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Investigating the mechanism(s) of antiviral action of ribosomally synthesized and post-translationally modified peptides with a focus on labyrinthopeptides

MSc. Biomolecular Science
Essay

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ABSTRACT

Ribosomally synthesized and post-translationally modified Peptides (RiPPs) are known for their structural and functional diversity. These peptides are modified after ribosomal synthesis, leading to the formation of unique stable structures. Lanthipeptides, a subgroup of RiPPs, are well-characterized for their antimicrobial activity and have the incorporation of noncanonical amino acids – Lanthionine and Methyllanthionine. This essay explores the structural dynamics and biosynthetic pathways of Lanthipeptides, with a particular focus on Class III lanthipeptides - the Labyrinthopeptides - LabyA1 and LabyA2. It investigates their structural and biosynthetic aspects, with an emphasis on their antiviral activity.

The mode of antiviral action of Labyrinthopeptides involves selective virolytic effects and lipid interaction, where Labyrinthopeptides act as entry inhibitors by targeting the lipid envelope, specifically phosphatidylethanolamine (PE). LabyA1 demonstrates dual anti-HIV and anti-HSV activity, outperforming established drugs in efficacy. Additionally, LabyA1 exhibits promising anti-ZIKV and anti-DENV activity by interfering with virus entry into target cells.

To understand mechanistic aspects of the antiviral action, a comparative analysis of labyrinthopeptides with other antiviral lanthipeptides, Duramycin and Cinnamycin, focusing on structural parallels is performed. The presence of specific hydrophobic and hydrophilic ends, along with potential Phosphatidylethanolamine-binding motifs, suggests similarities in their antiviral mechanisms. A comparison with other ribosomally synthesized and post-translationally modified peptides (RiPPs) provides insights into common structural elements associated with antiviral activity, such as unique bridges including Lysinoalanine and disulfide bridges, and the β-hydroxy modifications on Aspartic acid residue that help stabilize interactions with the lipid head group.

The essay underscores the importance of understanding these molecular interactions between labyrinthopeptides and viral envelope lipids. Structural comparisons and strategic mutations are proposed for further investigation. The study also highlights the potential of labyrinthopeptides, particularly LabyA1, as promising antiviral agents with low cytotoxicity, and a candidate for peptide engineering and therapeutic development.

TABLE OF CONTENTS

1. INTRODUCTION

Antimicrobial peptides (AMPs), particularly those produced by microorganisms, are an extensively researched class of secondary metabolites. They play a crucial role as the first line of defence in their niche for AMP-producing microorganism (Hassan et al., 2023; Mousavi Maleki et al., 2022). Such peptides have been regularly harnessed for their (potential) use in combating bacterial, fungal or viral infections (Mousavi Maleki et al., 2022). As predominantly protein-based compounds, these peptides face limitations in structural diversity due to the constraint of the 20 canonical proteogenic amino acids (Zhang et al., 2021). Nature has provided a solution to this limitation in the form of post-translational modifications as found in Ribosomally synthesised and Post-translationally modified Peptides – RiPPs.

RiPPs represent a class of highly modified peptides studied for, amongst other reasons, their antimicrobial properties against a diverse range of pathogens (Arnison et al., 2013). Following the biosynthesis of the main amino acid chain via the ribosomal pathway, these peptides undergo post-translational modifications to introduce thioether linkages between altered amino acids (Férir et al., 2014; Fu et al., 2021; Skinnider et al., 2016). Such modifications result in unique structures not readily achievable in natural ribosomal peptides with the limited canonical amino acids. Typically, the modifications give conformational rigidity to the peptides proving advantageous in enhancing target recognition, increasing metabolic and chemical stability, and improving protease resistance (Arnison et al., 2013; Férir et al., 2013). The resulting peptides are small, ranging from 19-38 aa (Oeyen et al., 2021), and are largely produced by bacteria belonging to the genera Staphylococci, Lactobacilli, and Actinomycetes (Meindl et al., 2010).

RiPPs are vastly diverse and can further be divided into distinct groups based on similar post-translational modifications and biosynthetic pathways (Fig. 1). One such subgroup are the Lanthipeptides (Fig. 1 – highlighted). They are characterized by the incorporation of the noncanonical amino acid lanthionine (Lan) or methyllanthionine (MeLan), derived from the dehydration of canonical amino acids serine and threonine, respectively (Arnison et al., 2013; Lohr et al., 2023). The resulting noncanonical amino acids contribute to the exceptional stability of these peptides through distinctive thioether cross-linkages or disulfide linkages (Meindl et al., 2010).

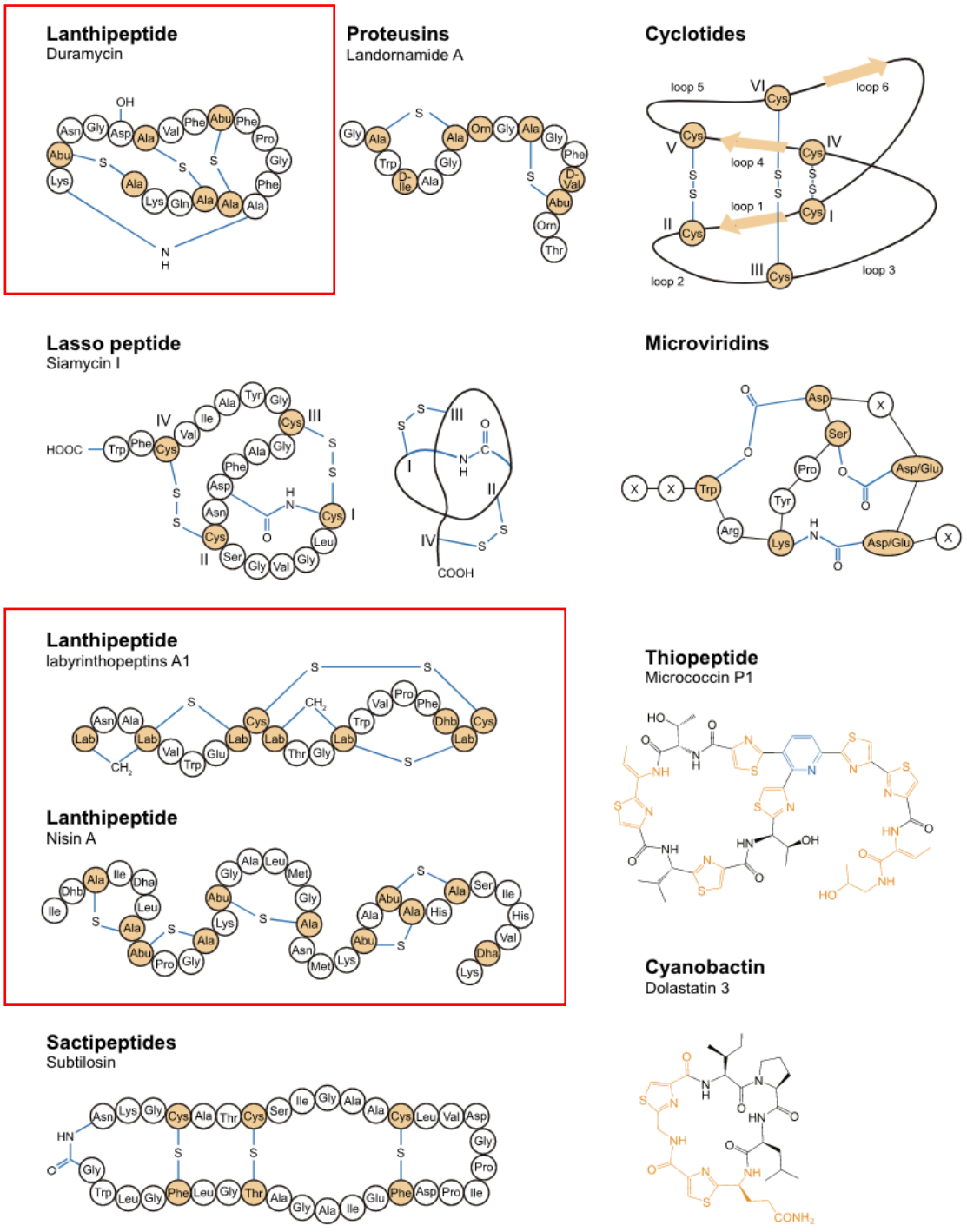

Figure 1: Representative compounds of eight selected RiPPs subgroups. Highlighted compounds belong to the Lanthipeptide subgroup (Fu et al., 2021).

Lanthipeptides stand out as the largest category within the RiPPs, notable for the antimicrobial activity of its subclass the lantibiotics. Among them, the FDA-approved commercial lantibiotic Nisin (Fig. 1), a class I lantibiotic, serves as a broad-spectrum antibacterial agent in the food preservation industry, particularly targeting bacterial membranes (Zhang et al., 2021). Within the vast diverse class of Lanthipeptides, a notable subgroup are the Labyrinthopeptides, discovered in 2010 as ribosomally synthesized peptides belonging to the class III family of lantibiotics (Villalaín, 2023). Produced by the filamentous actinomycete *Actinomadura namibiensis*, labyrinthopeptin derivatives A1, A2, and A3 have been identified with distinct disulfide bonds that result in the formation of unique noncanonical amino acids - Labionine (Section 2.3.) (Lohr et al., 2023).

Recent decades have seen the resurgence of viral infections, illustrated by outbreaks such as Zika and Dengue, and has therefore necessitated the development of effective antiviral drugs. The global Covid-19 pandemic in 2020 further accelerated the research against viral infections to prevent their spread (Luteijn et al., 2020).

In response to the increasing viral pandemics, the characterization and discovery of new antivirals with lower cytotoxicity against human cells, ease of production, and broader efficacy has gained importance (Martinez et al., 2015). Peptides, including lantibiotics, have thus become a focal point in the search for antiviral agents with their well characterised history of antimicrobial activity. Their unique attributes, such as increased interaction area, high-affinity bonding with targets, improved pore-formation capabilities, and resistance to proteases, make them promising candidates in the search for effective antiviral activity (Fu et al., 2021). Along with a relatively smaller size of 2-5 kDa these peptides are ideal candidates for novel drug development (Oeyen et al., 2021).

Among the various subgroups, the Labyrinthopeptides have particularly demonstrated their potential in combating viral infections such as HIV and HSV (Férir et al., 2014, 2013; Prochnow et al., 2020). These peptides exhibit wide-ranging antimicrobial and antiviral activity and demonstrate lower cytotoxicity in human cells when compared to other antiviral lanthipeptides like Duramycin (Blockus et al., 2020; Férir et al., 2013; Oeyen et al., 2021; Prochnow et al., 2020).

1.1. Aim

This essay aims to unravel the complexities of Class III lanthipeptides, specifically labyrinthopeptides, with a focus on LabyA1 and LabyA2. The exploration includes structural and functional aspects, focusing on antiviral activity and the mechanism of action. Key questions addressing the defining structural characteristics of lanthipeptides, their classification, biosynthetic pathways, and modifications are investigated. Furthermore, distinctive features of labyrinthopeptides, along with their unique structural dynamics associated with labionine amino acids, are studied. An analysis of the antiviral mechanisms of LabyA1 and LabyA2, acting as inhibitors of enveloped viruses, particularly through selective interaction with lipids on viral membranes, is conducted. Additionally, comparative structural analysis with established antiviral lanthipeptides is probed to understand potential lipid-binding mechanisms of Labyrinthopeptides. By addressing these questions, the essay aims to provide a comprehensive exploration of labyrinthopeptides, shedding light on their antiviral activity and potential mechanisms as therapeutic agents.

2. LANTHIPEPTIDES AND LABYRINTHOPEPTIDES

Lanthipeptides constitute one of the largest classes of Ribosomally synthesized and Post-translationally modified Peptides (RiPPs). The term "lanthipeptide" finds its roots in the Latin word "lana," meaning wool, and "thiol," which relates to sulfhydryl groups. Lanthionines, initially discovered in wool, serve as a key element in the etymology. The inclusion of "thiol" emphasizes the characteristic presence of sulphur bridges in these peptides (Moll et al., 2020). With over 15 unique post-translational modifications identified (Arnison et al., 2013; Meindl et al., 2010) and various biosynthetic pathways (Section 2.1.) there is a need for the classification of lanthipeptides.

Nisin, the first discovered lanthipeptide, belongs to the group of lanthionine-containing antibiotics referred to as lantibiotics. Nisin has been extensively studied for both its structure and mode of action (Lubelski et al., 2008). The elucidation of the unique amino acids present in the structure of Nisin led to the discovery of the noncanonical amino acids, meso-lanthionine and 3-methyllanthionine, that are now recognized as representative amino acids classifying lanthipeptides (Arnison et al., 2013).

Lanthionine, commonly denoted as Lan, and 3-methyllanthionine, denoted as MeLan, are amino acids that are unique, as they are not part of the canonical 20 amino acids (Meindl et al., 2010). Additionally, the presence of α,β- unsaturated amino acids such as dehydrobutyrine and dehydroalanine is also characteristic of the lanthipeptides (Ongey and Neubauer, 2016). The formation of the non-canonical Lan and MeLan amino acids occurs via a dehydration step involving the elimination of a hydroxyl group from proteogenic amino acid serine and threonine, respectively, resulting in the formation of a new amino acid (Lagedroste et al., 2020). The resulting unique amino acids are 2,3-didehydroalanine (Dha) formed through the dehydration of serine, and 2,3-didehydrobutyrines (Dhb) from the dehydration of threonine. Figure 2A illustrates an example of the dehydration step leading to the elimination of the side-chain hydroxyl group of threonine or serine, followed by a 1,4-conjugate addition of cysteine called the cyclization step, as depicted in Figure 2B, that gives rise to β-thioether cross links (Arnison et al., 2013; D. Hegemann and D. Süssmuth, 2020; Völler et al., 2012).

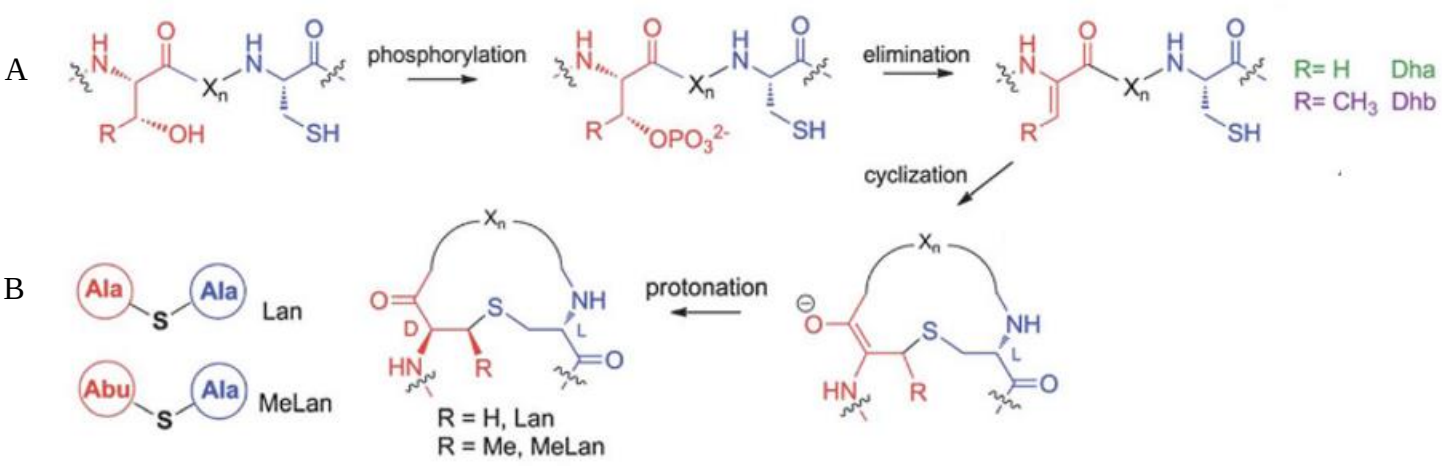

Figure 2. Illustration of the Two-Step Post-Translational Process in Lanthipeptide Formation - A) Dehydration Step and B) Cyclization Step with Cysteine Residue (Arnison et al., 2013).

2.1. Biosynthesis Of Lanthipeptides

Post-translationally modifies peptides like RiPPs have a general biosynthetic pathway that usually involves a precursor peptide that consists of a central core peptide which undergoes modifications to yield the final product. Additionally, there are sequences flanking the core peptide at the N-terminus (leader peptide) or C-terminus (follower peptide), or both (Oman and van der Donk, 2010). In eukaryotes, there may also be a signal sequence that guides the peptide to specialized compartments for modifications and secretions (Skinnider et al., 2016), but these signal sequences are not always found in prokaryotic RiPPs. The C-terminus can also at times contain a recognition sequence which has been shown to assist peptide cyclisation in cyanobactins, cyclotides and orbitides (Skinnider et al., 2016). Associated proteases are responsible for the removal of the leader and follower peptides during the maturation process. Further modifications customize the core peptide to produce various diverse constructs ranging from simple head to tail macrocyclization as seen in cyclic bacteriocins, to intricate thioether linkages as seen in lanthipeptides (Oman and van der Donk, 2010; Skinnider et al., 2016). Figure 3 illustrates the general biosynthetic pathway for RiPPs.

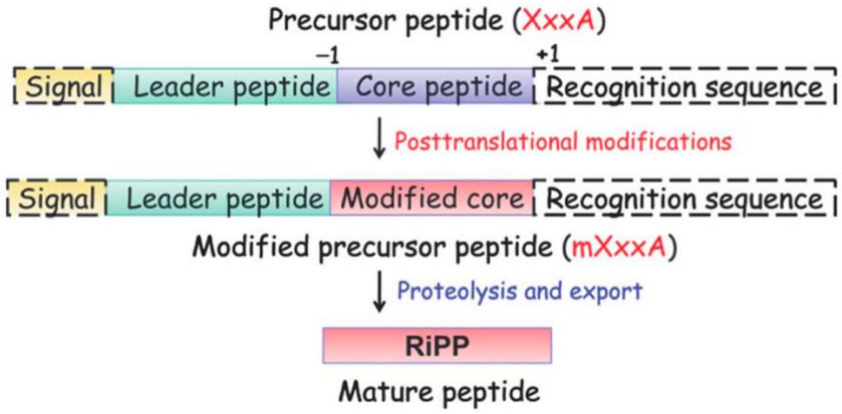

Figure 3: General Biosynthetic Pathway for RiPPs. Dotted regions are exclusive to specific RiPPs (Skinnider et al., 2016).

Lanthipeptides, belonging to RiPPs, generally follow a similar biosynthesis scheme. Specifically, the lanthipeptides have only an N-terminal Leader sequence and a C-terminal Core peptide. Notably, no follower peptides at the C-terminal have been reported for lanthipeptides (Repka et al., 2017). The precursor peptide, comprising the leader peptide and the core peptide, is referred to as LanA. “Lan” is a generic notation that symbolizes gene clusters that are involved in the biosynthetic pathways of lanthipeptides. LanA undergoes post-translational modifications, resulting in a modified precursor peptide called mLanA. Subsequently, mLanA undergoes proteolysis by serine protease LanP that removes the leader peptide, and the final product is exported as the mature lanthipeptide (Ongey and Neubauer, 2016; Repka et al., 2017).

The introduction of unique patterns of thioether cross-links gives lanthipeptides its high specificity and selectivity. The two N-terminal rings in the specific nisin ring pattern are responsible for the lipid II selectivity of Nisin, and phosphatidyl ethanolamine headgroup selectivity of Cinnamycin (Hsu et al., 2004; Meindl et al., 2010; Repka et al., 2017).

The regulatory system of the biosynthetic machinery involves the regulatory protein LanR, the histidine kinase LanK, and the ABC (ATP-binding cassette) transporter protein LanT. LanT plays a crucial role in

coordinating the modifying enzymes with the translocation of the bioactive peptides from the cell to their target environment where they function (Ongey and Neubauer, 2016).

Accordingly, lanthipeptides are categorized into four distinct classes based on variations to the general biosynthetic pathway, the enzymes involved in the maturation process and the modifications introduced to the core peptide.

2.1.1. Class I Lanthipeptides

Class I lanthipeptides include Nisin, the first discovered and characterized lantibiotic known to inhibit the growth of *Lactobacillus bulgaricus* (Repka et al., 2017). Studied as the representative molecule of Class I, Nisin consists of five thioether rings, one 2,3-didehydrobutyrine (Dhb), and two 2,3-didehydroalanine (Dha) residues (Repka et al., 2017). The stereochemistry of Nisin is also well-established, with (2S,6R) for the Lan residue and (2S,3S,6R) for the MeLan residue. Notably, all known structures of Class I lanthipeptides consist of the same stereochemistry (Repka et al., 2017). Apart from the characteristic PTMs observed in Lanthipeptides, compounds may also show N or C terminus modifications that improve the stability of the peptide or may change the overall charge of the peptide, leading to increased specificity. In Class I compounds, such examples of unique modifications include the C-terminal S-[(Z)-2-aminovinyl]-D-Cys (aviCys) found in epidermin, N-terminal 2-hydroxypropionate in epilancin 15x, and chlorination of a Trp residue and dihydroxylation of a Pro residue seen in microbisporicin, also known as NAI-107 (Fig. 4) (Repka et al., 2017).

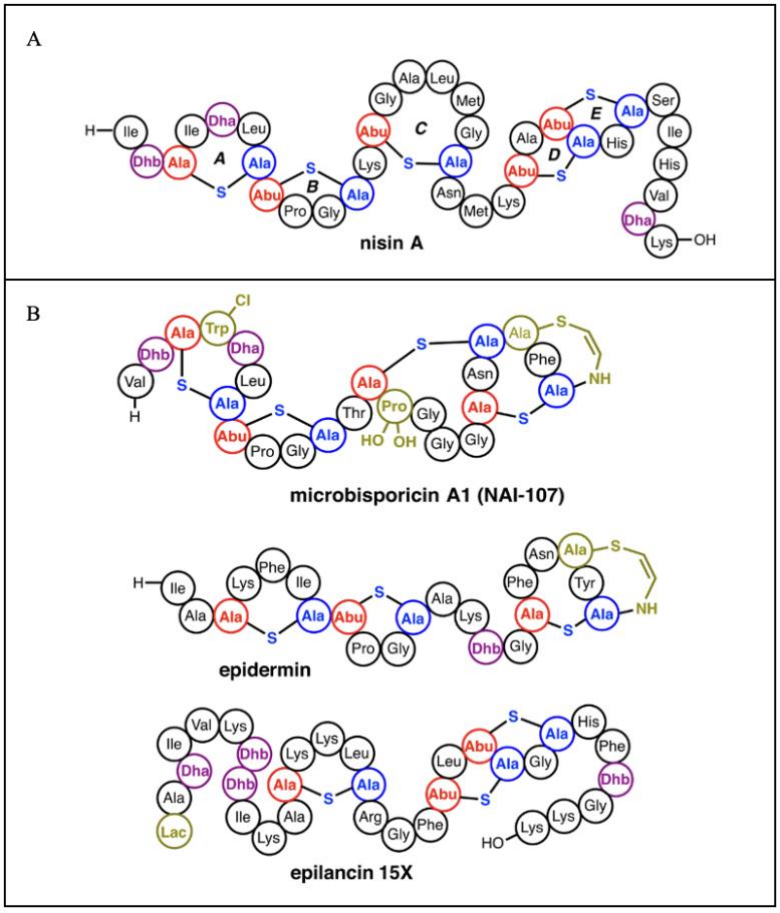

Figure 4: structure of some representative molecules of Class I lanthipeptides – A. Nisin with five thioether rings labelled A-E are present along with one Dhb and two Dha amino acid residues. B. Class I lanthipeptides with distinctive PTMs – microbisporicin A1 with chlorination of Trp and dihydroxylation of Pro residue, epidermin with aviCys modification and epilancin 15x with 2-hydroxypropionate at N-teminus (Repka et al., 2017).

Biosynthesis:

The enzymes involved in the biosynthetic pathway of Labyrinthopeptides share some common functions. The first enzyme participates in the dehydration of the lanthipeptide precursor, known as the dehydratase enzyme and another enzyme, sometimes another domain of the same enzyme, is responsible for cyclizing the precursor peptide, leading to the formation of the modified core peptide (Fig. 5A) (Arnison et al., 2013).

In Class I lanthipeptides, the dehydration step is initiated by glutamylation of Ser/Thr residues. Subsequently, the dehydration process ensues, leading to the formation of Dha and Dhb amino acids (Garg et al., 2013). Notably, in the case of nisin, it has been established that the leader peptide doesn't necessarily need to be attached to the core peptide, underscoring the flexible nature of this biosynthetic process (Arnison et al., 2013).

Additionally, Class I lanthipeptides have a dedicated split protein consisting of one glutamylation region and an elimination region (Fig. 5B). This enzyme is generically known as LanB and facilitates dehydration. The enzyme has specific designations such as NisB for nisin. The subsequent cyclization of Class I lanthipeptides is catalysed by a LanC cyclase. Evidence indicates that these proteins form a multienzyme complex, emphasizing the coordinated nature of the biosynthetic pathway (Arnison et al., 2013). There are also the LanFEGH proteins, characteristic of both Class I and Class II lanthipeptides, that play a crucial role in immunity by forming ABC transporters that act as protective mechanisms, shielding cells from potential attacks by the synthesized lanthipeptide product (Ongey and Neubauer, 2016).

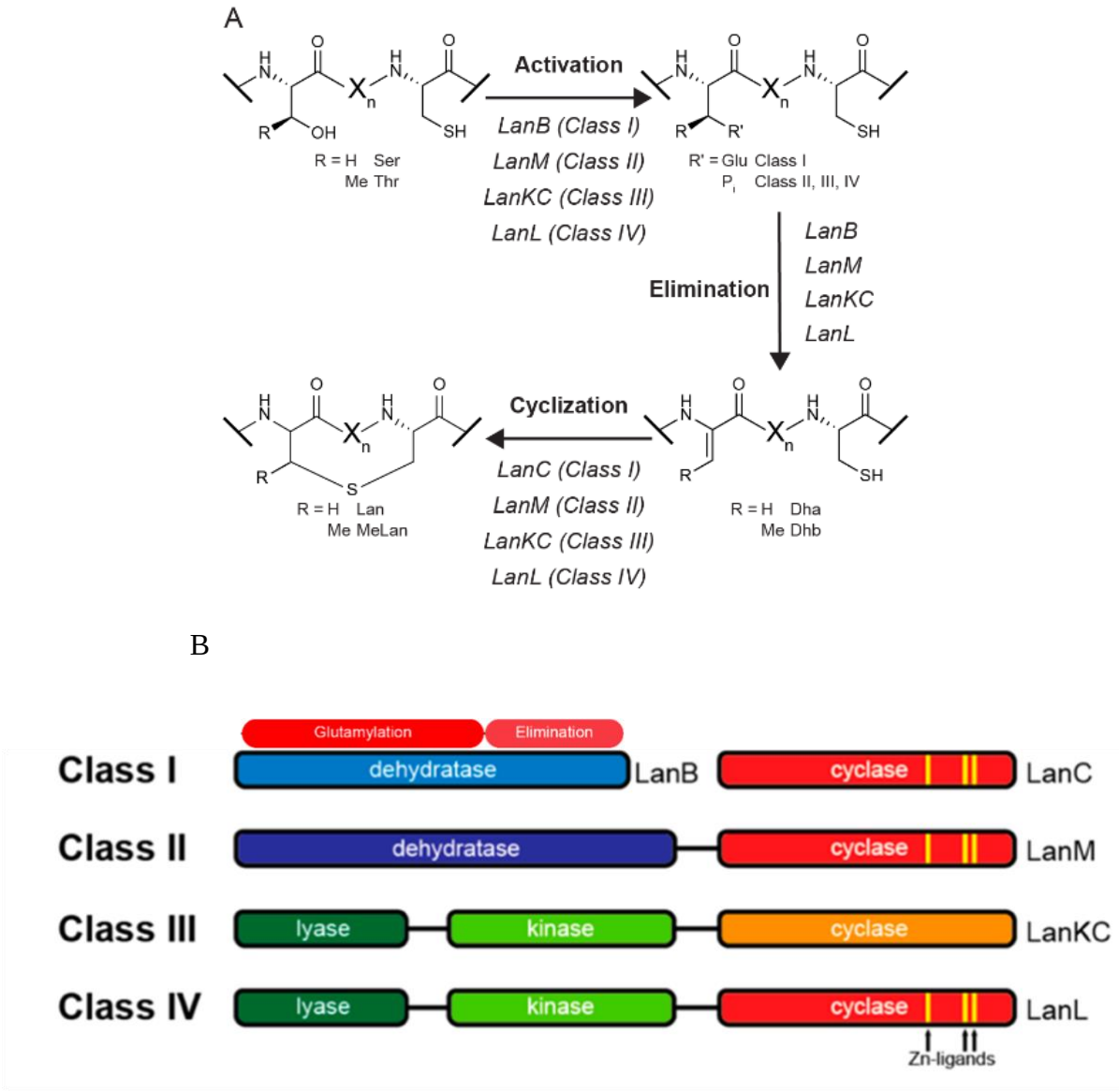

Figure 5: Biosynthesis of Lanthipeptides : A) Synthesis of the Dha/Dhb amino acids with the thioether cross links in the four different classes of Lanthipeptides. B) Enzymes and their respective domains involved in the installation of the thioether cross links in different classes of lanthipeptides (Ren et al., 2020).

2.1.2. Class II lanthipeptides

Duramycin and Cinnamycin are examples of class II lanthipeptides, characterized by a unique bridge link known as Lysinoalanine (lal) between lysine and dehydroalanine (dehydrated serine) (Fig. 6) (Meindl et al., 2010). These distinctive structural modifications confer these peptides with remarkably high affinity and specificity in targeting phosphatidylethanolamine (PE) within viral envelopes (Iwamoto et al., 2007). Duramycin, also known as Moli1901, has progressed to phase II clinical trials for the treatment of cystic fibrosis due to its ability to enhance chloride transport in airway epithelium (Meindl et al., 2010). Other examples include Lacticin 3147, that has demonstrated antimicrobial activity (Fig. 6) (Chatterjee et al., 2005).

Biosynthesis :

Class II biosynthetic enzyme – LanM encodes the dehydration, elimination, and cyclization domains on a single gene (Fig. 5B). They differ from LanB enzymes by requiring ATP and Mg2+ as cofactors (Chatterjee et al., 2005). Class II lanthipeptides, such as Lacticin 481, have also been shown not to require the attachment of the leader peptide to the core peptide (Arnison et al., 2013). Notably, the N-terminal dehydration domains of class II LanM lanthionine synthetases lack sequence homology with other enzymes in protein databases, while the C-terminal cyclization domains share homology with the LanC cyclases of class I (Repka et al., 2017). In contrast to LanB enzymes, the dehydration step relies on phosphorylation of serine and threonine residues instead of glutamylation, followed by subsequent elimination of the inorganic phosphate (Arnison et al., 2013).

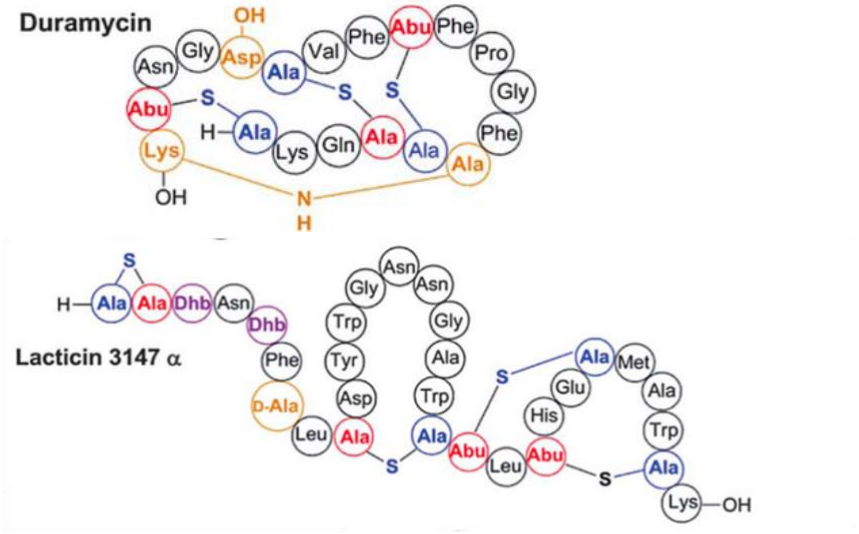

Figure 6: Class II lanthipeptide examples – Duramycin and Lacticin 3147 (Arnison et al., 2013).

2.1.3. Class III and IV lanthipeptides

Class III and IV lanthipeptides were more recently discovered, described only in 2010 (D. Hegemann and D. Süssmuth, 2020). Some examples of Class III lanthipeptides, like SapB and AmfS (Fig. 7), were well-known in the late 1900s but were recently re-classified as Class III with the discovery of labyrinthopeptides, introducing this new class (D. Hegemann and D. Süssmuth, 2020). In comparison to Class I and II lanthipeptides, these two classes exhibit weak antimicrobial activity but showcase various unique functions. For instance, Labyrinthopeptins and NAI-112 (Fig. 7) display antiallodynic functions (Iorio et al., 2014), lipolanthines demonstrate robust anti-MRSA activity, and Labyrinthopeptins also exhibit broad antiviral activity (D. Hegemann and D. Süssmuth, 2020). The biosurfactant function of SapB has been elucidated in the life cycle of Streptomycetes, adding to the functional variety of this class (Meindl et al., 2010).
While over 100 lanthipeptides have been characterized, there are only a few Class IV lanthipeptides. The first Class IV lanthipeptide - venezuelin, was discovered from *Streptomyces venezuelae*. Other reported Class IV lanthipeptides are structural homologs of venezuelin (Ren et al., 2020).

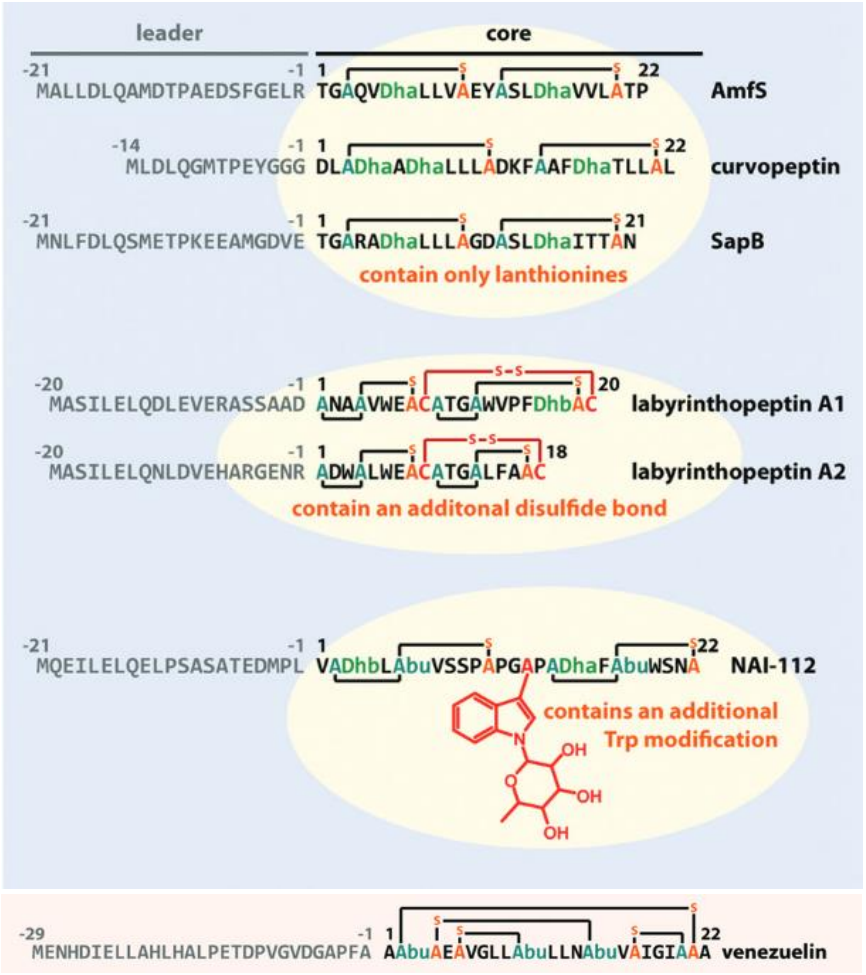

Figure 7 : Amino acid sequences and essential post-translational modifications of the Class III (blue) and Class IV (orange) lanthipeptides (D. Hegemann and D. Süssmuth, 2020).

Biosynthesis :

The biosynthetic enzymes of Class III and IV lanthipeptides are NTP-dependent and have a three-domain structure. These domains consist of an N-terminal Lyase domain, a central kinase domain, and a C-terminal cyclase domain (D. Hegemann and D. Süssmuth, 2020). In contrast to Class II lanthipeptides, where the dehydration enzyme (LanM) has one catalytic centre for both phosphorylation and β-elimination reactions, the Class III and IV dehydratase domain is split into separate lyase and kinase domains, each with its own catalytic centres (D. Hegemann and D. Süssmuth, 2020). The differentiating factor between the Class III and Class IV enzymes lies in the unique features of their cyclase domains. LanL, the cyclase domain of Class IV, has the representative Zn-binding motifs also seen in Class I and II cyclase domains, but these motifs are lacking in the Class III cyclases (Arnison et al., 2013; D. Hegemann and D. Süssmuth, 2020). Notably, a subset of Class III enzymes generates an additional carbon–carbon crosslink, termed Labionin (Lab), first identified in the labyrinthopeptins, which will be examined in greater detail in the upcoming section (Arnison et al., 2013).

2.2. Class III Labyrinthopeptides: introducing the unique amino acid – Labionine.

The structural features of labyrinthopeptides, particularly Labyrinthopeptin A1 (LabyA1) and Labyrinthopeptin A2 (LabyA2), showcase a unique amino acid—labionine, a post-translationally modified triamino acid (Fig. 8), setting them apart from other type III lantibiotics such as SapB that only contain the characteristic Lanthionine amino acid (Fig. 8) (Meindl et al., 2010; Völler et al., 2012).

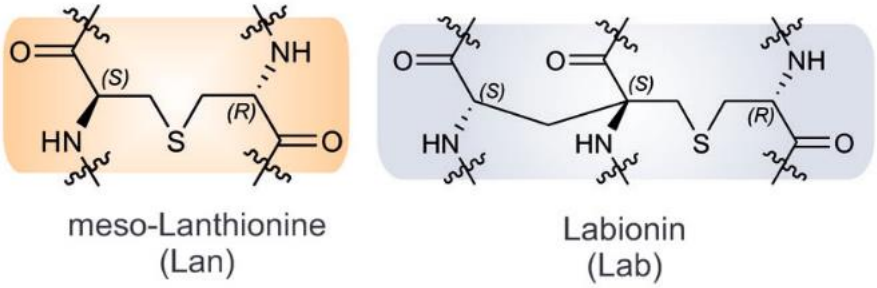

Figure 8: Chemical structural of Lanthionine and Labionine – unique non-canonical amino acids found in Lanthipeptides (Völler et al., 2012).

Labyrinthopeptins LabA1 and LabA2 were initially extracted from the Actinomycete *Actinomadura namibiensis* (Völler et al., 2012). The extracts from the bacteria contained a peptide that, upon mass spectrometry analysis, revealed a compound with a mass of 984.3333 Da. Additionally, amino acid analysis unveiled the presence of Gly and L-enantiomers of Ala, Thr, Leu, Asn/p, Cys, Phe, Glu/n, and Trp in a ratio of 1:1:1:2:1:2:1:1:2 (Meindl et al., 2010). The total molecular mass of the identified amino acid sequence differed from the measured molecular mass and could not be attributed to known lantibiotic post-translational modifications (Meindl et al., 2010). Subsequently, the X-ray structure at 1 A resolution provided analytical data revealing unique structural features resembling a maze or labyrinth. Due to this labyrinthine structure, the new compound was named labyrinthopeptin (Meindl et al., 2010).

Labyrinthopeptins exist in the variants -A1, A2, and A3 (Fig. 9), differing only in their amino acid sequence. A1 and A3 differ only in the presence of one additional Aspartic Acid residue at the N-terminal end of A3, which is not present in A1. A1 and A2 exhibit more differences in the amino acid sequence, with A1 being longer than A2, comprising 20 amino acids, while A2 has 18 amino acids (Fig. 9)(Villalaín, 2023).

The mature peptide structure of Labyrinthopeptin has a globular form primarily composed of hydrophobic amino acids. The structure of Labyrinthopeptides can be dissected into two peptide rings (Fig. 9). Each peptide bears a C-terminal Cys residue that forms a disulfide bond, a comparatively rare modification in lantibiotics but found in sublancin 168 from B. subtilis (Meindl et al., 2010).

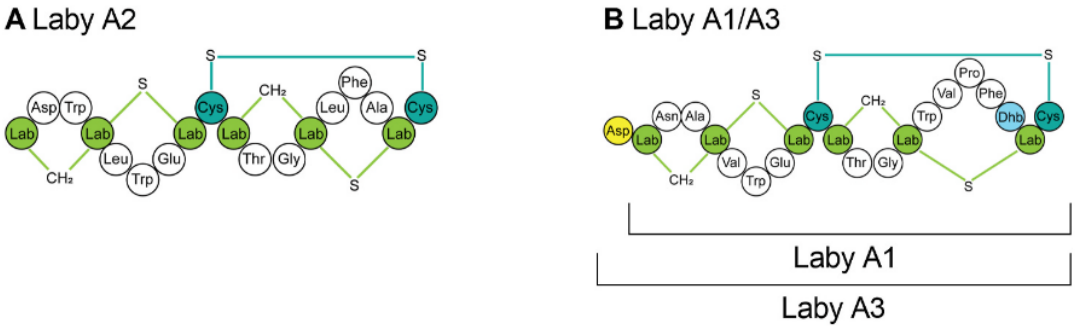

Figure 9: Amino acid sequence of Labyrinthopeptide derivatives - A) LabyA2 B) LabyA1 and LabyA3 (Oeyen et al., 2021).

The biosynthesis of Labionine involves a complex process wherein multiple dehydrations occur within the same peptide. This intricate mechanism results in the cross-linking of dehydroalanine (Dha) residues, ultimately leading to the generation of Labionin (Fig. 8). The core peptide of Labyrinthopeptides is bound by the enzyme LabKC (Fig. 10) through a recognition sequence in the N-terminal region of the leader peptide. This binding allows the peptide to remain attached to the enzyme until processing of the core peptide is completed. Similar to the biosynthetic pathway of other Lanthipeptides described in the previous section, the Labyrinthopeptide precursor peptide undergoes dehydration with the utilization of a GTP molecule, resulting in the formation of Dha residues (Fig. 11). Subsequent cyclization occurs, resulting in the formation of the characteristic Labionine residue. Further protease action and oxidation lead to the formation of the final mature peptide with the disulfide bridge (Fig. 11) (D. Hegemann and D. Süssmuth, 2020; Meindl et al., 2010; Müller et al., 2011).

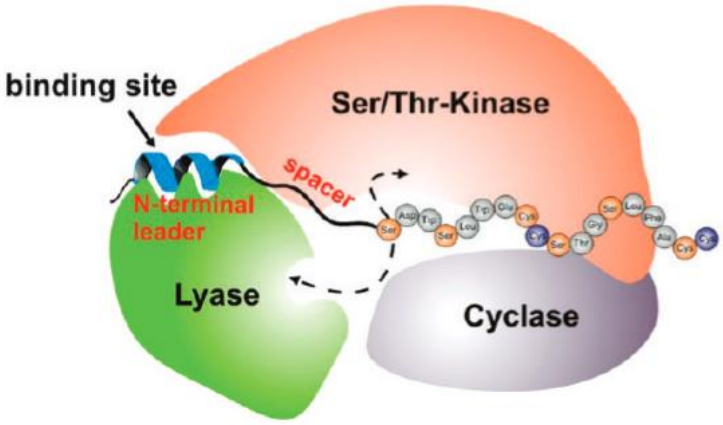

Figure 10: Schematic representation of the domains of LanKC with the binding site of the precursor Labyrinthopeptide (Müller et al., 2011).

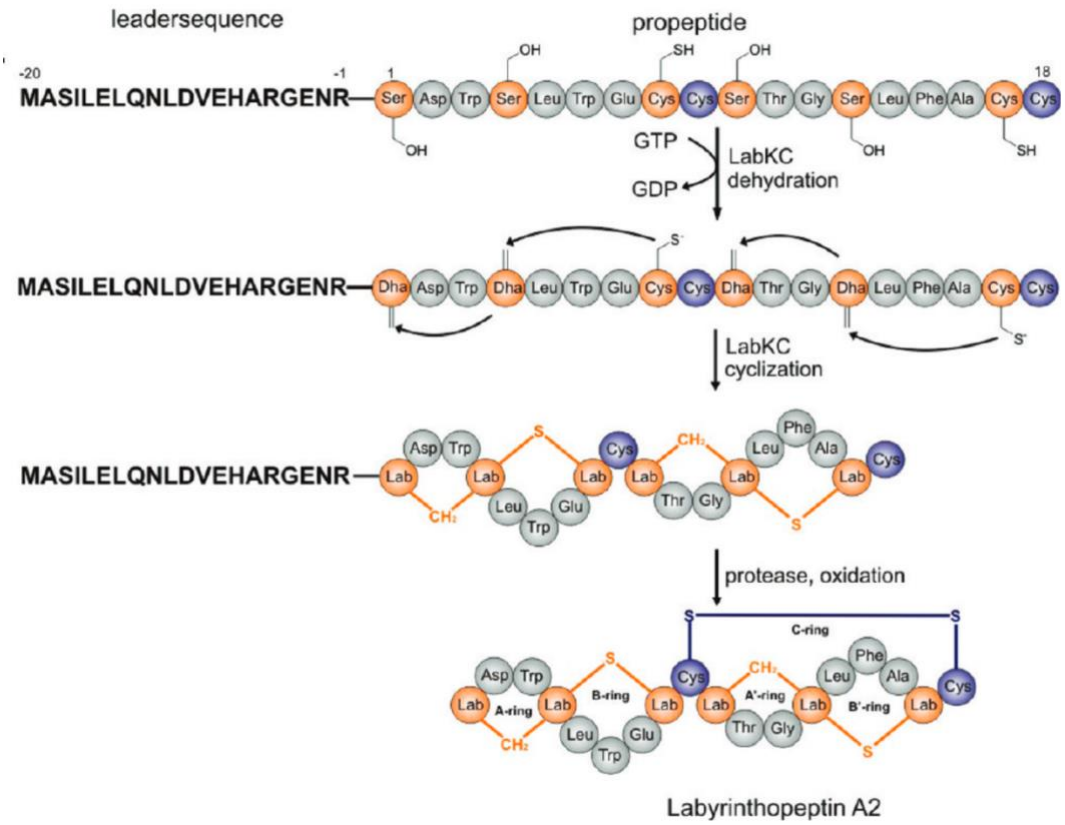

Figure 11: Biosynthetic pathway of Labyrinthopeptide. The enzyme LanKC performed dehydration and cyclisation on propeptide (precursor peptide) of Labyrinthopeptide resulting in the mature core peptide which is released following protease and oxidation action as the final mature product (Müller et al., 2011).

3. ANTI-VIRAL ACTION OF LABYRINTHOPEPTIDES – LabyA1 and LabyA2

Labyrinthopeptins are peptide isolated from the Actinobacterium - *Actinomadura namibiensis*, and akin to most lantibiotics are a part of the bacterial natural defence mechanism against competing microorganisms, enhancing its chances of survival in the same environment (Rupcic et al., 2018). The distinctive presence of labionine amino acid and di-sulfide bonds in labyrinthopeptides makes them intriguing candidates for further exploration regarding their biosynthesis and antimicrobial activities against common human pathogens. Notably, studies have demonstrated the broad-spectrum antiviral activity of these peptides which will be explored in subsequent sections (Blockus et al., 2020; Férir et al., 2014; Oeyen et al., 2021; Prochnow et al., 2020). Additionally, labyrinthopeptins have been reported to exhibit analgesic activities in a neuropathic pain mouse model, highlighting their potentially multifaceted therapeutic properties (Völler et al., 2012).

3.1. Labyrinthopeptins demonstrate broad-spectrum antiviral activity.

Labyrinthopeptin derivatives LabyA1 and LabyA2 serve as entry inhibitors against a diverse range of viruses by targeting the lipid envelope, particularly phosphatidylethanolamine (PE) (Fu et al., 2021). PE is uniquely present on the outer surface of the viral envelope but is mainly found in the inner leaflet of mammalian cell membrane, making PE a differentiating target for Labyrinthopeptins (Kuypers, 2007; Virtanen et al., 1998).

The need for broad-spectrum drugs has increased in recent times due to their potential in targeting re-emerging infections, especially for those infections where direct-acting antivirals are unavailable (Prochnow et al., 2020). Labyrinthopeptins have demonstrated antiviral activity against a wide range of enveloped viruses, including HIV - Human Immunodeficiency Virus, HSV - Herpes Simplex Virus, RSV - Respiratory Syncytial Virus, DENV - Dengue Virus, ZIKV - Zika Virus, WNV - West Nile Virus, HCV - Hepatitis C Virus, CHIKV - Chikungunya Virus, KSHV - Kaposi's Sarcoma-Associated Herpesvirus and CMV – Cytomegalovirus (Fu et al., 2021; Lohr et al., 2023; Villalaín, 2023). This comprehensive targeting demonstrates the broad-spectrum antiviral activity of Labyrinthopeptins. However, LabyA1 and LabyA2 are not active against non-enveloped viruses and lack activity against certain enveloped viruses such as Sindbis virus, (para)influenza and coronaviruses (Oeyen et al., 2021).

The subsequent subsections delve into specific aspects of labyrinthopeptins' antiviral mechanisms and their efficacy against these various viruses.

3.2. Mode of Action : Labyrinthopeptins' Selective Virolytic Effects and Lipid Interaction

Antiviral agents employ diverse mechanisms to fight viral infections, mainly focusing on two overarching strategies. The first strategy involves targeting the host proteins and pathways that are essential for viral replication. On the other hand, the second approach consists of directly acting on the virus at different stages of its infection life cycle (Fig. 12) (Fu et al., 2021).

Among these various strategies, labyrinthopeptides have been identified as entry inhibitors. Entry inhibitors interact with viral or cell surface receptors, thus eliminating the necessity for the antiviral agent to enter the cell. This characteristic, the absence of structural requirements to cross the cell membrane, enhances target

accessibility, allowing drugs of this kind to be more flexible in their structural and chemical characteristics. Thus, even small peptides, such as lanthipeptides, can be effective entry blockers. Furthermore, blocking viral entry is an effective strategy to prevent severe damage to the host cell from the later stages of the viral life cycle (Oeyen et al., 2021). Table 1 indicates some commonly reported antiviral lanthipeptides, and most of them are confirmed to operate as entry inhibitors. Alternatively, Nisin has been noted for its potential immunomodulatory effects (Fu et al., 2021), which might contribute to its antiviral activity. To investigate whether Nisin, like other reported antiviral Lanthipeptides, functions as an entry inhibitor, additional studies are required. However, this is beyond the scope of this essay.

Table 1: Reported Antiviral Lanthipeptides with their targets and potential mechanism of action (Fu et al., 2021).

RiPPs class	Peptides	Targeted virus	Mechanism of action
Lanthipeptide	Nisin	BVDV	Interact with the negatively charged viral capsid (hypothesis)
	Duramycin	Ebola/DENV/WNV/ZIKV	Prevent viral entry by binding viral envelope lipid PE
	Cinnamycin	HSV-1	Prevent viral entry by binding viral envelope lipid PE
	Labyrinthopeptin A1 Labyrinthopeptin A2	HIV/HSV/RSV/DENV/ZIKV/ WNV/HCV/CHIKV/KSHV/ CMV	Prevent viral entry mainly by binding viral envelope lipid PE; disrupt virus particle membrane integrity
	Divamide A	HIV	

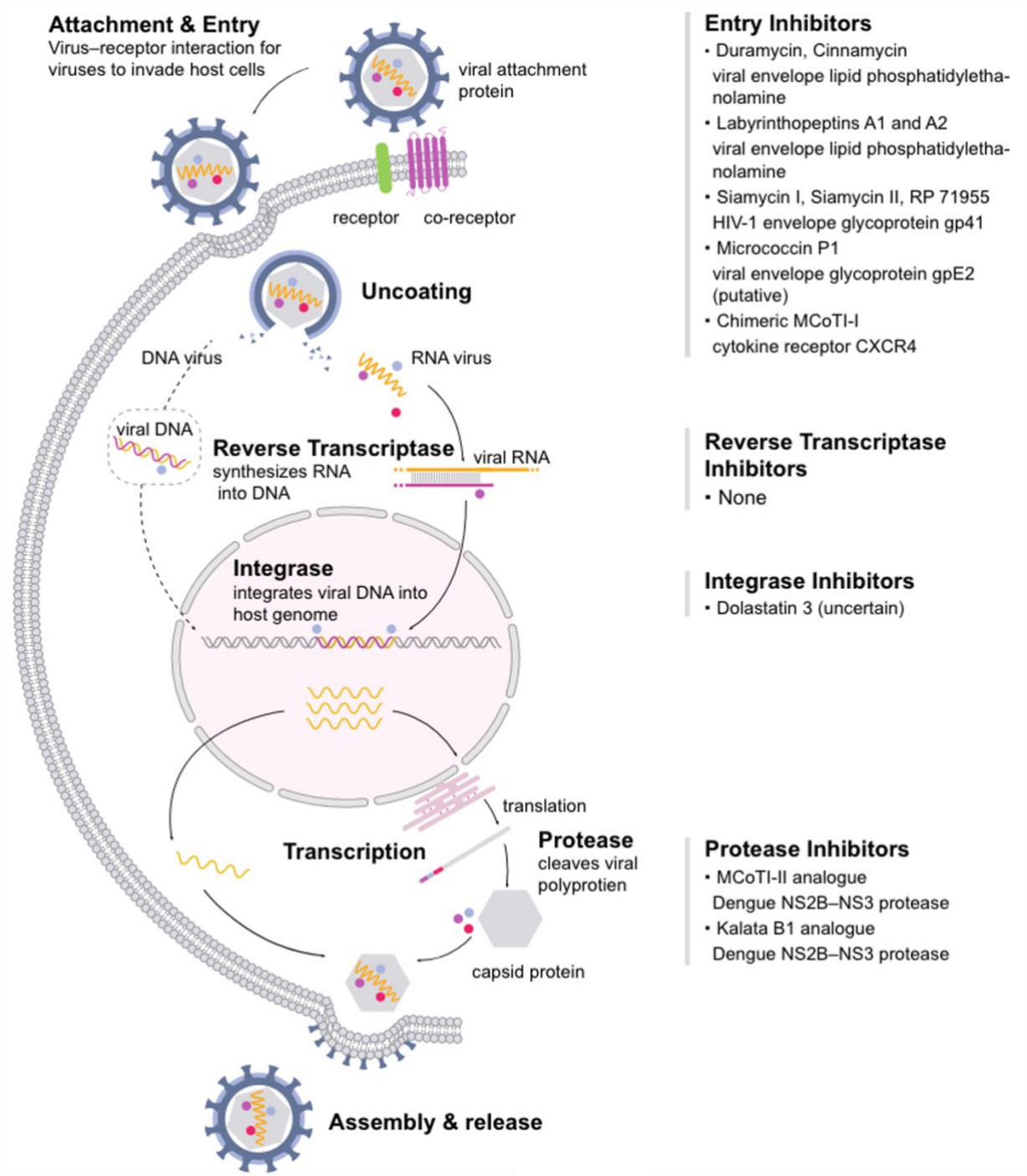

Figure 12: Schematic representation of the different mechanisms of viral infection (right) and respective antiviral drug action (left). Image adapted from (Fu et al., 2021).

LabyA1 and LabyA2 exhibit a broad-spectrum antiviral activity targeting a variety of viruses, all sharing a common characteristic—an envelope. Enveloped viruses, distinct from their non-enveloped counterparts, encapsulate their nucleocapsid with a lipid membrane. This lipid membrane, derived from the host cell's plasma membrane, varies in composition based on the viral replication pathway and the site of release. The diversity in lipid membrane composition may contribute to the selectivity of LabyA1 and LabyA2 within enveloped viruses (Luteijn et al., 2020). Thus, understanding the lipid selectivity of Labyrinthopeptides can help elucidate its mechanism of action and to know its antiviral spectrum.

3.2.1. Laby A1 and Laby A2 disrupt Respiratory Syncytial Virus (RSV) particle integrity by binding to phosphatidylethanolamine (PE).

To understand the mechanism of action and lipid selectivity of LabyA1/A2 against Respiratory Syncytial Virus (RSV), Blockus et al. (2020) conducted experiments involving HEP-2 cells infected with a clinical RSV strain (RSV-ON1-H1). The study involved the addition of LabyA1 or A2, along with the introduction of unilamellar vesicles composed of various lipids. The combinations included only phosphatidylethanolamine (PE)-containing liposomes, PE, and phosphatidylcholine (PC)-containing liposomes (40% DOPE and 60% DOPC), and liposomes composed of all three (40% DOPE/ 20% DOPC/ 20% DOPS) in one liposome. Additionally, liposomes containing PS and PC served as a control (20% DOPS/ 80% DOPC).

The cell-based screening approach identified LabyA1/A2 as effective inhibitors of RSV entry, disrupting viral particles and demonstrating their activity against RSV-ON1-H1. The results indicated that the presence of PS/PC-containing liposomes did not impact the antiviral activity of LabyA1 or A2 (Fig. 13A). On the other hand, in the presence of PE/PC-containing liposomes, the activity of both LabyA1 and A2 was attenuated (Fig. 13B). Contrary to expectations, the experiment revealed that the binding of PS by LabyA1/A2 was not responsible for the observed attenuation (Fig. 13A). As a result, PE in the liposomes competed against PE-binding sites on the virus, thereby reducing the antiviral efficacy (Blockus et al., 2020, (Luteijn et al., 2020). These results also indicate the inhibition of the infection during inoculation but not after 90 minutes post-infection, suggesting a mechanism of entry inhibition of RSV. Furthermore, the competition between LabyA1/A2 and liposomes composed of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) indicates the significance of PE-rich environments in the antiviral mechanism. This information underscores the potential of LabyA1/A2 in targeting enveloped viruses with abundant PE, giving valuable insights for further development and therapeutic strategies.

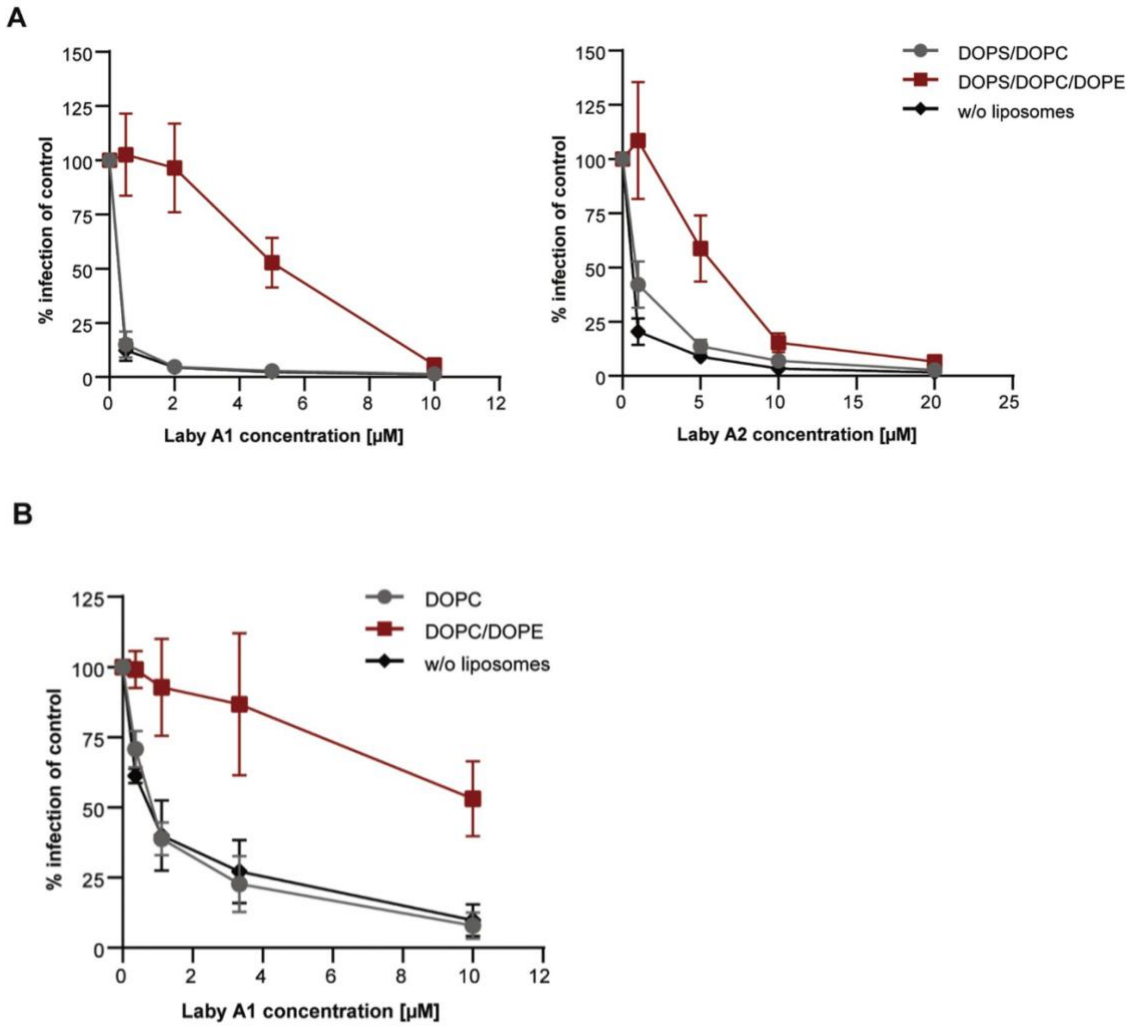

Figure 13: HEp-2 cells were pre-treated with increasing concentrations of Laby A1/A2 and a 10% fixed concentration of liposomes (with varying composition) before RSV-ON1-H1 infection. RSV-infected cell count was assessed 24 hours post-inoculation. A. Liposome compositions – DOPS/DOPC (grey circles), DOPS/DOPC/DOPE (red squares), no liposomes (black circles). B. Liposome composition – DOPC (grey circles), DOPC/DOPE (red squares) (Blockus et al., 2020).

3.2.2. LabyA1 shows dual anti-HIV and anti-HSV activity comparable to established drugs.

Effective antiviral agents against sexually transmitted viruses such as the Human Immunodeficiency Virus (HIV) and Herpes Simplex Viruses (HSV) are continuously being researched. The altered innate mucosal immunity due to HSV-2 infection has been identified as a cofactor contributing to an increased rate of HIV infection (Férir et al., 2014). Therefore, finding a broad range antiviral drug that can act against both HIV and HSV is of importance. In this context, the Labyrinthopeptide A1 (LabyA1) has been investigated by Ferir et al. 2013 for its broad antiviral activity against these two sexually transmitted diseases.

Drug activity assays reveal that LabyA1 demonstrates significant anti-HIV activity, exhibiting an EC50 (Effective concentration at which the drug activity is 50%) of 1.9 µM against HIV-1 strain and consistently displaying anti-HIV activity within the range of 0.70 to 3.3 µM against various strains. Additionally, LabyA1 retained comparable anti-HIV activity even against known resistant strains such as HIV-1 NL4.3. In contrast, other lantibiotics such as Nisin and LabyA2 exhibited higher EC50 values of 26 µM and 29 µM, respectively,

suggesting that LabyA2 is not as effective as an anti-HIV agent despite similar labionine amino acids and di-sulfide bonds as LabyA1 (Férir et al., 2013).

Similarly, Labyrinthopeptides were tested for their anti-HSV activity against various strains of HSV-1 and HSV-2. LabyA1 demonstrated anti-HSV activity within the range of EC50 = 0.29-2.8 µM, comparable to well-known anti-HSV drugs like acyclovir and cidofovir. Additionally, LabyA1 showed efficacy against acyclovir resistant HSV strains. LabyA2, though demonstrating anti-HSV activity, was a ten-fold less strong inhibitor than LabyA1 (Férir et al., 2013; Fu et al., 2021; Tiwari et al., 2020).

A time-of-drug-addition experiment was conducted to understand the antiviral target of LabyA1. Known benchmarks were used as controls, such as the polyanionic compound dextran sulfate 8000, that is recognized for inhibiting HIV replication exclusively during the time of infection. Similarly, AMD3100, an antagonist of CXCR4, which has been proven to hinder HIV replication, but its efficacy diminishes after two hours post-infection. And NNRTI, a reverse transcriptase inhibitor, that retains its full activity up to four hours post-infection. These benchmarks were employed to assess and compare the effectiveness of LabyA1 in inhibiting HIV infection. Using a p24 Ag ELISA test to measure HIV replication, the results indicated that LabyA1 inhibited the HIV viral entry process similar to AMD3100, suggesting LabyA1 to be an entry inhibitor of viral infection (Férir et al., 2013)

To understand the specific binding receptor of LabyA1 in HIV, surface plasmon resonance (SPR) study was conducted, revealing that LabyA1 shows a binding affinity to the gp120 of the HIV envelope which can be consistent with its antiviral activity. Although the authors indicated that glycans on gp120 are not the target binding site of LabyA1 they did not test if any other parts of the viral envelope interact with LabyA1. Further investigation is needed to establish the exact interaction with both HIV and HSV (Férir et al., 2013). Future studies, including experiments with knockout gp120 proteins and assessing the interaction of LabyA1 with viral envelope lipids, are essential to conclusively identify the binding site of LabyA1.

3.2.3. LabyA1 shows broad anti-ZIKV and anti-DENV activity by interfering with virus entry in target cells.

Dengue and Zika viruses, belonging to the class of Flavivirus, lack effective broad-spectrum antiviral treatments. Additionally, the FDA-approved DENV vaccine has many safety concerns (Halstead and Russell, 2016). In contrast, for other Flaviviruses - yellow fever and Japanese encephalitis effective and safe vaccines are available (Oeyen et al., 2021). Therefore, LabyA1 and LabyA2, known for promising activity against HIV and HSV, have been investigated for potential anti-DENV and anti-ZIKA activity.

LabyA1 has been shown to act as an entry inhibitor for viral particles (Prochnow et al., 2020). One advantage of entry inhibitors is the prevention of severe damage in the later stages of the viral life cycle. Hence, there are lower chances of late-stage damage to occur if the drug inhibits entry itself, rather than acting upon the virus after it has entered the host cell. DENV causes overactivation of the immune system in the later stages of its infection (Oeyen et al., 2021), highlighting the importance of studying Labyrinthopeptins, that are established as broad-spectrum entry inhibitors in other viruses (Férir et al., 2013).

The virucidal activity of LabyA1 and LabyA2 against dengue virus (DENV) and zika virus (ZIKA) in Vero cells was quantified by assessing cell viability after infection and treatment. LabyA1 consistently demonstrated inhibition of all studied strains, exhibiting an IC50 value in the low micromolar range of 0.51-0.99 µM against various Zika virus strains. In comparison, LabyA2 displayed antiviral activity against Zika virus strains but at a ten-fold lower efficacy than LabyA1. Against the DENV-2 strain LabyA1 demonstrated an IC50 of 0.8µM which falls in a similar range to that observed in ZIKA virus strains (Oeyen et al., 2021). Considering the structural differences between LabyA1 and LabyA2, the higher proportion of hydrophobic residues in LabyA1 suggests a potential mechanism that could contribute to a stronger binding affinity for phosphatidylethanolamine. This aspect is explored in more detail in the subsequent section.

To further elucidate LabyA1's mechanism as a potential entry inhibitor against flaviviruses, including ZIKA and DENV, a time of drug addition assay has been conducted. This involved infecting host cells with DENV-2 and ZIKA MR766 strain, followed by incubation with LabyA1 at different time points to assess the optimal antiviral activity time point (Oeyen et al., 2021). The effectiveness of labyrinthopeptins in inhibiting Dengue virus (DENV) infection diminishes when the compound is administered as early as 10 minutes after viral infection (Prochnow et al. 2020). Furthermore, Laby A1 demonstrates antiviral activity when introduced only before or at the same time as virus addition (time points -2 and 0) (Fig. 14 A, B). However, its efficacy declines significantly when added after infection with either Dengue virus (DENV) or Zika virus (ZIKV) (Oeyen et al., 2021). These results suggests that LabyA1 can only inhibit the virus before it enters the cell and starts the infection, elucidating its mechanism as an entry inhibitor.

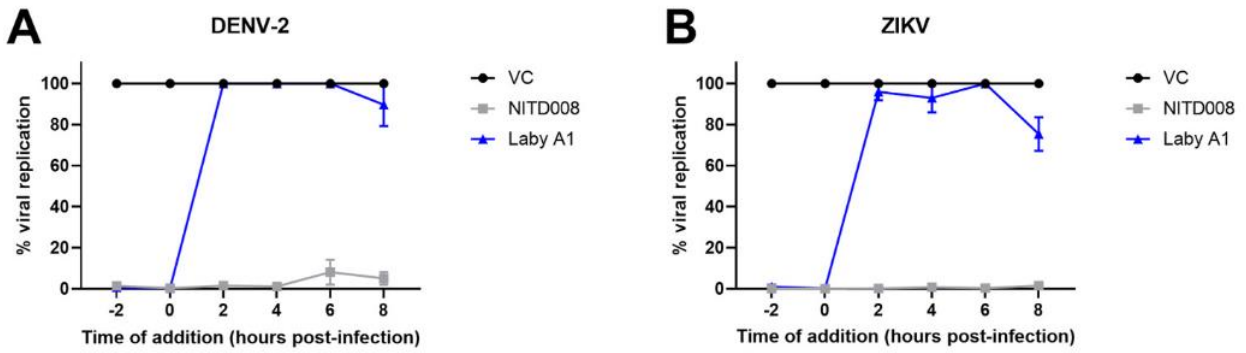

Figure 14: Percentage of Viral replication on addition of LabyA1, NITD008 – a well-known antiviral agent against flaviviruses, and VC – Viral Load. A – anti-DENV activity. B – anti-ZIKA activity (Oeyen et al., 2021).

Laby A1 in DENV and ZIKA selectively binds to liposomes containing phosphatidylethanolamine (POPE, kD: 4.1 ± 0.1 µM) and phosphatidylserine (POPS, kD: 4.8 ± 0.2 µM). Limited binding is observed with ceramide phosphoethanolamine (CPE)-containing liposomes (sphingomyelin with an ethanolamine head group, kD: 10.6 ± 0.1 µM), while no affinity is found for POPC or POPC mixtures with cholesterol or DOPA. This selectivity for PS and PE positions Laby A1 as an effective and non-toxic option for interacting with viral membranes enriched with these lipids (Oeyen et al., 2021), preventing cytotoxic activity in host cells consisting of PS and PE in the inner leaflet of the cell membrane. An interesting observation is that, unlike the binding specificity of LabyA1 to PE as described in the previous subsection, this study shows that LabyA1 also binds to PS. It is known that Duramycin binds only to PE, and this difference may indicate higher selectivity of Duramycin, but

broader selectivity of LabyA1 as it binds two different viral lipids. The specificity of LabyA1 may contribute to the lower IC50 of Duramycin (Blockus et al., 2020).

These findings further emphasise the broad-spectrum antiviral activity of LabyA1, while also underscoring the lower efficacy of LabyA2. Furthermore, there is an improved understanding of the binding of LabyA1 to both PE and PS in the viral envelope illustrating the specificity in lipid binding of Labyrinthopeptides.

3.2.4. LabyA1 has a higher selectivity index than Duramycin.

The antiviral efficacy and cytotoxicity of LabyA1 have been compared with those of Duramycin, a class II lanthipeptide known for its antiviral activity (Oeyen et al., 2021). Figure 15 provides insight into the long-term cytotoxicity evaluation in A549 cells, measuring the percentage of viable cells in each passaged subculture under the influence of LabyA1 or Duramycin. The figure highlights the sustained viability of A549 cells in the presence of 10µM of LabyA1 and in comparison, shows the noticeable decline observed with just 1µM of Duramycin. Thus, emphasizing the low cytotoxicity potential of LabyA1 as a prospective antiviral agent.

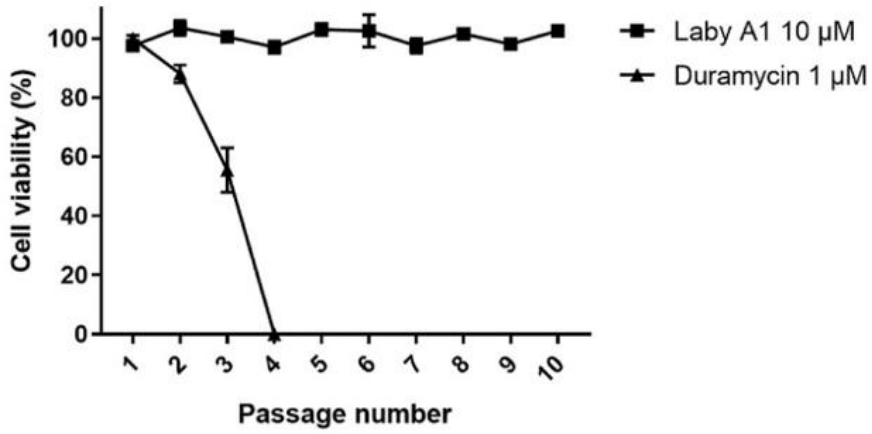

Figure 15: Evaluation of long-term cytotoxicity pressure of Laby A1 and duramycin in A549 cells. A549 cells were passaged every 3 days in the presence or absence of Laby A1 (10 µM) or duramycin (1 µM).

To effectively compare the two drugs, the selectivity index—a measure of drug safety—was calculated as the ratio of the 50% cytotoxic concentration (CC50) to the inhibitory concentration of 50% (IC50) (Indrayanto et al., 2021). A higher selectivity index is a measure of higher antiviral activity with minimal cell toxicity. The CC50 in Vero cells (cell line isolated from kidney epithelial cells) and the IC50 for various viruses in the presence of duramycin and labyA1 was measured (Table 2). The resulting selectivity index was significantly higher for LabyA1 than Duramycin. This implies that although Duramycin exhibits lower IC50 than LabyA1, indicating better antiviral activity, ultimately its higher toxicity to the cells than LabyA1 makes LabyA1 a more promising candidate for further exploration as an antiviral agent. (Oeyen et al., 2021)

Table 2: Selectivity index of LabyA1 and Duramycin (Oeyen et al., 2021).

	Laby A1		Duramycin	
CC_{50} (μM)[a]	124.6 ± 3.1		1.1 ± 0.1	
	IC_{50}[b] (μM)	SI[c]	IC_{50}[b] (μM)	SI[c]
ZIKV				
MR766	0.99 ± 0.1	126	0.09 ± 0.007	13
IBH 30656	0.51 ± 0.06	245	0.02 ± 0.003	61
PRVABC59	0.74 ± 0.12	169	0.12 ± 0.006	9
FLR	0.80 ± 0.18	156	0.06 ± 0.02	19
DENV-2	0.55 ± 0.02	227	0.22 ± 0.05	4.9

a Compound concentration required to inhibit Vero cell viability by 50%.

b Compound concentration required to inhibit viral replication in Vero cells by 50%

c Selectivity index = CC50 / IC50 against each virus in Vero cells

Additionally, previous research highlighted that labyrinthopeptins, including LabyA1, exhibited low cytotoxicity designating them as potential antiviral compounds with an unusual viral lipid targeting mechanism (Prochnow et al., 2020). Fu et al., 2021 also emphasized the advantages of LabyA1/A2 over Duramycin and Cinnamycin, citing low cytotoxicity and a lack of adverse drug effects, making labyrinthopeptins promising antivirals with efficacy against a broad range of viruses and a favourable therapeutic index over established antiviral agents.

4. COMPARATIVE ANALYSIS : STRUCTURAL CONTRIBUTIONS OF LABYRINTHOPEPTIDES IN INTERACTIONS WITH PHOSPHATIDYLETHANOLAMINE

The unique structure of Labyrinthopeptides includes the labionine amino acids and di-sulfide bridges (Section 2.1), setting them apart from other classes of Lanthipeptides with established thioether bridges. The mode of action section highlights the entry inhibitor role of Labyrinthopeptides through time-of-addition assays and its selective binding to phosphatidylethanolamine and phosphatidylserine in the viral envelope of various enveloped viruses. However, the molecular interactions between Labyrinthopeptides and these lipids remain inadequately studied. Important questions about whether the peptide binds to the lipid head group or tail, the stabilizing interactions between the peptide and the lipid, and how this binding leads to changes in the viral envelope leading to virolysis are yet to be explored. Drawing parallels with peptides exhibiting similar antiviral activity and structural features may offer insights, and this section aims to establish those connections.

Well-characterized lanthipeptides such as Cinnamycin and Duramycin have exhibited antiviral activity against various viruses (Fu et al., 2021). Nisin, another lanthipeptide, has also been reported to show antiviral properties specifically against Bovine Viral Diarrhea Virus (BVDV), but not against the viruses targeted by Labyrinthopeptides. Here, we aim to compare structural similarities of Duramycin and Cinnamycin with Labyrinthopeptide to better understand the structural contributions to its antiviral activity. Specifically, these peptides were selected for the comparative analysis as they have demonstrated antiviral efficacy against viruses like those targeted by Labyrinthopeptides (Table 1)

4.1. Structural Parallels: Comparing the structure of LabyA1 with Duramycin and Cinnamycin.

Duramycin and Cinnamycin are comprised of 19 amino acids each, forming a hydrophobic end with three phenylalanine residues and a hydrophilic end on the opposite side (Fig. 16) (Rzeźnicka et al., 2010). The key distinction between these peptides lies in the presence of a lysine residue at position 2 in Duramycin and an arginine residue at the same position in Cinnamycin (Fig. 16).

Moreover, Cinnamycin and Duramycin have been suggested to form a high-affinity complex with phosphatidylethanolamine (PE) (Iwamoto et al., 2007; Zhao, 2011). The hydrophobic region of Cinnamycin creates a pocket extending from Phe-7 to Ala-14, binding the ethanolamine head group of PE. This binding is further stabilized by ionic interactions between the amine group of ethanolamine and the β-hydroxy of Asp-15 (Fig. 17). Additional stability is provided by hydrophobic interactions involving Gly-8, Pro-9, Val-13, and two Phe-10 and 12 residues with the glycerol moiety of the lipid (Fig. 17) (Rzeźnicka et al., 2010).

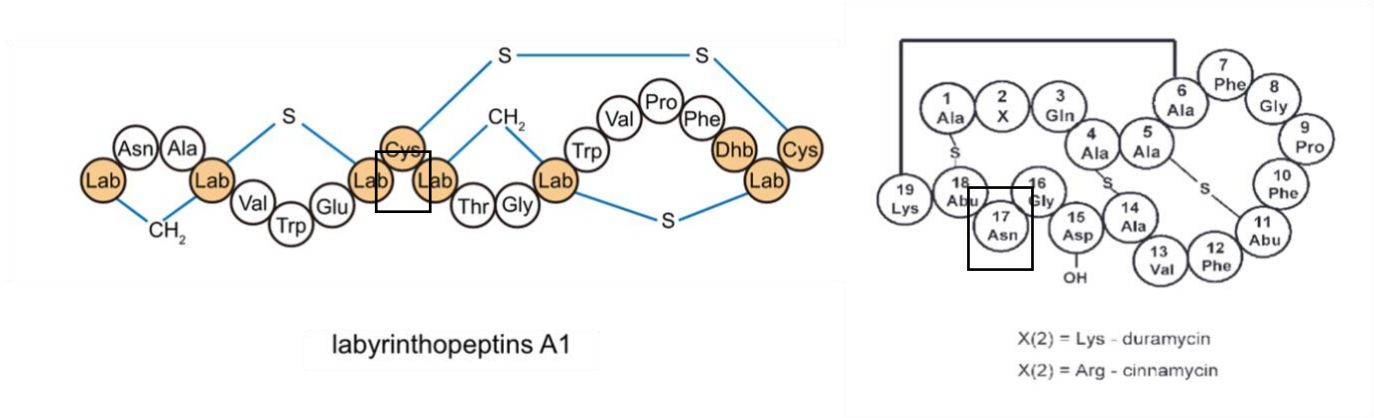

Figure 16: Amino acid sequence and post-translational modifications of Labyrinthopeptide A1(left) Duramycin/Cinnamycin (right) (Fu et al., 2021; Rzeźnicka et al., 2010).

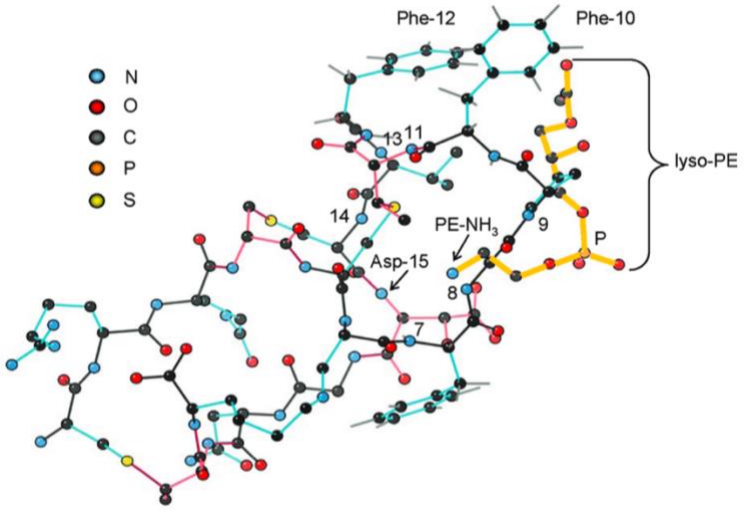

Figure 17: Structure of the Cinnamycin-PE complex (Rzeźnicka et al., 2010).

Comparing the structure of Cinnamycin/Duramycin with that of LabyA1 reveals common features, where LabyA1 also exhibits a hydrophobic end and a hydrophilic end (Fig. 16). From Figure 16, we can see that at the disulfide bridge end there is a hydrophobic ring starting from Trp-14 to Phe-17, and on the other end there are hydrophilic amino acids Asn-2 and Ala-2. These simple amino acid similarities might be coincidental and do not necessarily imply the PE binding ability of LabyA1. However, delving into further similarities, Cinnamycin, Duramycin, and LabyA1 share a commonality in their length, each consisting of 19-20 amino acids, and notably, they lack any free peptide ends. This structural characteristic serves as protection against proteolytic activity, potentially contributing to the peptides' stability and durability. Moreover, both Duramycin and Cinnamycin possess a β-hydroxy on the Asp-15, that is established as crucial for stabilizing the PE headgroup (Rzeźnicka et al., 2010). LabyA1, while lacking a β-hydroxy modification on Aspartic acid, has a Threonine residue located near the hydrophobic ring (Fig. 16) that consists of a hydroxyl group on its side chain. This hydroxyl group is akin to a β-hydroxy group and its presence may contribute to similar stabilising ionic interactions with the amine group of PE, suggesting potential parallels in their PE binding mechanisms. To test this hypothesis, a mutation could be done at the Threonine amino acid in LabyA1 to another amino acid lacking the OH group, such as Valine, but shares the same side chain structure as Threonine and substitutes the hydroxyl group with a methyl group. Alternatively, evaluating the stabilizing

effect of the beta hydroxyl group could involve replacing Threonine with Serine, which has a gamma hydroxyl group on its side chain. However, it is important to consider potential complications, such as the conversion of Serine to a Dha amino acid by the lanthipeptide enzymes and the hydrophobicity of the new amino acids as significant changes to the final mutated peptide, compared to wild-type LabyA1, could lead to an altered binding affinity to PE. This alteration may not be a direct result of the presence or absence of the hydroxyl group, highlighting the need for extensive considerations in peptide engineering.

Other parallels in the structure of these peptides include the presence of unique bridges not commonly found in lanthipeptides - the Lysinoalanine bridge in Duramycin and Cinnamycin and the disulfide bridge found in Labyrinthopeptides. While Duramycin and Cinnamycin feature a distinctive bridge link, Lysinoalanine (lal), between lysine and serine, contributing to their exceptionally high affinity and specificity in targeting phosphatidylethanolamine (PE) in viral envelopes (Meindl et al., 2010), the disulfide bridge in LabyA1 may similarly act to stabilize the hydrophobic binding pocket of LabyA1. To understand the importance of these bridges, structural studies with modifications to the disulfide bridge, such as replacing it with the Lysinoalanine bridge, could be conducted to compare their stability and contribution to antiviral activity.

The structural comparison of Cinnamycin, Duramycin, and LabyA1 highlights several shared features with potential implications for the antiviral mechanism of LabyA1. Further studies, including strategic mutations and structural analysis are essential for a comprehensive understanding of the antiviral activity.

4.2. Structural Parallels: Similarities between LabyA1 and selected peptides exhibiting antiviral activity.

Other RiPPs also exhibit antiviral activity, and analysing their structural features suggests that certain elements may be commonly associated with antiviral small peptides binding to viral envelope lipids.

Lasso peptides, a subset of RiPPs categorized into four classes, are particularly noteworthy. The first class, characterized by two disulfide bonds, is the only one displaying antiviral activity. This observation hints at the potential role of disulfide bonds in stabilizing the peptide or contributing to its antiviral binding—a characteristic that may extend to Labyrinthopeptides (Fu et al., 2021).

Additionally, the peptide CPXV012, derived from the cowpox virus, binds to phosphatidylserine (PS) by using electrostatic interactions between cationic residues in the peptide and the anionic head group of PS. This mechanism bears resemblance to the ionic interactions between β-hydroxy aspartic acid and the phosphatidylethanolamine (PE) head group. The presence of such stabilizing ionic interactions, especially when binding to charged lipid head groups, appears pivotal for the peptides' binding affinity to the viral envelope (Luteijn et al., 2020).

Moreover, a recently discovered lanthipeptide, Divamide A, isolated from symbiotic bacteria in marine tunicates of Papua New Guinea's Eastern Fields, exhibits anti-HIV activity. Structural analysis revealed a Lysinoalanine bridge, three methyl-lanthionine amino acids, and N-terminal trimethylation in the 20-amino acid peptide (Fig. 18). Divamide A consists of a hydrophobic ring of residues (Phenylalanine, Glycine, Isoleucine, and Valine) and thioether bonds between the Serine and Cysteine residues, resembling the

structure of Cinnamycin (Fig. 18) (Smith et al., 2018). The presence of a β-hydroxy aspartic acid in Divamide A further suggests a similar antiviral mechanism of binding to the phosphatidylethanolamine head groups on the HIV viral envelope (Smith et al., 2018).

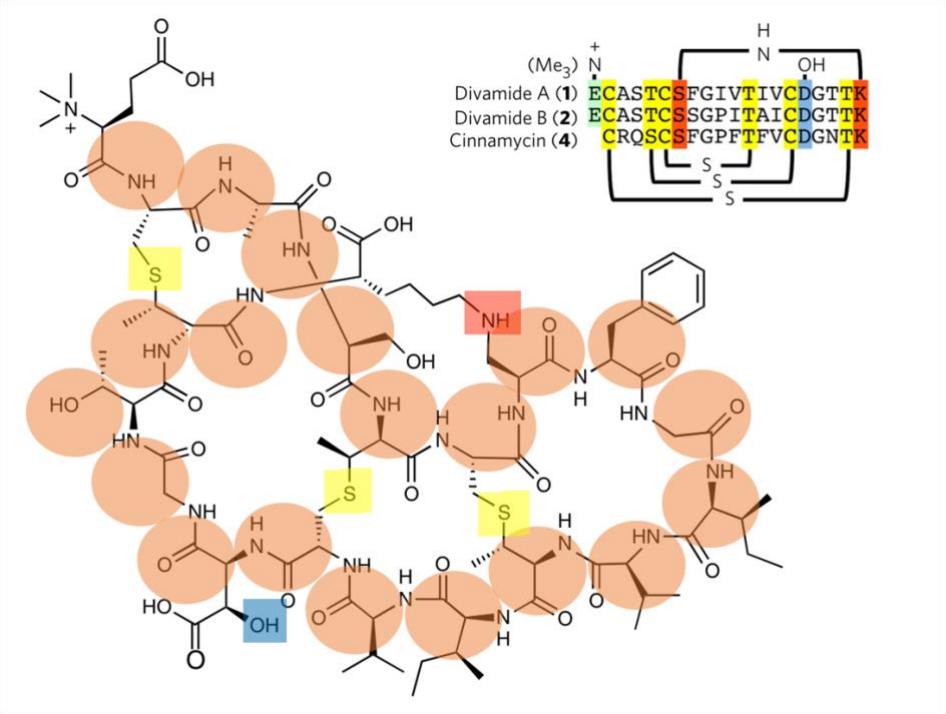

Figure 18: Structural Insight into Divamide A : Peptide Backbone Sketch (Orange), Thioether Bonds (Yellow), Lysinoalanine Bridge (Red), and β-Hydroxy Moiety of Aspartic Acid (Blue) [Image adapted from (Smith et al., 2018)]

Notably, another variant, Divamide B, isolated from a different tunicate colony, has certain differences in the amino acid sequence when compared to Divamide A (Smith et al., 2018). Although both share features like the Lysinoalanine bridges, methyllanthionine, N-trimethylation, and the β-hydroxy aspartic acid, Divamide B has a serine residue in place of phenylalanine in the hydrophobic pocket region (Fig. 18). This variation may account for its reported inactivity against HIV (Smith et al., 2018) and might imply the importance of the presence of a hydrophobic binding pocket to stabilize interactions with phosphatidylethanolamine.

These comparisons offer novel insights on some of the common structural features of antiviral Lanthipeptides that contribute to its viral lipid -PE- binding mechanism. Characterizing and studying these features for LabyA1 can provide valuable insights, potentially leading to peptide engineering for improved antiviral activity with lower cytotoxicity.

5. DISCUSSION, OUTLOOK, AND CONCLUSION ANALYSIS

Lanthipeptides, comprising one of the largest classes of Ribosomally synthesized and post-translationally modified peptides (RiPPs), stand out for their structural complexity and functional diversity. Notably, lanthipeptides exhibit over 15 unique post-translational modifications, with Nisin, the first-discovered lantibiotic, serving as a representative example.

The biosynthesis of lanthipeptides follows a general pathway involving precursor peptides, leader and follower peptides, and maturation processes. Lanthipeptides are categorized into four classes, each characterized by specific modifications and enzymatic processes. Class I, exemplified by Nisin, includes lanthionine-containing antibiotics, while Class II lanthipeptides, such as Duramycin, feature lysinoalanine bridges. More recently discovered Class III and IV lanthipeptides, including Labyrinthopeptins and venezuelin, introduce novel structural features, such as labionine amino acid and Zn-binding motifs in their cyclase domains.

Labyrinthopeptides, belonging to Class III, have garnered attention for their unique structural dynamics, particularly the presence of labionine. Labyrinthopeptin derivatives, such as LabyA1 and LabyA2, showcase distinctive amino acid sequences and exhibit promising antiviral and antimicrobial activities with lower cytotoxicity. The biosynthesis of labionine involves a complex process of multiple dehydrations and cyclizations within the same peptide, leading to the formation of labionine residues. The resulting mature peptides possess a globular structure with disulfide bonds, contributing to their stability.

In the ever-evolving landscape of infectious diseases, lanthipeptides, with their intricate structures and functional versatility, offer new avenues for the development of effective and targeted antimicrobial and antiviral agents. As research in this field progresses, the insights gained from studying lanthipeptides contribute to the ongoing quest for innovative solutions in the fight against emerging infectious challenges.

The labyrinthopeptides LabyA1 and LabyA2, isolated from *Actinomadura namibiensis*, exhibit remarkable antiviral properties, positioning them as promising candidates for therapeutic development. These peptides, belonging to the lanthipeptide Class III, play a vital role in the bacterial natural defence mechanism against competing microorganisms. LabyA1 and LabyA2 showcase broad-spectrum antiviral activity, particularly against enveloped viruses.

The antiviral mechanism of LabyA1 and LabyA2 involves their role as entry inhibitors, targeting the lipid envelope of viruses, specifically phosphatidylethanolamine (PE). This distinctive targeting has been demonstrated through extensive studies, particularly against RSV, HIV, HSV, DENV, and ZIKV. The peptides disrupt viral particles, preventing entry and subsequent infection. The specificity for PE-rich environments highlights the potential of labyrinthopeptides in targeting enveloped viruses with abundant PE, offering valuable insights for further therapeutic development. Notably, LabyA1 demonstrates dual anti-HIV and anti-HSV activity comparable to established drugs, showcasing its versatility against sexually transmitted viruses.

Moreover, LabyA1's effectiveness in inhibiting the entry of DENV and ZIKV into host cells presents a significant advantage by preventing late-stage damage in the viral life cycle. The selective binding of LabyA1 to liposomes containing phosphatidylethanolamine (PE) and phosphatidylserine (PS) contributes to its broad-spectrum antiviral activity. The high selectivity index of LabyA1, coupled with its low cytotoxicity,

positions it as a promising antiviral agent with a favourable therapeutic index over established drugs like Duramycin.

The multifaceted therapeutic potential of labyrinthopeptides extends beyond antiviral activity, as demonstrated by their reported analgesic effects in a neuropathic pain mouse model. This underscores the possibility of developing these peptides into drugs with multifunctional therapeutic properties.

The critical comparative structural assessments put forward for the first time in this essay, particularly with well-characterized antiviral lanthipeptides such as Duramycin and Cinnamycin, underscore potential parallels in their PE-binding mechanisms. LabyA1 shares structural features, including the absence of free peptide ends, hydrophobic and hydrophilic regions, and unique bridges, suggesting commonalities in their antiviral strategies. However, the presence of a β-hydroxy moiety in Duramycin and Cinnamycin, absent in LabyA1, poses intriguing questions regarding the role of this modification in stabilizing the PE headgroup, and should be explored further.

Furthermore, comparisons with other ribosomally synthesized and post-translationally modified peptides (RiPPs) exhibiting antiviral activity, such as lasso peptides and Divamide A, offer unprecedented insights into the mechanism of LabyA1. Shared characteristics like disulfide bridges, lysinoalanine bridges, and hydrophobic binding pockets suggests the importance of these structural elements in lipid binding on viral envelopes.

While LabyA1 demonstrates potent antiviral activity against a range of viruses, its lower cytotoxicity and higher selectivity index, compared to Duramycin, makes it a promising candidate for further exploration. However, extensive structural and functional studies, including targeted mutations and detailed binding analyses, are crucial to unravel the precise interactions between labyrinthopeptides and viral envelope lipids. Such insights will not only enhance our understanding of their antiviral mechanisms but also guide peptide engineering efforts to improve antiviral efficacy with minimal cytotoxic effects. Overall, labyrinthopeptides present a compelling avenue for the development of novel antiviral therapeutics with broad-spectrum activity and improved tolerability.

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