. Xia, J. P. Klinman, and C. M. Wilmot, “Nuclear Magnetic Resonance structure and binding studies of PqqD, a chaperone required in the biosynthesis of the bacterial dehydrogenase cofactor pyrroloquinoline quinone,” *Biochemistry*, vol. 56, no. 21, pp. 2735–2746, 2017.

[9] M. A. Ortega, Y. Hao, Q. Zhang, M. C. Walker, W. A. Van Der Donk, and S. K. Nair, “Structure and mechanism of the tRNA-dependent lantibiotic dehydratase NisB,” *Nature*, vol. 517, no. 7535, pp. 509–512, 2015.

[10] T. Sumida, S. Dubiley, B. Wilcox, K. Severinov, and S. Tagami, “Structural Basis of Leader Peptide Recognition in Lasso Peptide Biosynthesis Pathway,” *ACS Chem. Biol.*, vol. 14, no. 7, pp. 1619–1627, 2019.

[11] K. P. Yan *et al.*, “Dissecting the Maturation Steps of the Lasso Peptide Microcin J25 in vitro,” *ChemBioChem*, vol. 13, no. 7, pp. 1046–1052, 2012.

[12] S. Zhu *et al.*, “The B1 Protein Guides the Biosynthesis of a Lasso Peptide,” *Sci. Rep.*, vol. 6, no. August, p. 35604, 2016.

[13] J. I. Tietz *et al.*, “A new genome-mining tool redefines the lasso peptide biosynthetic landscape,” *Nat. Chem. Biol.*, vol. 13, no. 5, pp. 470–478, 2017.

[14] A. J. Dicaprio, A. Firouzbakht, G. A. Hudson, and D. A. Mitchell, “Enzymatic Reconstitution and Biosynthetic Investigation of the Lasso Peptide Fusilassin,” *J. Am. Chem. Soc.*, vol. 141, no. 1, pp. 290–297, 2019.

[15] T. A. Knappe *et al.*, “Introducing lasso peptides as molecular scaffolds for drug design: Engineering of an integrin antagonist,” *Angew. Chemie - Int. Ed.*, vol. 50, no. 37, pp.

8714–8717, 2011.

[16] M. Chen, S. Wang, and X. Yu, “Cryptand-imidazolium supported total synthesis of the lasso peptide BI-32169 and its d-enantiomer,” *Chem. Commun.*, vol. 55, no. 23, pp. 3323–3326, 2019.

[17] J. D. Hegemann *et al.*, “Rational improvement of the affinity and selectivity of integrin binding of grafted lasso peptides,” *J. Med. Chem.*, vol. 57, no. 13, pp. 5829–5834, 2014.

[18] K. Beis and S. Rebuffat, “Multifaceted ABC transporters associated to microcin and bacteriocin export,” *Res. Microbiol.*, no. xxxx, 2019.

[19] E. Gavrish *et al.*, “Lassomycin, a ribosomally synthesized cyclic peptide, kills mycobacterium tuberculosis by targeting the ATP-dependent protease ClpC1P1P2,” *Chem. Biol.*, vol. 21, no. 4, pp. 509–518, 2014.

[20] T. Ogawa *et al.*, “RES-701-2, -3 and -4, Novel and Selective Endothelin Type B Receptor Antagonists Produced by Streptomyces sp.: I. Taxonomy of Producing Strains, Fermentation, Isolation, and Biochemical Properties,” *J. Antibiot. (Tokyo).*, vol. 48, no. 11, pp. 1213–1220, 1995.

[21] Z. Feng, Y. Ogasawara, S. Nomura, and T. Dairi, “Biosynthetic Gene Cluster of a d-Tryptophan-Containing Lasso Peptide, MS-271,” *ChemBioChem*, vol. 19, no. 19, pp. 2045–2048, 2018.

[22] S. Zhu, C. D. Fage, J. D. Hegemann, D. Yan, and M. A. Marahiel, “Dual substrate-controlled kinase activity leads to polyphosphorylated lasso peptides,” *FEBS Lett.*, vol. 590, pp. 3323–3334, 2016.

[23] K. J. Hetrick and W. A. van der Donk, “Ribosomally synthesized and post-translationally modified peptide natural product discovery in the genomic era,” *Curr. Opin. Chem. Biol.*, vol. 38, pp. 36–44, 2017.

[24] S. Zhu *et al.*, “Insights into the unique phosphorylation of the lasso peptide paeninodin,” *J. Biol. Chem.*, vol. 291, no. 26, pp. 13662–13678, 2016.

[25] J. D. Hegemann, “Factors Governing the Thermal Stability of Lasso Peptides,” *ChemBioChem*, vol. Early View, 2019.

[26] J. D. Hegemann *et al.*, “XanthomoninsI-III: A new class of lasso peptides with a seven-residue macrolactam ring,” *Angew. Chemie - Int. Ed.*, vol. 53, no. 8, pp. 2230–2234, 2014.

[27] J. D. Hegemann *et al.*, “The ring residue proline 8 is crucial for the thermal stability of the lasso peptide caulosegnin II,” *Mol. Biosyst.*, vol. 12, no. 4, pp. 1106–1109, 2016.

[28] J. D. Hegemann, M. Zimmermann, X. Xie, and M. A. Marahiel, “Lasso Peptides: An Intriguing Class of Bacterial Natural Products,” *Acc. Chem. Res.*, vol. 48, no. 7, pp. 1909–1919, 2015.

[29] M. Zimmermann, J. D. Hegemann, X. Xie, and M. A. Marahiel, “Characterization of caulonodin lasso peptides revealed unprecedented N-terminal residues and a precursor motif essential for peptide maturation,” *Chem. Sci.*, vol. 5, no. 10, pp. 4032–4043, 2014.

[30] K. A. Wilson *et al.*, “Structure of microcin J25, a peptide inhibitor of bacterial RNA polymerase, is a lassoed tail,” *J. Am. Chem. Soc.*, vol. 125, no. 41, pp. 12475–12483, 2003.