Designing antimicrobial peptides

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Bachelor thesis

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CONTENTS

ABSTRACT

Antibiotic resistance is on the rise, with more and more multiple-antibiotic resistant bacteria being found. The classic way of semi synthetic antibiotics has long since yielded a completely new result, so we must find another way.

Eukaryote derived and microbe modified antimicrobial peptides are nature's way to, especially in marine organisms, fight off pathogenic bacteria and remain a healthy host. Some of these are modified to an extent not that is rarely seen in the world, with up to 30 modified amino acids in a peptide only 48 amino acids long. How can we use these types of peptides to further research into novel antimicrobial peptides? Here we find that antimicrobial peptides usually have a length of 30-40 amino acids and a charge of +3 to +4, along with all different kinds of modifications, even some only very recently described for the first time, which may help design anti-microbial peptides.

INTRODUCTION

Multi-antibiotic resistant bacteria are on the rise due to overuse of antibiotics worldwide. The discovery of new antibiotics has come to a halt and even the last resort antibiotics must be used in some cases. The question is, how do we combat this? Research into lantibiotics such as nisin reveals post-translationally modified peptides to be a suitable anti-microbial agent in, for example, food preservation.[1] A novel cytotoxic agent found in nature is a heavily modified peptide (proteusin) called a polytheonamide. This is found in the marine sponge *T. swinhoei* of the genus *Theonella* and family *Theonellidae*.[2] This sponge produces, in tight symbioses with several microorganisms, a peptide consisting of 48 amino acids of which 30 are modified in six different ways. This level of modification is unprecedented. [3,4] In this lies the challenge when it comes to polytheonamides. Because it is made in such a tight symbiosis between the sponge *T. swinhoei* and the *Entotheonella* bacterium, it is very hard to reproduce on a lab scale if we do not know the exact enzymes in play to modify the peptide to the extent that it is.[5,6] Because a lot of the amino acids found in a polytheonamide are non-proteinogenic and most antimicrobial peptides are non-ribosomally synthesized (with a few exceptions) it was long believed that polytheonamides were also non-ribosomally synthesized as the extend of modification is uncommon for ribosomally synthesized peptides..[4] The genes encoding for polytheonamides A and B, however, have been found in the genome of *T swinhoei* and proof was provided that they were, in fact, ribosomally synthesized.[3]

The questions answered in this thesis are: What do we, to this day, know about the different modifications found in anti-microbial peptides, about their structures and origin? And how can we use this knowledge to develop new antimicrobials?

DEFINITION OF RIPPS

Ribosomally synthesized and **P**ost-translationally modified **P**olypeptide**s** (RiPPs) are heavily modified peptides which are found in all the branches of the tree of life and fulfilling a lot of different tasks. The extent of their post translational modification allows them to fulfil roles not attainable by classic ribosomally synthesized peptides. Their biosynthetic gene cluster almost always has the same footprint, consisting of a signal, leader, core, and recognition sequence (figure 1). [7]

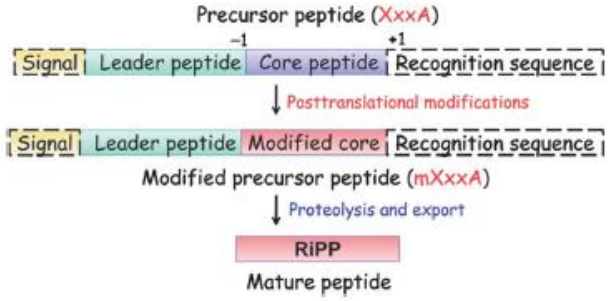


Figure 1: general structure of RiPPs genecluster | Arnison et. al 2013

A promising antimicrobial class of RiPPs are called proteusins. Proteusins are modified ribosomal peptides, but not every modified peptide is instantly also a proteusin, the definitions are somewhat hypothetical but a good starting point. First, the peptide needs to be ribosomally synthesized, this is important because non-ribosomal synthesized peptides (NRPs) are more prone to be modified to great extent and of course post translationally modified, resulting in the name: ribosomally synthesized and post translationally modified peptides (RiPPs). Often, they are encoded by a small biosynthetic gene cluster (BCG), in which both the precursor peptide and all its modifying enzymes are encoded.[7] Also, proteusins have very long leader sequences and large analog sequences between families.[8]

POLYTHEONAMIDES

First, the polytheonamides. Polytheonamides are highly cytotoxic agents with an inhibitory concentration of sometimes picomolar.[3] polytheonamides are the first discovered proteusin group, coming from the sponge *Theonella swinhoei* and its *Entotheonella* eubacteria. [3,5] How then, are polytheonamides synthesized. First, the peptide itself is produces in the sponge *T. swinhoei* and is then altered by the bacteria surrounding it, mostly of the *Entotheonella* types.[6] It then gets dehydrated by PoyF, C-methylated by PoyC, cleaved by PoyH, epiemerized by PoyD and N-methylated by PoyE. PoyH is inhibited by PoyG to reduce overcleaving of the polytheonamide.[9]

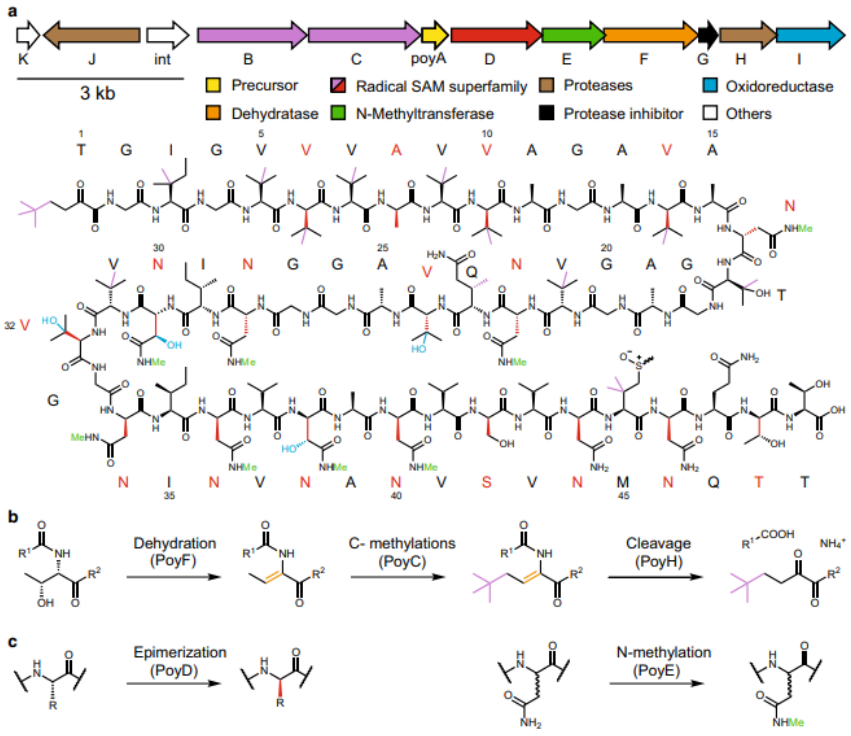


Figure 2: structure, genes and proposed biosynthetic pathway of a single modified amino acid. A: polytheonamides, a difference is made between A and B in residue 44. B: genome for production of the polytheonamide. C: proposed biosynthetic pathway of N-terminal threonine and epimerization carried out by PoyD. | Freeman et. Al 2019

LANDORNAMIDES

Landornamides are RiPPS from the bacteria *Kamptonema sp.* and is a newly defined proteusin. They differ from polytheonamides in, of course, their source organism, but also the extent of modifications undergone by this peptide.

Mainly the arginine to ornithine conversion described by Bösch et. Al, but also the D-amino acids and lanthionine rings present in the landornamide, combined with its potent anti-arenaviral actions make it a potent target for drug research. [10]

For landornamides, as previously stated, the arginase OspR is of huge importance, as it introduces the ornithine into the structure. Also notable is the epimerase OspD, which is responsible for the L to D epimerization of both the valine and isoleucine in the original structure. OspD is a member of the rSAM epimerase family, just as PoyD, the epimerase for polytheonamides. [3,10]

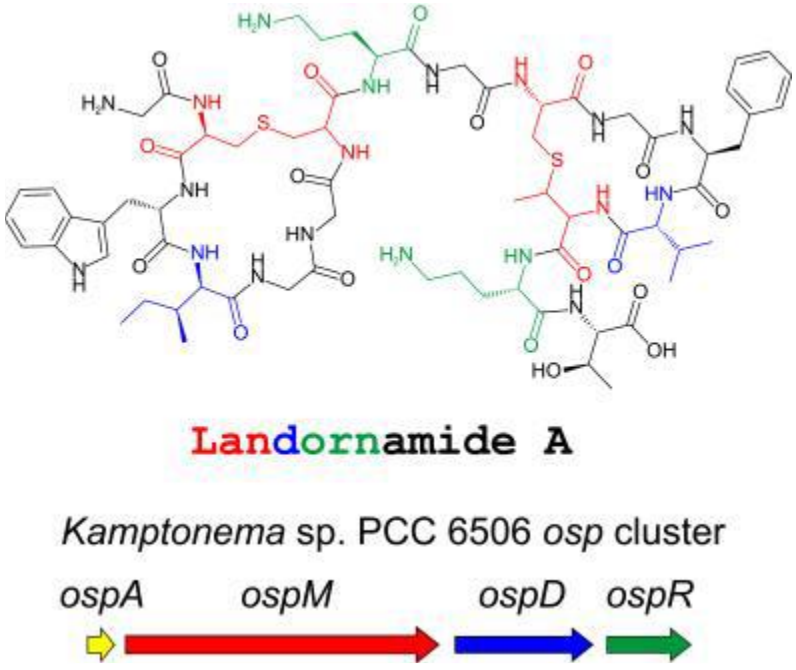


Figure 3: structure of landornamide A | Bösch et al. 2020

OspM is responsible for the formation on the lanthionine rings, although it is not clear whether it both facilitates the dehydration and the thiol-ether formation. Strangely enough, the antiviral capacity increases without the ornithine present (landornamide B does not have ornithines) indicating that ornithines are added to regulate the anti-viral capability. On the other hand, elimination of OspM and the resulting lack of lanthionine formation is completely detrimental for the anti-viral action.[10]

To this date, these are the only two described members of the proteusin group, as most bacteriocins are non-ribosomally synthesized.

STATISTICS OF AMPS

A lot of antimicrobial peptides (AMPs) have been identified, from all different kinds of sources (plants, fish, sponges etc.) although many of them are non ribosomally synthesized and/or not modified, we can learn a lot from their basal structure and key elements. Mainly the most common net charge and size of the peptide is of interest of course.

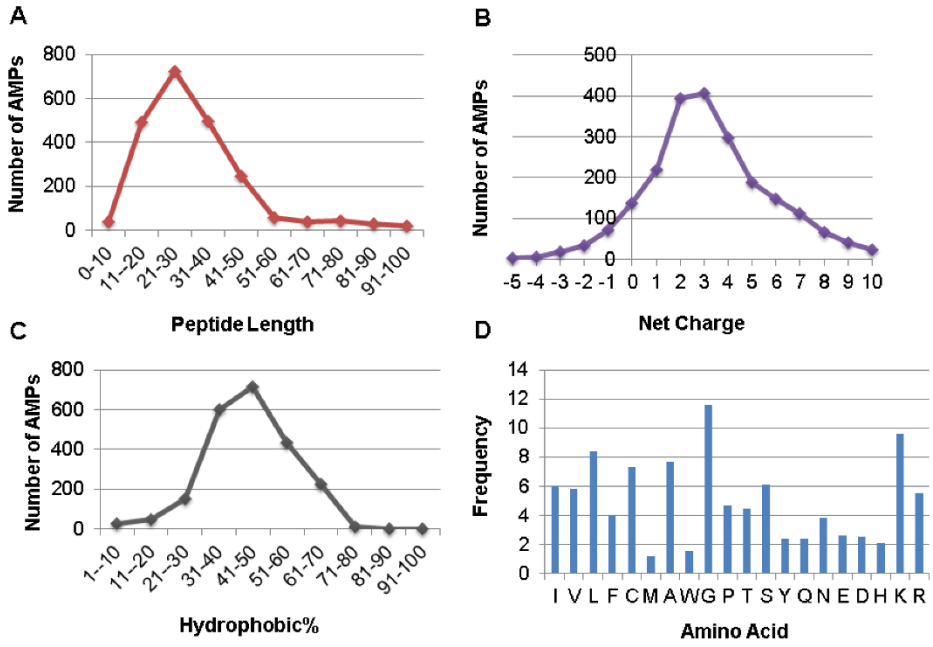


Figure 4: A) number of AM<P with a certain length. B) net charge of AMPs. C)relative amount of hydrophobic amino acids in AMPs. D) relative abundance of every (unmodified) amino acid in AMPs | Wang 2013

http://aps.unmc.edu/AP/main.php

In Figure 4 derived from data of the APD3[11–14] and obtained from the antimicrobial peptide database (APD3)[15], it becomes clear that the average AMP is about 30 amino acids in length, with a net charge of around +2 to +3 and a hydrophobicity count of around 50%. Especially the latter of those numbers, the positive charge and large amount of hydrophobic amino acids, is of importance, as most of the anti-microbial peptides with known function, as seen in figure 5 are membrane active, 263 out of 379 to be precise. For purely anti-bacterial peptides, the mean net charge is 3.54. [11–13]

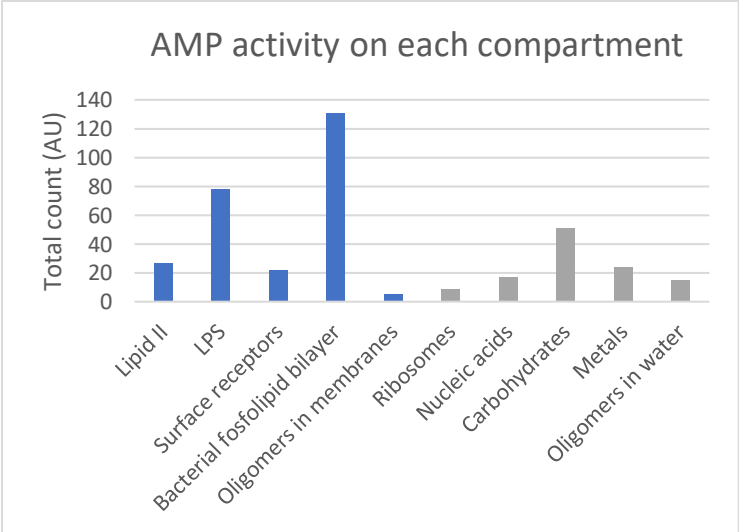


Figure 5: counts on which target the AMPs are active. if defined. Blue = active on membrane; gray = not active on membrane | data from: APD3

DIFFERENT CHEMICAL MODIFICATIONS TO PROTEINS

To properly determine the extent of modifications we can apply to peptides and proteins, we first need to identify all the currently possible/known chemical modifications to peptides. How they can be applied to a protein and what the consequences regarding protein structure and function are. Although the latter is mostly hypothetical.

EPIMERIZATION

Epimerization is a modification which alters the stereochemical conformation of a single amino acid. This action, performed by epimerases, results in L-amino acids instead of the naturally occurring D-amino acids.[16] Epimerization on alternating amino acids promotes formation of a β-helix.[17] In polytheonamides epimerization is performed by PoyD a radical adenosylmethionine peptide epimerase (rSAM).[18]

METHYLATION

Methylation is the addition of a methyl (~CH_3) group to an existing amino acid. It is possible on both N-residues (N-methylation) and C residues (C-methylation). In the case of polytheonamides, C-methylation is carried out by PoyB and PoyC and, sometimes to the extent that a t-butyl group is formed (figure 2). PoyE is also believed to catalyze n-methylation of asparagine.[3]

DEHYDRATION

Dehydration is the enzymatic removal of H_2O from any bond, for example: dehydration of serine resulting in dehydroalanine which can be used in lanthionine ring formation.[19,20] In polytheonamides, PoyF is responsible for this reduction as shown by Freeman et. Al (figure 6).[21]

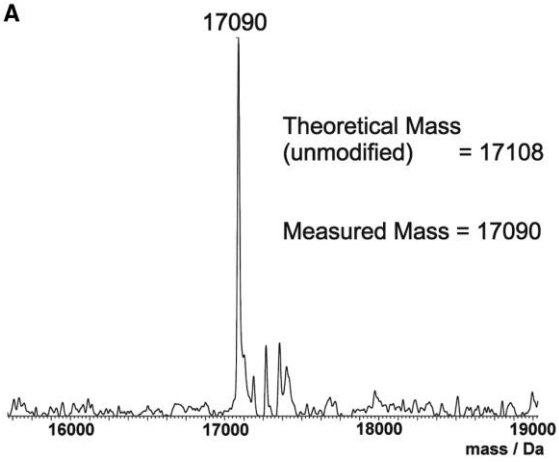


Figure 6: Mass spectrometric graph after dehydration with poyF. 18D missing, indicating loss of water. | Freeman et. Al 2012

CLEAVAGE

To cleave a peptide -or residue- is to remove part of it, in polytheonamides this is enzymatically carried out by PoyH and presumably PoyJ.[3] This can either cleave a protein into multiple peptides or cleave (part of) a residue of an amino acid. This can be a decarboxylase (as shown in figure 1) but is not limited to this.

A subclass of cleavage is arginine to ornithine conversion in ribosomal peptides, as recently discovered by Bösch et. Al.[10] As seen in figure 7 and 8, CN_2H_5 gets enzymatically removed.

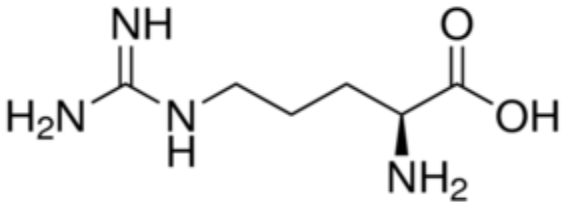


Figure 7: L-arginine | sigmaaldrich.com

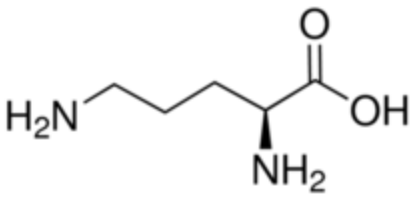


Figure 8: L-ornithine | sigmaaldrich.com

RING FORMATION

Sometimes, when a serine residue gets dehydrated, a dehydroalanine is formed, this can form a covalent bond with a cysteine residue, forming a thiol-ether bond. If this happens on the same strand of peptide, a lanthionine ring is formed. For example, nisin is formed using this. Lanthionine rings are also found in landornamides, where they are carried out by OspM, and many other anti-microbial agents, as it increases target apprehension.[10]

DEPSIPEPTIDE FORMATION

Depsipeptides are peptides where one or more of the peptide bonds is changed to an ester bond. Depsipeptides are commonly found in papuamides and other marine products.[22] This provides chemical stability and reduces the impact of peptide based resistance mechanisms in bacteria.

BETA-AMINO ACID INTRODUCTION

β-amino acids differ from α-amino acids in the position where the amine group is connected. In α-amino acids, both the carboxylic acid and the amino group are bound to the first carbon from the carboxylic acid (the Cα atom). In β-amino acids, the amino group binds to the β-amino acid, which is possible in all amino acids except for glycine, as it does not have a suitable Cβ atom.[23] as shown in figure 8.

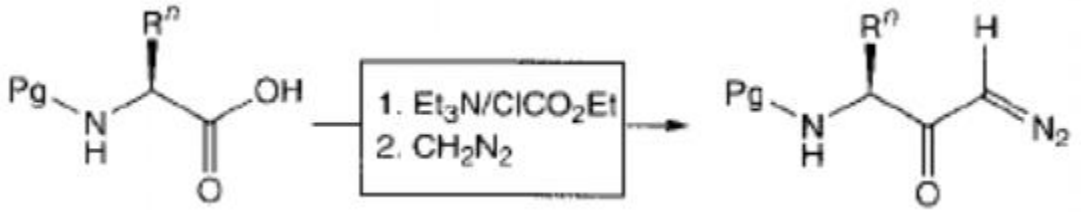


Figure 9: chemical α- to β-amino acid conversion | Dieter Seebach et. Al 1996

OTHER AMPS FOM *T. SWINHOEI.*

There is a broad Scala of similar antimicrobial polypeptides that are found in sponges. The sponge from which polytheonamides are isolated (*T. swinhoei*) is a potent producer of other, albeit non-ribosomally synthesized, AMPs.[24,25]

THEONELLAPEPTOLIDE

There are several theonellapeptolides known, mostly with immunosuppressant function. It is a non-ribosomally synthesized peptide with a number of modifications, including β-amino acids (R-group connected to β-carbon instead of the usual α-carbon), N-methylation and D-amino acids.[26] it is believed to be synthesized by symbiotic bacteria to the sponge.[24,26]

PAPUAMIDES

A papuamide is yet another cytotoxic depsipeptid derived from *Theonella swinhoei*. It is believed to be an anti-viral compound against HIV infected T-cells. It is also riddled with β-amino acids, D-amino acids and N-methylated groups.[25] The term depsipeptid means that at least one of its peptide bonds has been modified into an ester bond. This is most likely enzymatically performed by symbiotic bacteria.[27] Also, papuamides are strongly anti-fungal and their use in medication has been tested to counter infections with infections of *Candida albicans* fungi.[28]

THEOPALAUAMIDE

Theopalauamide is a bicyclic non ribosomally synthesized modified peptide with D-amino acids and a double ring formation coupled with a sugar group. It has been found to be modified by the symbiotic bacteria from *Theonella swinhoei*.[29]

NON-THEONELLA DERIVED MODIFIED AMPS

As previously stated, T. Swinhoei is not the only organism that produces a cytotoxic produced in collaboration with bacteria. It is not, however, the only sponge to do this. There are several other sponges

that, in collaboration with bacteria or other microorganisms, produce anti-microbial and/or anti-eukaryotic peptides and compounds.

Most of these sponges identified reside in the subclass *Heteroscleromorpha* or *Keratosa*. Most notably, the Dysidea sp. These produce a number of highly anti-microbial substances, including but not limited to: sesterterpenes, diterpenes and meteropenoids.[30–32]

POLYDISCAMIDE A-D

Polydiscamide A is an antibacterial metabolite form the sponge D*iscodermia kiiensis* with extensive N-methylation, bromination as well as sidechain methylation as the core modifications. Also, in this peptide, a *tert*-leucine (figure 10) has been identified. A non-natural amino acid. [33] this could, however, be a triple methylated alanine, as triple methylation is not unheard of.[3] Polydiscamides B-D are only slightly different from polydiscamide A.

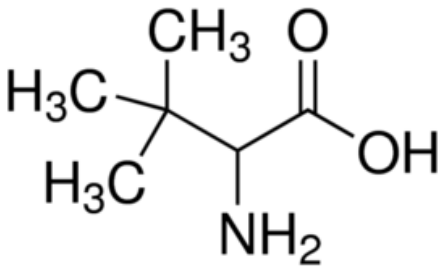


Figure 10: DL-*tert*-leucine | sigmaaldrich.com

JAMAICAMIDE A-D AND KERANAMIDE

Jamaicamides are cytotoxic peptides derived from the marine cyanobacterium *Lyngbya majuscula*. And is a heavily brominated, cyclical peptide. Although it is non ribosomally synthesized, it is a potent cytotoxic agent with numerous modifications which might also modify ribosomally synthesized peptides. Bromination in jamaicamides is performed by an unknown enzyme. However, in *Theonella swinhoei*, brominated peptides called keranamides are synthesized and their bromination is carried out by KrmI. [34]

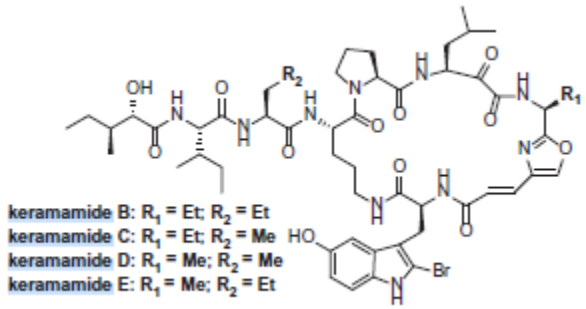


Figure 11: keranamides B-D | Gogineni and Hamann 2018

OTHER POSSIBLE PROTEUSINS

Where the class of proteusins currently consists of 2 subclasses, polytheonamides and landornamides, a lot more subclasses are expected to arise in the coming years. As more genes encoding for possible proteusins have been identified, just not researched.[8] We will discuss a few hypothesized proteusins here.

YAKU'AMIDES A AND B

Yaku'amides A and B are yet again sponge derived cytotoxic agents; this time form the sponge *Ceratopsion sp.* Their name is derived from the location this sponge was first observed, Yakushinsone. Both Yaku'amide A and B present potent cytotoxicity against murine leukemia cells.[35] The reason these compounds are

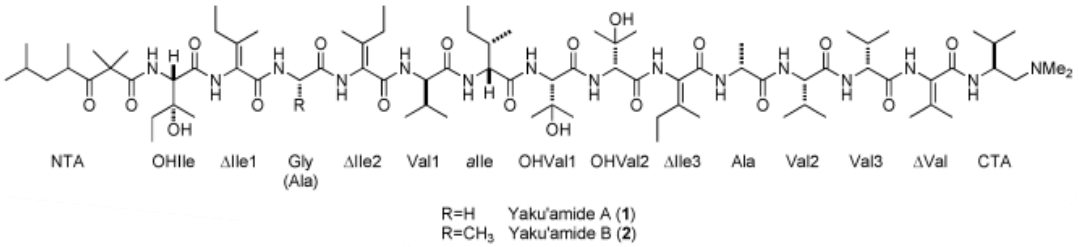
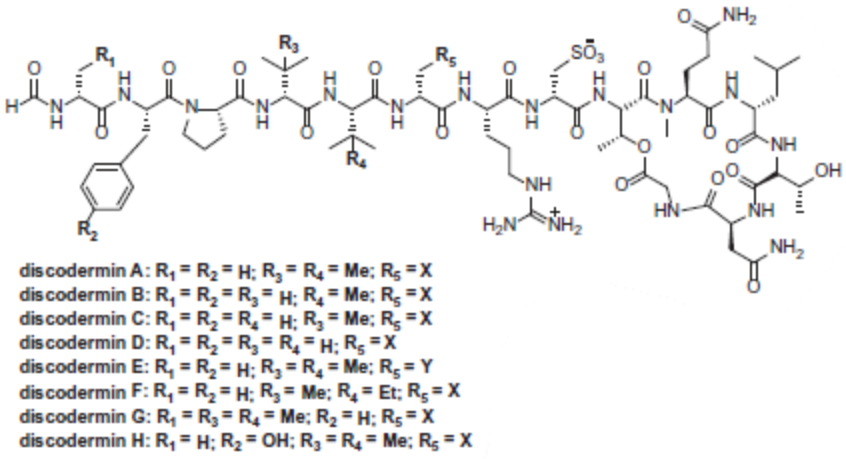


Figure 12: structure of yaku'amides A and B, only different in 3rd position. | Ueoka et al. 2010

believed to be in the proteusin class is the uncommonly long leader sequence on the peptide, as well as its many dehydrated amino acids (see figure 11 There is, however, no evidence suggesting that these are, in fact, ribosomally synthesized. This was also the case for polytheonamides, but they were later determined to be ribosomally synthesized.[3,4] Genome mining of the sponge *Ceratopsion* might reveal these peptides to be ribosomally synthesized as well.

DISCODERMINS

Another hypothesized subclass of proteusins are discodermins, these are derived from the same sponge as the previously discussed polydiscamide *Discodermia kiiensis.* This compound however is not also not witnessed to eb ribosomally synthesized, hence the lack of the proteusin label, but has the same characteristics of the long leader sequence and numerous modifications, such as D-amino acids and *tert*-leucines.[36]



discodermin A: $R_1 = R_2 = H$; $R_3 = R_4 = Me$; $R_5 = X$
discodermin B: $R_1 = R_2 = R_3 = H$; $R_4 = Me$; $R_5 = X$
discodermin C: $R_1 = R_2 = R_4 = H$; $R_3 = Me$; $R_5 = X$
discodermin D: $R_1 = R_2 = R_3 = R_4 = H$; $R_5 = X$
discodermin E: $R_1 = R_2 = H$; $R_3 = R_4 = Me$; $R_5 = Y$
discodermin F: $R_1 = R_2 = H$; $R_3 = Me$; $R_4 = Et$; $R_5 = X$
discodermin G: $R_1 = R_3 = R_4 = Me$; $R_2 = H$; $R_5 = X$
discodermin H: $R_1 = H$; $R_2 = OH$; $R_3 = R_4 = Me$; $R_5 = X$

Figure 13: Structure of Discodermin A-H | Gogineni and Hamann 2018

DISCUSSION

Here we set out to investigate several heavily modified antimicrobial or otherwise pharmaceutical peptides with a specific focus on the proteusin class of RiPPs to determine possible protein engineer possibilities for novel antimicrobial peptides. The point to be taken home is that most of the antimicrobial peptides have a positive net charge, preferably around +2 to +3, they are around 30-40 amino acids long and the more it gets modified the better it will be as an antimicrobial agent, although for antibacterial AMPs the mean is 3.54. the reason more modification may lead to a more potent AMP can be attributed to both a higher target specificity and a slimmer chance of resistance being developed against non-natural amino acids. Also a few possible new proteusins are described, although a lot of research is needed to prove these to be proteusins.

PROPOSAL FOR FUTURE RESEARCH

There still is a lot of research available in this area of molecular biology. A few research possibilities will be discussed here.

MODIFICATION OF EXISTING PEPTIDES

Can we, for instance, modify existing peptides (with antimicrobial features or not) with some of the modifications discussed in this paper to make them (more potent) anti microbials. Add arginine to ornithine conversion found in landornamides to polytheonamides might increase antimicrobial activity.[10]

Pros	**Cons**
Relatively fast and easy to do	Limited in sense of diversity within a peptide
Good benchmark as to extent of antimicrobial activity (original peptide)	

PRODUCING CHIMERIC PEPTIDES

Another possible is to fuse two known antimicrobial peptides into a new, chimeric peptide. Maybe to enhance peptide expression in *E. coli*[37]. Chimeric peptides might not work, but it is worth investigating.

Pros	**Cons**
Huge repertoire of antimicrobial peptides available	A lot of trial and error is involved

DESIGNING AMPS FROM THE GROUND UP

This is the most labor-intensive method of trying to create a novel anti-microbial peptide is by designing it amino acid by amino acid. As seen from the data of APD3, there is a trend to be witnessed among all anti-microbial peptides, maybe using this a s a template a novel AMP could be created

Pros	**Cons**
Huge diversity possible	Very labor intensive
	Implementing modifications could be tricky

Aside from using microbiology and gene editing, chemical approaches also pose great opportunities. Although in many cases, chemical solvents are still needed.

An example where chemical solvents are not needed, and thus a very good alternative for using bacteria, is click chemistry. Click chemistry is a way to perform reaction which are thermodynamically driven and produce little to no unwanted side products.[38] Click chemistry is already being used in peptide based drug design as a way to introduce modifications.[39] Using this method to introduce specific modifications may reduce the need to use complex enzyme systems.

Pros	**Cons**
Specific modifications without enzymes	Chemical reactions always have side products
Relatively fast	A lot of chemical knowledge required
Proven method in peptide base drug design	

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