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Discovery of antimicrobial peptides in two marine invertebrates, the sea anemone *Urticina eques* and the sea urchin *Echinus esculentus*

Isolation, characterisation, and structure-activity relationship studies

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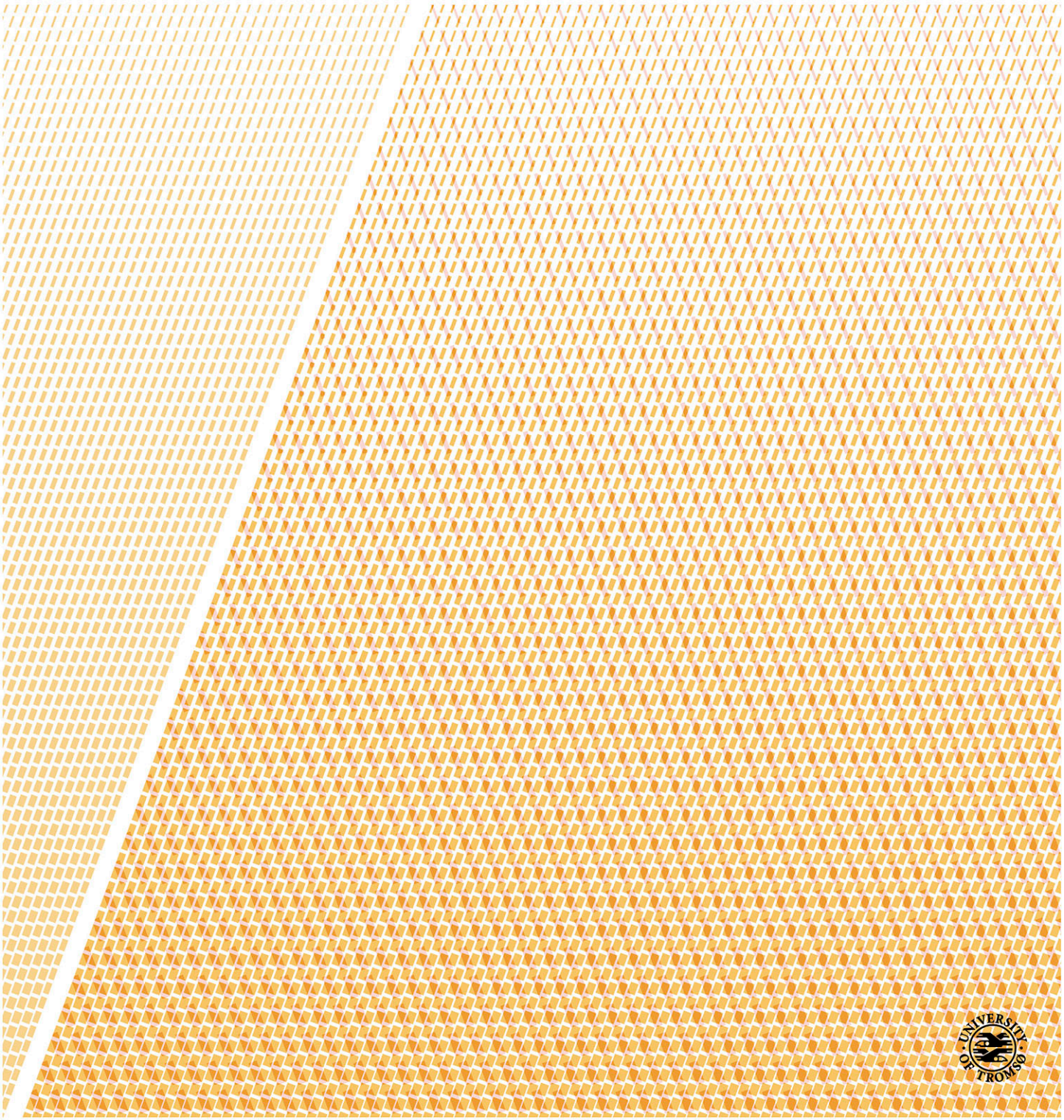

Summary

Bacterial resistance to antibiotics has become a serious global problem. Infections that once were easily cured with antibiotics have now become nearly impossible to treat. Thus, there is a desperate need for new antibacterial drugs. Antimicrobial peptides (AMPs) are a diverse group of compounds. Amongst their unique features are their ability to kill bacteria (often both Gram-positive and Gram-negative) as well as other microorganisms rapidly without toxicity to other cells. AMPs have been suggested as an option for treating bacterial infections where traditional antibiotics have little effect.

The overall aim of the study was to discover and characterise novel AMPs in Echinodermata and Cnidaria, and secondarily to map bioactivities, explore toxicity and perform structure-activity relationship studies.

The most potent AMPs were discovered via bioassay-guided purification in the edible sea urchin *Echinus esculentus*, killing bacteria at low µM-concentrations and fungi at somewhat higher concentrations. The AMPs were homologous to the centrocins and strongylocins of the green sea urchin *Strongylocentrotus droebachiensis* and were named EeCentrocins 1 and 2 (dimeric and with the antimicrobial activity located in the heavy chain), Ee4634 and EeStrongylocin 2. All AMPs were post-translationally modified on all Trp-residues with a bromine in the 6 position. Additional post-translational modifications involved C-terminal amidation on the light chains of EeCentrocins 1 and 2, N-terminal cyclic glutamate on EeCentrocin 2 and disulphide bonds on EeStrongylocin 2. None of the EeCentrocin HCs

displayed toxicity to human erythrocytes. Structure-activity relationship studies on EeCentrocin 1 heavy chain (30 amino acids) led to a truncated 12-mer AMP where Asp8 and Asn12 were replaced with Ala and Lys respectively. The AMP displayed potent antimicrobial activities. Additionally, an Ala-scan was performed identifying the two Trp-residues as crucial for activity towards Gram-negative bacteria, whereas the anti-Gram-positive activity was more dependent on the Trp in position two.

An AMP in *Urticina eques* was named τ-AnmTx Ueq 12-1 (short name Ueg 12-1) and characterised. This AMP was antibacterial exclusively towards the Gram-positive *Corynebacterium glutamicum* at 50 µM concentrations. Ueq 12-1 was bifunctional as it potentiated the TRPA1 ion channel in addition to its antibacterial activity. Analgesic activity was furthermore demonstrated *in vivo* in rats where the peptide reduced licking behaviour on a hot-plate test. The primary sequence of Ueq 12-1 appeared non-homologous to any currently known peptides, but the 3D-structure was somewhat homologous to the defensins.

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My office-mates Glen and Jonathan have been very much appreciated both for on- and off-topic discussion. The same goes for Erlend and Ida. Dr. Kine Ø. Hanssen also provided me with very valuable input when I was closing in on the finale.

Finally and most importantly, I would like to thank my wife and daughter for being very patient when I spent my afternoons working. Without their support, this would not have been possible.

Thank you all so much!

Runar G. Solstad, September 2016

List of publications

The foundation for this thesis is three papers, one of which is published, one is submitted for review and one is in the final stages of revision prior to submission.

Paper I

Runar Gjerp Solstad, Chun Li, Johan Isaksson, Jostein Johansen, Johan Svenson, Klara Stensvåg, Tor Haug. (2016).

Novel antimicrobial peptides EeCentrocins 1, 2 and EeStrongylocin 2 from the edible sea urchin *Echinus esculentus* have 6-Br-Trp post-translational modifications. Plos One **11** (3) e0151820.

Paper II

Runar Gjerp Solstad, Cecilie Johansen, Klara Stensvåg, Morten Bøhmer Strøm, Tor Haug

Structure-activity relationship studies of shortened analogues of the antimicrobial peptide EeCentrocin 1 from the sea urchin *Echinus esculentus*. (Manuscript).

Paper III

Yulia A Logashina, Runar Gjerp Solstad, Konstantin S Mineev, Yuliya V Korolkova, Irina V Mosharova, Igor A Dyachenko, Arkadii N Murashev, Victor A. Palikov, Yulia A. Palikova, Alexander S Arseniev, Sergey A Kozlov, Klara Stensvåg, Tor Haug,Yaroslav A. Andreev

New disulfide-stabilized fold provides sea anemone peptide to exhibit both antimicrobial and TRPA1 potentiating properties. (Submitted for review to the journal Structure).

Table of contents

Abbreviations

The abbreviations used in this thesis in order of appearance:

AMP – antimicrobial peptide

HDP – host defence peptide

APD – antimicrobial peptide database

SDS – sodium dodecyl sulfate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

POPC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine

PTM – post-translational modification

PHM – peptidylglycine α-hydroxylating monooxygenase

PAL – peptidyl-α-hydroxyglycine α-amidating lyase

HC – heavy chain

pI – isoelectric point

LC – light chain

1 Introduction

1.1 The problem

An antibiotic is a compound often produced by a microorganism, which is capable of killing or inhibiting the growth of bacteria. Antibiotics have been used as drugs since Alexander Fleming first discovered penicillin in the 1940s. It was because of antibiotics that once lethal infections came to be considered relatively harmless. A wide range of infections with potentially fatal outcomes, such as tuberculosis and pneumonia, were suddenly curable with relative ease (1).

However, bacteria tend to develop resistance towards compounds to which they are recurrently exposed. Therefore, as novel antibiotics has emerged, so has bacterial strains able to withstand their effects. This is becoming a gradually severe problem. According to the WHO global surveillance report, a comprehensive report on global antibiotic-resistant bacterial infections, the spread is critical and infections that were previously harmless can once again become lethal (2). Additionally, it is estimated that by the year 2050, 10 million will be at risk from drug-resistant infections unless the current prognosis is improved (3).

Currently, the most commonly used antibiotics originate from soil microorganisms, with penicillin being the first discovered antibiotic in this group. This propagated the discovery of many other antibiotics only to be swiftly followed by resistant bacterial strains (Fig 1). Although bacterial resistance is considered an ancient phenomenon (4), the dependence and usage of antibiotics raises the pressure on bacteria to adopt resistance (5).

Adding to this problem is the fact that antibiotic discovery and development has severely diminished over the last years as pharmaceutical companies to a large extent have discontinued their development (6). Thus, the need for novel drugs to aid in fighting bacterial infections is definite.

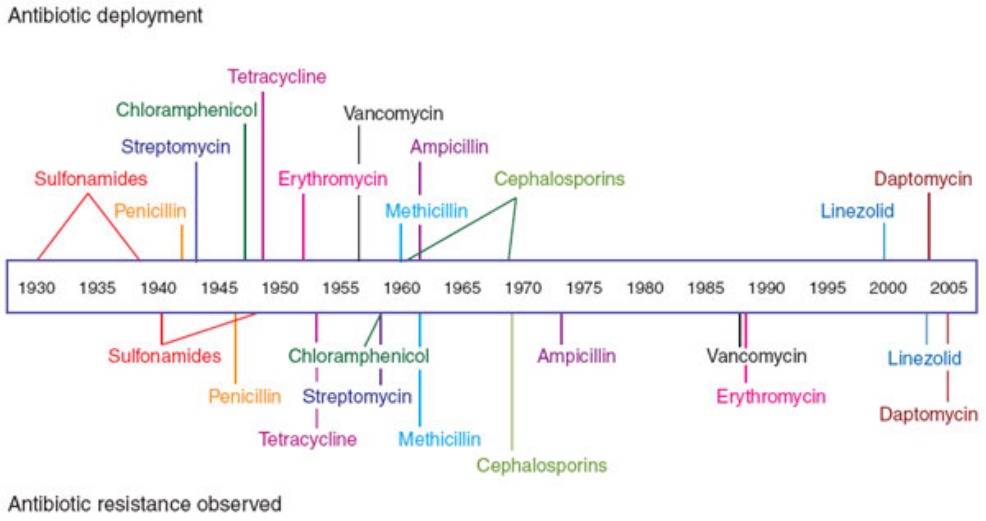

Fig 1: Antibiotic deployment (above timeline) and observed resistance to the antibiotic (below timeline) between 1930 and 2005. The figure is adopted from Clatworthy et al. (7).

1.2 Can antimicrobial peptides be the solution?

Antimicrobial peptides (AMPs), which are also referred to as host defence peptides (HDPs) when mentioned in the context of an immune response (8), are natural products and a uniform defence feature in all categories of animals, plants, fungi and bacteria. This suggests that AMPs are an ancient response mechanism (9). Reports suggest that AMPs are less prone than other antibacterial compounds to induce resistance in bacteria (10), and they frequently have a broad-spectrum antimicrobial activity (11). These are both attractive features in a drug and several AMPs are in clinical trials, indicating

their potential as alternatives to traditional antibiotics (12-15). Table 1 describes the AMPs in clinical and preclinical trials in 2013.

Table 1: Antimicrobial peptides in clinical trials. The table is modified from Fox (15).

Product	Indication	Phase
Magainin/pexiganan acetate	Diabetic foot ulcers	3
Omiganan	Rosacea	2
OP-145	Chronic bacterial middle-ear infection	2
Novexatin	Fungal infections of the toenail	1/2
Lytixar (LTX-109)	Nasally colonized MRSA	1/2
NVB302	*Clostridium difficile*	1
MU1140	Gram-positive bacteria (MRSA, *C. difficile*)	Preclinical
Arenicin	Multiresistant Gram-positive bacteria	Preclinical
Avidocin and purocin	Narrow spectrum antibiotic for human health and food safety	Preclinical
IMX924	Gram-negative and Gram-positive bacteria (improves survival and reduces tissue damage)	Preclinical

1.2.1 Structural properties of AMPs

1.2.1.1 General features

Although AMPs are a structurally diverse group, some generalisations based on their primary structures can be made. The sequences are often short, ranging most commonly from ~20-50 amino acids, but with both larger and smaller representatives. The hydrophobic content is considerable; almost 90% of the peptides in the antimicrobial peptide database (APD (16-18)) have

hydrophobic content over 30%. The final common generalisation is the net positive charge (~87% of the AMPs in the APD are positively charged) as most AMPs contain basic residues. The charge commonly ranges from +1 - +6.

1.2.1.2 Classes

Categories that are based on structural similarities among AMPs have been created. The three most common categories involve α-helical, β-sheet and extended peptides (Fig 2). An AMP will normally align to one of these categories although others such as cyclic, looped or combinations of the mentioned categories also exist (19,20).

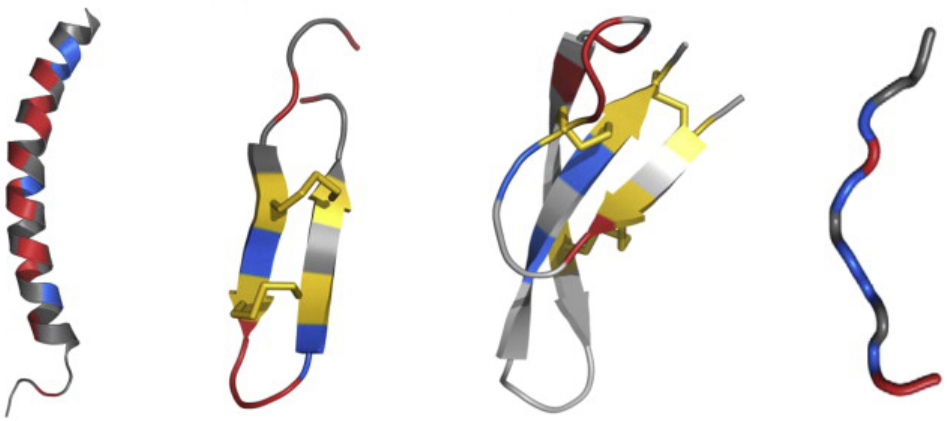

Fig 2: Representative AMPs of the three structural categories (from left to right) α-helical – LL-37 in Sodium dodecyl sulfate (SDS) micelles, β-hairpin/β-sheet – human α-defensin in aqueous HEPES buffer, porcine protegrin in POPC lipid vesicles, and the extended structure of bovine indolicidin in SDS micelles. The figure is edited from Takahashi et al. (20).

The α-helical peptides, e.g. magainins and LL-37 (20,21), commonly form an α-helix with two faces – a hydrophobic face that readily interacts with the lipid-core of the phospholipids and a hydrophilic face that readily interacts with the lipid head groups. Most α-helical peptides are disordered in aqueous solution and only form ordered helical structures when they interact with lipid bilayers (20). Some studies have even indicated that the formation of an α-helix preceding the membrane interaction, i.e. high helix propensity, can increase the AMP cytotoxicity (22,23). Common features for peptides in this group are also helix-stabilising residues like Ala, Leu and Lys (20). Many are not strictly α-helical throughout the entire peptide as they contain both terminally unstructured regions and internal hinges as can be provided by a Gly or a Pro near the centre. This group of AMPs is generally membrane active (19). The α-helical AMPs is the most common structural group (24).

The second structural group to be considered is the β-sheet AMPs. These AMPs will normally contain a number of Cys-residues and construct disulphide bonds to create the β-sheet structure (19). Defensins make up a large portion of the β-sheet AMPs. They are commonly constructed by 2-3 antiparallel β-sheets that are stabilised by intramolecular disulphide bonds. As with the α-helical AMPs, this group is commonly membrane active. AMPs with 2-4 Cys-residues commonly form β–hairpin structures, e.g. protegrins and human α-defensin (20).

The extended AMPs are rich in certain amino acids, such as Trp, Arg, Pro, Gly or His. They can adopt an array of different structures and are frequently not membrane-active (20). Tritrpticin and indolicidin fit in with the extended AMPs (25,26).

1.2.1.3 Post-translational modifications

Peptides and proteins are translated at the ribosome where a precursor is encoded by a structural gene. The precursor commonly contains an N-terminal signal sequence that is important for peptide-identification by enzymes performing post-translational modifications (PTMs) and for peptide relocation out of the cell or into granular vesicles (intercellular organelles) (27). The signal sequence is attached to what will be the mature peptide (propeptides that are not part of the signal sequence or the mature peptide is also common) and as the precursor binds to the modifying enzymes, the PTMs are formed. The signal sequence and propeptides are usually removed proteolytically at a late stage, leaving only the mature peptide (27). The expression of AMPs can be both constitutive or triggered by pathogen invasion (28).

Ranges of different PTMs are common in AMPs. The PTMs contribute in diversifying the structure of peptides and may enable them in recognising cellular targets such as cell walls, membranes, proteins and nucleic acids (29).

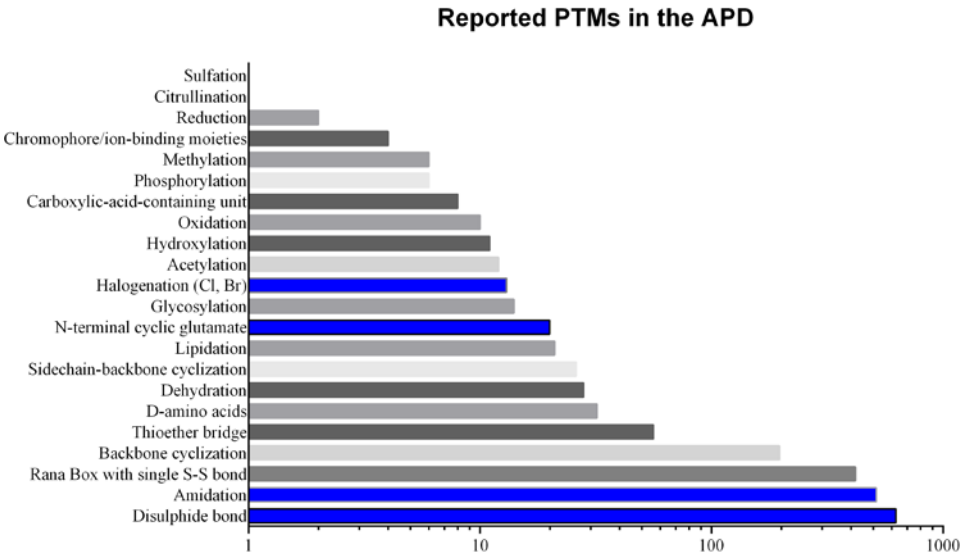

Fig 3: An overview of the different reported PTMs in the APD with their occurrence on a logarithmic scale. The most common PTMs are at the bottom and the least common at the top. Bars coloured blue indicate PTMs encountered in this thesis. The figure was constructed in September 2016 based on currently available AMPs in the database.

The PTMs encountered in this thesis are disulphide bonds, C-terminal amidation, N-terminal pyroglutamic acid formation and bromine halogenation (coloured blue in Fig 3). The first two are very common among AMPs while the latter two are not. Disulphide bonds are the formation of a covalent bond between the sulphur atoms of the thiol groups of two cysteines (30). The disulphide bonds can be involved in forming conformational rigidity, folding and stability in a peptide or protein (31). C-terminal amidation is a very common PTM, blocking the C-terminal of a peptide by amidating the free carboxylic acid and simultaneously reducing negative charge. The PTM can often be recognised by a C-terminal Gly-residue not

expressed in the mature peptide but present in its precursor peptide. Converting glycine to a C-terminal amide is an enzymatic process catalysed by the bifunctional enzyme peptidylglycine α-amidating monooxygenase which encodes the two enzymes peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL). PHM catalyses the stereospecific hydroxylation of the glycine α-carbon whereas PAL generates the α-amidated peptide and glyoxylate (32). N-terminal pyroglutamic acid (also known as 5-oxoproline and cyclic glutamate, abbreviated Glp) is the formation of a 5-membered heterocycle that can occur on an N-terminal glutamate or glutamine. Glp-formation can be both enzymatic and spontaneous and it effectively blocks the N-terminal e.g. from sequencing by Edman degradation as it only sequences α-carbons (33-35). Halogenation is the replacement of a hydrogen with a halogen (bromine is the most frequent in marine animals according to Wagner and König (36)) in place of a hydrogen. This most commonly occurs on the indole-ring on Trp-residues (29,37) and is often enzymatic either by haloperoxidases or halogenases (36).

Other PTMs that are frequently found in AMPs are rana boxes (a cysteine pair connected via a disulphide bond at the C-terminal end of the peptide), backbone cyclisation and sidechain-backbone cyclisation, thioether bridges (-C-S-C-), D-amino acids (the D-enantiomers of amino acids) and dehydration (a C=C double bond, usually between the Cα and Cβ (38)). Proteome-wide analyses on the occurrence of PTMs as recorded in the manually annotated Swiss-Prot database showed that acetylation, N-linked glycosylation and amidation were the most common PTMs whereas bromination was the least common PTM (37). Among AMPs (as dictated by the APD) amidation is very

common whereas glycosylation, acetylation and halogenation occurs less frequently and are all distributed somewhat equally (Fig 3).

1.2.2 Mechanisms of action of AMPs

1.2.2.1 Membrane activity

The first step in bacterial killing by AMPs is the attraction between peptide and the bacteria. This is commonly an electrostatic interaction due to opposite charges between the bacterial membrane and the AMP (39). The electrostatic attraction leads to AMP attachment to the bacterial outer membranes of Gram-negative bacteria or the cell wall of Gram-positive bacteria. This only occurs after circumnavigating an array of external factors like capsular polysaccharides (in Gram-negative and Gram-positive bacteria), teichoic and lipoteichoic acids (in Gram-positive bacteria). At low peptide/lipid ratios, this stage of attachment involves the adsorption and embedding into and parallel with the bacterial surface, spanning the bacteria in an inactive state, commonly known as the surface (S) state (39). When the peptide/lipid ratios increases, the arrangement of the peptides shifts from parallel to more perpendicular to the bacterial surface, which initiates the pore-forming I state. At high peptide/lipid ratios AMPs insert into the bacterial bilayer forming pores that span the bilayer. Three mechanisms for membrane permeabilisation is commonly proposed:

In the barrel-stave model, peptides form barrel-like pores in the membrane with a central cavity exposing the internal space of the bacteria (Fig 4A). Alamethicin is an example of an AMP making a barrel-stave pore (40,41).

In the carpet model, peptides are electrostatically attracted to the anionic head groups of the phospholipids at multiple sites (covering the bacteria like a carpet) and after a threshold concentration is reached, the bilayer is breached and phsopholipids are compartmentalised in micelles (Fig 4B). This opens up the bacterial wall in a detergent-like manner, i.e. the content is released unselectively (42,43). Ovispirin and BP-100 are proposed to act via the carpet model (44,45).

In the toroidal pore model, a pore lined with alternating lipid head groups and the hydrophilic regions of the peptide is formed. As the peptide concentration elevates, the phospholipids make a bend exposing only the phospholipid head groups in the pore (Fig 4C). Magainins and melittins are examples of AMPs exerting their activity via the toroidal pore mechanism (46-48). An addition to this mechanism is the disordered toroidal pore model in which only one or two peptides are in the centre of the water permeable pore (49).

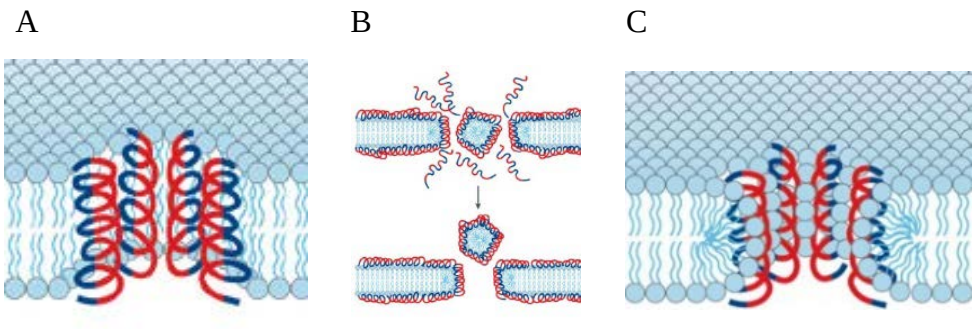

Fig 4: The three primary mechanisms for cell wall disruption induced by membrane active AMPs. barrel-stave (A), carpet (B) and toroidal pore (C). The figure is modified from Brogden (39).

In recent years, the nature of the three membrane permeabilisation mechanisms has been subject to some debate and uncertainty regarding their validity (8,49). However, the evaluation of the three mechanisms is not in the scope of this thesis and the debate is only mentioned here.

1.2.2.2 Intracellular activity

Some AMPs are capable of killing bacteria without permeabilising or disrupting the membrane and are shown to interact with intracellular targets (50,51). DNA or protein synthesis inhibition are two possible mechanisms for intracellular peptide action. PR-39, isolated from pig intestine, acts like a proteolytic agent and inhibits protein and DNA synthesis (52). A similar mechanism is observed for indolicidin which targets DNA synthesis (53). Protease inhibition is another common mechanism. This is displayed by the salivary AMP histatin 5 which inhibits a trypsin-like protease from *Bacteroides gingivalis*, stopping periodontal tissue destruction (54). Another example is eNAP-2 which displays selective microbial serine protease inactivation (55).

1.3 Marine bioprospecting

Extracts and natural products of plants, invertebrates, vertebrates, microbes and fungi have been used as drugs or remedies for a long time (56-58). Recently a 1,000-year-old remedy, likely used to treat sty in the 10th century, was found to have antistaphylococcal activity (59). These extracts and natural products contain antimicrobial compounds used to kill infecting bacteria. Bioprospecting, the process of discovering these compounds, was for a long time limited to terrestrial plants and animals. Marine bioprospecting arrived

later in comparison due to its more challenging availability (60,61). However, novel compounds from marine sources with various bioactivities and novel chemical scaffolds have been increasingly reported in the last years (62-74). This suggests that the marine habitat is also a likely candidate to discover novel AMPs.

1.3.1 AMPs in Echinoderms and cnidarians

Echinodermata and Cnidaria are two predominantly marine phyla with several species where the bioactivity is uncharacterised. As many of the species in these phyla are benthic and either sessile or slow-moving, the hypothesis is that they will have evolved chemical defences in order to thrive where they live and potentially produce AMPs.

Echinodermata (sea lilies, feather stars, sea stars, brittle stars, sea urchins and sea cucumbers) is a marine phylum consisting of animals with pentameric symmetry. In addition to the pentameric body plan, echinoderms have a coelomic water vascular system, which supports both locomotion and internal transport. The endoskeleton is made from calcite elements. The animals contain no joints nor head, and nearly all members are benthic (75,76).

Cnidaria (corals, sea anemones, jellyfish and hydroids) is a diverse phylum living predominantly in the marine environment, but with a few representatives in freshwater. Morphologically, they are the simplest extant metazoans, lacking a circulatory system and having a single opening acting as both mouth and anus (77). The defining trait of cnidarians is their ability to discharge hook-like cells called nematocysts, which are used for different purposes such as predation, adhesion and defence. The nematocyst discharge

contains a cocktail of bioactive peptides, proteins, enzymes and cytolysins (78). The phylum can be divided into the class Anthozoa (sea anemones, corals and sea pens) which live as sessile polyps and the subphylum Medusozoa (jellyfish, sea wasps and *Hydra*) of which many are capable of forming free-swimming medusa as well as polyps. Both groups display external radial symmetry although internally they can be asymmetrical (77).

A wide range of AMPs and other proteinaceous compounds has been reported from both echinoderm and cnidarian sources. Comprehensive lists of AMPs reported from the two phyla Echinodermata and Cnidaria are shown in tables 2 and 3. The task of making such a list can be challenging, as it is not always evident to what extent an AMP is characterised and to what extent the authors can be certain of the peptidic nature if a sequence is not obtained. In the lists, only indicated MWs ≤10 kDa or AMPs ≤100 amino acids are included.

Table 2: AMPs characterised from Echinodermata. Amino acids are abbreviated aa.

Species	AMP	Size	Reference	Organ
Class Asteroidea				
Asterias rubens	Fragments of histone H2A, actin and filamin A	1.8-4.7 kDa	(79,80)	Coelom
Class Echinoidea				
Strongylocentrotus droebachiensis	Strongylocin	48-52 aa	(81)	Coelom
	Centrocin	42 aa	(82)	Coelom
S. purpuratus	SpStrongylocin	48-52 aa	(83)	Recombinant
Paracentrotus lividus	β-thymosin fragments	11-20 aa	(84)	Coelom
	Paracentrin-1	11 aa	(85)	Coelom
Class Holothuroidea				
Cucumaria echinata	P332 (lectin-fragment)	20 aa	(86,87)	homogenate
C. frondosa	unsequenced	~6.0 kDa	(88)	Coelom
Apostichopus (*Stichopus*) *japonicus*	SjLys-C (part of lysozyme)	76 aa	(89)	Intestine/ recombinant
	A3	~6.5 kDa	(90)	Enteron
Holothuria tubulosa	Holothuroidin 1 and 2	12-14	(91)	Coelom

In extracts from coelomocytes of *A. rubens*, a number of partial sequences belonging to histone H2A, actin and filamin A was found to be antibacterial (79,80). The sea urchin *S. droebachiensis* produces two types of characterised AMPs, the centrocins and strongylocins, which were found to be antimicrobial at µM-concentrations towards Gram-positive and Gram-

negative bacteria. Additionally, the heavy chain of centrocin 1 displayed antifungal and antiyeast activities. Homologous sequences in *S. purpuratus* were also discovered, with the recombinant SpStrongylocin displaying antimicrobial activities at µM-concentrations towards Gram-positive and Gram-negative bacteria (83). The sea urchin *P. lividus* contain antimicrobial activities expected to come from any of three fragment-sequences of β-thymosin (84) of which one, the synthetic paracentrin-1, has displayed antimicrobial activities towards multiple *Staphylococcus* strains and one strain of *Pseudomonas aeruginosa* at 6.2-12.5 mg/ml. The AMP P332 of *C. echinata* is a 20 aa fragment of the CEL-III haemolytic lectin (87) with potent antibacterial activity towards Gram-positive bacteria ranging from 1-10 µM (86). Beauregard et al. (88) detected antimicrobial activity towards Gram-positive and Gram-negative bacteria in a coelomocyte extract from the orange-footed sea cucumber *C. frondosa*. The antimicrobial activity was observed in fractions containing ~6.0 kDa compounds, but also at lower MWs. Two individual studies have found antimicrobial activity of *A. japonicus* (previously *S. japonicus*). Firstly, a recombinant 76 amino acid peptide derived from the intestinal lysozyme displayed antimicrobial activity towards both Gram-positive and Gram-negative strains (89) and secondly, a purified fraction of ~6 kDa from the enteron displayed antimicrobial and antitumor activities (90). Two AMPs are isolated from *H. tubulosa*, namely holothuroidin 1 and 2. They are antimicrobial towards Gram-positive and Gram-negative bacteria in concentrations of 12.5 mg/ml (91).

Table 3: AMPs characterised from Cnidaria. Amino acids are abbreviated aa.

species	AMP	Size	Reference	Organ
Class Anthozoa				
Anemonia sulcata	(Phe-18)-BDS-I, (Leu-18)-BDS-I	43 aa	(92,93)	
	Neurotoxin 2 (=ATX-II)	47 aa	(94-96)	Tentacle
Phyllogorgia dilatata	*Pd*-AMP1 (partial characterisation)	29 aa	(97)	Homogenate
Pocillopora damicornis	Damicornin	40 aa	(98)	Ectoderm
Subphylum Medusozoa				
Hydra magnipapillata	Hydramacin-1	60 aa	(99)	
	c-arminin 1a (*in situ* hybridisation)	31 aa	(100)	Endoderm
	Kazal2	45 aa	(101)	Homogenized polyps
Aurelia aurita	Aurelin	40 aa	(102)	Mesoglea

Fewer AMPs have been isolated from cnidarian sources. However, the phylum has been bioprospected extensively primarily owing to the venoms demonstrated in its nematocysts displaying bioactivities towards various ion-channels. The two AMPs, (Phe-18)-BDS-I and (Leu-18)-BDS-I with antiviral properties, were sequenced from the sea anemone *A. sulcata* and later structurally elucidated by NMR spectroscopy (92,93). The same species produces a peptide toxin named ATX-II (95,96) that in recent years has given indications of also being antimicrobial in an experiment where extracts of the sea anemone displayed antimicrobial activity towards *Micrococcus lysodeikticus* (94). Alves de Lima et al. (97) presented antimicrobial activity

towards *S. aureus* in a fraction of *P. dilatata* containing a peptide, which was partially characterised and named *pd*-AMP1. The scleractinian coral *P. damicornis* was shown to produce an AMP of 40 amino acids with antimicrobial activities towards Gram-positive and Gram-negative bacteria in concentrations ranging from 1.25-20 µM and towards *Fusarium oxysporum* at 1.25 µM (98). Three AMPs are presented here from *H. magnipapillata*: hydramacin-1 displayed antimicrobial activity at µM-concentrations (0.2-14.3 µM) towards several strains of Gram-positive and Gram-negative bacteria (99), the AMP c-arminin 1a (derived from the C-terminal of arminin 1a) was shown to be antimicrobial at low µM-concentrations ranging from 0.1-1.6 µM towards an array of different multidrug resistant human pathogens (100). The third AMP, kazal2, a serine protease inhibitor, was antistaphylococcal at 0.7-0.8 µM. Auerlin is an AMP isolated from *A. aurita* with antimicrobial activity towards *Listeria monocytogenes* at 22.64 µg/ml and towards *Escherichia coli* at 7.66 µg/ml (102).

1.4 Aim

The primary aim of this study has been to discover novel AMPs in selected marine invertebrates from the phyla Echinodermata and Cnidaria, namely *Echinus esculentus* and *Urticina eques*.

Secondary aims have been to:

- Describe the primary structure of the AMPs discovered.
- Characterise their antimicrobial and haemolytic bioactivity profile.
- Identify the pharmacophore by performing SAR studies on selected AMPs.

2 Summary of papers

Paper I: Novel Antimicrobial Peptides EeCentrocins 1, 2 and EeStrongylocin 2 from the Edible Sea Urchin *Echinus esculentus* have 6-Br-Trp Post-Translational Modifications

The global problem of microbial resistance to antibiotics has resulted in an urgent need to develop new antimicrobial agents. Natural antimicrobial peptides are considered promising candidates for drug development. Echinoderms, which rely on innate immunity factors in the defence against harmful microorganisms, are sources of novel antimicrobial peptides. This study aimed to isolate and characterise antimicrobial peptides from the Edible sea urchin *Echinus esculentus*. Using bioassay-guided purification and cDNA cloning, three antimicrobial peptides were characterised from the haemocytes of the sea urchin; two heterodimeric peptides and a cysteine-rich peptide. The peptides were named EeCentrocin 1, 2, and EeStrongylocin 2, respectively, due to their apparent homology to the published centrocins and strongylocins isolated from the green sea urchin *Strongylocentrotus droebachiensis*. The two centrocin-like peptides EeCentrocin 1 and 2 are intramolecularly connected via a disulphide bond to form a heterodimeric structure, containing a cationic heavy chain of 30 and 32 amino acids and a light chain of 13 amino acids. Additionally, the light chain of EeCentrocin 2 seems to be N-terminally blocked by a pyroglutamic acid residue. The heavy chains of EeCentrocins 1 and 2 were synthesised and shown to be responsible for the antimicrobial activity of the natural peptides. EeStrongylocin 2 contains 6 cysteines engaged in 3 disulphide bonds. A fourth peptide (Ee4635) was also discovered but not fully characterised. Using mass spectrometric and NMR analyses, EeCentrocins 1 and 2, EeStrongylocin 2 and Ee4635 were all shown

to contain post-translationally brominated Trp residues in the 6 position of the indole ring.

Paper II: Structure-activity relationship studies of shortened analogues of the antimicrobial peptide EeCentrocin 1 from the sea urchin *Echinus esculentus*

Increased microbial resistance to commercial antibiotics has led to an extensive search for novel antimicrobial agents to overcome this challenge. Antimicrobial peptides (AMPs) have the ability to kill bacterial pathogens, often with low toxicity to mammalian cells, and have therefore attracted interest as novel antimicrobial lead compounds. EeCentrocin 1 is a potent AMP, originally isolated from the marine sea urchin *Echinus esculentus.* The AMP has a hetero-dimeric structure with the pharmacophore located in its largest monomer (the heavy chain, HC), containing 30 amino acids. The aim of the present study was to locate the pharmacophore within the HC and to perform structure-activity relationship studies with sequence modification of the pharmacophore. The experiments consisted of 1) truncation of the heavy chain, 2) replacement of amino acids unfavourable for *in vitro* antimicrobial activity and 3) alanine scanning of the truncated and modified AMP. The heavy chain was truncated to less than half its initial size retaining most of its original antimicrobial activity. The optimized lead peptide consisted of the 12 N-terminal amino acids from the original AMP sequence with two amino acid replacements and a C-terminal amidation. Results from the alanine scan indicated that the lead peptide contained the optimal sequence for antibacterial activity. The lead peptide was superior in antifungal activity compared to the other peptides with minimal inhibitory concentrations

(MICs) in the low micromolar range. In addition, the peptide displayed minor haemolytic activity.

Paper III: New disulfide-stabilized fold provides sea anemone peptide to exhibit both antimicrobial and TRPA1 potentiating properties

Sea anemones are known producers of biologically active peptides and the sea anemone *Urticina eques* was hypothesized to contain biologically active peptides in the nematocyst cells that are discharged as a defensive feature. The sea anemone was stimulated electrically while exposed to air, which forced it to discharge the nematocyst cells. The discharged exudate was collected, desalted and found to inhibit bacterial growth of *Corynebacterium glutamicum* at 0.08 mg/ml and inhibit the transient receptor potential ankyrin 1 (TRPA1) ion channel at 0.1 mg/ml. Purification by RP-HPLC led to the discovery of a peptide, τ-AnmTx Ueq 12-1 (short name Ueg 12-1), which both inhibited *C. glutamicum* and potentiated the TRPA1 ion channel. Ueq 12-1 was identified as a 45-residue, cysteine-rich peptide of 4.8 kDa. The cDNA was extracted and the protein sequence elucidated, but no homology to known peptides or proteins could be established. The disulphide bridges and secondary structure of the peptide were elucidated via NMR spectroscopy. Ueq 12-1 seems to be packed in a W-shaped structure where the core of the structure is formed by a 3-strand antiparallel β-sheet, a small 2-strand parallel β-sheet and one turn of a 3-10 helix. Additionally, there are three large loops including eight β-turns of various types and finally the structure is stabilized by 5 disulphide bridges (C1-C8, C11-C42, C17-C35, C22-C43 and C29-C44). The 3D-structure of Ueq 12-1 is homologous to that of the defensins.

3 Discussion

The work presented in this thesis includes the isolation and characterisation of five AMPs (EeCentrocins 1 and 2, Ee4635, EeStrongylocin 2 and τ-AnmTx Ueq 12-1) and initial SAR studies (truncation and alanine scan) of EeCentrocin 1. Four AMPs were isolated from the echinoderm *E. esculentus* (paper I and SAR studies in paper II) and one from the cnidarian *U. eques* (paper III).

3.1 Echinoderm AMPs

The echinoderm AMPs are homologous to the previously isolated centrocins and strongylocins of the green sea urchin *S. droebachiensis* (81,82). The isolated AMPs of *E. esculentus* share the feature of bromination in the 6 position of the Trp-residues at or near the N-terminal end. The centrocin homologues were named EeCentrocin 1 and 2, the hypothesised heavy chain (HC) of a third centrocin homologue was named Ee4635 and the strongylocin 2 homologue was named EeStrongylocin 2 (paper I). All the AMPs display potent activity towards both Gram-positive and Gram-negative bacteria.

Of the isolated AMPs, the centrocin family proved ideal for structure-activity relationship (SAR) studies as the HC, uninterrupted by disulphide bridges, was shown to be the antimicrobial section of the peptides. C-terminal truncation and alanine scan analysis were performed on the HC of EeCentrocin 1 (paper II). The truncated analogue HC(1-12)A8K12 (amino acids 1-12 of the HC, Asp8 and Asn12 exchanged with Ala8 and Lys12 and a C-terminal amidation) was the shortest analogue proven to retain potent antimicrobial activity compared to the native HC. The HC analogue was

subsequently chosen as template for an alanine-scan. The alanine-scan identified the individual residues of importance to antibacterial activity. Trp2 was identified as a crucial residue in the antibacterial activity towards both Gram-positive and Gram-negative bacteria whereas substituting Trp3 reduced antibacterial activity towards Gram-negative bacteria only.

3.2 Cnidarian AMP

The studies on the cnidarian AMP (paper III) was a collaborative effort where the peptide τ-AnmTx Ueq 12-1 (short name Ueq 12-1), isolated from defensive mucus secretions, was shown to potentiate the transient receptor potential ankyrin 1 (TRPA1) ion channel in addition to being antibacterial towards a Gram-positive strain. Additionally, the five disulphide bonds from 10 Cys-residues were elucidated via recombinant production and NMR-spectroscopy. Ueq 12-1 and its cDNA sequence are not homologous to any known protein sequences, but its spatial structure resembles that of the defensins.

3.3 Bioactivity

3.3.1 Purified AMPs compared to extracts and homologues

Table 4 compares the antibacterial activity (MIC) of the eluates and the isolated AMPs of *E. esculentus* and *S. droebachiensis* (81-83). For ease of comparing eluates and purified AMPs, all the concentrations are presented as µg/ml instead of µM. The *E. esculentus* 40% SPE eluate's antibacterial activity concentration-range (Paper I, Table 4) is separated by six titre steps from 313 µg/ml towards the least sensitive bacterial strains (*S. aureus*) to 9.8 µg/ml towards the most sensitive (*C. glutamicum*). The concentration-range

of antimicrobial activity of the individual AMPs is smaller, being antibacterial generally from 0.5-3.8 µg/ml (three titre step separation). *C. glutamicum* is the most sensitive bacterial strain, both in assays with eluates and purified AMPs. The shorter activity range of purified AMPs compared with eluates can be explained by the fact that an eluate will contain an array of different compounds, some of which will be inactive, perhaps potentiate or inhibit bioactivities or other inter-compound interactions. In addition, the concentration of any AMP will normally be lower in the eluate. Compared to the centrocins and strongylocins of the green sea urchin *S. droebachiensis*, the AMPs discovered in *E. esculentus* are antimicrobial at lower µg-concentrations.

Table 4: Minimum inhibitory concentration (MIC; µg/ml) of eluates containing AMPs and purified AMPs of *E. esculentus*, *S. droebachiensis* and recombinant AMPs of *S. purpuratus.*

eluate/AMP	*C. glutamicum*	*S. aureus*	*P. aeruginosa*	*E. coli*
40% SPE	9.8	313	39.1	156
EeCentrocin 1	3.8	3.8	3.8	0.5
EeStrongylocin 2	9.2	18.5	9.2	4.6
SdCentrocin 1	5.8	11.2	Nt	5.8
SdCentrocin 2	5.7	22.0	Nt	11.0
SdStrongylocin 1	14.0	14.0	Nt	28.0
SdStrongylocin 2	14.5	14.5	Nt	28.9
SpStrongylocin 1*	42.0	84.0	Nt	42.0
SpStrongylocin 2*	22.8	45.0	Nt	90.0

Nt: Not tested, *: recombinant peptide.

A similar table as Table 4 for the Cnidarian AMP cannot be made as the antimicrobial activity is towards one strain and therefore no concentration-range of individual AMPs exist. However, the crude mucus extract of *U. eques* is antibacterial towards *C. glutamicum* at 80 µg/ml. Compared to Ueq 12-1, which is antibacterial at 240 µg/ml; it is likely that other antibacterial components exist in the crude mucus extract (Paper III, Table 5).

Table 5: Minimum inhibitory concentration (MIC; µg/ml) of the crude mucus extract compared with the AMP Ueq 12-1 of *U. eques*.

Extract/AMP	*C. glutamicum*
Crude mucus extract	80
Ueq 12-1	240

3.3.2 Isoelectric point and net charge

The *E. esculentus* peptides have a few typical traits indicative of AMPs. Namely, an abundance of positively charged and hydrophobic amino acids and the *pI* is around 10 for all peptides. Conversely, Ueq 12-1 has a significantly lower *pI*. The *pI* has been suggested as a superior characteristic of AMPs than net charge is as it correlates better with antimicrobial activity (103,104). The *pI* values (as calculated by the ExPASy server, http://web.expasy.org/compute_pi/) of the EeCentrocin and EeStrongylocin peptides are all very similar: EeCentrocin 1: 10.04, EeCentrocin 1 HC: 11.57, EeCentrocin 2: 10.24, EeCentrocin 2 HC: 10.67, Ee4635 (an expected HC): 11.13 and EeStrongylocin 2: 9.51. However, these calculations do not consider the bromination and folding present in the peptides that may influence the *pI*. The calculated values of Ueq 12-1 is lower at 5.28.

Consistent with the cited literature, the antimicrobial activity is more pronounced among the *E. esculentus* AMPs compared with Ueq 12-1.

3.4 Structures

The structures of the different AMPs have been established to variable degrees. There is evidence suggesting a random structure of the monomer EeCentrocin 1 HC (Paper I) in water, which might also apply to the dimers EeCentrocin 2 and Ee4635. The disulphide bond connectivity of EeStrongylocin 2 (Paper I) that dictates its secondary structure is unknown. However, the five disulphide bonds of Ueq 12-1 (Paper III) has been established by NMR to be Cys1-Cys8, Cys11-Cys42, Cys17-Cys35, Cys22-Cys43 and Cys29-Cys44. The AMP forms a W-shaped structure in aqueous solution with the core formed by a three-strand antiparallel β-sheet (Tyr16-Glu18, His27-Asp28 and Arg41-Cys44).

3.4.1 EeCentrocins and Ee4634

It is commonly accepted that α-helix forming AMPs only adopt the helical structure in a helix-inducing environment such as the surface of a bacteria (39). Reports have even suggested that the propensity to form an α-helix in non-inducing environments (i.e. without a negatively charged surface or similar inducers) can be suitable for AMP-toxicity (22,23).

The synthetic HC of EeCentrocin 1 (Paper I) was readily soluble in water, but proved challenging to solubilise in SDS micelles as it would precipitate upon their addition. This meant that no helix-inducing environment could be formed. In water, the HC had the typical appearance of an unfolded peptide

with poorly separated HN correlations. Calculated order parameters based on the chemical shifts of the peptide were used to predict the secondary structure using TALOS+ (Fig 5).

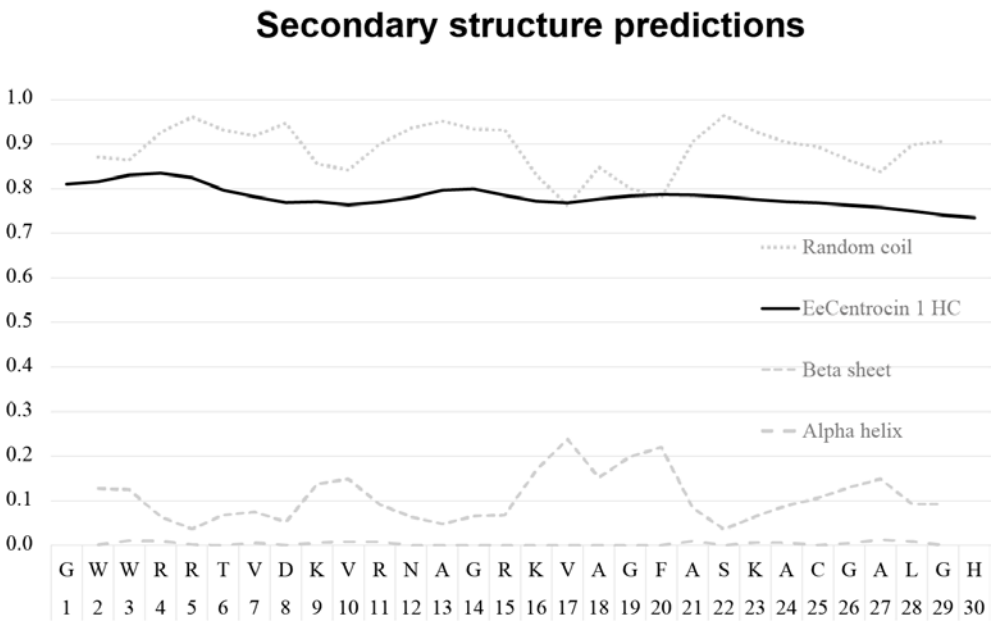

Fig 5: TALOS+ predictions of order parameters for EeCentrocin 1 HC with different secondary structures (grey) and generalised order parameters based on the available backbone chemical shifts of the synthetically produced EeCentrocin 1 HC acquired in H2O:D2O, 9:1 (black). The generalised order parameters are as predicted for a random coil structure.

The secondary structure of EeCentrocin 1 HC has only been established in water, but the helical projections that dictate how a secondary structure α-helix would look like, provide a very convenient amphipathic structure with the positive charges and the hydrophobic phase distinctly separated. It is thus

appealing to hypothesise that the EeCentrocin 1 HC forms an α-helix in helix-inducing environments. Helical projections have been made for the EeCentrocin 2 HC and Ee4635 with similar results (Fig 6, made using the Pepweel software available from: http://www.bioinformatics.nl/cgi-bin/emboss/pepwheel).

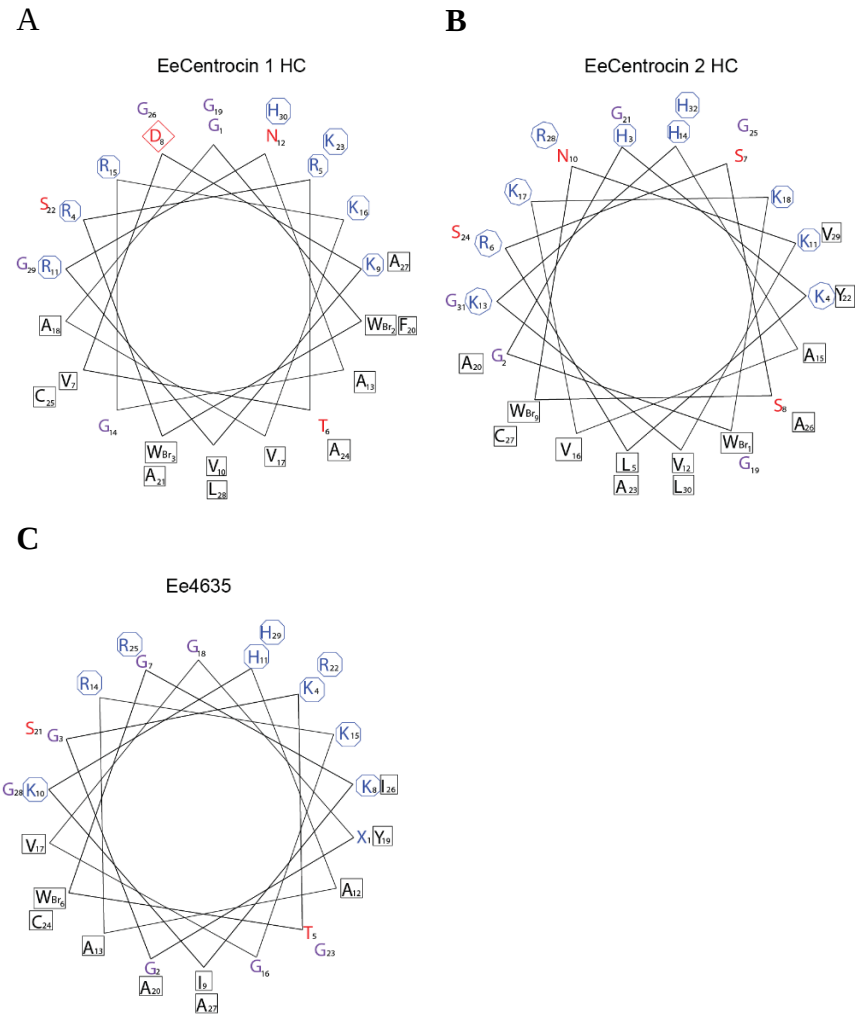

Fig 6: Helical wheel projections of the HCs of EeCentrocin 1 (A), 2 (B), and the expected HC of the partially sequenced Ee4635 (C). All the projections are made with the basic residues on top with octagonal frames in blue colour and the hydrophobic residues at the bottom with square frames in black. The negatively charged Asp (D) is placed in a diamond coloured red.

At the centre or near the centre of all the dimeric peptides (EeCentrocins 1 and 2) and the expected dimeric peptide (Ee4635) presented in Paper I, there

is a Gly-residue. Centred Gly-residues and Pro-residues are known inducers of the helix-hinge-helix motif commonly found in cathelicidins (105-107) but also other AMPs (108). The structure-prediction tool PEP-FOLD3 at the mobyle portal (available from: http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#welcome) predicts all the peptides presented in Fig 7 to have a hinge in this region. The prevalence of hinge-inducing residues points to some significance and indications to the importance in antimicrobial activity have been made as the hinge provides flexibility and might support the AMP span the lipid bilayer (109,110). When truncating EeCentrocin 1 (paper II), antibacterial activity tended to decrease as the peptide became shorter but no conclusive argument regarding the significance of Gly14 can be made as potent antibacterial activity was demonstrated for the substituted 12-mer HC(1-12)A8K12.

Acidic residues, like glutamic acid and aspartic acid, are more commonly found in anionic AMPs (39,111). However, there is an aspartic acid residue in position 8 in the HC of EeCentrocin 1. The nature of this residue is uncertain but it may be involved in a salt bridge with a positively charged residue, making a bend in the peptide, as is demonstrated in the antimicrobial peptide LL-37 (112). When a salt-bridge is formed, the acidic residue is normally $i \pm 3$ or $i \pm 4$ positions away from a basic residue (108). All of these positions except $i + 4$ are Arg-residues in EeCentrocin 1. A salt-bridge may assist in helix- or other secondary structure stabilisation (113-115).

3.4.2 Ueq 12-1

The primary structure of Ueq 12-1 (Paper III) is to a degree atypical of AMPs if one considers the APD to be the benchmark of what is typical appearances

in this respect. The typical primary structures of AMPs are more frequently laden with positively charged and hydrophobic residues whereas this is not the case for Ueq 12-1. The AMP is markedly less potent compared to the centrocins and the strongylocins, which may be connected with the atypical AMP structures. A possible explanation to this phenomenon can be that the primary task to which the peptides are produced by the organism is in fact not to be antimicrobial, but other functions that remain to be elucidated. Ueq 12-1 for example is not only antimicrobial as it displays agonistic activity of the TRPA1 ion-channel as well.

3.4.3 Post-translational modifications

Some post-translational modifications (PTMs) are associated with proteolytic protection of the peptides (116-119). This is stimulated by bacteria evolving new strategies to survive in the presence of AMPs or supress them. Two of the AMPs presented in this thesis – EeStrongylocin 2 (Paper I) and Ueq 12-1 (Paper III) – contain between three and five disulphide bonds. Intramolecular disulphide bonds are important for the peptide structure, but have also been suggested to be part of the protection against proteolytic degradation of the peptides (116). The EeStrongylocin 2, EeCentrocins and Ee4635 all contain brominations on all their Trp-residues in the mature peptides, a PTM which is frequently seen in marine peptides and has been connected with proteolytic protection as well. Shinnar et al. (117) performed a molecular modelling study where overlaps of van der Waals radii were observed for all possible bromotryptophan regioisomers (2-, 4-, 5-, 6-, and 7-Br-Trp) making it a poor fit in the active site of chymotrypsin. A mechanistic explanation for this was provided by Karstad et al. in two research papers (120,121) where synthetic peptides became stable towards both trypsin and chymotrypsin when a bulky

side group was attached to one of the hydrophobic amino acids. This furthered the distance of the amide bonds to the activated Ser-residue, which led to peptides not being cleaved. The proteolytic cleavage of EeCentrocin 1 (Paper I) resulted in only one observable fragment in the mass spectrometer, namely the N-terminal $GW_{Br}W_{Br}R$. This observation could substantiate the bromination as having a proteolytic protective function.

The light chain (LC), by constructing a dimeric peptide, might also provide protection from proteolysis on the C-terminal side of the HC. This has been proposed for other dimeric peptides like the halocidin-derived peptide HG1 of the tunicate *Halocynthia aurantium* and distinctin of the amphibian *Phyllomedusa distincta* (122,123). It has also been suggested that dimeric peptides make larger pores in microbial membranes than monomers (124). The LCs of *E. esculentus* are frequently found to be amidated and in one instance to contain an N-terminal pyroglutamic acid. These modifications are also linked to proteolytic protection (118,119).

3.5 Structure-activity relationship (SAR) studies

SAR studies of EeCentrocin 1 HC (Paper II) showed that, similar to eluate antibacterial activity, *S. aureus* displayed a markedly higher intrinsic resistance towards the AMPs than the other strains of sensitive bacteria. This effect was amplified during truncation. As the SAR studies exclusively truncated the C-terminal of the peptide, one obvious assumption is that this terminal was an important contributor in the antibacterial activity overall and especially towards *S. aureus*. Interestingly, the importance of the N-terminal Gly1 was discovered early in the truncation process with the AMP HC(2-16)A7 being markedly less antimicrobial compared to HC(1-16)A8, whose

difference is only Gly1. A Gly-residue in position one is among very few AMP motifs that Tossi et al. (108) found to be prevalent across a wide range of AMPs. This indicates some importance of the residue.

Gram-positive bacteria such as *S. epidermidis* and *S. aureus* are known to contain defensive strategies towards antimicrobial peptides (125,126). Common strategies are the alteration of membrane teichoic acids and subsequent reduction of the electrostatic interactions between the cationic peptide and the negatively charged membrane (39,127,128) and secretion of proteolytic enzymes (129,130). Additionally *S. aureus* produces staphylokinase, an extracellular AMP-binding compound primarily attacking the human α-defensin (125,131). However, defensive strategies are not exclusively found in Gram-positive bacteria (132).

3.6 Bioinformatics

The interchains (ICs) of the centrocin peptides are conserved through the three species of sea urchins where such data exist, i.e. *S. droebachiensis*, *S. purpuratus* and *E. esculentus* (82,133). From a total sequence length of 24 amino acids, 15 amino acids are identical across all three species with the rest often resembling pysiochemically. Naturally, the rate of identical residues is higher in the individual species: *E. esculentus*: 24/25, *S. droebachiensis*: 21/25 and *S. purpuratus*: 19/25. Combined, the ICs share ~60% of the amino acids. Additionally, the IC sequences on their own will prove homologous to the other centrocins and centrocin-homologues from other organisms using the BLAST search engine. The conserved sequence suggest some evolutionary importance and could perhaps be used as a template when bioprospecting other species of sea urchins or closely related species.

The HC and LC sequences are very diverse in sequence and can generally not reach significant homology results alone. However, the BLAST functionality in the LAMP database (134) seems to operate by slightly different parameters using a smaller dataset (concentrated only on AMPs) and is able to connect the EeCentrocin 1 and 2 HCs with the centrocin peptides of *S. droebachiensis*. Additionally, the peptide Ee4635 is also suggested to be a centrocin homolog in this database. This can be considered contributing – but not conclusive – evidence in the hypothesis that Ee4635 is a centrocin homolog.

The characterised AMP of *U. eques* differ greatly from the ones of *E. esculentus* on the topic of homology as Ueq 12-1 (Paper III) is novel in primary structure and does not yield homologous sequences in BLAST. Some homology to the 3D-structure of defensins and defensin-like peptides has been indicated.

4 Conclusion

Several antimicrobial peptides have been identified and characterised in the course of this project. In *E. esculentus* two centrocin homologues named EeCentrocin 1 and 2 were identified, in addition to an expected centrocin homologue named Ee4634 and a strongylocin homologue named EeStrongylocin 2. In *U. eques* an AMP with no detectable sequence homology named τ-AnmTx Ueq 12-1 was identified. All peptides except Ee4634 have also had their cDNA sequences elucidated.

All the mature AMPs display varying degrees of antibacterial and/or antifungal activities from the low micromolar range to >100 µM. Haemolytic activity also varies, but is commonly low around the MIC-concentrations. One AMP, Ueq 12-1, potentiates the TRPA1 ion channel and produces analgesic and anti-inflammatory effects *in vivo*.

The pharmacophore of EeCentrocin 1 was identified in a SAR study, resulting in a potent AMP of 12 amino acids.

5 References

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