Heterologous Expression of a Novel Lasso Peptide

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Abstract

Lasso peptides are a fairly recently-discovered but rapidly expanding classification of ribosomally assembled and post-translationally modified peptides found within various bacterial strains. Lasso peptides are a natural product of interest due to their unique biosynthesis and "lasso" topology, which consists of a tail of amino acid residues threaded through a ring of amino acid residues, as well as their high potential for medical application, particularly through antimicrobial activity.[1] The most efficient way of isolating and assessing the function of these peptides is often through heterologous expression, in which engineered plasmids are introduced to host bacteria and used to express foreign genes. Our general goal was to use heterologous expression in order to produce a lasso peptide encoded within the genome of an *Enterobacter* strain. Because of its amino acid sequence similarities to lasso peptides involved in bacterial RNA polymerase inhibition, we suspected that this novel lasso peptide has similar inhibitory effects and could therefore have antimicrobial properties. After heterologous expression, we had also planned to use liquid chromatography to isolate the peptide of interest and then assess its activity and structure through antimicrobial assays and nuclear magnetic resonance spectroscopy, respectively.

Project Context and Rationale

As antibiotic resistance has become an increasingly prevalent topic within modern medicine, unique and functionally distinct antimicrobial compounds with the potential to circumvent this resistance have become highly sought after. While many medical compounds have artificial origins, a plethora of natural products produced by organisms have contributed greatly to the repertoire of antimicrobial drugs available today.[2] Lasso peptides are a class of ribosomally synthesized and post-translationally modified peptides, or RiPPs, which undergo a complex series of modifications in order to achieve a distinct "lariat knot" shape consisting of an isopeptide ring, a loop, and a tail threading through the ring.[1] The unique processing and topology of these peptides offers insight into biosynthetic pathways as well as the physical chemistry behind peptide structure. In addition, lasso peptides' qualities make them prime targets for antimicrobial drug discovery. While specific lasso peptides have shown potential medical relevance ranging from inhibition of HIV[3] to the restriction of cancer cell migration,[4] a frequent trend among lasso peptides is antimicrobial activity caused by inhibition of prokaryotic RNA polymerase, which may lead to medical innovation through the creation of safe and effective antibiotics.

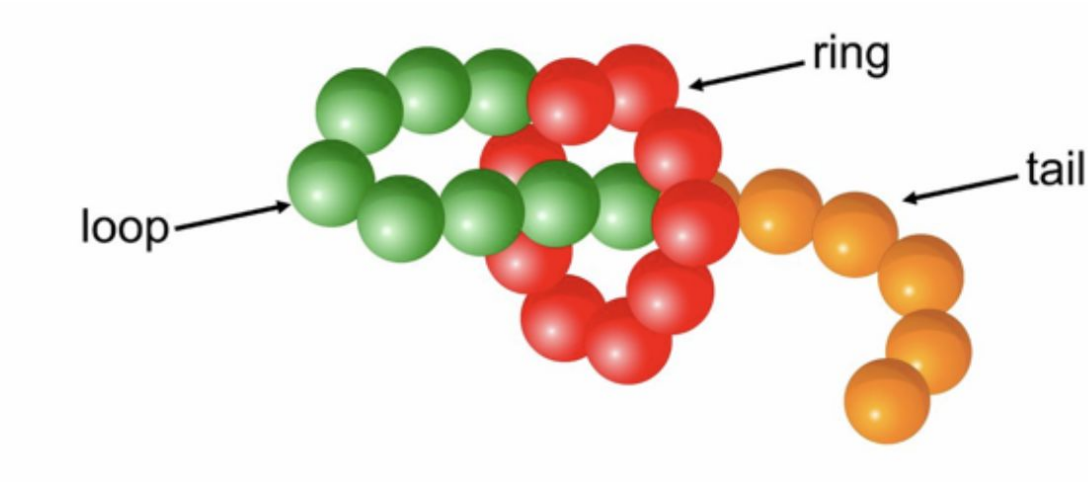

Fig. 1: Lasso peptide topology

Notable progress has been made in recent years regarding the discovery of new lasso peptide genes as well as the characterization of certain lasso peptide variants. Genome mining allows for relatively rapid discovery of biosynthetic gene clusters (BGCs) within bacterial strains, each containing a lasso peptide gene and genes for associated proteins and enzymes involved in lasso peptide production or function. After gene discovery, these BGCs can be heterologously expressed in order to conduct research on the biochemical novelty and medical viability of a given lasso peptide variant.

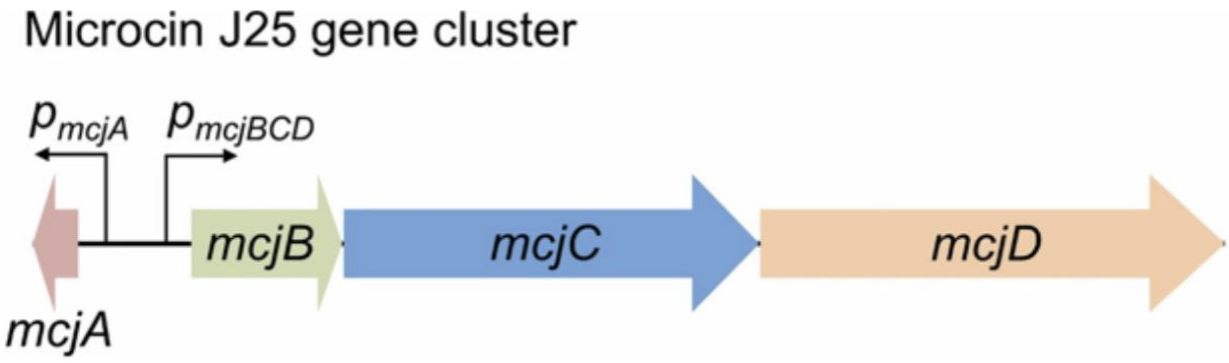

Fig. 2: Architecture of a model lasso peptide gene cluster

Although the advent of genome mining has revolutionized the process of identifying BGCs and has therefore allowed for the discovery of numerous putative lasso peptides and associated enzymes, expressing and characterizing each of these individual lasso peptides remains a lofty task. Put simply, while many lasso peptide gene clusters are known to exist, little is known about many of them because they have not been reproduced and researched in the laboratory. Because of this, a major priority within the field of lasso peptide research is simply expressing and characterizing new lasso peptide varieties found in nature in order to facilitate further research, whether it is checking for biological or chemical significance, assessing potential medical value, or gathering knowledge about the growing class of peptides as a whole.[1]

This research project was an attempt to bridge the gap between knowing of a given gene cluster's existence on paper and being able to conduct meaningful research on it. Specifically, we set out to reconstruct and express a novel lasso peptide gene cluster discovered in *Enterobacter cloacae sp.* LB3, a hospital strain of gut bacteria. Due to similarities to citrocin, an antimicrobial lasso peptide recently discovered by the Link Lab, it is hypothesized that this novel peptide also has antimicrobial effects, potentially as a mechanism for the host organism to eliminate competition from other *Enterobacter* strains within its native microbiome.[5] Because *Enterobacter* is a common cause of human disease and is frequently antibiotic-resistant, the novel peptide may be able to serve as an effective narrow-spectrum antibiotic where other molecules fail. This antimicrobial activity, which is also seen in examples such as microcin J25 and capistruin, is due to inhibition of RNA polymerase through interactions between the ring and tail regions of the lasso peptide and the secondary channel of the RNAP. After expression, assays of antimicrobial activity could be performed in order to determine if the hypothesis that the Enterobacter lasso

peptide also exhibits this behavior holds true.[6, 7] In this sense, reconstruction of the gene cluster and heterologous expression of the lasso peptide provide a foundation or framework that allows deeper questions to be explored in the future.

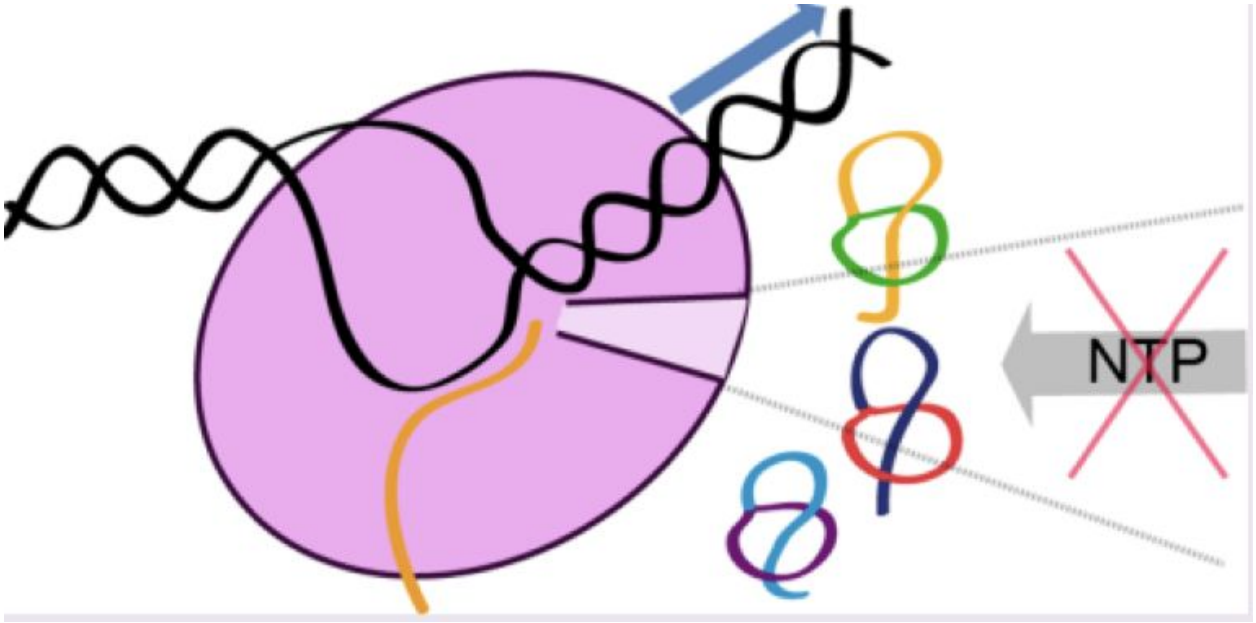

Fig. 3: Inhibitory interactions with RNA Polymerase.

Along with the antimicrobial potential of the novel peptide, elucidation of the peptide's structure, function, and biosynthesis on a molecular level may also yield valuable insights. The novel lasso peptide gene cluster is fairly typical, consisting of an A gene (precursor lasso peptide), a B gene (first maturation enzyme, a cysteine protease), a C gene (second maturation enzyme, an isopeptide bond-forming enzyme) and a D gene (ATP-binding cassette transporter, or ABC transporter).[1] Like many other lasso peptides, the precursor peptide in this case is thought to be cleaved by the first maturation enzyme, leaving behind a leader sequence. After being processed, the remaining core peptide is looped into a lariat knot conformation and then

locked in place by the second maturation enzyme, which forms a ring out of numerous amino acid residues on one end of the peptide.[1] The mature lasso peptide is then carried out of the cell into the surrounding environment through the ABC transporter. Because of the in-depth biosynthetic pathway undergone by these peptides and the complexity of the resulting lasso topology, future characterization and structural elucidation of the peptide of interest may also yield impactful results.

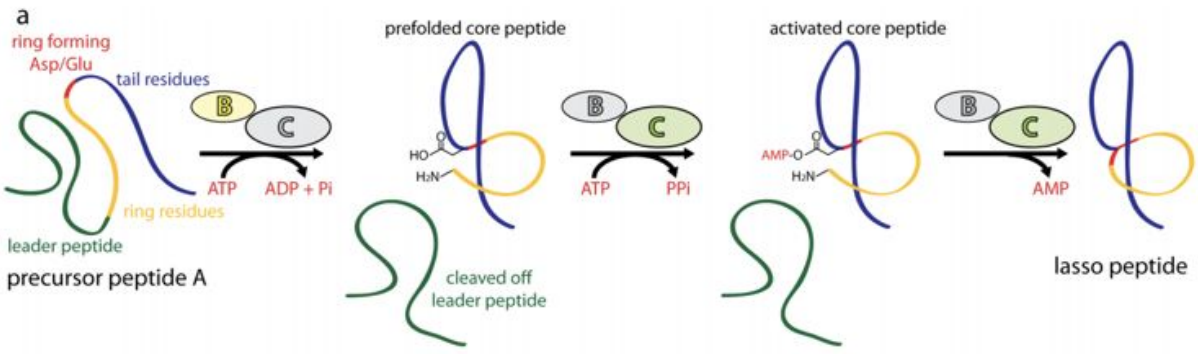

Fig. 4: Post-translational maturation of a precursor lasso peptide

Methodology

In order to produce the peptide of interest for further study, it was necessary to perform heterologous expression via *E. coli*. The plasmid pQE80, commonly used for recombinant DNA techniques, was used to recreate the ABCD gene cluster through the addition of two inserts: one stretch of DNA consisting of the A gene and another consisting of the B, C, and D genes. The A gene was constructed through PCR amplification involving multiple overlapping DNA primers. This gene and the pQE80 vector were digested using the restriction endonucleases and ligated together with DNA ligase, creating a new plasmid containing the gene for the precursor peptide, which was then mailed in for sequence confirmation. The same general format of gene assembly

through PCR, digestion of the gene and vector, ligation, and sequencing was used in order to construct the BCD region as an insert for the second plasmid. Three DNA sequences were used in overlap extension PCR in order to make a single stretch of DNA consisting of the remaining three genes of the gene cluster. Cultures of XL1-Blue, an *E. coli* strain, were used in order to clone plasmids for cellular extraction. Afterwards, a separate *E. coli* strain used for plasmid expression, BL21, was used for large-scale lasso peptide production. Spin column and agarose gel techniques were used to purify and visualize DNA from PCR and cellular extraction.

After expression of the target lasso peptide, the product is to be purified through a combination of reverse-phase HPLC (high performance liquid chromatography) and LCMS (liquid chromatography-mass spectroscopy). In HPLC, the peptide solution extracted from the cells is first washed and treated with water and methanol and then resuspended in acetonitrile. Afterwards, the peptide solution is injected into a stream of pressurized fuel and run through a column, and the contents of the solution are separated into aliquots as they move through the column at different rates. In LCMS, the mass-charge ratio of each aliquot's contents is measured, allowing identification of the aliquot containing the purified peptide given its theoretical mass-charge ratio.

After extraction and purification, we had planned to characterize the antimicrobial activity, or lack thereof, of the novel peptide. Specifically, RNA polymerase inhibition would be measured by recording bacterial density under set conditions and exposing them to varying concentrations of the peptide. Based on changes in transcription leading to cell death, the general impact of the lasso peptide on RNA polymerase activity in an *in vivo* environment can be inferred.

Though time was a limiting factor in the research project, we had also originally planned to begin structural elucidation of the lasso peptide using nuclear magnetic resonance spectroscopy. In this process, various forms of 2D NMR (COSY, TOCSY, NOESY) are used in order to measure the response of hydrogen atoms in a molecule to magnetism. Because the graphical features of these hydrogen atoms differ depending on the other atoms in their proximity, NMR can be used to create a detailed reproduction of the lasso peptide's molecular structure and spatial orientation.

Results

Genome mining has allowed us to identify the desired Enterobacter gene cluster and determine the amino acid sequences encoded by its constituent genes, including the precursor peptide encoded by gene A:

MDVKNKHALTQRNEKALNAVSITRIPVKASKI|TR|**GHSVDRIPE|YFGPPLPGPVLFYS**

Where the unbolded portion of the sequence is the leader sequence that is cleaved off during post-translational processing, threonine (T) and arginine (R) at the end of the leader sequence are a highly conserved section essential to the processing pathway, the bolded sequence represents the final peptide after cleavage of the leader, with the first segment being the ring and the second segment being the tail, and the underlined tyrosine (Y) and phenylalanine (F) are the residues contributing to the putative antimicrobial properties of the peptide. Although no concrete results regarding the structure or function of the peptide itself can be provided at this time due to time

constraints, its numerous similarities to citrocin suggest that this novel peptide may have similar qualities.

Although the peptide of interest has not yet been characterized, our primary goal of heterologous expression has been achieved and we are now capable of reliably expressing the peptide in BL21 *E. coli*. Two plasmids were constructed in order to facilitate heterologous expression: pAK1 and pAK2. pAK1 uses pQE80 as a template vector and contains the A (precursor peptide) gene in its multiple cloning site. *Eco*RI-HFand *Hind*III were the restriction enzymes used to clone the A gene into the template vector. pAK2 uses pAK1 as a template vector and has the BCD gene cluster inserted with the use of the restriction enzymes *Nhe*I and *Nco*I. While several bacterial clones containing pAK2 were produced, they all initially contained point mutations, insertions, and deletions, and the finalized pAK2 plasmid produced is the result of digestion of separate pAK2 clones and ligation with one another in order to remove mutated pieces. With the completion of the finalized pAK2 plasmid (Fig. 5), the entire lasso peptide gene cluster has been reproduced, allowing expression of the peptide and its associated proteins.

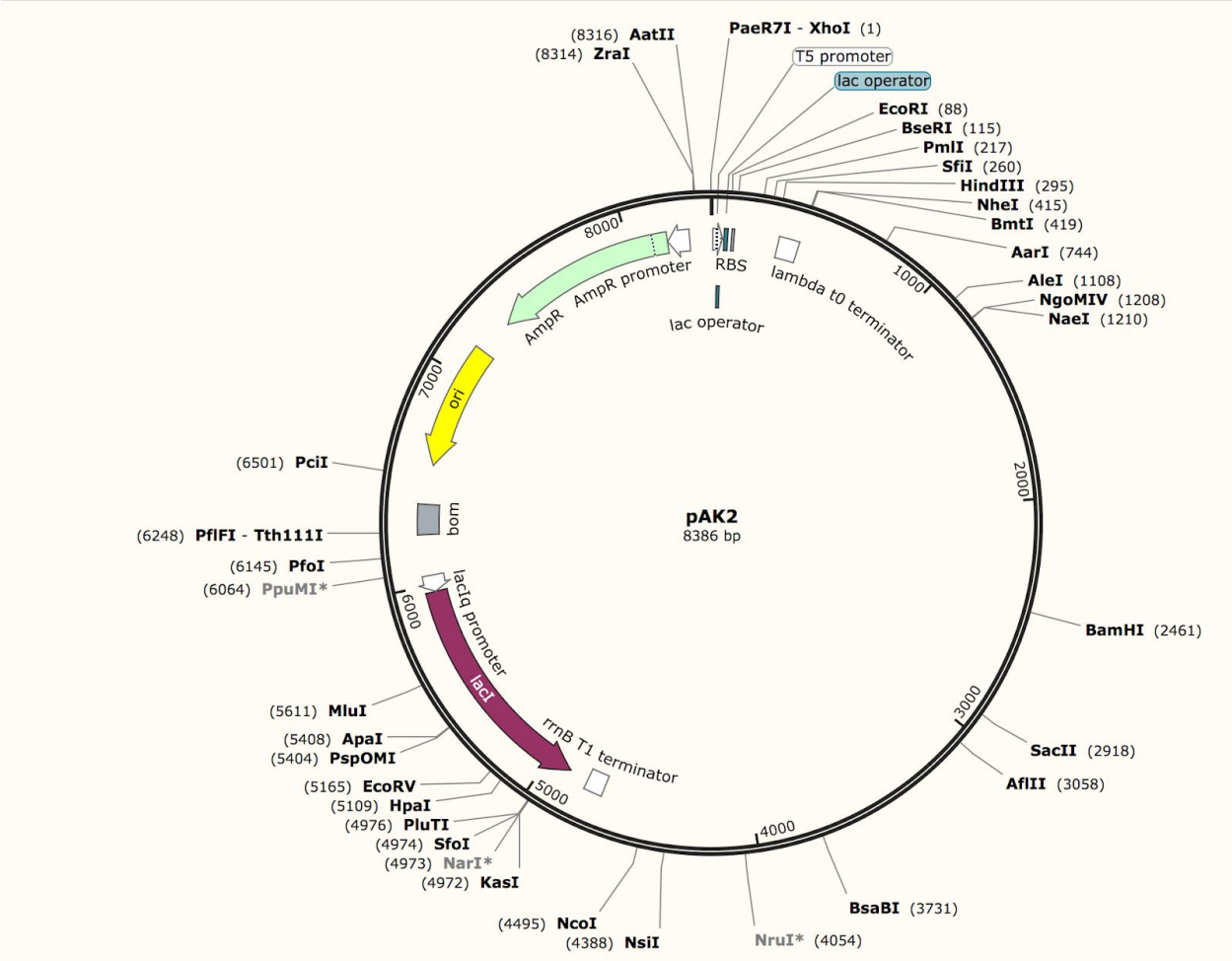

Fig. 5: SnapGene plasmid map of pAK2

Impact and Future Directions

Although structural elucidation and analysis of antimicrobial behavior was outside of the scope of this summer, this research project has paved the way for further research centered around the *Enterobacter* lasso peptide. While heterologous expression often serves as a barrier towards the study of elusive natural products and proteins due to the time associated with it, this project has created a suitable foundation that can be used to produce meaningful results and insights in the near future. Alongside the potential investigation into the structure, biosynthesis and maturation, and antibiotic function of the novel lasso peptide which has already been

discussed at length, other experiments could be used to investigate the novel natural product on a molecular level.

The physical and chemical properties of the novel lasso peptide could be investigated more thoroughly. While structural elucidation of the lasso peptide through NMR has been discussed, further testing may also be able to determine how the lasso structure forms as well as the properties conferred by that structure. For example, steric interactions involved in the formation of the ring, loop, and tail could be studied in order to reach a greater understanding of the unique conformation of lasso peptides. Additionally, denaturation under different conditions, such as various temperatures, could be studied in order to see how close the novel lasso peptide conforms to the generalization that many lasso peptides have very high thermal stability.

Mutagenesis could be used in order to create variants of the peptide precursor (peptide A) as well as its associated enzymatic processors and transporters (proteins B, C, and D). By inducing controlled mutations in the lasso peptide gene, the effect of specific amino acid residues on the overall structure and function of the peptide can be inferred.[3] Likewise, controlled mutations in the three associated genes in the gene cluster can show the importance of specific residues in the post-translational processing and export of the peptide. If the characteristics of the lasso peptide or its biosynthetic pathway change in some way as a result of mutation--or if expression halts altogether--it is a good first indicator that the given amino acid residue deserves further investigation.

References

1) Julian D. Hegemann, Marcel Zimmermann, Xiulan Xie, and Mohamed A. Marahiel. "Lasso Peptides: An Intriguing Class of Bacterial Natural Products." *Accounts of Chemical Research* 2015 *48* (7), 1909-1919 DOI: 10.1021/acs.accounts.5b00156

2) Khan, Riaz A. "Natural Products Chemistry: The Emerging Trends and Prospective Goals." *Saudi Pharmaceutical Journal*, vol. 26, no. 5, July 2018, pp. 739–753., doi:10.1016/j.jsps.2018.02.015.

3) Martin-Gómez, Helena, and Judit Tulla-Puche. "Lasso Peptides: Chemical Approaches and Structural Elucidation." *Organic & Biomolecular Chemistry*, vol. 16, no. 28, 27 June 2018, pp. 5065–5080., doi:10.1039/c8ob01304g.

4) Sangkeun Son, Mina Jang, Byeongsan Lee, Young-Soo Hong, Sung-Kyun Ko, Jae-Hyuk Jang, Jong Seog Ahn. "Ulleungdin, a Lasso Peptide with Cancer Cell Migration Inhibitory Activity Discovered by the Genome Mining Approach." *Journal of Natural Products,* 2018, *81* (10) , 2205-2211. DOI: 10.1021/acs.jnatprod.8b00449.

5) Cheung-Lee, Wai Ling, et al. “Discovery and Structure of the Antimicrobial Lasso Peptide Citrocin.” *Journal of Biological Chemistry*, vol. 294, no. 17, 2019, pp. 6822–6830., doi:10.1074/jbc.ra118.006494

6) Braffman, Nathaniel R., et al. “Structural Mechanism of Transcription Inhibition by Lasso Peptides Microcin J25 and Capistruin.” *Proceedings of the National Academy of Sciences*, vol. 116, no. 4, 2019, pp. 1273–1278., doi:10.1073/pnas.1817352116.

7) Metelev, Mikhail, et al. “Acinetodin and Klebsidin, RNA Polymerase Targeting Lasso Peptides Produced by Human Isolates of Acinetobacter Gyllenbergii and Klebsiella Pneumoniae.” *ACS Chemical Biology*, vol. 12, no. 3, 2017, pp. 814–824., doi:10.1021/acschembio.6b01154.

Fig. 1 and 2) Maksimov, M. O., et al. “Precursor-Centric Genome-Mining Approach for Lasso Peptide Discovery.” *Proceedings of the National Academy of Sciences*, vol. 109, no. 38, 2012, pp. 15223–15228., doi:10.1073/pnas.1208978109.

Fig. 3) See citation 7.

Fig. 4) See citation 1.