RATIONEL DESIGN OF ALPHA-HELICAL ANTIMICROBIAL PEPTIDES AND PEPTIDOMIMETICS

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Rational Design of Alpha-helical Antimicrobial Peptides and Peptidomimetics

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Paper 1: **Rational Design of alpha-helical antimicrobial peptides – DOs and DON'Ts**.
Lars Erik Uggerhøj, Tanja Juul Poulsen, Jens Kristian Munk, Marlene Fredborg, Teis Esben Sondergaard, Niels Frimodt-Moller, Paul Robert Hansen, Reinhard Wimmer
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Paper 2: **Structural Features of Peptoid-Peptide Hybrids in Lipid-Water Interfaces**.
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This thesis has been submitted for assessment in partial fulfillment of the PhD degree. The thesis is based on the submitted or published scientific papers which are listed above. Parts of the papers are used directly or indirectly in the extended summary of the thesis. As part of the assessment, co-author statements have been made available to the assessment committee and are also available at the Faculty. The thesis is not in its present form acceptable for open publication but only in limited and closed circulation as copyright may not be ensured.

1. Preface

This work has been conducted in the framework of the Danish Centre for Antimicrobial Research and Development (DanCARD, www.dancardproject.dk). The main part of the work has been conducted at the Department of Biotechnology, Chemistry, and Environmental Engineering at Aalborg University from September 2010 to December 2013. Lesser parts of the work have been conducted at the Department of Basic Sciences and Environment and Department of Pharmacology at the University of Copenhagen, Statens Serum Institut, and the Danish Centre for Food and Agriculture.
The academic supervisor was Associate Professor Reinhard Wimmer, affiliated to the Department of Biotechnology, Chemistry and Environmental Engineering at Aalborg University.

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Lars Erik Uggerhøj

2. Summary

For more than three decades, the increasing occurrence of infections caused by multiresistant bacteria has been the driving force for exploration of antimicrobial peptides (AMPs) as an alternative type of antibiotics. A large effort has been made in order to understand the relationship between structure/composition and activity (both antibacterial and cytotoxic) of the AMPs which are believed to interact with the bacterial membranes in an unspecific way (i.e. no target molecule). Literature suggests many structural parameters, which can explain the activity in some studies, but none of these have proved to be generally applicable for a given class of AMPs. This thesis presents our effort and results in obtaining a better understanding of the interaction between cationic α-helical AMPs (and peptidomimetics thereof) and membranes. The research is focused on structural studies of AMPs interacting with primarily DPC micelles as the membrane mimetic. Information regarding this interaction is obtained through standard NMR techniques and the use of paramagnetic relaxation enhancement, which yields information regarding the tilt, rotation, and insertion depth of the investigated peptides upon interaction with the membrane mimetic.

These studies revealed the importance of side chain properties as well as the orientation of these relative to the overall structure and orientation of the peptide. It is shown how the side chains of some residues can bend towards their most favorable environment, while others cannot due to their position in the peptide sequence. It is also shown how alterations in peptide sequence can lead to extension and rotation of the helix.

In a larger study of analogues of the short, cationic, and helical AMP, anoplin, correlations were sought between activity and the structural parameters: Hydrophobicity, hydrophobic moment/amphipathicity, charge, polar angle, rotation, tilt angle, and insertion depth. However, none of these proved useful for explaining the antibacterial activity, hemolytic activity or selectivity for the set of peptides. Correlations were only found when observing the hydrophobicity of the hydrophobic face and polarity of the hydrophilic face separately. An increase the hydrophobicity of the hydrophobic face increases the activity against all membranes: At low hydrophobicity no membrane activity is present, at moderate hydrophobicity decent activity against Gram-negative bacteria can be achieved, at higher hydrophobicity good activity can be achieved against both Gram-positive and Gram-negative bacteria while hemolytic activity becomes significant, and at high hydrophobicity the hemolytic activity becomes dominant which removes selectivity of the AMPs. Regarding the polarity of the hydrophilic face, an increase in polarity will increase the activity against especially Gram-negative bacteria, while it appears that it does not influence selectivity of the AMPs. When utilizing the found correlations to optimize a longer peptide, it was found that the correlations hold true, but that the mean hydrophobicity of the hydrophobic face should be lowered in order to avoid strong hemolytic activity. Based on our findings, the usefulness of the structural parameters used in literature is discussed.

The importance of side chain properties and their orientation was kept in mind during the interpretation of the solved peptidomimetic structures. When inserting a peptoid monomer into the helical AMP, maculatin-G15, an equilibrium between the *cis* and *trans* conformers was observed. This equilibrium was highly shifted, depending on the site of insertion of the hydrophobic peptoid monomer. For two of the analogues, only one conformer was observed and for one of the analogues the conformer was found to be the *trans* conformer. For the two other maculating-G15 analogues, an almost equal distribution between the *cis* and *trans* conformers was found. This led to a hypothesis that the backbone fold of the *trans* conformer might be energetically more favorable, and that the peptoid side chain properties plus site of insertion might be determining for the distribution of the *cis* and *trans* conformers.

3. Resume

Igennem mere end tre artier har den stigende mængde infektioner, der er forårsaget af multiresistente bakterier, været den drivende kraft bag forskning i hvordan man kan anvende antimikrobielle peptider (AMPer) som et alternative til konventionel antibiotika. Inden for den gruppe af peptider der menes at interagere med bakteriemembraner på en uspecifik måde er der blevet gjort en stor indsats for at forstå hvordan peptidernes opbygning og struktur hænger sammen med deres antimikrobielle og hæmolytiske aktivitet. I den henseende er der blever foreslået mange strukturelle parametre i litteraturen som i de givne studier korrelerer med peptidernes aktivitet, men som ikke har vist sig at kunne forklare aktiviteten i andre studier med samme klasse af peptider. I denne tese præsenteres indsatsen i- og resultaterne fra vores arbejde med at opnå en bedre forståelse for interaktionen mellem kationiske α-heliske AMPer (og peptidlignende analoger) og membraner. Vores forskning har fokuseret på strukturelle studier af AMPer der interagerer med miceller dannet af primært DPC. Der er anvendt standard NMR teknikker såvel som paramagnetisk relaksationsforstærkning til at indhente informationer om hældning, rotation og indsættelsesdybde af peptiderne mens de er bundet til micellerne.
Disse studier tydeliggjorde vigtigheden af sidekædernes egenskaber såvel som deres placering i forhold til den overordnede struktur or orientering af peptiderne. De viste hvordan nogle aminosyrers sidekæde kan bøje mod det miljø der giver den mest favorable interaktion, mens andres sidekæde ikke kan på grund af deres placering in peptid sekvensen. Studierne viste også hvordan substitutioner i peptidets sekvens kan forårsage rotation of forlængelse af dets heliks.
I et større studie af analoger til det korte, kationiske og helikale AMP, anoplin, blev der søgt efter korrelationer mellem aktivitet og de strukturelle parametre: Hydrofobicitet, hydrofobe moment, ladning, polære vinkel, rotation, hældning og indsættelsesdybde. Ingen af disse parametre kunne dog forklare den antibakterielle aktivitet, hemolytiske aktivitet eller selektivitet for gruppen af peptider. Der blev kun fundet korrelationer når man enkeltvis betragtede hydrofobiciteten af den hydrofobe side og polariteten af den hydrofile side. Forøges hydrofobiciteten af den hydrofobe side forøges aktiviteten mod alle membraner. Ved lav hydrofobicitet opnås ingen membran aktivitet. Ved moderat hydrofobicitet kan der opnås god aktivitet mod Gram-negative bakterier. Ved højere hydrofobicitet kan der opnås god aktivitet mod både Gram-positive og Gram-negative bakterier men der vil også være betydelig hæmolytisk aktivitet. Ved høj hydrofobicitet vil den hæmolytiske aktivitet blive dominerende og al selektivitet vil forsvinde for AMPerne. Angående polariteten af den hydrofile side vil en forøgelse i polaritet forøge activiteten mod specielt Gram-negative bakterier, imens det ser ud til at selektiviteten ikke bliver påvirket. Ved at anvende disse korrelationer til at optimere aktiviteten af et længere peptid blev det eftervist at korrelationerne er alment gældende, men forsøgene vist også at den gennemsnitlige hydrofobicitet af den hydrofobe side skal reduceres i takt med at længden af AMPet forøges for at undgå stærk hæmolytisk aktivitet. Baseret på disse resultater er brugbarheden af de forskellige strukturelle parametre blever diskuteret.
vigtigheden af sidekædernes egenskaber og placering blev inddraget i fortolkningen af de løste peptidlignende strukturer. Når en peptoid monomer indsættes i det helikale AMP, maculatin-G15, se en ligevægt mellem *cis*- og *trans* konformerer. Denne ligevægt forskydes kraftigt alt afhængigt af hvor peptoid monomeren indsættes. For to af analogerne kan kun en af konformererne observeres, og for den ene af disse analgoer blev det bevist at det var *trans* konformeren. For de to andre maculatin-G15 analoger blev der fundet en næsten ligelig fordeling af *cis*- og *trans* konformererne. Dette ledte til en hypotese om at peptidernes foldning af rygraden er energimæssigt mest favorabel for *trans* konformeren, og at peptoid monomerens sidekæde egenskaber og indsættelses sted er afgørende for fordelingen mellem *cis*- og *trans* konformererne.

4. Table of Contents

5. Abbreviations and Nomenclature

Amino acids are abbreviated to three or four letters because of the extensive use of non-classic amino acids. Peptide analogues are named by the way they differ from the wild type molecule with classic amino acids abbreviated to one letter and non-classic amino acids abbreviated to three or four letters as follows: The anoplin analogue 8K is anoplin where residue 8 is substituted to Lys, and 4Orn is anoplin where residue 4 is substituted with the non-classic amino acid ornithine.

Citations in the articles refer to only the reference list at the end of that article. Citations in the thesis refer to the reference list at the end of the thesis.

AMP: antimicrobial peptide
Aoc: L- 2-amino octanoic acid
BCA: bicinchoninic acid
βNal: β-2-naphthylalanine
CCA: α-cyano-4-hydroxycinnamic acid
CD: circular dichroism
Cha: β-cyclohexylalanine
cmc: critical micelle concentration
CFU: colony forming units
COSY: correlation spectroscopy
Dab: 2,4-diaminobutyric acid
Dap: 2,3-diaminopropionic acid
DIC/DIPCDI: N,N´-diisopropylcarbodiimide
DIEA: N,N-diisopropylethylamine
DPC: dodecyl phosphocholine
EC50: effective concentration at which 50% of the red blood cells are lysed
Epa: 2-amino,3-ethylpentanoic acid
Fmoc: fluorenylmethyloxycarbonyl chloride
Gd(DTPA-BMA): Gadolinium diethylenetriaminepentaacetic acid bismethylamide
HATU: N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridine-1-yelmethylene]-N-methylmethanaminium hexafluoro-phosphate N-oxide
HOAt: 1-hydroxy-7-aza-benxotriazole
Hle: homoleucine
HPLC: high-performance liquid chromatography
Hser: Homoserine
HSQC: heteronuclear single quantum coherence
MALDI: matrix-assisted laser desorption ionization
MBC: minimum bacteriocidal concentration
MIC: minimum inhibitory concentration
MS: mass spectrometry
Nle: norleucine
Nleu: [(2-methylpropyl)amino]acetic acid
NMP: N-methyl-2-pyrrolidone
NMR: nuclear magnetic resonance
NOE: nuclear Overhauser effect

NOESY: nuclear Overhauser effect spectroscopy
Nva: norvaline
O-Me-Ser: O-methyl-serine
Orn: Ornithine
PBS: phosphate buffered saline
PLS: partial least squares
PRE: paramagnetic relaxation enhancement
QSAR: quantitative structure-activity relationship
RMSD: root mean square deviation
SAR: structure-activity relationship
SDS: sodium dodecyl sulfate
TFA: trifluoroacetic acid
TFE: triflouroethanol
TIS: triisopropyl silane
TOCSY: total correlation spectroscopy
TOF: time of flight

6. Introduction

During the last few decades, a vast amount of research has been conducted on elucidating the structure-activity relationships (SARs) of antimicrobial peptides (AMPs)[1–5]. AMPs have been deemed as one of the most promising classes of molecules for development of new antibiotics[1,2,6]. This is due to the fact that they have always existed as part of the innate immune system, without causing development of significant resistance mechanisms in the invading pathogens[1,6–8]. The goals of the SAR research are to understand the biochemical interactions of AMPs with microbes, and to be able to make rational designs of AMPs instead of producing huge combinatorial libraries, which are both costly and labor intensive[8].
In general, AMPs can be divided into classes based on which organism they have been isolated from; mammalian defensins[9], plant defensins[10], and bacteriocidins[11]. Furthermore, AMPs are normally also categorized based on their biophysical characteristics, where the major distinction is whether they are cationic or anionic AMPs. The group of cationic AMPs is significantly larger than the anionic group[12,13]. Finally, AMPs are also categorized based on their structural characteristics into α-, β-, αβ-, non-αβ-, and θ-defensins (cyclic AMPs)[8,14].

As of May 2014, the amount of AMPs reported in the antimicrobial peptide database[13] is 2407, and of these, 2106 are cationic. A search based on their structural characteristics reveals that the major group of AMPs is the cationic α-helical peptides with a total of 311 entries (many of the entries have not been structurally investigated). Approximately half of these AMPs have been isolated from amphibians and insects[13].

In general, AMPs can have both a direct antimicrobial effect and have the ability to activate the adaptive immune system[15]. When only considering the direct antimicrobial effect, many different mechanisms of action have been proposed for AMPs, but, in general, AMPs are either membrane interacting or membrane penetrating. The membrane interacting AMPs are believed to either cause lysis of the cells through pore formation/dissolution of the membrane or to bind to the membrane or a membrane component (both specific lipids and proteins) in a way that prevents normal function of the cell[6,16].The membrane penetrating AMPs are proposed to interact with an intracellular target, which in most cases has been proposed to be DNA[6,17]. A recent review of the proposed mechanisms of action has been made by Nguyen *et al.*[18].

The cationic α-helical peptides (one of the major groups of AMPs) are generally characterized as having an extended structure in solution, but forming an amphipathic helix upon interaction with phospholipid membranes[6,17]. However, reports have also been made of compounds that appear to adapt a globally amphiphilic distribution of its residues without the formation of a secondary structure upon interaction with membranes[19]. Many SAR studies have been performed on the cationic α-helical AMPs in order to increase their activity against microorganisms and/or reduce their toxicity towards human cells.

Parameters used for evaluation of AMP potency

Normally, the studies evaluate the potency of the AMPs by using the minimum inhibitory concentration (MIC) of the peptide in vitro, i.e. the peptide concentration which is required to prevent microbial growth in a selected media. However, in some studies also the minimum bacteriocidal concentration (MBC) is used instead of the MIC. In many studies, the aim has been to obtain more potent analogues in order to use lower amount of drugs for a treatment. However, many of these potent analogues have also showed a high hemolytic activity which in turn increases the health risks. The hemolytic activity is often measured as an EC50 value,

is the concentration of peptide required to lyse 50% of the red blood cells in vitro. In the end, it is the combination of the antimicrobial and hemolytic activities which determines whether an AMP is a good candidate to become an antimicrobial drug. The aim is to obtain a high specificity, i.e. a high ratio of EC50/MIC, and this ratio has been termed the therapeutic index or selectivity index. For a good AMP, the EC50 value is high and the MIC value is low, and thus a good therapeutic index is high. It should be noted that the absolute value of the therapeutic index varies depending on the method of choice (for instance the initial amount of CFU in the microorganism suspension used for the experiments.

Structural parameters used to explain activity and selectivity of AMPs

The structural parameters which have been investigated in order to obtain more potent AMPs include; charge, hydrophobicity, hydrophobic moment/amphipathicity, helicity, and polar angle[1,2,6,17,20].

The charge of the cationic α-helical peptides normally lies in the range of +2 to +9 and is believed to be responsible for the initial electrostatic attraction to the negatively charged phospholipid membranes of the microorganisms[17]. Mutation studies have shown that there is a correlation between increased charge and increased antimicrobial activity and selective toxicity towards microorganisms[2,5]. However, this correlation is not always found[21], and increasing the charge above a certain amount can reduce the antimicrobial activity and increase the toxicity towards host cells[5].

The hydrophobicity of a peptide is estimated in several ways in the literature. It is sometimes defined as the percentage of hydrophobic residues within a peptide, and is normally in the range of 40-50%[8,17]. The hydrophobicity can also be defined as the sum of the hydrophobicity of each residue in the peptide (several different hydrophobicity scales are used, which yield distinct values for a given peptide[22–27]). In order to be able to compare the hydrophobicity between peptides of different length, the mean hydrophobicity has been introduced, which is simply the sum of the hydrophobicity of each residue divided by the number of residues[2,28]. Another suggested parameter regarding the hydrophobicity is the sum of the residual hydrophobicities of the hydrophobic helix surface[5].

The hydrophobicity is essential for the interaction between the AMP and a membrane, but for all definitions of hydrophobicity presented here, an increased level of hydrophobicity is strongly correlated with increased toxicity towards mammalian cells and thus loss of specificity[1,2,5,8,17,29].

The composition of amino acids in known AMPs has been plotted against the AMP classification as either antibacterial, anticancer, antifungal, antiviral, or toxic towards mammalian cells[8]. It shows that the AMPs that are toxic towards mammalian cells are characterized as having the highest degree of hydrophobicity. In order to reduce the toxicity of AMPs to mammalian cells, the hydrophobicity should be reduced to below 50% hydrophobic residues[8].

The hydrophobic moment of a helical AMP is used as a quantitative measurement for its amphipathicity[17] and is calculated as the vectorial sum of the individual amino acid hydrophobicities, normalized to an ideal helix[24,30]. Like with the hydrophobicity of AMPs, an increased hydrophobic moment has been found to correlate strongly with an increased activity against mammalian cells and thus, a reduced specificity[1,3].

The helicity of peptides is the percentage of α-helical content in the secondary structure, predicted on the basis of circular dichroism (CD) measurements. Helicity has been determined both in solutions containing the helix inducer, triflouroethanol (TFE), and in detergents such as SDS[2,21]. From studies including helicity as a parameter, it has been found that increased helicity can increase the antimicrobial activity of an AMP, but it increases the toxicity toward cells composed of neutral phospholipids even more[1,2,20].

The polar angle

Based on the distributions of the amino acids in the helical wheel model, the polar angle has been defined as the angle which spans the hydrophilic residues plus half the distance from the outermost hydrophilic residue to the first hydrophobic residue on each side. This parameter has also been investigated in relation to AMPs activity and selectivity, and it has been found that a reduced polar angle (i.e. larger hydrophobic face of the helix) increases the activity of some AMPs[3,4,31].

Quantitative structure-activity relationships

A different approach on how to develop AMPs with superior abilities was initiated in the 1980's, where continuous physico-chemical properties of the 20 amino acids were described[32]. This property data matrix was soon after extended and subjected to principal component analysis, from which 3 principal properties were derived. These 3 descriptors were then successfully used in a partial least squares (PLS) regression analysis in order to determine quantitative structure-activity relationships (QSARs) between peptides and their activities[33].
Later on, this principle has found widespread use[34–39], and even descriptors for non-naturally occurring amino acid have been derived[40].

The initial derivation of QSARs and the prediction of more potent peptide analogues had some downsides, as information about sequence and secondary structure was not implemented in the amino acid descriptors. Thus, the derived QSARs only yielded information about the general content of amino acids in a peptide[32–34]. This caused researchers to include peptide descriptors such as helicity (as determined by circular dichroism), hydrophobicity (not percentage of hydrophobic residues but the sum of the hydrophobicities of all residues), and charge together with the amino acid descriptors, which resulted in improved prediction abilities[34]. More recently, the inclusion of the interaction between residue neighbors has successfully been included in the PLS modeling[36], and predictions by the use of neural networks have also been presented[41].

Motivation for this work

Despite all the mutation studies and all the QSAR investigations, the molecular changes which result in increased or decreased antimicrobial activity and specificity have not yet been understood[41]. Already in 1992, Bessalle *et al.* stated that parameters such as helicity and amphipathicity are rather indirect, and that information regarding the actual structures of AMPs and their association with bacteria is needed[21]. Almost a decade later, Giangaspero *et al.* stated that the interrelationships between size, sequence, charge, helicity, overall hydrophobicity, amphipathicity, angles subtended by hydrophobic and hydrophilic faces are a key to understand how AMPs function, but that these interrelationships are often not taken into account in SAR studies[2].

We, too, believe that the key to understanding the function of AMPs lies within the molecular interaction between the AMP and the bacterial membrane, and that a more detailed knowledge about this interaction is needed in order to rationally design the optimal AMP based on a given lead structure.

Paramagnetic relaxation enhancement

Recent advances within nuclear magnetic resonance (NMR) spectroscopy have made it possible to obtain very accurate and detailed structural data regarding the interaction between a peptide and a membrane mimic. This is done by determining the insertion depth of individual

(e.g. each H^{α}) of the peptide in micelles consisting of the widely accepted membrane mimic dodecylphosphocholine (DPC)[42,43].

Paramagnetic relaxation enhancement (PRE) is commonly used in NMR to obtain long-range distance information which is very helpful when determining the tertiary structure of large proteins. The closer a paramagnetic agent is to an excited nucleus, the faster the nucleus will return to its equilibrium state. Thus, by measuring the signal intensities at different time points, the relaxation rate of each atom can be measured and converted to relative distances to the paramagnetic agent.
In our technique, this principle is used by adding chelated Gd ions to the NMR samples containing AMPs bound to DPC micelles. The Gd complex, Gd(DTPA-BMA), has been found not to interact with either DPC or peptides and to be bulky enough not to be able to reach the interior of a DPC micelle. Thus, the experimental conditions can be considered to consist of a DPC micelle with one or more helical AMPs inserted into the micelle surface and surrounded by water and Gd(DTPA-BMA) complexes, as illustrated in Figure 6.1.

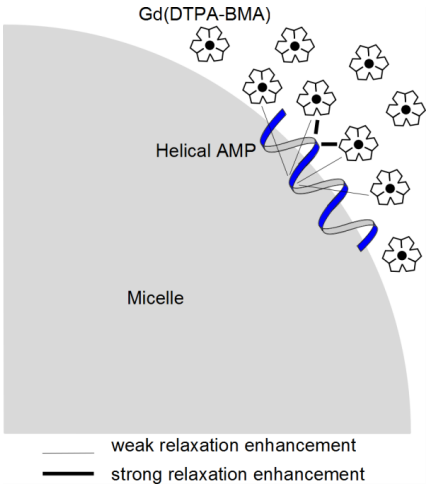

Figure 6.1: Illustration of how the paramagnetic agents influence the relaxation of the different H^{α} of the peptide with different magnitudes.

As illustrated by the thickness of the lines between the Gd(DTPA-BMA) complexes and the peptide backbone, the part of the AMP which is exposed to the water will experience a strong relaxation enhancement while the parts buried in the micelle interior will experience a weaker relaxation enhancement. The only atoms of the peptide which can be used for these calculations are the ones which do not exhibit hydrogen exchange (thus ruling out many H^{N}). However, due to the side chain flexibility the insertion depth accuracy decreases the further away from the backbone a hydrogen is located, and thus only the H^{α} atoms are used. This allows for the most well defined orientation of the peptide backbone relative to the micelle interior.

7. Aim and content of this work

The overall aim of this work has been to use PRE to study in detail the interaction between helical AMPs and DPC micelles (membrane mimic) and through this explain how the different structural parameters correlate with their antimicrobial and hemolytic activity. In addition, PRE is used to study the structural consequences of implementing a peptoid monomer into a helical AMP (insertion of peptoid monomers increases the proteolytic stability of a given AMP).

This work was initially conducted on analogues of the cationic helical AMP Anoplin, but regarding the peptidomimetics the major part of the work has been conducted on analogues of the cationic helical AMP maculatin. Each of these studies has resulted in a separate manuscript to be submitted.
However, some of the work was not appropriate to include into these publications, and thus a section has been included in this thesis containing further results and discussion of these.

Based on the new knowledge obtained during this study, I reexamined various SAR parameters used in literature to understand, how they arose and how useful they really are. This has resulted in a section containing a discussion of commonly used SAR parameters in the light of the results presented in this thesis.
Finally, the major conclusions of this thesis are given together with my thoughts about further research, changes in methodology, and prospects for future application of PRE in relation to the work of AMPs and peptidomimetics presented in this thesis.

8. Paper section

Paper 1: **Rational Design of alpha-helical antimicrobial peptides – DOs and DON'Ts**.
Lars Erik Uggerhøj, Tanja Juul Poulsen, Jens Kristian Munk, Marlene Fredborg, Teis Esben Sondergaard, Niels Frimodt-Moller, Paul Robert Hansen, Reinhard Wimmer
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FEBS Letters 2014 Aug 25;588(17):3291-7

Paper not included

Synthetic analogs of anoplin show improved antimicrobial activities.
Munk, J, Uggerhøj, LE, Poulsen, TJ, Frimodt-Møller, N, Wimmer, R, Nyberg, NT & Hansen, PR
Journal of Peptide Science, vol 19, nr. 11, s. 669-75.

Rational Design of alpha-helical antimicrobial peptides – DOs and DON'Ts

Lars Erik Uggerhøj[a], Tanja Juul Poulsen[a], Jens Kristian Munk[b], Marlene Fredborg[c], Teis Esben Sondergaard[a], Niels Frimodt-Moller[d], Paul Robert Hansen[b], Reinhard Wimmer*[a]

Antimicrobial peptides (AMPs) are promising candidates to battle multiresistant bacteria. Despite extensive research, structure-activity relationships of AMPs are not fully understood, and there is a lack of structural data of AMPs in lipids. Here, we present the NMR structure of anoplin (GLLKRIKTLL-NH_2) in micellar environment. A vast library of substitutions was designed and tested for antimicrobial and hemolytic activity as well as changes in structure and lipid interactions. This showed that improvement of antimicrobial activity without concomitant introduction of strong hemolytic activity can be achieved by subtle increases in the hydrophobicity of the hydrophobic face or subtle increases in the polarity of the hydrophilic face of the helix, or – most efficiently – a combination of both. Based on the results, a set of guidelines are given on how to modify cationic α-helical AMPs in order to control activity and selectivity. The guidelines are finally tested on a different peptide.

Introduction

Since the discovery of penicillin, the occurrence of infections by drug resistant bacteria has become more and more frequent. Today, the development of bacterial resistance towards the known antibiotics has become an extensive problem, and there have even been examples of infections by bacteria which are resistant to all approved antibiotics.[1] In an attempt to obtain new drugs for treating multiresistant bacterial infections, a vast amount of research has been conducted regarding AMPs. This effort has been going on for more than three decades, but as of now, only a few AMPs have been approved for use; ambicin (nisin – a food additive), polymyxin B and E (last-resort antibiotic due to its heavy side effects), and gramicidin S (topical treatment of wounds).[2] Much of the work regarding AMPs has been focused on understanding how to make potent AMPs with little toxicity towards the host cells. A major class of AMPs is the cationic helical peptides,[3] which are the focus of this work.

Many of the older studies regarding the cationic helical AMPs introduced structural parameters (hydrophobicity, charge, hydrophobic moment/amphipathicity, angle of hydrophobic and hydrophilic face, and helicity), which were useful for explaining the observed difference in antibacterial activity and in some cases cytotoxicity.[4–8] However, none of these parameters were sufficient for making a general guideline on how to improve antibacterial activity without causing increased cytotoxicity. In later been suggested that this is because of the parameters being interconnected:[9] e.g. an increase in charge or hydrophobicity will result in an altered amphipathicity.

It is now known that increases in charge and hydrophobicity in general increase antibacterial activity, but that both can increase cytotoxicity, this goes especially for hydrophobicity.[5,7,10] Is has also been shown that an increase in hydrophobicity will result in a larger increase in activity against Gram-positive bacteria compared to Gram-negative bacteria.[5,11] Helicity is believed to correlate with the cytotoxicity of the AMPs, but helicity is often measured by circular dichroism (CD) in solutions of trifluoroethanol (TFE) which is a helix-inducing solvent.[9] The helicities obtained from such experiments are not directly comparable to the degree of helicity that will be induced in the AMPs upon interaction with actual membranes.

In some of the later research, great care has been taken to keep certain parameters constant while varying one or two other parameters, and the actual interaction of a given AMP with the membrane has been taken into consideration. Here, a principle called the "snorkel effect" has been considered, which states that the insertion of a peptide is dependent on the side chain length of the polar residues. The long aliphatic side chains of Arg and Lys thus allow the peptide to be deeper inserted into the membrane while the side chain

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charge is still able to interact with the lipid head groups on the surface.[9]

Despite a vast amount of data, the structure-activity relationship of cationic helical AMPs is still not fully understood, and a generally applicable set of guidelines on how to increase AMP activity while retaining or improving selectivity has not yet been achieved.

Unlike many other studies, we approach the development of structure-activity relationships by including experimentally determined high-resolution structures of peptides bound to micelles into the study, because we think that a key to understanding SAR of AMPs is the interaction between peptides and lipids.

For this study, we focus on the simplest helical AMP found to date, anoplin, isolated from the venom sack of the solitary spider wasp, *Anoplius samariensis*. Anoplin is a decapeptide AMP, GLLKRIKTLL-NH_2, amidated at the C-terminus. It has an extended conformation in water, but upon binding to membrane mimics it folds into (at least partly) an alpha helix.[12] The actual structure of the peptide has not previously been published. The activity for anoplin and derivatives thereof has been reported in several sources, where the most frequently used strain is *E.coli* ATCC 25922.[12–17] However, the reported MIC values are not consistent for this peptide, which gives rise to some concern about how active this peptide really is.

This work is a continuation of previous studies on anoplin.[13,14] Initially an Ala scan conducted on anoplin showed that increased activity resulted from insertion of Ala in positions 5 and 8.[13] This was further explored by insertion of strongly hydrophobic aromatic amino acids in these positions. The analogues 5W (Trp inserted in position 5 of anoplin), 5F8W, and 5K8W all showed a significant increase in antibacterial activity, but also an increase in hemolytic activity. For 5W and 5F8W this increase in haemolytic activity is so high, that the analogues lose their selectivity.[13,14]

The onset of this work was to structurally study anoplin together with the analogues 5W, 5F8W, and 5K8W in order to find an explanation for their differences in antibacterial and haemolytic activity. This culminated in three successive studies. Firstly, a structural study, utilizing a recently developed NMR technique for studying the actual orientation and insertion depth of peptides into micelles,[18] was performed on the selected anoplin analogues. These results showed structural differences in the interaction between the peptide and the DPC micelle of the three anoplin analogues. This gave rise to a hypothesis explaining the activities of the three analogues compared to anoplin. Secondly, a larger set of peptides was designed using conservative substitutions in order to investigate how the separate modulation of hydrophobicity of the hydrophobic face and polarity of the hydrophilic face influenced the activity of anoplin. It was also investigated how these changes affected the insertion depth of selected analogues into the DPC micelle. Thirdly, the found correlations between structure and activity of the anoplin analogues were used to design two optimized anoplin analogues as well as to design optimsed analogues of a different helical AMP, citropin. This verified that the discovered correlations could be used for rational design and that they apply to other helical AMPs as well.

The two optimized anoplin analogues proved to be the most active and selective analogues of anoplin found to date and confirm our theories regarding how to optimize the activity and selectivity of cationic α-helical AMPs. The optimized citropin analogues also supports our theories, but showed that hydrophobicity per turn of the helix must be reduced for longer peptides in order to maintain selectivity of the AMPs. Both the improved anoplin and citropin analogues showed broad spectrum activity, and high activity against two multiresistant bacterial strains. In conclusion we present a list of what to do and what not to do when attempting to improve the activity of a cationic α-helical AMPs.

Results and Discussion

Results are presented in three parts:
Part 1 describes the structure ensembles of anoplin, 5W, 5K8W, and 5F8W and compares the observations with previous publications on the structure of anoplin. The structural behavior of these analogues upon insertion into DPC micelles is then discussed, leading to a hypothesis on two different types of polar residues in α-helical AMPs.

Part 2 describes all results related to the antibacterial and hemolytic activity of anoplin and its analogues. This part also includes the results of our structural studies of selected analogues, as well as our hypothesis, which explains their activities through various structural parameters.

Part 3 describes how the findings from part 2 are used in designing optimized analogues of anoplin and citropin, and what was discovered from this small set of peptides.

Part 1: Structural analysis

The structures of anoplin, 5W, 5F8W, and 5K8W were solved in the presence of DPC micelles using classical NOE constraints (see Figure S1), Talos derived angle restraints, and weighted PRE-derived distance restraints to the micelle center. Subsequently the structure ensembles were energy minimized in a DPC micelle surrounded by water.

anoplin	GLLKRIKTLL-NH_2
5W	GLLK**W**IKTLL-NH_2
5F8W	GLLK**F**IK**W**LL-NH_2
5K8W	GLLK**K**IK**W**LL-NH_2

The refined structures have been submitted to the Protein Data Bank Japan (PDB) (anoplin: 2MJQ, 5W: MJR, 5K8W: 2MJS, 5F8W: 2MJT). Statistics for the structure ensembles can be found in Table S2.

Helical content of anoplin

All 4 peptides folded into a regular α-helix for residues 2-10 plus the C-terminal amide and featured a flexible N-terminal glycine residue, as exemplified by anoplin in Figure 1. This high degree of helicity is in contradiction with the helical content of anoplin reported in literature from CD measurements: Between 32% and 77% in membrane mimicking solvents (up to 50% TFE or 8.5mM sodium dodecyl sulfate (SDS)).[12,13,19] As stated in the introduction, and supported by Zelezetsky et al.[9], the helicities obtained in solutions of TFE are not comparable to the degree of helicity that will be induced upon interaction with actual membranes. SDS has a critical micelle concentration (cmc) of 8.5mM (dependent on salt concentration). Thus, under the conditions reported in the literature, there was no or only very small amount of micelles present. In our NMR samples, the concentration of DPC is more than 100 times its cmc. This difference in experimental conditions might explain why complete helices are observed in our study and not in previous publications. The use of TFE and submicellar SDS concentrations will not represent the actual degree of helicity a peptide will adopt upon binding to a lipid membrane. However, the low helicities obtained from CD might also be caused by the method itself, as CD is known to have limited sensitivity for short helical sequences[15]. In any case, the NOE data on anoplin and the three analogues provide clear evidence for the presence of an α-helix ranging from residue 2 to 10 (Figure S1).

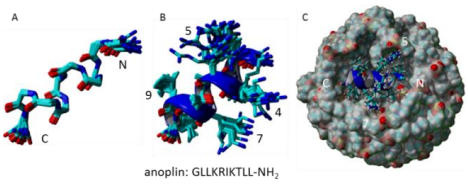

Figure 1. Structures in the final ensemble of anoplin. A: Comparison of the backbone of all 20 structures shows very little variation, except for the terminal residues "N" and "C" denote the N- and C-termini of the peptide, respectively. B: Illustration of the flexibility of the side chains and their orientation relative to the helix. Residue numbers are labeled. C: Illustration of the location of the first three structures of the anoplin ensemble in the interface between the micelle and the surrounding water. It is noteworthy that the nitrogen atoms of Arg 5, Lys 7, and the N-terminus are located close to the phosphate groups of the lipids. The illustration has been made with a water accessible surface using YASARA [20].

Peptide insertion into DPC micelle

The determined insertion depth profiles of anoplin, 5W, 5F8W, and 5K8W are shown in Figure 2. The insertion depth values for each H^{α} are listed in Table S3 along with their standard deviations. The insertion depth profile is very similar for anoplin, 5W, and 5K8W. There is a variation in the insertion depth of the N-terminus (residues 2 and 3) with anoplin being the least inserted followed by 5K8W and then 5W, which correlates with the increasing hydrophobicity of these analogues. The variation in position 5 of these three peptides shows that Lys5 is less deeply and Trp5 is more deeply inserted than Arg5. However, these changes are approximately of the same size as the experimental uncertainties (RMSD limits shown in Table S3), and should be interpreted with caution. On the other hand, 5F8W has a significantly different insertion depth profile, which shows this analogue is oriented quite differently in the micelle. Furthermore, 5F8W is significantly deeper inserted than the other analogues.

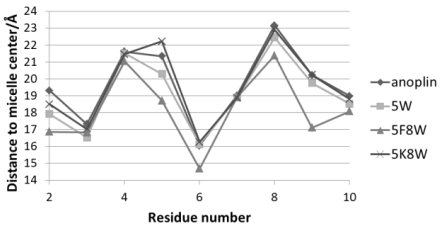

Figure 2. Insertion depth profiles of anoplin and the three analogues 5W, 5K8W and 5F8W, showing the distance of each H^{α} from the micelle center. The micelle radius is ≈22.7 Å. When comparing the influence of the 5W mutation, the overall insertion depth profile is conserved except for residue 5. There is a slightly deeper insertion of Leu2 and Leu3 in the 5W analog, but their insertion depth relative to each other is conserved. For 5K8W, the substitution from Thr8 to Trp has not altered structure and micelle interaction at the C-terminus at all. The mutation from Arg5 to Lys only resulted in a slightly lesser insertion of H^{α} 5. The insertion depth profile of the double mutation 5F8W is significantly different from the others: 5F8W is deeper inserted into the membrane, and the changes in the insertion depth profile correspond to a rotation around the helical axis.

To visualize the differences in insertion of the peptides into the membrane mimetic, a geometrical analysis was performed on the refined structure ensembles. Here, the angle of the geometric center of each side chain relative to the direction to the micelle center were determined and used to draw the helical wheel plots of the four AMPs shown in Figure 3.

The helical wheel projection of anoplin corresponds well with the helical wheel model except for a slight compression of the hydrophobic face (the hydrophobic side chains are moved closer to each other than expected from the helical wheel projection). For 5W, a slight clockwise rotation of the N-terminal part of the helix is seen, while the Trp side chain is oriented much closer to the membrane interior compared to the direction in the standard helical wheel. For 5F8W, the whole helix has been rotated about 30° clockwise and Trp8 side chain is likewise bent towards the membrane

interior. For 5K8W, the orientation of all side chains corresponds to the standard helical wheel.

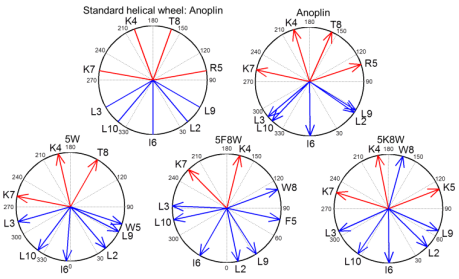

Figure 3. Helical wheel projections based on experimental data together with the standard helical wheel projection of anoplin. The arrows represent the direction from the micelle center to the geometric mean of each side chain. The plots are oriented in a way that the direction towards the micelle center is down (defined as 0°), i.e. Ile6 of anoplin points directly towards the micelle center. The grid with small labels shows the angle of rotation relative to the micelle center. Hydrophilic side chains are illustrated by red arrows, and hydrophobic side chains are illustrated with blue arrows. No experimental data regarding the insertion depth of Gly1 could be obtained, and the structure ensembles show that Gly1 is highly disordered for all analogues. Thus, Gly1 has not been included in the helical wheel projections.

All of these observations can be explained by the fact that a given side chain will orient itself in the direction which reduces the total energy of the system to its minimum. In anoplin, the side chains are grouped closer together on the hydrophobic face, which can be caused by either a slight extension of the helix or by a bending of the hydrophobic side chains in order for them to be more directly inserted into the micelle interior. For 5W, the Trp residue is bent significantly towards the interior of the micelle with a resulting twist of the backbone for residues 2-5. This indicates that the insertion of the Trp side chain into the hydrophobic interior of the micelle is so favorable that a small distortion of the backbone is of less importance. When looking at the standard helical wheel of anoplin, one might have assumed that the substitution of residue 5 from charged to hydrophobic side chain would cause a rotation of the whole peptide, in order for the hydrophobic face to point directly into the micelle. However, the experimental data show that this is not the case. It is not possible for a single substitution to influence the structure of the helix beyond the turn of which it is embedded in (assuming a reasonable high amphipathicity of each turn). Thus, only residues 2-5 are influenced on the backbone level, and only by a small amount, whereas the remaining residues are still locked completely in position by the strongly amphipathic motif of anoplin. Almost all the structural change is restricted to the orientation of the side chain. For 5F8W, the rotation of the whole helix allows the now hydrophobic residues 5 and 8 to be located closer to the micelle interior relative to the two remaining charged residues. Like for 5W, this analogue also show a bending of the Trp side chain towards the micelle interior, although it is not as significant as for 5W. In this peptide, both turns of the helix have had a substitution on the same side of the helix, which is why there is now a rotation of the whole molecule as opposed to the 5W analogue. In Figure 4, the change in orientation of Trp in 5W and 5F8W can be seen relative to the orientation of the corresponding side chain in anoplin.

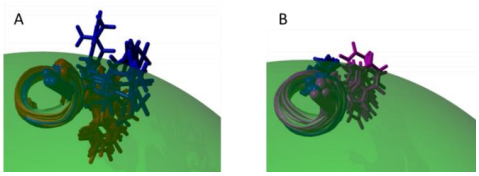

Figure 4. A: Structure ensembles of anoplin and 5W inserted into a spheric model of a membrane with a radius of 22.7Å, where only the side chains of residue 5 are displayed. This shows how the change in side chain 5 orientation of 5W, schematically shown in Figure 3, affects its orientation relative to Arg5 and the micelle surface. B: Structure ensembles of anoplin and 5F8W inserted into a spheric model of a membrane with a radius of 22.7Å, where only the side chains of residue 8 are displayed. This shows how the rotation of 5F8W and bending of the Trp 8 side chain affects its orientation relative to Thr8 and the micelle surface.

In 5K8W, Trp is inserted on the right hand side of the second turn of the helix (in the projection). Here, it is oriented further towards the bulk water phase compared to the other two analogues with Trp. In this position neither rotation nor bending of the side chain takes place. The structure of this analogue shows that residue 8 is too far away from the micelle interior for it to be able to bend and reach the micelle interior and through that obtain a more energetically favored configuration. When comparing this with the observation from the structural ensemble of anoplin, where residue 5 and 7 were often found in close proximity of a lipid head group and that residue 4 and 8 were primarily pointing directly into the solvent, it appears that there are two different types of the hydrophilic residues in these AMPs: in each turn of the helix, one of the hydrophilic side chains is primarily interacting with the membrane, whereas the other primarily interacts with the solvent.

An important result from this study is that there is a significant deviation between the theoretical helical wheels of the peptides and their actual helical wheel projections. This effect is quite clear, despite the fact that the α-helix of anoplin only consists of three turns. It could well be imagined that this effect becomes more significant with increasing length of the helix. Thus, care should be taken, when using the helical wheel model to design optimized analogues.

Part 2: Antimicrobial and hemolytic activity studies

In order to understand how the activity of anoplin correlates with its structure and physiochemical properties, we designed a stringent set of peptides, where only one parameter was modified at a time. This set of analogues was made by only performing conservative substitutions, where charge, polarity, and chain length at the hydrophilic face and hydrophobicity and branching at the hydrophobic face were modified. Based on the structural studies already described, we ensured to keep a positive charge in position 5 and 7, in order to not disrupt their interaction with the lipid head groups. Furthermore, we did not perform modifications on the central residue of the hydrophobic face (Ile6).

Due to the discrepancies in the reported MIC data of anoplin[12–14], we revised the standard methodology related to the activity assays in order to ensure the reliability of our data. Peptide synthesis, purification, and validation were done according the standard procedures as described in the methods section. Regarding concentration determination, we discarded the standard procedures as they either depend on 100% completion of chemical reactions (amino acid analysis, Bradford, modified Lowry, and bicinchoninic acid (BCA)) or a compatible amino acid composition with the standard protein sample (Bradford, modified Lowry, bicinchoninic acid (BCA), and UV/Vis). Weighing of the peptides was also discarded due to insufficient knowledge regarding the amount of remaining counterions from the chromatographic purification. For anoplin (four positive charges), the presence of four trifluoroacetate ions would account for 40% of the weight. We decided to use quantitative NMR with triflouroethanol (TFE) as an internal standard. This allows for the quantification of trifluoroacetate by ^{19}F-NMR in addition to quantifying the peptide by ^{1}H-NMR. Furthermore, TFE can easily be removed by lyophilisation. We mainly compared the peaks of H^{ε} (CH_2 group) of Lys and H^{δ} of Arg residues and the peaks for the CH_2 group in TFE, which was our internal standard. These peaks proved to be very well separated in the 1D spectrum as shown in Figure S4. For the determination of antimicrobial activity we have included an activity study using an oCelloScope for real time optical measuring[21] in addition to the standard broth micro-dilution assay[22]. The oCelloScope allows for a semi-continous microscopic monitoring of the bacterial cells, thus enabling the identification of irregularities during the assay. In order to enable comparison of our activity data with previously reported activity values of anoplin analogues, the whole set of peptides were tested against the most frequently used bacterial strain in anoplin studies, *E.coli* ATCC 25922.Testing of the hemolytic activity was performed using the standard procedures as described in the methods section.

The antibacterial and hemolytic activity of the whole set of anoplin analogues is shown in Table S5, while the subsets of analogues used for the discussion are shown in Tables 1-5. The full names of the non-classic amino acids are given in the abbreviation list, and their structures are shown in Figure S6.

MIC value of anoplin

The MIC value of anoplin was measured to be 100μM (the tested concentrations were 75μM, 100μM, and 150μM) against $5x10^5$ CFU/mL of E.coli ATCC 25922. This value is twice as high as the reported MIC value for anoplin at low salt concentration against $1x10^5$ CFU/mL[12]. Our experimental conditions are very similar to those of Konno et al.[12], except for the initial concentration of bacteria. Small studies were performed to investigate the importance of bacterial growth phase and initial bacterial concentration on the measured MIC values. It was found that only the initial bacterial concentration influenced the MIC value: each dilution of bacterial concentration by a factor of 5 ($1.25x10^7$, $2.5x10^6$, $5x10^5$, $1x10^5$ CFU/mL), reduced the MIC value by a factor of 2. Based on this, our activity value for anoplin is in complete agreement with those published in the first paper of anoplin[12].

Substitutions on the hydrophilic face

When increasing the polarity in position 4 by rearrangement of side chain atoms, shortening of side chain, and increase in charge, a general increase in antimicrobial activity is observed (Table 1). An increase in activity is not observed for every single modification, but it is highly plausible that the changes in activity occur, which are smaller than the concentrations steps used in the MIC assay. The only exception for this trend is 4Dap, but this might be caused by the ability of the charge on the side chain of Dap to interact with the backbone[23]. Thus, this amino acid should not be used in the design of AMPs. Based on these results, the presence of a charge in position 4 is not essential for maintaining the activity, but the residue should be highly polar. However, the presence of a charge in position 8 is very important, while the less drastic increase in polarity to 8S did not show a measurable effect. The change from Arg to Lys in position 5 did not result in a measurable change in antimicrobial activity. The 4,8Dab analogue illustrates the combined effect of using charged residues with a reduced chain length (increased polarity compared to Lys). It features an 8-fold increase in activity compared to anoplin, yet without introducing hemolytic activity within the range of concentrations tested (≤500μM).

Table 1. Activities of anoplin analogues with substitutions on the hydrophilic face.

	MIC [μM]	EC_{50} [μM]	Selectivity (EC_{50}/MIC)	Hydrophobicity
4T	200	>500	>2.5	7.12
4Hser	150	>500	>3.3	~7.12[a]
4S	100	>500	>5	6.82
Anoplin	**100**	**>500**	**>5**	**5.87**
4Orn	100	>500	>5	5.75
8S	100	>500	>5	5.57
4Dab	75	>500	>6.7	5.52
4Dap	100	>500	>5	5.26
5K8K	25	>500	>20	4.64
8K	25	>500	>20	4.62
4,8Dab	12.5	>500	>40	3.92

[a] The hydrophobicity is estimated by extrapolation of the loss or gain in hydrophobicity upon removing or adding a CH_2 group to a functionally identical side chain.

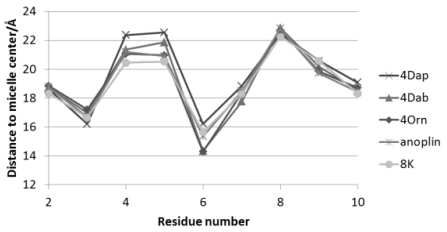

Figure 5. Insertion depth profiles of 4Orn, 4Dab, 4Dap, anoplin, and 8K, showing the distance of each H^{α} from the micelle center. The micelle radius is ≈22.7 Å. It shows that the decrease in chain length of residue 4 gradually pulls residue 4 and 5 further out of the micelle, and that for 4Dap this even influences the insertion depth of the whole molecule. Furthermore, the increase in chain length in position 8 allows for a deeper insertion of the peptide, especially around residues 4 and 5.

This improved activity upon increasing the polarity of the hydrophilic face by adding a charge to position 8 (8K) and shortening of the side chains in position 4 and 8 (4,8Dab) is intriguing. Is position 8 more prone to interact with lipid head groups compared to residue 4? Does the shortening of these side chains increase the possibility for these residues to interact with lipid head groups? Based on the structural studies in part 1 of this study, we estimated that residues 4 and 8 were primarily solvent interacting. If this is true, does the charge then create a better interaction with the solvent? Or does the increased charge increase the efficiency of the peptide in reaching the lipid part of the bacterial membrane?

In an attempt to find an answer to these questions, the structures and insertion depth profiles of 4Orn, 4Dab, 4Dap, and 8K were determined and compared to anoplin. In Figure 5, it can be seen how a decrease in chain length of residue 4 causes the peptide to be less deeply inserted in that turn of the helix. Going from the anoplin to 4Dap, the insertion depth of residue 4 changes with about 1.5Å and becomes very similar in insertion depth to Thr8. The analogue 8K is slightly deeper inserted than anoplin and 4Orn. Previous studies have considered the so-called snorkel effect, where the length of the polar side chains is believed to influence the insertion depth of the helix.[9] The observations in our data confirm this theory: The longer the side chains are on the hydrophilic face, the deeper the AMP can be inserted. In our data, we can only state this for the two solvent interacting residues, as we do not have data on analogues with shorter chain lengths of residues 5 and 7. The insertion depth data does, however, not correlate with the antimicrobial activity of the analogues. EC50 was not measurable for any of these analogues.

The refined structures and helical wheel projections of these analogues did not yield an explanation for their differences in antimicrobial activity. In this respect, it can be debated whether the DPC micelles are an appropriate model system for the negatively charged bacterial membrane of *E.coli*, and whether the use of a more accurate model system would reveal an explanation for their activities.

The fact is that the zwitterionic nature of the DPC micelles resembles the surface charge of human cells more than that of the negatively charged bacterial cell membranes. Thus, DPC micelles are not the ideal model system. However: DPC is available in deuterated form, and it forms relatively small aggregates to keep transverse nuclear magnetic relaxation from becoming too fast. SDS would fulfil those criteria, too, and have a negative charge, but previous experiments have shown that the PRE-based method for determining insertion depths does not yield reliable results with SDS micelles. In order to obtain further understanding of the interaction of these AMPs with membranes, further developments in solution state NMR compatible membrane mimics are required.

In the end, we can conclude that an increased polarity of the hydrophilic face of anoplin increases its antimicrobial activity against *E.coli*, but not conclude why.

Substitutions on the hydrophobic face

As stated in the introduction, an increase in hydrophobicity of a compound is often associated with enhanced antimicrobial- and hemolytic activity, the latter being enhanced the most. Our results show that anoplin is no exception, however, our careful tuning of hydrophobicity allows for a more detailed view on this.

The hydrophobic face of the helix was altered conservatively by gradually increasing or decreasing hydrophobicity of the two ends of the peptide (residues 2, 3, 9, and 10). The amino acids used to substiute the Leu residues were (O-Me-Ser)-Val-Met-Nva-Leu-Nle-Epa-Hle-Aoc-Cha-βNal, listed by increasing hydrophobicity. The resulting 2,10 and 2,3,9,10 series presented in Table 2 show that even a slight reduction in hydrophobicity removes membrane activity, while an increase in hydrophobicity increases both antibacterial and hemolytic activity. Any increase in hydrophobicity compared to the wild type, save for 2,10Epa, results in a measurable hemolytic activity (EC_{50} drops below 500μM). For the 2,10 series, it can be seen that the hemolytic activity increases much more steeply than the antibacterial activity after the 2,10Hle analogue (two- to threefold reduction in selectivity). It is also interesting to notice the increase in both antibacterial and hemolytic activity when going from the branched 2,10Epa analogue to the longer and less branched 2,10Hle analog, which have equal hydrophobicities. A similar trend is not observed when going from anoplin (Leu) to the linear Nle analogues which also have equal hydrophobicities. The 2,10Hle analogue is the most selective of the anoplin analogues with substitutions in both ends of the peptide. It should be noted that anoplin, 2,10Nle, and 2,10Epa might have a higher selectivity, but it could not be determined due to the very low hemolytic activities. Based on this, we can conclude that the hydrophobicity of the hydrophobic face is essential for the selectivity of anoplin, and this is probably valid for other cationic α-helical AMPs as well.

Table 2. Activities of anoplin analogues with substitutions of the hydrophobic face.

	MIC [μM]	EC_{50} [μM]	Selectivity (EC_{50}/MIC)	Hydrophobicity[e]
2,10 substitutions series				
2,10(O-Me-Ser)	>200	>500	-	~2.29[a]
2,10V	>200	>500	-	4.91
2,10M	>200	500	<2.5	4.93
2,10Nva	>200	>500	-	5.21
Anoplin	100	>500	>5	5.87
2,10Nle	100	>500	>5	5.87
2,10Epa	50	>500	>10	~6.57[a]
2,10Hle	12.5	380	30.4	~6.57
2,10Aoc	6.25	71	11.4	~7.27[a]
2,10Cha	6.25	100	16	7.91
2,3,9,10 substitution series				
2,3,9,10(O-Me-Ser)	>200	>500	-	~0.11[a]
2,3,9,10V	>200	>500	-	3.95
2,3,9,10M	>200	>500	-	3.99
2,3,9,10Nva	>200	>500	-	4.55
Anoplin	100	>500	>5	5.87
2,3,9,10Nle	100	>500	>5	5.87
2,3,9,10Hle	12.5	125	10	~7.27[a]
2,3,9,10Aoc[b]	-	20	-	~8.67[a]

[a] The hydrophobicity is estimated by extrapolation of the loss or gain in hydrophobicity upon removing or adding a CH_2 group to a functionally identical side chain. [b] Sample could not have its MIC value measured reliably due to poor solubility.

The question is then: How should the hydrophobicity of the hydrophobic face be increased in order to obtain the best selectivity? The results in Table 2 demonstrate an interesting feature of the anoplin analogues regarding this. When comparing 2,10Hle with 2,3,9,10Hle, no change in antibacterial activity is observed, while a threefold increase in hemolytic activity occurs. An approximate threefold increase in hemolytic activity is also observed when going from 2,10Aoc to 2,3,9,10Aoc, but here the MIC value could not be determined reliably, due to aggregates in the solution during the assay (this was only detected by inclusion of the oCelloScope measurements). The further increase in hydrophobicity by also increasing the size of residues 3 and 9 does not benefit the antibacterial activity but significantly increases the hemolytic activity and the tendency of the peptides to form aggregates (maybe self-aggregates). This trend is supported by our set of single substitutions presented in Table 3.

Table 3. Activities of anoplin analogues with single substitutions on the hydrophobic face which increases the hydrophobicity.

	MIC [μM]	EC_{50} [μM]	Selectivity (EC_{50}/MIC)	Hydrophobicity
Anoplin	100	>500	>5	5.87
2Cha	25	500	20	6.89
10Cha	12.5	350	28	6.89
2(βNal)	6.25	300	48	7.25

[a] The hydrophobicity is estimated by extrapolation of the loss or gain in hydrophobicity upon removing or adding a CH_2 group to a functionally identical side chain.

This series of peptides also demonstrates the correlation between hydrophobicity and membrane activity discussed for the 2,10 and 2,3,9,10 series. However, this series also demonstrates that the effect on peptide activity depends on the site of modification, as seen by comparing 2Cha with 10Cha. When comparing 2Cha and 10Cha with 2,10Cha, it can be seen that the single substitutions exhibit better selectivities. 2(βNal) supports this trend, as its hydrophobicity is comparable to that of 2,10Aoc and 2,3,9,10Hle but displays a three- to fivefold higher selectivity, respectively.

These observations lead us to the conclusion that an increase in hydrophobicity on the hydrophobic face of the helix should not be divided equally on each turn of an AMP, as this increases the general membrane affinity of the whole peptide and makes it unselective through increased hemolytic activity. This is substantiated by the findings of Slootweg et al.[17], where the Lipophilic amino acid derivative ((S)-2-aminoundecanoic acid) was incorporated into positions 2 of anoplin. This amino acid side chain contains 5 extra carbons compared to Leu, and the hydrophobicity of the analogues are thus comparable to that of 2,3,9,10Hle and 2,10Aoc. They reported a fairly high selectivity of 10-20 against *E.coli* ATCC 873ˆ

substitution at position 2. However, they did see a decrease in selectivity when inserting their lipophilic amino acid in positions 6 and 10, indicating that the site of insertion is important. It is important to notice that our data set is not sufficient to say where in an AMP the residue with increased hydrophobicity should be inserted. In our dataset, there are only small differences in activity when Cha is inserted in position 2 or 10, but larger differences were found in other studies[14,17].

Modulation of hydrophobicity profile

A small set of the anoplin analogues was designed in order to investigate how the activity of anoplin would change if the hydrophobicity distribution on the hydrophobic face was altered. βNal was inserted in position 6, while the side chains of residues 2,3,9, and 10 were shortened. The analogues with similar or lower hydrophobicity than anoplin lost their activity, whereas the ones with higher hydrophobicity were able to retain the same activity as anoplin. However, their hydrophobicities are comparable to that of 2,10Hle, which shows a markedly higher antibacterial and hemolytic activity. This confirms that the antibacterial and hemolytic activity is very dependent on a high membrane affinity of the whole molecule and not just a single turn of the helix.

Table 4. Activities of anoplin analogues where the hydrophobicity profile of the hydrophobic face has been modulated.

	MIC [μM]	EC_{50} [μM]	Selectivity (EC_{50}/MIC)	Hydrophobicity
2,10A6(βNal)	>200	>500	-	4.37
2,3,9,10-Nva6(βNal)	>200	>500	-	5.83
Anoplin	100	>500	>5	5.87
2,10Nva6(βNal)	100	>500	>5	6.49
3,9Nva6(βNal)	100	>500	>5	6.49

Non-conservative mutations

The activity data for the three non-conservative analogues which were the outset of our work (5K8W, 5W, and 5F8W) exhibit increasing hemolytic activities with increasing hydrophobicity. It would be tempting to ascribe this increase in hemolytic activity solely to the increase in hydrophobicity. However, 5K8W is still relatively selective, while 5W and 5F8W have lost selectivity completely. The observation that might explain the activity of these analogues is that the inserted aromatic residues in 5W and 5F8W can interact with the membrane interior whereas the Trp in 5K8W cannot. Thus, mutations at the membrane-water interface (positions 5 and 7 in anoplin) can have a far more dramatic consequence on the hemolytic activity. This should be kept in mind when designing analogues.

Table 5. Activities of the non-conservative anoplin analogues.

	MIC [μM]	EC_{50} [μM]	Selectivity (EC_{50}/MIC)	Hydrophobicity
Anoplin	100	>500	>5	5.87
5K8W	12.5	130	10.4	7.88
5W	37.5	20	0.5	9.13
5F8W[a]	-	2	-	10.66

[a] Sample could not have its MIC value measured reliably due to poor solubility.

The question remains: If the Trp in position 8 does not interact directly with the membrane, why does it increase both activity and hemolytic activity of that peptide compared to anoplin? The activity of these α-helical AMPs might be governed by more than just their interaction with the lipid part of the membrane, but as of now we do not have an answer to what that might be.

From the set of analogues with substitutions on the hydrophobic face of the helix, 2Cha, 10Cha, 2,10Hle, 2,10Aoc, and 2,10Cha were chosen for structural investigation in order to see if an explanation could be found for their differences in activity. These analogues showed very similar insertion depth profiles as shown in Figure 6. The insertion depth of these analogues weakly correlates with their hydrophobicity. However, these data are not so clear, as the experimental uncertainty is in the same order of magnitude as the changes in insertion depth observed. The peptide with the deepest insertion of this set of peptides is 2,10Cha – the most hydrophobic of the investigated analogues. When comparing the insertion depth of this analogue with those of the peptides with non-conservative analogues 5W and 5F8W in Figure 2, a similar insertion depth is found for 5W, but a much deeper insertion depth is found for 5F8W. This shows that the increased hydrophobic face of this analogue places it in a class of its own, regarding its insertion depth.

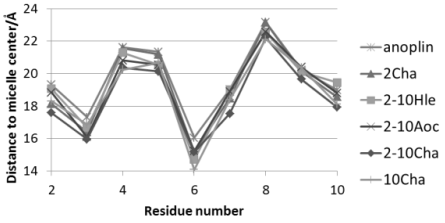

Figure 6. The insertion depth profiles of the analogues which were structurally investigated and are not shown in Figures 1 or 6, showing the distance of each H^{α} from the micelle center. The micelle radius is ≈22.7 Å. These analogues were all made with conservative substitutions, and they show that there is only very slight changes in insertion depth profiles and that the insertion depths are similar.

After solving the structures of these anoplin analogues and performing the restrained refinement, an experimental helical wheel projection

analogues was made. These helical wheels were very similar to each other, with no large deviations from the theoretically predicted helical wheel, and are thus not shown.

Solubility issues of anoplin analogues

From the standard MIC determination by the broth micro-dilution assay, we found that a few of the peptides could not have their MIC value determined reliably. To understand what the cause for this was, we studied what happens in the first 6 hours of the MIC assay by using an oCelloScope. The images generated via the oCelloScope showed that not all peptides were completely solubilized. Very large aggregates of different kinds were observed in samples containing 2,3,9,10Aoc (filamentous) and 5F8W (crystals) as shown in Figure S7. These two peptides did not yield reproducible MIC values in either of the used assays.

Smaller and much more scarce aggregates were found in samples containing 2,10Aoc, 5W, 5K8W, 2,3,9,10Hle, 2,10Cha, and 2,9Cha at high peptide concentrations. These peptides yielded reproducible MIC values, so it is believed that these smaller aggregates are not detrimental to the assay. Aggregation seems to occur at hydrophobicities > ≈ 7, when using the octanol-water partition coefficient derived scale from Fauchère et al [24].

How bacteria are killed by anoplin and its analogues

The images from the oCelloScope measurements showed a difference in bacterial growth of 5W and 5F8W compared to the rest of the analogues. In general, when the peptide concentration is at or above the MIC concentration, bacterial growth is observed for approximately 2 hours, after which the bacterial cell count drops. The cell growth is measured as surface area of cells, and upon inspection of the images of the bacterial population it is clear that a large part of the detected initial growth is caused by elongation of the cells rather than cell division, but there also appears to be an increase in the amount of cells. This is illustrated in Figure 7. When peptide concentration was below MIC, growth occurred with varying growth rates depending on concentration and peptide analogue for about 2 hours. After this, either a small drop in amount of cells, a plateau of constant amount of cells, or continued growth at the same rate was observed. This is assumed to be a result of how close the peptide concentration is to the actual MIC value. After 4 hours, growth was observed in all of these samples.

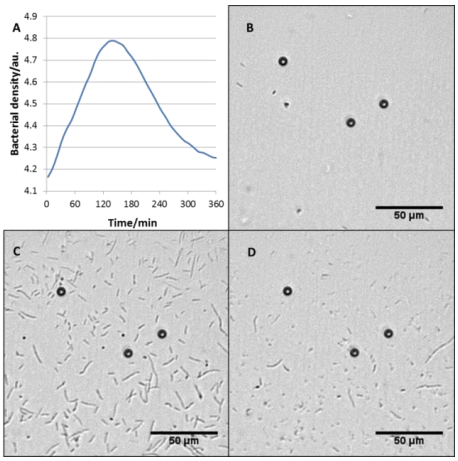

Figure 7. oCellosope images of the growth of *E.coli* in the presence of 2Cha at the MIC. Panel A shows the development of the bacterial density (arbitrary unit) in the well over time. Initially, both an increase in number and size of cells occurs. After approximately 2 hours, no more growth occurs, but instead a steady decline in intact cells is observed. Panel B: Still image from the beginning of the experiments showing very few cells. Panel C: Still image at the maximum optical density showing both an increase in number of cells and filamentation. Panel D: Still image at the end of the experiment showing few normal and elongated cells and a lot of particles smaller than *E.coli* cells, probably cell debris.

For 5W and 5F8W there was either growth or no growth – no plateau or intermediate growth rates were observed. These two peptides did not give rise to the appearance of elongated bacteria, either. Growth curves for *E.coli* at different peptide concentrations for 5W and 2,3,9,10Hle are shown in Figure 8.

These observations have led us to believe that anoplin does not work by direct lysing of the bacterial cells. This is in accordance with previous studies of anoplin, which shows that the primary mode of action is not through membrane lysis[19,25]. Instead it somehow lowers the growth rate of the bacteria and causes a deficiency in the ability of the bacteria to divide, which causes some of the cells to grow very long (filamentation). This is, however, not the case for 5W and 5F8W: They both completely prevent bacterial growth from the start and show very high hemolytic activities, which have led us to believe that these two analogues are indeed cell lysing peptides. This cannot be explained by their hydrophobicity alone, as the hydrophobicity of 5W is fairly close to that of 2,3,9,10Aoc. It is more likely a result of the increased hydrophobic interaction of these analogues with the interior of the membranes. The fact that 5K8W does not cause cell lysis while 5W does, supports that residues 4 and 8 in anoplin cannot interact with the membrane interior, as opposed to residues 5 and 7.

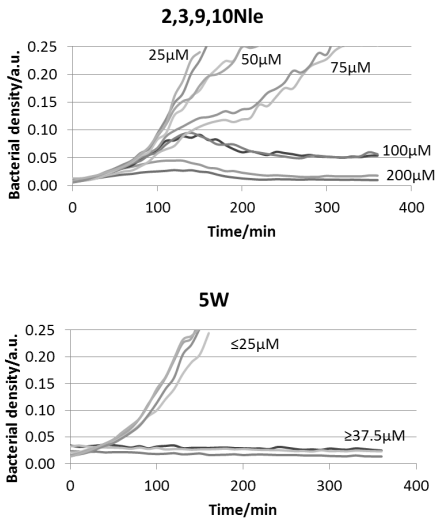

Figure 8. Growth curves obtained from oCelloScope measurements of *E.coli* in the presence of 2 different peptides. They illustrate the difference in method of inhibition which is observed throughout the whole data set. 2,3,9,10Hle (upper panel) allows for growth for about 2 hours, after which cell death occurs at concentrations ≥MIC. For concentrations below MIC, the peptide still slows down the growth of the bacteria in a concentration dependent manner. 5W (lower panel), however, inhibits growth immediately upon interaction with the cells at concentrations ≥MIC, whereas no slowing of growth is seen at concentrations below MIC: There is either growth or no growth. Data are shown from two replicates.

Applicable structure-activity relationship parameters

In order to explain the activity of all the anoplin analogues presented thus far, correlations were sought between their activity and the classical structural parameters; hydrophobicity, charge, hydrophobic moment/amphipathicity, and polar angle, but neither were sufficient for explaining the behavior of the set as a whole. From the structural studies, we calculated the experimentally derived polar angle, hydrophobic angle, tilt angle, rotation of Ile6 relative to the micelle center, and mean insertion depth, but neither of these proved useful as well.

The only useful parameters we found were those used in the description of the results: polarity of the polar face and hydrophobicity of the hydrophobic face. An increased polarity of the hydrophilic face of the peptide is beneficial for antimicrobial activity, and an increase in the hydrophobicity of the hydrophobic face yields a higher activity against all membranes until a threshold is reached. Exceeding this threshold for hydrophobicity will primarily increase the hemolytic activity of the AMP, and is thus detrimental to its selectivity.

Part 3: Designing optimal AMPs

After obtaining the data presented this far, we decided to see how applicable our results were in the design of an "optimal" anoplin analogue, and how the principles used to improve the activity of anoplin could be transferred to another helical AMP. Furthermore, we tested whether the anoplin motif could be extended in order to obtain longer and more active yet non-hemolytic AMPs. For these analogues, all the polar residues were replaced with Lys. In order to estimate how useful our optimized anoplin and citropin analogues could be as potential drugs, we decided to measure the antibacterial activity of this set of peptides against a wider selection of clinically important bacterial strains. We selected two Gram-positive and two Gram-negative bacteria, where one strain of each type is classified as a multiresistant strain. The MIC values were determined using the broth micro-dilution assay and the data are shown in Table 6.

The optimal anoplin analogues

Based on the observations of the presented set of peptides, 4 substitutions on the hydrophobic face appeared appealing: 2,10Hle, 2Cha, 10Cha, and 2(βNal), as they all had very promising selectivities. Of these, we chose to use 2,10Hle due to its similarity of the Leu residues originally located in these positions and due to the very slight modification that it actually is. On the hydrophilic face, Lys or Dab should be in position 4 and 8 as these were the most beneficial substitutions.

The results showed that our two optimized anoplin analogues 8K-2,10Hle and 4,8Dab-2,10Hle indeed had higher activity and improved selectivity against *E.coli*. Furthermore, these compounds showed equally high activities against the other bacterial strains which classify these analogues as broad spectrum compounds. These are the most active and selective analogues of anoplin published to date, and the show how the careful trimming of polarity of the hydrophilic face and of the hydrophobicity of the hydrophobic face can be used to optimize a cationic α-helical AMP.

Optimized citropin analogues

Based on the insertion depth data on Citropin (GLFDVIKKVASVIGGL-NH_2) obtained by Franzmann et al.[26], three analogues were designed using only the classic amino acids which had a higher polarity on the hydrophilic face and a stronger hydrophobic face. These substitutions gradually replaced Ser, Val, and Gly with Lys on the hydrophilic face and Ala and Val with Leu on the hydrophobic face.

As can be seen from the data in table 6, citropin is already quite active against Gram-positive bacteria, but show poor activity against Gram-negative and exhibits a high hemolytic activity. The first two ra

designed analogues of citropin significantly increased the activity against the Gram-negative bacteria, thus making it into a broad spectrum compound. These two analogues also have a moderately increased activity against the Gram-positive bacteria. However, the last analogue only shows a very modest increase in activity against one strain (*E.coli*). All the designed analogues showed increases in hemolytic activity, which was to be expected due to an increased hydrophobicity of the hydrophobic face. Citropin was already quite hemolytic before modification of its sequence, and thus AMPs with high selectivity were not obtained. This is despite the fact that the hydrophobicity per turn of the helix is lower for citropin than for anoplin.

Extension of the 8K motif

The extension of the anoplin motif (anoplin+2 turns, resulting in GLLKRIKKLLKKIKKLL-NH_2) showed an increase in activity compared to 8K by a factor of two, but it also showed a drastic increase in hemolytic activity. In fact, anoplin+2 turns shows less activity than 4,8Dab-2,10Hle, but 25-fold higher hemolytic activity. When increasing the length of the peptide even further (anoplin+4 turns, GLLKRIKKLLKKIKKLLKKIKKLL-NH_2), the compound becomes highly toxic with EC_{50} < 1μM. At the same time, the MIC values could no longer be measured reliably, which resembles the data for 5F8W and 2,3,9,10Aoc, which showed a strong tendency to self-aggregate.

Despite having the same amphipathicity per turn (i.e. the same strength of both the hydrophobic and hydrophilic face), the selectivity of the extended anoplin analogues is significantly reduced. This is a result of making the peptide too hydrophobic. It was observed in the first set of peptides, that when traversing a given threshold in hydrophobicity, selectivity is very quickly destroyed due to drastic increase in hemolysis. The longer the peptide becomes, the less hydrophobic each turn of the helix should be in order to prevent loss of selectivity and ultimately self-aggregation.

Window of useful hydrophobicity

For anoplin, it appears that there is a minimum and maximum limit to how hydrophobic the hydrophobic face of the helix may be, if selectivity should be retained. This is illustrated in Figure 9.

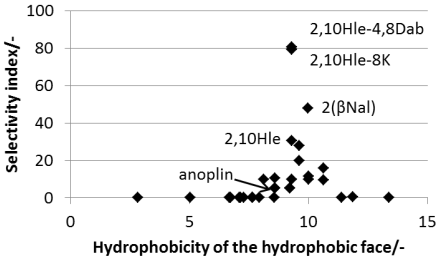

Figure 9. Plot of the hydrophobicity of the hydrophobic face of each analogue versus their selectivity for *E.coli* over red blood cells. Peptides with a MIC value above 200μM have had their selectivity set to 0, and peptides with EC50 value above 500μM have had their selectivity calculated as if the EC50 value was 500μM. The plot is based on all the anoplin analogues with substitutions on the hydrophobic face including the non-conservative substitutions 5W, 5F8W, and 5K8W.

When reducing hydrophobicity too much, activity is lost, and when increasing hydrophobicity too much, the hemolytic activity becomes too high. Staying within this window of useful hydrophobicity and modulating the polarity of the hydrophilic face allows for analogues with maximum selectivity, as illustrated by our optimized anoplin analogues. Similar (but not identical) limits are probably found for all α-helical AMPs. As seen from citropin and the extended anoplin peptides, the mean hydrophobicity of the hydrophobic face should be reduced upon increasing the length of the peptide in order to maintain selectivity.

Conclusions – the DOs and DON'Ts of Rational Design of short α-helical AMPs

The general activity of an α-helical AMP is modulated by the strength of the interaction of its hydrophilic face with the lipid head groups and the solvent, and by the strength of the interaction of its hydrophobic face with the membrane interior.

Based on the data presented here for anoplin, we propose a list of DOs and DON'Ts when trying to improve activity of a cationic α-helical AMP. It is important to keep in mind that this is only valid for the specific group of short cationic α-helical AMPs which presumably act by unspecific membrane interaction – our conclusions are not applicable to pore forming peptides or AMPs with specific targets.

Table 6. Activity data on the final set of peptides, which were designed based on the results from the peptides presented in Table 1.

	EC_{50} [µM]	MIC [µM] (selectivity)[e] *S.aureus*[a]	MIC [µM] (selectivity)[e] *E.faecium*[b]	MIC [µM] (selectivity)[e] *E.coli*[c]	MIC [µM] (selectivity)[e] *P.aeruginosa*[d]
8K-2,10Hle	500	12.5 (40.0)	12.5 (40.0)	6.3 (79.4)	6.3 (79.4)
4,8Dab-2,10Hle	250	6.3 (39.7)	6.3 (39.7)	3.1 (80.6)	3.1 (80.6)
citropin[f]	40	12.5 (3.2)	6.3 (6.3)	50 (0.8)	200 (0.2)
10L 11,12K[g]	22	12.5 (1.8)	6.3 (3.5)	6.3 (3.5)	50 (0.4)
9,10L 11,12K[g]	9	3.1 (2.9)	1.6 (5.6)	6.3 (1.4)	12.5 (0.7)
9,10L 11,12,14K[g]	6	3.1 (1.9)	1.6 (3.8)	3.1 (1.9)	12.5 (0.5)
anoplin+ 2 turns[h]	9	12.5 (0.7)	6.3 (1.4)	12.5 (0.7)	12.5 (0.7)
Anoplin+ 4 turns[i]	<1	-	-	-	-

[a] Methicillin resistant *S.aureus* ATCC 33591. [b] Vancomycin resistant *E.faecium* ATCC 700221. [c] *E.coli* ATCC 25922. [d] *P.aeruginosa* ATCC 27853. [e] The selectivity is defined as EC_{50}/MIC. [f] Citropin sequence: GLFDVIKKVASVIGGL-NH_2. [g] Citropin analogues. [h] Anoplin+2 turns sequence: GLLKRIKKLLKKIKKLL-NH_2. [i] Anoplin+4 turns sequence: GLLKRIKKLLKKIKKLLKKIKKLL-NH_2.

- Don't rely on the helical wheel model when designing AMP analogues, especially when making non-conservative mutations.

- Do identify the role of the residues: define for each residue whether its role is the interaction with the membrane interior, lipid head groups, or the bulk solvent.

- Do increase polarity of residues interacting with bulk solvent: An increase in polarity by shortening the chain length and increasing charge of the hydrophilic face will in general increase the activity of the peptide. These changes have only little effect on the hemolytic activity. For anoplin these are residues 4 and 8. The shorter lysine analogue Dab is clearly beneficial, but not the even shorter analogue Dap.

- Do change hydrophobicity of the hydrophobic face in small steps: An increase in hydrophobicity of the hydrophobic face increases the activity of the peptide, but when exceeding a peptide-length dependent threshold value it greatly increases the hemolytic activity, thus making the AMP unspecific. For anoplin a high degree of hydrophobicity is needed in all positions of the hydrophobic face, but on longer AMPs the hydrophobicity per residue should be lower.

- Do change hydrophobicity of the hydrophobic face only in a few positions: It seems to be more beneficial to increase hydrophobicity of only a few residues instead of spreading the increase across the whole hydrophobic face. This might depend on where the increase in hydrophobicity is located; our data set is not comprehensive enough on this point.

- Don't substitute residues interacting with the lipid head groups with hydrophobic residues: Higher activity of a peptide can be achieved by inserting hydrophobic residues instead of hydrophilic ones. However, when this is done in positions which enables the hydrophobic side chain to interact with the membrane interior (by bending), the peptide will lose selectivity due to large increases in hemolytic activity.

Experimental Section

Materials

All standard Fmoc protected amino acids were purchased from Iris Biotech, all non-classic amino acids from Novabiochem, α-cyano-4-hydroxycinnamic acid (CCA) from Bruker Daltonics, DPC-d_{38} (98% D) from Cambridge Isotope Laboratories, and the remaining chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Peptide Synthesis

All peptides shown in Tables 1-5 were synthesized by Fmoc solid state peptide synthesis[27] using TentaGel S (200 mg RAM resin with 0.24 mmol/g) as solid support. Coupling was performed twice for each amino acid using Fmoc protected amino acid (3 equiv.), *N,N'*-Diisopropylcarbodiimide (DIC) (3 equiv.), and 1-hydroxy-7-aza-benzotriazole (HOAt) (3 equiv.) dissolved in *N*-methyl-2-pyrrolidone (NMP) for 1.5 hours. Deprotection was done using 20 % piperidine in NMP first for 3 min and then for 7 min. Between each coupling and deprotection step, the resin was washed 7 times with NMP. Cleavage of peptides from resin was performed in 95% trifluoroacetic acid (TFA)/5% triisopropyl silane (TIS) for 2 hours. The resin was then washed with 100% TFA. Solutions from cleavage and washing were collected and pooled. The peptides were subsequently precipitated and washed twice in diethyl ether, before lyophilization.

All peptides shown in Table 6 were purchased from GenScript (USA inc, www.genscript.com), purified to >98%.

HPLC Purification

The lyophilized peptides were dissolved in 1mL of 50% acetonitrile in water, to which two drops of 99.8% acetic acid were added. Purification was done using a Grace C18-reverse-phase column (10-15 µm dp, 300 Å pore size) on a Waters system and Empower Pro software.

Mass Spectrometry

Masses of the purified peptides were measured using matrix-assisted laser desorption ionization-Time of flight mass spectrometry (MALDI-TOF MS) using a Bruker Microflex A099-01 MALDI. The samples were prepared using CCA as matrix.

NMR quantification

The purified peptide samples were lyophilized and dissolved in a freshly made solution (550µl) of TFE (5 mM) in D_2O and transferred to NMR tube. The samples were measured using the standard 1D ^1H Bruker pulse sequence with a relaxation delay between scans of 30s. Quantification was done by integrating resonances of lysine H^{ε} and Arg H^{δ} (CH_2 group) using the CH_2 group of TFE as internal standard.

Broth micro-dilution assay:

The MIC of the peptides were either tested on *E.coli* ATCC 25922 alone or on the four strains listed in Table 2 by the broth micro-dilution method [22] in 96-well polypropylene micro titer plates. The strain suspensions used in the test were diluted to reach a concentration at $5x10^5$ CFU/mL in Mueller-Hinton II bouillon. The peptides were diluted in a series of twofold dilutions ranging from 256 to 0.25 µg/mL in Mueller-Hinton II bouillon. The bacterial strains were incubated for 18 hours after which the MIC values were read as the lowest concentration of peptide where there was no visible growth. The antibiotic gentamycin was used as control.

oCelloScope

Experiments were performed on the Gram-negative facultative aerobe bacterium, *E.coli* ATCC 25922. An overnight culture (0.1 mL) was inoculated in 8 mL of Müller-Hinton broth (Merck, VWR, Herlev, Denmark) for 2 h (37°C) to reach the exponential phase. The cell suspensions were standardized by adjusting the concentration of inoculates to $2.3x10^7$ cells/mL, determined by measurements of the optical density at 600 nm (UV-3100 PC spectrophotometer; VWR, Herlev, Denmark), and subsequently diluting to a final bacterial cell suspension of $5x10^5$ cells/mL. Beads were added to bacterial cell suspensions in order to focus the microscope ($2×10^4$ 6-µm beads/mL, microsphere standard, B-7277; Invitrogen, Naerum, Denmark) and loaded onto an F-base microtiter plate (50 µL/well) (Sigma-Aldrich, Brøndby, Denmark) either untreated (control samples) or treated with antibiotic peptides of interest.

Antibiotic susceptibility tests were performed using the oCelloScope detection system (Unisensor A/S, Allerød, Denmark). Each well was scanned repeatedly every 10 min. Time-lapse experiments, digital analysis, and image processing were conducted by use of the pixel histogram summation algorithm as previous described [21]. The oCelloScope was placed within an Innova 44 incubator (New Brunswick Scientific) in order to keep the temperature constant at 37ºC.

An initial screening was performed with peptides in a series of twofold dilutions ranging from 256 to 0.25 µg/mL. Then, two series were performed with peptides in concentrations of 50%, 75%, 100%, 150%, and 200% of the MIC value observed in the initial screening.

Hemolytic activity assays

Human erythrocytes (0 rhesus positive) were washed three times with phosphate buffered saline (PBS) (0.15 M), followed by centrifugation for 2x8 minutes at 3000 rpm and 1x8 minutes at 4000 rpm. After washing, the erythrocytes were diluted to 0.5% v/v in 0.15 M PBS. 0.75µL of the erythrocyte solution was added to a 96-well polypropylene plate with V-shaped bottoms together with peptide solution (0.75 µL). The peptides were diluted in a series of twofold dilutions ranging from 500 to 0.98 µg/mL. The plate was then incubated at 37°C for 1 hour. After incubation, the plate was centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a 96-well polystyrene microtiter plate and the absorbance was read with an ELISA reader at 414 nm. Triton-X-100 (0.2%) was used as positive control (100% hemolysis)

NMR Spectroscopy

For structural analysis, each peptide was dissolved to 3 mM, 5% D_2O, NaN_3 (2 mM) and DPC-d_{38} (98% D) (150 mM) in phosphate buffer (10 mM), pH 6.5.

All spectra were recorded on a Bruker DRX 600 spectrometer with a 5mm TXI(H/C/N) or a 5mm TCI (H/C/N) probe, and on a BRUKER AV 900 spectrometer with a 5mm TCI (H/C/N) probe. For all peptides, NMR data were recorded at 37ºC. However, in some cases, additional NMR data measured at 20 ºC were used for resolving overlapping spin systems. TopSpin v. 1.3 was used for recording and TopSpin v. 2.1 was used for processing NMR data. The following spectra were recorded: ^1H-^1H-TOCSY with 75 ms mixing time, ^1H-^1H-NOESY with 60 ms mixing time, ^1H-^{13}C-HSQC at natural abundance, and occasionally ^1H-^1H-COSY. Watergate was used for water suppression in homonuclear 2D-spectra.

The individual spin systems were assigned in the ^1H-^1H-TOCSY spectra using CARA v. 1.8.4 with the aid of the ^1H-^{13}C-HSQC, ^1H-^1H-COSY, and ^1H-^1H-NOESY spectra. Subsequently, integration of NOE cross peaks were performed in the NEASY subroutine of CARA v. 1.5.5.[28] The C^{α} and C^{β} chemical shifts were obtained from the ^1H-^{13}C-HSQC spectra and used to calculate backbone torsion angle restrains using the program TALOS+.[29]

NMR assignments have been submitted to the BioMagResBank (anoplin: 11551, 5W: 11552, 5K8W: 11553, 5F8W: 11554).

PRE constraints

PRE constraints were derived as described by Franzmann et al.[18] Eight inversion recovery NOESY spectra with recovery delay times of 1, 50, 150, 400, 700, 1200, 2600, and 4000 ms were recorded at 600 MHz in a pseudo-3D manner for each of the four Gd(DTPA-BMA) titration points: 0, 2, 5, 10 mM. All the reliable peaks with H^{α} in the indirect dimension were integrated in all spectra in the NEASY subroutine of CARA v. 1.5.5. R_1 relaxation rates were determined, and by a linear fit of the relaxation rates for the 4 titration points the PRE values were determined. The PRE values were then averaged for each H^{α} and converted to distance restraints to the micelle center. These restraints were used as upper and lower distance restraints with values of plus and minus 1Å, respectively. The PRE distance restraints were weighted with 10% compared to the NOE distance restrains, in order to prevent distortion of the peptide structure.

On the basis of the NOE-derived distance constraints, angle restraints, and PRE-derived distance restraints, 80 structures of each peptide were calculated using CYANA v. 2.1,[30] after which, the 20 structures with the lowest target function value were chosen for restrained refinement.

Restrained refinement

The 20 output structures from CYANA were energy minimized using YASARA.[20] The peptide structures were loaded one at a time into a simulation cell together with a DPC micelle obtained from the homepage of Professor Peter Tieleman at the University of Calgary (http://moose.bio.ucalgary.ca).[31] For each peptide, 10 repetitions of the refinement were performed, and for each repetition, the DPC micelle was oriented randomly before simulation start in order to insert the peptide at different locations. Then the peptide is pulled into the micelle by adding the PRE distance restraints. Subsequently, the molecular dynamics simulation is turned on in order to minimize the van der Waals repulsion between the atoms of the soup. This energy minimization is initially done *in vacuo* using the NOVA force field. After this initial refinement, the simulation cell is filled with water molecules. The,n another round of restrained molecular dynamics simulation is performed using the YASARA force field, and final structure statistics are calculated.

Finally, out of the 10 refinement repetitions for each input peptide structure, the structure with the lowest constraint violation energy is selected for the final ensemble.

Geometrical analysis

The orientation of the side chains in each of the final structures were calculated in two steps: first, the helical axis was calculated by the GroupLine command in YASARA, returning a regression line optimally fitting the backbone atoms ($N+C^{\alpha}+C$) of residues 2-10 (the ones that were folded into helix). From this line, the direction towards the geometric mean of each side chain or H^{α} relative to the direction towards the micelle center was calculated.

Abbreviations

AMP: antimicrobial peptide, Aoc: L- 2-amino octanoic acid, BCA: bicinchoninic acid, βNal: β-2-naphthylalanine, CCA: α-cyano-4-hydroxycinnamic acid, Cha: β-cyclohexylalanine, cmc: critical micelle concentration, COSY: correlation spectroscopy, Dab: 2,4-diaminobutyric acid, Dap: 2,3-diaminopropionic acid, DIC: N,N´-diisopropylcarbodiimide, DPC: dodecyl phosphocholine, Epa: 2-amino,3-ethylpentanoic acid, Fmoc: fluorenylmethyloxycarbonyl chloride, Gd(DTPA-BMA): Gadolinium diethylenetriaminepentaacetic acid bismethylamide, HOAt: 1-hydroxy-7-aza-benxotriazole , Hle: homoleucine, HPLC: high pressure liquid chromatography, Hser: Homoserine, HSQC: heteronuclear single quantum coherence, MIC: minimum inhibitory concentration, Nle: norleucine, NMP: *N*-methyl-2-pyrrolidone, NMR: nuclear magnetic resonance, NOE: nuclear Overhauser effect, NOESY: nuclear Overhauser effect spectroscopy, Nva: norvaline, O-Me-Ser: O-methyl-serine, Orn: Ornithine, PBS: phosphate buffered saline, PRE: paramagnetic relaxation enhancement, RMSD: root mean square deviation, SDS: sodium dodecyl sulfate, TFA: trifluoroacetic acid, TFE: triflouroethanol, TIS: triisopropyl silane, TOCSY: total correlation spectroscopy.

Acknowledgements

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Keywords: anoplin · antimicrobial peptides · NMR spectroscopy · paramagnetic relaxation enhancement · structure-activity relationships

[1] R. Laxminarayan, A. Duse, C. Wattal, A. K. M. Zaidi, H. F. L. Wertheim, N. Sumpradit, E. Vlieghe, G. L. Hara, I. M. Gould, H. Goossens, et al., *Lancet Infect. Dis.* **2013**, *13*, 1057–98.

[2] J. Bradshaw, *BioDrugs* **2003**, *17*, 233–40.

[3] G. Wang, X. Li, Z. Wang, *Nucleic Acids Res.* **2009**, *37*, D933–937.

[4] Y. Chen, C. T. Mant, S. W. Farmer, R. E. W. Hancock, M. L. Vasil, R. S. Hodges, *J. Biol. Chem.* **2005**, *280*, 12316–12329.

[5] A. Giangaspero, L. Sandri, A. Tossi, *Eur. J. Biochem.* **2001**, *268*, 5589–5600.

[6] M. Dathe, T. Wieprecht, H. Nikolenko, L. Handel, W. L. Maloy, D. L. MacDonald, M. Beyermann, M. Bieı *FEBS Lett.* **1997**, *403*, 208–212.

[7] M. Dathe, H. Nikolenko, J. Meyer, M. Beyermann, M. Bienert, *FEBS Lett.* **2001**, *501*, 146–150.

[8] T. Wieprecht, M. Dathe, E. Krause, M. Beyermann, W. L. Maloy, D. L. MacDonald, M. Bienert, *FEBS Lett.* **1997**, *417*, 135–140.

[9] I. Zelezetsky, S. Pacor, U. Pag, N. Papo, Y. Shai, H.-G. Sahl, A. Tossi, *Biochem. J.* **2005**, *390*, 177–188.

[10] R. Bessalle, H. Haas, A. Goria, I. Shalit, M. Fridkin, *Antimicrob. Agents Chemother.* **1992**, *36*, 313–317.

[11] N. Yang, T. Lejon, O. Rekdal, *J. Pept. Sci.* **2003**, *9*, 300–11.

[12] K. Konno, M. Hisada, R. Fontana, C. C. Lorenzi, H. Naoki, Y. Itagaki, A. Miwa, N. Kawai, Y. Nakata, T. Yasuhara, et al., *Biochim. Biophys. Acta* **2001**, *1550*, 70–80.

[13] D. Ifrah, X. Doisy, T. S. Ryge, P. R. Hansen, *J. Pept. Sci.* **2005**, *11*, 113–121.

[14] J. K. Munk, L. E. Uggerhøj, T. J. Poulsen, N. Frimodt-Møller, R. Wimmer, N. T. Nyberg, P. R. Hansen, *J. Pept. Sci.* **2013**, *19*, 669–675.

[15] A. Won, S. Pripotnev, A. Ruscito, A. Ianoul, *J. Phys. Chem. B* **2011**, *115*, 2371–2379.

[16] K. Meinike, P. R. Hansen, *Protein Pept. Lett.* **2009**, *16*, 1006–1011.

[17] J. C. Slootweg, T. B. van Schaik, H. (Linda) C. Quarles van Ufford, E. Breukink, R. M. J. Liskamp, D. T. S. Rijkers, *Bioorg. Med. Chem. Lett.* **2013**, *23*, 3749–3752.

[18] M. Franzmann, D. Otzen, R. Wimmer, *Chembiochem* **2009**, *10*, 2339–2347.

[19] M. P. Dos Santos Cabrera, M. Arcisio-Miranda, S. T. Broggio Costa, K. Konno, J. R. Ruggiero, J. Procopio, J. Ruggiero Neto, *J. Pept. Sci.* **2008**, *14*, 661–669.

[20] E. Krieger, G. Koraimann, G. Vriend, *Proteins* **2002**, *47*, 393–402.

[21] M. Fredborg, K. R. Andersen, E. Jørgensen, A. Droce, T. Olesen, B. B. Jensen, F. S. Rosenvinge, T. E. Sondergaard, *J. Clin. Microbiol.* **2013**, *51*, 2047–2053.

[22] C. Thornsberry, L. K. McDougal, *J. Clin. Microbiol.* **1983**, *18*, 1084–1091.

[23] S. Padmanabhan, E. J. York, J. M. Stewart, R. L. Baldwin, *J. Mol. Biol.* **1996**, *257*, 726–734.

[24] J.-L. Fauchère, M. Charton, L. B. Kier, A. Verloop, V. Pliska, *Int. J. Pept. Protein Res.* **1988**, *32*, 269–278.

[25] L.-N. Zhu, C.-Y. Fu, S.-F. Zhang, W. Chen, Y.-T. Jin, F.-K. Zhao, *J. Pept. Sci.* **2013**, *19*, 566–74.

[26] M. Franzmann, *Novel Applications of Constrast Agents in Structural Biology of Peptides and Detergents.*, Aalborg University, Ph.D. Thesis, **2010**.

[27] G. B. Fields, R. L. Noble, *Int. J. Pept. Protein Res.* **2009**, *35*, 161–214.

[28] R. Keller, *The Computer Aided Resonance Assignment Tutorial*, CANTINA Verlag, Goldau (Switzerland), **2004**.

[29] Y. Shen, F. Delaglio, G. Cornilescu, A. Bax, *J. Biomol. NMR* **2009**, *44*, 213–223.

[30] B. López-Méndez, P. Güntert, *J. Am. Chem. Soc.* **2006**, *128*, 13112–22.

[31] D. P. Tieleman, D. van der Spoel, H. J. C. Berendsen, *J. Phys. Chem. B* **2000**, *104*, 6380–6388.

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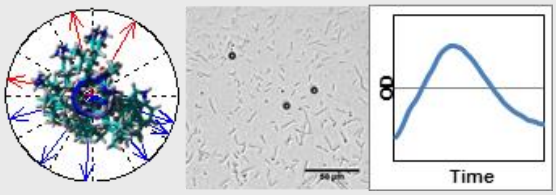

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Rational Design of alpha-helical antimicrobial peptides – DOs and DON'Ts

There are lots of examples on how the activity of α-helical antimicrobial peptides has been enhanced. However, general guidelines have not yet been reported. Here, we investigate the effect of mutations on the structure and membrane insertion of peptides, present structure-based guidelines to enhance activity and selectivity of small α-helical antimicrobial peptides, and put them to the test.

Supplementary Material

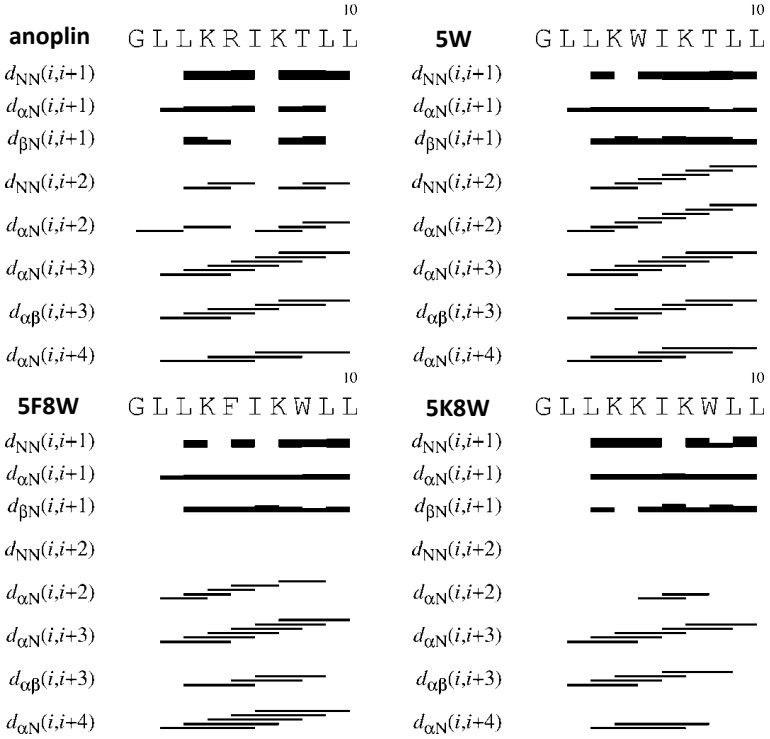

Figure S1: Graphical overview of the NOEs used for the structure calculations of each peptide.

	Anoplin	R5W	R5F_T8W	R5K_T8W
Number of distance constraints				
Intra-residue	44	49	52	64
Sequential (i-j=1)	22	33	28	28
Medium-range (1<i-j<5)	28	59	27	15
To micelle center	15	14	15	12
TALOS+ derived dihedral angle constraints				
ϕ Angles	6	8	7	7
Ψ Angles	6	8	7	7
RMSD for residue 2-10				
Average backbone (N + C^{α} + C)	0.19±0.06	0.16±0.04	0.24±0.10	0.24±0.10
Average heavy atoms	1.07±0.23	0.79±0.17	1.18±0.21	1.04±0.27
Constraint violations				
No of NOE constraint violations > 0.1Å	0	0	0	0
Maximum NOE violation	0.1	0.099	0.1	0.1
No of Dihedral angle constraint violations > 5°	0	1	4	0
Maximum PRE constraint violation	0.31	0.15	0.22	0.29
Ramachandran plot statistics				
Residues in favored regions	96.9%	100%	97.5%	99.4%
Residues in additional allowed regions	1.3%	0%	0.6%	0.6%
Residues in generally allowed regions	0%	0%	0.6%	0%
Residues in disallowed regions	1.9%	0%	1.3%	0%
Average energy results (kJ/mol)				
Total energy	-36718±926	-33967±1261	-35013±1132	-34664±2543
Electrostatic solv. Energy	-23044±1211	-22507±980	-22728±1223	-22414±1022
Van der Waals solv. energy	-4077±217	-4107±185	-4014±247	-4041±143

Table S2: A list of the amount and types of constraints used for calculation of the peptide structures followed by statistics for the ensembles of structures after restrained refinement in YASARA. The PRE derived distance constraints to the micelle center are mainly from H^{α}, but in cases where the H^{N} shows no water exchange peak, distance constraints from H^{N} to the micelle center were also included. The PRE constraint violations are of higher magnitude than the NOE constraint violations. This is a consequence of the weighting of the PRE constraints relative to NOE constraints. The maximum PRE constraint violation was 0.31 Å and is thus quite small in relation to the length of the constraints, where the minimum distance was approximately 14 Å.

	anoplin					**R5W**				
residue	insertion depth	No. of peaks	RMSD	lower limit	upper limit	insertion depth	No. of peaks	RMSD	lower limit	upper limit
2	19.31	2	0.013	19.03	19.56	17.92	5	0.008	17.64	18.17
3	17.32	7	0.006	17.05	17.57	16.53	6	0.008	16.13	16.89
4	21.61	3	0.007	21.55	21.66	21.52	3	0.013	21.40	21.63
5	21.34	5	0.055	20.77	21.80	20.29	6	0.044	19.56	20.85
6	16.04	4	0.009	15.48	16.52	16.13	3	0.009	15.61	16.58
7	19.01	7	0.018	18.56	19.40	18.89	2	0.000	18.88	18.89
8	23.16	3	0.045	22.98	23.33	22.45	1	-	-	-
9	20.23	4	0.014	20.02	20.42	19.75	5	0.033	19.10	20.28
10	18.99	3	0.011	18.72	19.25	18.52	3	0.002	18.47	18.56
	R5F_T8W					**R5K_T8W**				
residue	insertion depth	No. of peaks	RMSD	lower limit	upper limit	insertion depth	No. of peaks	RMSD	lower limit	upper limit
2	16.87	5	0.007	16.54	17.17	18.5	1	-	-	-
3	16.82	7	0.004	16.63	17.00	17.03	4	0.002	16.94	17.12
4	21.05	4	0.009	20.95	21.14	21.46	4	0.027	21.21	21.68
5	18.71	5	0.005	18.58	18.83	22.22	4	0.029	22.03	22.39
6	14.68	6	0.007	14.01	15.25	16.26	3	0.002	16.14	16.39
7	18.92	5	0.009	18.70	19.12	18.88	5	0.019	18.38	19.31
8	21.38	1	-	-	-	22.91	4	0.066	22.60	23.18
9	17.11	4	0.003	17.00	17.22	20.24	3	0.032	19.73	20.66
10	18.08	3	0.002	18.00	18.15	18.79	3	0.012	18.49	19.07

Table S3: Raw data obtained from the paramagnetic relaxation enhancement experiments for anoplin and the three analogs R5W, R5F_T8W, and R5K_T8W. The quantity of paramagnetic relaxation enhancement is defined as the slope of the linear fit to the relaxation rates obtained from the titration steps. This slope is calculated for each useful H^{α} NOESY cross peak for each residue. The insertion depth is then calculated based on the average slope for each residue. The RMSD value is calculated for the slopes, and when subtracting and adding this value from the mean slope, the lower and upper limits of insertion depth are calculated.

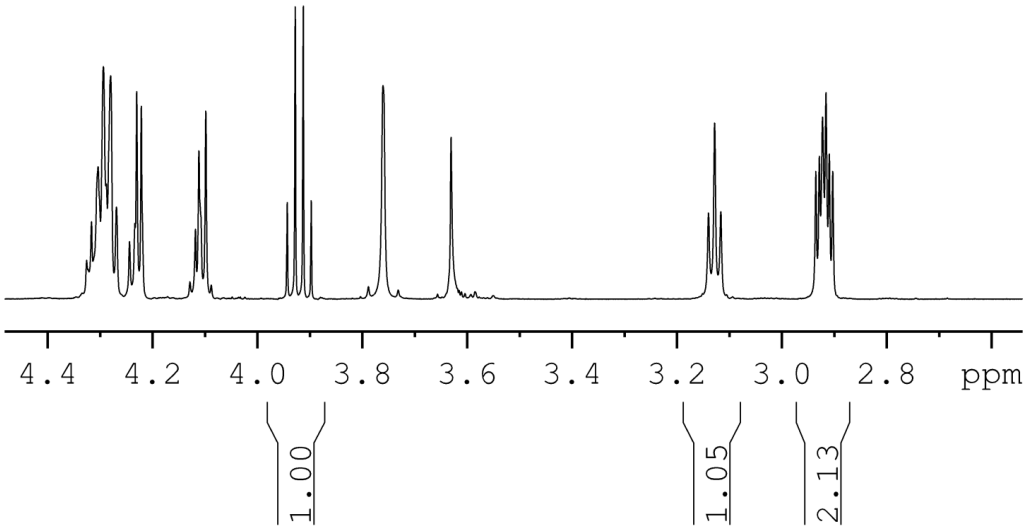

Figure S4: Example for ^{1}H-NMR spectra used to quantify peptides: region of the 1D NMR spectrum of 10Cha in the presence of 5mM TFE. The peak integrals have been normalized to of the CH_2 group of TFE (integral=1.00). The triplet at 3.13 ppm (integral 1.05) is the signal of Arg5 H^{δ} and the multiplet at 2.84 ppm (integral 2.13) is the signal of H^{δ} of both Lys4 and Lys7.

Table S5. Overview of the activities of the anoplin analogs[a].

Peptide	Sequence	MIC	EC_{50}	Selectivity index[d]	Hydrophobicity[e]
Substitutions on the hydrophilic face[f]					
4T	GLL**T**RIKTLL-NH_2	200	>500	>2.5	7.12
4Hser	GLL**Hser**RIKTLL-NH_2	150	>500	>3.3	~7.12
4S	GLL**S**RIKTLL-NH_2	100	>500	>5	6.82
Anoplin	GLLKRIKTLL-NH_2	100	>500	>5	5.87
4Orn	GLL**Orn**RIKTLL-NH_2	100	>500	>5	5.75
8S	GLLKRIK**S**LL-NH_2	100	>500	>5	5.57
4Dab	GLL**Dab**RIKTLL-NH_2	75	>500	>6.7	5.52
4Dap	GLL**Dap**RIKTLL-NH_2	100	>500	>5	5.26
5K8K	GLLK**K**IK**K**LL-NH_2	25	>500	>20	4.64
8K	GLLKRIK**K**LL-NH_2	25	>500	>20	4.62
4,8Dab	GLL**Dab**RIK**Dab**LL-NH_2	12.5	>500	>40	3.92
Substitutions on the hydrophobic face					
2,3,9,10(O-Me-Ser)	G**(O-Me-Ser)(O-Me-Ser)**KRIKT**(O-Me-Ser)(O-Me-Ser)**-NH_2	>200	>500	-	~0.11
2,10(O-Me-Ser)	G**(O-Me-Ser)**LKRIKTL**(O-Me-Ser)**-NH_2	>200	>500	-	~2.29
2,3,9,10V	G**VV**KRIKT**VV**-NH_2	>200	>500	-	3.95
2,3,9,10M	G**MM**KRIKT**MM**-NH_2	>200	>500	-	3.99
2(O-Me-Ser)	G**(O-Me-Ser)**LKRIKTLL-NH_2	>200	>500	-	~4.43
2,3,9,10Nva	G**NvaNva**KRIKT**NvaNva**-NH_2	>200	>500	-	4.55
2,10V	G**V**LKRIKTL**V**-NH_2	>200	>500	-	4.91
3,9M	GL**M**KRIKT**M**L-NH_2	>200	>500	-	4.93
2,10M	G**M**LKRIKTL**M**-NH_2	>200	500	<2.5	4.93
2,10Nva	G**Nva**LKRIKTL**Nva**-NH_2	>200	>500	-	5.21
2M	G**M**LKRIKTLL-NH_2	200	400	2	5.4
Anoplin	GLLKRIKTLL-NH_2	100	>500	>5	5.87
2,10Nle	G**Nle**LKRIKTL**Nle**-NH_2	100	>500	>5	5.87
2,3,9,10Nle	G**NleNle**KRIKT**NleNle**-NH_2	100	>500	>5	5.87
2,10Epa	G**Epa**LKRIKTL**Epa**-NH_2	50	>500	>10	~6.57
2,10Hle	G**Hle**LKRIKTL**Hle**-NH_2	12.5	380	30.4	~6.57
2Cha	G**Cha**LKRIKTLL-NH_2	25	500	20	6.89
10Cha	GLLKRIKTL**Cha**-NH_2	12.5	350	28	6.89
2(βNal)	G**βNal**KRIKTLL-NH_2	6.25	300	48	7.25
2,3,9,10Hle	G**HleHle**KRIKT**HleHle**-NH_2	12.5	125	10	~7.27
2,10Aoc	G**Aoc**LKRIKTL**Aoc**-NH_2	6.25	71	11.4	~7.27
2,9Cha	G**Cha**LKRIKT**Cha**L-NH_2	12.5	120	9.6	7.91
2,10Cha	G**Cha**LKRIKTL**Cha**-NH_2	6.25	100	16	7.91
2,3,9,10Aoc[g]	G**AocAoc**KRIKT**AocAoc**-NH_2	-	20	-	~8.67
Modulation of hydrophobicity profile					
2,10A6(βNal)	G**A**LKR**βNal**KTL**A**-NH_2	>200	>500	-	4.37
2,3,9,10Nva6(βNal)	G**NvaNva**KR**βNal**KT**NvaNva**-NH_2	>200	>500	-	5.83
Anoplin	GLLKRIKTLL-NH_2	100	>500	>5	5.87
2,10Nva6(βNal)	G**Nva**LKR**βNal**KTL**Nva**-NH_2	100	>500	>5	6.49
3,9Nva6(βNal)	GL**Nva**KR**βNal**KT**Nva**L-NH_2	100	>500	>5	6.49
Non-conservative substitutions					
Anoplin	GLLKRIKTLL-NH_2	100	>500	>5	5.87
5K8W	GLLK**K**IK**W**LL-NH_2	12.5	130	10.4	7.88
5W	GLLK**F**IKTLL-NH_2	37.5	20	0.5	9.13
5F8W[g]	GLLK**F**IK**W**LL-NH_2	-	2	-	10.66

[a] The full names of the non-classic amino acids are given in the abbreviation list, and their structures are shown in Figure S4. [b] Measured against *E.coli* ATCC 25922. [c] Hemolytic activity measured against human erythrocytes (0 rhesus positive). [d] The selectivity index is defined as EC_{50}/MIC. [e] The residual hydrophobicities are obtained from the work of Fauchère[24]. This scale was selected because it contained experimental data on some of the non-classic amino acids. The hydrophobicities with a "~" in front are estimated by extrapolation of the loss or gain in hydrophobicity upon removing or adding a CH_2 group to functionally identical side chains. [f] For this set of peptides, a reduction in hydrophobicity equals an increase in polarity of the polar face. [g] Sample could not have their MIC value measured reliably due to poor solubility.

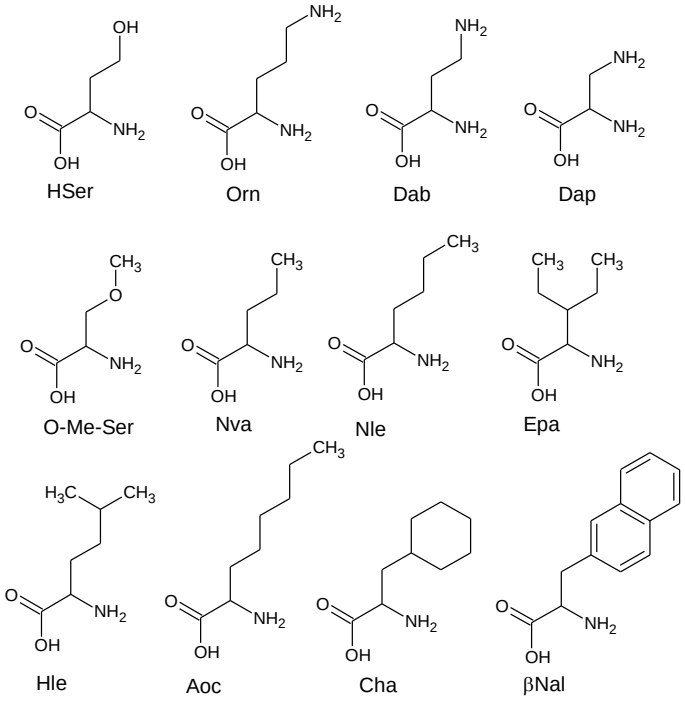

Figure S6: structures of the non-classic amino acids used in this paper. HSer=homoserine, Orn=ornithine, Dab=2,4-diaminobutanoic acid, Dap=2,3-diaminopropanoic acid, O-Me-Ser=*O*-methyl-serine, Nva=norvaline, Nle=norleucine, Epa=2-amino,3-ethylpentanoic acid, Hle=homoleucine, Aoc=2-aminooctanoic acid, Cha=3-cyclohexylalanine, βNal=3(2-naphtyl)alanine

A B

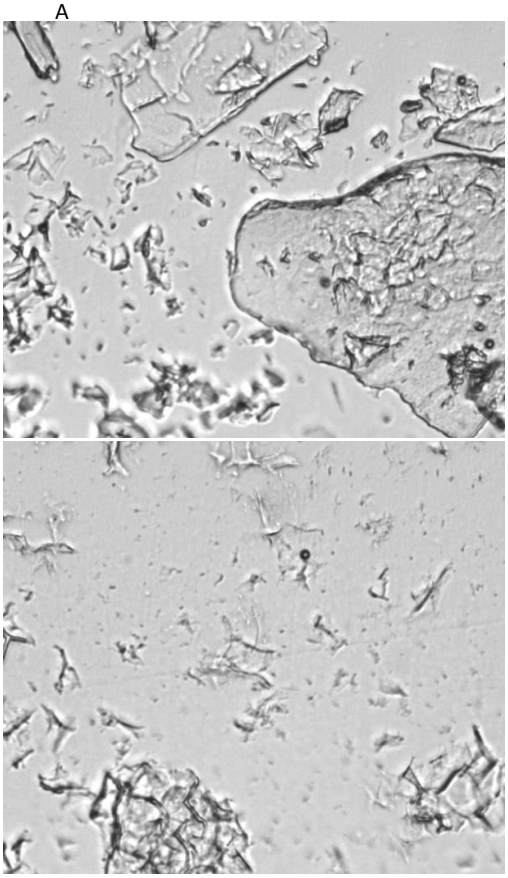

Figure S7: Microscopy images of the sample solutions obtained from the oCelloScope experiments. A: Obtained from 100µM of 5F8W dissolved in Mueller Hinton II bullion. B: Obtained from 100µM of 2,3,9,10Aoc dissolved in Mueller Hinton II bullion.

Structural Features of Peptoid-Peptide Hybrids in Lipid-Water Interfaces

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Abstract
The inclusion of peptoid monomers into antimicrobial peptides (AMPs) increases their proteolytic resistance, but introduces conformational flexibility (reduced hydrogen bonding ability and *cis/trans* isomerism). We here use NMR spectroscopy to answer how the insertion of a peptoid monomer influences the structure of a regular α-helical AMP upon interaction with a DPC micelle. Insertion of [(2-methylpropyl)amino]acetic acid in maculatin-G15 shows that the structural change and conformational flexibility depends on the site of insertion. This is governed by the micelle interaction of the amphipathic helices flanking the peptoid monomer and the side chain properties of the peptoid and its preceding residue.

Keywords
Antimicrobial peptides, peptoids, NMR, maculatin, paramagnetic relaxation enhancement

Abbreviation
AMP: antimicrobial peptide, CCA: α-cyano-4-hydroxycinnamic acid, COSY: correlation spectroscopy, DIPCDI: Diisopropylcarbodiimide, DPC: dodecyl phosphocholine, Fmoc: fluorenylmethyloxycarbonyl chloride, Gd(DTPA-BMA): Gadolinium diethylenetriaminepentaacetic acid bismethylamide, HPLC: high pressure liquid chromatography, HSQC: heteronuclear single quantum coherence, Nleu: [(2-methylpropyl)amino]acetic acid, NOE: nuclear Overhauser effect, NOESY: nuclear Overhauser effect spectroscopy, PRE: paramagnetic relaxation enhancement, RMSD: root mean square deviation, SDS: sodium dodecyl sulfate, TFA: trifluoroacetic acid, TFE: triflouroethanol, TIS: triisopropyl silane, TOCSY: total correlation spectroscopy.

Highlights
- A high-resolution structure of a peptoid-peptide hybrid ("peptomer") is presented
- The peptoid residue breaks the helix and displays *cis*/*trans* isomerism
- Maculatin-Nleu11 displays only the *trans* isomer
- Maculatin-Nleu13 displays almost equimolar amounts of *cis* and *trans* conformers
- Peptide amphipathicity and peptoid hydrophobicity govern the *cis*/*trans* isomerism

Introduction
Antimicrobial peptides (AMPs) hold great potential as future antibiotics, as they show high antimicrobial activity against even multiresistant bacteria. However, AMPs are prone to proteolytic degradation and thus have short life times in the body. In order to increase proteolytic stability of AMPs, several peptidomimetics are researched. These include D-amino acids, peptoids, β-peptides, and hybrids hereof.[1–6]

Single peptoid (N-substituted glycine[7]) residues in a peptide chain are conformationally flexible, as backbone hydrogen bonding is impossible due to the absence of H^N atoms, which are a major participant in stabilizing secondary structures. Furthermore, the *cis* and *trans* conformations can be equally favorable, causing the presence of both conformations.[8]

Hybrids of peptides and peptoids are called "peptomers"[9] and examples of these have been found in nature, e.g. cyclosporine. (The term "pepto

however, also used for polymers of peptides without any peptoid residues.[10]) Artificial peptomers have been constructed as mimics of bacterial quorum sensing signals[11], synthetic inhibitors of a kinase[12], or as novel pharmaceuticals[13]. Lee et al introduced peptoid residues into folded ribonuclease A and demonstrated that the resulting peptomer still retained some activity.[14]

Peptoid residues were also successfully incorporated into AMPs: Incorporation of two alanine peptoid residues into the hydrophobic face of an α-helical AMP significantly reduced its hemolytic activity but not its antibacterial activity.[15] Substituting some of the leucine resiudes in the zipper motif of melittin with different peptoid residues resulted in helix disruption, but the antibacterial activity was still intact (while hemolysis was significantly reduced).[16] Also hybrids with alternating peptide/peptoid building blocks were shown to have antimicrobial activity.[3,17–19]

In this work, we investigate the effect of a single peptoid substitution on the structure of an α-helical AMP bound to a micelle. We have chosen maculatin as model AMP. Wild type maculatin 1.1, is a cationic 21 amino acid AMP (GLFGVLAKVAAHVVPAIAEHF-NH_2) extracted from the Australian frog *Litoria genimaculata*.[20] Maculatin exhibits antimicrobial activity against various microbial strains. It is unstructured in water, but in the presence of 50% TFE or DPC micelles it folds into an α-helix with a slight kink at Pro 15.[21] Maculatin-G15 (P15G mutation)[22] was found to fold into a complete α-helix in the presence of DPC micelles (Figure 1A). We use the continuous α-helix in maculatin-G15 as the scaffold for studying both the local and global structural consequences of inserting a peptoid monomer into a regular α-helical AMP. As model peptoid residue, we chose Nleu, [(2-methylpropyl)amino]acetic acid (Figure S1), as its side chain is identical to that of leucine, a very frequent amino acid in AMPs.

Materials and Methods

Materials

All standard Fmoc protected amino acids, 99.5% Isobutylamine, 99% bromoacetic acid, trifluoroacetic acid (TFA), and triisopropyl silane (TIS), were purchased from Fluka. Piperidine and N,N'-Diisopropylcarbodiimide (DIPCDI) from Iris Biotech. α-cyano-4-hydroxycinnamic acid (CCA) from Bruker Daltonics. DPC and SDS from Avanti Polar Lipids and DPC-d_{38} (98% D) and SDS-d_{25} (98% D) from Cambridge Isotope Laboratories, and the remaining chemicals were purchased from Sigma-Aldrich.

Peptide Synthesis

Peptomers were synthesized using Fmoc solid phase peptide synthesis and the submonomer approach[23,24], purified by HPLC, and verified by mass spectroscopy as described before.[3]

Calculation of Expected Short Distances

Starting from the structures of *cis* and *trans*-maculatin-Nleu11, respectively, distances between atoms of interest were calculated while systematically varying one or two dihedral angles. Dihedral angles were defined as following with Nleu as residue *i*:
ϕ_i: C_{i-1}-N_i-$C^{\alpha}{}_i$-C'_i, ψ_i: N_i-$C^{\alpha}{}_i$-C'_i-N_{i+1}. In the case of Nleu, χ_1 was defined as C_{i-1}-N_i-$C^{\beta}{}_i$-$C^{\gamma}{}_i$.

NMR Spectroscopy

Each peptide was dissolved to 3 mM in 10 mM phosphate buffer, pH 6.5, containing 5% D_2O, 2 mM NaN_3 and, 150 mM DPC-d_{38} or SDS-d_{25}.
Spectra were recorded on Bruker DRX600 spectrometers at 37ºC. Additional spectra measured at 20 ºC were used for resolving overlapping spin systems. TopSpin v. 1.3 and 2.1 were used for recording processing NMR data. The following spectra were recorded: ^{1}H-^{1}H-TOCSY (75 ms mixing time), ^{1}H-^{1}H-NOESY (60 ms mixing time), ^{1}H-^{1}H-COSY and ^{1}H-^{13}C-HSQC (natural abundance). Excitation sculpting[25] was used for water suppression in homonuclear 2D-spectra.
The individual spin systems were assigned in the ^{1}H-^{1}H-TOCSY spectra using CARA v. 1.8.4 with the aid of the ^{1}H-^{13}C-HSQC, ^{1}H-^{1}H-COSY, and ^{1}H-^{1}H-NOESY spectra. Subsequently, integration of NOE cross peaks were performed in the NEASY subroutine of CARA v. 1.5.5 [26]. C^{α} and C^{β} chemical shifts were obtained from the ^{1}H-^{13}C-HSQC spectra and used to calculate backbone torsion angle restrains using the program TALOS+.[27] The peptoid residue itself and the residues preceding and succeeding the peptoid residue, were excluded from TALOS+ analysis.

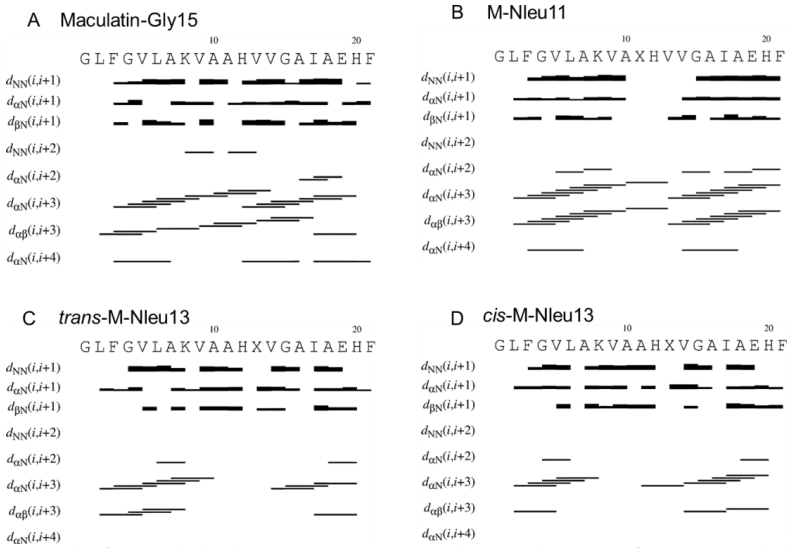

Figure 2: Plots of sequential and medium-range NOEs vs. sequence visualizing secondary structure information contained in the NOESY spectra from different maculatin derivatives: A: Maculatin-Gly15 in DPC micelles, B: M-Nleu-11 in DPC micelles, C: *trans*-M-Nleu-13 in SDS micelles, D: *cis*-M-Nleu-13 in SDS micelles. "X" denotes the peptoid residue Nleu. In case of chemical shift degeneracy between atoms of M-Nleu-13 in the *cis* and *trans* conformers, the cross peaks were assumed to be present in both conformations.

PRE Constraints

PRE constraints were derived as described by Franzmann et al.[28] Eight inversion recovery NOESY spectra with recovery delay times of 1, 50, 150, 400, 700, 1200, 2600, and 4000 ms were recorded in a pseudo-3D manner for each of the four Gd(DTPA-BMA) titration points: 0, 2, 5, 10 mM. All peaks with H^{α} in the indirect dimension were integrated in all spectra. R_1 relaxation rates were determined, and by a linear fit of the relaxation rates for the 4 titration points the PRE values were determined. PRE values for each H^{α} were then converted to distance restraints to the micelle center as described[28]: If there was more than one PRE value for a given H^{α}, we used the average value of obtained distances. For H^{α} yielding three or more PREs, we also calculated the standard deviation of the distances. Standard deviations for the PRE derived distances were between 0.1 and 1.0 Å. Thus, all PRE derived distances were used as upper and lower distance restraints with values of average distance ±1 Å, respectively, also for atoms yielding less than three PREs, not permitting the calculation of standard deviations. PRE-derived distance restraints were weighted with 10% compared to the NOE-derived distance restraints.[28]

Structure Calculation

A pseudoatom representing the micelle center was attached to the C-terminal end of the peptide by a ≈70Å flexible linker consisting of pseudoatoms (CYANA residues -LL-LL2-LL2-$(LL5)_{11}$-$(LL2)_4$-LL-). On the basis of the NOE-derived distance constraints, angle restraints and PRE-derived distance restraints, 80 structures of each peptide were calculated using CYANA v. 2.1[29]. The 20 structures with the lowest target function value were included in the final structure ensemble. For overlapping NOE peaks between the *cis* and *trans* conformer, 90% of the total peak intensity was used. The resulting integral values were then split according to the ratio of 1.3 between the *trans* and *cis* conformer. This ratio was found as an average based on the peak intensities of completely resolved peaks in the TOCSY spectra.

Results

It is advantageous for the structure calculation of a peptomer that the conformation of the peptoid monomer (*cis* or *trans)* is determined to start with.

Cis and *trans* conformations each show characteristic short distances: the distance between $H^{N}_{(i-1)}$ and H^{α}_{i} (with *i* denoting the peptoid residue) is in the *trans* conformation bigger than 3.6 Å, while this distance in the *cis* conformation can be <2.5 Å, depending on $\psi_{(i-1)}$. Independent of $\phi_{(i-1)}$ and $\psi_{(i-1)}$, this distance will always be shorter in the *cis* than in the *trans* conformation. This behavior is opposite for the distance between $H^{N}_{(i-1)}$ and H^{β}_{i}. Likewise, the distances between $H^{\alpha}_{(i-1)}$ and $H^{\alpha 2,3}_{i}$ are shorter in the *cis* conformation, while the distances $H^{\alpha}_{(i-1)}$ and $H^{\beta 2,3}_{i}$ are shorter in the *trans* conformation (Figure S2).

The sequences of the two maculatin-G15 analogs investigated in this study are:

M-Nleu11 GLFGVLAKVA-Nleu-HVVGAIAEHF-NH_2

M-Nleu13 GLFGVLAKVAAH-Nleu-VGAIAEHF-NH_2

M-Nleu11 showed only one conformer when bound to SDS or DPC micelles and was found to have a *trans* conformation of the Nleu residue based on the NOESY cross peaks from H^{α} Ala10 to H^{β} Nleu11 (Figure 2). The insertion of the molecule into the DPC

was determined by paramagnetic relaxation enhancement (PRE) experiments. These data are shown in Figure 3 and were used as restraints for the structure calculations.

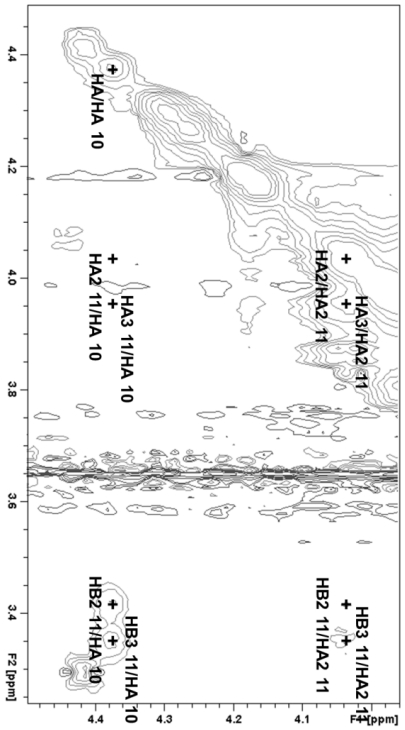

Figure 3: Region of a NOESY spectrum of M-Nleu11 in DPC micelles containing the NOEs for distinguishing *cis* and *trans* peptoid conformation. It shows the absence of $H^{\alpha}_{(i-1)} - H^{\alpha}_{(i)}$ NOEs and the presence of the $H^{\alpha}_{(i-1)} - H^{\beta}_{(i)}$ NOEs (with i denoting the peptoid residue). The NOESY section also shows weak cross peaks between $H^{\alpha 2}$ and $H^{\beta 2/3}$ of Nleu 11.

M-Nleu13 exhibited an almost equal distribution between *cis* and *trans* isomers of Nleu, and both structures were solved bound to SDS micelles. Useful PRE data could not be obtained for this analog, because of H^{α} chemical shift degeneracy between the two conformations.

NMR assignments and structure ensembles of M-Nleu11 in DPC micelles and M-Nleu13 (*cis* and *trans*) in SDS micelles have been submitted to the PDB and BMRB databases. The structural statistics and accession codes are given in Table 1.

Analysis of NOE patterns show that all three structures showed a well-defined α-helix in both ends of the molecule, with a flexible region around the Nleu residue (Figure 1).

It was possible to obtain insertion depth data for M-Nleu11 in DPC, which determined the orientation of the two terminal α-helices relative to each other. The structure ensemble resulting from the use of PRE derived restraints is rigid around the peptoid residue, yielding lower average backbone RMSD for M-Nleu11 than for M-Nleu13. The final structure ensemble of M-Nleu11 is shown in Figure 4C. Its hydrophobic residues are oriented towards the micelle center, the polar and Gly residues are oriented towards the lipid head groups and solvent surrounding the micelle (except for His20), and the Ala residues are primarily located in an orientation parallel with the micelle surface (Figure 5A).

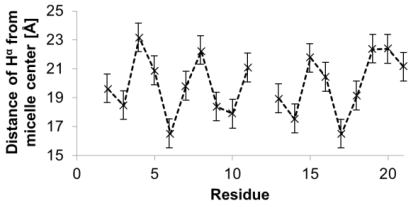

Figure 4: Distance from the micelle center to H^{α} atoms of M-Nleu11 in DPC micelles obtained from PRE experiments. For structure calculations, these values were used as distance restraints with ±1 Å and a weight of 10% relative to NOE distance restraints.

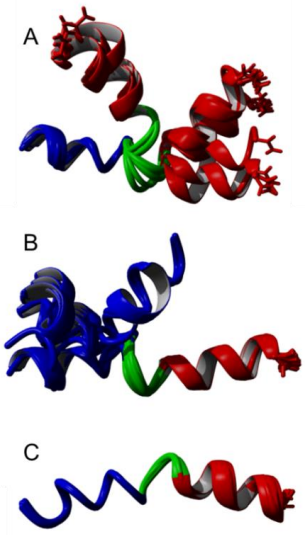

Figure 5: Structure ensembles of M-Nleu11 in DPC micelles with Nleu in the *trans* conformation. A: superposition of residues 2 to 10 calculated without PRE restraints. B: superposition of residues 12-21 calculated without PRE restraints. C: superposition of residues 2 to 21 calculated with PRE restraints. Residues 1-9 are colored blue, residues 10-12 are colored green, and residues 13-21 plus the C-terminal amide are colored red. The image was generated using the POVRay plugin to YASARA.[33]

In order to understand why one of the maculatin analogs is found in only one conformation while the other has two conformations, the hypothetical structure of M-Nleu11 with *cis*-Nleu was calculated using all experimental data, but forcing Nleu 11 into the *cis* conformation. Figure 5 shows the orientation of the Nleu side chain in the *trans* and the hypothetical *cis* conformation. In the *trans* conformation, the side chain is buried in the micelle, and in the hypothetical *cis* conformation, the side chain would be exposed to the bulk water.

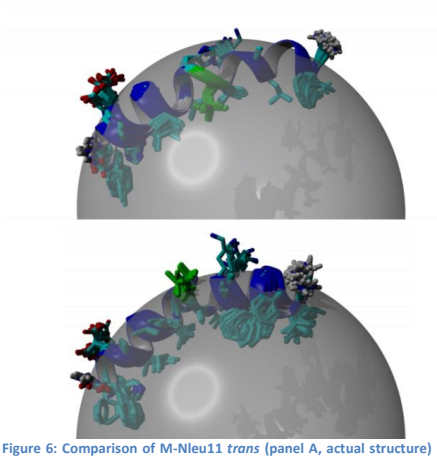

Figure 6: Comparison of M-Nleu11 *trans* (panel A, actual structure) and *cis* (panel B, hypothetical structure) in DPC micelles. Only the *trans* conformation was observed experimentally, the *cis* conformation was calculated by forcing ω_{Nleu11} to 0°, otherwise the same restraints were used. The micelle is indicated by a semi-transparent grey sphere with a radius of 22.7 Å. Both in the *trans* and the *cis*-structure, the charges at the N-terminus, Lys 8 and E19, are located at the micelle-water interface. So are the uncharged C-terminal amide and His 12, while His 20 is immersed deeper into the micelle. Other residues at the micelle surface include Gly 4, Ala 7 and Gly 15. The hydrophobic residues (Phe, Leu, Val, Ile) point towards the hydrophobic interior of the micelle. The peptoid residue, shown in green, points towards the solvent in the *cis*-conformation, but towards the micelle interior in the *trans* conformation. The figure was created with YASARA[33].

Discussion

Distinguishing between *cis* and *trans* conformation

Initially, we attempted to solve the structures of both maculatin analogs in a solution of SDS. However, due to identical chemical shifts of H^{α} Ala10 and H^{β} Nleu11 in M-Nleu11 bound to SDS, the presence of NOESY cross peaks between these atoms could not be established. Thus, a M-Nleu11 sample using DPC as the membrane mimic was used, where these peaks were resolved. In this sample, NOESY cross peaks from H^{α} Ala10 to the side chain of Nleu11 were present (see Figure 2), thus establishing that this analog contains a *trans* peptoid bond. The presence of a smaller amount of *cis* conformation cannot be ruled out completely, but additional spin systems were not present. Based on the signal-to-noise ratio of the strongest signals in the NOESY spectrum, we estimate that an eventually present *cis* conformation would be populated to less than 5%. When calculating the structure of M-Nleu11 in both the *trans* and *cis* conformation, the distance restraints from H^{α} Ala10 to the Nleu11 side chain were the only ones that could not be fulfilled by both conformations. Very recently, a computational study of conformational preferences of peptomers was published, investigating the optimum backbone dihedral angles of an alanine residue preceding a peptoid residue (N-methyl-glycine).[30] The optimum angles are found within the regions adopted by pre-proline residues in the PDB database from the "Richardson top 8000" database.[31] Figure 6 shows the φ/ψ angle distribution of the amino acid preceding the peptoid residue in the structures presented here. While the φ/ψ angles of *trans*-M-Nleu-11 are close to what can be expected for an amino acid preceding a peptoid, the angles do not fit for *cis*-M-Nleu-11, further substantiating the presence of a *trans* conformation.

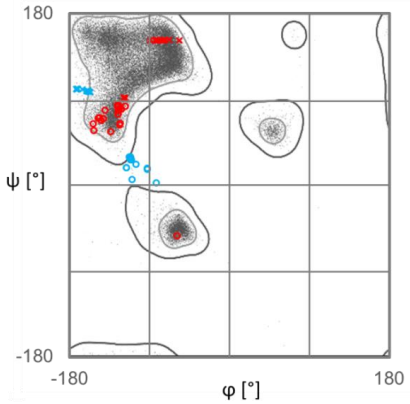

Figure 7: Ramachandran plot showing the distribution of the backbone torsion angles of the amino acid preceding the peptoid residue in maculatin-based peptomers: Ala 10 in M-Nleu-11 (blue) and His 12 in M-Nleu-13 (red). Circles denote *cis* conformation of the peptoid, crosses denote *trans* conformation of the peptoid. The black and white background shows the distribution of φ/ψ angles in amino acid residues preceding proline according to "Richardon's top 8000" database[31].

In M-Nleu13, one of the conformers was found to have a NOESY cross peak from H^{α} His12 to the Nleu13 side chain, thus being the *trans* conformer. The other conformer had weak NOESY cross peaks from H^{α} His12 to H^{α} Nleu13 (i.e. backbone to backbone), which should only be found in the *cis* conformer.

Structural evaluation

For both M-Nleu11 and M-Nleu13, the insertion of the peptoid monomer exerts a helix breaking effect. From the NOESY spectra this can be seen directly due to very weak or missing $H^{\alpha}(i) - H^{N}(i+3)$ NOESY cross peaks across the Nleu residue (Figure 1). Furthermore, the helix breaking effect is clearly visible from the structure ensembles of both analogs (without PRE-derived restraints), as they are characterized by well-defined helices at both termini with a very flexible region around the Nleu residue. The helix breaking effect is likely due to the steric repulsion between the Nleu side chain and the side chain of the previous residue as well as the loss of the hydrogen bonding H^{N} atom.

After inclusion of the PRE-derived restraints for M-Nleu11, the RMSD for the structure ensemble becomes quite low. Despite the lack of regular secondary structure around the peptoid residue, the position of the two terminal helices relative to each other is well defined as a consequence of the restraints to the micelle center. In α-helical cationic AMPs, the peptides fold into an amphipathic structure where the hydrophobic residues are inserted i

membrane interior and the polar residues are interacting with the lipid head groups and the surrounding solvent.[28,32] Therefore, the 20 structures of M-Nleu11 become very similar: the two helical ends of the molecule insert into the membrane mimic and lock the otherwise flexible region in place. This demonstrates the usefulness of PRE experiments to determine the insertion depth of each residue. Without this information, it is not possible to determine the orientation of the two helical ends of the molecule relative to each other (Figure 4, panel A and B).

The structures of M-Nleu13 were solved without the use of PRE-derived restraints. The high degree of similarity of chemical shifts between the two conformers and resulting overlap of peaks made it impossible to obtain distinguishable relaxation rates. The lack of insertion depth data allows for highly variable orientations of the two helices relative to each other (Figure 7). Thus, only the fact that both ends fold into a helical structure, and that this molecule has an almost equal tendency to adopt a *trans* and *cis* conformation can be concluded.

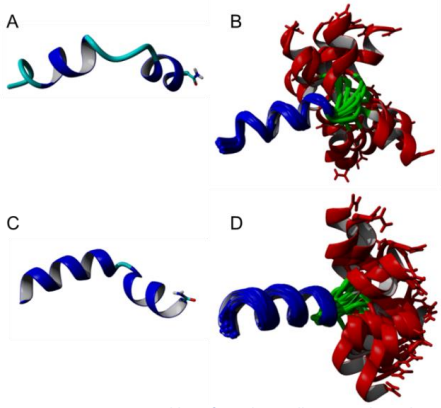

Figure 8: Structure ensembles of M-Nleu13 illustrating how the peptide folds into two α-helices for both the *trans* (A and B) and *cis* (C and D) conformation of Nleu13, as well as how flexible the middle region around the Nleu residue is. In panels A and C, the backbone structure of a single conformer from the bundle is displayed to illustrate the helical structure. Residues in blue are classified by YASARA [33] as helical whereas residues in cyan are not. In panels B and D, the entire bundle of 20 conformers is displayed as a superposition of residues 2-10. Residues 1-11 are colored blue, reisdues 12-14 are colored green, and residues 15-21 plus the C-terminal amide are colored red.

Why is the *trans* conformation preferred in M-Nleu11?

In the two maculatin analogs presented here, the Nleu residue was inserted at two different positions. One of these positions yields a molecule which prefers only a *trans* conformation of the Nleu residue, whereas the other has an equal tendency to adopt both *cis* and *trans* conformations of the Nleu residue.

In the well-defined structures of M-Nleu11, a closer look on the Nleu residue shows that the side chain properties as well as the place of insertion of the peptoid monomer might be the determining factors for preferring the *trans* conformation:

In M-Nleu11, the orientation of the peptide on the micelle-water interface is determined by the amphipathicity of the two helical segments. They will orient on the micelle surface such as is most favorable for them. Under these conditions, the Nleu side chain will be oriented towards the solvent in the *cis* conformation but towards the membrane interior in the *trans* conformation, as shown in Figure 5. This difference in orientation might explain why only the *trans* conformation is found for this peptide.

Why are both *cis* and *trans* conformation present in M-Nleu13

By following the argumentation for M-Nleu11, the reason why the *cis* and *trans* conformations are present in almost equimolar amounts in M-Nleu13 is that both conformations achieve an energetically equally favorable structure upon interaction with the membrane mimic.

The Nleu side chain is located on the opposite side of the helix in M-Nleu13, where the backbone of the residue in wild-type maculatin (Val) is inserted into the hydrophobic interior of the micelle. It is possible that both the *cis* and *trans* conformation allows for the hydrophobic Nleu side chain to be inserted into the hydrophobic interior of the micelle, but we cannot conclude on this based on structures without PRE-derived constraints. In addition, Nleu-13 follows the bulky His-12, while Nleu-11 follows the less bulky Ala-10. Distances between side chain atoms of a peptoid and the side chain atoms of its preceding residue are generally shorter for the *trans* conformer. Figure 8 shows the distribution of shortest inter-sidechain distances in all 20 structures of all four molecules calculated. *Trans*-M-Nleu-13 shows distances <2.4 Å (twice the VdW radius of hydrogen), leading to steric clashes, while *cis*-M-Nleu-13 displayed a wide range of distances both favorable and unfavorable. This might also be a reason for this molecule to partly adopt a *cis* conformation. Very short distances are also present in *trans*-M-Nleu-11, but to a lesser extent.

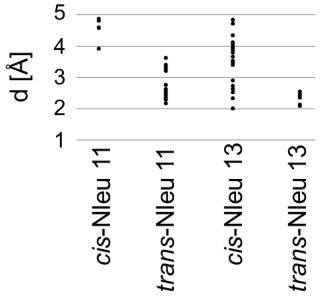

Figure 9: Shortest distance found between any two hydrogen atoms of the peptoid side chain and the preceding peptide side chain in all 20 calculated structures of both peptomers studied in their *cis* and *trans* conformations. The Van der Waals radius of hydrogen is 1.2 Å, thus making distances closer than 2.4 Å energetically unfavorable.

Conclusion

The insertion of a peptoid monomer into an α-helical AMP disrupts the helix. Inserting the Nleu residue can result in both a *cis* and *trans* conformation of the peptide. The conformations can be determined experimentally by the presence of NOESY cross peaks from H^{α} of the preceding residue to either the side chain H^{β} (*trans*) or backbone H^{α} (*cis*) of the peptoid residue. As the side chain of the peptoid monomer is shifted counter-clockwise in the helical wheel, hydrophobic peptoid monomers should be placed on the left-handed side of the helical wheel (looking down the helical axis from the N-to the C-terminus, with the helix oriented such that the membrane interior points downwards) near the middle of maculatin in order for only the *trans* conformer to be present. When inserting Nleu on the right-handed side of the helical wheel near the middle of an AMP, both the *cis* and *trans* conformers can be present. In addition, *trans* conformers can be favored by not placing the peptoid after a bulky residue, as bulky residues favor the *cis* conformation in subsequent peptoids. Furthermore, we have demonstrated the usefulness of PRE experiments for determining the global structure of peptomers bound to micelles.

Acknowledgements

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References

1. Miller SM, Simon RJ, Ng S, Zuckermann RN, Kerr JM, Moos WH (1994) Proteolytic studies of homologous peptide and N-substituted glycine peptoid oligomers. Bioorg Med Chem Lett 4:2657–2662.

2. Godballe T, Nilsson LL, Petersen PD, Jenssen H (2011) Antimicrobial β-peptides and α-peptoids. Chem Biol Drug Des 77:107–16.

3. Meinike K, Hansen PR (2009) Peptoid analogues of anoplin show antibacterial activity. Protein Pept Lett 16:1006–1011.

4. Pripotnev S, Won A, Ianoul A (2010) The effects of L- to D-isomerization and C-terminus deamidation on the secondary structure of antimicrobial peptide Anoplin in aqueous and membrane mimicking environment. J Raman Spectrosc 41:1645–1649.

5. Olsen CA, Bonke G, Vedel L, Adsersen A, Witt M, Franzyk H, Jaroszewski JW (2007) Alpha-peptide/beta-peptoid chimeras. Org Lett 9:1549–52.

6. Chongsiriwatana NP, Patch JA, Czyzewski AM, Dohm MT, Ivankin A, Gidalevitz D, Zuckermann RN, Barron AE (2008) Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. Proc Natl Acad Sci U S A 105:2794–9.

7. Simon RJ, Kania RS, Zuckermann RN, Huebner VD, Jewell DA, Banville S, Ng S, Wang L, Rosenberg S, Marlowe CK, et al. (1992) Peptoids: a modular approach to drug discovery. Proc Natl Acad Sci 89:9367–9371.

8. Moure A, Sanclimens G, Bujons J, Masip I, Alvarez-Larena A, Pérez-Payá E, Alfonso I, Messeguer A (2011) Chemical modulation of peptoids: synthesis and conformational studies on partially constrained derivatives. Chem Eur J 17:7927–39.

9. Ostergaard S, Holm A (1997) Peptomers: a versatile approach for the preparation of diverse combinatorial peptidomimetic bead libraries. Mol Divers 3:17–27.

10. Robey FA, Kelson-Harris T, Roller PP, Robert-Guroff M (1995) A helical epitope in the C4 domain of HIV glycoprotein 120. J Biol Chem 270:23918–21.

11. Fowler SA, Stacy DM, Blackwell HE (2008) Design and synthesis of macrocyclic peptomers as mimics of a quorum sensing signal from Staphylococcus aureus. Org Lett 10:2329–32.

12. Murugan RN, Park J-E, Lim D, Ahn M, Cheong C, Kwon T, Nam K-Y, Choi SH, Kim BY, Yoon D-Y, et al. (2013) Development of cyclic peptomer inhibitors targeting the polo-box domain of polo-like kinase 1. Bioorg Med Chem 21:2623–34.

13. Ovadia O, Linde Y, Haskell-Luevano C, Dirain ML, Sheynis T, Jelinek R, Gilon C, Hoffman A (2010) The effect of backbone cyclization on PK/PD properties of bioactive peptide-peptoid hybrids: the melanocortin agonist paradigm. Bioorg Med Chem 18:580–9.

14. Lee B-C, Zuckermann RN (2011) Protein side-chain translocation mutagenesis via incorporation of peptoid residues. ACS Chem Biol 6:1367–74.

15. Song YM, Park Y, Lim SS, Yang S-T, Woo E-R, Park I-S, Lee JS, Kim J Il, Hahm K-S, Kim Y, et al. (2005) Cell selectivity and mechanism of action of antimicrobial model peptides containing peptoid residues. Biochemistry 44:12094–106.

16. Zhu WL, Song YM, Park Y, Park KH, Yang S-T, Kim J Il, Park I-S, Hahm K-S, Shin SY (2007) Substitution of the leucine zipper sequence in melittin with

residues affects self-association, cell selectivity, and mode of action. Biochim Biophys Acta 1768:1506–17.

17. Patch JA, Barron AE (2003) Helical peptoid mimics of magainin-2 amide. J Am Chem Soc 125:12092–3.

18. Ryge TS, Frimodt-Moller N, Hansen PR (2008) Antimicrobial activities of twenty lysine-peptoid hybrids against clinically relevant bacteria and fungi. Chemotherapy 54:152–6.

19. Ryge TS, Hansen PR (2006) Potent antibacterial lysine-peptoid hybrids identified from a positional scanning combinatorial library. Bioorg Med Chem 14:4444–51.

20. Rozek T, Waugh RJ, Steinborner ST, Bowie JH, Tyler MJ, Wallace JC (1998) The Maculatin peptides from the skin glands of the tree frogLitoria genimaculata: a comparison of the structures and antibacterial activities of Maculatin 1.1 and Caerin 1.1. J Pept Sci 4:111–115.

21. Chia BC, Carver JA, Mulhern TD, Bowie JH (2000) Maculatin 1.1, an anti-microbial peptide from the Australian tree frog, Litoria genimaculata solution structure and biological activity. Eur J Biochem 267:1894–908.

22. Ambroggio EE, Separovic F, Bowie JH, Fidelio GD, Bagatolli LA (2005) Direct visualization of membrane leakage induced by the antibiotic peptides: maculatin, citropin, and aurein. Biophys J 89:1874–1881.

23. Zuckermann RN, Kerr JM, Kent SBH, Moos WH (1992) Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis. J Am Chem Soc 114:10646–10647.

24. Chann WC, White PD Fmoc Solid Phase Peptide Synthesis: A Practical Approach. Oxford Univ. Press; 2000.

25. Hwang TL, Shaka AJ (1995) Water Suppression that Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. J Magn Reson Ser A 112:275–279.

26. Keller R The computer aided resonance assignment tutorial. 1 st edn. Goldau (Switzerland): CANTINA Verlag; 2004.

27. Shen Y, Delaglio F, Cornilescu G, Bax A (2009) TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. J Biomol NMR 44:213–223.

28. Franzmann M, Otzen D, Wimmer R (2009) Quantitative Use of Paramagnetic Relaxation Enhancements for Determining Orientations and Insertion Depths of Peptides in Micelles. Chembiochem 10:2339–2347.

29. López-Méndez B, Güntert P (2006) Automated protein structure determination from NMR spectra. J Am Chem Soc 128:13112–22.

30. Butterfoss GL, Drew K, Renfrew PD, Kirshenbaum K, Bonneau R (2014) Conformational Preferences of Peptide-Peptoid Hybrid Oligomers. Biopolymers in press.

31. Richardson JS, Keedy DA, Richardson DC "The Plot" Thickens: More Data, More Dimensions, More Uses. In: Bansal M, Srinivasan N, editors. Biomolecular Forms and Functions. A Celebration of 50 Years of the Ramachandran Map. Singapore: World Scientific; 2013. pp. 46–61.

32. Vad B, Thomsen LA, Bertelsen K, Franzmann M, Pedersen JM, Nielsen SB, Vosegaard T, Valnickova Z, Skrydstrup T, Enghild JJ, et al. (2010) Divorcing folding from function: How acylation affects the membrane-perturbing properties of an antimicrobial peptide. Biochim Biophys Acta - Prot Proteomics 1804:806–820.

33. Krieger E, Koraimann G, Vriend G (2002) Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. Proteins 47:393–402.

Supplementary Material

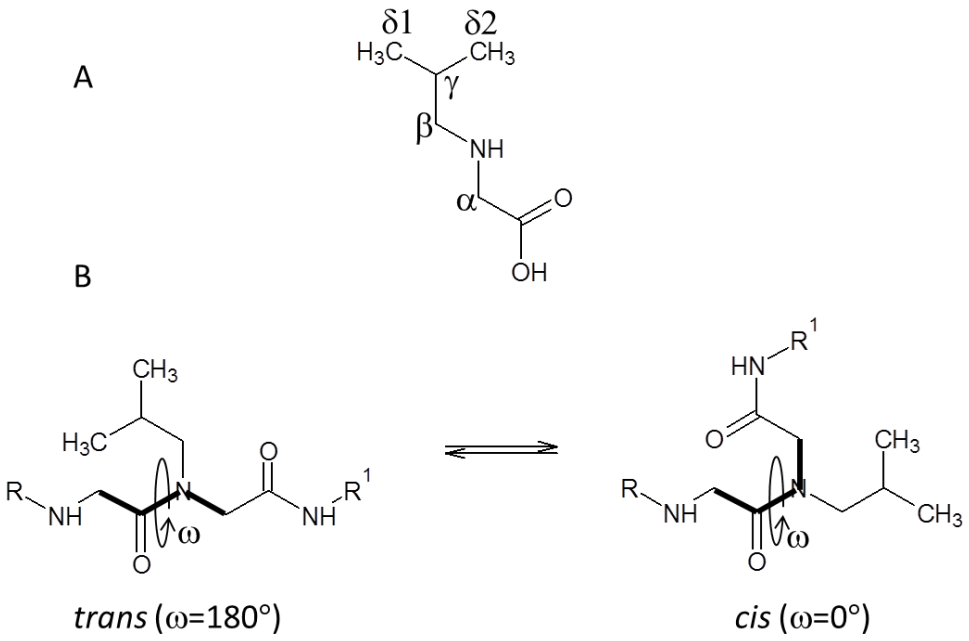

Figure S1: A: Chemical structure of Nleu with atom nomenclature used. B: Illustration of *cis* and *trans* isomerism of Nleu inserted into a peptide chain.

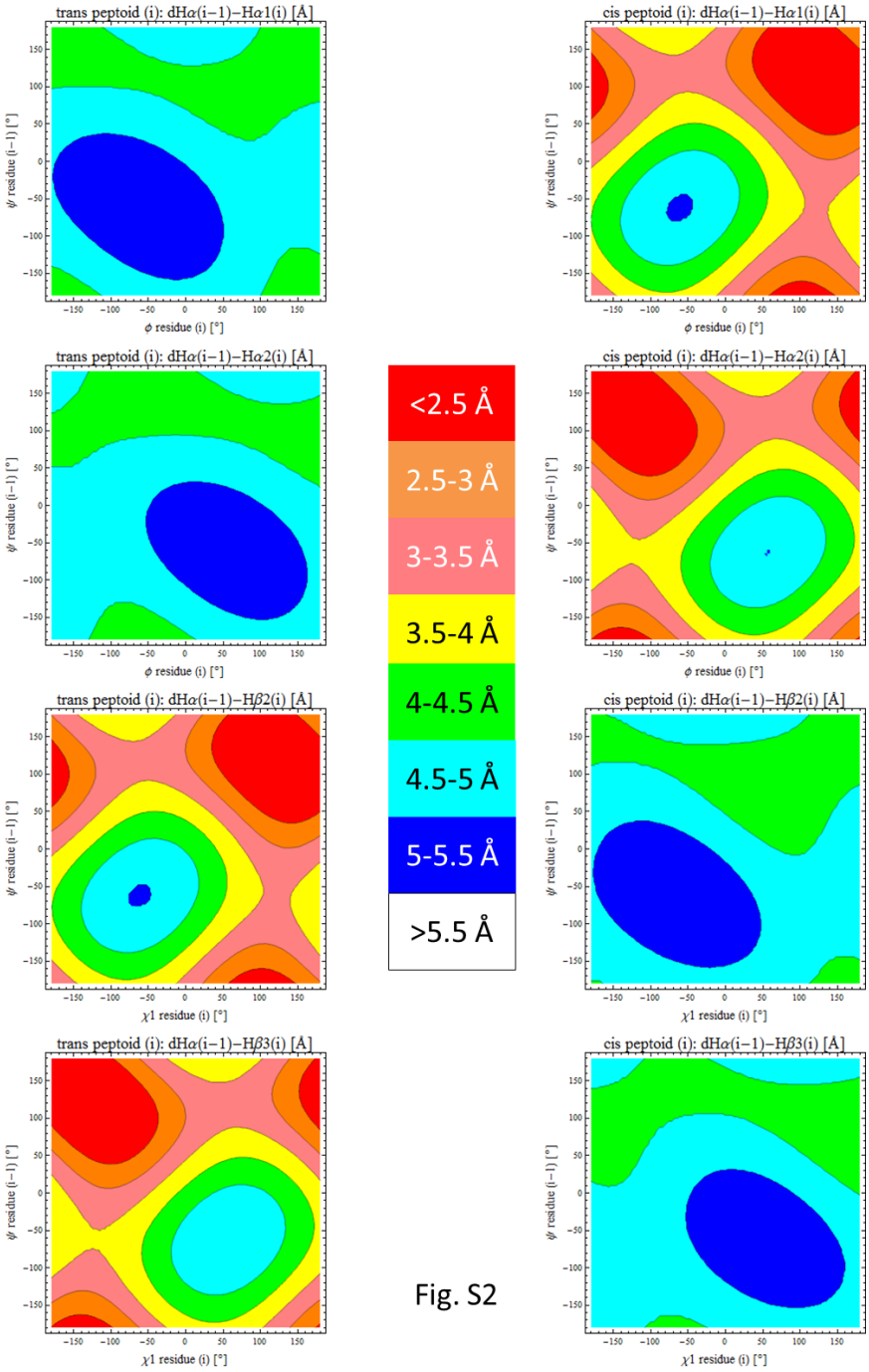
trans peptoid (i): dHα(i−1)−Hα1(i) [Å]
cis peptoid (i): dHα(i−1)−Hα1(i) [Å]
trans peptoid (i): dHα(i−1)−Hα2(i) [Å]
cis peptoid (i): dHα(i−1)−Hα2(i) [Å]
trans peptoid (i): dHα(i−1)−Hβ2(i) [Å]
cis peptoid (i): dHα(i−1)−Hβ2(i) [Å]
trans peptoid (i): dHα(i−1)−Hβ3(i) [Å]
cis peptoid (i): dHα(i−1)−Hβ3(i) [Å]
ψ residue (i−1) [°]
ϕ residue (i) [°]
χ1 residue (i) [°]
<2.5 Å
2.5-3 Å
3-3.5 Å
3.5-4 Å
4-4.5 Å
4.5-5 Å
5-5.5 Å
>5.5 Å

Fig. S2

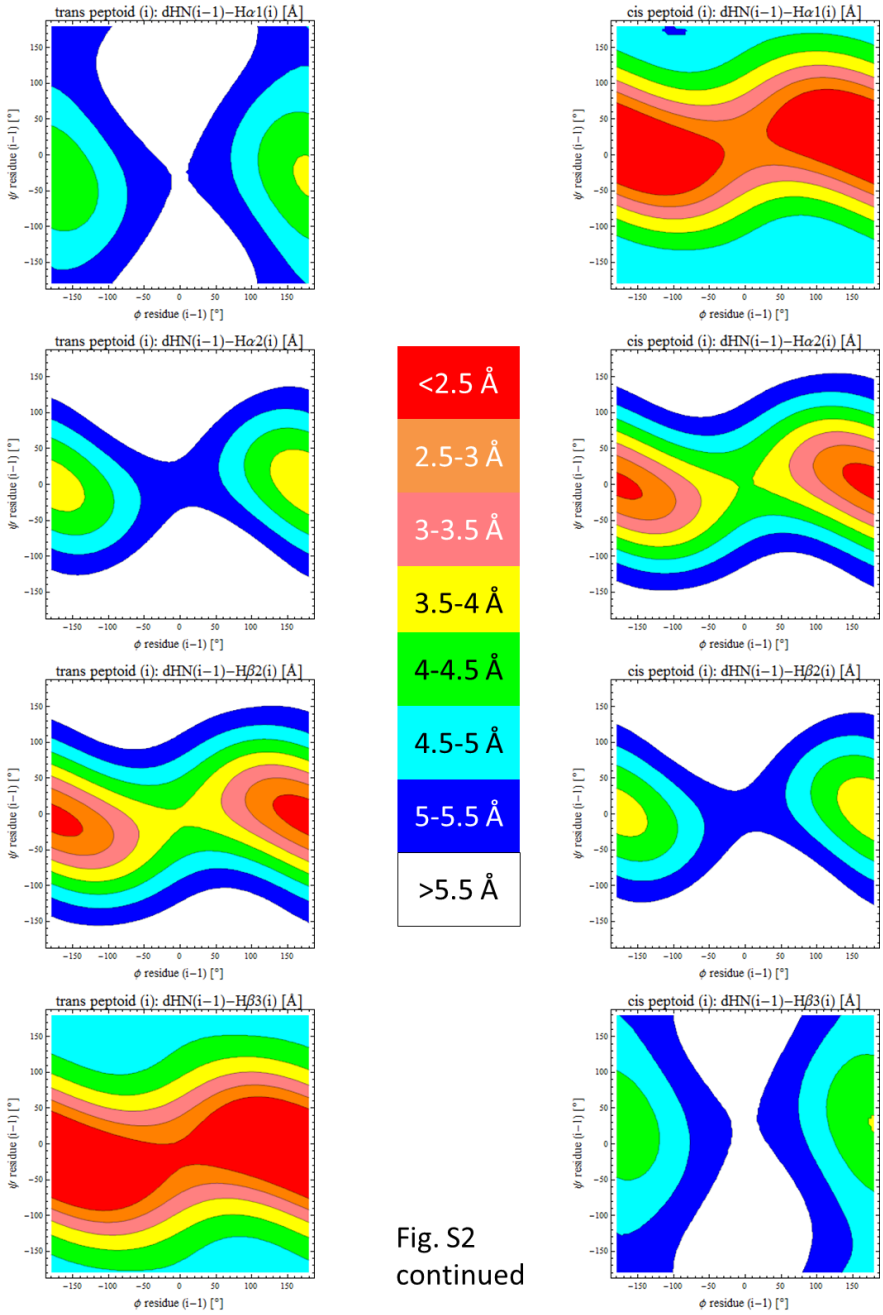

Fig. S2 continued

Figure S2: Contour plots showing the distances between atoms of the peptoid residue *i* and the residue preceding the peptoid residue (*i-1*), depending on the *cis*/*trans* conformation of the peptoid bond and other dihedral angles.

9. Further results and discussion

Circular Dichroism9

All peptides studied in this work were measured by circular dichroism (CD) prior to activity assays and NMR measurements. CD measurements were performed in the wavelength range 190-260nm on a Chirascan plus spectropolarimeter using a peptide concentration of 50µM dissolved in 2 mM phosphate buffer, pH 6.5 at 20˚C. The pathlength through the cuvette was 1mm, and DPC and/or SDS (both from Avanti Polar Lipids) was gradually added until the CD curve no longer changed upon further addition of lipid (titration steps varied between sample types). For all samples which were measured with both DPC and SDS, no differences in CD profiles were observed.

The purpose of these measurements was to see whether secondary structure was induced in the peptides upon interaction with the zwitterionic (DPC) and anionic (SDS) lipids, before using either of these membrane mimics in the NMR samples.

All peptides presented in the two articles showed an induced secondary structure (α-helical) upon binding to the membrane mimics. Many of these peptides showed an isosbestic point, displaying a direct transition from the unstructured conformation in 2mM phosphate buffer to the structured conformation upon binding to the micelles following a two-state model. An example of this is given in Figure 9.1. However, a peptide such as 5F8W did not have an isosbestic point as shown in Figure 9.2, and already showed some degree of secondary structure before adding lipids to the sample. This is in accordance with the observation in Paper 1, where it was found that 5F8W has a strong tendency to self-aggregate. The CD signal of 5F8W never becomes purely α-helical, despite the fact that this structure was solved via NMR and found to fold into a complete α-helix. Another peptide which has been discussed a lot in paper 1 is 5W, and its CD spectra are shown in Figure 9.3. Like 5F8W, this peptide has some degree of secondary structure before the addition of lipids, but oppositely to 5F8W this peptide shows a high degree of helicity upon addition of lipid. The information obtained from CD cannot be used to predict activity as such, but it is noteworthy that the peptides with the highest degree of hemolysis show a different CD profile from the majority of tested peptides. CD might provide a fast way to qualitatively determine their hemolytic activity (unspecific membrane interaction) and whether the peptide has a tendency for self-aggregation.

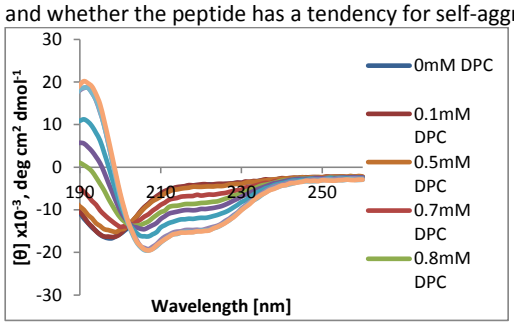

Figure 9.10: CD spectra of 50µM anoplin in 2mM phosphate buffer, pH 6.5, 20°C at various concentrations of DPC.

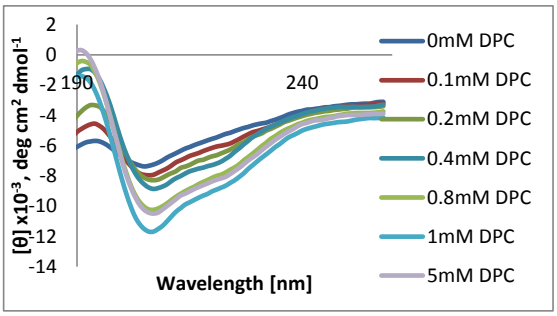

Figure 9.2: CD spectra of 50µM 5F8W in 2mM phosphate buffer, pH 6.5, 20°C at various concentrations of DPC.

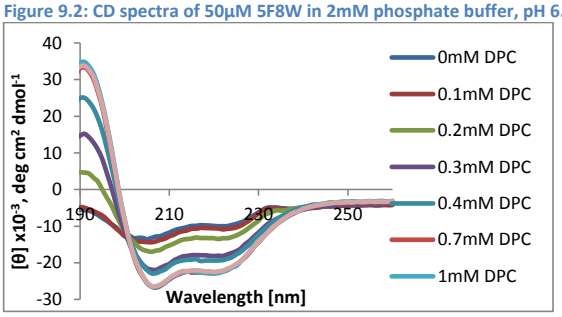

Figure 9.3: CD spectra of 50µM 5W in 2mM phosphate buffer, pH 6.5, 20°C at various concentrations of DPC.

In relation to the work with peptide/peptoid hybrids several smaller peptides were investigated, where a N-(indol-3-yl-ethyl)-glycine was inserted into position 3, 5, and 9 in anoplin. For these hybrids, the CD spectra showed a significant loss of structure compared to anoplin, and the structure was only determined for the analogue with peptoid monomer in position 5. The CD spectrum for this anoplin analogue is shown in Figure 9.4. Despite the poor quality of the CD spectrum, it can be seen that a partly helical profile is present at the high SDS concentrations (minima around 208nm and 220nm).

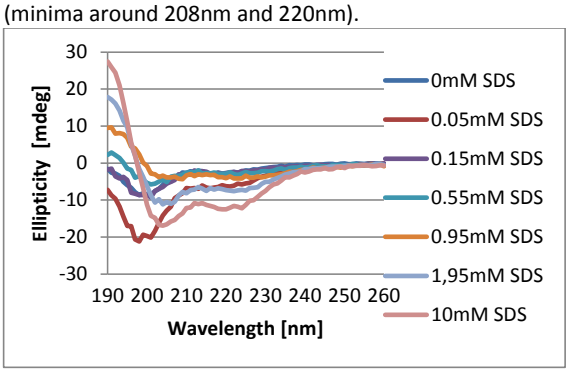

Figure 9.11: CD spectra at various SDS concentrations of the anoplin analogue with an N-(indol-3-yl-ethyl)-glycine inserted in position 5. The spectra are of poor quality because they were recorded on an older CD spectropolarimeter (Jasco J-715).

Structural studies of peptides containing peptoid monomers

Anoplin-peptoid chimeras

The structure of the anoplin analogue containing N-(indol-3-yl-ethyl)-glycine in position 5 was determined using NMR. One of the calculated structures is shown in Figure 9.5. The peptide structure was not easily obtained. Firstly, the peptide was split into a major and minor conformer. Secondly, spin systems from the N-terminal part of the analogue were weak (some not even found) while the spin systems of the C-terminus were strong. Thirdly, exchange peaks between the two conformers were found showing that a transition between the two conformers takes place. In general, HN for residue 1 is never observed in the NMR spectra of peptides, due to fast exchange of the proton with the water protons. For a flexible terminus this can also be the case for several of the following residues due to mobility reasons. In this peptide, HN for Leu2 could not be seen, and the HA peaks were likely hidden under some of the stronger LEU spin systems. Furthermore, there is no HN on peptoid monomers. It has on several occasions been observed that the insertion of a peptoid monomer can cause the HN signal to disappear from nearby residues. In this analogue, this was the case for Ile6. Due to all these reasons, only a few classical NOEs expected for α-helices were found for the C-terminal part of the anoplin analogue (residues 6 to 10), and only very sparse NOEs at all in the N-terminal part (residues 1-5). This can be seen from the sequence plot in Figure 9.5. Consequently, the final structure (calculated from the major solution conformer in the trans configuration) displays a flexible N-terminus and an α-helical C-terminus (Figure 9.6), thus explaining why the CD spectrum of this analogue showed a high certain degree of α-helix. This means that small peptides can maintain at least some of their secondary structure upon insertion of a helix-disrupting peptoid monomer. However, this is likely dependent on the side chain properties and site of insertion, as concluded in Paper 2. The inability of forming an α-helix on the N-terminal side of the peptoid monomer was the onset for studying the structural impacts of inserting a peptoid monomer in a much longer helical AMP (maculatin).

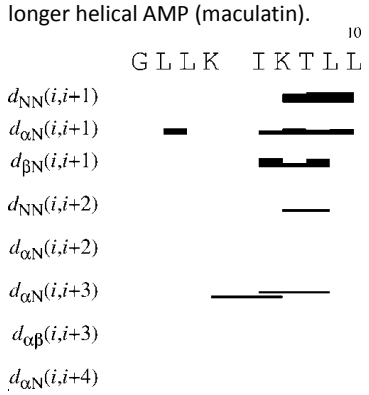

Figure 9.5: Sequnce plot of the anoplin analogue containing N-(indol-3-yl-ethyl)-glycine in position 5 (unlabeled in the sequence).

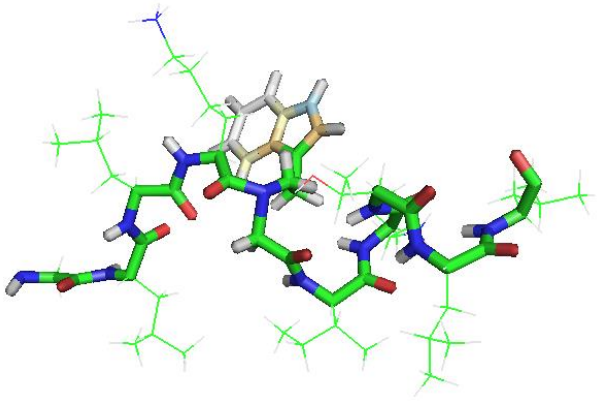

Figure 9.6: NMR structure of the anoplin analogue which has an N-(indol-3-yl-ethyl)-glycine inserted in position 5. The backbone and peptoid monomer is shown in sticks, and the standard amino acids with side chains are shown in lines. The N-terminus is to the left. No regular secondary structure is found for the N-terminus, but an α-helical structure is observed for the C-terminus. Structure is visualized via PYMOL.

Maculatin-peptoid chimeras

In the study of the maculatin-peptoid hybrids, 4 analogues were originally designed and synthesized. The Nleu monomer was inserted into positions 9, 10, 11, and 13.

Maculatin-G15	GLFGVLAKVAAHVVGAIAEHF-NH2
M-Nleu13	GLFGVLAKVAAHNleuVGAIAEHF-NH2
M-Nleu11	GLFGVLAKVANleuHVVGAIAEHF-NH2
M-Nleu10	GLFGVLAKVNleuAHVVGAIAEHF-NH2
M-Nleu9	GLFGVLAKNleuAAHVVGAIAEHF-NH2

In all these analogues, Nleu was inserted in place of aliphatic amino acids (Ala or Val) in the center of the maculatin sequence. These sites of insertion were chosen because we wished to see if the same structural motif would occur regardless of insertion site, because we wished to make the substitutions relatively conservative, and because the peptoid monomer should be located so it allows for helix formation on both sides of the insertion site.

Maculatin-G15 was shown by Franzmann[44] to fold into a continuous α-helix throughout the whole peptide. By using his NMR spectra, the PRE data were revised and the final insertion depth data (Figure 9.7) were used for obtaining an experimentally derived helical wheel plot, similar to the ones obtained for anoplin in Paper 1. The experimentally derived helical wheel plot is shown in Figure 9.8 together with the theoretical helical wheel for maculatin-G15.

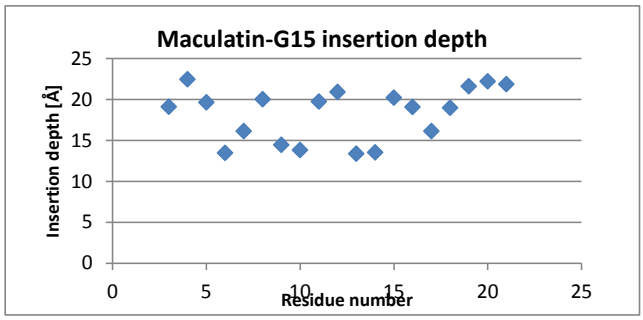

Figure 9.7: Insertion depth profile of maculatin-G15.

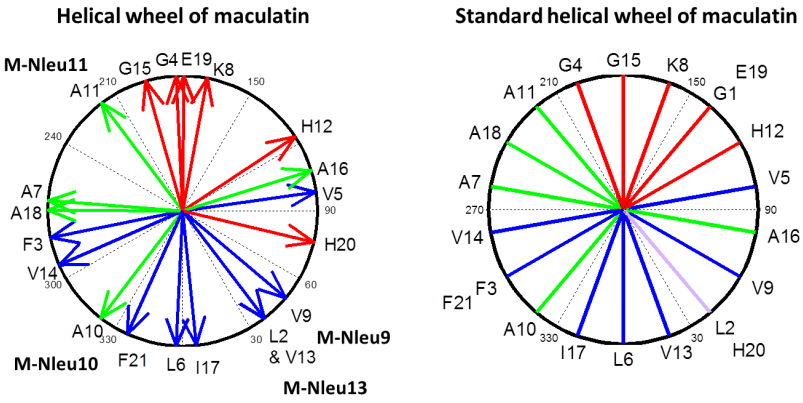

Figure 9.8: Experimentally derived and theoretical helical wheel projection of maculatin-G15 oriented such that 0 degrees is the direction towards the micelle interior. Hydrophobic residues are in blue, polar and Gly residues in red, and Ala residues in green. The purple line in the theoretical helical wheel indicates that both a polar and hydrophobic residue have this orientation. In the experimentally derived helical wheel projection, the sites of insertion of peptoid monomers have been shown by the peptide analogue names.

By now, we have realized that the helical wheel plot is not a very accurate model, as the side chain properties can distort the backbone of the helix away from the 100 degrees per residue, thus compressing or stretching the helix. This is also the case for maculatin, where the actual structure is more extended than predicted by the helical wheel model. This is most easily seen by comparing the orientations of for instance G4 and G15 or H12 and E19 between the two helical wheel plots.

The use of a peptoid monomer will displace the side chain of that residue with about 33 degrees counterclockwise, which means that both Nleu10 and Nleu11 will have their side chain oriented further towards the micelle interior, whereas both Nleu9 and Nleu13 will have their side chain oriented further towards the micelle exterior (if the overall structure and orientation of the peptide does not change).

The structures were only succesfully solved for M-Nleu11 and M-Nleu13, due to poor spectra quality of the M-Nleu9 and M-Nleu10 samples. During the structural work on M-Nleu9 and M-Nleu10 it was however clear that M-Nleu9 consists of two conformers similarly to M-Nleu13 in the presence of SDS-micelles and that M-Nleu10 consists of only one conformer similarlu to M-Nleu11. This confirms the statement in Paper 2 that insertion of the hydrophobic peptoid monomer, Nleu, on the left-handed side of the helical wheel plot allows for one very beneficial conformation (*trans* for M-Nleu-11 and unknown for M-Nleu10), whereas both *cis* and *trans*

conformation are equally favorable when inserting the peptoid monomer on the right-handed (M-Nleu9 and M-Nleu13).
It would be desireable to know whether the *trans* conformer is in general the most beneficial conformation for a peptoid monomer inserted into an α-helical AMP, and if the orientation of the Nleu side chain in the *cis* and *trans* conformation is always as found for the *trans* and hypothetical *cis* conformer of M-Nleu11 in paper 2. If this is the case, the presence of only one conformation in M-Nleu10 and M-Nleu11 would be explained by the favorable interaction of the hydrophobic side chain in *trans* conformation with the membrane interior. At the same time, it would also explain why the *cis* conformation is present in M-Nleu9 and M-Nleu13, as the *trans* conformation would orient the hydrophobic side chain away from the micelle interior, thus making the *cis* conformer relatively more favorable.
A verification of these assumptions would yield a useful guideline about where to insert peptoid monomers (with non-rigid side chains) in order for only one structure to occur (hydrophobic side chains on the left-handed side and hydrophilic on the right-handed side). However, a more comprehensive structural investigation is required, as well as studies on how the insertion of the peptoid monomers affect the activity of the AMPs.

α-peptide-β-peptoid chimeras

Some initial work was also done on peptidomimetics consisting of a sequence with alternating α-amino acids and β-peptoid monomers. One of these were the octamer shown in Figure 9.9.

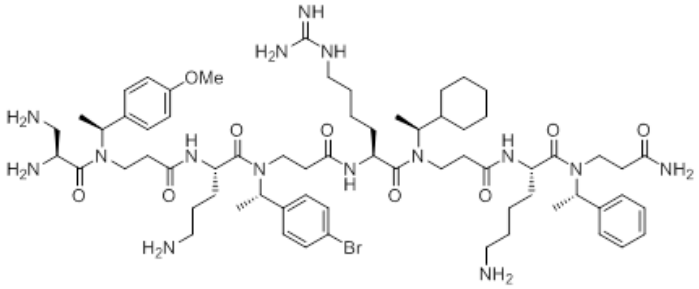

Figure 9.9: Chemical structure of the octamer used in the study of α-peptide-β-peptoid chimeras.

This work proved to be of such complexity, that the attempts to solve their structures were stopped. The reason was that the NMR spectra showed such a high amount of different conformers, that the spins systems could not be separated. An accurate amount of conformers could not even be determined, but theoretically, each β-peptoid monomer could take 2 conformations, leading to a total of 16 different conformations for this compound based on *cis*/*trans* isomerism.

10. Discussion of the usefulness of commonly used SAR parameters

As stated in the introduction, some of the usual parameters investigated in relation to the activity of cationic α-helical AMPs are hydrophobicity, mean hydrophobicity, hydrophobic moment/amphipathicity, charge, helicity, and polar angle. These parameters have been suggested by various authors and more or less accepted by the scientific community. At one point of my work, these structural parameters were tested against our set of peptides to see if they could explain the observed activities, but no useful correlations were found. For the anoplin analogues, we also included parameters such as tilt of the helix, rotation of the helix, and average insertion depth to no avail. We thus started from scratch and came to the parameters and conclusions presented in Paper 1. Based on that work I took another look at these structural parameters, and I now believe that these parameters should either be revised or discarded, as their definitions are stated without proper restrictions to their use or they are simply not parameters relevant for the activity of this class of AMPs.

Helical wheel

The helical wheel model has been used for more than half a century[45], and is an excellent way to inspect the properties of α-helical structures. In the work of helical AMPs, this model has been used extensively in the design of improved analogues and even to calculate structural parameters such as hydrophobic moment and polar angle. However, the helical wheel is only a model. It does not necessarily match the actual helical structure of a given peptide. In this work, we have shown that side chains can be oriented quite differently from the theoretical orientation, and that the compactness of the helix is influenced by the side chain distribution upon AMP interaction with a membrane mimic. It is common knowledge, that helices can be compressed (3_{10} helix), and we showed experimentally that the helix of maculatin was extended slightly throughout the helix. This means that the helical wheel model becomes less accurate with increasing length of the AMP under study.

Based on this, the structural parameters based on the helical wheel will be prone to error, and prediction of beneficial substitution sites might be based on false assumptions. The longer the peptides, the larger the errors can be. Furthermore, the helical wheel model is difficult to use in relation to helices containing proline residues (or even D-amino acids), as the kink they induce makes it difficult to predict the orientation of the two helical parts relative to each other (e.g. wild type maculatin).

With the PRE method used in our study[42], the actual structure of a helix can be determined experimentally, and this should (in my opinion) be done instead of relying only on the helical wheel model.

Hydrophobicity

Hydrophobicity is used in two ways: the sum of hydrophobicities of all residues and the mean hydrophobicity of all residues. The sum of hydrophobicities is only meaningful when comparing peptides of the same length whereas the mean hydrophobicity can (in theory) be used to compare peptides of various lengths.

The first issue regarding hydrophobicity is that there are so many scales for it, and they do not rank the amino acids in the same order[46]. In order to make use of hydrophobicity as a

parameter, it must be standardized which scale to use, and this scale should be expanded to include all non-classic amino acids as well, as this is a severe limitation in some of these scales. This expansion is important, as more and more work using non-classic amino acids is being performed in the attempt to make peptides more resistant to degradation.
The second issue regarding hydrophobicity is that it can be changed in different ways. In paper 1, the insertion of aromatic compounds in various positions of anoplin showed that it makes a huge difference whether they were inserted instead of a water-interacting residue or a lipid head-group interacting residue. The latter would result in a much higher loss of selectivity due to the ability of that residue to orient itself inward in the micelle and interact with the hydrophobic interior. Our experimental data are very clear on this point, but they are based on a small set of peptides and should be documented further (see perspectives).
Due to the positional difference in activity and selectivity of an inserted hydrophobic residue, the parameter hydrophobicity should be restated to "hydrophobicity of the hydrophobic face", and then the effect of insertion of hydrophobic/non-polar residues pointing towards the bulk water (solvent) should be investigated as a separate concept.
This would perhaps yield a parameter useful for comparing peptides of different lengths as well. The results of paper 1 showed that a less strong hydrophobic face is required as the length of the helix increases. i.e. the mean hydrophobicity of the hydrophobic face should be reduced as the length of the peptide increases. Due to the very limited set of peptides in our study, it is not yet possible to determine this relationship quantitatively. A useful design of peptides should include a hydrophilic face which has a conserved charge and polarity per extension, after which the strength of the hydrophobic face should be modulated by choosing between Phe, Trp, Ile, Leu, Val, Ala, and Gly at various positions. This will allow for determination of the useful range of hydrophobicity of the hydrophobic face required to achieve activity (hydrophobic enough) without significant loss of selectivity (too high hydrophobicity). It should be noted that this hydrophobicity window only can yield a range of useful hydrophobicities, as our data and other studies have shown that the distribution of the hydrophobicity also is important.

Mean hydrophobicity

The mean hydrophobicity has (until now) been a measure which could be used to compare peptides of different lengths, as opposed to the hydrophobicity which is just a summation. However, the mean hydrophobicity is influenced by changes on both the hydrophilic- and the hydrophobic face of an AMP, and we have shown in Paper 1 that the hydrophobicity/polarity of these can be modulated with different effects. Thus, the mean hydrophobicity cannot be used as a parameter in itself as it depends on the contribution from two, more fundamental, parameters. It might be appropriate to defined a parameter like "average hydrophobicity of the hydrophobic face", which might be useful when comparing peptides of different lengths. However, as shown and discussed in Paper 1, the degree of hydrophobicity of the hydrophobic face should be reduced when increasing the length of an AMP, thus requiring such a parameter be some sort of peptide-length-function.

Hydrophobic moment/amphipathicity

The hydrophobic moment is a complex descriptor, which is dependent on the properties and orientation (in the helical wheel model) of the individual amino acids[30]. The parameter hydrophobic moment is also referred to as the amphipathicity of the helix. It is the vectorial sum of the hydrophobicities of each residue, where the vectorial directions are taken from the helical wheel model. The issue regarding the differences between the various hydrophobicity scales mentioned under the hydrophobicity section is also applicable here. The strength of the

hydrophobic moment (as a structural parameter) is that it takes into account whether a hydrophobic residue is oriented towards membrane interior or bulk solvent. It is easy to design studies, where this parameter can be useful (this is actually the case for the main part of the peptides published in paper 1), but the parameter cannot be used on all studies as it depends on several other parameters.

In order to increase the hydrophobic moment of a peptide, three possibilities exist, but commonly only two of them are used: either the polarity of a hydrophilic residue is increased (shown in paper 1 not to strongly increase hemolytic activity) or the hydrophobicity of a hydrophobic residue is increased (strongly increases hemolytic activity). The third (and mostly unexplored) way is to swap residues or atoms between side chains on either the hydrophobic- or hydrophilic face in a way which directs the most polar and most hydrophobic residues oppositely of each other (shown in paper 1 under "modulation of hydrophobicity profile" to have quite an impact on activity). The hydrophobic moment does not take into consideration the distribution of the various residues along the helical axis. Thus, it cannot differentiate between a peptide with an even distribution of hydrophobic resides and a peptide which has one strongly hydrophobic residue (lipophilic anchor) and correspondingly weaker hydrophobic residues on the hydrophobic face. This was shown in paper 1 to yield different activities. When these three types of alterations yield distinct changes in activity and selectivity, the hydrophobic moment/amphipathicity of a helix should NOT be used as a structural parameter in SAR studies. Much in line with the parameters defined for hydrophobicity, amphipathicity should be divided into describing the hydrophilic and hydrophobic faces separately, as well as observing each turn of the helix separately.

I cannot think of one parameter which can integrate these three considerations into one parameter. It would perhaps be useful to consider the faces separately, where the hydrophobic face is already considered in "hydrophobicity of the hydrophobic face" and its opposite was termed "polarity of the hydrophilic face". Both of these showed correlations with activity and selectivity in the study of paper 1, but neither takes into consideration how to distribute the hydrophobicity and polarity along the helical axis.

Charge

An increase in charge has often been found to increase antimicrobial activity without increasing cytotoxicity. In some papers, the increase in charge has even been found to increase selectivity by reducing the hemolytic activity[1,2,5]. However, a few papers[5,21] have been cited for showing that an increase in charge will not always be beneficial, and that it can increase the hemolytic activity to increase the charge above a certain point. These papers do not show that an increase in charge for a given peptide is unfavorable. In the work done by Bessalle et al., the increase in charge is done by extending the AMP of interest by a poly cationic segment, and thus the modified peptides cannot be compared to the original peptide[21]. In the work of Dathe et al., the increase in charge is followed by an increase in the hydrophobicity of the hydrophobic face (hydrophobicity of the non-cationic helix surface) of the AMP of interest in order to maintain hydrophobicity, hydrophobic moment, and polar angle (angle subtended by cationic residues) [5]. Thus, the increase in hemolytic activity is in my opinion purely a result of the hydrophobicity of the hydrophobic face, which is in accordance with their observations. As such, when increasing the polarity of the hydrophilic face of an α-helical AMP without changing its length, I have found no contradictions to the statement: An increase in charge will increase the activity of the AMP.

Whether the increase in charge can increase selectivity of a given α-helical AMP is still uncertain to me. In our experiments, where the increase in charge does not influence the hydrophobicity of the hydrophobic face, selectivity is not improved. Thus, I believe that an increase in charge or polarity will only increase the selectivity of a given AMP if it replaces a residue which was

contributing to the hydrophobicity of the hydrophobic face. The results presented in the study of Dathe et al.[5] can all be explained by this, but I cannot say for sure whether the increase in selectivity (by reduction of hemolytic activity) is caused solely by removal of hydrophobic residues that are able to interact with the membrane interior.

Helicity

Helicity is an often mentioned parameter in relation with SAR studies, and quite a lot of conclusions have been drawn on the basis of helicity measurements[5,21,28,47–52]. The most common conclusion is that the increase in helicity correlates with antimicrobial activity to some degree, and highly with cytotoxicity[52]. This is also the case for studies on anoplin[28,49–51].

First of all, I do not think that the helicity measurements conducted in many studies actually measures the degree of helix, a peptide adopts in the presence of a biological membrane. When measuring the helicity using CD, the commonly used and helix inducing solvent, TFE, does not yield information of how much a given peptide will fold into a helix upon interaction with actual membranes[52]. The use of DPC or SDS is a much more viable option, although the lipid composition and curvature of these micelles differs significantly from that of an actual membrane.
The question is; *what do these helicity measurements actually tell us about?*
The reported helicities are most likely a measure of the peptide's affinity for inserting into a membrane, which is dependent on the hydrophobicity of the hydrophobic face.
An increase in helicity is often associated with an increase in hydrophobicity of the peptide or an increased retention time on the HPLC, while a decrease in helicity is often associated with disrupting the secondary structure of otherwise perfectly amphipathic helices by the use of for instance D-amino acids [5,28,47,51,52]. The effects of increasing the hydrophobicity of a peptide have already been discussed. The retention time on a HPLC column depends on how large a hydrophobic interaction that can take place between the peptide and the carbon chains in the column. The size of this interaction would be greatest when most of the hydrophobic residues in the peptide are bound to the carbon chains, which for amphipathic helical AMPs would be when the hydrophobic face of the helix is bound to the column interior. The disruption of the secondary structure of such a peptide would then prevent the formation of the strongest possible hydrophobic face, thus lowering the hydrophobic interaction within the HPLC column.

This means that the observed correlation between helicity and hemolytic activity is most likely caused by the increase in hydrophobicity of the hydrophobic face (increased affinity for the hydrophobic interior of all membranes). The correlation sometimes observed between helicity and activity is caused by this as well, as an increase in hydrophobicity of the hydrophobic face will also increase the activity against the bacterial membranes at the same time as it increases the affinity against erythrocytes. This also explains why increase in helicity has not been reported to correlate with increased selectivity, as an increase in hydrophobicity of the hydrophobic face will eventually remove all selectivity of the AMP.

The following walkthrough of the published data on anoplin serves as a great example to substantiate my claim that the measured helicities does not say anything about the actual structure of a peptide in the presence of a biological membrane.

Helical content of anoplin

Until now, several reports regarding the helical content of anoplin have been given in literature, and the values are summarized in Table 11.1. Furthermore, molecular dynamics (MD)

simulations indicated that the anoplin helix involves residues 4-9 [49] and 4-7 [50]. Furthermore, it has been stated that "the helical content of peptides like anoplin cannot exceed 50-60% due to the terminal effects" [51].

Table 11.1: Reported helical content of anoplin	
Spectroscopic method	Helical content [membrane mimic]
CD[a] [49]	45% [30% TFE] 65% [160μM SDS]
CD[a] [28]	77% [50% TFE]
CD[a] [50]	32-43% [40% TFE] 32-43% [8mM SDS]
Resonance Raman[51]	9% [50% TFE]
[a] CD is known to have limited sensitivity for short helical sequences[51].	

In opposition to these data, all anoplin analogues studied by NMR in the presence of approximately equimolar amounts of DPC micelles (150mM DPC to 3mM AMP) were shown to fold into a regular α-helix for residue 2-10 plus the C-terminal amide while the N-terminal glycine residue was flexible (>90% helicity). Despite the differences in the experimental conditions, these data are quite contradictory to previous results regarding the helicity of anoplin.

This is why I question the classical way to measure helicity: The degree of helicity obtained for solutions of TFE or lipid concentrations of SDS and DPC below their CMC values are not comparable to the degree of helicity that will be induced upon interaction with actual membranes. These helical AMPs fold into their amphipathic structure due to the greatly favorable condition where the hydrophobic side chains are burried within a membrane and polar side chains interacting with lipid head groups or solvent. This condition is not fulfilled for peptides interacting with the isotropic solvent, TFE, or with individual surfactant molecules. In our NMR samples, the surfactant concentrations are more than 100 times the cmc, and the NMR data provide clear evidence for the presence of an α-helix ranging from residue 2 to 10.

In relation to the above discussion, it should be noted, that a result like "50% helicity" only means that 50% of the peptide bonds are in a helical structure. This could mean that 50% of the residues in each peptide are folded into a helix, but it could also mean that 50% of the peptide molecules in the ensemble are fully helical (e.g. bound to a membrane), while 50% are random coil (e.g. not bound to a membrane). It is not possible to distinguish between these two situations (and anything in between) using CD. The helicities obtained from CD are most likely just a measure of the propensity of the AMP to interact with a membrane. This would fit with the observed correlations between helicity and cytotoxicity – the more helicity, the higher the affinity for inserting into a membrane, and the higher hemolytic activity. Recall that increases in hydrophobicity are reported to result in increased helicity, and thus the increase in hydrohobicity of the hydrophobic face is, in my opinion, the actual cause for the increase in hemolytic activity.

When helicity is a measure of the propensity for an AMP to interact with a membrane, it would be thinkable that the degree of helicity measured by CD could still be an accurate measure of how much peptide is actually folded into helix. However, this is not the case, based on our experiments. The low helicities measured by CD of these analogues (which were shown by NMR to have >90% helicity) indicates that a large part of the peptides does not bind to the micelles. The PRE data contradicts this, as the distance dependence of the PRE would cause such a high relaxation rate on all peptides not bound to micelles that the PRE differences between buried residues and exposed residues of the micelle bound peptides would vanish.

Structural impact of kinks in a helix

When it has just been argued why helicity is not related to the actual helical structure of a peptide, then why are correlations found between helicity and hemolytic activity when disrupting the helical structure by the use of helix breaking residues?

I believe that it is not the kink itself (i.e. a residue which does not obtain helical phi and psi angles) that reduces the hemolytic activity. It is rather the structural impact that follows from the kink, where some of the hydrophobic residues that used to be able to interact with the membrane interior are forced into another orientation by steric hindrances and lack of hydrogen bonds in the backbone. This way, the hydrophobic strength of the hydrophobic face will be reduced, and a lower membrane affinity results. This is probably the reason why a lower hemolytic activity is obtained. The measured reduction in helicity upon introduction of a kink can be caused by two things then: The kink residue will not contribute to the helical signal in the CD measurement and the lower membrane affinity can cause a reduction in the number of molecules adopting a helical configuration in the sample.

The study by Shai and Oren of the cationic α-helical peptide TApar (GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE-$(NH_2)_2$) showed a gradual decrease in hemolytic activity upon reducing the helicity (measured in 40% TFE)[47]. However, this reduction of helicity was achieved by the substitution of Pro7, Leu18, and Leu19 with D-amino acids. These substitutions will break the helical structure, but the reduced hemolytic activity is probably a result of the structural changes around these substitutions, which prevents the hydrophobic residues from interacting with the membrane interior. Our data show that it is the strength of the interaction between the hydrophobic face and the membrane interior which determines the degree of hemolytic activity (Paper 1). In their study, they alter the direction of two of the most hydrophobic residues (Leu18 and Leu19) and/or introduced a kink between two pairs of very hydrophobic residues (LIPKII). A structural study of these analogues would show how much these changes displace the strongly hydrophobic residues, and would likely explain why a lower affinity (=lower hemolytic activity) for the membrane interior arises. In accordance with our results, the native TApar peptide is already too hydrophobic in order to exhibit a decent selectivity, and similar activity data as published by Zhai and Oren could likely be achieved by simply reducing hydrophobicity in the areas which were affected by the insertion of D-amino acids. Their insertion of D-amino acids yields a similar effect as we observed for 5K8W (paper 1), i.e. use of hydrophobic residues which are not able to interact with the membrane interior, and thus the resulting peptide retains a higher selectivity.

A similar, but more comprehensive, study was made by Dathe et al. using the peptide KLALKLALKALKAAKLA-NH_2 (KLAL)[48]. Here, the amino acids were pairwise substituted from the N-terminus with their D-analogues. It was found that the helicity (CD in 50%TFE) decreases the most when substituting in the middle of the sequence and that helicity correlates with dye release from neutrally charged large unilamellar vesicles (corresponding to their hemolytic activity). The introduction of the D-amino acids in the middle of the sequence will have a higher impact on the helical structure at the termini, and this is probably the reason that a lower hemolytic activity is observed for these analogues. The structural changes prevent some of the hydrophobic residues from interacting with the membrane interior, and a greater disruption (and thus reduction in the strength of the hydrophobic face) takes place when substituting in the middle of the sequence. This is in accordance with their own observations where they write “We suggest that double D-amino acid substitution modifies the size of the hydrophobic helix domain thus influencing hydrophobic peptide-lipid interactions” [48].

Furthermore their study also included a few analogues with reduced hydrophobicity (Leu to Ala substitutions). These analogues were found to exhibit lower hemolytic activity and reduced helicity as well. It is likely the strength of the hydrophobic face which again is the cause for the increased hemolytic activity.

To conclude

If a kink is introduced in an α-helical AMP, the resulting changes in side chain orientations will simply yield a smaller hydrophobic face, thus resulting in lower hemolytic activity. If the side chains were subsequently swapped around to allow for all hydrophobic side chains to again be directed towards the membrane interior, I believe that a basically unaltered hemolytic activity compared to the original AMP would be observed. Furthermore, reduction in hydrophobicity of the hydrophobic face is found to correlate with reduced hemolytic activity and helicity, which substantiates my hypothesis that helicities are most likely a measure of the peptide's affinity for inserting into a membrane, which is dependent on the hydrophobicity of its hydrophobic face.

Polar angle

The polar angle/hydrophobic angle is not a structural parameter which in itself is related to an α-helical AMPs activity. In a study such as Paper 1, the polar- or hydrophobic angle would be the same for all the conservative analogues, which display quite significant variations in both activity and selectivity. One could imagine that the experimentally determined polar angle might differ from analogue to analogue, but this was found not to be the case.
Furthermore, the polar angle is a "dependent variable", which means that the polar angle cannot be altered without also altering other (and more fundamental) properties of the peptide.

The polar angle is a geometrical concept based on a projection of expected orientations of each residue in an α-helix. In my opinion, the fact that this is a projection is often forgotten or not considered very well. When looking at a helical wheel projection of a helical AMP and attempting to increase the polar angle, this is can be done by either non-conservative substitutions or rearranging the locations of the polar residues. This is best explained by examples. In Figure 10.1, a few helical wheel projections are given of peptides published by Dathe et al.[3].

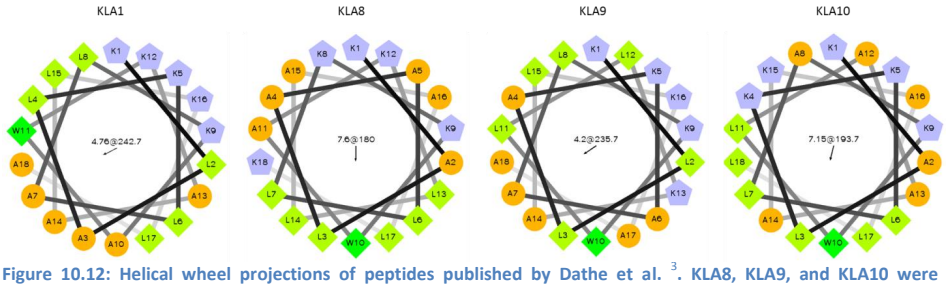

Figure 10.12: Helical wheel projections of peptides published by Dathe et al. [3]. KLA8, KLA9, and KLA10 were designed to be analogues which vary only in polar angle. Projections are made using the online service at: http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.
Note: KLA8, KLA9, and KLA10 were designed by Dathe et al. to be identical in respect to hydrophobicity and nearly identical in respect to hydrophobic moment (reported hydrophobic moments were 0.291, 0.295, and 0.299, respectively). They all consist of the exact same amount of amino acids 6 Ala, 6 Leu, 1 Trp, and 5 Lys, and were designed in order to compare the effect of increasing the polar angle as an isolated parameter. From the helical wheel projections shown here, it is obvious that KLA9 cannot have a similar hydrophobic moment to that of KLA8 and KLA10. The size and direction of the hydrophobic moment is actually given in the center of each projection. The hydrophobic moments reported in the article are thus erroneous.

Non-conservative substitutions

The polar angle is extended either with a continuous polar face, or with a polar face with apolar residues in between. From KLA1 (in Figure 10.1), an increase in a continuous polar angle would result from substituting L8 or L2 with a polar amino acid, whereas a polar angle with hydrophobic residues in between would result from substituting L15 or A13 with a polar residue. Of course, this is under the assumption that the helical wheel projection is in agreement with the actual helical structure of the peptide.

These alterations are actually not just an increase in polar angle, but also an increase in polarity of the hydrophilic face and a reduction of the hydrophobicity of the hydrophobic face of the helix. I believe that the two latter are the actual cause for changes in activity, and not the increased polar angle as such.

Rearrangement of residues

Examples of how the polar angle is increased by rearranging the polar residues can be seen from KLA8, KLA9, and KLA10 compared with KLA1. The problem of this type of modification is that it looks fine on the helical wheel projection, but in fact, this type of modification drastically alters the amphipathicity of each turn of the helix. Both KLA8 and KLA10 are left with one turn devoid of polar residues, creating a very hydrophobic segment (which makes it highly attracted to all membranes), while KLA9 has two very strongly hydrophobic residues oriented towards the bulk water (assuming that the peptide adapts a helical structure and inserts itself into a membrane with the polar residues oriented out of the membrane). I am actually not convinced that KLA9 will actually fold into a regular α-helix, as Ala does not have a high affinity for a membrane interior, whereas Leu does. I believe that this analogue would be more or less unstructured, as this will allow for the highly hydrophobic residues to interact with the membrane interior. This is supported in part by their helicity measurements, where KLA9 has the lowest degree of helicity of all the KLA analogues in that paper. Keep in mind that helicity measurements in TFE does not show whether a helix will actually be formed upon membrane interaction.

KLA8, KLA9, and KLA10 all show about a factor of 5 higher hemolytic activities than KLA1, while only altering the antimicrobial activity with about a factor of 2. According to our theories, this increase in hemolysis should be caused by a stronger interaction of the hydrophobic face of the peptide with the membrane interior. This is easily explained for KLA8 and KLA10 due to the turn without hydrophilic residues and due to the highly hydrophobic residues being directed towards the membrane interior (in KLA1, the Ala residues are directed towards the membrane interior and the Leu residues towards the interface between membrane and solvent, according to the projections). For KLA9, a stronger interaction of the Leu residues with the membrane interior would be possible if a regular helix is not formed, but experimental data is needed to conclude on this. In the work of Dathe et al., it was pointed out (and included in the abstract) that a polar angle above 80˚ was associated with a significant increase in hemolytic activity, which I believe is a misinterpretation for the reasons explained above.

To conclude

Our results from Paper 1 and the principles exemplified from literature clearly show that the polar angle is not a parameter which can be used in SAR studies. It is rather a phenotype which can result from several types of modifications of the peptides.

Even if people do not agree with these points, we have already shown in paper 1 and 2 that the side chain orientations are not in complete agreement with the helical wheel model, and that the helix of longer peptides can be extended (and maybe compressed) depending on the sequences. Thus, polar angles calculated based on the helical wheel model are not necessarily in accordance with the actual structure of the peptides.

11. Conclusion

The SAR derived from the anoplin study

The relationship between the activity of a cationic helical AMP and its structure is surprisingly simple, considering the variety of parameters used in the literature so far. In order to increase the general membrane affinity of an AMP, the hydrophobicity of its hydrophobic face should simply be increased. This will, however, be an unbiased increase in membrane affinity, and after crossing a peptide-length dependent threshold value, further increases in hydrophobicity will quickly cause the loss of selectivity towards bacterial membranes. This knowledge also gives the answer on how to increase the selectivity of an already highly hemolytic helical AMP: reduce the hydrophobicity of its hydrophobic face (within reason). This will always increase the selectivity for highly hemolytic helical AMPs. In order to increase the activity of a cationic helical AMP in selective manner, the focus should be on the hydrophilic face of the helix. We have demonstrated in paper 1 that there should be a distinction between the residues oriented such that their side chains cannot interact with the membrane interior and the residues which are in the interface between the membrane interior and the surrounding solvent. It was shown that increasing the polarity (which includes the addition of charge) will for both types create analogues with higher antibacterial activities. For some analogues, this increase in activity was followed by an equal increase in hemolytic activity, thus maintaining the selectivity. However, due to low hemolytic activities, it could not be seen if all increases in charge are followed by an equal increase in hemolytic activity or if an increase in activity can be achieved without an increase in hemolytic activity.

The simple “to do” is thus: First trim the hydrophobicity of the hydrophobic face until hemolysis is at an acceptable level, and then increase the polarity of the hydrophilic face until further increases cannot increase the antibacterial activity any further.

Although this very simple SAR exists for the cationic helical AMPs, there are various other ways to alter the sequence (and in some cases structure as well) of these AMPs. One can use hydrophobic residues on the hydrophilic face, polar or charged residues on the hydrophobic face, replace a given residue with its D-amino acid counterpart or peptoid counterpart, or even use a mixture of these types of changes. Further research is still needed to answer on how all these kinds of changes will influence the activity and selectivity of the cationic helical AMPs.

12. Perspectives

AMP testing in the future

The use of the MIC assay in evaluating the potency of AMPs is in principle a good way to evaluate the effect of given substitutions compared to the initial AMP lead structure. However, many of the studies presented in literature evaluate the antimicrobial activity at peptide concentration increments of a factor of 2 or more. This brings about a great deal of uncertainty of how big a change in activity is actually achieved by a given substitution. As an example, AMP1 has an actual MIC of 95µM and AMP2 has an actual MIC of 105µM. If tested in a factor 2 dilution series starting at 200µM, AMP1 would be measured with a MIC of 50µM and AMP2 would be measured with a MIC of 100µM. They are thus observed to have a factor 2 difference in activity, despite that the actual difference is only a factor of 1.1. It is important for this type of studies to use small increments in peptide concentration in the MIC assay, and this becomes very essential in the structure activity relationship studies where the structural changes are very small.

In newer literature, a higher degree of consensus regarding the parameters which are important for AMP activity and selectivity is found[53], but despite promising *in vitro* experiments it is still uncertain whether these compounds are of any use *in vivo*. After spending 3 years on SAR studies, I cannot help but pondering about the usefulness of the MIC and hemolysis assay in relation to evaluating AMPs as potential antibiotics. For some of my compounds the selectivity (EC50/MIC) reaches a factor of 80, but is this enough? What is a good selectivity? Upon administration of a drug, the local concentration should never reach a toxic level, and in my opinion, even 5-10% hemolysis is pretty bad. The gap between the MIC and the concentration at which no hemolysis is observed is significantly less than the calculated selectivity. Furthermore, the fact that hemolysis actually does occur at high concentrations of a given AMP indicates that the AMP has some affinity for the mammalian cells, which will cause adsorption of the AMP to non-bacterial cells and thus reducing the effective concentration of that AMP.
It is in my opinion essential that we develop new methods for routine testing of AMP usefulness. The most straightforward approach to evaluating the MIC and selectivity of an AMP *in vivo* is to better mimic the conditions of an infection in the blood. By performing the MIC testing in the presence of human blood cells, the adsorption of AMP to the mammalian cells will be taken into account in the determination of the MIC value. Furthermore, by using a microscope technique to monitor the growth of bacterial cells as well as the destruction of the erythrocytes, it will be possible to measure whether a MIC concentration without significant side effects can be obtained. This would give a reasonable guideline for which AMPs are selective enough to be considered potential for drug development. The current developments with the oCelloScope, already makes such a combined assay technically possible.
This does, however, not take into consideration the rate of degradation of AMP, which is essential according to our experiments. They show that the anoplin analogues should be present at a concentration above the effective MIC for more than 2 hours before killing of bacterial cells is initiated, and for more than 4 hours for effective killing of bacterial cells (dilution of our peptide to below MIC after 4 hours of incubation allowed for bacterial cells to continue growth). The inclusion of host AMP degradation in an *in vitro* experiment would be a huge improvement in a MIC assay.

If these changes could be implemented in the routine testing of AMPs, I believe this would give a rather realistic evaluation of the usefulness of an AMP for combatting bacterial infections in the blood by intravenous administration. However, it will not be possible to extrapolate these results to infections in tissues or by other means of administration, as the transport of AMP to the bacterial cells becomes much more complex.

Future experimental designs

During the work performed in this Ph.D. study, I came across a lot of questions regarding AMP structure-activity relationships which I could not answer based on the set of peptides which were produced and tested in the project. In order to answer these questions, a series of smaller studies should be performed, which I present in this section.

Bending of side chains

From the set of anoplin analogues containing Trp, the structural studies showed a bending of the side chain of the Trp residue for 5W and 5F8W (the ones which places Trp as an extension of the hydrophobic face), but not for 5K8W (Trp inserted instead of a solvent interacting residue). These analogues were the onset for thinking that there are two types of polar residues in an amphipathic helical AMP. However, the bending is very significant for a Trp residue, as the orientation of the asymmetric indole moiety is highly influential on the direction to the geometric mean of the side chain. It is uncertain whether an equally significant bending will be observed when using for instance a Leu residue. The data from our study are unambiguous, but the results are still obtained from a very small set of peptides, and only by using Trp as the hydrophobic residue. In order to validate our findings, a more extensive study which includes a more symmetric hydrophobic side chain should be performed. For this study I propose the use of 5,8K as the reference compound in which Trp and Leu are systematically inserted instead of the Lys residues to investigate the structural behavior in all positions occupied by a polar residue in the wild type anoplin.

Reference compound	Trp series	Leu series
GLLKKIKKLL-NH2 (anoplin 5,8K)	GLL**W**KIKKLL-NH2	GLL**L**KIKKLL-NH2
	GLLK**W**IKKLL-NH2	GLLK**L**IKKLL-NH2
	GLLKKI**W**KLL-NH2	GLLKKI**L**KLL-NH2
	GLLKKIK**W**LL-NH2	GLLKKIK**L**LL-NH2

Lipid head group interacting versus a water interacting residues

It was observed that inserting the Trp residue as an extension of the hydrophobic face of anoplin (5W) was detrimental to the selectivity of these analogues due to a very large rise in hemolytic activity. However, insertion of the Trp residue instead of a solvent exposed residue (5K8W) was less detrimental to the selectivity due to a lower rise in hemolytic activity. However, the resulting analogues also varied significantly in mean hydrophobicity which prevents a conclusive answer to why the insertion of Trp in position 8 is less detrimental to the selectivity. In extension to the “Bending of side chain” study, the analogues should be tested for antimicrobial and hemolytic activity to verify that there is a difference in selectivity between inserting a hydrophobic residue instead of a lipid head group interacting and instead of a solvent oriented residue. However, the reference compound (5,8K) has an EC50 of above 500μM, which makes it impossible to estimate how much the selectivity is affected, despite it will be easily observed which analogues will be most hemolytic. To quantify the loss in selectivity, a slightly more hemolytic analogue should be used as reference, such as 2,10Hle5,8K, which was found to have an EC50 below 500μM.

Reference compound	Trp series	Leu series
G-Hle-LKKIKKL-Hle-NH2 (2,10Hle5,8K)	G-Hle-L**W**KIKKL-Hle-NH2 G-Hle-LK**W**IKKL-Hle-NH2 G-Hle-LKKI**W**KL-Hle-NH2 G-Hle-LKKIK**W**L-Hle-NH2	G-Hle-L**L**KIKKL-Hle-NH2 G-Hle-LK**L**IKKL-Hle-NH2 G-Hle-LKKI**L**KL-Hle-NH2 G-Hle-LKKIK**L**L-Hle-NH2

The role of Gly and Ala in AMPs

For longer peptides, Gly and Ala become more prevalent. They are often found on the polar face or at the the interface between the hydrophobic and polar face. In the study of Ifrah et al., the Ala scan of anoplin showed that antimicrobial activity and hemolytic activity was increased when inserting Ala instead of the polar residues 5, 7, and 8, but not upon substitution of polar residue 4 [28]. I believe that Ala mainly plays a role as an interface residue, which contributes only little to the strength of the hydrophobic face, whereas Gly does not contribute to the strength of the hydrophobic face at all. Gly is in my opinion used on the polar face as a "neutral" residue in order to reduce charge repulsion between the charged side chains – in other words: just a filling residue.

In order to confirm these thoughts, I would perform an Ala scan and Gly scan only on the polar residues while using 5,8K as the reference compound. The results from the Ala scan would help in validating whether there are two different types of polar residues, as well as being useful for estimating how much Ala actually contributes to the strength of the hydrophobic face. The Gly scan would be useful for estimating whether Gly can actually contribute to the strength of the hydrophobic face, and estimate the importance of charge in each of the polar positions.

Reference compound	Ala series	Gly series
GLLKKIKKLL-NH2 (anoplin 5,8K)	GLL**A**KIKKLL-NH2 GLLK**A**IKKLL-NH2 GLLKKI**A**KLL-NH2 GLLKKIK**A**LL-NH2	GLL**G**KIKKLL-NH2 GLLK**G**IKKLL-NH2 GLLKKI**G**KLL-NH2 GLLKKIK**G**LL-NH2

Rotation of a helical AMP

In our study we observed rotation of anoplin around the helical wheel axis with respect to the micelle surface when double substituting with hydrophobic residues. In our experiments, these were highly hydrophobic residues, and the equal "pull" towards the micelle interior of all residues makes rotation and the observed amount of rotation highly plausible. However, in many AMPs the distribution of hydrophobic residues is not as symmetric as in anoplin. Thus, it would be beneficial to see how much the rotation depends on the hydrophobic strength of the hydrophobic residues. Furthermore, it would be interesting to know if this rotation can take place in both directions, and whether the terminal groups influence the ability to rotate. As a preliminary study, I propose to structurally study two types of double substitutions in anoplin (4,7X and 5,8X as shown in Figure 12.1) by using either Leu, Val, or Ala for each analogue. By using Leu, it will be possible to see if the helix has an equal propensity for rotation in both directions when all the hydrophobic residues have an equal hydrophobicity. By using Val and Ala, it will be possible to see how the magnitude of hydrophobicity influences the degree of rotation.

Reference compound	Leu series	Ala series	Gly series
GLLKKIKKLL-NH2 (anoplin 5,8K)	GLLK**L**IK**L**LL-NH2 GLL**L**KI**L**KLL-NH2	GLLK**A**IK**A**LL-NH2 GLL**A**KI**A**KLL-NH2	GLLK**G**IK**G**LL-NH2 GLL**G**KI**G**KLL-NH2

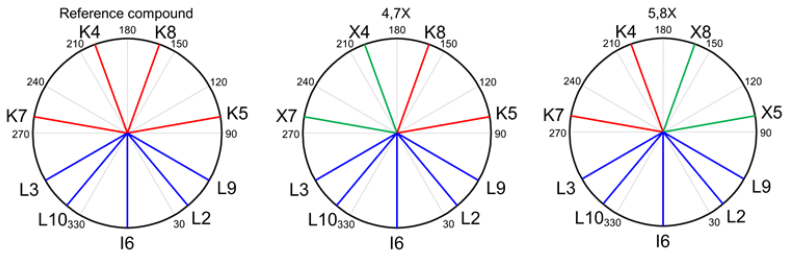

Figure 12.1: Helical wheel plots, illustrating the locations of the double substitutions used in the experiments to investigate rotation of the helix compared to the reference compound. Hydrophobic residues are in blue, hydrophilic residues in red, and substitution sites in green.

The knowledge obtained from the Gly and Ala substitution series as well as these rotation studies will provide crucial information regarding whether to replace an Ala and Gly residue in an AMP with a polar or hydrophobic residue when modulating the activity. Furthermore, this will probably enable one to predict the results from Ala scan experiments, thus rendering these experiments unnecessary in the future.

Site of insertion of a lipophilic anchor

The results of my studies where Cha was inserted at different locations in anoplin showed a tendency that the site of insertion influenced the antimicrobial and hemolytic activities in different ways. This was estimated to be within experimental error, and thus nothing could be concluded on the basis of these data. However, the studies of Munk et al. and Slootweg et al. showed similar results, where the MIC and EC50 are dependent on the site of Leu to X substitution[54,55]. The study of Munk et al. was however designed with very large increments in peptide concentrations in the MIC assay (jumps with a factor of 3), which makes it uncertain how big the difference in MIC actually is while the study of Slootweg et al. was based on a small subset of peptides.

Thus, in order to answer whether the site of insertion really does play a significant role in the antimicrobial and hemolytic activity, a more systematic study should be performed. I suggest to increase hydrophobicity of each of the hydrophobic residues in turn, using e.g. Hle, Epa, Phe, Cha, and (S)-2-aminoundecanoic acid (the lipophilic anchor used by Slootweg et al.[54]). Further choices of side chains with increasing hydrophobicity can of course be included.

Reference compound	Substitution series	Suggested residues
GLLKKIKKLL-NH2 (anoplin 5,8K)	G**X**LKKIKKLL-NH2	Hle
	GL**X**KKIKKLL-NH2	Epa
	GLLKK**X**KKLL-NH2	Phe
	GLLKKIKK**X**L-NH2	Cha
	GLLKKIKKL**X**-NH2	(S)-2-aminoundecanoic acid

Anoplin is an excellent model peptide for an initial study on hydrophobic anchors, but due to its very short length a large change in hydrophobicity in one position becomes very influential for the whole peptide. This will make the findings from a study on anoplin very hard to extrapolate to longer peptides. Thus, this type of study should also be performed on a peptide of a significant length to distinguish between an increase in hydrophobicity at a terminal, near a terminal, and in the middle of an AMP. The choice of AMP should be one which shows only a slight degree of hemolysis in the reference sequence, as all increases in side chain hydrophobicity will increase the hemolytic activity and the propensity for self-aggregation.

Maculatin-G15 studies with inclusion of peptoid monomers

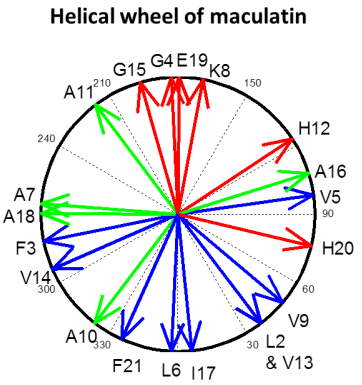

Figure 12.2: Experimentally derived helical wheel of maculatin-G15 (GLFGVLAKVAAHVVGAIAEHF-NH2).

Is *trans* in general the most favorable conformer?

From the structural studies of maculatin-G15 analogues with a peptoid monomer (Nleu) substituted into position 9, 10, 11, and 13 showed that upon insertion of the analogue into a micelle, only one conformer was observed for M-Nleu10 and M-Nleu11 (*trans* for M-Nleu-11 and unknown for M-Nleu10), while the *trans* and *cis* conformers were almost equally favorable for M-Nleu9 and M-Nleu13. The cause for this is puzzling to me. Could it be that the trans conformer in general is more favorable than the cis conformer (perhaps due to the differences in backbone folding), but that the trans conformer always directs the Nleu side chain significantly counter-clockwise (>100˚) in the helix relative to an amino acid side chain, as seen for M-Nleu11? If this is the case, the trans conformer of M-Nleu9 and M-Nleu13 would have the Nleu side chain directed towards the lipid head groups or solvent surrounding the micelle. This would be energetically unfavorable, thus making it relatively more favorable to adopt the cis conformation. This could be the explanation for the presence of both *cis* and *trans* conformers for these two analogues.

Before trying to prove this hypothesis, it should however be tested if this is a general trend, and not just a coincidence within this small subset of analogues. In order to test whether there is a trend is to expand the set of analogues with a Nleu residue inserted. My suggestions for the sites of substitution are marked by underline and bold in the maculatin-G15 sequence:

GLFG**VLA**KVAAHV**V**G**AIA**EHF-NH2

It is important to notice that the insertion of the peptoid monomer close to the terminals of the peptide increases the risk that a helical secondary structure will not be formed on both sides of the peptoid monomer, and that the smaller segment might not be amphipathic enough to insert itself into the micelle. However, if helical structure and insertion into micelle is obtained for both ends of the analogues, a larger set and variety of peptoid monomer orientations can be tested for prevalence of the *cis* and *trans* conformer. From Figure 12.2, it can be seen that residues 5, 6, and 7 are oriented similarly to residues 16, 17, and 18, and significantly different from the already investigated analogues (residues 9, 10, 11, and 13).

A different approach for testing whether a general trend for *trans* being the most favorable conformer is observed is to use a charged peptoid monomer such as Nlys (peptoid monomer with side chain identical to that of Lys) in the same positions as were tested with the Nleu residue. This would invert the side chain properties and should thus make the *trans* conformer unfavorable for positions 10 and 11, while making it favorable for positions 9 and 13. The benefit

of this approach is that the two helical segments surrounding the peptoid monomer will be formed (although the degree of helix breaking can vary from what has been observed in our studies). Of course, the set of analogues with this polar peptoid monomer can be expanded to all the sites suggested for Nleu.
NMR is a fast method for determining the distribution of the *cis* and *trans* conformers of the various analogues. A quick estimation of the amount of spin systems in the TOCSY spectrum reveals whether one or two conformers are present, and after assignment of residues to the different spin systems, the NOESY spectrum reveals whether it is the *cis* or *trans* conformer. The distinction is based on the presence of NOEs from the H^{α} of the residue preceding the peptoid monomer to the backbone or the side chain of the peptoid monomer. This is relatively easy to perform, compared to calculating the actual structure for each analogue, thus making this a worthwhile study to perform.

Further characterization of the maculatin peptide/peptoid hybrids

The study of the maculatin analogues in this work has solely been focusing on structural characterization. Thus, other properties of these analogues have not been investigated, which leaves unanswered questions regarding especially the activity (MIC and EC50) and proteolytic stability.

MIC and EC50 study
When studying how the insertion of the Nleu peptoid monomer influences the activity of the AMP, it is highly interesting to notice whether the presence of one or two conformers significantly changes the activity and selectivity. It would be highly interesting to compare M-Nleu13 with M-Nleu14 (both are Val to Nleu substitutions), as these analogues have the same overall properties but are expected to have different conformer distributions. M-Nleu13 has already been shown to adopt two conformers, and residue 14 is oriented between the residues 10 and 11 which only adopted one conformer.

Proteolytic stability study
It is well known that the insertion of a peptoid monomer increases the proteolytic resistance of an AMP, but it is not well described how this increase in proteolytic resistance is influenced by the length of the AMP, the site of insertion of the peptoid monomer, and the number of inserted peptoid monomers. For such a long AMP, it is likely that the proteolytic resistance is only slightly increased upon insertion of a peptoid monomer at one of the ends of the AMP, as a major part of the helix will be unaffected by this substitution.
The measurements of the proteolytic stability of these hybrid compounds should be done both in an aqueous environment and in a sample where interaction with lipid micelles is possible. Depending on the membrane affinity for a given compound, the different conditions can yield very different degradation rates. Finally, it would be highly interesting to relate the presence of one or two conformers with the proteolytic resistance of the AMPs in order to evaluate what is most favorable.

From all of these studies, the hope is to be able to formulate a set of guidelines on where to insert hydrophobic and polar peptoid monomers in order to modulate the activity and selectivity of the AMP while increasing the proteolytic stability as much as possible.

Prospects for using PRE in future research

As shown in paper 1, the use of PRE to derive distance restraints was of high importance for analogues which were non-conservatively modified, as it showed how the orientation of side

chains and peptide as a whole changed. For the conservative substitutions, only slight changes in insertion depth were observed, and for conservative changes on the hydrophobic face, these insertion depth changes did not correlate well with the activity of the analogues. In my opinion this demonstrates that PRE derived distance restraints are not useful for deriving SAR parameters. However, PRE is highly beneficial to use on the lead compound of interest in order to characterize its orientation and to classify its residues (solvent interacting, lipid head group interacting, or membrane interior interacting). This knowledge is very important for rationally designing improved analogues, and an important benefit from using PRE.

The perhaps largest strength in our use of PRE is its ability to yield structural information of unstructured membrane interacting peptides and peptide segments. For unstructured segments, this technique will yield information on which residues are embedded into the membrane and which are exposed to the solvent. This knowledge will assist in understanding how these AMPs are bound to the membrane and which requirements to their sequence that must be fulfilled in order for this membrane binding to take place (if any requirements exists).

Many α-helical AMPs contain a Pro residue in their sequence which is generally believed to cause a kink in the helix. Kinks are also expected upon insertion of D-amino acids or peptoid monomers, which are primarily done in order to increase proteolytic resistance of the AMP of focus. As demonstrated in our work on maculatin analogues with an embedded peptoid monomer, PRE derived distance constraints are essential for determining the global orientation of the two segments which are separated by a kink. This even provided an explanation of why only the *trans* conformer was found for M-Nleu11. Thus, PRE is of great potential in structural studies of membrane interacting peptides with kinks or structurally undefined middle regions.

13. References

1. Chen Y, Mant CT, Farmer SW, Hancock REW, Vasil ML, Hodges RS (2005) Rational design of alpha-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. J. Biol. Chem. 280:12316–12329.

2. Giangaspero A, Sandri L, Tossi A (2001) Amphipathic α helical antimicrobial peptides. Eur. J. Biochem. 268:5589–5600.

3. Dathe M, Wieprecht T, Nikolenko H, Handel L, Maloy WL, MacDonald DL, Beyermann M, Bienert M (1997) Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides. FEBS Lett. 403:208–212.

4. Wieprecht T, Dathe M, Krause E, Beyermann M, Maloy WL, MacDonald DL, Bienert M (1997) Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. FEBS Lett. 417:135–140.

5. Dathe M, Nikolenko H, Meyer J, Beyermann M, Bienert M (2001) Optimization of the antimicrobial activity of magainin peptides by modification of charge. FEBS Lett. 501:146–150.

6. Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3:238–50.

7. Nizet V (2006) Antimicrobial peptide resistance mechanisms of human bacterial pathogens. Curr. Issues Mol. Biol. 8:11–26.

8. Wang G Antimicrobial Peptides : Discovery, Design and Novel Therapeutic Strategies. Wallingford, Oxon, GBR: CABI Publishing; 2010.

9. Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. Nat. Immunol. 6:551–557.

10. Broekaert WF, Terras FR, Cammue BP, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol. 108:1353–1358.

11. Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12:39–85.

12. Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1462:11–28.

13. Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res. 37:D933–937.

14. Michael E. Selsted (2004) Theta-Defensins: Cyclic Antimicrobial Peptides Produced by Binary Ligation of Truncated alpha-Defensins. Curr. Protein Pept. Sci. 5:365–371.

15. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395.

16. Reddy KVR, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. Int. J. Antimicrob. Agents 24:536–547.

17. Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. 55:27–55.

18. Nguyen LT, Haney EF, Vogel HJ (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol. 29:464–472.

19. Mowery BP, Lindner AH, Weisblum B, Stahl SS, Gellman SH (2009) Structure-activity relationships among random nylon-3 copolymers that mimic antibacterial host-defense peptides. J. Am. Chem. Soc. 131:9735–9745.

20. Dathe M, Wieprecht T (1999) Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochim. Biophys. Acta - Biomembr. 1462:71–87.

21. Bessalle R, Haas H, Goria A, Shalit I, Fridkin M (1992) Augmentation of the antibacterial activity of magainin by positive-charge chain extension. Antimicrob. Agents Chemother. 36:313–317.

22. Engelman DM, Steitz TA, Goldman A (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Annu. Rev. Biophys. Biophys. Chem. 15:321–353.

23. Rose G, Geselowitz A, Lesser G, Lee R, Zehfus M (1985) Hydrophobicity of amino acid residues in globular proteins. Science. 229:834–838.

24. Eisenberg D (1984) Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. 179:125–142.

25. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105–132.

26. Cornette JL, Cease KB, Margalit H, Spouge JL, Berzofsky JA, DeLisi C (1987) Hydrophobicity scales and computational techniques for detecting amphipathic structures in proteins. J. Mol. Biol. 195:659–685.

27. Fauchère J-L, Charton M, Kier LB, Verloop A, Pliska V (1988) Amino acid side chain parameters for correlation studies in biology and pharmacology. Int. J. Pept. Protein Res. 32:269–278.

28. Ifrah D, Doisy X, Ryge TS, Hansen PR (2005) Structure-activity relationship study of anoplin. J. Pept. Sci. 11:113–121.

29. Li X, Li Y, Han H, Miller DW, Wang G (2006) Solution structures of human LL-37 fragments and NMR-based identification of a minimal membrane-targeting antimicrobial and anticancer region. J. Am. Chem. Soc. 128:5776–5785.

30. Eisenberg D, Weiss RM, Terwilliger TC (1982) The helical hydrophobic moment: a measure of the amphiphilicity of a helix. Nature. 299:371–374.

31. Uematsu N, Matsuzaki K (2000) Polar angle as a determinant of amphipathic alpha-helix-lipid interactions: a model peptide study. Biophys. J. 79:2075–2083.

32. Sjöström M, Wold S (1985) A multivariate study of the relationship between the genetic code and the physical-chemical properties of amino acids. J. Mol. Evol. 22:272–277.

33. Hellberg S, Sjoestroem M, Skagerberg B, Wold S (1987) Peptide quantitative structure-activity relationships, a multivariate approach. J. Med. Chem. 30:1126–1135.

34. Lejon T, Strøm MB, Svendsen JS (2001) Antibiotic activity of pentadecapeptides modelled from amino acid descriptors. J. Pept. Sci. 7:74–81.

35. Jenssen H, Gutteberg TJ, Lejon T (2005) Modelling of anti-HSV activity of lactoferricin analogues using amino acid descriptors. J. Pept. Sci. 11:97–103.

36. Jenssen H, Fjell CD, Cherkasov A, Hancock REW (2008) QSAR modeling and computer-aided design of antimicrobial peptides. J. Pept. Sci. 14:110–114.

37. Jenssen H, Gutteberg TJ, Rekdal Ø, Lejon T (2006) Prediction of activity, synthesis and biological testing of anti-HSV active peptides. Chem. Biol. Drug Des. 68:58–66.

38. Taboureau O (2010) Methods for building quantitative structure-activity relationship (QSAR) descriptors and predictive models for computer-aided design of antimicrobial peptides. Methods Mol. Biol. 618:77–86.

39. Jenssen H (2011) Descriptors for antimicrobial peptides. Expert Opin. Drug Discov. 6:171–184.

40. Sandberg M, Eriksson L, Jonsson J, Sjöström M, Wold S (1998) New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. J. Med. Chem. 41:2481–2491.

41. D. Fjell C, E.W. Hancock R, Jenssen H (2010) Computer-Aided Design of Antimicrobial Peptides. Curr. Pharm. Anal. 6:66–75.

42. Franzmann M, Otzen D, Wimmer R (2009) Quantitative Use of Paramagnetic Relaxation Enhancements for Determining Orientations and Insertion Depths of Peptides in Micelles. Chembiochem 10:2339–2347.

43. Respondek M, Madl T, Göbl C, Golser R, Zangger K (2007) Mapping the orientation of helices in micelle-bound peptides by paramagnetic relaxation waves. J. Am. Chem. Soc. 129:5228–5234.

44. Franzmann M Novel applications of constrast agents in structural biology of peptides and detergents. Ph.D. thesis: Aalborg University; 2010.

45. Schiffer M, Edmundson AB (1967) Use of Helical Wheels to Represent the Structures of Proteins and to Identify Segments with Helical Potential. Biophys. J. 7:121–135.

46. Tossi A, Sandri L, Giangaspero A (2002) New consensus hydrophobicity scale extended to non-proteinogenic amino acids. Peptides:416–417.

47. Shai Y, Oren Z (1996) Diastereomers of Cytolysins, a Novel Class of Potent Antibacterial Peptides. J. Biol. Chem. 271:7305–7308.

48. Dathe M, Schümann M, Wieprecht T, Winkler A, Beyermann M, Krause E, Matsuzaki K, Murase O, Bienert M (1996) Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. Biochemistry 35:12612–12622.

49. Konno K, Hisada M, Fontana R, Lorenzi CC, Naoki H, Itagaki Y, Miwa A, Kawai N, Nakata Y, Yasuhara T, et al. (2001) Anoplin, a novel antimicrobial peptide from the venom of the solitary wasp Anoplius samariensis. Biochim. Biophys. Acta 1550:70–80.

50. Dos Santos Cabrera MP, Arcisio-Miranda M, Broggio Costa ST, Konno K, Ruggiero JR, Procopio J, Ruggiero Neto J (2008) Study of the mechanism of action of anoplin, a helical antimicrobial decapeptide with ion channel-like activity, and the role of the amidated C-terminus. J. Pept. Sci. 14:661–669.

51. Won A, Pripotnev S, Ruscito A, Ianoul A (2011) Effect of point mutations on the secondary structure and membrane interaction of antimicrobial peptide anoplin. J. Phys. Chem. B 115:2371–2379.

52. Zelezetsky I, Pacor S, Pag U, Papo N, Shai Y, Sahl H-G, Tossi A (2005) Controlled alteration of the shape and conformational stability of alpha-helical cell-lytic peptides: effect on mode of action and cell specificity. Biochem. J. 390:177–188.

53. Jiang Z, Vasil AI, Vasil ML, Hodges RS (2014) “Specificity Determinants” Improve Therapeutic Indices of Two Antimicrobial Peptides Piscidin 1 and Dermaseptin S4 Against the Gram-negative Pathogens Acinetobacter baumannii and Pseudomonas aeruginosa. Pharmaceuticals 7:366–391.

54. Slootweg JC, van Schaik TB, Quarles van Ufford H (Linda) C, Breukink E, Liskamp RMJ, Rijkers DTS (2013) Improving the biological activity of the antimicrobial peptide anoplin by membrane anchoring through a lipophilic amino acid derivative. Bioorg. Med. Chem. Lett. 23:3749–3752.

55. Munk JK, Uggerhøj LE, Poulsen TJ, Frimodt-Møller N, Wimmer R, Nyberg NT, Hansen PR (2013) Synthetic analogs of anoplin show improved antimicrobial activities. J. Pept. Sci. 19:669–675.