

Structure-Function Analysis of the Antimicrobial Peptide Latarcin 5

Struktur- und Funktionsanalyse des antimikrobiellen Peptids Latarcin 5

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

von der KIT-Fakultät für Chemie und Biowissenschaften
des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

von

M.Sc. Kieron Laqua-Acosta

1. Referentin: Prof. Dr. Anne S. Ulrich

2. Referentin: Dr. Claudia Muhle-Goll

Tag der mündlichen Prüfung: 20.04.2026

Declaration of Authorship

Ich versichere hiermit wahrheitsgemäß, diese Arbeit selbständig verfasst, alle benutzten Hilfsmittel vollständig und genau angegeben und alles kenntlich gemacht zu haben, was aus Arbeiten andere unverändert oder mit Abänderungen entnommen wurde sowie die Satzung des KIT zu Sicherung guter wissenschaftlicher Praxis in der jeweils gültigen Fassung beachtet zu haben.

I hereby declare that I have prepared this work independently, that all aids and sources used have been fully and accurately acknowledged, and that all passages taken from the work of others, whether quoted directly or adapted, have been clearly identified. I further declare that I have obeyed the KIT statutes on safeguarding good scientific practice in their currently applicable version.

Date

Signature

Acknowledgement

First of all, I would like to thank Prof. Dr. Anne S. Ulrich for the opportunity to do my master and PhD thesis in her working group. Your excellent scientific input and uplifting spirit made my time in your group a pleasure. Thank you!

My thanks go out to Dr. Birgid Langer for always acting in the best interest of the students in our group and keeping the ship afloat.

My special thanks to Dr. Parvesh Wadhwani for his excellent and competent guidance even in stressful times throughout the period of this work. Even when your own wellbeing was uncertain, you consistently made time for your student, a generosity I will never forget and for which I am deeply grateful.

Thank you, Dr. Erik Strandberg, for providing significant input and help on all circular dichroism and NMR matters. It was a pleasure to work with you.

Thank you to Dr. Sergii Afonin for his help and input during DSC measurements. I will miss our little chats on the freezing cold balcony.

Special thanks to Dr. Tamta Turdzeladze. Whenever a question with regard to organisation comes up you are the person everyone runs to. More than that, you always help with a smile.

I would also like to thank Bianca Posselt, Andrea Eisele, Kerstin Scheubeck and Stefanie Vollmer for their excellent supervision provided in the CD-lab, synthesis-lab and the biology-lab, respectively.

Furthermore, I would like to thank all members of the working group for providing competent input and a friendly work environment on a daily basis. Special thanks in that regard to Dennis Winkler, Rebecca Vivien Ernst, Mark Herbert and Marnie Baumeister.

Thank you to my lab partners during the Enzyme kinetics practicum, Dr. Marcel Huhn and Nataliia Petlovana. Thank you for some of the best memories during my time as a PhD student.

My thanks go out to my childhood friend group, the Anti-Stress-Komitee (ASK e.V). With you guys, I can always be myself and feel like I am back in tenth grade. I look forward to many more ski and hiking trips with each and every one of you, for as long as our legs will carry us. Grüße gehen raus.

Thank you to my family Jannick Laqua, Sarah Doren Laqua, Claudia Laqua and Dr. Gerhard Laqua for their support and late-night talks in the sunroom or the sauna. You are the best family one could wish for.

Finally, I would like to thank my wife, Jenny-Marie Laqua-Acosta, for her constant and loving support and for taking weight off my shoulders day after day. I would not have been able to do it without you by my side. You believe in me more than I believe in myself and keep pushing me to become better in every aspect of my life in a unique and loving way.

I will always love you! (Pew, Pew, Pew)

Table of Contents

1	<i>Abbreviations</i>	1
2	<i>Zusammenfassung</i>	3
3	<i>Introduction</i>	5
3.1	Bioactive peptides from animal venom	5
3.2	Antimicrobial peptides from animal venoms	8
3.3	AMPs derived from spider venoms	11
3.4	Latarcins, AMPs from the central Asian spider <i>Lachesana tarabaevi</i>	13
4	<i>Aim</i>	16
5	<i>Structure analysis of Latarcin 5</i>	18
5.1	Results	21
5.1.1	Peptide synthesis	21
5.1.2	CD and OCD spectroscopy	22
5.1.3	Antimicrobial activity	27
5.1.4	Hemolytic activity	28
5.1.5	Vesicle leakage	30
5.1.6	Solid-state NMR spectroscopy	33
5.1.7	Structure calculation	39
5.2	Discussion	44
6	<i>Trapping the membrane-active state of Latarcin 5 in lipid bilayers</i>	57
6.1	Results	61
6.1.1	Peptide synthesis	61
6.1.2	CD and OCD spectroscopy	61
6.1.3	Vesicle leakage	65
6.1.4	Solid-state NMR spectroscopy	67
6.2	Discussion	75
7	<i>Functional role of aromatic residues in Latarcin 5</i>	81
7.1	Results	83
7.1.1	Peptide synthesis	83

7.1.2	CD and OCD spectroscopy	83
7.1.3	Antimicrobial activity	88
7.1.4	Hemolytic activity	89
7.1.5	Vesicle leakage	90
7.2	Discussion	92
8	<i>Role of the N-terminal hydrophobicity in Latarcin 5</i>	102
8.1	Results	106
8.1.1	Peptide Synthesis	106
8.1.2	CD and OCD spectroscopy	106
8.1.3	Antimicrobial activity	111
8.1.4	Hemolytic activity	112
8.1.5	Vesicle leakage	114
8.2	Discussion	115
9	<i>Unified Mechanism of the Antimicrobial Peptide Latarcin 5</i>	124
9.1	Results	126
9.1.1	Minimal bactericidal concentration of Latarcin 5	126
9.1.2	Membrane depolarisation induced by Latarcin 5	128
9.1.3	In vivo membrane disruption induced by Latarcin 5	130
9.1.4	Anionic lipid clustering caused by Latarcin 5	132
9.2	Discussion	135
10	<i>Conclusion and Outlook</i>	140
11	<i>Appendix</i>	142
11.1	Instrumentation and Materials	142
11.2	Buffers and solutions	146
12	<i>Methods</i>	148
12.1	Peptide synthesis	148
12.2	Solid-state NMR spectroscopy	149
12.3	Circular dichroism and oriented circular dichroism	150
12.4	Antimicrobial activity assays	153

12.5	Hemolysis assay	155
12.6	Vesicle leakage assay	156
12.7	Membrane depolarisation assay	157
12.8	Membrane disruption assay	158
12.9	Anionic lipid clustering assay	159
13	<i>Theory</i>	159
13.1	NMR-Spectroscopy	159
13.2	Solid-state NMR spectroscopy	164
13.3	Structural fitting	165
13.4	Circular dichroism	167
13.5	Oriented circular dichroism	169
13.6	Fluorescence	171
14	<i>Appendix</i>	173
15	<i>List of Tables</i>	181
16	<i>List of Figures</i>	181
17	<i>List of References</i>	183
18	<i>List of publications</i>	199

1 Abbreviations

Ala-d ₃	Deuterated Alanine
AMP	Antimicrobial peptide
Amino acids abbreviation	The typical three letter code was used for all canonical amino acids
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt
ATP	Adenosine triphosphate
ACS	Acute coronary syndrome
CD	Circular dichroism
ChA	Cyclohexyl alanine
CL	Cardiolipin
CPP	Cell penetrating peptides
Da	Dalton
DiSC ₃ (5)	3,3-dipropylthiadicarbocyanine iodide
2D _c	Thickness of the hydrophobic core of a lipid bilayer
D _{HH}	Headgroup to headgroup lipid bilayer thickness
DErPC	1,2-dierucoyl-glycero-3-phosphocholine
DErPG	1,2-dierucoyl-glycero-3-phosphoglycerol
DLPC	1,2-dilauroyl-glycero-3-phosphocholine
DLPG	1,2-dilauroyl-glycero-3-phosphoglycerol
DMPC	1,2-Dimyristoyl-glycero-3-phosphocholine
DMPG	1,2-Dimyristoyl-glycero-3-phosphoglycerol
DPX	p-Xylene-bis(pyridinium bromide)
DD	Double distilled
DSC	Differential scanning calorimetry
ED	Effective dose
EGFR	Epidermal Growth Factor Receptor
ESI	Electrospray ionization
FDA	Food and Drug Administration
FID	Free induction decay
FRET	Förster resonance energy transfer
H _I	Hexagonal lipid phase
H _{II}	Inverse hexagonal lipid phase
HT	High Voltage
HBTU	2 (1H-benzotriazol 1 yl) 1,1,3,3 tetramethyluronium hexafluorophosphate
HER2	Human Epidermal Growth Factor Receptor 2

HOBt	1-hydroxybenzotriazole
HPLC	High performance liquid chromatography
ITC	Isothermal titration calorimetry
KIT	Karlsruhe Institute of Technology
L _α	Lamellar lipid phase
LC-MS	Liquid chromatography–mass spectrometry
Ltc	Latarcin
LUV	Large unilamellar vesicle
LPS	Lipopolysaccharide
MBC	Minimal bactericidal concentration
MIC	minimal inhibitory concentration
MLV	Multilamellar vesicles
MRE	Mean residue ellipticity
M _w	Molecular weight
NMR spectroscopy	Nuclear-magnetic-resonance spectroscopy
NTA	N-terminally truncated analogue
OD	Optical density
OCD	Oriented circular dichroism
PB	Phosphate buffer
PG	Phosphatidylglycerol
PI	Propidium iodide
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), sodium salt
P/L	Peptide to lipid molar ratio
PS	Phosphatidylserine
Rh	Rhesus
RMSD	Root mean square deviation
RT	Room temperature
SUV	Small unilamellar vesicle
ss	Solid-state
TD	Toxic dose
T _m	Lipid phase transition temperature
TI	Therapeutic index
v/v	Volume to volume ratio
WT	Wild type

2 Zusammenfassung

Latacin 5 (Ltc5) ist ein 28 Aminosäuren langes, lineares, α -helikales, amphipathisches, membranaktives Peptid aus der zentralasiatischen Spinne *Lachesana tarabaei* mit hoher antimikrobieller Aktivität und geringen Nebenwirkungen. Ein Alanin-Scan zeigte, dass der Austausch von individuellen Aminosäuren durch Alanin gut toleriert ist und keine nennenswerten Struktur- oder Funktions Unterschiede verursacht. Da Struktur und Mechanismus bei antimikrobiellen Peptiden oft korrelieren, wurde in dieser Arbeit die Sekundärstruktur und die Orientierung von Ltc5 in Membranen aus synthetischen Lipiden untersucht um den Wirkmechanismus von Ltc5 nachzuvollziehen. Eine hochpräzise Bestimmung von Orientierung und Dynamik gelang mittels einer Festphasen- ^2H -NMR-Analyse in vollständig hydrierten Lipid Doppelschichten. Diese ergab, dass Ltc5 eine nahezu durchgängige α -Helix mit N- und C-Terminalem Abschnitt bildet, die nahezu identische Helix Neigungswinkel τ (86° N-Terminus, 85° C-Terminus), aber deutlich unterschiedlichen azimuthalen Rotationswinkel ρ (99° N-Terminus, 85° C-Terminus) aufweisen. Beide Helix Segmente zeigen wenig Mobilität im Neigungs- sowie im Rotations-Winkel. Die α -Helix liegt flach auf der Membran wobei der Hydrophobe Sektor von Ltc5 in Richtung des hydrophoben Kerns der Membran orientiert ist. NMR-Messungen in Membranen unterschiedlicher Dicke und bei variierenden Peptidkonzentrationen zeigten zudem eine weitgehende Unabhängigkeit der Helix Orientierung von beiden Faktoren. Daher wird der oberflächengebundene S-state als membranaktive Orientierung von Ltc5 angenommen. Weitere Experimente zeigten, dass die Hydrophobizität für die Aktivität wichtiger ist als die spezifische Chemie der in der Primärsequenz von Ltc5 häufig vorkommenden aromatischen Aminosäuren. Dennoch dürften aromatische Reste aufgrund ihrer spezifischen physikalischen Eigenschaften zu einer spezifischen Peptid-Membran Interaktion beitragen, die evolutionär an die natürlichen Ansprüche an die Funktion von Ltc5 angepasst ist. Das gehäufte Auftreten hydrophober Reste am N-Terminus in Ltc5 scheint besonders für die initiale Membranbindung relevant zu sein. Eine systematische N-terminale Verkürzung veränderte die Aktivität des Peptids deutlich. So konnte ein neues AMP, Ltc5 5-28, mit starker Selektivität gegen Gram-positive Bakterien und minimalen hämolytischen Nebenwirkungen identifiziert werden. In weiteren Experimenten konnten membrandepolarisierende Effekte in *E. coli*, induziert durch Ltc5, nachgewiesen werden. Diese treten ohne nennenswerte Membranschäden auf. Als möglicher Mechanismus konnte mittels kalorimetrischer Messungen die Formation von anionischen "Lipid-Clustern" durch Ltc5 nachgewiesen werden. Neben den oben genannten Mechanismen liegt auch ein intrazellulärer Wirkmechanismus als Bestandteil des Wirkspektrums von Ltc5 weiterhin im Bereich des Möglichen.

3 Introduction

3.1 Bioactive peptides from animal venom

In nature, complex mechanisms involving bioactive peptides are optimised over millions of years to exert a specific activity. Through trial and error, evolution developed bioactive peptides with effective killing mechanisms in venomous predators. Analysing these peptides with regard to their function and possible applications in human medicine has proven to be a rich source for new bioactive peptides.^{1,2} Their spectrum of action ranges from pain relief and inhibiting inflammatory reactions, to support for the immune system in its fight against intruding organisms or cancerous cells.³

Instead of developing novel drugs, it has proven to be more efficient to take naturally occurring molecules and screen for potential bioactivity. Conventional antibiotics are one of the most successful examples using this approach.^{4,5} Animal venoms can contain cocktails ranging from a dozen to thousands of different peptide components, each with a unique activity.³ A large fraction of animal venoms is represented by proteins or peptides. Mass-spectrometric and proteo-transcriptomic studies show that cone snail venoms are predominantly composed of peptide toxins, with non-proteinogenic modifications occurring only in a small minority of components.⁶ The variability in activity of bioactive peptides is broad. They can complement the activity of other components, without harming a target organism themselves,³ or they can show direct activity, giving them the potential for a standalone drug.^{2,3} Such components can act by binding selectively to cellular targets such as ion channels, membrane receptors, the membrane itself, enzymes or components of the extracellular matrix.³

After collecting the animal venom, peptide components are purified and classified. Once classified, the peptides can be produced en masse by biological or chemical synthesis, ensuring availability for screening processes.

First, the potential bioactivity has to be tested. This can include assays testing for metabolic differences in cells treated with venom peptides or toxicity screens against eukaryotic and prokaryotic cells.⁷

Once the bioactivity of a peptide is identified, its structure and in-depth function are characterized to shed light on possible mechanisms of action underlying its bioactivity.⁷ The activity and selectivity of promising candidates can be optimised further before

entering clinical trials and ultimately being approved for medical application. A typical pipeline for bioactive peptides derived from animal venoms is depicted in **Figure 1**.

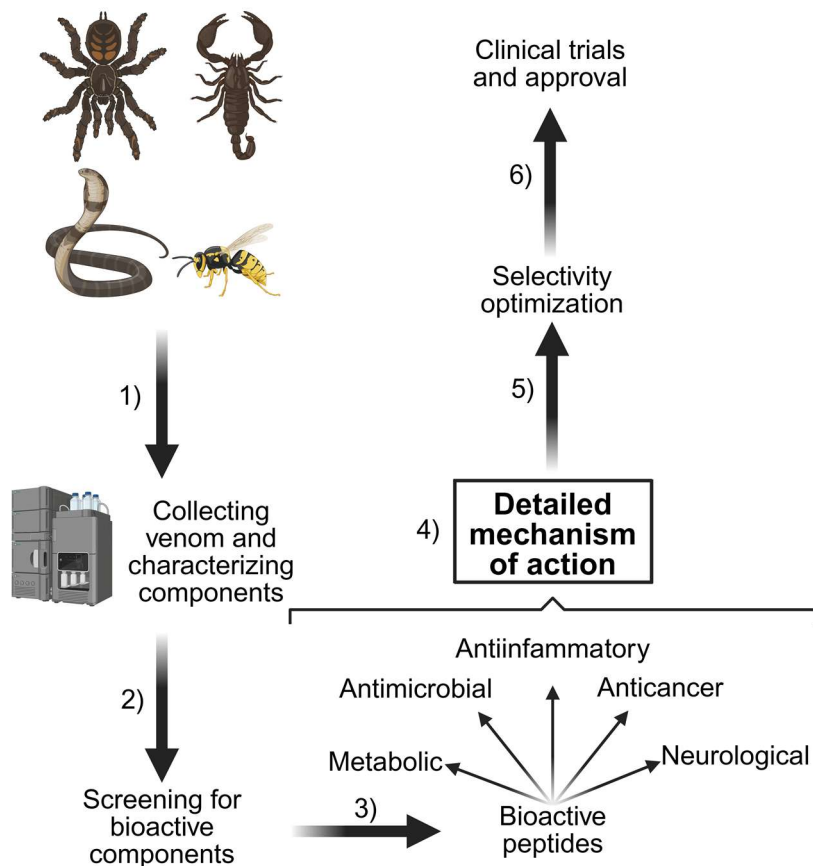


Figure 1: Scheme of drug development pipeline based on animal venoms.

1) Collecting venom and characterizing of venom components. 2) Identification of bioactive components. 3) Bioactive peptides for different areas of application. 4) Determination of a detailed mechanism of action. 5) Optimizing the selectivity of the peptide (reduce possible side effects, improve bioavailability). 6) Clinical trials. Created in <https://BioRender.com>.

Table 1 contains a list of bioactive venom peptides approved by the Food and Drug Administration (FDA). The table lists peptides from different origins, together with their primary sequences and medical applications.⁸

Table 1: List of clinically approved bioactive peptides derived from animal venom of different species.

The table contains the name of the component, its species of origin, primary sequence of the bioactive peptide and medical use.

Name	Origin	Sequence	Medical use
Ziconotide ⁹	<i>Conus magus</i>	CKGKGAKCSRMLYDCCTGSCRSGKC	Pain medication
Eptifibatide ⁸	<i>Sistrurus miliarius</i>	CXGDWPC (X=hArg)	cardiovascular, antithrombotic
Exenatide ¹⁰	<i>Lacertilia</i>	HGEGTFTSDLKQMEEEAVRLFIEWLKNGGPSSGAPPS	Metabolic disease, diabetes
Lixisenatide ¹¹	<i>Heloderma suspectum</i>	HGEGTFTSDLKQMEEEAVRLFIEWLKNGGPSSGAPPS KKKKK	Metabolic disease, diabetes
Hainantoxin ¹²	<i>Haploplema hainanum</i>	MKASMFALTGLALLFVVCYASESEEKEFSNELLSSVL AVDDNSKGEERECLGFKGKCNPSNDQCKSSNLVCSRK HRWCKYEIGK	Ion-Channel Neurotoxin
Melittin ^{13,14}	<i>Apis mellifera</i>	GIGAVLKVLTTGLPALISWIKRKRQQ	Antimicrobial, Immunotherapy

The listed bioactive peptides in **Table 1** illustrate the variety of different activities found in bioactive peptides derived from animal venom and emphasise their potential for medical purposes.^{8–14} To illustrate this diversity, the following paragraphs highlight three venom-derived peptides that represent distinct therapeutic areas: neurogenic pain (ziconotide), acute cardiovascular indications (eptifibatide), and immunotherapeutic properties (melittin).

Ziconotide from cone-snail venom (**Table 1**, row 1) is an approved pain-relieving drug prescribed to patients with severe pain or chronic inflammatory pain.^{3,9} It blocks calcium uptake in neurons, thereby inhibiting the release of neurotransmitters and stopping pain signals from reaching the brain.⁸

Whereas ziconotide exemplifies ion-channel modulation in the nervous system, eptifibatide demonstrates how venom peptides can be leveraged to interfere with

receptor-mediated platelet activation in cardiovascular disease. Eptifibatide (**Table 1**, row 2) inhibits platelet aggregation by blocking the binding of adhesive proteins and coagulation factors to glycoproteins IIb and IIIa, which are located on the surface of activated platelets.¹⁵ It reduces ischemic cardiac events and is approved for the treatment of acute coronary syndrome (ACS), during which the blood vessels supplying oxygen to the heart become clogged.^{8,15}

Beyond highly selective, single-target inhibitors such as ziconotide and eptifibatide, some venom peptides display broader, pleiotropic activities that may be therapeutically valuable in multiple contexts. Melittin from bee venom (**Table 1**, row 6) has been reported to affect metabolic parameters. Melittin has shown glucose- and lipid-lowering properties in the blood stream.^{13,14} It suppresses the phosphorylation of Epidermal Growth Factor Receptor (EGFR) and Human Epidermal Growth Factor Receptor 2 (HER2), which are overactive in different types of cancer cells, and reduces the viability of leukaemia cells by inducing apoptosis, giving it anticancer properties.^{13,14} Melittin also shows antimicrobial properties. In an in vivo study on mice infected with methicillin-resistant *S. aureus* (MRSA), full recovery was observed after treatment with melittin.¹⁶ Thus far, it is only approved for the treatment of illnesses affecting the lymphatic system.⁸

3.2 Antimicrobial peptides from animal venoms

Among the diverse activities of venom peptides, antimicrobial activity has one of the most promising applications. Peptides demonstrating activity against microbes, such as the previously mentioned melittin, are also called antimicrobial peptides (AMPs). In recent years the need for alternatives to conventional antibiotics is increasing rapidly^{17–19} to combat the rise of bacterial strains with multiple resistances.^{20,21} AMPs are often linear, α -helical and amphipathic peptides with a high number of positive charges. They are mostly unordered in solution and begin to fold into their amphipathic secondary-structure upon binding to membranes.²² The cationic properties of an AMP facilitate the initial electrostatic attraction towards the anionic lipid head groups. Negatively charged lipids are prevalent in prokaryotic membranes, making these membranes an ideal target for AMPs.^{22–24} The mentioned anionic properties of bacterial membranes are based on phospholipids with negatively charged headgroups like

phosphatidylglycerol (PG), cardiolipin (CL) or phosphatidylserine (PS) present in the lipid bilayers surrounding their cellular contents.^{23,24}

Even though AMPs are promising alternatives to conventional antibiotics, there are several challenges that exclude many AMPs from entering clinical trials. These challenges include, but are not limited to, bioavailability and selectivity.²⁵ The pipeline for AMP development is not yet optimised, leading to high research and development costs. Funding from large pharmaceutical companies is therefore often used alternatively. If observed trends continue and the need for antimicrobial agents increases further, this might change in the near future.^{25–28}

Cationic AMPs have historically been postulated to exert their primary activity via disruptive mechanisms against anionic bacterial membranes. An α -helical, amphipathic AMP can damage bacterial membranes at elevated concentrations through soap-like mechanisms. However, robust bactericidal activity is frequently observed at substantially lower concentrations, indicating alternative mechanisms of action that do not require membrane disruption. In **Table 2**, a small exemplary selection of AMPs derived from animal venoms or skin secretions is depicted.

Table 2: List of exemplary AMPs from diverse origins.

The table includes peptide names, organism of origin, primary sequence and mechanisms of action/target. All listed peptides are venom peptides, with the exception of magainin 2, which is derived from frog skin secretions.

Name	Organism of origin	Sequence	Mechanism / Target
Mastoparan ²⁹	Wasp	INLKALAALAKKIL-NH ₂	Pore forming
Pilosulin 1 ³⁰	Ant	MKLSCLLLTLTIIFVLTIVHAPNVEAKDLADPESEAVGF ADAFGEADAVGEADPNAGLGSVFGRRLARILGRVIPKVAK KLGPKVAKVLPKVMKEAIPMAVEMAKSQEEQQPQ	Membrane disrupting
Crotamine ³¹	Snake	FVQWFSKFLGRIL	Membrane disrupting
Syphaxin ³²	Frog	GVLDILKGAAKDLAGHVATKVINKI	Pore forming
Api137 ³³	Bee (Derivative)	gu-ONNRPVYIPRPPHRL gu=N,N,N',N'-tetramethylguanidino O=L-ornithine	Intracellular Membrane
Magainin 2 ^{34,35}	Frog	GIGKWLHSAKKFGKAFVGEIMNS	Pore forming

One example of a well-understood pore-forming AMP is magainin 2 (row 6, **Table 2**). Magainin 2 is a canonical amphipathic α -helical AMP derived from frog skin that kills bacteria primarily by membrane permeabilization. Multiple studies name the formation of toroidal pores as its primary mechanism of action against bacterial membranes.^{34,35} In live-cell experiments, one study explicitly contrasts its action on bacterial versus mammalian cells, describing permeabilization consistent with a toroidal pore-forming mechanism in bacteria and a more carpet-like interaction against mammalian membranes.³⁵ During a carpet-like mechanism, an AMP is oriented parallel to the membrane-normal when interacting with the membrane. The peptide helix is oriented in such a way that the amphipathic character of the helix is matched with the membrane amphipathicity. Once a certain threshold concentration is reached, the peptide begins to tilt into the membrane and forms peptide lipid clusters, disrupting the membrane in the process.^{36,37} In situ spectroscopy work further supports concentration-dependent membrane orientation, with magainin 2 adopting different orientations at low versus higher concentrations in anionic bilayers—behaviour that maps onto threshold-driven permeabilization.^{34,38}

In contrast to magainin 2, Api137 (row 5, **Table 2**) is a known AMP with intracellular targets in Gram-negative bacteria. Api137 is a proline-rich designer AMP derived from apidaecin 1b in honey bee venom.³⁹ It inhibits translation by binding to the ribosomal exit tunnel and inhibiting release factors.³⁹ Peptide synthesis comes to a halt through ribosomal arrest at stop codons, as the finished peptide can no longer be released from the ribosome. Api137 is a prime example of an AMP acting on intracellular targets rather than through membrane lysis.

These two examples, together with those summarised in **Table 2**, indicate the diverse activities found in AMPs. The variety of mechanisms remains substantially underexploited and, in many cases, not fully understood. In contrast, many conventional antibiotics entering the market are derivatives of established scaffolds and frequently act via closely related mechanisms.^{19,32} Therefore, bacteria are already equipped with defence mechanisms, lowering their efficacy significantly. By comparison, our understanding of AMP mechanisms of action is still at an early stage, despite their considerable therapeutic promise and broad functional versatility. A more detailed mechanistic understanding of AMPs is expected to directly improve rational

peptide design, enabling the development of increasingly effective peptide-based antimicrobials in the near future.^{32,33}

3.3 AMPs derived from spider venoms

A rich source of AMPs is spider venom, where they are primarily reported to perforate membranes by pore formation or carpet-like mechanisms.⁴⁰ When considering the origin of AMPs from spider venom, it cannot be said if their evolutionary task is to permeate membranes and allow other venom components like neurotoxins to reach their targets or whether the perforation of the membrane is itself a primary mode of killing.⁴⁰ Most likely, both effects contribute to increasing the overall activity of the venom. Synergistic properties between components from spider venom have been reported previously.^{40,41} The antimicrobial activity of AMPs present in spider venom also assures that microbes cannot proliferate in the venom glands, thereby protecting the glands from infection and loss of function.^{36,40} A list of AMPs derived from spider venoms is given in **Table 3**.

Table 3: List of AMPs derived from spider venom.

The list gives an overview over important representatives and includes their name, origin, sequence and mechanism / target.

Name	Origin	Sequence	Mechanism / Target
Lycotoxin I ^{42,43}	<i>Lycosa carolinensis</i>	IWLTALKFLGKHAAKHLAKQQLSKL-NH ₂	ATP synthase inhibition
Cupiennin 1a ^{44,45}	<i>Cupiennius salei</i>	GFGALFKFLAKKVAKTVAKQAAKQGAKYVVKQME-NH ₂	Inhibition of nitric oxide synthase
LyeTx-I ⁴⁶	<i>Lycosa erythrognatha</i>	IWLTALKFLGKNLGKHLAKQQLAKL-NH ₂	Membranolytic
Latarcin 2a ^{2,47}	<i>Lachesana tarabaei</i>	GLFGKLIKKFGRKAISYAVKKARGKH-COOH	Highly membranolytic

Lycotoxin I (**Table 3**, row 1) (and II, not mentioned here) are among the first reported AMPs derived from spider venom in 1998. The ability of lycotoxin I to collapse ion gradients across membranes, depolarising them, was already described in 1998 along

with pore-forming properties.⁴² More selective targets were described only 18 years later. In 2016, its ability to inhibit the ATP synthase of *E. coli* was demonstrated.⁴³

Cupiennin 1a (**Table 3**, row 2) resembles a typical AMP and is derived from the venom of the spider *Cupiennius salei*. It is a cationic, amphipathic peptide and is proposed to have a helix-hinge-helix secondary structure with broad activity against bacteria and fungi.^{44,45} It was used as a blueprint for many design studies to better understand the mode of action of AMPs. In addition to its unselective membranolytic activity, it has shown the ability to inhibit the nitric oxide synthase in *E. coli*, which is part of the stress response to external stimuli.^{44,46}

LyeTx-I (**Table 3**, row 3) represents another template peptide that found application in the optimisation of AMPs. The wild type (WT) shows high antimicrobial activity (minimal inhibitory concentration (MIC) 2-8 μ M, minimal bactericidal concentration (MBC) 2-16 μ M). Its activity is most likely based on its membranolytic properties and its ability to displace membrane-associated divalent cations.⁴⁸ LyeTx-I was used as a template to create several truncated and optimised peptides with varying activity.^{49,50} One such truncated derivative showed increased antimicrobial activity, while its membranolytic activity against erythrocytes decreased, therefore increasing its therapeutic index compared to the WT.⁵¹

The therapeutic index (TI) describes a drug's safety margin by comparing the dose (or concentration) that produces toxicity to the dose (or concentration) that produces the desired therapeutic effect. It is commonly expressed as a ratio of the toxic dose (TD) to the effective dose (ED). $TI = TD_{50}/ED_{50}$. Larger values indicate a wider separation between effective and toxic effects. Drugs with a small TI are considered "narrow therapeutic index" agents and generally require closer dosing and monitoring. During clinical trials, this value is not sufficiently meaningful, as it does not include other possible side effects.⁵¹ During preclinical trials, however, it should be taken into consideration when assessing the potential of an AMP. As red blood cells make up a larger proportion of the total cell count in humans⁵², it is often taken as a benchmark for the TD_{50} in preclinical phases. A low TI might be a reason to exclude a peptide from further studies. A main focus of preclinical studies can be to optimise the TI of an AMP by either increasing the antimicrobial activity or decreasing the hemolytic activity through site specific mutations of the WT.

The remaining AMP in **Table 3** (row 4) is called Latarcin 2a (Ltc2a). It is a representative of a class of AMPs derived from the venom of the spider *Lachesana tarabaei* called Latarcins. Ltc2a has a predominantly α -helical structure in contact with lipid bilayers (81 % determined using circular dichroism spectroscopy (CD)), is highly cationic with a net charge of plus ten and is antimicrobially active (MIC of 2-4 $\mu\text{g/mL}$) with a postulated membranolytic mode of action.⁴⁷ The latter was tested against erythrocytes and membrane mimicking lipid bilayers made from different lipid compositions.^{2,47} As its membranolytic activity stayed high across different testing conditions, it is considered to have indiscriminate activity against lipid bilayers and membranes.^{2,47} Oriented CD (OCD) measurements in combination with solid-state (ss) ^{15}N -NMR analysis additionally showed a strong tilt into the membrane, supporting the postulated pore-forming mechanism.⁴⁷ As Ltc2a shows activity both against erythrocytes (hemolysis) and bacteria (MIC), its therapeutic index is expected to be low, making it an ideal candidate for optimisation studies rather than a candidate to enter clinical trials without previous optimisation.

3.4 Latarcins, AMPs from the central Asian spider *Lachesana tarabaei*

The mentioned group of latarcins (Ltc) consists of seven highly α -helical, cationic, and amphipathic peptides with lengths varying from 20 to 34 amino acids. Functionally, these membrane-active peptides display potent broad-spectrum antimicrobial and cytolytic activity against bacteria, yeast, and even mammalian cells at micromolar concentrations.^{2,47} However, recent comparative research has revealed diverse membrane interactions for different latarcins, suggesting that each peptide may operate via distinct modes of action rather than a uniform mechanism.⁴⁷

In a study by Wadhwani et al.⁴⁷, all seven latarcins were evaluated for their lipid induced secondary structure by CD and ^{15}N -NMR, their orientation by oriented circular dichroism, their antimicrobial activity by MIC assay, and their membrane disrupting capacity by vesicle leakage and hemolysis assays. In the latter two assays, the peptide's ability to disrupt lipid bilayers was assessed by measuring the release of vesicle contents from model vesicles or hemoglobin from erythrocytes.

In **Table 4**, a complete list of all seven latarcins is provided with their respective names, primary sequence, molecular weight (Mw, in Da), and their net positive charge.^{2,47}

Table 4: Latarcins, seven AMPs derived from spider venom.

All seven latarcins are listed in this table. The table includes their names, primary sequence, Mw in Dalton (Da) and their net charge.

Name	Sequence	Mw [Da]	Net charge
Ltc1	SMWSGMWRRKLKKLRNALKKKLKGE-COOH	3073.79	+9
Ltc2a	GLFGKLIKKFGRKAISYAVKKARGKH-COOH	2902.57	+10
Ltc3a	SWKSMAKKLKEYMEKCLKQRA-NH ₂	2483.05	+6
Ltc4a	GLKDKFKSMGEKCLKQYIQTWKAKF-NH ₂	2902.50	+6
Ltc5	GFFGKMKEYFKKFGASFRRFANLKKRL-NH ₂	3430.18	+10
Ltc6a	QAFQTFKPDWNKIRYDAMKMQTSLGQMKKRFNL-COOH	4051.77	+5
Ltc7	GETFDKLKEKLTIFYQKLVEKAEDLKGDLKAKLS-COOH	3942.61	+2

Ltc1, 2a and 5 stand out among the latarcins. They have the highest net charges and show the highest antimicrobial activity (MIC 2 µg/mL to 8 µg/mL) against the bacterial strains tested in the work by Wadhwani et al.⁴⁷ The membrane interactions, however, seem to differ between the three peptides:

Ltc1 consists of 25 amino acids and has the second to least helical content of all Ltcs (66 % determined using CD) but is still predominantly α -helical.⁴⁷ Only Ltc6a has a similarly low helical content with 65 %. Ltc1 lies flat on the membrane surface, as determined by OCD and ss-¹⁵N-NMR, and has high antimicrobial activity against Gram-positive and Gram-negative bacteria strains with MIC values of 4 µg/mL. As it shows very little leakage in LUVs composed of DMPC/DMPG (7:3) (7 % at a peptide to lipid molar ratio (P/L) of 1:50), only moderate hemolysis (36 % at 256 µg/mL) and lies flat on the membrane surface (OCD, ¹⁵N-NMR) it was postulated to traverse the membrane without disturbing its integrity, following an intracellular mechanism of action.⁴⁷ The combination of low MIC and moderate hemolysis values indicates a decent therapeutic index, even though values were not calculated in the study by Wadhwani et al.⁴⁷

The previously mentioned Ltc2a is 26 amino acids long and shows the highest antimicrobial activity (MIC 2-4 µg/mL) of all Ltcs. It has an α -helical content of 81 %

(CD-spectroscopy) but shows a tilted orientation in membranes as determined by OCD and ss-¹⁵N-NMR. In contrast to Ltc1, Ltc2a causes high amounts of leakage in membrane mimetics and erythrocytes.⁴⁷ The combination of the tilted orientation and the high amount of leakage/hemolysis indicates a pore forming-mode of action according to Wadhwani et al.⁴⁷ Whilst being the most active among the Ltcs against microbes, it also disrupts membranes of varying composition, including membranes of erythrocytes.⁴⁷ Therefore, the expected therapeutic index for Ltc2a is inferior to that of Ltc1.

Ltc5 shows even better properties than Ltc1. CD revealed a helical content of 85 % with the helix lying flat on the membrane surface as determined by OCD and ss-NMR.⁴⁷ It is highly antimicrobial, with a MIC of 4 µg/mL against three of the tested strains and 8 µg/mL against *E. coli*. Ltc5 induces minimal damage to membrane mimetics and human erythrocytic membranes. With its 27 % hemolysis at a peptide concentration of 256 µg/mL, it induces significantly less damage to human erythrocytes compared to Ltc1, increasing its expected therapeutic index. This combination of high antimicrobial activity and low to moderate hemolytic activity makes it the most promising AMP out of the Ltc family to pass clinical trials and be approved as an alternative to conventional antibiotics in medical use. It will therefore be the main focus of this work.

4 Aim

It is well known that animal venom is a natural source of therapeutic molecules used in the treatment of bacterial infections, pain, cancer, and several neurological disorders.³³ The ongoing rise in microbial resistance has led to an acute shortage of effective antimicrobial agents.²⁸ Together, these factors have driven the isolation, identification, and characterization of antimicrobial peptides derived from animal venom. Latarcins, first identified in 2006 by Kozlov et al.², are one such group of seven α -helical, membrane-active antimicrobial peptides, isolated from the venom of the spider *Lachesana tarabaevi*. Our group has recently addressed the membrane alignment and biological properties of latarcins.⁴⁷ Among the seven Ltcs, Ltc5 in particular shows great potential as a future antimicrobial drug due to its high antimicrobial activity and low cytotoxicity toward human cells. However, neither the structure nor the mechanism of action of Ltc5 is known. This work therefore aims to advance the understanding of the selectivity of Ltc5 for prokaryotic membranes while sparing eukaryotic membranes.

The main aim of this thesis is to elucidate the mechanism of action of the membrane-active antimicrobial peptide Ltc5. Since the mechanism of action is closely linked to the structure of the peptide in the membrane, the primary objective is to investigate both the structure of Ltc5 and its structure-function relationships. The specific objectives are as follows:

- 1) The first chapter aims to determine the structure of Ltc5 in the membrane. To achieve this aim, every residue of Ltc5 was replaced by Ala-d₃ individually. The resulting 28 peptides were analysed with regard to their structure and function using a range of biophysical and biological methods to identify conserved positions. The same Ala-d₃ analogues were next used in a solid-state ²H-NMR spectroscopy study to determine the precise structure, orientation and dynamics of Ltc5 in membranes.
- 2) Once the structure and orientation are determined, the next aim is to trap the membrane-active state of Ltc5 by varying experimental conditions, such as peptide concentration and hydrophobic matching with lipid bilayers. Combined with results from the first aim, the data should allow for the development of a model describing how Ltc5 is oriented in the membrane while exerting its mechanism of action.

3) Ltc5 is characterized by a high proportion of aromatic residues on the hydrophobic face of its helix. This chapter aims to understand the role of these aromatic residues on the helix alignment and antimicrobial activity of Ltc5. Therefore, the aromatic residues in Ltc5 were replaced by either alanine, leucine, or cyclohexylalanine. This systematic variation simultaneously addresses not only the role of aromatic residues but also the influence of increasing or decreasing hydrophobicity along the helix of Ltc5 on the therapeutic value of the peptide.

4) Many of the hydrophobic residues in Ltc5 are concentrated in the N terminal region, making the N terminus highly hydrophobic. Since hydrophobicity is often associated with increased hemolytic side effects, the main aim of this chapter was to remove the N terminal hydrophobic residues and to study their influence on the therapeutic index. By analysing the resulting structure activity relationships, we expect to (a) obtain Ltc5 analogues with increased therapeutic potential and (b) to identify the role of N-terminal hydrophobicity on antimicrobial activity.

5) Given the inherently low lipid disturbance shown by Ltc5, even at high peptide concentrations, the aim of this final chapter is to investigate non-membranolytic, membrane-focused mechanisms of action underlying the antimicrobial activity of Ltc5. Furthermore, all information obtained from the previous aims will be combined in order to develop a unified mechanism of action for Ltc5.

Through these five aims, this study seeks to characterise the membrane-bound structure of Ltc5, including detailed information about helix orientation and side-chain positioning. It also aims to identify significant residues for the structure and function of Ltc5 and assess the impact of aromaticity and hydrophobicity on the biological activity of Ltc5. By examining both membrane-perturbing and non-perturbing mechanisms, this thesis ultimately aims to present a unified model for the mechanism of action of the natural membrane-active peptide Ltc5.

5 Structure analysis of Latarcin 5

Ltc5 is a 28-residue AMP with a net positive charge of ten, which adopts a highly α -helical, amphipathic secondary structure upon interaction with membranes.⁴⁷ A distinctive feature of Ltc5, setting it apart from the other latarcins, is its unusually high content of aromatic residues distributed along the hydrophobic face of its amphipathic α -helix: 25 % of its 28 amino acids are aromatic. Ltc5 is the most promising membrane-active antimicrobial peptide (AMP) among the seven latarcins isolated from the venom of the Central Asian spider *Lachesana tarabaei*. Its combination of high antimicrobial efficacy and relatively low hemolytic activity makes it an attractive candidate for future clinical applications.

Figure 2 shows the helical wheel (left) and horizontal helical mesh (right) representations of Ltc5 following the Ulrich group's protein origami scheme.⁵³ The helical wheel provides a view along the helical axis and facilitates the identification of helical sectors, highlighting hydrophobic and polar regions. The helical mesh corresponds to a longitudinally cut and unrolled helix, highlighting periodicity, side-chain registry, and nearest-neighbour relationships along the sequence. This representation makes local patterns between adjacent residues particularly apparent.

In the helical-wheel projection (left, **Figure 2**), a well-defined hydrophobic sector is visible in the lower-right quadrant, consisting of hydrophobic residues (yellow). Residues outside this sector are predominantly polar (blue or red). This sector consists of ten positively charged residues, seven Lys and three Arg, and one negatively charged residue, namely Glu. Notably, the polar regions also contain small non polar residues such as Gly and Ala. The helical-mesh representation (right, **Figure 2**) reproduces these features: the yellow area delineates the hydrophobic sector, within which Arg20 and Arg27 interrupt the otherwise continuous hydrophobic patch. Ser16 and Gly14 lie at the interface between the hydrophobic and polar sectors of Ltc5, as evident in both depictions. Within the first three helical turns of the N-terminus of Ltc5, four aromatic residues are found, namely Phe2, Phe3, Tyr9, and Phe10. Together with Met6, they form a highly hydrophobic N-terminal region in the α -helical state of Ltc5.

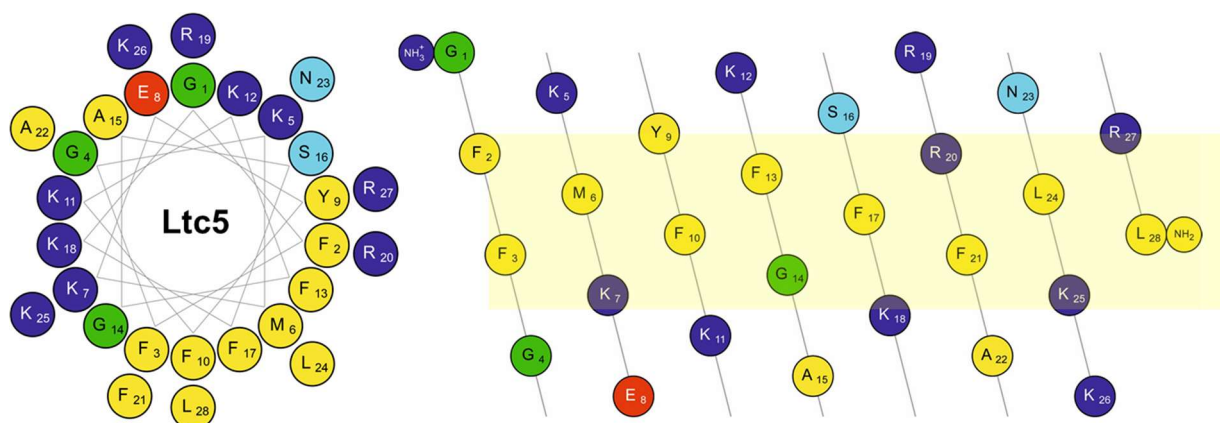


Figure 2: Helical wheel (left) and helical mesh (right) representations of Ltc5.

The helical wheel illustrates the peptide as viewed along its helical axis, providing a rapid overview of the spatial orientation of individual amino acid side chains. Colour code: yellow = hydrophobic; dark blue = positively charged polar residues; light blue = polar uncharged residues; red = negatively charged polar residues; green = glycine. The helical mesh can be envisioned as the helix being “unwrapped” lengthwise and flattened, allowing a straightforward assessment of which residues are positioned in close proximity. Both representations highlight clusters of hydrophobic and polar residues. The yellow highlighted box shows the hydrophobic strip of the peptide, which represents the area of the helix mainly interacting with the hydrophobic parts of lipid bilayers. The figure was generated using the Protein Origami web application.⁵³

The contribution of each individual amino acid to the structure and function of Ltc5 is so far unknown. To investigate whether single-residue substitutions alter the properties of Ltc5, an alanine scan (Ala-scan) was performed in which all 28 residues of the primary sequence of Ltc5 were individually replaced by deuterated alanine (Ala-d₃). The rationale for using Ala-d₃ instead of natural alanine will be explained below. The resulting 28 analogues were then analysed with respect to their structure and function and compared to Ltc5 WT. Significant changes in either structure or activity indicate that the corresponding substitution is not tolerated and that the respective position should be considered important for the structure or function of Ltc5 WT. The properties examined in this study include the secondary-structure of the Ala-d₃ analogues as determined by CD, their orientation on lipid bilayers by OCD, their antimicrobial activity by MIC assays, and their ability to induce membrane damage in membrane mimetics (vesicle leakage) and in human erythrocytes (hemolysis).

Structure and function are closely linked in peptides and proteins.³⁷ However, the structure of Ltc5 in its membrane-associated state has not yet been fully characterized. So far, only secondary-structure and orientational information have been obtained from CD, OCD, and ¹⁵N-NMR spectroscopy, which indicate that Ltc5 adopts a predominantly α -helical conformation upon association with lipid bilayers and lies on the membrane surface without significant insertion or tilting into the bilayer.⁴⁷ A detailed high-resolution structure under these conditions is still lacking. Membrane-associated peptides can be investigated in more detail using ssNMR. The nuclei most commonly used for structural and orientational analysis in ssNMR include ²H, ¹³C, ¹⁵N, and ¹⁹F. The group of Professor Anne S. Ulrich has focused primarily on ²H-, ¹⁵N-, and ¹⁹F-NMR to characterise membrane-active peptides in their membrane bound state.^{54–56}

In this work, ²H-NMR is the method of choice. To perform ²H-NMR experiments on peptides, the peptides first have to be labelled with deuterated amino acids. Ala is an ideal label with a CD₃-group rigidly attached to the peptide backbone. The three deuterons of its methyl group give rise to a single, well-defined quadrupolar splitting that is straightforward to analyse. The splitting reports on the orientation of the C α -C β -bond. By synthesising the Ltc5 analogues used for the Ala-scan with Ala-d₃ instead of non-deuterated Ala as mentioned previously, the same peptides can be used both for the functional screening and for the NMR analysis. Additionally, the Ala-scan provides crucial information for the subsequent ss-NMR study. If any of the Ala-d₃-labelled peptides display significant structural or functional artefacts compared to the Ltc5 WT, their NMR data should not be included in the structural calculations, because the orientation and structure of such analogues would no longer be representative of the Ltc5 WT peptide.

Using the described methods, the aims of this chapter are twofold. First, to identify any significant positions in the primary sequence of Ltc5 with regard to structure and function using the Ala-scan. Such positions, once identified, can help to elucidate structure-function relationships and may guide future rational optimisation and design efforts. An identified important position might also be essential for interaction with potential molecular targets, thereby providing hints about the mechanism of action of Ltc5. Second, to determine a detailed structure of Ltc5 in the presence of membranes. For AMPs and proteins, the basic principle “form follows function” applies. Thus,

obtaining a high-resolution structure is expected to yield important insights into the mechanism of action of Ltc5.

To reach this aim, a total of 28 analogues were synthesised. Each amino acid, one at a time, was replaced by Ala-d₃. Table 5 includes the Ltc5 WT and the first 4 Ala-d₃ analogues for illustration purposes. The complete list, containing the 28 Ala-d₃ analogues, is provided in the appendix (**Table A 1**).

Table 5: Analogues used for the Ala-scan of Ltc5.

The Ltc5 analogues (e.g. G1Ala-d₃ meaning glycine at position one was exchanged for deuterated alanine) are specified together with their assigned names used in this work, primary sequences, molecular weights (MW, in Daltons), and net charges. For illustration, only the Ltc5 WT and the mutations at the first four positions are shown here. The complete set of analogues is provided in the appendix. (**Table A 1**).

Mutation	Peptide Name	Sequence	Mw [Da]	Net charge
Wildtype	WT	GFFGKMKEYFKKFGASFKRRFANLKKRL-amide	3430.18	+10
G1Ala-d ₃	G1	(Ala-d ₃)FFGKMKEYFKKFGASFKRRFANLKKRL-amide	3447.20	+10
F2Ala-d ₃	F2	G(Ala-d ₃)FGKMKEYFKKFGASFKRRFANLKKRL-amide	3357.10	+10
F3Ala-d ₃	F3	GF(Ala-d ₃)GKMKEYFKKFGASFKRRFANLKKRL-amide	3357.10	+10
G4Ala-d ₃	G4	GFF(Ala-d ₃)KMKEYFKKFGASFKRRFANLKKRL-amide	3447.17	+10
K5Ala-d ₃	K5	GFFG(Ala-d ₃)MKEYFKKFGASFKRRFANLKKRL-amide	3376.08	+9
.....				
L28Ala-d ₃	L28	GFFGKMKEYFKKFGASFKRRFANLKKR(Ala-d ₃)-amide	3391.10	+10

The data shown in the subsequent results chapter includes the data of eleven Ala-d₃ analogues, namely Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22, and L24 which were measured during the master thesis of the author.⁵⁷ As these data are part of the complete Ala-scan and add to the accuracy of the structure determination, it is included in this work.

5.1 Results

5.1.1 Peptide synthesis

The peptides were prepared by automated Fmoc-solid-phase peptide synthesis and purified using reverse-phase (RP) HPLC as previously described (see methods, Chapter 12). Synthesis was performed by A. Eisele, K. Scheubeck and the author of

this work. In the appendix, the LC-MS spectra of the Ltc5 WT, Ltc5 A15 and Ltc5 A22 are given (**Figure A 1**, A 2 and A 3). Other peptides gave similar results.

The peptides Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and L24 were synthesized during the master thesis of the author.⁵⁷

5.1.2 CD and OCD spectroscopy

The conformation of a peptide constrains its ability to interact with potential targets (e.g., lipid bilayers, proteins). Thus, establishing the secondary-structure is essential. Ltc5 has previously been reported to adopt a predominantly α -helical conformation in contact with lipid bilayers.^{2,47} These results were reproduced for the Ltc5 WT. For the Ala-d₃ analogues, a variety of potentially structure-altering changes were introduced into the primary sequence. For example, switching Arg to Ala changes charge, steric-hindrance and polarity in that position. Replacing Phe or Tyr by Ala-d₃ residues changes aromatic properties, steric-hindrance and hydrophobicity. To investigate whether the introduction of these changes leads to structural differences in the resulting analogues, they were analysed using CD spectroscopy. If the changes are well tolerated with regard to the secondary-structure, little to no change would be expected.

CD measures the difference in absorption of left- versus right-circularly polarized light by the peptide backbone. When passing through the sample, left- and right-circularly polarized light is absorbed to different extents, resulting in elliptically polarized light. When the backbone forms regular α -helices or β -sheets, this results in distinctive CD spectra. Therefore, a CD spectrum contains information about the secondary structure, which can be further analysed by performing a secondary structure deconvolution on the CD spectrum. Each measurement was conducted twice on different samples (Methods, Chapter 12).

The peptide concentration was determined before the measurements using UV/Vis-spectroscopy to ensure constant P/L ratios in all measurements. Measurements in the absence of lipids were performed under two conditions. 1) in distilled water without pH control and 2) in 10 mM phosphate buffer (PB) at pH 7. Since previous studies on Ltcs have reported phosphate-dependent aggregation⁴⁷, these measurements were

conducted to test whether Ltc5 or its Ala-d₃ analogues form oligomers or aggregates in the presence of phosphate ions (PO₄³⁻). The PO₄³⁻ ions shield the high number of positive charges of the peptides, therefore reducing repulsive electrostatic forces, leading to more favourable conditions for peptide-peptide interactions.

The results of the measurements in water and PB are presented in the appendix (**Figure A 5**). The spectra indicate the absence of any dominating secondary-structure and a high content of unordered peptides. In PB, the CD spectra show a slight increase in possibly ordered secondary structure compared to the measurements in water. Even with PB, the spectra still indicate mostly unordered peptides. To determine precise secondary-structure distributions, the spectra were deconvoluted to quantify their content of α -helix, β -sheet, turns and unordered residues. Deconvolution of the spectra was done using the web tool and databases of DichroWeb.^{58,59}

In 10 mM PB, deconvolution of the CD spectra of Ltc5 WT and all tested Ala-d₃ analogues showed an unordered secondary structure as the largest fraction (Table A 3). On average, the spectra contained ~40 % unordered, ~30 % β -strands, ~20 % turns and ~10 % α -helix. This distribution can be used to explain the line shape of the spectra. Signals induced by the partially folded and unfolded peptides cancel each other out, resulting in the observed low intensity spectra. The deconvolution in water did not give reliable results and is therefore not included. However, the spectral shapes indicate a similar distribution of secondary structures as in PB.

For CD measurements in the presence of lipids, small unilamellar vesicles (SUVs) consisting of 1,2-dimyristoyl-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-glycero-3-phosphoglycerol (DMPG) in a molar ratio of 7:3 were used at a peptide-to-lipid molar ratio (P/L) of 1:50. Whenever ratios are mentioned in this work, they refer to molar ratios, unless stated otherwise. The respective CD spectra are presented in **Figure 3**. Measurements were conducted at 30°C, which is above the phase transition temperature (T_m) of the lipids used. The CD spectra of all Ala-d₃ analogues measured in the presence of vesicles show the characteristic features of highly α -helical peptides, namely the distinct positive band at 197 nm and two negative bands at 208 nm and 222 nm. This positive band at 197 nm shows a slight red shift compared to other latarcins⁴⁷, which typically display a positive band between 193 nm and 195 nm. The positive band at 197 nm was nearly always more than twice as

intense as the negative band at 208 nm, also indicating pronounced α -helical folding.^{60,61} The CD spectra in the presence of SUVs were deconvoluted using the web tool and database of DichroWeb.^{58,59} The results are shown in **Table A 3**. Ltc5 WT has a predominantly α -helical structure with 89 % helicity. All analogues tested retained α -helicity levels above 80 % helicity, with the lowest at 81 % and the highest at 92 %. Even substitutions of charged or sterically more demanding amino acids, such as Phe or Leu, by Ala-d₃ did not result in significant changes to the peptide's secondary-structure compared to the WT.

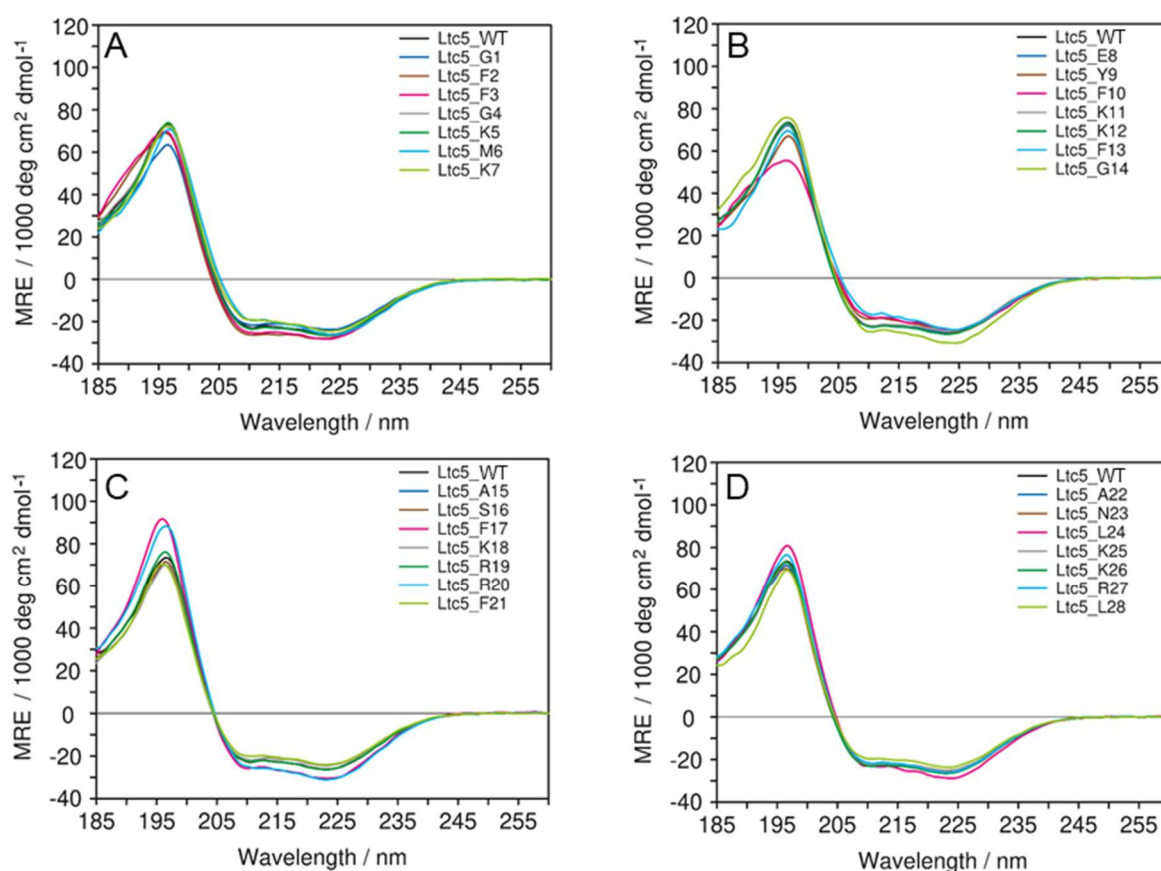


Figure 3: CD spectra of all Ala-d₃ analogues measured in small unilamellar vesicles (SUVs)

SUVs were made of DMPC/DMPG (7:3) and P/L of 1:50. The measurements were conducted above the T_m of the used lipids at 30°C. Each panel contains the spectra of peptides with Ala-d₃ by positions A) 1 to 7 B) 8 to 14 C) 15 to 21 and D) 22 to 28. Every panel additionally contains the spectrum of the Ltc5 WT as a reference. Data of the Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and L24 were previously obtained during the master thesis of the author.⁵⁷

In addition to solution CD, the Ala-d₃ analogues were also analysed using OCD. This method is used as a fast preliminary screen to assess how α -helical peptides, such as Ltc5 WT and its Ala-d₃ analogues, orient in a lipid bilayer. OCD spectra also contain information about the secondary-structure of the measured peptide and therefore act as a link between the previous CD measurement and the following NMR analysis, complementing both methods.

During sample preparation, peptide and lipid are co-solubilised in organic solvents (MeOH/CHCl₃, 1:1) and the mixture is spread onto a quartz glass slide. The samples are dried to remove the last traces of organic solvent. The dry films are subsequently rehydrated overnight at 30°C and 97 % relative humidity to ensure full hydration and formation of lipid bilayers. Under these conditions, the peptides orient within the aligned lipid bilayers. With this sample preparation, anisotropic information about the peptide is preserved during the measurement. Samples are placed in the spectrometer with the membrane-normal parallel to the incoming light beam. Unlike in solution CD, peptides have a uniform orientation with respect to the incoming light. Therefore, the orientation of peptides with respect to the membrane-normal can be analysed.⁶² Each measurement was repeated a second time to ensure reproducibility (see Methods, Chapter 12).

A peptide forming a pore is most likely present in an inserted state (I-state), at a 90° angle to the bilayer normal or a strongly tilted state (T-state), between 90° and 0° with regard to the bilayer normal. If a peptide does not form a transmembrane pore, a surface bound state (S-state) is often observed. The orientational information gained through OCD therefore gives initial insights into the possible mode of action of an AMP.

OCD measurements were performed in DMPC/DMPG (7:3) at a P/L of 1:100 at 30°C. The humidity was maintained at 97 % relative humidity before and during the measurements. The sample preparation of the OCD samples therefore closely resembles that of the NMR samples used in Chapter 5.1.6, increasing the comparability of the resulting data. The OCD spectra are shown in **Figure 4**.

To allow direct comparison, each spectrum was normalized at 222 nm to -15 ellipticity/mdeg. Across all Ala-d₃ analogues, the spectra were highly similar to that of the Ltc5 WT and displayed the characteristic helical features observed in solution CD, indicating that once membrane-bound, the analogues retain both the helical

conformation and the orientation in the membrane of the Ltc5 WT. The reduced band intensity at 208 nm relative to 222 nm observed in all spectra would typically suggest a slight tilt of the α -helix in the lipid bilayers. However, this has been attributed to an artefact caused by the high number of aromatic residues in Ltc5 WT by Wadhwani et al. and is in line with the literature.^{47,60,63} Wadhwani et al. determined the orientation of Ltc5 WT as an S-state in previous ¹⁵N-NMR studies.⁴⁷ By the same rationale, all Ala-d₃ analogues are likewise considered to adopt an S-state configuration, with their helical axes aligned perpendicular to the membrane-normal. None of the spectra indicate the

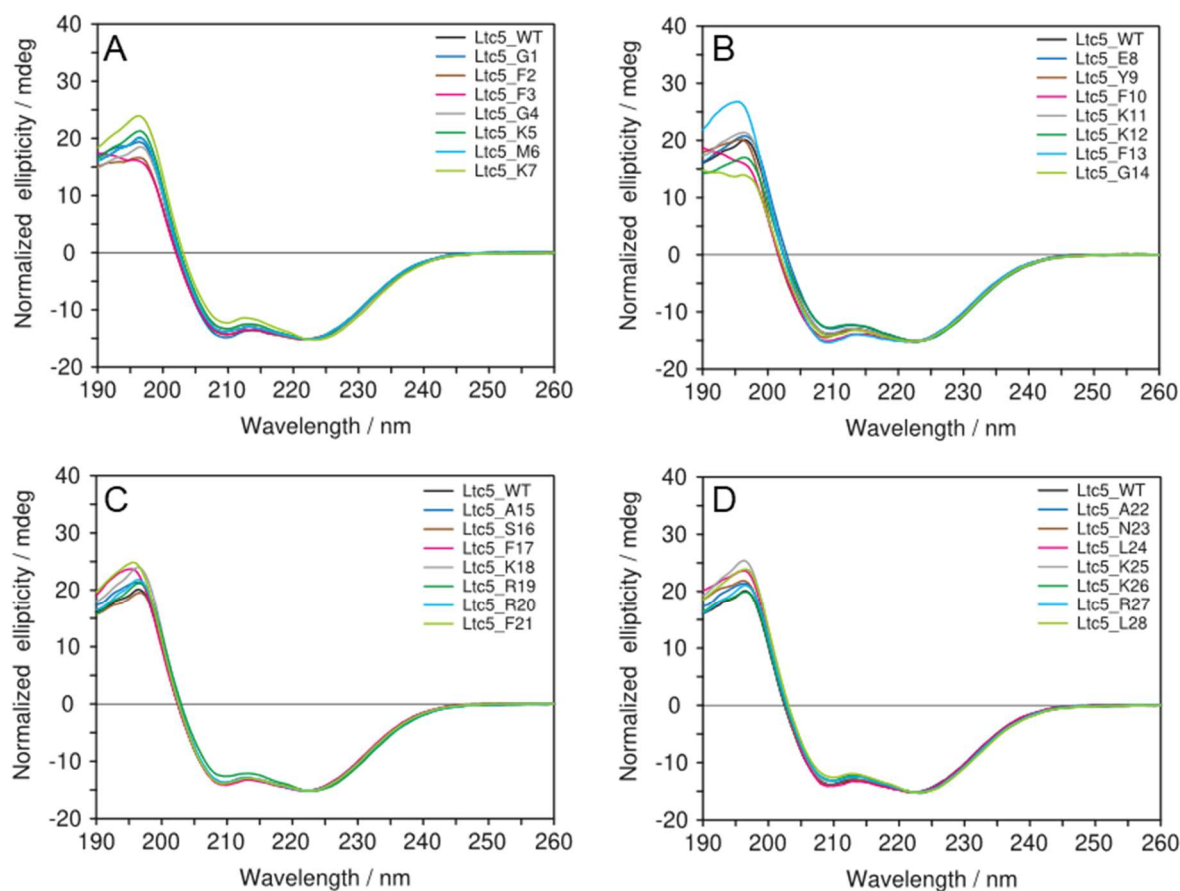


Figure 4: Normalized OCD spectra of the Ltc5 WT and all Ala-d₃ analogues.

Measurements were conducted in lipid bilayers made from DMPC/PG (7:3) and a P/L of 1:100 at a hydration rate of 97% measured at 30°C. Each panel contains the spectra of peptide with Ala-d₃ by positions A) 1 to 7 B) 8 to 14 C) 15 to 21 and D) 22 to 28. Every panel additionally contains the spectrum of the Ltc5 WT as a reference. Data of the Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and presence of aggregated peptides in the samples. It can be concluded that substitutions at all positions by Ala-d₃ are tolerated without any considerable disturbance of peptide structure or orientation in the membrane.

5.1.3 Antimicrobial activity

Antimicrobial activity is the defining property of an AMP and can be considered its most important functional attribute. To test whether the introduction of an Ala-d₃ label affects the antimicrobial activity of Ltc5 WT, all 28 synthesized analogues were evaluated in a minimal inhibitory concentration (MIC) assay. The MIC assay was performed on different bacterial strains to test potential strain-dependent activities. Differences in activity can arise from distinct metabolic pathways or from variations in enzymatic and protein compositions involved in key cellular processes among different bacteria. Another important factor is the difference in membrane architecture between Gram-positive and Gram-negative bacteria. Therefore, two Gram-negative strains, *Escherichia coli* and *Acinetobacter sp.*, as well as two Gram-positive strains, *Bacillus subtilis subsp. spizizenii* and *Staphylococcus xylosus*, were included in the MIC assays in this work.

The MIC assay was conducted as previously described (see Methods, Chapter 12) and repeated twice to ensure reproducibility. The results of the MIC assay are shown in **Table 6**.

Several analogues showed an increase in activity by one dilution step, whereas others displayed a modest decrease, typically also limited to a single dilution step. Notably, even mutations that introduce substantial changes in charge, polarity, side-chain volume/steric bulk, or aromatic character (e.g., replacement of phenylalanine or arginine for Ala-d₃) still preserved the high antimicrobial activity observed for the Ltc5 WT. Moreover, the activity appeared to be largely strain-independent, as MIC values remained within a comparable range for both Gram-positive and Gram-negative strains. Taken together with the structural information obtained from CD and OCD measurements, these findings indicate that the single-point mutations tested here do not substantially affect the antimicrobial activity of Ltc5.

Table 6: MIC assay performed in a two-fold peptide dilution series.

The assay was performed in a concentration range of 256 µg/mL to 2 µg/mL on all Ala-d₃ analogues of Ltc5. The antibacterial activity was tested against two Gram-negative strains, *E. coli* and *Acinetobacter sp.* and two Gram-positive strains, *B. spizizenii* and *S. xylosus*. Data of the Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and L24 were previously obtained during the master thesis of the author.⁵⁷

Bacteria strain	MIC [µg/mL]							
	WT	G1	F2	F3	G4	K5	M6	K7
<i>E.coli</i>	4	8	8	4	8	4	4	8
<i>Acinetobacter sp.</i>	4	8	4	8	8	8	4	8
<i>B. Spizizenii</i>	4	4	8	4	8	8	2	8
<i>S. xylosus</i>	2	8	4	8	8	8	2	8

	WT	E8	Y9	F10	K11	K12	F13	G14
<i>E.coli</i>	4	8	4	4	4	4	8	8
<i>Acinetobacter sp.</i>	4	8	4	4	4	4	2	4
<i>B. Spizizenii</i>	4	8	2	2	4	4	2	8
<i>S. xylosus</i>	2	8	2	2	4	4	2	4

	WT	A15	S16	F17	K18	R19	R20	F21
<i>E.coli</i>	4	4	8	4	8	8	8	4
<i>Acinetobacter sp.</i>	4	4	8	4	8	8	4	4
<i>B. Spizizenii</i>	4	2	4	2	8	8	4	4
<i>S. xylosus</i>	2	4	8	4	8	8	4	4

	WT	A22	N23	L24	K25	K26	R27	L28
<i>E.coli</i>	4	4	4	4	4	8	4	4
<i>Acinetobacter sp.</i>	4	4	8	4	8	4	8	8
<i>B. Spizizenii</i>	4	4	4	4	4	4	4	8
<i>S. xylosus</i>	2	2	8	4	8	4	8	8

5.1.4 Hemolytic activity

Erythrocytes are the most abundant cell type in the human body, accounting for roughly 80% of all cells.⁵² Because of this predominance, it is crucial that new antimicrobial agents exhibit minimal or no toxicity toward human red blood cells in order to limit potential side effects for any therapeutic application. Ltc5 has shown low hemolytic activity in previous studies.^{47,64} It is therefore of interest to determine whether single-residue substitutions to Ala-d₃ alter this property in any way or whether the low hemolytic activity of the Ltc5 WT peptide is retained.

In this assay, the release of hemoglobin caused by membrane disruption upon exposure to an AMP is measured as a function of peptide concentration.^{65,66} The extent of haemoglobin release directly reflects the degree of hemolysis: the greater the membrane damage caused by a peptide, the higher the hemolysis, and consequently the lower the therapeutic index of the AMP. Each measurement was performed in triplicate and repeated twice (see Methods, Chapter 12). The results of the hemolysis assay are presented in **Figure 5**.

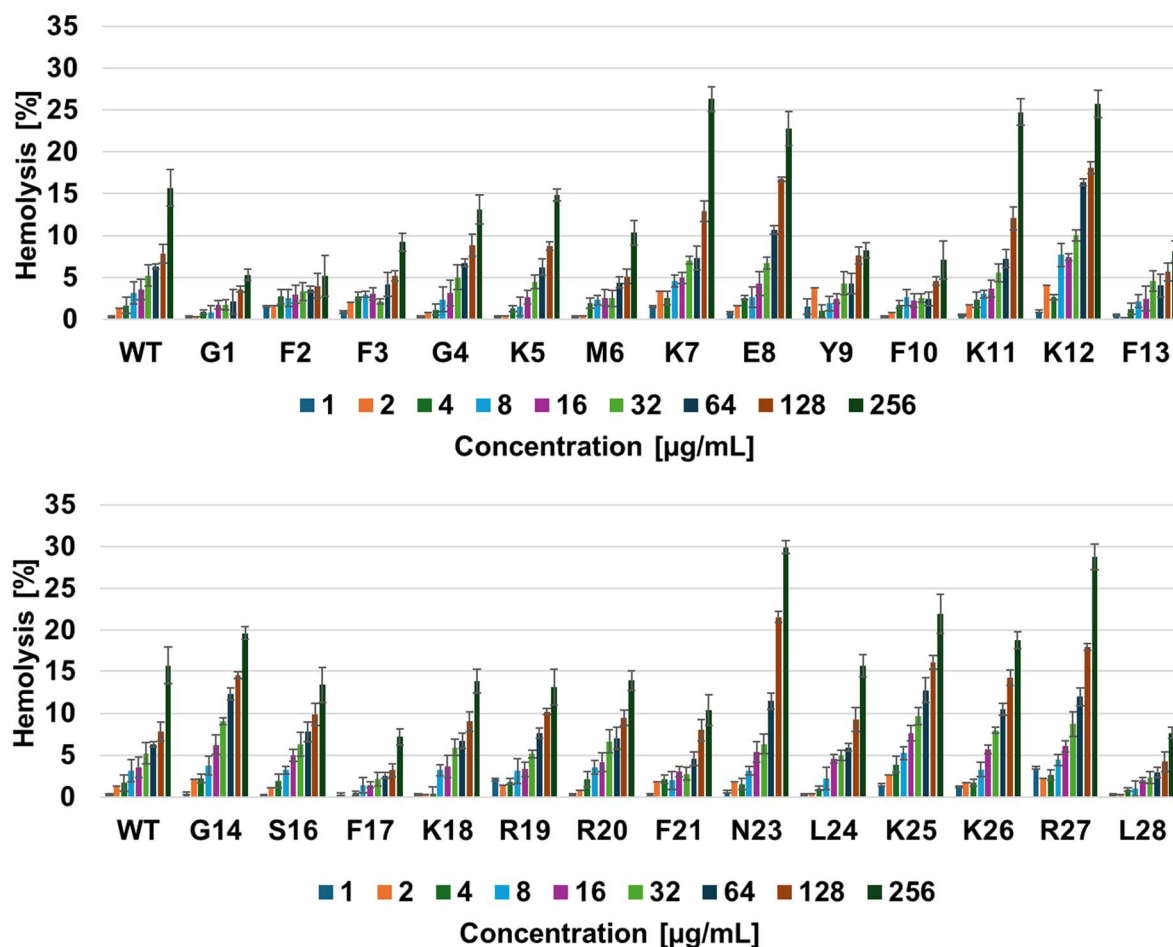


Figure 5: Concentration dependent hemolysis of all Ala-d₃ analogues.

The assay was performed using a serial dilution of peptides in the concentration range between 256 µg/mL and 1 µg/mL using the Ltc5 WT as reference peptide. Blood type was A, Rh(D) positive. Data of the Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and L24 were previously obtained during the master thesis of the author.⁵⁷

Hemolytic activity of an AMP often correlates with increasing peptide hydrophobicity.⁶⁷ In the alanine scan, each residue was substituted with Ala-d₃, thereby reducing hydrophobicity in analogues where Phe, Tyr, Leu, or Met was replaced, and increasing hydrophobicity when Gly, Lys, Arg, Glu, Ser, or Asn was substituted. In addition, the

overall charge of the peptide is altered in analogues where either Lys, Arg, or Glu was replaced by Ala-d₃. As Ltc5 already carries a high positive net charge of +10, a moderate reduction in charge is expected to be well tolerated in the Ala-d₃ analogues and should not markedly affect their hemolytic activity. In contrast, further increasing the net positive charge, as in the case of Ltc5 E8, would be expected to slightly increase the activity of the respective analogue.

The experimental data show that at the average MIC of 4 µg/mL, hemolysis for all analogues remained below 5 %, including those with increased hydrophobicity. Only at the highest tested concentration (256 µg/mL) did hemolysis reach values of up to 30 %. The highest hemolysis levels were primarily observed for analogues with increased hydrophobicity, such as K7, E8, K11, K12, N23, K25, K26, and R27. However, this trend was not consistent across all polar substitutions, as K5, K18, R19, and R20 did not show increased hemolysis despite their higher hydrophobicity.

5.1.5 Vesicle leakage

The vesicle leakage assay is a fluorescence-based method used to investigate how an AMP affects the organisation and integrity of lipid membranes. It allows the use of model membranes with well-defined lipid compositions that mimic prokaryotic or eukaryotic membranes. Bacterial membranes typically contain a high density of negatively charged phospholipid headgroups, whereas cholesterol is found exclusively in eukaryotic cell membranes. With this assay, the influence of the individual Ala-d₃ replacements on the membranolytic activity of Ltc5 towards membrane mimetics is investigated.

In the leakage assay, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS) and p-xylene-bis(pyridinium bromide) (DPX) are employed as a fluorophore and quencher, respectively, as described previously.⁶⁸ A quencher reduces a fluorophore's emission by interacting with its excited state, either by energy transfer, electron transfer, or formation of a non-fluorescent complex, so that the fluorophore relaxes without emitting light when the quencher is in close proximity. Typically, ANTS and DPX are co-encapsulated inside large unilamellar vesicles (LUVs). When the model membrane (here the LUVs) is disrupted by the peptide, their contents leak out, increasing the distance between ANTS and DPX molecules. This reduces quenching

efficiency and results in an increase in ANTS fluorescence (see methods, Chapter 12). Measurements were repeated three times on separate days.

To investigate the concentration-dependent leakage caused by Ltc5 WT and to identify suitable testing conditions, P/L ratios of 1:100, 1:50, 1:25, and 1:10 were tested in advance of the vesicle leakage assay for Ltc5 WT in POPC/POPG (1:1). The induced vesicle leakage at the different P/L ratios is shown in **Figure 6**.

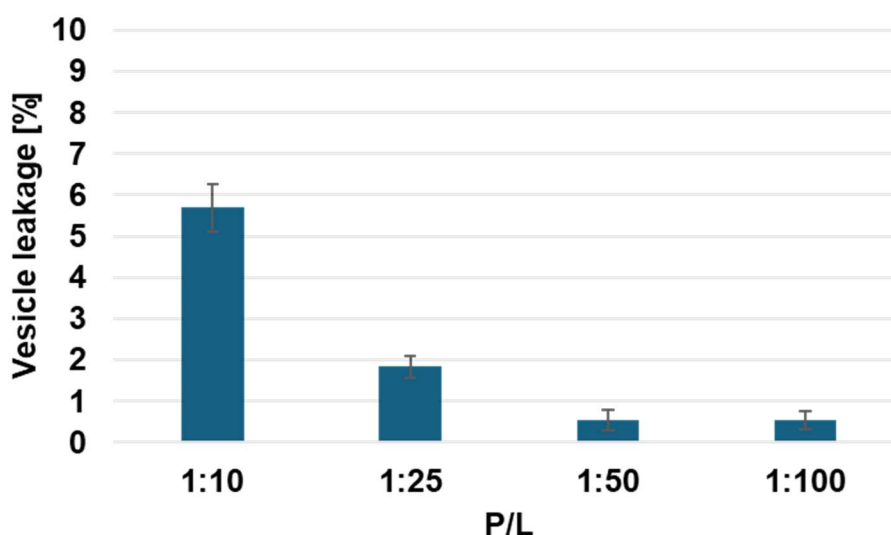


Figure 6: Concentration-dependent Vesicle leakage of Ltc5 WT.

The assay was performed in LUVs made from POPC/POPG (1/1) at different P/L to find optimised testing conditions for consecutive experiments.

Increasing the P/L to 1:10 resulted in only 5.7 % vesicle leakage. Ltc5 can thus be considered to induce only minor membrane damage, even at high peptide concentrations. A P/L of 1:25 was ultimately chosen to assess all Ala-d₃ analogues for their ability to cause membrane damage. Charge neutrality between Ltc5 (charge of plus ten) and POPC/POPG (-1/2) is reached at a P/L lower than 1:20. Therefore, all peptides are expected to bind at a lower P/L than 1:20.

All 28 Ala-d₃ analogues and Ltc5 WT were tested, as previously described, in vesicles composed either of POPC/POPG (1:1), mimicking prokaryotic membranes, or in cholesterol-containing LUVs, composed of POPC/POPG/Chol (2:2:1) (see Methods, Chapter 12). Since eukaryotic membranes contain cholesterol, these vesicles serve as

eukaryotic model membranes.^{23,24} The results of the vesicle leakage assay are shown in **Figure 7**.

For both vesicle compositions, all Ala-d₃ analogues exhibited very low leakage, well below 10 %. In POPC/POPG (1:1), the Ltc5 WT caused vesicle leakage of ~2 %. Under these conditions, only two analogues showed a notable increase compared to the Ltc5 WT, namely K18 and K25, with the highest measured leakage values of ~6 %.

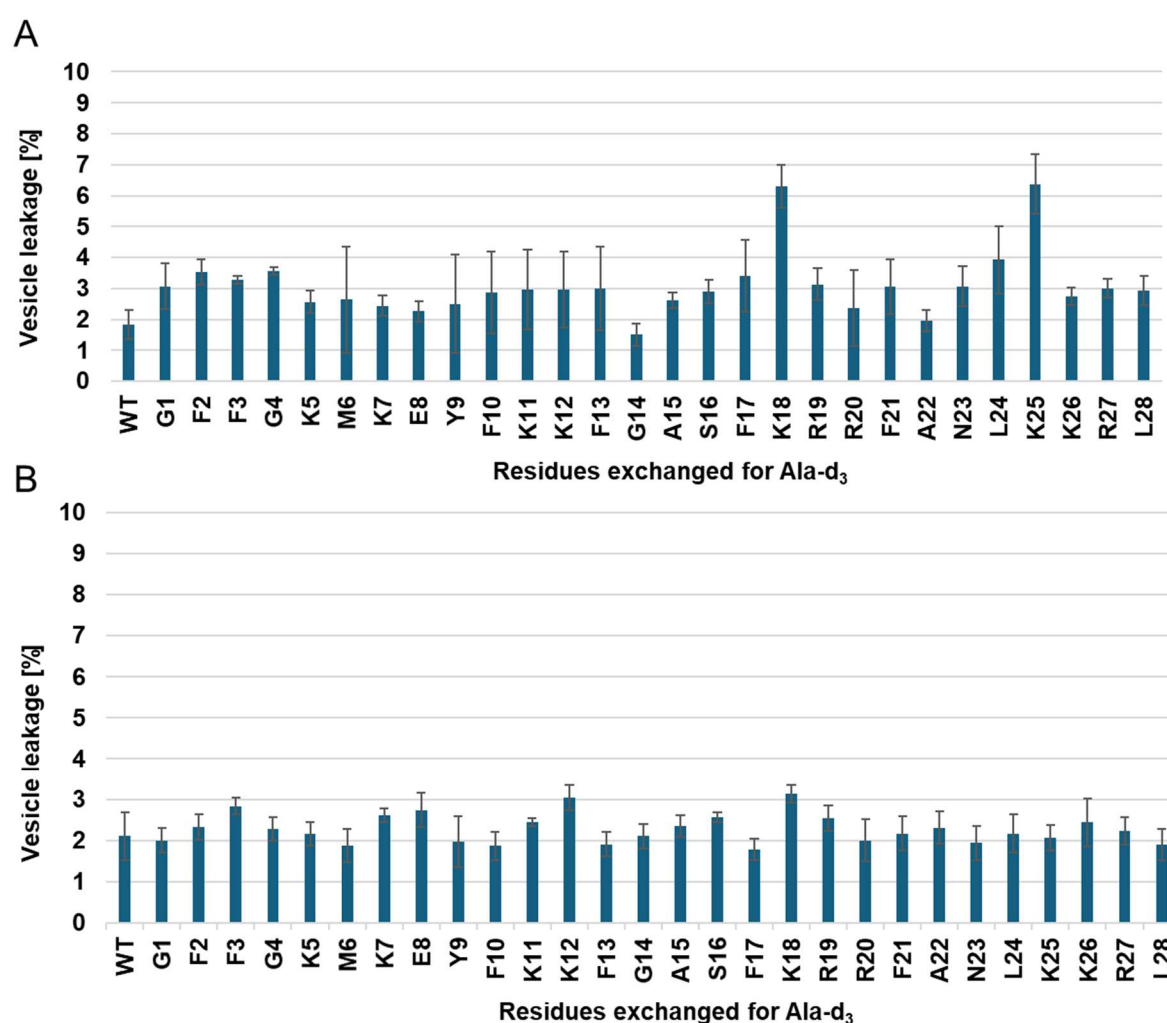


Figure 7: Results of the vesicle leakage assay performed on all Ala-d₃ analogues of Ltc5.

Ltc5 WT and all 28 Ala-d₃ analogues were tested for their membranolytic activity towards large unilamellar vesicles composed of A) POPC/PG (1:1) or B) POPC/POPG/Chol 2:2:1. Measurements were conducted at 30 °C at a P/L of 1:25. Data of the Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and L24 were previously obtained during the master thesis of the author.⁵⁷

All other analogues remained below 4 %. In POPC/POPG/Chol LUVs, the Ltc5 WT induced leakage of ~2 %, which is the same range as in POPC/POPG vesicles.

None of the 28 Ala-d₃ analogues caused significantly higher leakage than the Ltc5 WT in the cholesterol-containing LUVs, with the highest observed value being ~3 % for K12 and K18. All differences observed fall within the experimental error of the method.

Since the Ltc5 WT and all Ala-d₃ analogues showed only very low hemolytic activity (**Figure 5**) and their secondary structure and orientation remained unchanged (**Figure 3**, **Figure 4**), it can be concluded that membrane disturbance caused by Ltc5 does not change significantly due to single Ala-d₃ mutations.

Overall, all positions in the primary sequence of Ltc5 seem to tolerate replacement by Ala-d₃ in the methods applied during the Ala-scan. The structure and function of all Ala-d₃ analogues closely resemble those of Ltc5 WT. Therefore, all Ala-d₃ analogues were considered suitable for use in the ssNMR analysis.

5.1.6 Solid-state NMR spectroscopy

A precise structure and orientation of Ltc5 within lipid bilayers have not been determined previously. As the structure of peptides and proteins is closely related to their function, we hope to get insights into the mechanism of action of Ltc5 by determining the structure of Ltc5 in membranes. Solid-state ²H-NMR measurements were performed on all Ala-d₃ analogues which were previously analysed during the Ala-scan. The ssNMR samples were prepared as previously described (see methods, Chapter 12).^{54,69,70} All ssNMR measurements were performed on a Bruker Avance 500 MHz spectrometer at 35 °C.

Lipid bilayers in eukaryotic and prokaryotic membranes form lamellar (L_α) phases, which must be reproduced in ssNMR experiments to ensure comparability. To achieve membrane-like conditions during measurement, a well-oriented L_α phase of the lipid bilayers is essential. To ensure the presence of a stable L_α phase in the sample, ³¹P spectra were measured before and after every ²H-NMR measurement, using a flat coil dual resonance probe head with a Hahn echo pulse sequence consisting of a 3.9 μs 90° pulse and a 7.8 μs 180° pulse, a 30 μs echo time and phase cycling. 17 kHz ¹H-SPINAL64 decoupling was used to filter any proton couplings during the ³¹P measurements.⁷¹ To ensure full hydration of the sample, before and after each ²H measurement, a ¹H spectrum was measured. Water gives rise to a distinct ¹H

signal, whose intensity can be monitored and used as a readout of the sample's hydration level. The water signal observed in the initial ^1H measurement is taken as the reference corresponding to a fully hydrated lipid bilayer. A ^2H -goniometer probe head was used to perform the ^2H -NMR measurements on the respective Ala- d_3 analogue. Every sample was measured twice, glass plates and thus also the membrane-normal oriented at 0° and once at 90° with respect to the magnetic field. A 90° - 90° solid echo pulse sequence was used with a $4.45\ \mu\text{s}$ 90° pulse and $70\ \mu\text{s}$ echo time.⁷²

A simple and efficient way to confirm the mobility of a peptide in the bilayer is to compare the spectra measured with the membrane-normal at 0° and 90° sample tilt with respect to the external magnetic field B_0 . If the quadrupolar splitting at 90° sample tilt is reduced by $-1/2$ compared to the value at 0° , this indicates fast rotational diffusion of the peptide around the membrane-normal. Aggregated peptides are immobile or move significantly slower than monomeric α -helical peptides in a lipid bilayer. Therefore, a reduction of the quadrupolar splitting by $-1/2$ would not be expected. In this case, a powder spectrum would be expected at a 90° tilt, with a broader line shape and a maximum splitting of $\sim 37\ \text{kHz}$.⁷³ No change in the quadrupolar splitting between 0° and 90° would highly suggest the formation of oligomers by the peptides. In a dry sample the lamellar orientation of the lipid bilayer is usually lost, deeming the peptide orientation in that particular sample as inconclusive. In such a case, new samples have to be made.

Each of the Ala- d_3 labelled analogues was measured in lipid bilayers composed of DMPC/DMPG (7:3) at $35\ ^\circ\text{C}$ and with an identical P/L to that used in OCD (1:100) experiments to ensure comparability of the results gained from both methods. The measured quadrupolar splittings were later used for precise structural calculations.

The resulting NMR spectra are shown in Figure 8 and Figure 9.

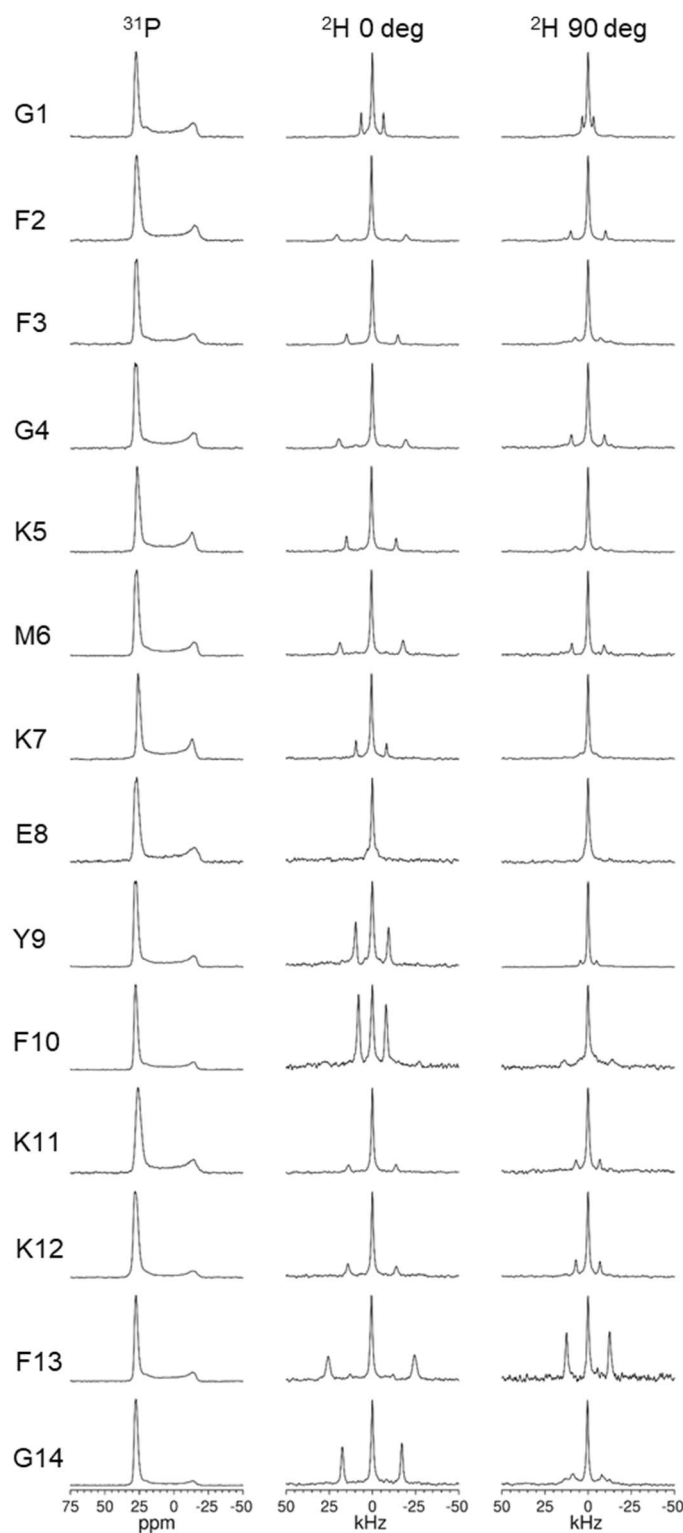


Figure 8: Solid-state NMR spectra of Ala-d₃ labelled Ltc5 at positions one to fourteen.

Three data sets are shown next to each other. In every data set the left column shows the ^{31}P -spectrum before the measurement. The middle column shows the ^2H -spectrum at 0° sample tilt. The right column shows the ^2H -spectrum at 90° sample tilt. The respective analogue name, made up from the residues exchanged for Ala-d₃ and the position of this residue, is written on the left of each row. Lipid composition DMPC/DMPG (7:3), P/L of 1:100, measured at 35°C .

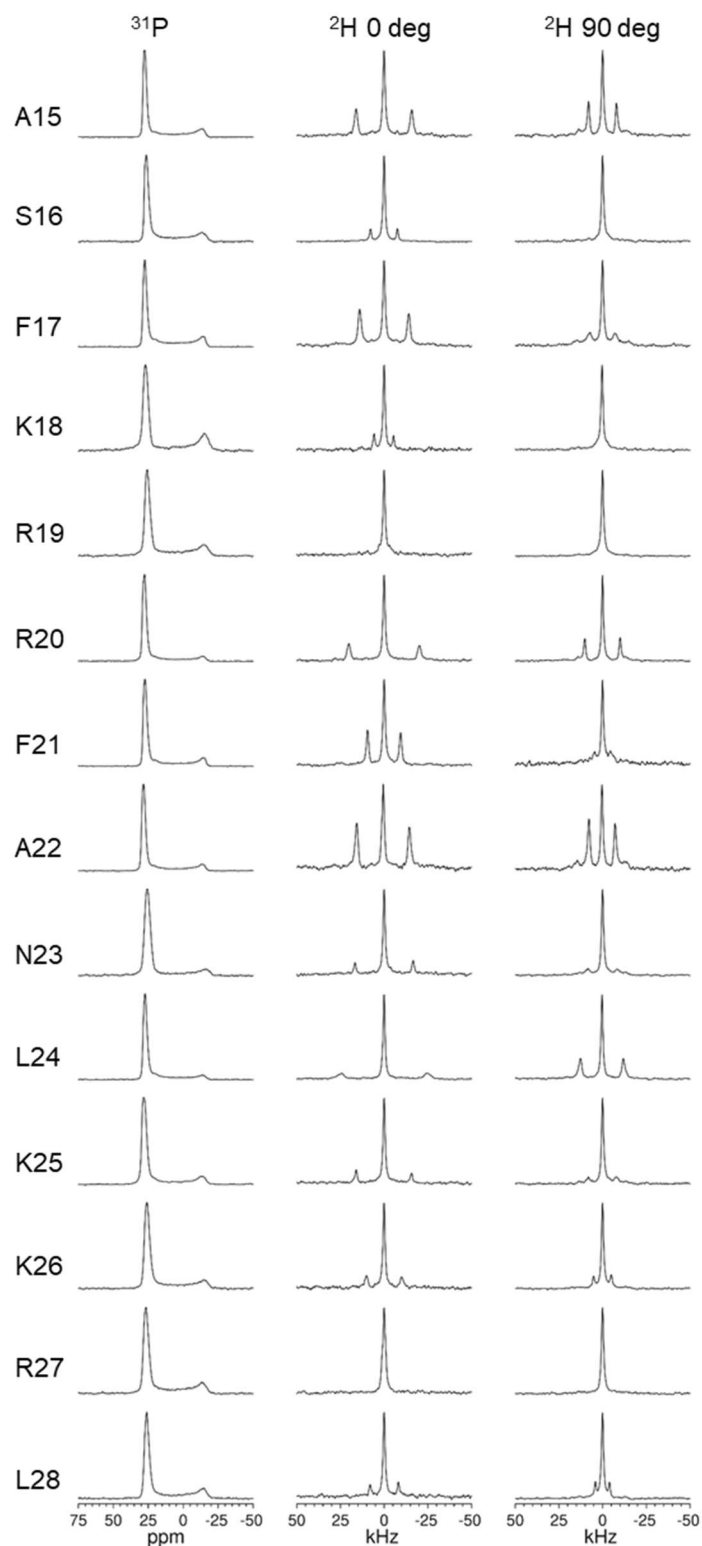


Figure 9: Solid-state NMR spectra of Ala-d₃ labelled Ltc5 at positions 15 to 28.

Three data sets are shown next to each other. The left column of each data set shows the ³¹P spectrum before the measurement. The middle column shows the ²H spectrum at 0° sample tilt. The right column shows the ²H spectrum at 90° sample tilt. The respective analogue name, made up from the residues exchanged for Ala-d₃ and the position of this residue, is written on the left of each row. Lipid composition DMPC/DMPG (7:3), P/L of 1:100, measured at 35 °C.

Lipids oriented in an L_α phase result in a signal around 29 ppm. Samples usually also contain a small powder component (<10 %) giving rise to a signal around -16 ppm. The two described signal types can be observed in every ^{31}P spectra measured (see **Figure 8** and **Figure 9**) with the dominant peak at 29 ppm indicating a well oriented lipid bilayer predominantly present in an L_α phase.

^2H spectra at 0° sample tilt show a central peak from naturally occurring partly deuterated water (HOD) and a quadrupolar splitting from the Ala- d_3 label in the peptides. At 90° sample tilt, the ^2H spectra are typically scaled by a factor of -1/2, indicating that the peptide is mobile and the quadrupolar splitting can be used for the structure calculations. Values of the quadrupolar splittings are given in **Table 7**.

The labelled peptides R19 and R27 showed small quadrupolar splittings at 0° sample tilt. When tilting these samples by 90° the splittings could not be resolved. The absence of a resolved splitting can either indicate that the deuterium labels of these peptides are oriented close to the magic angle of 54.74° with respect to the external magnetic field during the measurement, so that the splitting is smaller than the width of the central peak and can therefore not be resolved. Alternatively, some experimental problem might have occurred. To ensure no errors were made during sample preparation or measurement for these peptides, the respective measurements were reproduced with new samples. As a first assumption, the data for the three peptides were considered to be valid and were used in the structural calculations.

Table 7: Values of quadrupolar couplings of all Ala-d₃ analogues.

Measurements were conducted in DMPC/DMPG (7:3) at a P/L of 1:100 and 35°C by ²H-NMR. Each sample was measured at 0° and 90° sample tilt with respect to B₀. Halved splittings in the 90° tilted sample indicate mobile peptides. Data of the Ltc5 M6, Y9, F10, F13, G14, A15, F17, R20, F21, A22 and L24 were previously obtained during the master thesis of the author.⁵⁷

Name of mutant	Quadrupolar splitting 0° [kHz]	Quadrupolar splitting 90° [kHz]
G1Ala-d ₃	13.0	6.6
F2Ala-d ₃	39.9	20.2
F3Ala-d ₃	29.8	14.4
G4Ala-d ₃	38.9	19.2
K5Ala-d ₃	28.7	14.3
M6Ala-d ₃	36.6	18.2
K7Ala-d ₃	17.7	8.8
E8Ala-d ₃	11.1	Unresolved
Y9Ala-d ₃	19.0	9.5
F10Ala-d ₃	16.0	8.1
K11Ala-d ₃	27.6	13.6
K12Ala-d ₃	28.2	14.1
F13Ala-d ₃	50.0	25.0
G14Ala-d ₃	34.5	17.2
A15Ala-d ₃	32.1	16.1
S16Ala-d ₃	14.8	7.4
F17Ala-d ₃	28.4	14.2
K18Ala-d ₃	10.3	5,2
R19Ala-d ₃	5.7	Unresolved
R20Ala-d ₃	40.5	20.3
F21Ala-d ₃	19.0	9.5
A22Ala-d ₃	30.1	15.0
N23Ala-d ₃	32.8	16.5
L24Ala-d ₃	48.6	24.2
K25Ala-d ₃	31.0	15.6
K26Ala-d ₃	20.0	10.1
R27Ala-d ₃	1.5	Unresolved
L28Ala-d ₃	16.2	8.1

5.1.7 Structure calculation

To determine the membrane orientation of Ltc5 WT in lipid bilayers, quadrupolar splittings obtained from ^2H -NMR of the site-specific Ala- d_3 -labelled Ltc5 analogues were used. The resulting data were fitted using a model that assumes an ideal α -helix as a reference. This method characterises the tilt angle Tau (τ) and the rotation angle Rho (ρ), which together define the exact orientation of a peptide in a lipid bilayer. τ is defined as the angle between the helix axis and the bilayer normal. Accordingly, a perfectly horizontal α -helix corresponds to $\tau = 90^\circ$. ρ is defined as the azimuthal rotation angle around the helix axis, with $\rho = 0^\circ$ assigned when residue 12 of the analysed peptide (here, Ltc5) lies in the x-y plane. A schematic definition of τ and ρ is shown in **Figure 10**.

The structural calculations were carried out according to the dynamic GALA (Geometric Analysis of Labelled Alanines) method developed by Ulrich et al.^{54,69,70} The theory behind this method is described in detail in Chapter 13. For these calculations, the quadrupolar splittings at 0° sample tilt with respect to B_0 were used. The quadrupolar splittings at 0° sample tilt and 90° sample tilt are summarised in **Table 7**.

The experimental quadrupolar splittings were plotted against the residue number and compared to a theoretical quadrupolar wave based on an ideal α -helix. To assess helical periodicity, the data were also plotted as a function of the angular position around the helix. Best-fit curves and RMSD plots were generated to identify the most probable τ and ρ angles of each segment relative to the membrane, with the lowest RMSD regions indicating the best agreement between model and experiment.

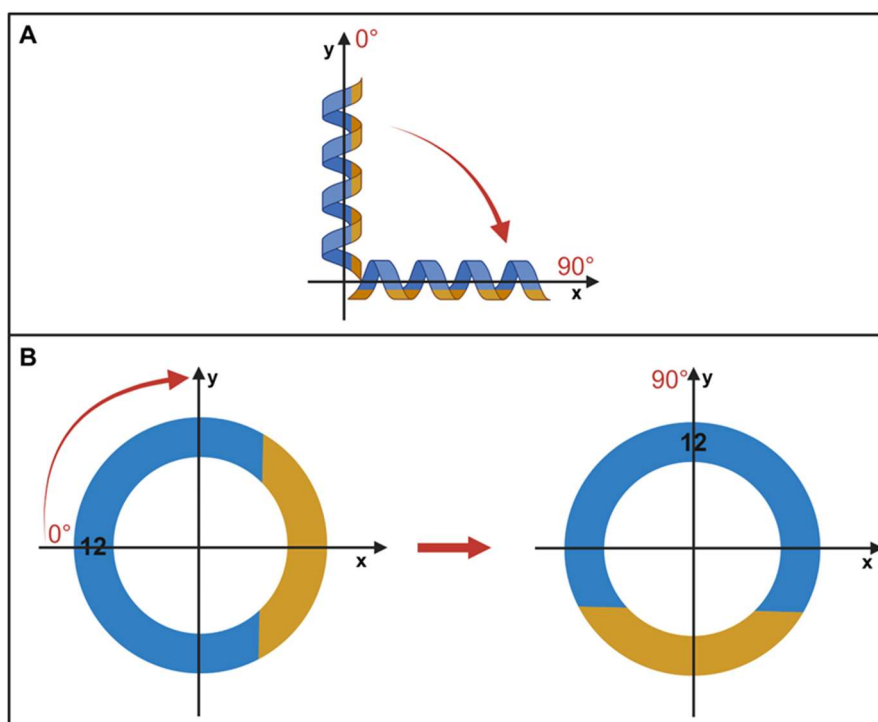


Figure 10: Definition of the helical orientation angles τ and ρ used for peptide helices.

A) τ is the helix tilt angle relative to the membrane normal, varying from 0° for a helix aligned with the normal to 90° for a helix lying parallel to the membrane plane. B) ρ is the azimuthal rotation of the helix about its long axis, here defined using residue 12 as the reference. the example shows rotation from 0° to 90° .^{54,69,60} Created in <https://BioRender.com>

Initially, all labelled positions were included in a single global fit. This resulted in a relatively high root mean square deviation (RMSD > 6 kHz), which nevertheless still indicated an acceptable overall agreement with a single helical model for the peptide. A list of the most important fitting results is provided in Table 8.

Table 8: Calculated values based on the quadrupolar splittings from the Ala- d_3 analogues of Ltc5.

Given are the rotational angle (ρ), the tilt angle (τ), the root mean square deviation RMSD/kHz and the σ values of τ and ρ .

Positions included in fit	tilt angle (τ) [°]	rotational angle (ρ) [°]	RMSD [kHz]	σ of τ [°]	σ of ρ [°]
1-28	87	93	6.1	0	8
2-27 without 18 and 20	86	92	5.8	0	9
2-11	87	98	2.6	0	10
2-11 without 8	86	99	1.7	9	6
12-27 without 16 and 20	85	85	1.8	9	4

To refine the analysis and identify deviating data points, a sequential fitting approach was applied. Starting from the N-terminus, residues were added stepwise one at a time to the fit (e.g., positions 1-5, then 1-6, 1-7, and so on). This procedure allowed to monitor how the RMSD evolved as additional positions are included, and thereby to pinpoint the exact residues at which the agreement with a single helical model begins to deteriorate.

A good fit was obtained for positions 2-11, with an improved RMSD of 2.6 kHz (**Table 8**). Position 1 was excluded as residues at the ends of a helix are often more mobile and less rigid due to missing stabilizing hydrogen bonds.⁷⁴ They therefore deviate from an ideal helical geometry, which the fitting algorithm assumes to be present. Including such edge residues typically worsens the agreement of the data with a fitting curve, as was indeed observed when position 1 was included. Inspection of the fitting curve revealed that position E8 was an overall poor fit compared to the other residues. Substitution of Glu by Ala introduced substantial changes: the only negatively charged residue in the WT sequence is removed, a highly hydrophilic carboxyl group is replaced by a slightly hydrophobic methyl group, and the steric hindrance of the side-chain is significantly reduced. These factors likely caused subtle structural changes in the E8 to Ala-d₃ analogue. Although these perturbations do not appear to affect the functional properties of the analogue, they seem to induce local changes in the helix structure around this position. These structural changes seem to be too subtle to be detected by CD spectroscopy. Consequently, this analogue is not representative of the Ltc5 WT peptide's conformation and was excluded from further structural calculations. Omitting E8 from the fit for positions 2-11 yielded an excellent RMSD of 1.7 kHz, as shown in **Figure 11**.

Extending the fit beyond position 11 led to a sudden increase in RMSD of >5 kHz. Fits that included both positions 11 and 12 always showed increased RMSD values. Based on these results, a C-terminal fit was first attempted for positions 12-27. Position 28 was excluded for the same reasons as position 1 in the N-terminal fit, namely the increased mobility and imperfect α -helicity of terminal residues (see **Table 8**).

This initial fit yielded an RMSD > 4 kHz. Inspection of the fitting curve revealed two major outliers: positions S16 and R20 deviated strongly from the optimal fit. When S16 and R20 were omitted from the fit, the resulting analysis for positions 12-27 showed a

pronounced improvement, with the RMSD dropping by more than 2 kHz to 1.8 kHz (**Table 8**). This fit is therefore considered the most plausible for the C-terminal part of the peptide and is shown in **Figure 12**.

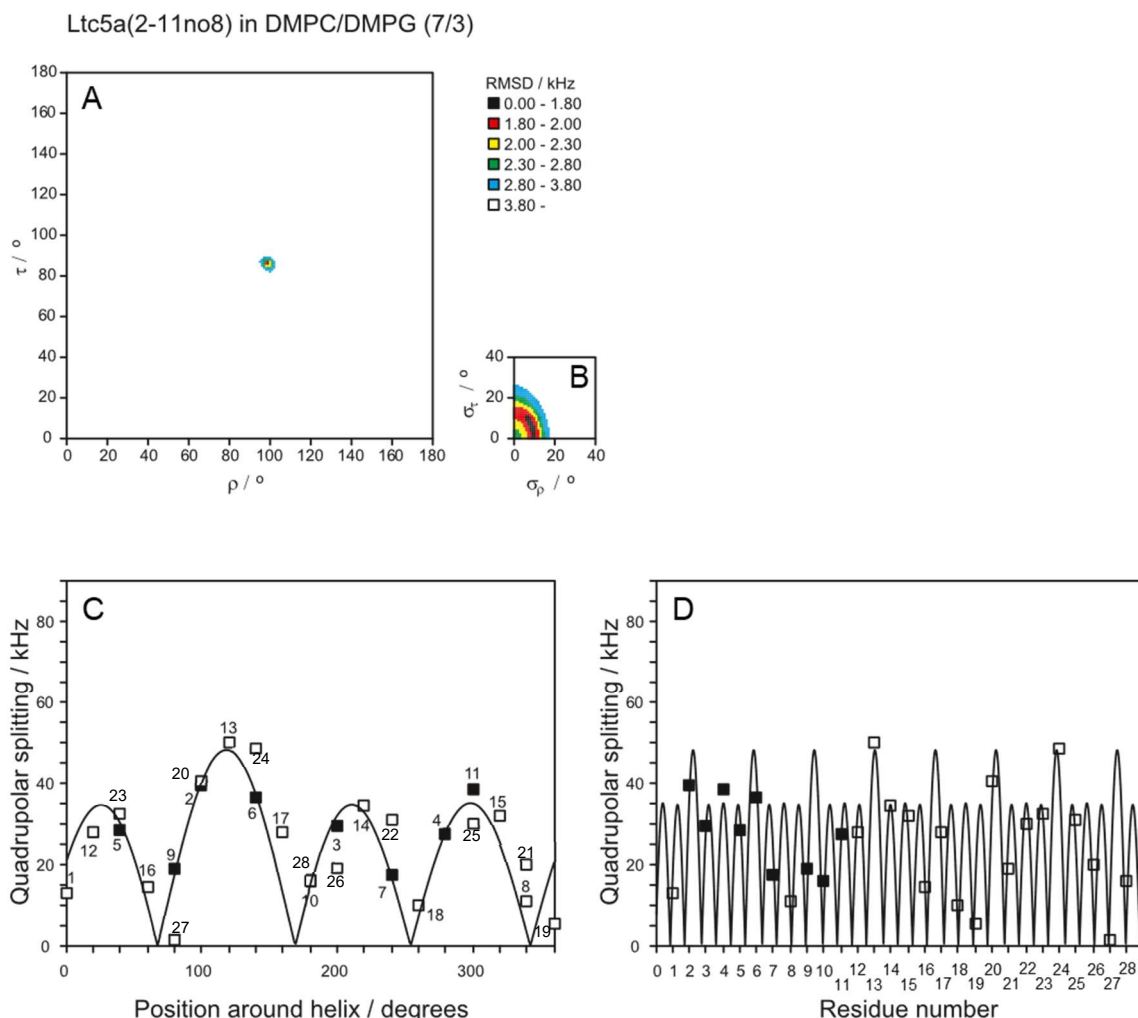


Figure 11: Fit of the quadrupolar splitting including positions 2-11 without position 8.

A), B), C) and D) in both fits stand for the following: A) Error plot of the RMSD as a function of τ and ρ using the values generated by the vertical distance from each data point to the helical wave curve for given values of τ and ρ while taking σ_τ and σ_ρ into consideration. B) Error plot indicating the most likely values of σ_τ and σ_ρ for the respective fit. C) Quadrupolar splitting plotted against the position around the helix. The calculated curve corresponds to an ideal helix. The closer the splittings are to the curve, the better does the structure of the peptide resemble a helix. D) Quadrupolar splitting plotted against the position in the primary sequence. The squares in B and C are the data points available for the fit. White data points not used, black data points used in the fit.

Extending this fit beyond position 12 by including position 13 again led to an increase in RMSD. One factor that further increases the reliability and structural relevance of

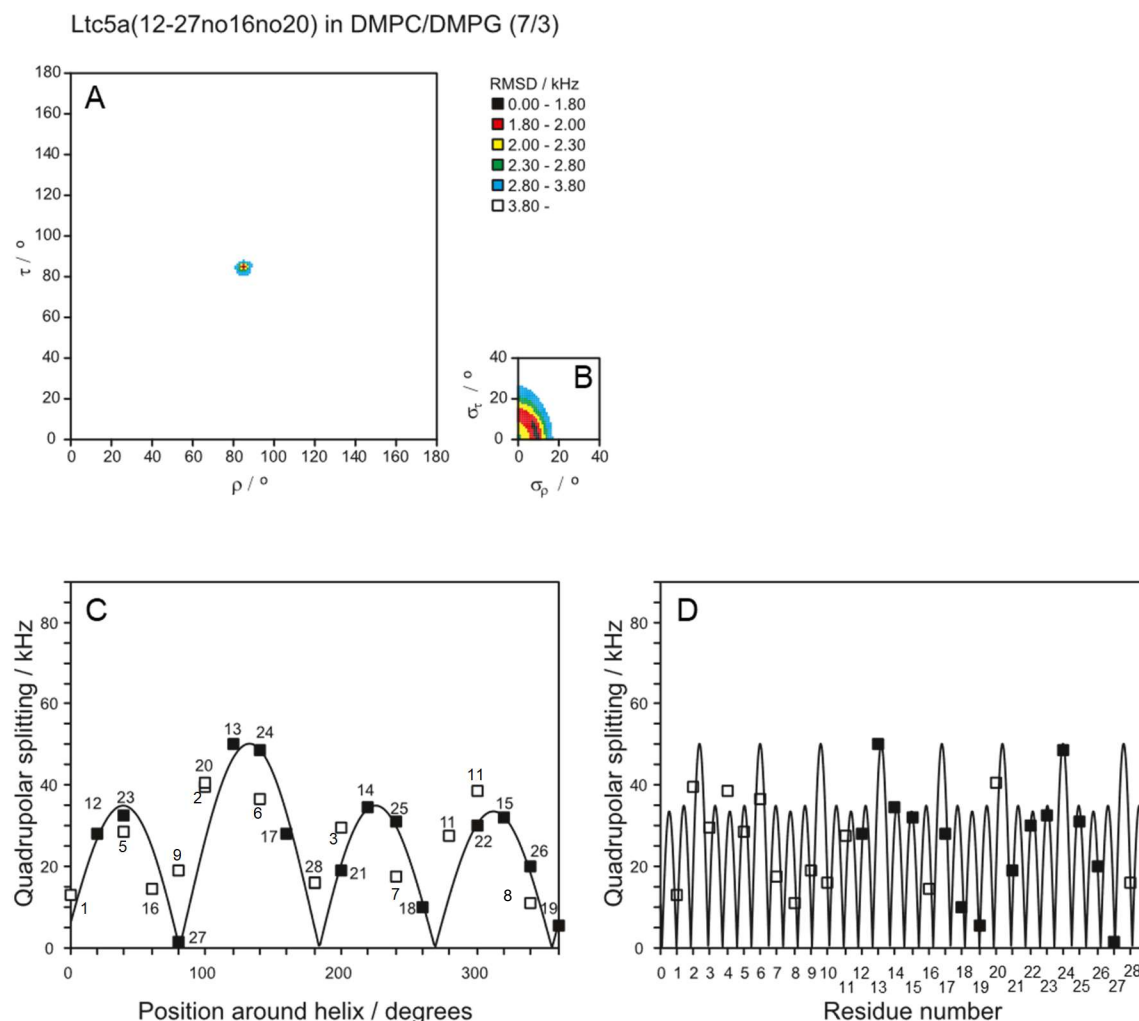


Figure 12: Fit of the quadrupolar splitting including positions 12-27 without positions 16 and 20.

A) Error plot of the RMSD. B) Error plot indicating the most likely values of σ_τ and σ_ρ for the respective fit. C) Quadrupolar splitting plotted against the position around the helix. D) Quadrupolar splitting plotted against the position in the primary sequence. The squares in B and C are the data points available for the fit. White data points not used, black data points used in the fit. For more information see Figure 10.

the C-terminal fits is the accuracy of positions 15 and 22. The analogues Ltc5 A15 and Ltc5 A22, with Ala replaced by Ala- d_3 , are not expected to introduce any significant artefacts, as these substitutions do not substantially alter the physicochemical properties. Consequently, residues 15 and 22 should align closely with the fitted curve, as they effectively represent near-perfect structural analogues of the Ltc5 WT peptide in this region.

5.2 Discussion

The first aim of this chapter was to determine whether Ltc5 has any single positions that are significant for its structure and function. This aim was addressed by replacing every residue in the primary sequence of the Ltc5 WT peptide by Ala-d₃ in a so-called Ala-scan. If the replacement of any single residue by Ala-d₃ is not tolerated, the respective analogue is expected to show significant differences in structure or function compared to the Ltc5 WT. The WT was previously shown to be unordered in solution and predominantly α -helical in contact with lipid bilayers.⁴⁷ The first method applied to analyse the secondary-structure of the Ala-d₃ analogues, was CD spectroscopy. All analogues were measured in double distilled (DD) water, 10 mM PB and in the presence of SUVs composed of DMPC/DMPG (7:3).

It is important to distinguish between aggregation, which refers to peptides forming highly unordered assemblies in solution without a defined secondary-structure, and oligomerisation, which describes the formation of peptides with ordered secondary-structure that interact with one another. An increase in defined secondary structure in 10 mM PB compared to DD water is expected due to the presence of salts and controlled pH, which reduce electrostatic repulsion between peptides and may move the peptide closer to its isoelectric point. These effects promote peptide-peptide association and could stabilise β -sheet or α -helix rich oligomers, whereas in low-ionic-strength DD water, the peptides remain more separated and less prone to secondary-structure formation.

The CD spectra of the Ltc5 WT and all Ala-d₃ analogues measured in water (**Figure A 5**) show a high degree of similarity. All spectra indicate the presence of unordered peptides and do not show spectral features associated with peptide aggregation or oligomerisation. Deconvolution of the CD spectra yielded high deviations or no results at all, depending on the data set used. The deconvolution data is thus not included in this work. As mentioned previously, peptides have an increased probability to aggregate or oligomerise in PB compared to DD water. Most assays applied in this work are either performed in buffer or salt containing media. Therefore aggregation and oligomerisation can affect their outcome and must be addressed. The CD spectra of the Ltc5 WT and all 28 Ala-d₃ (**Figure A 5**) analogues again showed high similarities to one another. All CD spectra measured in PB are consistent with highly unordered

peptides and do not show spectral features associated with peptide aggregation. Deconvolution supports the observations made in the CD spectra. All Ala-d₃ analogues exhibit an unordered fraction above 35 % similar to the Ltc5 WT, which is 36 % unordered (**Table A 2**). Interestingly, the deconvolution in PB showed an increase in β -strand content from 20 % to 40 % for the Ala-d₃ analogues. Ltc5 WT showed 22 %. This could indicate the formation of unstable oligomers in PB, which can dissociate from one another in a rapid manner. As the overall share of unordered peptides in PB is also around 40 % on average for all measured peptides, aggregation events in Ltc5 WT and all 28 Ala-d₃ analogues are deemed unlikely in solution, prior to lipid binding. Most importantly, the CD spectra of all Ala-d₃ analogues showed highly similar behaviour to that of the Ltc5 WT.

In the presence of SUVs composed of DMPC/DMPG (7:3) at a P/L of 1:50, the peptides fold predominantly into α -helices. Deconvolution of the secondary-structure revealed α -helical contents between 81 % and 92 % for the Ala-d₃ analogues and 89 % for the Ltc5 WT (**Table A 3**). Even substitution of charged or sterically more challenging amino acids such as Phe or Leu with Ala-d₃ were well tolerated and did not result in significant changes in secondary structure compared to the Ltc5 WT. This high α -helical content can be attributed to the peptide's amphipathicity. Ltc5 and the Ala-d₃ analogues can fully express their amphipathic character when they adopt an α -helical conformation. In this conformation, the hydrophobic sector, as illustrated in the helical wheel depiction of Ltc5 WT in **Figure 2**, can interact with the hydrophobic core of the lipid bilayer, while the polar face remains oriented toward the surrounding aqueous phase. The Gibbs free energy of the peptide in the membrane-bound α -helical state relative to the peptide in bulk buffer is significantly reduced. Consequently, the α -helical secondary-structure is strongly favoured.⁷⁵ As the major share of the secondary-structure is unordered in PB, the observed predominantly α -helical secondary-structure in the presence of lipids also indicates sufficient binding of the peptide to lipid bilayers. This has been shown experimentally in previous studies.^{76,77}

In CD spectroscopic measurements, all 28 Ala-d₃ analogues showed high similarities compared to the Ltc5 WT in every condition tested. The changes introduced to the analogues by the introduction of single Ala-d₃ residues do not seem to affect the analogue's ability to bind to membranes and fold into α -helices in a significant manner as they all retain a predominantly α -helical conformation in the presence of SUVs

(**Table A 3**). As the net positive charge of Ltc5 WT is high with plus ten, even lost positive charges caused by the mutation of Arg or Lys to Ala-d₃ can be compensated by the remaining charge of plus nine, keeping the attraction towards negatively charged lipid bilayers high. The replacement of single amino acids by Ala-d₃ does not seem to significantly alter the secondary-structure of the respective analogues compared to the Ltc5 WT and thus seem to be well tolerated.

To analyse the orientation of the Ltc5 WT and the Ala-d₃ analogues in lipid bilayers, OCD measurements were conducted (Figure 4). The orientation in lipid bilayers can give insights into the possible modes of action of membrane-active peptides like Ltc5. Pore forming α -helical peptides are expected to show a tilted T- or even a fully inserted I-state in oriented lipid bilayers. A peptide showing a surface bound S-state in oriented lipid bilayers is unlikely to form toroidal- or barrel-stave-like pores.

The OCD spectra of the Ltc5 WT and all 28 Ala-d₃ analogues exhibit the characteristic double minima at 208 nm and 222 nm and the strong positive band at 197 nm, indicative of an α -helical fold. This is consistent with the previous observations of predominantly α -helical secondary-structures made during CD spectroscopy in the presence of lipids. The OCD spectrum of the Ltc5 WT shows an α -helical secondary structure that lies flat on the membrane surface in an S-state. The OCD spectra of the Ala-d₃ analogues measured in this work indicate a similar orientation in lipid bilayers to the Ltc5 WT. The results do not indicate a pore forming mechanism for Ltc5 WT, as the peptide lies flat on the membrane surface in an S-state. However, this does not exclude the possibility of a pore forming mechanism. Pores could form only under specific conditions, for example higher concentrations. For those analogues in which Phe or Tyr (amino acids with aromatic residues) substituted by Ala-d₃, a clear trend emerged: the negative band at 208 nm intensifies slightly, which aligns with the explanation of a reduced band caused by the high number of aromatic residues in Ltc5. This hypothesis was previously proposed by Wadhwani et al.⁴⁷ Accounting for this artefact, all Ala-d₃ analogues tested can be assigned to an S-state, with an orientation comparable to that of Ltc5 WT. Observed differences are within the margin of error of the method. Single Ala-d₃ substitutions seem to be well tolerated with regard to the orientation of the respective analogues within oriented lipid bilayers as proven here by OCD spectroscopy.

The minimum inhibitory concentration (MIC) assay demonstrated that all tested Ala-d₃ analogues exhibit high antimicrobial activity against both Gram-positive and Gram-negative bacterial strains, similar to the Ltc5 WT. All analogues tested in this chapter have essentially the same MIC values (between 2 µg/mL and 8 µg/mL, see **Table 6**), within one dilution step, which can be considered as within the error of the method. No single residue in the primary sequence of Ltc5 can therefore be considered essential for the antimicrobial activity of Ltc5.

To analyse the membrane disruptive capabilities of Ltc5 and possible differences in the Ala-d₃ analogues against human erythrocytes, a hemolysis assay was conducted. Neither structure (CD), orientation in lipid bilayers (OCD), nor antimicrobial activity (MIC) changed for any of the Ala-d₃ analogues. Thus, a similarly low impact on the hemolytic activity is expected for the Ala-d₃ analogues. Increasing hydrophobicity by substituting polar residues with Ala-d₃ generally enhanced the hemolytic activity of the respective analogues compared to the Ltc5 WT peptide (**Figure 5**). Such effects are well known for other AMPs.⁶⁷ Analogues with increased hydrophobicity that did not show a clear gain in hemolytic activity at least retained activity comparable to the Ltc5 WT. When polar or charged residues on the polar face of Ltc5 are replaced with alanine, the overall helix becomes more hydrophobic while its polarity and charge density decreases. Red blood cell membranes largely consist of zwitterionic lipids and are rich in cholesterol, and therefore tend to interact more strongly with peptides that have a higher average hydrophobicity. Conversely, decreasing hydrophobicity by replacing more hydrophobic residues with Ala-d₃ in the hydrophobic sector led to a reduction in hemolytic activity. This slightly increased the therapeutic index of the corresponding analogues, as their antimicrobial activity remained unchanged compared to the Ltc5 WT. Only for Ltc5 Phe2, Phe3, and Leu28 is the reduced hemolytic activity offset by a concomitant decrease in antimicrobial activity (**Table 6**). Polyansky et al.⁷⁸ showed that alterations in the N-terminal region of the Ltc5 WT peptide have a pronounced impact on the hemolytic activity of resulting analogues. By replacing Phe2 or Phe3 with Ala-d₃, the N-terminal hydrophobicity is reduced, which could be the reason for the observed decrease in hemolytic activity in the respective analogues.

Overall, the effects of replacing individual amino acids with Ala-d₃ are detectable, but small. Even at the highest peptide concentration tested during the hemolysis assay,

none of the substitutions to Ala-d₃ led to a significant change of more than 10 % in hemolytic activity compared to the Ltc5 WT. At MIC-relevant concentrations (2 µg/mL - 8 µg/mL), the impact of Ala-d₃ substitutions is even smaller, with differences generally remaining below 5 %. With respect to hemolytic activity, replacing a single amino acid in the primary sequence of Ltc5 is well tolerated.

A second method applied in this work to analyse the ability of a peptide to disrupt membranes is the vesicle leakage assay. The previous hemolysis assay focused on the disruption of eukaryotic cells, which is considered a side effect in AMPs. The vesicle leakage assay is performed on LUVs mimicking prokaryotic membranes. Therefore, it reflects on possible membrane disrupting modes of action of AMPs against bacterial membranes. High vesicle leakage would indicate a membrane disrupting mechanism of action, possibly through pore formation. Differences between the Ala-d₃ analogues and the Ltc5 WT would therefore reflect different membrane interactions. As both the Ltc5 WT and the Ala-d₃ analogues lay flat on the membrane surface in an S-state (OCD) no significant differences are expected in the Ala-d₃ analogues.

Replacing individual amino acids with Ala-d₃ did not produce any significant change in membranolytic activity (**Figure 7**). At a P/L of 1:25, all Ala-d₃ analogues induced vesicle leakage to a similar extent to Ltc5 WT. Any apparent differences fall within the experimental error of the assay and therefore cannot be considered significant. This trend was observed for both LUV compositions tested. POPC/POPG (1:1) vesicles were used to mimic prokaryotic membranes, whereas POPC/POPG/Chol (2:2:1) vesicles were used to mimic eukaryotic membranes. The combined results of the hemolysis and vesicle leakage assays indicate that Ltc5 does not follow a membranolytic mechanism of action.

To summarise the results of the Ala-scan, it can be said that all Ala-d₃ analogues exhibit properties broadly similar to those of the Ltc5 WT. Single Ala-d₃ substitutions are well tolerated with regard to structure and activity by Ltc5. Although minor differences and trends were observed in the MIC and hemolysis assays, these effects were not substantial enough to disqualify any of the analogues from subsequent ssNMR analysis. Unexpectedly, even Ala-d₃ substitutions introducing significant differences in hydrophobicity, charge, steric hindrance or aromatic properties did not cause

significant differences between the respective analogues and the Ltc5 WT. Therefore, all Ala-d₃ analogues are considered suitable for ssNMR studies.

As all Ala-d₃ analogues are expected to behave broadly similarly to the Ltc5 WT with regard to structure and function, ssNMR was applied to gain a detailed structure of Ltc5 WT in membranes. The antimicrobial activity of AMPs, as well as their side effects, often correlate directly with their structure. We hope to gain insight into the mechanism of action used by Ltc5 to exert its antimicrobial activity. Even though membrane disruption is not observed for Ltc5 (low hemolysis, low vesicle leakage), non-membranolytic but membrane focused mechanisms of actions are still conceivable. Even if Ltc5 ultimately proves to act via a non-membrane-focused mechanism, such as targeting an intracellular component, it must still cross the membrane to reach that target. Thus, determining the detailed structure and orientation of Ltc5 within lipid bilayers may indicate how the peptide traverses' membranes. In any case, resolving its membrane-bound structure will be a key to elucidating the mechanism of action of the AMP Ltc5.

As shown in **Figure 2**, the initial assumption was that Ltc5 possesses a single continuous hydrophobic sector spanning the entire helix. However, the calculated fits indicate that Ltc5 is better described as comprising two separate helices with almost identical tilt angles (86° for residues 2-11 and 85° for residues 12-27) but markedly different rotational angles (99° for residues 2-11 and 85° for residues 12-27).

The results of the structural calculations indicate that Ltc5 consists of one helix. This helix is subdivided in two parts with different rotational angles ρ , with 14°, but almost identical tilt angles τ with only 1° difference. The described N- and C-terminal helical segments of Ltc5 are depicted in **Figure 13** and separated by the black dotted line.

The dotted red lines in **Figure 13** approximately indicate the centre of each hydrophobic sector. In a simplified model of peptide-membrane interaction, these can be regarded as lines of maximum hydrophobicity. It can be expected that the helix tends to align so that the area of the peptide corresponding to the red dotted line has maximum contact with the hydrophobic part of the membrane, resulting in the observed rotational shift of 14° determined between the two helical segments.

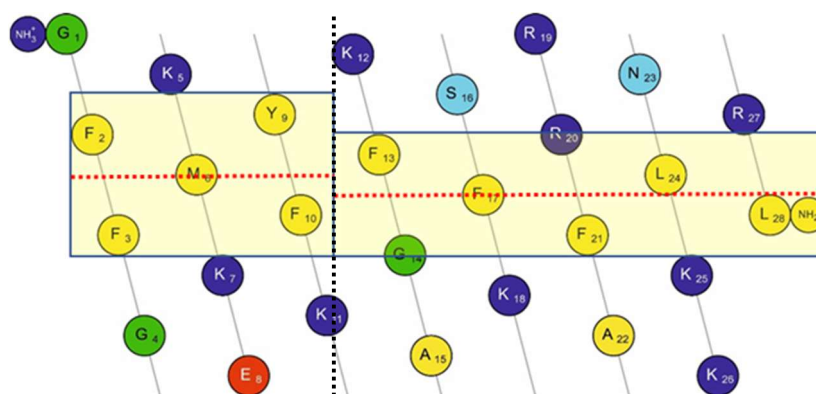


Figure 13: Helical mesh of Ltc5 with separate N- and C-terminal part.

The yellow highlighted boxes show the adjusted hydrophobic sectors of the peptide as determined according to the results of the structural calculations. When folded into an α -helix, this sector forms the hydrophobic face of the amphipathic peptide which interacts primarily with the hydrophobic face of lipid membranes. The dotted red lines show an estimation of the centres of the peptides hydrophobic sectors. The black dotted line marks the border between the two helical parts.

These differences in rotational orientation are driven by the distinct hydrophobic sectors of the two helical segments. The N-terminal helix has a broader hydrophobic sector, whereas the C-terminal helix features a narrower hydrophobic sector forming a single band of hydrophobic residues, as highlighted by the yellow boxes in **Figure 13**. As mentioned, the hydrophobic residues within each sector favour maximal contact with the membrane in order to minimize exposure to surrounding water molecules. At the same time, the polar and charged residues at the interface between the hydrophobic and polar face of the peptide α -helix prefer the surrounding bulk water rather than the hydrophobic membrane core. Consequently, the rotational angle of the helix depends on the width of the hydrophobic sector, the position of its centre of attraction (indicated by the dotted red lines in **Figure 13-15**) and on the charged and polar residues in the interface region. Even though there is a free energy cost when distorting the helix, this seems to be lower than the free energy cost of placing the hydrophobic and charged residues in an unfavourable environment. Therefore, the two

segments of the helix orient as depicted in **Figure 14** with a rotational difference of 14° .

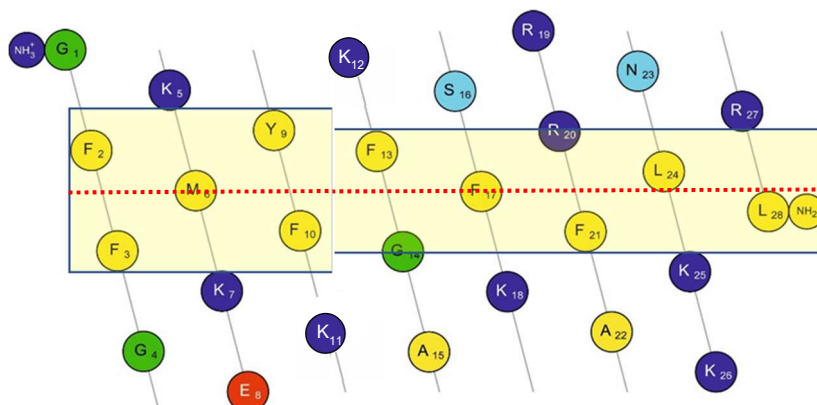


Figure 14: Depiction of Ltc5 aligned according to the centres of hydrophobicity.

The two helical segments are aligned in accordance with the centres of their hydrophobic sectors (red dotted line).

In order to understand how the substitutions at positions 16 and 20 introduce significant changes to the respective analogues and therefore have to be excluded from the structural fits, it is necessary to consider the shape of the hydrophobic sector in these cases. When the amino acids at positions 16 or 20 are replaced by a hydrophobic residue (alanine in this case), the broad hydrophobic sector of the N-terminus is effectively extended toward these positions, as illustrated in **Figure 15**.

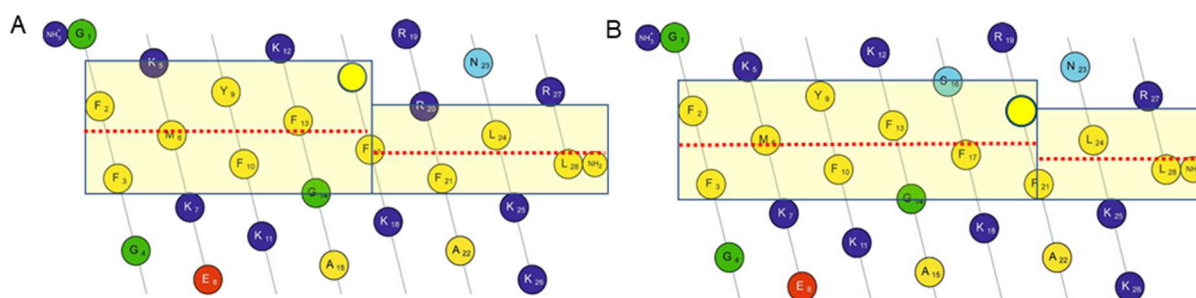


Figure 15: Helical meshes of the Ltc5 WT showing the artefacts found for Ltc5 S16 and Ltc5 R20.

In both A) and B), the yellow boxes indicate the hydrophobic sectors of the N- or C-terminal helix respectively. The red dotted lines indicate the centre of the hydrophobic sectors. The hydrophobic residues are indicated by yellow circles. A) In position 16, Ser was replaced by an Ala-d₃, elongating the hydrophobic sector of the N-terminus until position 16. B) In position 20, Arg was replaced by Ala-d₃ elongating the hydrophobic sector of the N-terminus until position 20.

Following the previous reasoning, this changes the free energy cost and makes elongation of the helix with the N terminal rotational angle up to position 16 or 20 more favourable.

This theory is supported by the positions of Ser16 and Arg20 when overlapping the fitting curves of the N- and C-termini as shown in **Figure 16**.

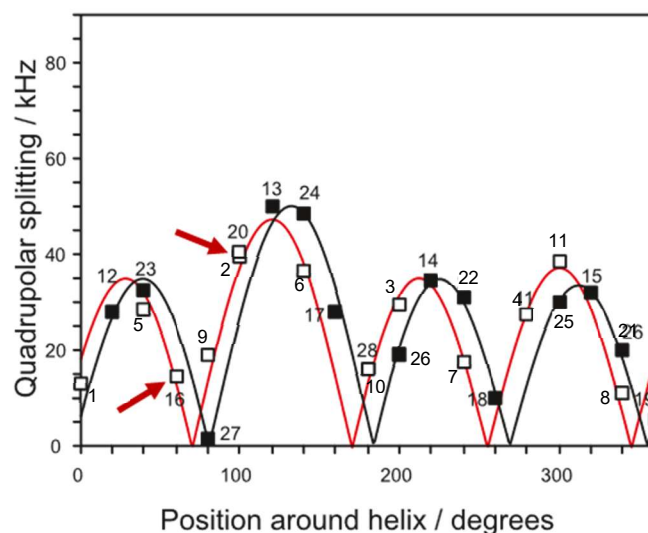


Figure 16: Comparison N- and C-terminal fitting curve.

The black line represents the fitting curve of the C-terminal fit and the red line that of the N-terminal fit. Residue Ser16 and Arg20 (red arrows), even though located in the C-terminal part of Ltc5's helix are located on the fitting curve of the N-terminal helix.

The curves shown correspond to the best fits for the C-terminal (black) (**Figure 11**) and the N-terminal (red) (**Figure 12**) helices, respectively. As can be seen, the data points for positions 16 and 20 align more closely with the N-terminal fit (red arrows, **Figure 16**) even though they lie in the C-terminal region of the helix, whereas all intervening positions (19, 18, 17) and preceding residues (15, 14, 13, 12) match almost perfectly with the C-terminal helix. This strongly supports the assumption that the substitutions at positions 16 and 20 effectively elongate the hydrophobic sector of the N-terminal helix.

The effect described above is only observed at positions 16 and 20. These residues are in the interface region between the polar and the hydrophobic sectors (see helical wheel of Ltc5, **Figure 3**). Replacing them with hydrophobic Ala therefore shifts the border and changes the balance in the crucial region between the N- and the

C-terminal domains. Arg27 is also on the boundary of the helix but is not in the interface between domains. The same applies to Lys7.

An α -helix is intrinsically rigid^{62,79,80} due to the network of backbone hydrogen bonds characteristic of an α -helix (**Figure 17**). The free energy for a relative rotation of the C- and N-terminal helices, as suggested here, must therefore be lower than leaving the residues Ser16 and Arg20 in unfavourable environments.

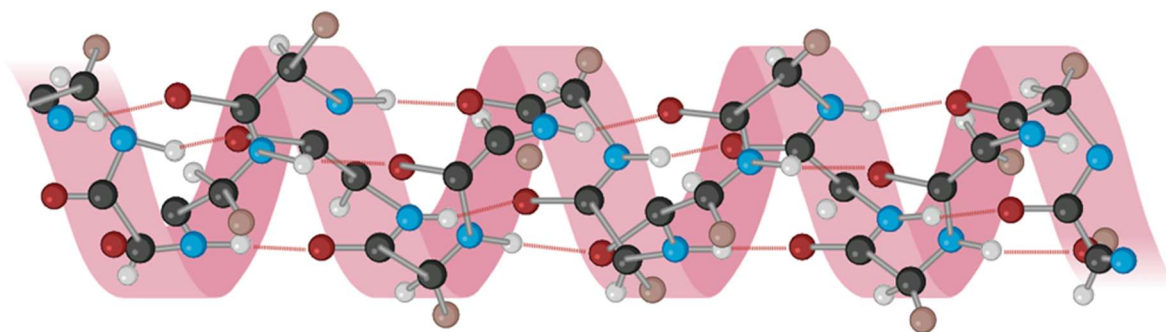


Figure 17: Depiction of a peptide α -helix.

The hydrogen bond responsible for the rigidity of a peptides α -helix. Red dots represent oxygen atoms, blue dots nitrogen, grey dots hydrogen and black dots carbon. The hydrogen bonds are indicated by the red lines between oxygen and hydrogen atoms. Created in <https://BioRender.com>

The conformational dynamics of the helical regions of Ltc5 can be evaluated from the deviations (σ) of the τ (tilt) and ρ (rotation) angles. These parameters represent the possible fluctuations in τ and ρ derived from the structural fits. Low σ values correspond to a helix with limited motion. The respective values are summarised in **Table 8**. The σ_ρ values are 6° and 4° for the N-terminal and C-terminal helical parts, respectively, indicating that both segments are highly restricted with respect to rotational motion, with the C-terminal helix being slightly more constrained. This can be attributed to the sharp border between polar and hydrophobic sectors. We can note that the shift in ρ between the N- and C-domains is larger than σ_ρ . For σ_τ , both helices show a deviation of 9° , suggesting comparably small dynamic behaviour in terms of tilt relative to the membrane plane. A σ_τ of 9° indicates that both helical segments are structurally stable, exhibiting only minor fluctuations in their membrane tilt.

Panel A of **Figure 18** shows Ltc5 in a helical conformation with angles consistent with those of an ideal α -helix. Panel B of the same figure shows the α -helix of Ltc5 with the adjusted τ and ρ angles determined in this chapter. Ltc5 is presented in side view, with the hydrophobic sector facing the reader. This representation can be compared with

the helical mesh model of Ltc5 and the adjusted hydrophobic sectors of the N- and C-termini shown in **Figure 14**. A 14° shift between the two N-termini can be observed when comparing the helix in panel A with the helix in panel B. This shift aligns the hydrophobic faces of the two helical segments. In this orientation, the amphipathic character of the Ltc5 α -helix becomes fully apparent, allowing for an optimal interaction between membrane and peptide.

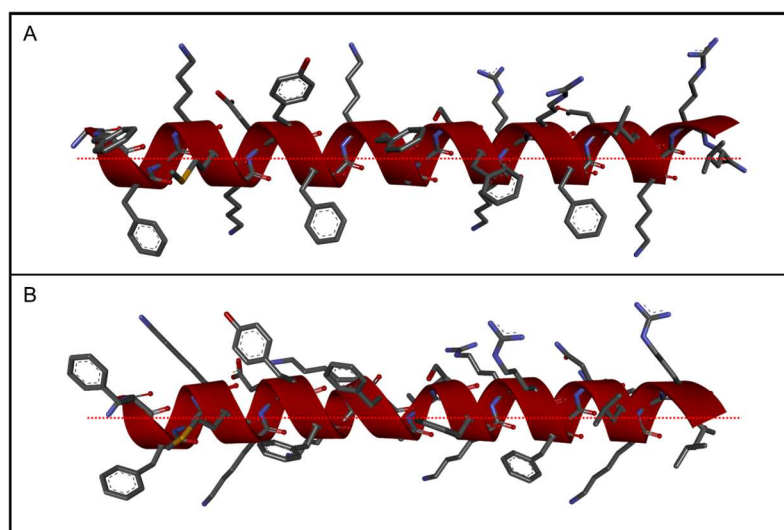


Figure 18: 3D-cartoon depiction of the α -helix of Ltc5.

A) α -helix of Ltc5 without adjusted τ and ρ angles. The depicted helix has the ideal backbone angles of a right-handed peptide α -helix. B) α -helix of Ltc5 with adjusted τ and ρ angles. The angles had to be adjusted by hand and are therefore not completely accurate. The dotted red lines indicate the centre of the hydrophobic sectors. Created using BioVia Discovery Studio 2025.

The final results of the structural fittings are summarised in **Figure 19**. The figure shows a cartoon representation of the α -helix of Ltc5 in three different perspectives. A) lying on a lipid bilayer as viewed from above. B) Viewed from the side with the τ angle given above the respective helix segment. C) Viewed along the helix axis starting from the N-terminus with the ρ angles given. As mentioned previously, the helix of Ltc5 lies flat on the membrane surface in an S-state and is divided into two parts: an N-terminal part, including residues 1-11 and a C-terminal part, including residues 12-28. The two parts have nearly identical tilt angles with only 1° difference, but have a significant difference of 14° in their rotational angles. The hydrophobic sectors of the two helix segments are oriented towards the hydrophobic core of the membrane, while the polar residues are oriented away from the membrane, towards surrounding bulk water.

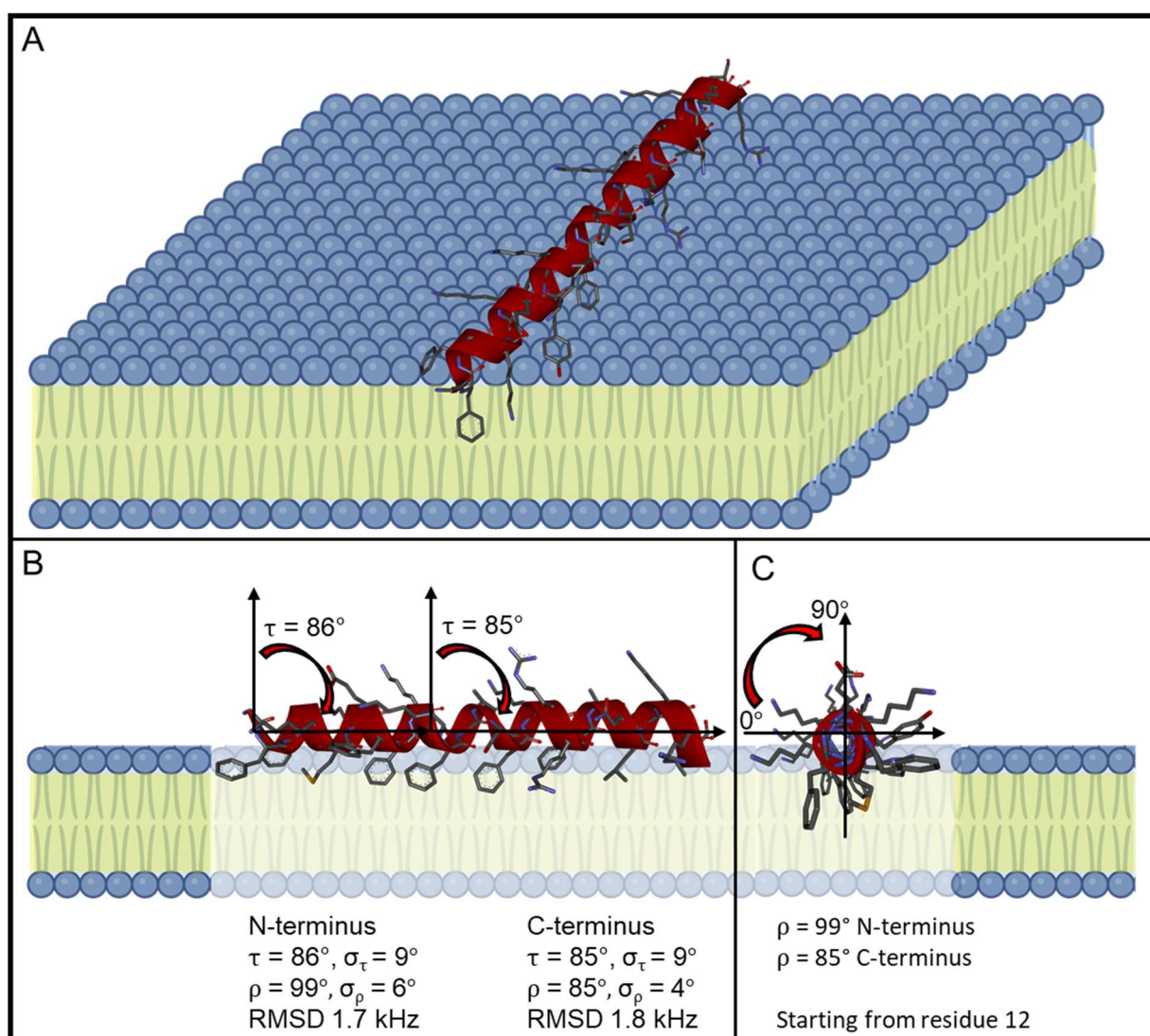


Figure 19: Cartoon representation of Ltc5 from three different perspective.

A) Top view of the helix laying on the membrane surface in an S-state. B) Side view of the helix on a lipid bilayer. C) View along the helix axis starting from the N-terminus. Created in <https://BioRender.com> and BioVia Discovery Studio 2025.

Combining the structural information with the previously analysed functional data provides insights into the possible mechanisms of action of Ltc5. The calculated tilt angles supported by the OCD results indicate that Ltc5 lies flat on the membrane surface rather than inserting into the membrane. Significant membrane damage at low peptide concentrations would require the peptide to insert into the membrane to form stable pores. However, since Ltc5 remains on the membrane surface, it is unable to cause substantial perforation, resulting in low leakage and hemolysis values. Even the formation of temporary pores is highly unlikely due to the dynamic properties indicated

by the low σ_τ and σ_ρ values. These values would have to be much higher to allow a reasonable probability for pore formation of any kind. Despite this, Ltc5 demonstrates high antibacterial activity, as evidenced by the MIC assays. One plausible explanation is that Ltc5 can translocate across the bacterial membrane without significant disruption and subsequently interferes with essential intracellular metabolic processes. Another plausible mechanism of action could involve membrane depolarisation. Such a depolarisation could be the result of increased local membrane permeability for ions in the presence of Ltc5 due to membrane thinning or changes in lateral lipid arrangement. Since membrane polarisation is essential for various metabolic functions, such depolarisation events would result in bacterial cell death.^{23,24} This would also explain the combination of low leakage and hemolysis values with high antimicrobial activity.

In conclusion, the structural fitting revealed that Ltc5 can be described as a continuous helix from the N- to the C-terminus, with distinct rotational angles. The N-terminal helix consists of residues 2 to 11 with a tilt angle τ of 86° , a rotational angle ρ of 99° , a σ_τ of 9° and a σ_ρ of 6° . The C-terminal part includes residues 12 till 27 with a tilt angle τ of 85° , a rotational angle ρ of 85° , a σ_τ of 9° and a σ_ρ of 6° . The low σ_τ and σ_ρ values indicate a rigid helix with little movement in the membrane. These findings are in line with previous ^{15}N -NMR⁴⁷ and OCD results. This surface-bound, low-mobility S-state makes the formation of permanent or transient pores as the primary mechanism of action for Ltc5 is unlikely. This interpretation is further supported by the very low hemolytic activity and minimal vesicle leakage observed for Ltc5 (**Figure 5**, **Figure 6**). Possible modes of action against bacteria that are consistent with these findings are therefore non-membranolytic and may involve mechanisms such as membrane depolarisation or intracellular targets. Another possibility is that Ltc5 acts on intracellular targets and crosses the membrane without causing significant disruption. Membrane translocation may involve different peptide orientations that are only observable under specific conditions. This possibility will be investigated in the next chapter.

6 Trapping the membrane-active state of Latacin 5 in lipid bilayers

The membrane-active state of an AMP describes the conformation, orientation, membrane insertion and aggregation state that it adopts while exerting its activity on a membrane.²² Ltc5 has so far been described as an α -helix that is surface-bound, without any indications of a tilt of the α -helix in the membrane. It is therefore unclear if this S-state already corresponds to its membrane-active state, or whether Ltc5 adopts a different orientation under conditions where it exerts its full activity. There are several ways to entrap the membrane-active state of an AMP in lipid bilayers, which will be discussed in the following paragraphs. To entrap the membrane-active state of a peptide, the most commonly adjusted conditions are the lipid composition of bilayers, adjustment of temperature below T_m or an increase of peptide concentration.^{81,82} In this chapter, the “entrapment” and analysis of the membrane-active state of Ltc5 will be attempted.

BP100 is one example of an AMP where different orientational states were found by Strandberg et al.⁸¹ It is a small 11-mer with a net charge of +6. Many different mechanisms of action have been proposed for BP100. In fact, the membrane orientation and therefore also the mechanism of action of BP100 seems to strongly depend on the conditions it is placed into. BP100 inherently lies flat on membranes in an S-state in lamellar oriented bilayers, above T_m of the respective lipids. BP100 was found to adopt an I-state in the membrane when lowering the temperature during measurements and adjusting the thickness of the lipid bilayers used for sample preparation. The latter influences the hydrophobic match between peptide and lipid bilayer.⁸¹ The NMR analysis in the previous chapter was carried out in a single lipid system, namely DMPC/DMPG (7:3). The orientation of Ltc5 in contact with lipid bilayers of that composition was determined to be an S-state, forming a single continuous α -helix with different rotational orientations of the N- and C-terminal segments, as shown by ²H-NMR (Chapter 5). DMPC/DMPG mixtures are widely used as a simplified model of bacterial membranes. The combination of zwitterionic DMPC and anionic DMPG approximates the overall charge density and bilayer thickness of bacterial cytoplasmic membranes.⁸³ With 30% anionic headgroups, it captures the initial electrostatic attraction force between cationic AMPs and anionic lipids.⁸³ In

addition, this lipid composition provides a well-defined, stable, fluid, and homogeneous bilayer.

To investigate the effects of different degrees of hydrophobic match, Ltc5 was analysed in three different lipid systems: 1,2-dilauroyl-glycero-3-phosphocholine (DLPC) / 1,2-dilauroyl-glycero-3-phosphoglycerol (DLPG), DMPC/DMPG and 1,2-dilauroyl-glycero-3-phospho (DErPC) / 1,2-dierucoyl-glycero-3-phosphoglycerol (DErPG) were selected, forming bilayers with increasing thickness in that order. The hydrophobic match between peptide and lipid bilayer therefore differs in each lipid system. All mixtures were used at a molar PC/PG ratio of 7:3, thereby maintaining comparable electrostatic properties across the three lipid systems.

The different phospho-lipids used in this chapter and their corresponding bilayer thickness from headgroup to headgroup (D_{HH}) and thickness of the hydrophobic core ($2D_c$) are listed in **Table 9**.^{84–86}

Table 9: Phospholipids of varying length used to test the behaviour of Ltc5 in different bilayer thicknesses.

D_{HH} represents the bilayer thickness from headgroup to headgroup while $2D_c$ corresponds to the thickness of the hydrophobic core. The measurements or simulations were conducted for fully hydrated bilayers in a fluid lipid phase.^{84–86}

Lipid	D_{HH} [Å]	$2D_c$ [Å]	T_m [°C]
DLPC	30.8	20.9	-2
DLPG	30.6	20.2	-3
DMPC	35.3	25.4	24
DMPG	35.4	23.6	23
DErPC	47	35.7	-15
DErPG	~ 43-44	~ 34	~ -14

The thicknesses given in **Table 9** were determined by X-ray and neutron scattering, NMR, or molecular dynamics simulations for fully hydrated bilayers above their phase transition temperature (T_m), i.e. in the fluid phase.^{84–86} Measurements in this chapter were performed at 35 °C, which is above the T_m for all lipids used.

Ltc5 is 28 residues long with 26 residues (residues 2-27) being part of the α -helix. For an ideal α -helix, a pitch of 1.5 Å per residue is commonly assumed,^{23,24} giving an estimated total length of ~39 Å for Ltc5. Since an ideal helix is rarely realized in practice and peptide helices exhibit some intrinsic flexibility,^{23,24} a length of approximately 37-41 Å is considered realistic for Ltc5 in this work.

Another important parameter besides the hydrophobic match is the P/L, directly correlating with the peptide concentration on the membrane. In an attempt to entrap the membrane-active orientation of Ltc5 the concentration in the samples were increased. For many AMPs proposed to follow a carpet-like mechanism, peptides initially lie flat on the membrane in an S-state and begin to tilt into the membrane once a threshold surface concentration is reached.^{63,87,88} For example, PGLa and related helices undergo a transition from a surface-bound S-state at low P/L to a tilted T-state at higher P/L. This tilt often correlates with membrane permeabilization.⁸⁹ By performing concentration series in the different lipid systems it was attempted to entrap a membrane orientation differing from the previously determined S-state for Ltc5. The threshold concentration can vary substantially depending on the properties of the respective AMP. In this work, P/L ratios of 1:100, 1:50, 1:25, and 1:10 were selected.

A third approach to trap the membrane-active state of an AMP is to decrease the temperature to T_m or below T_m . For PGLa an I-state was found to be trapped during measurements below the T_m in DMPC/DMPG (3:1) using ^{19}F -NMR by Afonin et al.⁸² When increasing the temperature above T_m , the trapped I-state was released and the peptide was found in a T-state. When increasing the temperature even further, PGLa was found to adopt an S-state. This strategy was also explored in the three lipid systems during this work but yielded inconclusive data and will therefore not be discussed further.

The differences in membrane orientations were monitored using ssNMR. To allow for ^2H -NMR measurements, the two alanines in the primary sequence of Ltc5 were replaced with Ala- d_3 . This yields two labelled Ltc5 peptides which do not show any artefacts introduced by the labels, as proven during the Ala-scan in Chapter 5. The alanines replaced are at position 15 and 22. Introducing two labels at distinct positions allows conclusions to be drawn about potential changes in the orientation of Ltc5 under different experimental conditions compared to the previous measurements in

DMPC/DMPG (7:3). If the quadrupolar splittings during ^2H -NMR measurements remain the same for both labelled peptides under the new conditions, the Ltc5 WT is expected to adopt the same orientation as determined previously in Chapter 5. If the quadrupolar splitting changes, a different orientation is to be expected.

The respective peptides were synthesized using automated Fmoc solid phase synthesis and purified using RP-HPLC. **Table 10** includes the primary sequence, Mw, net charge, and retention time of the two Ala- d_3 labeled peptides.

Table 10: Ala to Ala- d_3 labelled analogues of Ltc5.

The labeled peptides were used for the analysis in different lipid systems and the concentration dependent tilt of Ltc5. The table includes the labels introduced, the name of the labelled peptide and the primary sequence.

Peptide name	Primary sequence	Mw [Da]	Net Charge	Ret. Time
Ltc5A15	GFFGKMKEYFKKFG (Ala-d_3) SFKRRFANLKKRL-amide	3433.18	+10	12.3
Ltc5A22	GFFGKMKEYFKKFGASFRRF (Ala-d_3) NLKKRL-amide	3433.18	+10	12.3

To complement the NMR measurements and confirm that the labeled analogues retain the same properties as Ltc5 WT across the three lipid systems, the two Ala- d_3 -labeled analogues were also examined in all three systems with respect to secondary structure by circular dichroism (CD), membrane orientation by oriented circular dichroism (OCD), and membrane-disruptive activity using a vesicle leakage assay. Conditions applied in these assays were kept the same as in Chapter 5, to allow comparisons

The combined aim of this chapter is to investigate the behaviour of Ltc5 WT at increasing peptide concentrations and in lipid bilayers providing different hydrophobic matching. If Ltc5 is found to adopt different orientation in lipid bilayer based on concentration of hydrophobic matching, this could indicate the presence of membrane active orientations differing from the previously determined surface bound S-state in Chapter 5.

6.1 Results

6.1.1 Peptide synthesis

The peptides used in this chapter were synthesized as part of the Ala-scan described in Chapter 5. Fmoc solid phase synthesis and consecutive RP-HPLC purification were performed by A. Eisele and K. Scheubeck at IBG-2, KIT. The corresponding liquid chromatograms and mass spectra of the peptides are provided in the appendix (**Figure A 2** and **Figure A 3**).

6.1.2 CD and OCD spectroscopy

Potential changes in secondary structure were assessed using CD spectroscopy in the three lipid systems. The three peptides Ltc5 WT, Ltc5 A15 and Ltc5 A22 were measured in DLPC/DLPG, DMPC/DMPG and DErPC/DErPG at a P/L of 7:3. The Ala-d₃ labelled peptides are expected to show highly similar behaviour to the Ltc5 WT in all three lipid systems. All measurements were conducted at a P/L of 1:50 above the T_m of the respective lipids ensuring a fluid L _{α} phase, resembling in vivo conditions. Measurements were performed twice to ensure reproducibility.

In **Figure 20**, the spectra of both Ala-d₃ labelled Ltc5 peptides and the Ltc5 WT peptide, measured in all three lipid systems, are shown.

Panel A in **Figure 20** shows the spectra of Ltc5 WT, Ltc5 A15 and Ltc5 A22 measured in DLPC/DLPG (7:3) at a P/L of 1:50. This lipid composition forms the thinnest bilayers examined in this study. The measurements in DMPC/DMPG (7:3), conducted at a P/L of 1:50 and 30°C, are shown in panel B of **Figure 20**. This lipid composition forms bilayers with a thickness intermediate between that of POPC/POPG and DErPC/DErPG. In Panel C, the CD spectra measured in SUVs composed of DErPC/DErPG (7:3) are shown. This lipid system forms the thickest bilayers used here and provides the most favourable hydrophobic match between Ltc5 and the lipid bilayer among the three systems tested (see **Table 9**). Panel D includes the Ltc5 WT spectra measured in the three discussed lipid systems.

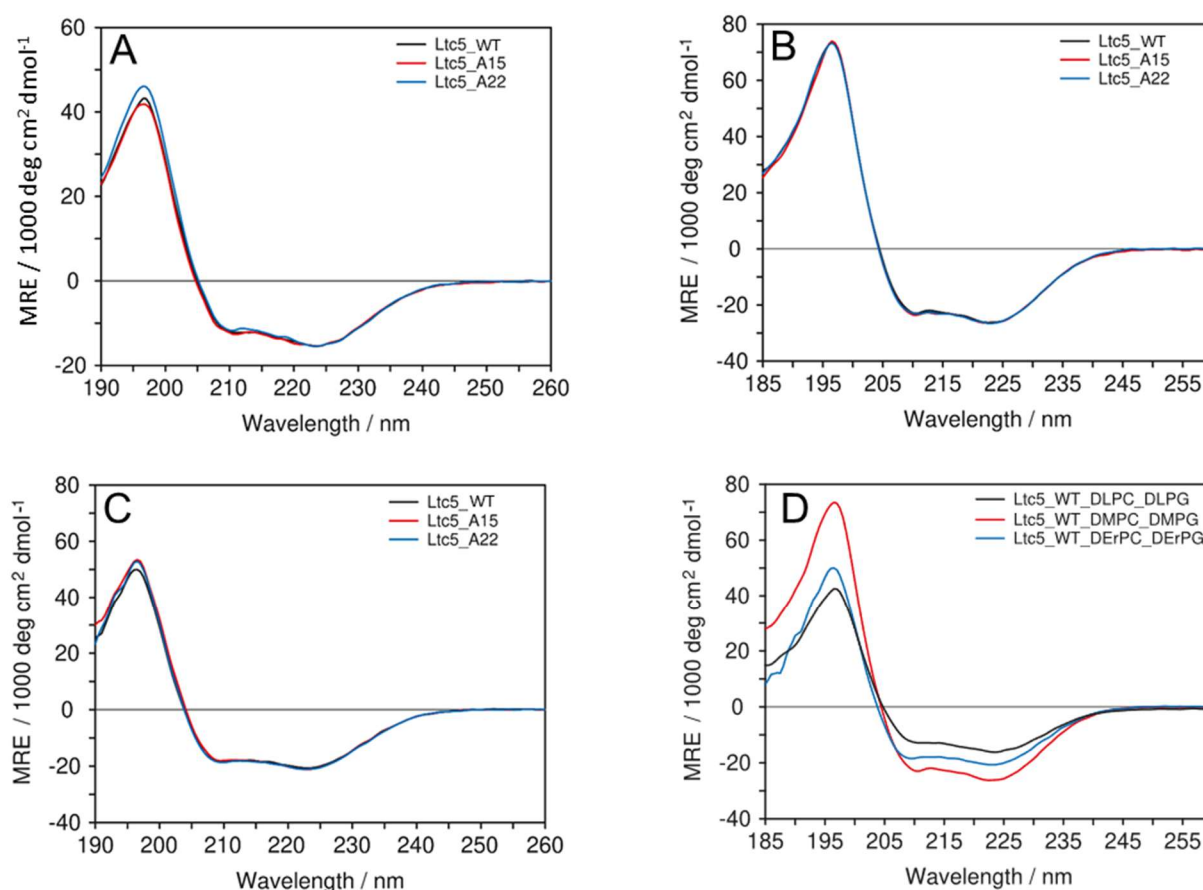


Figure 20: CD spectra of Ltc5 A15, Ltc5 A22 and Ltc5 WT in three different lipid systems.

The three peptides were measured in SUVs of three different lipid compositions. A) DLPC/DLPG B) DMPC/DMPG C) DErPC/DErPG with a PC/PG ratio of 7:3, P/L of 1:50 and above T_m of the respective lipids at 30 °C. Panel D) shows the spectra of the Ltc5 WT in all three lipid systems.

The spectra in panels A, B and C show high similarities between the labeled peptides and the Ltc5 WT. All peptides show the typical indications for predominantly α -helical peptides. Namely the negative bands at 222 nm and 208 nm as well as the strong positive band at 198 nm. The spectra of the Ltc5 WT in all three lipid systems show some deviations in intensity from one another. Especially the spectra obtained in DMPC/DMPG shows higher intensities. Aside from the intensity differences, there are no major differences observed between the spectra. As mentioned previously, all indicate the presence of a predominantly α -helical peptide.

DErPC/DErPG contains unsaturated acyl chains, which show increased absorbance at lower wavelengths and thus lead to a reduced signal-to-noise ratio at lower

wavelengths corresponding to the increased absorption of the double bonds. All three spectra seen in panel C (**Figure 20**) display increased noise at wavelengths of 198 nm and below. To compensate for the loss in signal, the CD spectrometer automatically increases the high voltage (HT) at the photomultiplier tube, which in turn amplifies noise in the resulting spectra. To minimise this issue, the spectra in panel C were recorded on a Jasco J-1500 instrument with improved optics. Owing to the higher light intensity reaching the sample, the overall signal is increased, allowing the HT to remain lower than on the Jasco J-815, which was used for all other CD measurements in this work.

The CD spectra were next deconvoluted using the web tools DichroWeb and the associated reference datasets.^{58,59} The resulting secondary-structure estimates are summarised in **Table 11**.

Table 11: Deconvolution of CD spectra in three different lipid systems.

SUVs were made from either DLPC/DLPG (7:3), DMPC/DMPG (7:3) or DErPC/DErPG (7:3) at P/L of 1:50. The table includes the distribution of secondary-structures in percent. Included secondary-structures are helix, strand, turn and unordered.

Ltc5 WT	DMPC/DMPG	DLPC/DLPG	DErPC/DErPG
Helix [%]	89	87	79
Strand [%]	6	4	3
Turn [%]	3	5	6
Unordered [%]	3	4	13

A15	DMPC/DMPG	DLPC/DLPG	DErPC/DErPG
Helix [%]	85	82	81
Strand [%]	3	4	4
Turn [%]	4	4	5
Unordered [%]	9	10	11

A22	DMPC/DMPG	DLPC/DLPG	DErPC/DErPG
Helix [%]	83	88	81
Strand [%]	4	3	1
Turn [%]	5	3	6
Unordered [%]	9	6	11

As expected from the CD spectra, all three peptides exhibit predominantly α -helical structures in each of the lipid systems tested. For Ltc5 WT, the α -helical content exceeds 80 % in all cases except in DErPC/DErPG (7:3). Here Ltc5 WT showed 79 % α -helical content in DErPC/DErPG, corresponding to the lowest α -helical content observed. The highest helicity (89 %) was found for Ltc5 WT in DMPC/DMPG (7:3). The differences observed within each lipid system are within the error of the method.

The orientation of Ltc5 WT and the two analogues Ltc5 A15 and Ltc5 A22 on lipid bilayers composed of DLPC/DLPG (7:3), DMPC/DMPG (7:3), or DErPC/DErPG (7:3) was investigated using OCD spectroscopy at A P/L of 1:100. The resulting spectra are shown in **Figure 21**.

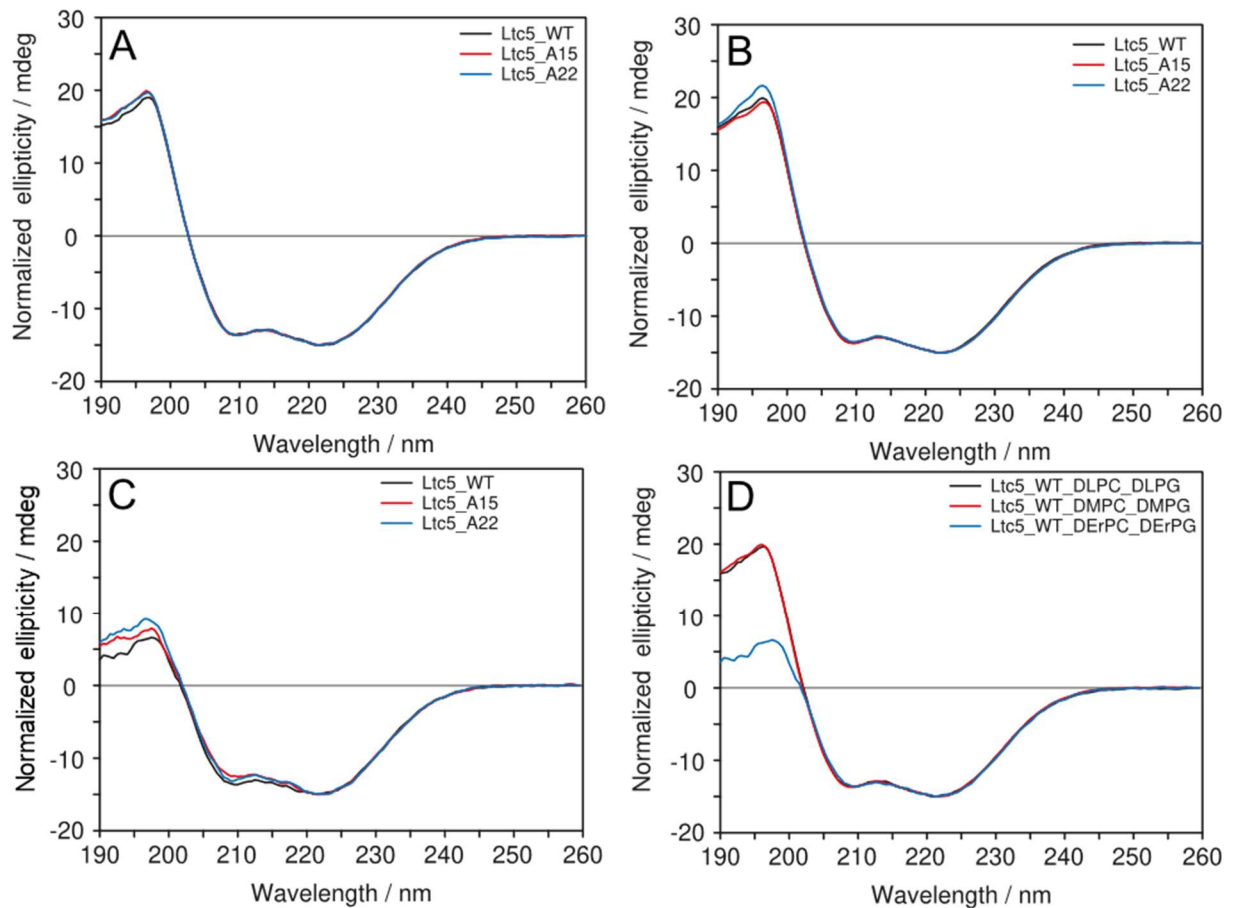


Figure 21: Normalized OCD spectra of Ltc5 A15, A22 and Ltc5 WT measured in three different lipid systems.

The three peptides were measured in three different lipid compositions. A) DLPC/DLPG B) DMPC/DMPG C) DErPC/DErPG with a PC/PG ratio of 7:3, P/L of 1:100, relative hydration of 97 % and above T_m of the respective lipids at 30°C. Panel D) shows the spectra of the Ltc5 WT in all three lipid systems.

Previous OCD measurements demonstrated a surface-bound orientation of Ltc5 WT in DMPC/DMPG (7:3). Panel A in Figure 21 shows the spectra of the three peptides in DLPC/DLPG (7:3), panel B in DMPC/DMPG (7:3) and panel C in DErPC/DErPG (7:3). In every lipid system the spectra of the labelled peptides and the Ltc5 WT closely overlap with one another indicating little difference in their orientation in the membrane.

The spectra again show the artefact caused by the high number of aromatic residues as previously described by Wadhwani et al.⁴⁷, which causes a reduction of the negative band at 208 nm. The spectra of all three peptides exhibit clear features of an α -helical secondary-structure in every lipid system tested, consistent with the CD measurements. The ratio between the 208 nm and the 222 nm signal indicates a surface-bound orientation on the lipid bilayer for every peptide in every lipid system.

Measurements in DErPC/DErPG (panel C **Figure 21**) show a strong line flattening at lower wavelength which can be attributed to the increased absorption of the unsaturated side chains. The OCD measurement cell is only compatible with a Jasco J-810 spectrometer and cannot be used with the J-1500 to improve the signal-to-noise ratio, as previously done for the CD measurements.

Panel D show that the Ltc5 WT spectra strongly overlap, except for the mentioned line flattening for the spectrum measured in DErPC/DErPG. For the three lipid systems used in this chapter the orientation of the Ltc5 WT seems to be independent of the lipid system. The peptide stays in an S-state in all cases.

6.1.3 Vesicle leakage

The effect of hydrophobic matching between the peptide and the hydrophobic core of lipid bilayers on the membrane-disruptive potential of Ltc5 WT and the two labelled analogues, Ltc5 A15 and Ltc5 A22, was investigated next. The best hydrophobic match between Ltc5 and lipid bilayer is given in DErPC/DErPG. As tilting of AMPs in lipid bilayer can depend on the ability of the peptide to span across the membrane, as shown for model KIA peptides⁷⁶, the highest probability of a tilt and consequent pore formation or membrane disruption is expected in DErPC/DErPG. As previous OCD measurements did not reveal peptide tilting as a function of membrane thickness, any membrane-disruptive effects would likely have to arise from non-pore-forming

mechanisms. A carpet-like mechanism could account for such effects.³³ Since Ltc5 WT did not exhibit significant changes in either CD or OCD measurements across the different lipid systems, membrane disruption and, consequently, vesicle leakage were not anticipated.

To assess whether Ltc5 WT or the two labelled analogues Ltc5 A15 and Ltc5 A22 are capable of inducing membrane damage depending on hydrophobic matching vesicle leakage assays were performed in LUVs composed of DLPC/DLPG, DMPC/DMPG and DErPC/DErPG with constant PC/PG ratios of 1:1. Previous leakage experiments employed LUVs composed of POPC/POPG (1:1), as this lipid system provides highly stable vesicles and highly reproducible data.⁷⁶ Vesicle leakage was monitored using a ANTS/DPX dye release assay as previously described (see Methods, Chapter 12). To ensure comparability with the earlier experiments in POPC/POPG (1:1), a P/L of 1:25 and 30°C were used for all measurements.

The obtained vesicle leakage data is shown in Figure 22. Each measurement was repeated four times with freshly prepared LUVs.

The observed leakage remained low throughout measurements in all three lipid systems. In the thinnest bilayers used in this work, namely DLPC/DLPG (panel A **Figure 22**), the observed leakage is very low with values of <1% for all three peptides. In DMPC/DMPG with intermediate thickness, the highest leakage among the three tested lipid systems was recorded with values beneath 3% for all three peptides. In the thickest lipid bilayers, composed of DErPC/DErPG, the recorded vesicle leakage remained below 2% for all three peptides. The differences observable between the lipid systems can be attributed to the inherent stability of LUVs composed of the different lipids as well as the inherent error of the method. Neither the Ltc5 WT nor the two Ala-d₃ labelled peptides induce significant vesicle leakage in any of the three tested lipid systems.

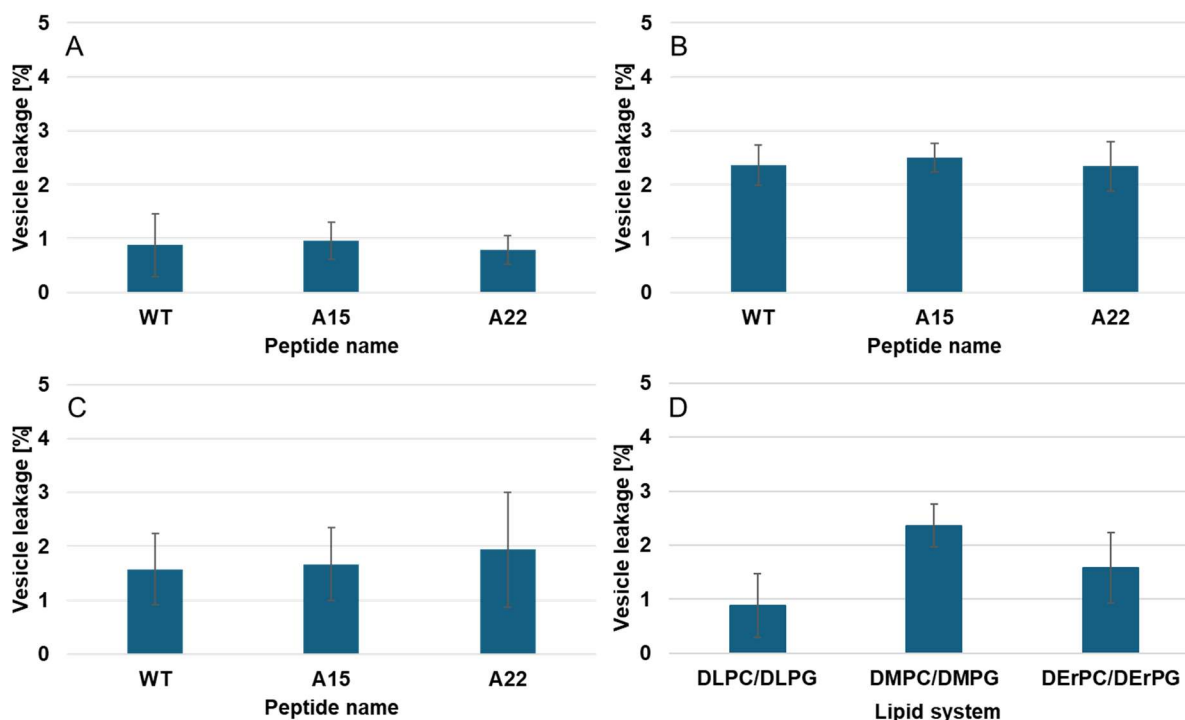


Figure 22: Vesicle leakage of Ltc5 A15, Ltc5 A22 and Ltc5 WT measured in three different lipid systems.

The assays were conducted using LUVs made from lipids forming increasingly thicker bilayers. Leakage results in A) DLPC/DLPG, B) DMPC/DMPG and C) DErPC/DErPG were used at a PC/PG ratio of 1:1, a P/L of 1:25 and at 30 °C. D) includes the data of the Ltc5 WT measured in all three lipid systems to

6.1.4 Solid-state NMR spectroscopy

In the previous chapter, the structure of Ltc5 was analysed by solid-state NMR (ssNMR) in DMPC/DMPG (7:3) at a P/L of 1:100. Under these conditions, Ltc5 adopted a stable surface bound S-state. However, it remains unclear whether variations in hydrophobic match between the peptide and the hydrophobic core of the lipid bilayer, or an increase in peptide concentration, affect the orientation of Ltc5 within the membrane. To address this question, ssNMR measurements were performed in DLPC/DLPG (7:3), DMPC/DMPG (7:3), and DErPC/DErPG (7:3), which form bilayers of increasing thickness in this order, to examine the orientation of Ltc5 in membranes with different degrees of hydrophobic match. For each lipid system, a concentration series comprising P/L ratios of 1:100, 1:50, 1:25, and 1:10 were conducted. If the quadrupolar splitting, determined by ^2H -NMR, remains unchanged across the different P/L ratios and lipid systems at both labelled positions (Ltc5 A15 and Ltc5 A22), this would indicate that Ltc5 adopts the same orientation in the tested bilayers as under the

conditions used for the structural determination described in Chapter 5. A change in orientation could indicate a different membrane-active state, depended on the measurement conditions. In DMPC/DMPG (7:3) and at a P/L of 1:100, Ltc5 A15 showed a quadrupolar splitting of 32.1 kHz and Ltc5 A22 a splitting of 30.1 kHz (Table 7). The results will again be presented in order of increasing bilayer thickness.

The results obtained in the thinnest bilayers tested, composed of DLPC/DLPG (7:3), at four different P/L ratios are shown in **Figure 23**. The ssNMR measurements of the two Ala-d₃ labelled Ltc5 peptides in lipid bilayers composed of DLPC/DLPG (7:3) show similar behaviour to that previously observed in DMPC/DMPG (7:3) (see Chapter 5).

For Ltc5 A15, the ³¹P spectra recorded before (1 A, D, G, **Figure 23**) and after (1 C, F, I, **Figure 23**) the ²H measurements at a P/L of 1:100, 1:50 and 1:25 indicate a predominantly oriented lamellar bilayer, as evident by a strong signal at 27 ppm. The weak signal at -13 ppm is caused by a small fraction of unoriented lipids present in the sample.

At P/L 1:25 (before ²H measurement: 1 G, after ²H measurement: 1 I, **Figure 23**) a small signal at 7 ppm appears. This signal is indicative of a hexagonal (H_I) phase beginning to form at this P/L. At the highest P/L of 1:10 (before ²H measurement: 1 J; after ²H measurement: 1 L, **Figure 23**), the signal at 7 ppm increases markedly, while the signals at 27 ppm and -13 ppm are no longer detectable. The pronounced peak at 7 ppm, which persists after the ²H measurement, indicates the transition from a lamellar (L_α) into a predominantly H_I phase of the lipids in the sample.

The ²H spectra of Ltc5 A15 in DLPC/DLPG (1 B, E, H, K, **Figure 23**) show a constant quadrupolar splitting up to a P/L of 1:25 of ~32.1 kHz. At a P/L of 1:10 (1 K, **Figure 23**) no quadrupolar splitting is observed. This finding can be rationalised in light of the ³¹P data: once the lipids adopt an exclusively H_I phase, the peptides experience a broad distribution of orientations, leading to orientational averaging and a signal decrease in the ²H spectrum.

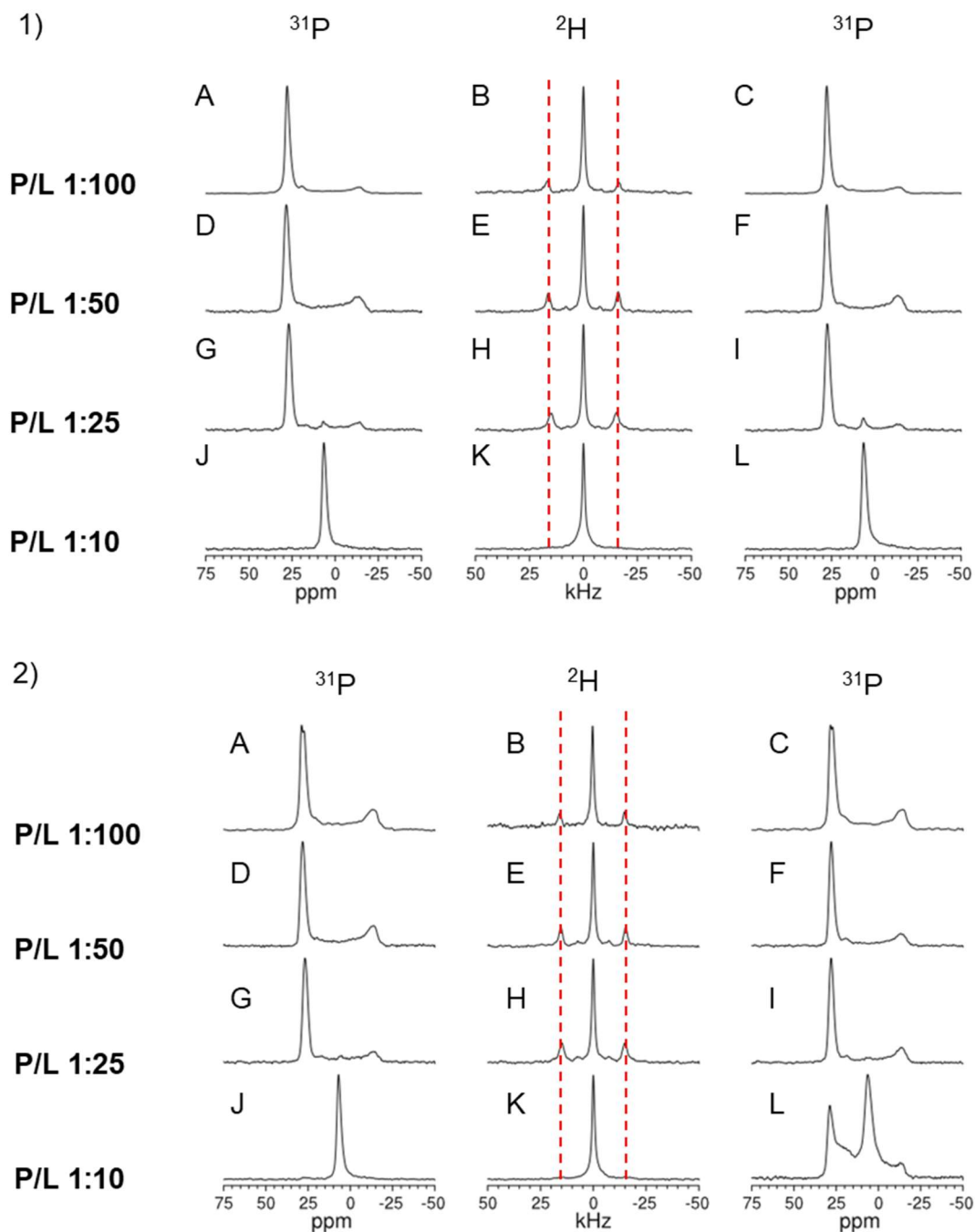


Figure 23: Solid-state ^{31}P - and ^2H -NMR spectra of 1) Ltc5 A15 and 2) Ltc5 A22 in DLPC/DLPG (7:3).

For each peptide a concentration series including the P/L ratios 1:100, 1:50, 1:25 and 1:10 was conducted. The respective P/L is given to the left of each row of spectra. Before (A, D, G, J) and after (C, F, I, L) every ^2H measurement a ^{31}P measurement was conducted to monitor the orientation of lipid bilayers in the sample.

The ssNMR measurements of Ltc5 A22 measured in the three different lipid systems also showed high similarities to the previous measurements from Chapter 5. The ^{31}P spectra before (2 A, D, G, **Figure 23**) and after (2 C, F, I, **Figure 23**) at P/L 1:100, 1:50 and 1:25 shows characteristics of a well oriented lipid in an L_α phase. At a P/L of 1:25 the rise of a signal at 7 ppm can be seen again, as for Ltc5 A15. At a P/L of 1:10, the ^{31}P spectrum recorded before the ^2H measurement (2 J, **Figure 23**) is dominated by the signal at 7 ppm, indicative of an H_I phase, with only minor contributions from lamellar (27 ppm) and unoriented (-13 ppm) lipid populations. The intensity of the 7 ppm signal decreases in the spectrum recorded after the ^2H experiment (2 L, **Figure 23**), accompanied by an increase in the signals at 27 ppm and -13 ppm. This behaviour suggests partial reorientation of the lipids from an H_I into a L_α phase.

The quadrupolar splittings in the ^2H spectra of Ltc5 A22 remain essentially unchanged, with values of approximately 30.1 kHz, for P/L ratios up to 1:25 (2 B, E, H, **Figure 23**). At the highest P/L of 1:10 (2 K, **Figure 23**), no intense quadrupolar splitting is observed. Only a very small signal can be seen. This supports the reasoning for the absence of a quadrupolar splitting previously given for Ltc5 A15 of orientational averaging. The peptides, which are located in the partly reoriented L_α phase, regain a stable orientation. This share of oriented peptide in the sample gives rise to the small quadrupolar splitting observable in the ^2H spectrum of Ltc5 A22 (2 K, **Figure 23**).

With increasing bilayer thickness, Ltc5 WT and the two Ala- d_3 analogues were next examined in DMPC/DMPG (7:3). This lipid system was previously employed in Chapter 5 to determine the precise orientation of Ltc5 in membranes at a P/L of 1:100. **Figure 24** shows the NMR concentration series in DMPC/DMPG (7:3), including P/L ratios of 1:100, 1:50, 1:25, and 1:10 for Ltc5 A15 and Ltc5 A22.

The ^{31}P spectra recorded before and after the ^2H measurements for Ltc5 A15 and Ltc5 A22 (**Figure 24**) indicate a predominantly oriented lamellar lipid phase up to a P/L of 1:25. Increasing the P/L to 1:10 (1 and 2 J, **Figure 24**) leads to the appearance of a second peak at approximately 7 ppm again indicating the formation of an H_I lipid phase. A fraction of the lamellar phase remains present, as evident by the signal at 27 ppm. In the ^{31}P spectrum recorded after the ^2H measurement at a P/L of 1:10 (1 and 2 L, **Figure 24**), the signal attributed to the H_I phase is no longer detectable. Instead, the sample shows an almost complete reorientation into a L_α phase, as

indicated by the prominent peak at 27 ppm. Notably, this peak exhibits signs of lipid segregation within the sample. Rather than a single resonance, a second, partially overlapping peak emerges. This may reflect a separation of PC and PG headgroups into distinct local lipid domains, giving rise to individual signals rather than a single averaged resonance. Since the lipids are thoroughly mixed during sample preparation, a single averaged peak is expected and indeed observed at lower P/L ratios. The appearance of two partially resolved peaks is therefore unlikely to be an artefact of sample preparation and can instead be attributed to the presence of the peptide in the lipid bilayers. A similar peak splitting is observed in some spectra even at lower P/L. For example, spectrum 1 G in **Figure 24**, at a P/L of 1:25, also shows indications of lipid phase separation.

Another effect observed for Ltc5 A15 in the ^{31}P spectra is that the degree of lipid orientation appears to increase with rising P/L up to a ratio of 1:25. The intensity of the signal corresponding to the unoriented fraction at approximately -13 ppm decreases as the P/L increases. This trend is evident in the ^{31}P spectra recorded both before and after the ^2H measurements (1 A $\rightarrow$ D $\rightarrow$ G and 1 C $\rightarrow$ F $\rightarrow$ I, **Figure 24**).

The ^2H spectra of Ltc5 A15 and Ltc5 A22 (1 and 2 B, E, H, K, **Figure 24**), do not change significantly with increasing P/L. Even at the highest P/L of 1:10 (1 and 2 K, **Figure 24**), the original quadrupolar splitting of 32.1 kHz for Ltc5 A15 and 30.1 kHz for Ltc5 A22 is still detectable, although the overall signal intensity is markedly reduced compared to lower P/L ratios. This reduction in signal intensity can be attributed to the presence of the H_i phase observed in the ^{31}P spectra as discussed previously.

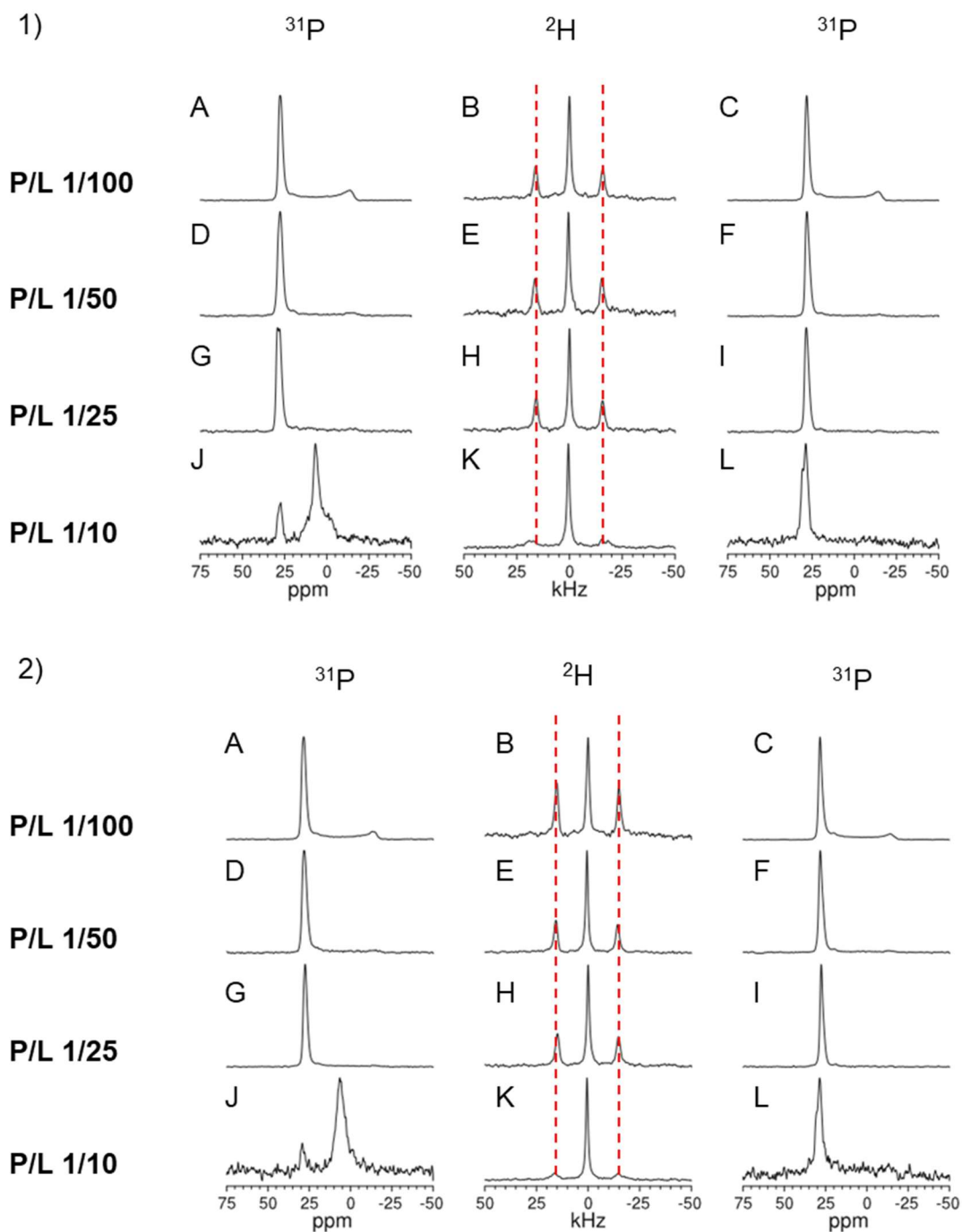


Figure 24: Solid-state ^{31}P - and ^2H -NMR spectra of 1) Ltc5 A15 and 2) Ltc5 A22 in DMPC/DMPG (7:3).

For each peptide a concentration series including the P/L ratios 1:100, 1:50, 1:25 and 1:10 was conducted. The respective P/L is given to the left of each row of spectra. Before (A, D, G, J) and after (C, F, I, L) every ^2H measurement a ^{31}P measurement was conducted to monitor the orientation of lipid bilayers in the sample.

The final concentration series was conducted in DErPC/DErPG (7:3). This lipid system forms the thickest bilayers used in this chapter and provides the best hydrophobic matching between bilayer and Ltc5. It is therefore considered the system in which Ltc5 is most likely to populate a T-state or I-state if the hydrophobic matching indeed matters for the orientation in/on the bilayer. The corresponding spectra are shown in **Figure 25**.

The ^{31}P spectra of samples containing Ltc5 A15 or Ltc5 A22 recorded before (1 and 3 A, D, G, **Figure 25**) and after (1 and 2 C, F, I, **Figure 25**) the ^2H measurements at P/L ratios from 1:100 up to 1:25, show highly oriented bilayers with the characteristic lamellar signal at 27 ppm and a small fraction of unoriented lipids, indicated by a signal at -13 ppm. At a P/L of 1:10, the ^{31}P spectra recorded before and after the ^2H measurement (1 and 2 J, L, **Figure 25**) exhibit the emergence of a second peak in the vicinity of 27 ppm, consistent with minor phase separation of PC and PG lipids. In contrast to the behaviour in DMPC/DMPG and DLPC/DLPG, no pronounced signal attributable to an H_i phase is observed and a substantial fraction of the lipids remain in an L_α phase even at a P/L of 1:10. However, the overall degree of lamellar orientation is clearly reduced as the average signal intensity between 22 ppm and -21 ppm, corresponding to random lipid orientations, increases.

Overall, the Ala- d_3 labelled peptides Ltc5 A15 and Ltc5 A22 show very similar behaviour across all lipid systems tested. The quadrupolar splittings observed in the ^2H spectra deviate only slightly from those obtained under the conditions used for structural determination in Chapter 5. Pronounced changes in the NMR spectra occur only at the highest P/L tested, namely P/L 1:10, where H_i -like phases appear in a manner that seem to depend on bilayer thickness and thus on the degree of hydrophobic matching.

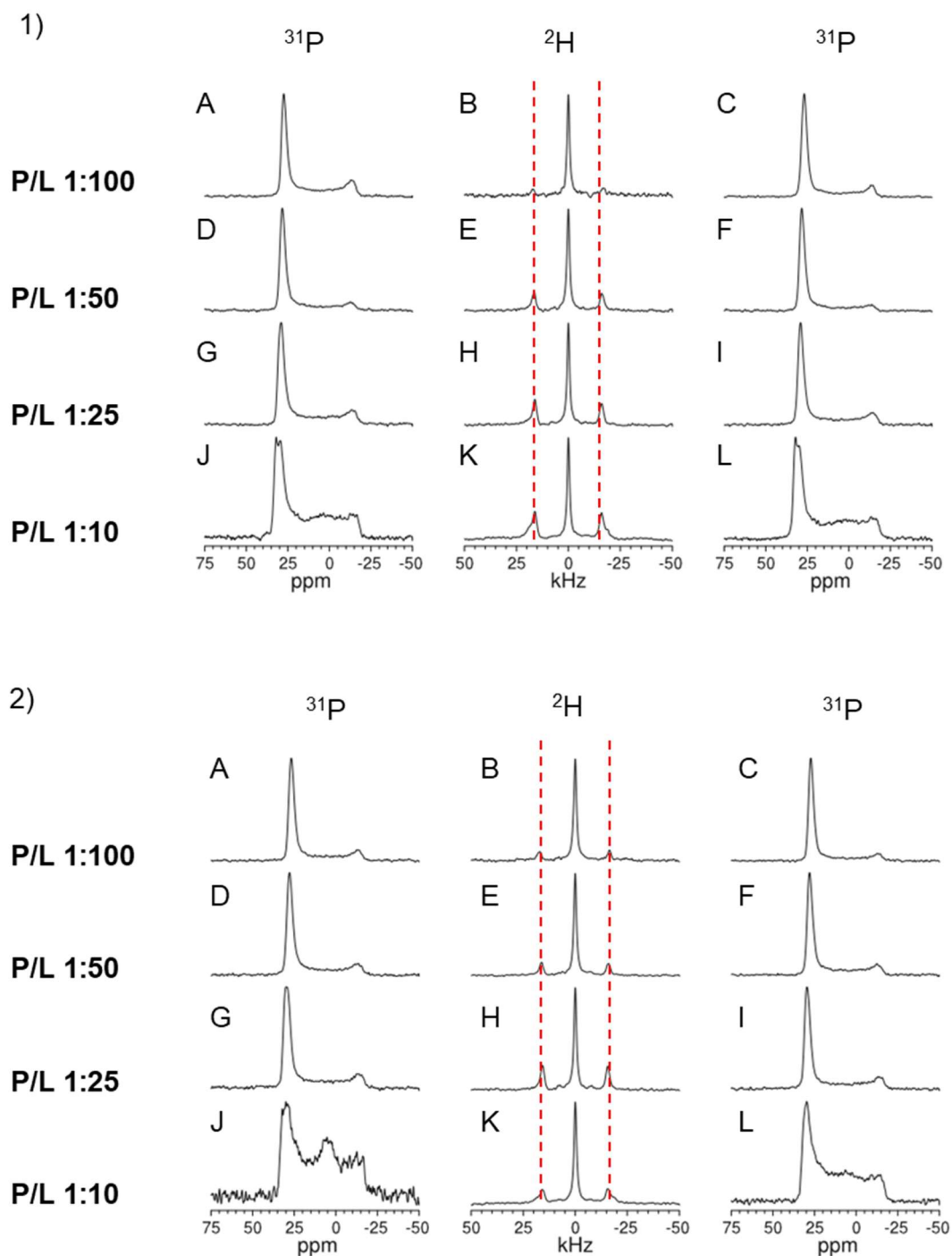


Figure 25: Solid-state ^{31}P - and ^2H -NMR spectra of 1) Ltc5 A15 and 2) Ltc5 A22 in DErPC/DErPG (7:3).

For each peptide a concentration series including the P/L ratios 1:100, 1:50, 1:25 and 1:10 was conducted. The respective P/L is given to the left of each row of spectra. Before (A, D, G, J) and after (C, F, I, L) every ^2H measurement a ^{31}P measurement was conducted to monitor the orientation of lipid bilayers in the sample.

6.2 Discussion

AMPs are known to adopt different orientations in lipid bilayers depending on their mechanisms of action. One impactful property influencing this orientation is the hydrophobic match between peptide length and membrane thickness.^{55,89} A model peptide derived from MSI-103, comprising a repeated (KIAGKIA)₃ sequence, was previously shown to adopt different membrane orientations depending on the degree of hydrophobic matching between the peptide and the lipid bilayer.⁷⁶ Under conditions of optimal hydrophobic match, the peptide adopted a fully inserted I-state. In lipid bilayers that were too thin to accommodate a full transmembrane span of the peptide, it compensated by adopting a tilted T-state.⁷⁶ Another key factor influencing orientation of peptide in lipid bilayers, is the peptide concentration, here expressed through the P/L.^{55,89}

The aim of this chapter is to investigate the behaviour of Ltc5 at increasing peptide concentrations and in lipid bilayer with changed hydrophobic matching. If Ltc5 undergoes orientational changes under specific conditions, this may provide insight into its membrane-active state. Conversely, if Ltc5 retains the same orientation at elevated peptide concentrations and in membranes of different hydrophobic thickness, the previously identified surface bound S-state may be considered the membrane-active state of the peptide. The difference in membrane thickness and peptide length, i.e., the hydrophobic match in case of transmembrane helices, would therefore not be important for Ltc5. To address this aim, Ltc5 WT and the two deuterium-labelled peptides Ltc5 A15 and Ltc5 A22 were analysed in respect to their secondary-structure (CD), orientation in the lipid bilayer (OCD), and ability to induce membrane disruption (vesicle leakage) in lipid systems of varying hydrophobic thickness. Solid-state NMR studies were conducted to monitor precise deviations from the previously determined orientation in Chapter 5. The ssNMR studies additionally included concentrations series in the different lipid systems.

All three peptides were first examined by CD spectroscopy in SUVs composed of DLPC/DLPG (7:3), DMPC/DMPG (7:3), or DErPC/DErPG (7:3) (**Figure 20**). The previously used lipid system during the Ala-scan and ssNMR analysis in Chapter 5 was DMPC/DMPG (7:3). DLPC/DLPG forms thinner bilayers than DMPC/DMPG, whereas DErPC/DErPG forms thicker bilayers and provides the best hydrophobic match

between lipid bilayer and Ltc5 (**Table 9**). In all three lipid systems, the CD spectra showed the expected high degree of similarity between the Ltc5 WT peptide and the labelled analogues Ltc5 A15 and Ltc5 A22. All peptides showed spectral features characteristic of a predominantly α -helical content (panels A, B and C, **Figure 20**) under every tested condition. Furthermore, the spectra of the Ltc5 WT measured in the three lipid systems also showed insignificant deviation from one another, indicating that the secondary-structure of Ltc5 is independent of hydrophobic matching with the lipid bilayer. This observation is upheld by deconvolution (**Table 11**), which shows α -helical shares between 79 % and 89 % in all lipid systems and for all three peptides.

As all peptides adopt a predominantly α -helical conformation in the three lipid systems during CD analysis, their orientation in the membrane could next be analysed using OCD. OCD serves as a fast preliminary screening for the following ssNMR analysis. The OCD spectra in all three lipid systems at a P/L of 1:100 do not indicate any major orientational changes correlating with bilayer thickness for either Ltc5 WT or the two Ala- d_3 labelled Ltc5 A15 and Ltc5 A22 (**Figure 21**). Even in the lipid system exhibiting the closest hydrophobic match, namely DErPC/DErPG, no tilt was detected. In principle the likelihood to observe a tilt of Ltc5 was expected to be the highest in this lipid system. A tilt, depending on hydrophobic matching was not observed for Ltc5 during the OCD measurements.

Membrane damage induced by the three peptides in bilayers composed of DLPC/DLPG (7:3) (**Figure 22**), DMPC/DMPG (7:3) and DErPC/DErPG (7:3) (**Figure 22**) was assessed using a vesicle leakage assay with ANTS/DPX-loaded LUVs at a P/L of 1:25. Leakage values for all three peptides were below 3% throughout all three lipid systems of varying thickness. These results support a non-membranolytic mechanism of action that appears to be independent of hydrophobic matching. The very low leakage values also suggest that pore formation is unlikely for Ltc5. Small residual leakage may arise from generic bilayer perturbations upon binding or from translocation events, but the assay does not allow distinguishing between these possibilities. These observations are in line with the S-state determined during OCD measurement.

Finally, the two Ala- d_3 labelled peptides Ltc5 A15 and Ltc5 A22 were used to conduct ss- ^{31}P - and ^2H -NMR studies to investigate orientational changes of Ltc5 depending on

hydrophobic matching and peptide concentration. While OCD data only allows for a crude estimation of the membrane orientation, ssNMR allows for the detection of even minor orientational changes compared to the orientation determined in Chapter 5. During the measurements the lipid amount was kept constant while the peptide amount was adjusted according to the required P/L. The effects of increased P/L on the lamellar orientation of lipid bilayers were monitored by ^{31}P -NMR while changes in peptide orientation were monitored using the Ala- d_3 labels in Ltc5 A15 and Ltc5 A22 for ^2H -NMR. Again, the three different lipid systems DMPC/DMPG, DLPC/DLPG and DErPC/DErPG in PC/PG ratios of 7:3 were used to investigate the effects of hydrophobic matching. The concentration series of Ltc5 A15 and Ltc5 A22 conducted during this work indicate that Ltc5 WT does not induce major disturbance to the lamellar orientation of lipid bilayers up to a P/L of 1:25. In **Figure 23**, **Figure 24** and **Figure 25** one can see, that the ^{31}P spectra before (1 and 2 A, D, G) as well as after (1 and 2 C, F, I) the ^2H measurement show a predominantly lamellar orientation of the lipids in a bilayer indicating no differences between low (P/L 1:100) and high (P/L 1:25) peptide concentrations.

The ^2H spectra showed a consistent quadrupolar splitting at a P/L of 1:25 and lower independent of peptide concentration and bilayer thickness (**Table 12**). ~32.1 kHz for Ltc5 A15 and ~30.1 kHz for Ltc5 A22 were observed. The unchanged quadrupolar splittings, corresponding to two different positions in the α -helix of Ltc5, allow for the conclusion, that up to a P/L of 1:25 the orientation of Ltc5 is the same in all three tested lipid systems. Furthermore, the quadrupolar splittings observed for both peptides are the same as in Chapter 5, indicating that the orientation observed here is the exact same as determined previously (**Table 8**). In **Table 12** the quadrupolar splittings at different P/L and in the three different lipid systems are summarised.

Table 12: Quadrupolar splittings ($\Delta_{\nu Q}$) of Ltc5 A15 and Ltc5 A22 measured in three different lipid compositions.

Measurements were conducted in DMPC/DMPG, DLPC/DLPG and DErPC/DErPG using ss^2H -NMR. PC/PG ratios of 7:3 were used for every measurement. The measurements were performed at P/L of 1:100, 1:50, 1:25 and 1:10 at 35 °C. The P/L ratios of the respective $\Delta_{\nu Q}$ are provided in the leftmost column. The respective peptide and the lipid systems are provided in the first row.

Ltc5 A15	$\Delta_{\nu Q}$ DMPC/DMPG [kHz]	$\Delta_{\nu Q}$ DLPC/DLPG [kHz]	$\Delta_{\nu Q}$ DErPC/DErPG [kHz]
P/L 1:100	32.2	32.1	32.1
P/L 1:50	31.9	32.1	32.1
P/L 1:25	32.1	32.0	32.0
P/L 1:10	31.8	unresolved	31.8

Ltc5 A22	$\Delta_{\nu Q}$ DMPC/DMPG [kHz]	$\Delta_{\nu Q}$ DLPC/DLPG [kHz]	$\Delta_{\nu Q}$ DErPC/DErPG [kHz]
P/L 1:100	30.1	30.2	30.1
P/L 1:50	30.1	30.1	30.0
P/L 1:25	29.9	29.9	30.1
P/L 1:10	30.2	unresolved	30.1

Only at a P/L of 1:10 a significant change in the quadrupolar splitting of the lipids could be observed. In all three lipid systems, and therefore at three different bilayer thicknesses, the labelled analogues Ltc5 A15 and Ltc5 A22 induced the formation of a signal at 7 ppm. The observed hexagonal H_I phase is induced to different extents. The most prominent H_I phase can be observed in DLPC/DLPG, which forms the thinnest lipid bilayers out of the three lipid systems tested (~ 31 Å). Here the H_I phase dominates and is the only lipid orientation observable in the ^{31}P spectra (1 and 2 J, L, **Figure 23**). Furthermore, a small signal corresponding to the presence of an H_I phase can already be observed at a lower P/L of 1:25. This is only the case in the thinnest bilayer composed of DLPC/DLPG. With increasing bilayer thickness, the H_I phase is reduced. In theory, the signal at 7 ppm could correspond to a hexagonal H_I phase or an inverse hexagonal H_{II} phase. As the signal decreases with increasing lipid chain length, the H_I phase is more plausible. At a P/L of 1:10 Ltc5 is highly crowded on the lipid bilayer. It pushes the lipid head groups aside, creates cavities underneath. These can lead to a more favourable energetic state when forming a H_I phase, as depicted in **Figure 26**. The cavities described, form at the membrane water interface, and can therefore be better compensated for by longer lipid chains. A H_I phase is therefore more in line with

the observed lipid chain length dependency as an H_{II} phase. The absence of a detectable quadrupolar splitting in the 2H spectra when the lipids are predominantly oriented in an H_I phase can be explained through orientational and motional averaging. When the lipids adopt a lamellar L_α phase (e.g., at $P/L \leq 1:25$), Ltc5 resides in the surface-bound S-state. In this configuration, the peptide is expected to undergo two-dimensional lateral diffusion within the membrane plane (x-y). Upon conversion to the H_I phase, the lipids assemble into hexagonally packed cylinders lined by lipid monolayers. The polar headgroups face the aqueous surrounding, whereas the acyl chains point inwards. In such a geometry, peptides located at the headgroup-water interface can experience additional motional degrees of freedom associated with diffusion and reorientation along the cylindrical channels. If these motions are sufficiently rapid, on the 2H -NMR timescale, they can substantially average the quadrupolar interaction of an Ala- d_3 label, leading to strong attenuation or collapse of the quadrupolar splitting. In return detecting the anisotropic 2H signal becomes impossible (1 and 2 K; **Figure 23**). In **Figure 26** the two relevant lipid phases L_α and H_I are depicted in a cartoon like manner.

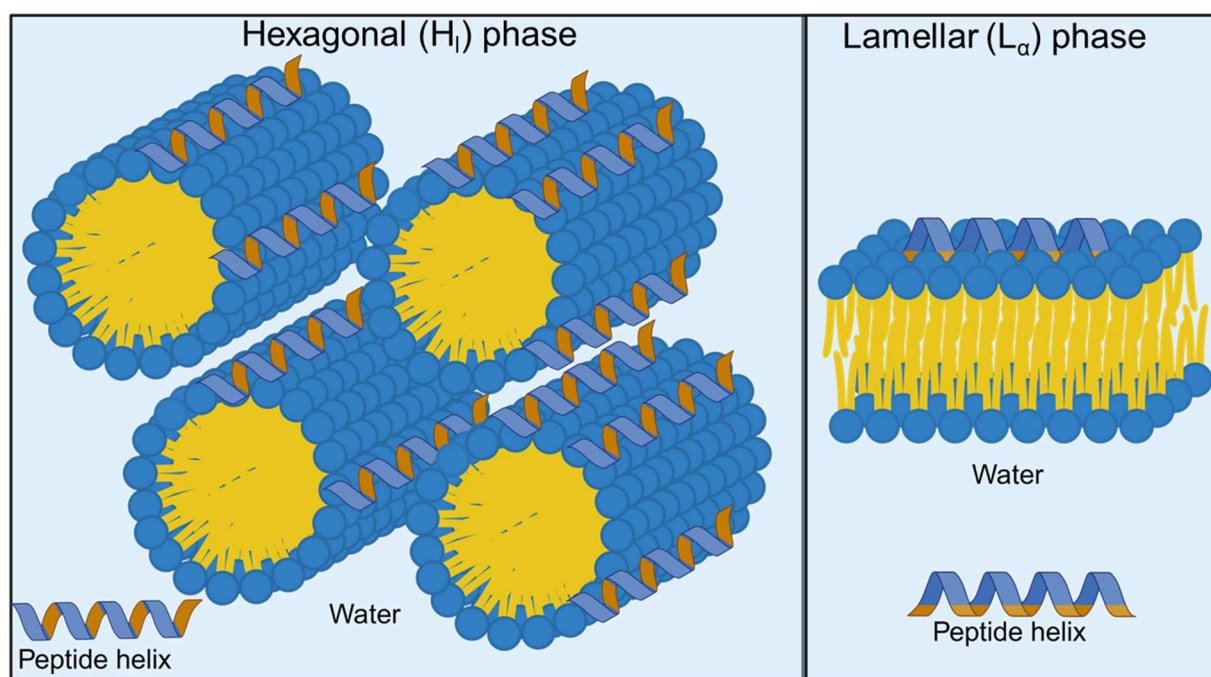


Figure 26: Cartoon depiction of the two lipid phases hexagonal (H_I) and lamellar (L_α).

The figure includes depictions of peptide α -helices orienting in the respective phase. Created in <https://BioRender.com>

At this point, it is important to take the resulting structural and organizational state of the lipid systems into consideration. In an S-state, the 28-residue Ltc5 lies flat on a single leaflet of the bilayer, so its interfacial footprint is the relevant geometric parameter to consider. Using the canonical α -helical rise of 0.15 nm per residue^{23,24}, the α -helix length is approximately $28 \times 0.15 \text{ nm} \approx 4.2 \text{ nm}$. A typical peptide α -helix has an effective outer diameter of roughly 1.0 nm when side chains are included.^{23,24} Approximating the surface-bound helix as a $4.2 \text{ nm} \times 1.0 \text{ nm}$ cylinder on the membrane yields a hydrophobic contact area of $\sim 4.2 \text{ nm}^2$. For fluid PC bilayers, neutron/X-ray scattering and simulations estimated an area-per-lipid headgroup of 0.6 nm^2 to 0.66 nm^2 . Taking 0.63 nm^2 is a commonly used estimation.^{90,91} The number of lipids required to cover one flat peptide on that leaflet is $4.2 / 0.63 \approx 6.7$, i.e. ~ 7 lipids. Because a P/L of 1:10 refers to total bilayer lipids, each leaflet contains ~ 5 lipids per peptide. With an area per lipid of 0.63 nm^2 , the available lipid area per leaflet is $\sim 3.2 \text{ nm}^2$. The $\sim 4.2 \text{ nm}^2$ interfacial footprint of an S-state helix would then occupy $\sim 58 \%$ of the local leaflet surface, resembling a highly crowded surface in which peptide-lipid packing effects may drive phase transitions towards the H_I phase. In other words, at a high P/L of 1:10, the lipid bilayer experiences major crowding with peptides. In vivo crowding events of such extent are not required for the antibacterial action of Ltc5 (Low MIC of $2 \mu\text{g/mL}$ to $4 \mu\text{g/mL}$). Nevertheless, the mechanism underlying the L_α to H_I phase transition could be part of membrane binding or membrane traversing mechanisms of Ltc5.

In summary, the membrane orientation of Ltc5 appears to be largely independent of lipid chain length and peptide concentration. Ltc5 exhibited comparable behaviour in lipid bilayers of different thicknesses at P/L ratios of up to 1:25. Only at the highest concentration tested (P/L of 1:10) was a disruption of the lamellar alignment of the lipid bilayers observed. Given that Ltc5 displays strong antimicrobial activity at low concentrations ($\text{MIC} \leq 4 \mu\text{g/mL}$), a P/L of 1:10 likely represents an extreme condition, which is not required for antibacterial activity. The membrane-active state of Ltc5 is therefore proposed to correspond to a surface bound S-state. In this orientation, the peptide could, for example, promote anionic lipid clustering. This could modulate membrane properties in a manner, that facilitates ion leakage or the interruption of lipid domains, detrimental to bacterial viability. This will be investigated in Chapter 9.

7 Functional role of aromatic residues in Latarcin 5

Ltc5 contains an unusually high number of aromatic residues, seven out of 28, accounting for 25% of its amino acids. Four of these aromatic residues are located toward the N-terminus in neighbouring positions (Phe2 and Phe3, Tyr9 and Phe10). The remaining three are situated closer to the centre of the peptide as part of the C-terminal helix (Phe13, Phe17 and Phe21). The key property, distinguishing aromatic amino acids from other hydrophobic residues, is the π -electrons of their aromatic rings, which might enable unique interaction modes with membranes or intracellular targets. In the previous chapters it was shown, that Ltc5 causes only minor membrane permeabilization under standard conditions and lies flat on the membrane surface in a so-called S-state, independent of bilayer thickness. In this chapter, we address whether aromaticity itself is required for the activity of Ltc5, or if hydrophobicity is sufficient. With this aim, three specific analogues were synthesised: 1) An Ala analogue in which all phenylalanines (Phe) were replaced by Ala, thereby reducing both hydrophobicity and steric bulk. In this Ala-rich analogue, the Tyr9 was retained to allow UV-based concentration determination. 2) A Leu analogue in which all aromatic residues, including Tyr9, were replaced by leucine, thereby fully removing aromatic character while introducing only modest changes in hydrophobicity and steric bulk. 3) A ChA analogue in which all aromatic residues were replaced by cyclohexyl alanine (ChA). ChA lacks aromaticity and ring planarity but retains a bulky six-carbon ring. Various structure-hydrophobicity correlations suggest that ChA is at least as hydrophobic as Phe, and likely more so, although no quantitative scale is available in the peptide context.⁹² The helical wheel and helical mesh of Ltc5, with the replaced residues marked with orange circles, is shown in **Figure 27**.

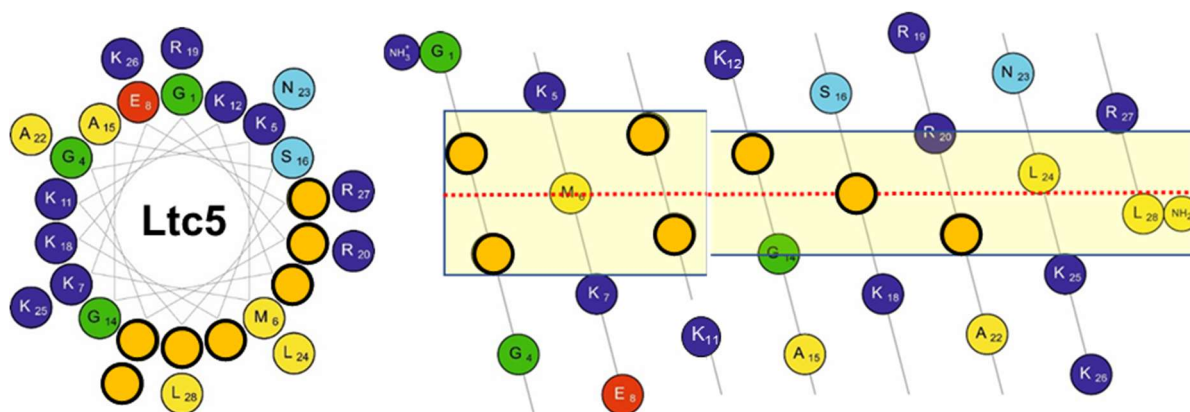


Figure 27: Helical wheel and horizontal mesh of Ltc5 with all aromatic residues marked by an orange circle.

In total, Ltc5 has seven aromatic residues located in the hydrophobic sector. All of them were replaced by either Ala, Leu or ChA. The aromatic residue Tyr9 was retained in the Ltc5 Ala analogue to allow concentration determinations of the peptide. Created using Protein Origami.⁵³

Table 13 includes the peptides name, primary sequence, molecular weight (Da), net charge, and HPLC retention times, of the peptides used in this chapter.

Table 13: List of peptides with aromatic residues replaced.

Aromatic residues were replaced by either Ala, Leu or ChA. The table includes the nomenclature of the analogues, their primary sequence, molecular weight (Mw) in Dalton [Da], their net charge and the HPLC retention time (RT) in minutes. X=ChA.

Peptide Name	Primary sequence	Aromatic residues [n]	Mw [kDa]	Net Charge	RT [min]
Ltc5 WT	GFFGKMKEYFKKFGASFRRRFANLKKRL-amide	7	3430.17	10	12.3
Ltc5 Ala	GAAGKMKEYAKKAGASAKRRAANLKKRL-amide	1	2973.55	10	10.4
Ltc5 Leu	GLLGKMKELLKKLGASLKRRLANLKKRL-amide	0	3176.06	10	18.9
Ltc5 ChA	GXXGKMKEXXKKXGASXKRRXANLKKRL-amide	0	3456.51	10	14.4

The analogues were first characterised by CD and OCD spectroscopy to determine whether their secondary-structure or membrane alignment differed from that of Ltc5 WT due to their changed properties. This was followed by three functional assays: MIC, hemolysis, and vesicle leakage. The combined structural and functional data from these experiments should help to clarify the role of the aromatic residues in Ltc5.

7.1 Results

7.1.1 Peptide synthesis

All analogues were successfully obtained by Fmoc solid-phase synthesis and purified by RP-HPLC (see Chapter 12).

7.1.2 CD and OCD spectroscopy

CD measurements were performed to assess whether the three synthesised peptides display differences in secondary-structure under the tested conditions and therefore allow conclusions about the correlations between aromatic residues and the structure of Ltc5 WT. The amphipathic α -helical conformation is generally considered important for the activity of α -helical AMPs and is likely essential for the mechanism of action of Ltc5 as well. If removal of the aromatic residues were to alter the secondary-structure substantially, this might already suggest that aromaticity is required for the correct folding of the peptide and therefore for its activity. All measurements were carried out under the same conditions as those used for the alanine-scan in Chapter 5. The peptides were measured either in distilled water (no pH control), in 10 mM phosphate buffer (PB) at pH 7, or in the presence of SUVs composed of DMPC/DMPG (7:3) at a P/L of 1:50 in 10 mM PB at pH 7. The measurements in H₂O and 10 mM PB were performed to test for phosphate-dependent oligomerisation or aggregation, which is known to affect AMP activity and has been suggested for some peptides from the latacin family.⁴⁷ In this work, we distinguish oligomerisation (small, functional complexes with a defined structure) from aggregation (large, heterogeneous, inactive assemblies, of unordered coils or β -sheets). The respective spectra are presented in **Figure 28**. Each measurement was repeated at least once to ensure reproducibility.

In distilled water (**Figure 28**, panel A), the spectra of Ltc5 WT and Ltc5 Ala exhibit the features of an unordered conformation, with no significant indications for any predominant secondary-structure. The spectrum of the Ltc5 Leu analogue mainly resembles an unordered secondary structure, with a possible population known as poly-proline-helix-II-like structure with a pronounced minimum at 198 nm and a steep signal decrease starting at 214 nm. In contrast, Ltc5 ChA shows spectral characteristics indicative of α -helical content. Mainly the two minima at 208 nm and 222 nm, accompanied by the strong positive signal at 190 nm. The minimum at 222 nm is not as pronounced as expected for a predominantly α -helical peptide and the positive

signal at lower wavelength is blue shifted. Both effects could correlate to a certain unorder in the peptides secondary-structure. The results of the secondary-structure deconvolution of the CD spectra using the web tool and databases of DichroWeb^{58,59,93} is shown in **Table 14**. The deconvolution supports the previously made observations. Ltc5 WT and Ltc5 Ala are mainly unordered but show roughly 30 % of β -sheet formation. Ltc5 Leu is predominantly unordered (80 %) while Ltc5 ChA shows an unexpectedly high helical population of 44 %.

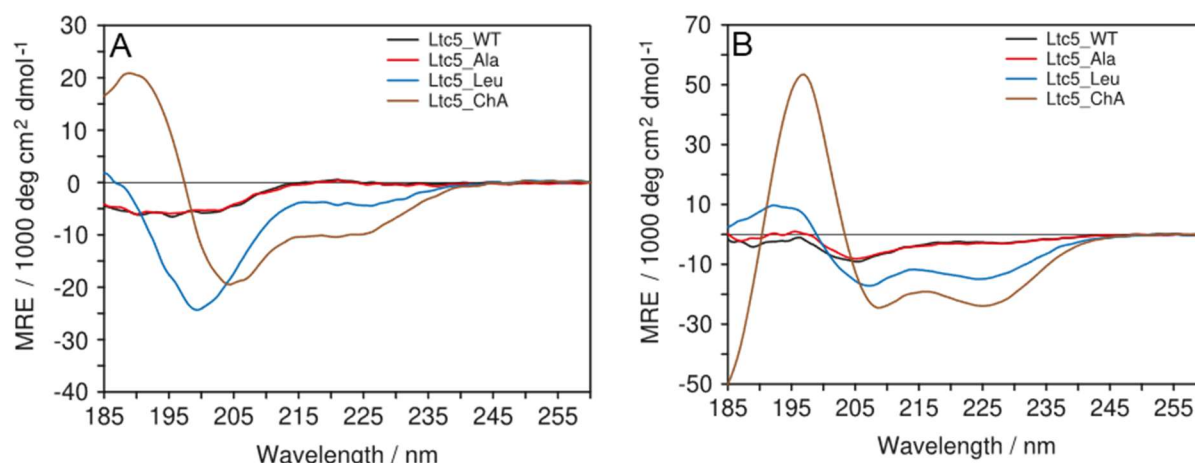


Figure 28: CD spectra of Ltc5 analogues with all aromatic residues replaced.

Aromatic residues were replaced by either Ala (except Tyr9), Leu or ChA. Measured were conducted A) in H₂O at 25 °C or B) in 10 mM PB at 25 °C.

The CD spectrum of the Ltc5 WT measured in 10 mM PB correlates to a highly unordered secondary structure (**Figure 28**, B) with no specific spectral features and overall low intensity. Unexpectedly, deconvolution revealed a secondary structure share of 36 % unordered with roughly 20 % of turn and β -strand, respectively. The CD spectra of Ltc5 WT and Ltc5 Ala, measured in PB, closely overlap once more, indicating structural similarities between the two peptides. Deconvolution supports these observations. The secondary structure in 10 mM PB of Ltc5 WT was determined to be 16 % α -helical and 36 % unordered while Ltc5 Ala is 18 % α -helical and 37 % unordered in PB. As the secondary structure of Ltc5 WT and Ltc5 Leu both have high contents of different secondary structure in water and in PB. The signals of these shares cancel each other out, resulting in the observed CD spectra of the two peptides. The spectra of Ltc5 Leu and Ltc5 ChA are consistent with a partly α -helical population, as evident by the two characteristic minima at approximately 208 nm and

222 nm. The overall share of helicity seems to be lower for Ltc5 Leu compared to Ltc5 ChA, as the intensity of the positive signal at 195 nm is diminished and the zero-crossing of the spectrum is substantially blue-shifted, both characteristic features of an increased proportion of unordered content. Deconvolution again verified the observations (**Table 14**). In 10 mM PB Ltc5 Leu showed an α -helical content of 50 % and Ltc5 ChA a highly α -helical content with 82 % α -helix.

Measurements in the presence of lipids were conducted above the lipid T_m , at 30°C. The spectra obtained in PB and in SUVs composed of DMPC/DMPG (7:3) at a P/L of 1:50 are presented in **Figure 29**. Measurements were repeated twice with different samples.

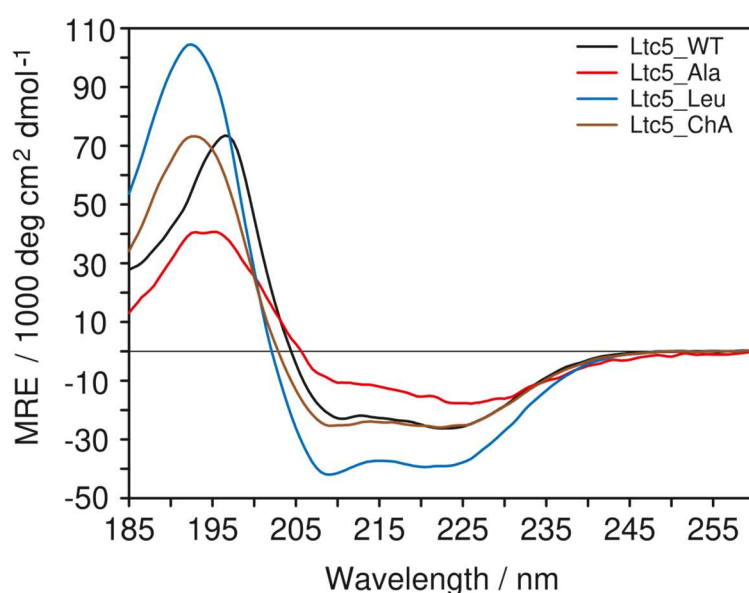


Figure 29: CD spectra of Ltc5 analogues with all aromatic residues replaced.

Aromatic residues were by either Ala, Leu or ChA measured in 10 mM PB in the presence of SUVs composed of DMPC/DMPG 7:3 at a P/L of 1:50 and at 30°C (above T_m of the lipids).

For all peptides, except Ltc5 Ala, the relative intensities of the negative double minima at 208 nm and 222 nm and the positive band at 195 nm are characteristic of a predominantly α -helical secondary structure for the measurement with SUVs (**Figure 29**). For Ltc5 Ala, the two characteristic minima are less pronounced and the positive band near 195 nm is strongly diminished, indicating lesser α -helical content relative to the other peptides. In contrast, Ltc5 ChA and Ltc5 WT exhibit the typical CD signatures of α -helical structures, including a prominent positive band at 195 nm. Their spectra are highly similar, indicating similar secondary structures. Minor differences, such as a

small blue shift of the zero-crossing point and the positive band for Ltc5 ChA, are likely explained by the higher aromatic residue content of Ltc5 WT, which can introduce a relative red shift.

Table 14: Deconvolution of the CD spectra of Ltc5 WT and the Ltc5 analogues with replaced aromatic residues.

Aromatic residues were by Ala (except Tyr9), Leu or ChA. The results shown correspond to the spectra measured in either H₂O, 10 mM PB or 10 mM PB in the presence of SUVs composed of DMPC/DMPG 7:3 at a P/L of 1:50. Deconvolution includes the secondary structural motifs helix, strand, turn and unordered.

In water	Ltc5 WT	Ltc5 Ala	Ltc5 Leu	Ltc5 ChA
Helix [%]	3	5	7	44
Strand [%]	29	27	7	9
Turn [%]	19	13	5	18
Unordered [%]	48	53	80	29

In PB	Ltc5 WT	Ltc5 Ala	Ltc5 Leu	Ltc5 ChA
Helix [%]	16	18	50	82
Strand [%]	22	23	5	5
Turn [%]	25	22	16	9
Unordered [%]	36	37	29	5

In SUVs+PB	Ltc5 WT	Ltc5 Ala	Ltc5 Leu	Ltc5 ChA
Helix [%]	89	66	93	93
Strand [%]	6	14	3	1
Turn [%]	3	9	1	2
Unordered [%]	3	11	3	5

Ltc5 Leu shows an essentially textbook α -helical spectrum, as indicated by the zero-crossing point and positive-band position. Both features are slightly blue-shifted relative to Ltc5 WT, with the y-axis crossed at 202 nm while the positive band is found at 193 nm. Notably, the intensity of the Ltc5 Leu spectra is significantly increased compared to all other peptides measured. Deconvolution of the secondary structure revealed that Ltc5 Ala had the lowest observed α -helicity with only 66 %. For Ltc5 WT (89 %), Ltc5 Leu (93 %) and Ltc5 ChA (93 %), high α -helical contents were found. The difference in intensity between Ltc5 Leu and Ltc5 ChA contradicts the observed α -helical content of 93 % for both peptides on first glance. Aggregation occurring in the

Ltc5 ChA samples, which can cause light scattering artefacts and therefore lower the signal intensity, could explain the differences.

The impact of the aromatic residues on the orientation of Ltc5 in lipid membranes was analysed using OCD. Aromatic residues are well known to act as membrane anchors that stabilise and hold peptides at the lipid-water interface.^{94,95} Removing the aromatic residues from Ltc5 WT is therefore expected to alter this property substantially and may permit orientations in lipid bilayers, other than the S-state described in Chapter 5. Any change in orientation could indicate a change in the mode of action and thus provide further insight into the functional role of the aromatic residues in Ltc5. The orientation of the three Ltc5 analogues depleted of aromaticity and the Ltc5 WT in lipid bilayers was therefore investigated using OCD spectroscopy. The respective OCD spectra are shown in **Figure 30**. Measurements were repeated twice on different samples. The OCD spectra of all measured peptides have the typical two minima at 222 nm and 208 nm and the strong positive signal between 190 nm and 197 nm corresponding to an α -helical secondary structure in the samples.

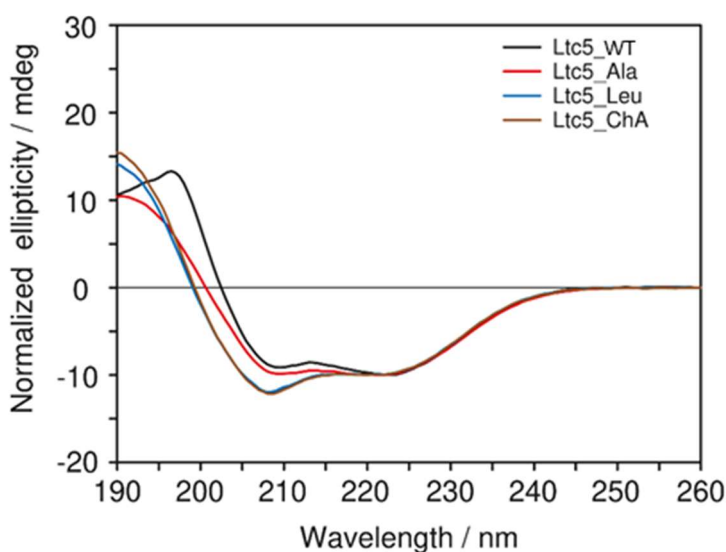


Figure 30: Normalized OCD spectra of the Ltc5 analogues without aromatic residues.

The aromatic residues were by either Ala (keeping Tyr9), Leu or ChA. Measured in DMPC/DMPG 7:3 molar ratio and a P/L molar ratio of 1:100 at a hydration level of 97%. The measurements were conducted at 30°C.

Ltc5 WT was previously proven to be present in an S-state in Chapter 5. The increased intensity of the negative signal at 208 nm, usually corresponding to a T-state, was described previously⁴⁷ as an artefact caused by the high number of aromatic residues

in Ltc5 WT. Additionally, the positive signal at lower wavelengths experiences a red shift compared to the other spectra. This effect was already observed during the solution CD measurements and can also be attributed to the high number of aromatic residues. As both effects described correlate with the number of aromatic residues, they are not observed in the spectra of the peptides with depleted aromaticity, supporting the assumption made by Wadhwani et al. Therefore, the spectra of the peptides with reduced or depleted aromaticity can be analysed with the usual standards for OCD measurements with regard to peptide orientation. The spectrum of Ltc5 Ala shows a decrease of the intensity of the signal at 208 nm. This decrease suggests that Ltc5 Ala is oriented in a slightly tilted T-state rather than an S-state. For Ltc5 Leu and Ltc5 ChA the minimum at 208 nm is lower than the minimum at 222 nm, indicating a surface bound orientation of both peptides.

In summary, the OCD spectra show that Ltc5 WT, Ltc5 Leu, and Ltc5 ChA reside in the lipid bilayer in an S-state, whereas the Ltc5 Ala analogue begins to tilt slightly into the membrane.

7.1.3 Antimicrobial activity

The antimicrobial activity is of particular interest in this set of experiments. If the aromatic residues are required primarily for interactions with specific targets, the analogues could retain their secondary-structure and membrane orientation yet still lose antimicrobial activity. To test this hypothesis, the antimicrobial activities of Ltc5 Ala, Ltc5 Leu and Ltc5 ChA were evaluated using a MIC assay (Chapter 12). The results of the MIC assay are summarised in Table 15. Measurements were repeated twice in triplicate, using independently grown cultures.

The antimicrobial assays revealed no detectable activity up to 256 µg/mL for Ltc5 Ala and Ltc5 ChA (MIC >256 µg/mL). Ltc5 Leu (MIC 16 µg/mL) showed a reduction in activity compared to the Ltc5 WT (MIC 2 µg/mL-4 µg/mL), showing an increased MIC by two dilution steps for *E. coli*, *Acinetobacter sp.*, *B. spizizenii* and by three dilution steps for *S. xylosus*. The observed activities for all peptides were strain-independent.

Table 15: MIC assay performed with the Ltc5 analogues depleted of aromaticity.

The assay was conducted on two Gram-negative strains, namely *E. coli* and *Acinetobacter sp.*, and two Gram-positive strains, namely *B. spizizenii* and *S. xylosus*, using the Ltc5 analogues with replaced aromatic residues for either Ala (except Tyr9), Leu or ChA. The MIC assay was performed in a two-fold serial dilution starting at 256 µg/mL down to 2 µg/mL.

Bacteria strains	MIC [µg/mL]			
	Ltc5 WT	Ltc5 Ala	Ltc5 Leu	Ltc5 ChA
<i>E.coli</i>	4	>256	16	>256
<i>Acinetobacter sp.</i>	4	>256	16	>256
<i>B. spizizenii</i>	4	>256	16	>256
<i>S. xylosus</i>	2	>256	16	>256

7.1.4 Hemolytic activity

As Ltc5 falls into the category of membrane-active peptides, the next two assays examined the ability of the three Ltc5 analogues with depleted aromaticity to induce membrane disruption in erythrocyte membranes. Changes in hemolytic activity give insights into the role of the aromatic residues when interacting with membranes. Furthermore, an increase in hemolytic activity worsens the therapeutic index of the analogues. The hemolytic activity must therefore be evaluated to determine, whether the Ltc5 analogues with depleted aromaticity remain suitable candidates for further optimisation in future work. Both Ltc5 Leu and Ltc5 ChA are more hydrophobic than Ltc5 WT, a property often associated with enhanced hemolysis. The HPLC-RT of the respective analogues is expected to directly correlate with their hydrophobicity. Based on their increased hydrophobicity (**Table 13**), we expected a modest increase in hemolysis for both Ltc5 Leu and Ltc5 ChA. The hemolytic activity was tested using concentrated human erythrocytes as already described in Chapter 5. The respective data are given in **Figure 31**.

The hemolysis assay revealed marked differences between the three analogues and the Ltc5 WT. Ltc5 Ala showed virtually no hemolytic activity at any tested concentration, reaching values below 3 % hemolysis at the highest peptide concentration tested. In contrast, Ltc5 Leu displayed a modest increase in hemolytic activity compared to the Ltc5 WT across all concentrations. At 256 µg/mL, Ltc5 Leu reached 23 % hemolysis, corresponding to an increase of roughly 7 % relative to the

Ltc5 WT. The Ltc5 ChA analogue, however, exhibited a pronounced and unexpectedly high increase in hemolytic activity, reaching 99 % hemolysis at a moderate peptide concentration of 16 $\mu\text{g/mL}$. At this concentration, the Ltc5 WT caused only 4 % hemolysis, corresponding to more than a 27-fold increase for Ltc5 ChA. At peptide concentrations above 16 $\mu\text{g/mL}$, Ltc5 ChA caused 100 % hemolysis. Measurements were repeated three times to ensure reproducibility.

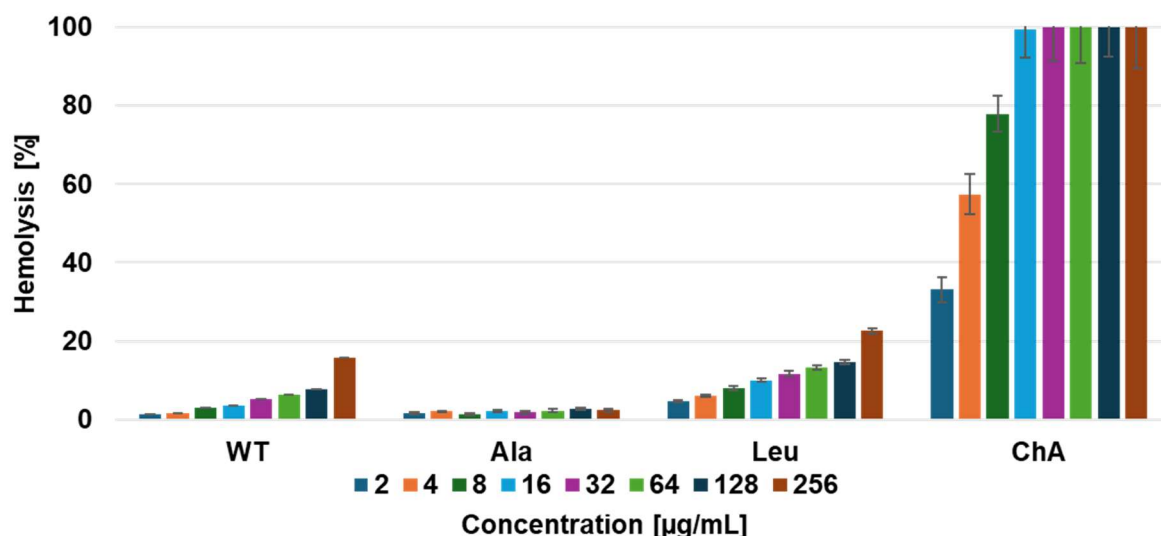


Figure 31: Concentration dependent hemolysis assay performed on Ltc5 analogues depleted of Aromaticity.

Aromatic residues were by either Leu, ChA or Ala (Tyr9 not replaced in Ltc5 Ala), using a two-fold serial dilution of peptides from 256 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$. Ltc5 WT data is included for reference. The different peptide concentrations are shown in different colours with the unit $\mu\text{g/mL}$. Beneath every concentration series the name of the respective peptide used is given. Leu refers to the analogue Ltc5 Leu, ChA to Ltc5 ChA and Ala for Ltc5 Ala.

7.1.5 Vesicle leakage

The ability of the Ltc5 analogues with depleted aromaticity to induce membrane damage in model membranes of different compositions was examined next. Vesicle leakage assays were performed on LUVs composed of POPC/POPG (1:1) or POPC/POPG/Chol (2:2:1) at a P/L of 1:25. The cholesterol-free LUVs mimic prokaryotic cytoplasmic membranes, whereas the cholesterol-containing LUVs mimic eukaryotic plasma membranes. The vesicle leakage assay on LUVs containing

cholesterol was performed to test whether the cholesterol present in the membranes of erythrocytes enables Ltc5 ChA to selectively target such membranes, thereby explaining the observed high hemolysis values and low antimicrobial activity. The results of the vesicle leakage assays are shown in Figure 32.

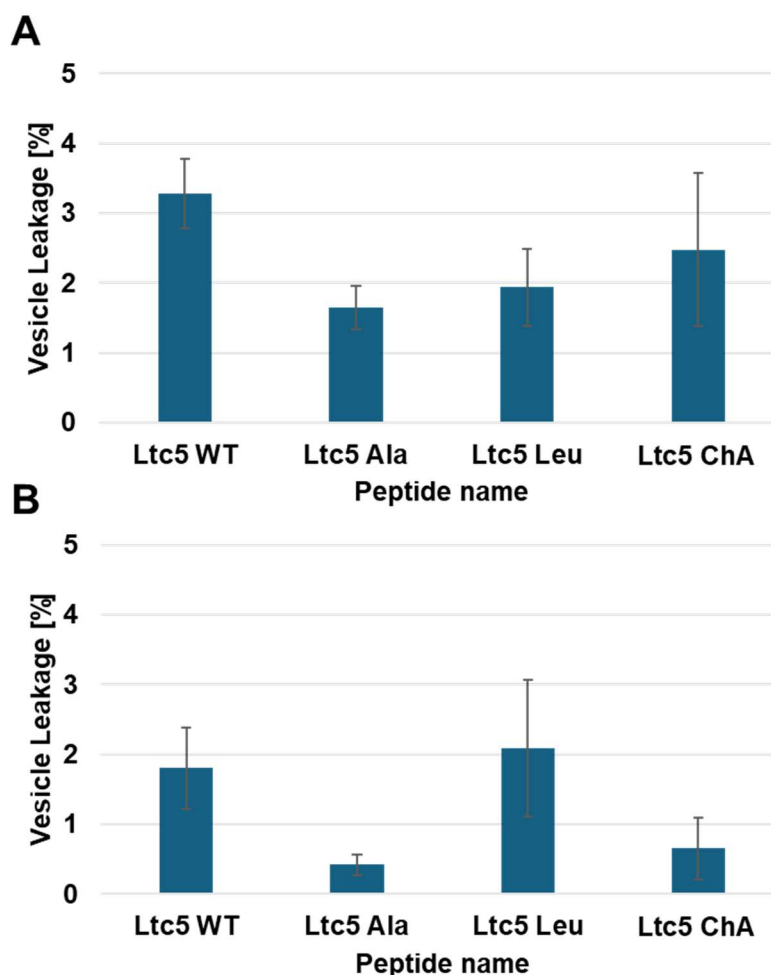


Figure 32: Vesicle leakage assay performed on the Ltc5 analogues depleted of aromaticity.

The assay was performed in LUVs made from A) POPC/POPG (1:1) or B) POPC/POPG/Chol (2:2:1) at a P/L of 1:25 at 30°C. All Ltc5 analogues with depleted aromatic residues were tested along with the Ltc5 WT as reference. Leu resembles the peptide Ltc5 Leu, ChA the Ltc5 ChA and Ala Ltc5 Ala.

All three analogues showed very low leakage (<4 %) in POPC/POPG (1:1) (Panel A, **Figure 32**), comparable to or lower than the Ltc5 WT peptide. Within experimental error, no substantial differences in vesicle permeabilization were observed between the Ltc5 analogues with no aromatic residues and the Ltc5 WT peptide.

The results for the cholesterol containing LUVs are shown in panel B) of **Figure 32**. No significant differences were observed between LUVs with and without cholesterol. The measured values remained below 3 % for all peptides tested.

7.2 Discussion

The aim of this chapter is to investigate the role of the 7 aromatic residues in Ltc5 by replacing them with either Ala, which is less hydrophobic and less sterically demanding, or Leu, which has increased hydrophobicity and comparable steric bulk, or with ChA, which retains steric hindrance similar to the replaced aromatic residues but is non-planar and more hydrophobic. All analogues except Ltc5 Ala do not have any aromatic residues in their primary sequence. Ltc5 Ala has a Tyr in position 9, initially retained to allow determination of peptide concentrations in stock solutions. In all three cases, any antimicrobial mechanism that relies on unique properties of the π -electron systems present in the aromatic side chains should no longer be operative. If, however, the mechanism of action depends primarily on hydrophobicity rather than on genuine aromatic character, the Ltc5 Ala analogue would be anticipated to display reduced or no activity compared with Ltc5 WT, whereas the Ltc5 ChA and Ltc5 Leu analogues would be expected to show activities comparable to Ltc5 WT.

First, the secondary structures of the Ltc5 WT and the three Ltc5 analogues with depleted aromaticity will be discussed. In 10 mM PB, AMPs can exhibit phosphate-dependent formation of secondary structure.⁴⁷ Assay conditions including PB approximate the behaviour of peptides in bulk aqueous solution before they interact with membranes or lipid bilayers. This is of interest because most activity-based assays are performed in buffered solutions. The results of the CD measurements can be found in **Figure 28** and **Figure 29**, while the deconvolution results are summarised in **Table 14**. The Ltc5 WT showed an α -helical content of 3 % when measured in H₂O. In 10 mM PB, this increased to 16 %. As previously described in Chapter 5, a helical content of 16 % cannot be considered an indication for the formation of oligomers. Measurements in the presence of SUVs made from DMPC/DMPG (7:3) at a P/L of 1:50 revealed an α -helical content of 89 % for the Ltc5 WT. The peptide is therefore expected to interact with the lipid bilayers of the SUVs and fold into a predominantly α -helical secondary structure. Ltc5 Ala seems to behave in a very similar way to the

Ltc5 WT with regard to its secondary structure in different environments. Ltc5 Ala shows 5 % α -helix in water and 18 % in PB. Only in the presence of SUVs does the structure of Ltc5 Ala deviate significantly from the Ltc5 WT. Here Ltc5 Ala only shows an α -helical content of 66 % compared to the 89 % of the Ltc5 WT. This difference is also reflected by the shape of the CD spectrum of Ltc5 Ala. The signals at 195 nm, 208 nm and 222 nm are significantly reduced relative to those of the Ltc5 WT. Such a decrease in helicity is most likely caused by a reduced binding affinity of Ltc5 Ala for the SUVs due to the reduced hydrophobicity. This is supported by the shorter HPLC RT compared to the Ltc5 WT (see **Table 13**). As linear amphipathic AMPs fold into α -helices upon membrane binding, the decrease in helical content is an indication of reduced membrane binding.^{62,96} Ltc5 Leu exhibits a low helical content of 7 % in water and an increase to 50 % α -helical content in PB. Phosphate dependent folding seems to occur.

Increasing the hydrophobicity of an amphipathic α -helical peptide on its hydrophobic face is known to increase the likelihood of oligomerisation in aqueous solution.⁹⁷ As a reminder, in this work we distinguish oligomerisation (small, functional complexes with a defined structure) from aggregation (large, heterogeneous, inactive assemblies, of unordered coils or β -sheets). Ltc5 Leu has an increased hydrophobicity compared to the Ltc5 WT (HPLC RT). The observed increase in helical content in PB most likely correlates with the formation of oligomers caused by an increase in hydrophobicity. The peptide most likely forms compact assemblies that minimize the exposure of hydrophobic residues to bulk water by orienting the hydrophobic residues of different monomers toward one another, while the polar residues remain exposed to the aqueous environment.^{98,99} Such oligomerisation events could have unexpected effects on the peptide's efficacy in activity-based assays.

In the presence of SUVs, the α -helical content of Ltc5 Leu is increased further to 93 %. Therefore, it can be concluded that the oligomers, even though favourable compared to an unordered structure in PB, are less favourable than the interaction with the hydrophobic core of lipid bilayers. Ltc5 ChA already showed 44 % α -helical content in water and 82 % α -helical content in PB. The moderate α -helical content of Ltc5 ChA in H₂O and high content in PB is a clear indication for the formation of α -helical oligomers following the same reasoning as for Ltc5 Leu. Judging from the 82 % α -helical content of Ltc5 ChA in PB compared to the 50 % of Ltc5 Leu, the oligomers formed by

Ltc5 ChA might be considerably more stable than those of Ltc5 Leu. This is in line with the higher hydrophobicity of ChA compared to Leu. Furthermore, ChA is a known assembly promoting residue.¹⁰⁰ Alhattab et al. showed the formation of hydro-gels involving fibre-like structures in a small four residue peptide with two ChA residues.¹⁰⁰

The HPLC RT does not show the expected increase, following the increased hydrophobicity for Ltc5 ChA (Ltc5 ChA 14.4 min, Ltc5 Leu 18.9 min). The RT can be rationalised by the previously mentioned formation of oligomers. Under HPLC conditions (mostly aqueous) the formation of oligomers does not happen for Ltc5 Leu (7 % helix in H₂O). For Ltc5 ChA however, even under HPLC conditions oligomers form (44 % helix in H₂O). In its oligomeric state Ltc5 ChA shields its hydrophobic residues from the hydrophobic solid phase of the RP-HPLC column. This shielding is not complete, resulting in an increased RT compared with the significantly less hydrophobic Ltc5 WT, but it appears to be sufficient to prevent the expected increase relative to the slightly less hydrophobic Ltc5 Leu analogue. Aggregation instead of oligomerisation can be dismissed. Literature suggests, that aggregation events are commonly associated with increased retention times, fronting or broadening effects on the HPLC column, neither of which was observed here.^{23,24,101} The HPLC peak of Ltc5 ChA is monodisperse, showing no indication of aggregation artefacts. The LC-MS spectra of Ltc5 ChA, which show high similarities to the HPLC spectra, are shown in **Figure A 4** in the appendix. Therefore, oligomerisation in solution appears to be the most plausible explanation based on the accumulated data. Oligomers can stabilise the secondary structure in solution and decrease the activity of an AMP by reducing its probability to interact with the lipid bilayers of target cell.⁹⁷ All effects described for the measurements in water and PB correlate peptide hydrophobicity rather than aromaticity.

When evaluating the reason for an increased α -helical secondary structure, the intrinsic helix propensity must also be considered in addition to the oligomerisation effects described above for Ltc5 Leu and Ltc5 ChA. The decrease in helical content for Ltc5 Ala cannot be explained with the helix propensity as Ala promotes helix formation to a greater extent than Phe or Tyr. Here the decrease in hydrophobicity is likely the leading cause as explained above. Both Leu and ChA are known to promote helix formation. According to Tsai et al.¹⁰², who derived per-residue helical propensities as log-odds [log(HPNA)] from a protein database comprising 1.7 million helical peptides

and proteins, leucine exhibits a higher helix propensity than Phe and Tyr (Leu: 0.175, Phe: -0.016, Tyr: 0.122). Therefore, the substitution of six Phe residues and one Tyr residue with Leu is expected to increase the helical propensity of the analogue slightly. This in fact is observed for the Ltc5 Leu analogue measured in the presence of SUVs (89 % Ltc5 WT, 93% Ltc5 Leu). For ChA, such a quantitative value has not yet been determined due to the limited amount of available structural data. However, there are reports of highly helical peptides containing ChA residues.¹⁰³ Thus, the slightly increased helicity of the Ltc5 ChA analogue, measured in SUVs, compared to the Ltc5 WT (89 % Ltc5 WT, 93 % Ltc5 ChA) is consistent with the reported behaviour of ChA in peptides.

The effects on the orientational alignment within lipid bilayers due to the replacement of the aromatic residues were analysed using OCD spectroscopy. In Chapter 5, an artefact in the OCD spectra attributed to the high number of aromatic residues in Ltc5 was described, as previously postulated.⁴⁷ The Ltc5 WT spectrum resembles a T-state, but the peptide is in fact present in an S-state. This was proven in Chapter 5. Removing aromatic residues should restore intensity of the signal at 208 nm. The OCD spectra of the Ltc5 analogues without aromatic residues can therefore be interpreted as standard OCD spectra. Ltc5 Ala only has one out of seven aromatic residues left in its primary sequence, namely Tyr9. The OCD spectrum of Ltc5 Ala should therefore still experience a slight reduction in signal intensity of the negative band at 208 nm compared to the spectra of the two analogues without any aromatic residues. When comparing the three spectra of Ltc5 Ala with those of Ltc5 Leu and Ltc5 ChA, the signal reduction at 208 nm is much more pronounced than expected. Thus, the comparatively reduced intensity of its 208 nm band suggests that Ltc5 Ala is slightly tilted within the membrane. This behaviour was not anticipated. The spectra of Ltc5 Leu and Ltc5 ChA closely overlap. In both cases, the negative band at 208 nm is increased compared to the Ltc5 WT spectrum, consistent with the absence of the aromaticity-related artefact discussed above. Both spectra therefore strongly indicate an S-state.

In OCD samples, as prepared in this study, only very small amount of bulk aqueous phase is available for the peptide to partition into. The peptide is therefore effectively forced into association with the lipid bilayers. Ltc5 Ala showed a decrease in helical content in CD experiments in the presence of SUVs compared to the Ltc5 WT, which was interpreted as less effective membrane association. This effect is compensated

during OCD measurements because of the sample preparation. The peptide is forced into an environment it does not preferentially occupy, due to a lack of alternatives. Therefore, its OCD spectrum resembles that of a highly α -helical peptide. The Ltc5 ChA analogue is also affected by the different sample preparation. During CD measurements in the presence of SUVs, the spectra of Ltc5 ChA and Ltc5 Leu showed significant differences in intensity. It was proposed that the signal intensity in the spectrum of Ltc5 ChA is reduced due to oligomerisation. During OCD, the peptides are co-solubilized in organic solvent. Under such conditions, the driving force for the formation of oligomers, the bulk water, is missing. Therefore, Ltc5 ChA primarily interacts with the lipid bilayers and does not form oligomers. The orientational alignment during the OCD measurements of Ltc5 ChA and Ltc5 Leu is consequently highly similar.

The antimicrobial activity data (**Table 15**) showed that the three tested peptides are all less active than the Ltc5 WT, which has a MIC of 2 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$. The Ltc5 Ala peptide suffered a complete loss of antimicrobial activity in the concentration range tested during this work (MIC >256 $\mu\text{g/mL}$, **Table 15**). Ltc5 Ala has decreased hydrophobicity compared to Ltc5 WT and shows a reduced helical content in the lipid bilayers (SUVs during CD) relative to the Ltc5 WT (**Table 14**). This decrease could either be caused by a smaller fraction of the total peptide population in the sample binding to lipid bilayers or by the peptide adopting a less helical conformation when bound to the lipid bilayer.^{62,93,96} It is most plausible that Ltc5 Ala binds less efficiently to the lipid bilayer due to its reduced hydrophobicity. This would also explain the loss of activity observed in the MIC assay. As the ability of the Ltc5 Ala peptide to bind to the bacterial membranes is reduced, its antimicrobial efficacy is likewise reduced. The peptide is unable to exert the same mechanism of action as the Ltc5 WT.

The Ltc5 Leu peptide displays reduced activity compared to Ltc5 WT, but can still be considered antimicrobially active with a MIC of 16 $\mu\text{g/mL}$ against all four tested strains. Ltc5 Leu showed some signs of oligomerisation with 50 % helical content in PB. These oligomers, consisting of helical monomers, cause a decrease of free energy while the peptide is in bulk water. Even though interaction with lipid bilayers is still more favourable (93 % helical in the presence of SUVs according to CD) the peptide has the alternative of remaining in a reversible oligomeric state in the bulk water surrounding the SUV. This is mainly enabled by the increased hydrophobicity of Ltc5 Leu compared

to the Ltc5 WT. Thus, the MIC is slightly increased, but the peptide retains some antimicrobial efficacy.

Ltc5 ChA shows a complete loss of activity at all tested concentrations in the MIC assay. However, the reasoning differs from that for Ltc5 Ala. The loss of antimicrobial activity observed for Ltc5 ChA might be caused by the formation of stable oligomers in solution. The possibility of oligomerisation was proposed based on the high helical content observed in the CD measurements in 10 mM PB and H₂O (**Table 14**). Unlike Ltc5 Leu, Ltc5 ChA is stuck in a stable oligomeric state due to the analogue's higher hydrophobicity increasing oligomeric stability.

The MIC data indicate that the right amount of hydrophobicity seems to be required for optimal antimicrobial efficacy. The hydrophobicity of Ltc5 Ala is too weak to enable strong membrane interactions. Thus, its activity against bacteria is diminished. Ltc5 Leu is slightly too hydrophobic to exert optimal antimicrobial efficacy. The increased hydrophobicity enables a second state besides the membrane bound state. Namely, the oligomeric state in bulk aqueous solution. Therefore, the equilibrium between membrane-bound and unbound peptide states is slightly shifted toward the unbound state, reducing its activity. The hydrophobicity of Ltc5 ChA is increased to a point at which the equilibrium between the membrane-bound and unbound state is strongly shifted towards the unbound-state. It can form stable oligomers in bulk aqueous solution and does not prefer membrane binding over the unbound state. Therefore, its activity is diminished. The Ltc5 WT seems to be evolutionarily optimised for the task of bacterial membrane interaction. Its hydrophobicity is sufficient to promote membrane interaction, but not so pronounced as to favour oligomerisation. Its equilibrium therefore appears to lie toward the membrane bound state. Aromaticity in itself does not seem to be a requirement for the antimicrobial activity of Ltc5 WT. Rather, it seems like the aromatic residues confer a specific amount of hydrophobicity and therefore provide an ideal balance of hydrophobicity in the Ltc5 WT peptide for antimicrobial activity.

Hemolytic activity in general tends to correlate with the hydrophobicity of AMPs.¹⁰⁴ While other factors such as charge distribution and amphipathicity also influence hemolytic activity, these parameters were not altered in the analogues tested here. The hydrophobicity of a peptide correlates with its HPLC RT. Ltc5 Ala has reduced

hydrophobicity, as evidenced by its shorter RT compared to Ltc5 WT (Ltc5 Ala 10.4 min, Ltc5 WT 12.3 min, Table 13) and shows reduced hemolytic activity (Ltc5 Ala 2 % at 256 $\mu\text{g/mL}$, Ltc5 WT: 16 % at 256 $\mu\text{g/mL}$, Figure 31). The marked loss of activity of Ltc5 Ala in both MIC and hemolysis assays is therefore proposed to result from a reduced tendency to bind to lipid bilayers due to its decreased hydrophobicity. Ltc5 Leu has increased hydrophobicity, confirmed by its longer RT (18.9 min, Table 13) and its slight increase in hemolytic activity of 7 % (23 % at 256 $\mu\text{g/mL}$, Figure 31) compared to Ltc5 WT. The hypothesized reversible oligomerisation also does not factor in as strongly, as the erythrocyte membrane is an easier target compared to bacterial membranes. They lack protective barriers like peptidoglycan layers or additional lipid bilayers. As expected, the increased hydrophobicity yielded higher hemolytic activity. Among all tested Ltc5 analogues, Ltc5 ChA showed the greatest increase in hydrophobicity relative to Ltc5 WT. The proposed formation of stable oligomers and the resulting shift towards the unbound state would therefore be expected to lead to low hemolytic activity. However, this is not the case: Ltc5 ChA shows 99 % hemolysis at a moderate peptide concentration of 16 $\mu\text{g/mL}$ and should therefore be considered highly hemolytic. It exceeds the hemolytic activity of the Ltc5 WT by nearly 25-fold at the given concentration. To a certain extent, this is in line with the increase in hydrophobicity in this analogue but contradicts the idea of dysfunctional oligomerisation at first glance. The difference in activity may arise from the distinct compositions of bacterial and erythrocytic membranes. One potential contributing factor could be the high cholesterol content of erythrocyte membranes.¹⁰⁵ This possibility will be discussed further in the next paragraph.

The results of the vesicle leakage assay (**Figure 32**) in LUVs composed of POPC/POPG (1/1) and POPC/POPG/Chol (2/2/1) show less than 3 % vesicle leakage for all tested peptides (Ltc5 WT, Ltc5 Ala, Ltc5 Leu, and Ltc5 ChA). For Ltc5 Ala, this outcome was expected, as the reduced hydrophobicity of the peptide most likely diminishes its interaction with lipid bilayers and thereby its overall ability to disturb the lipid bilayers. Ltc5 Leu, which displays increased hydrophobicity, behaved similarly to Ltc5 WT in all assays, showing slightly reduced antimicrobial activity and slightly increased hemolytic activity. Consequently, it was anticipated to exhibit vesicle leakage comparable to Ltc5 WT, with possibly slightly enhanced leakage due to the increased hydrophobicity. The differences observed in the vesicle leakage assay (**Figure 32**)

between Ltc5 Leu and Ltc5 WT fall within the experimental error, matching the expectations. The Ltc5 ChA analogue also showed low vesicle leakage in both lipid compositions. Thus, the presence of cholesterol, as mentioned above, in erythrocytic membranes cannot account for the selective activity of Ltc5 ChA towards erythrocytes by itself. A lipid composition dependent selectivity for the interaction of Ltc1 with membranes was recently postulated by Mironov et al.¹⁰⁶ A similar selectivity could also apply to the Ltc5 ChA peptide. The composition of erythrocytic membranes includes cholesterol, sphingomyelin and other components that differ from bacterial membranes. These components can form domains with specific physical properties. Such domains create lateral heterogeneity in erythrocyte membranes. At the interface between different lipid domains, line tension and packing defects occur¹⁰⁷, which allow AMPs better access to the hydrophobic core of the membrane.¹⁰⁷ This could lower the energy requirement for membrane insertion and therefore shift the equilibrium from an unbound, oligomeric state towards the membrane bound state for Ltc5 ChA in erythrocytes.

Another interesting aspect, linked to the aromaticity and hydrophobicity of the amino acids, is the depth of insertion into a membrane. MacCallum et al. performed simulations to calculate the interaction energies of almost all canonical amino acids with different regions of lipid bilayers.¹⁰⁸ They determined the minimum of the potential of mean force (PMF), that is, the free-energy profile $G(z)$ for moving an amino acid side-chain analogue along the membrane-normal (depth z) through a lipid bilayer. The zero of free energy was set in bulk water, so negative values indicate favourable partitioning relative to water, and the depth at which $G(z)$ is the lowest corresponds to the most thermodynamically favoured position in the membrane. Leu exhibits its energetic minimum in the centre of the hydrophobic core, whereas aromatic residues such as Trp and Tyr have their minima at the lipid-water interface. Phe shows a comparatively broad energy distribution with its minimum located between the hydrophobic core and the membrane water interface.¹⁰⁸ This property makes Phe significant compared to other aromatic or purely hydrophobic residues. This property may be key to the optimised antimicrobial activity of Ltc5.

A summary of the properties shown by the Ltc5 analogues with depleted aromaticity is shown in **Table 16**.

Table 16: Summary of the results of all Ltc5 analogues with depleted aromaticity.

The table includes the Peptides hydrophobicity as derived from their HPLC RT, α -helical content (CD), orientation in membranes (OCD), Antimicrobial activity (MIC), hemolytic and vesicle leakage activity and their proposed oligomeric state based on the combined data gathered in this chapter.

	Ltc5 WT	Ltc5 Ala	Ltc5 Leu	Ltc5 ChA
Hydrophobicity	++	-	+++	+++++
α -helical content in membrane [%]	++++	++	++++	++++
Orientation in membrane	S	S	S	S
Antimicrobial activity	+++	- - -	++	- - -
Hemolytic activity	+	- - -	++	+++++
Vesicle leakage	- - -	- - -	- - -	- - -
Suggested oligomerisation state	Monomer	Monomer	Oligomer	Oligomer

The results of this chapter can be summarised as follows: the Ltc5 Ala analogue showed no sign of oligomerisation or aggregation. The decrease in hydrophobicity in this analogue leads to a decrease in the peptide's ability to interact with bacterial membranes, thus rendering the analogue inactive in all assays conducted during this work. The Ltc5 Leu analogue showed only limited evidence of oligomerisation, which may account for its slightly reduced antimicrobial activity compared to the Ltc5 WT. The oligomeric state in bulk solution offers an alternative to the membrane-bound state and therefore slightly shifts the equilibrium of the analogue from the membrane-bound towards the unbound state. Ltc5 ChA has the highest increase in hydrophobicity. It was thus hypothesized that the formation of stable oligomers is responsible for the significant decrease in antimicrobial activity. These stable oligomers shift the peptide's equilibrium even further towards the unbound state than for Ltc5 Leu. Its ability to cause hemolysis is attributed to the different membrane compositions of erythrocytes and bacteria, allowing a favourable interaction between Ltc5 ChA and erythrocyte membranes.

In conclusion, the data suggest that aromaticity is not a strict requirement for the antimicrobial activity of Ltc5. Hydrophobicity appears to be sufficient to preserve

activity, as demonstrated by the Ltc5 Leu analogue. However, increasing hydrophobicity also enhances hemolytic side effects, as observed for the Ltc5 Leu and Ltc5 ChA analogues. Thus, the hydrophobicity of Ltc5 WT appears to be a crucial determinant of the balance between antimicrobial efficacy and hemolytic toxicity, whereas insufficient hydrophobicity results in a complete loss of both activities. Overall, the aromatic residues in Ltc5 WT seem to be fine-tuned with regard to their hydrophobicity, thereby enabling antimicrobial activity while minimizing hemolytic side effects.

The following two paragraphs will provide a brief outlook on possible future experiments.

The analogues synthesised in this chapter only allow examination of an extreme case, namely peptides with a complete loss of aromatic residues. Future peptide design and accompanying experiments should therefore include segment-specific replacement of aromatic residues. For example, the cluster of aromatic residues at the N-terminus could be selectively replaced to analyse the specific contribution of the high number of aromatic-residue cluster in this region. The Ala-scan in Chapter 5 showed that individual replacements with Ala are well tolerated. However, replacing two or more of the aromatic residues at a time with alanine could be of interest as well.

Additionally, erythrocyte morphology could be monitored over time by light microscopy to directly compare the temporal effects of the Ltc5 ChA analogue with those of Ltc5 WT. This would help determine whether Ltc5 ChA acts through echinocyte formation, membrane blebbing, or direct lysis. To further investigate the hypothesis of irreversible or highly stable oligomerisation of Ltc5 ChA, a MIC assay in the presence of ethanol could be performed. Ethanol may interfere with oligomer formation by interacting with both the hydrophobic and polar residues of the peptide, thereby shifting the equilibrium towards the membrane-bound state. Ethanol concentrations of up to 25 % are reportedly well tolerated by bacteria in MIC assays.¹⁰⁹ If the Ltc5 ChA analogue exhibits antimicrobial activity under these conditions, this would strongly support the oligomerisation hypothesis for Ltc5 ChA.

8 Role of the N-terminal hydrophobicity in Latacin 5

In a study by Polyansky et al., truncation of the N-terminus of Ltc2a and Ltc5 by three amino acids resulted in a pronounced decrease in hemolytic activity and cellular toxicity against the human leukaemia cell line K562.⁷⁸ The N-terminal truncations thus led to an increase in the therapeutic index in both cases. The amino acids truncated from Ltc5 were Gly1, Phe2 and Phe3. Thus, two highly hydrophobic and aromatic residues, Phe2 and Phe3, were removed, reducing the hydrophobicity of the N-terminus in the process. In this work Ltc5 will be truncated systematically based on the structural analysis performed in Chapter 5 to achieve further reduction of hemolytic side effects while maintaining the antimicrobial activity of the Ltc5 WT. The aim in this chapter is therefore to find a Ltc5 analogue with an increased therapeutic index compared to the already decent Ltc5 WT.

The helical wheel and horizontal mesh, according to the structure of Ltc5 as determined in Chapter 5, are shown again in **Figure 33**.

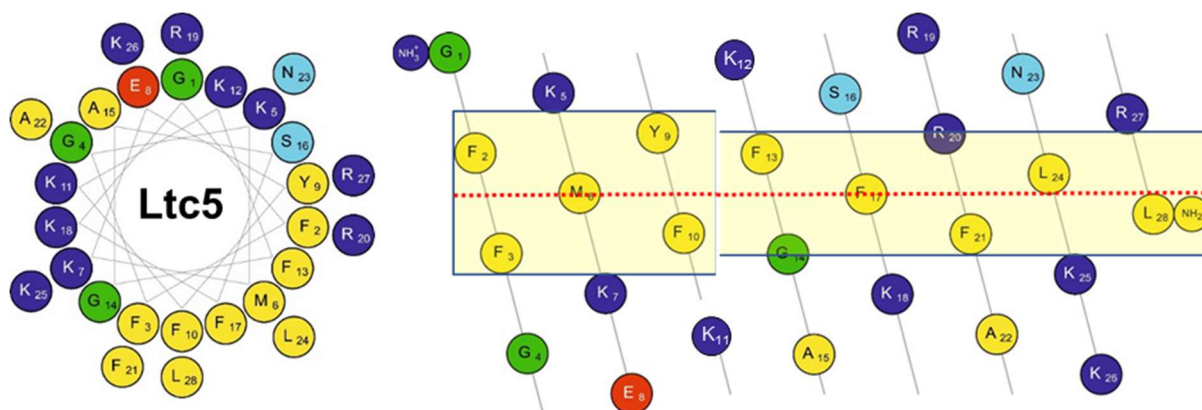


Figure 33: Helical wheel and horizontal helical mesh of Ltc5 with adjusted N- and C-terminal helix part.

The red dotted line in the helical mesh shows the centre of attraction of each helix part. This sequence was shortened one amino acid at a time starting at position 4 until an analogue with a length of 18 amino acids was reached (Ltc5 11-28). Ltc5 11-28, being the shortest analogue synthesized, truncates almost the entire N-terminal helix. The figure was created using Protein origami.⁵³

In Chapter 5, the structure of Ltc5 was shown to comprise a single α -helix with two distinguishable parts: an N-terminal part (residues 1-11) and a C-terminal part (residues 12-28). The N-terminal part contains a 2-1-2 pattern of hydrophobic residues, consisting of two hydrophobic and aromatic amino acids (Phe2, Phe3), one hydrophobic but non-aromatic amino acid (Met6) and two more hydrophobic and aromatic residues (Tyr9, Phe10). As hydrophobic and aromatic residues are known to act as membrane anchors,^{94,95} inserting into the interfacial region of lipid bilayers, the highly hydrophobic N-terminal region of Ltc5 may play a crucial role in its membrane-interaction mechanisms. In the Chapter 7, it was proposed that sufficient hydrophobicity is most likely needed for membrane binding in Ltc5. The data of the Ltc5 Ala analogue with highly reduced hydrophobicity showed clear indications of insufficient membrane binding to exert activity. In the work by Polyansky et al.⁷⁸ removal of the first three residues, including residues Phe2 and Phe3, led to a substantial reduction in hydrophobicity of the new N-terminus in the truncated Ltc5 analogue. In this chapter, the N-terminus of Ltc5 will be truncated by 4 to 10 residues, removing one additional residue at a time and thereby decreasing the hydrophobicity at the N-terminus of the resulting analogues even further. The shortest truncated analogue lacks almost the entire N-terminal helix as defined in Chapter 5. Only residue 11 is kept, as it provides hydrogen bonding partners that help stabilise the remaining C-terminal helix part.

The truncation after position 4 was chosen as a starting point, as the first three residues had already been removed in the work of Polyansky et al.⁷⁸ One helical turn corresponds to approximately 3.6 amino acids.^{23,24} Removing the first four residues therefore eliminates the first helical turn of Ltc5, comprising Gly1, Phe2, Phe3 and Gly4. The corresponding analogue is referred to as Ltc5 5-28.

A complete list of all truncated analogues, including their molecular weights (Da), net charges, HPLC RTs and number of hydrophobic and aromatic residues, is provided in Table 17. The peptides were prepared by solid-phase peptide synthesis at IBG-2, KIT, by A. Eisele and K. Scheubeck.

Table 17: List of Ltc5 analogues with a truncated N-terminus.

The table includes the nomenclature of the N-terminally truncated Ltc5 analogues (NTAs), their primary sequence, molecular weight (Mw) in Dalton [Da], net charge, number of hydrophobic residues (HR) and aromatic residues (AR) [n], as well as the HPLC retention time (RT). A total of seven shortened Ltc5 analogues were synthesised.

Peptide name	Primary sequence	HR/AR [n]	Mw [Da]	Net Charge	HPLC RT
Ltc5 WT	GFFGKMKEYFKKFGASFKRRFANLKKRL-amide	12 / 7	3430.17	+10	12.3
Ltc5 5-28	KMKEYFKKFGASFKRRFANLKKRL-amide	10 / 5	3021.71	+9	9.9
Ltc5 6-28	MKEYFKKFGASFKRRFANLKKRL-amide	10 / 5	2893.55	+8	9.6
Ltc5 7-28	KEYFKKFGASFKRRFANLKKRL-amide	9 / 5	2762.35	+8	9.3
Ltc5 8-28	EYFKKFGASFKRRFANLKKRL-amide	9 / 5	2634.17	+7	9.7
Ltc5 9-28	YFKKFGASFKRRFANLKKRL-amide	9 / 5	2505.06	+8	9.4
Ltc5 10-28	FKKFGASFKRRFANLKKRL-amide	8 / 4	2341.88	+8	9.1
Ltc5 11-28	KKFGASFKRRFANLKKRL-amide	7 / 3	2194.71	+8	8.7

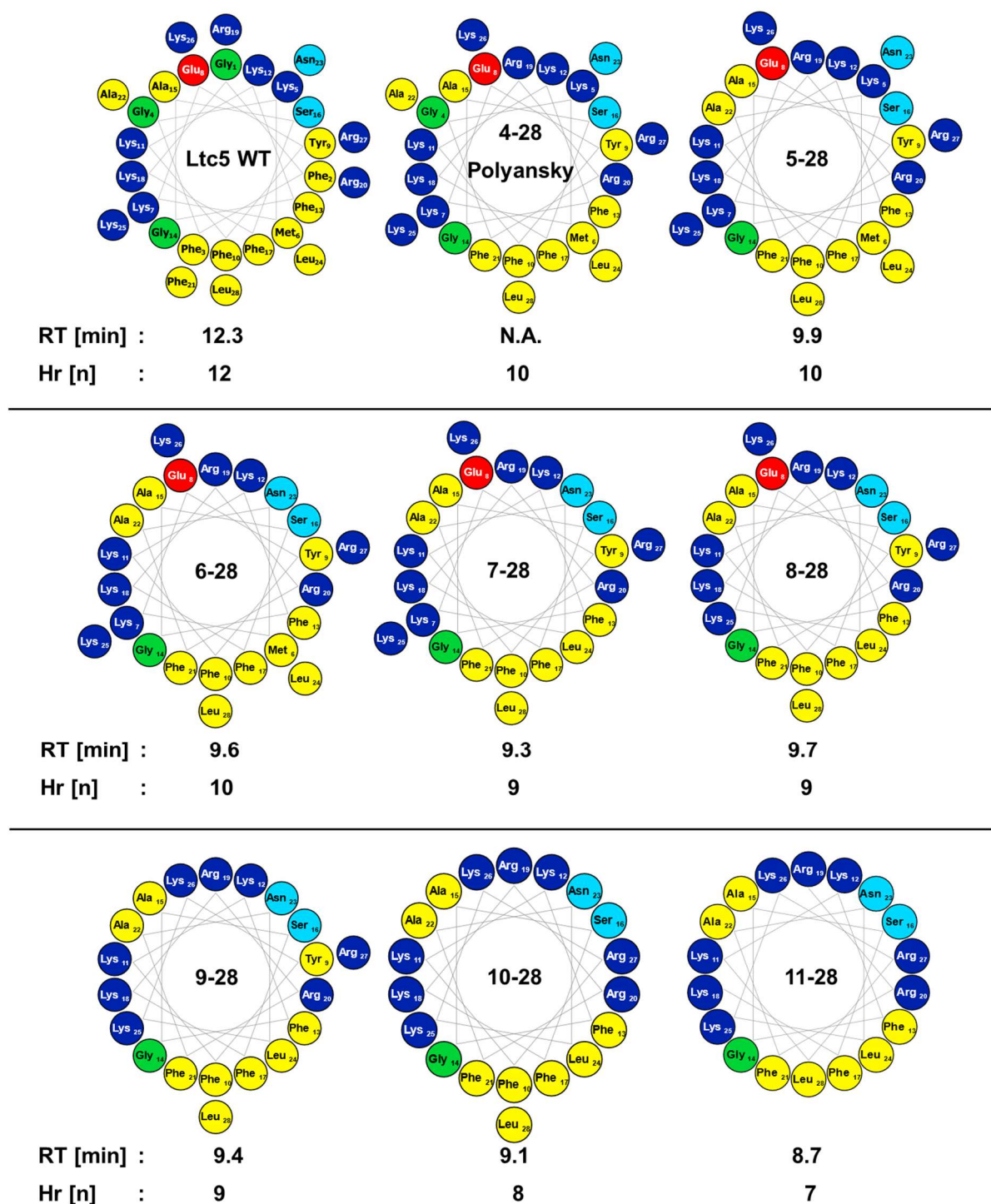


Figure 34: Helical wheels of all seven truncated analogues of Ltc5.

The list includes the truncated analogue previously described by Polyansky et al. and that of the Ltc5 WT. The positions of the remaining residues are shown in the peptide's name. All truncated analogues retain the amphipathic character of the WT peptide. The HPLC RT and the number of hydrophobic residues are provided below each helical wheel.

The helical wheel projections of all seven Ltc5 analogues with a truncated N-terminus (NTAs) are shown in **Figure 34** alongside the previously analysed analogue by Polyansky et al. and the Ltc5 WT.

In the helical wheel projection, the distinct hydrophobic sector (yellow residues) can be identified in every truncated analogue. All truncated analogues retain an amphipathic character, if folded into an α -helix. Whether the NTAs are still able to fold into α -helical secondary structures will be monitored using CD spectroscopy. Induced changes in hydrophobic matching and polarity caused by the truncations may have the potential to alter the orientation of the shorter peptides within the membrane. This aspect will be investigated using OCD spectroscopy. Effects on the activity caused by truncating the N-terminus of Ltc5 will be analysed using MIC assays, hemolysis assays and vesicle leakage assays. The results of these methods will determine whether truncating the N-terminus of Ltc5, beyond position 3, increases the therapeutic index of the resulting analogues, thus fulfilling the aim of this chapter. Additionally, the question of whether the highly hydrophobic N-terminal region, featuring the 2 1 2 pattern of hydrophobic residues, is required for the structure and antimicrobial efficacy of Ltc5 will be answered.

8.1 Results

8.1.1 Peptide Synthesis

All analogues were successfully obtained by Fmoc solid-phase synthesis and purified by RP-HPLC (see Chapter 12 for details).

8.1.2 CD and OCD spectroscopy

Truncations at the highly hydrophobic N-terminus reduce the overall hydrophobicity of the resulting Ltc5 analogues. Five of the 12 hydrophobic residues in Ltc5 are located within the N-terminal helix. As discussed in Chapter 7, hydrophobicity is required for the activity of Ltc5. The decreased hydrophobicity could potentially influence the interaction of the truncated Ltc5 analogues with lipid bilayers as well as their behaviour in solution.

One way to probe AMP interactions with lipid bilayers, as well as their behaviour in solution, is CD spectroscopy. The secondary-structure observed in CD spectra recorded in the presence of SUVs reflects the peptide's membrane interaction to a certain extent, whereas secondary structures observed in solution reflect a peptide's tendency to oligomerise or aggregate. The secondary structures of the truncated analogues were investigated under two different conditions. 1: in aqueous solution containing 10 mM PB, and 2: in the presence of SUVs composed of DMPC/DMPG (7:3). Measurements in 10 mM PB were aimed at probing potential phosphate dependent aggregation or oligomerisation. The overall hydrophobicity of the NTAs is decreased compared to the Ltc5 WT (see HPLC RT, **Table 17**). This decreases the likelihood of aggregation and oligomerisation.¹¹⁰ Ltc5 WT showed no signs of aggregation or oligomerisation in either H₂O or 10 mM PB (see Chapter 5). If the NTAs do not exhibit aggregation or oligomerisation tendencies in PB, their likelihood of forming aggregates or oligomers in water is expected to be low as well. Therefore, measurements in water will only be performed if indications of aggregation or oligomerisation are observed in PB. The measurements in 10 mM PB were performed at 25°C as described previously (see methods, Chapter 12). The resulting spectra in PB are shown in **Figure 35**. Peptide concentration was determined using UV spectroscopy prior to all CD measurements.

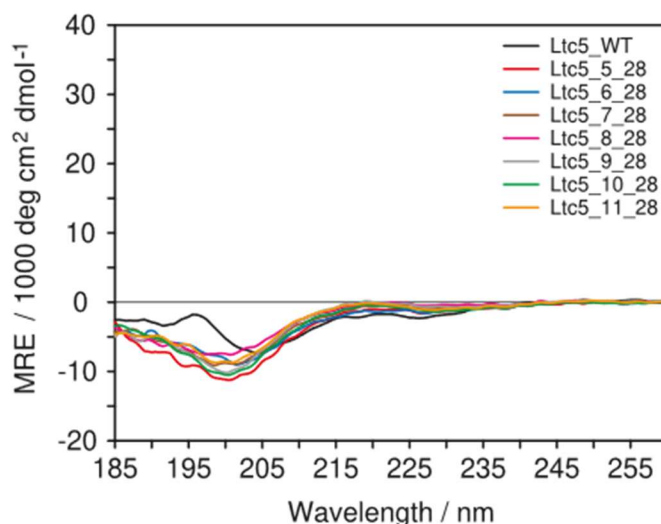


Figure 35: CD spectra of Ltc5 analogues with a truncated N-terminus.

Measurements were conducted in 10 mM PB at 25°C.

The CD spectra measured in 10 mM PB of all NTAs show characteristics of predominantly unordered secondary structures. The NTAs of Ltc5 do not show any

tendencies to form phosphate dependent secondary structures. The CD spectra were deconvoluted using the online tool and databases of DichroWeb.^{58,59,93} The results of the deconvolution are shown in **Table 18** and support the observations made in the CD spectra. The Ltc5 WT is 36 % unordered and 16 % α -helical in PB. The NTAs of Ltc5 show unordered content between 55 % and 60 % and α -helical contents between 2 % and 4 %. Based on these results, measurements in water were not conducted.

The CD spectra of the truncated analogues, measured in 10 mM PB in the presence of SUVs composed of DMPC/DMPG (7:3) and at a P/L of 1:50, are shown in **Figure 36**.

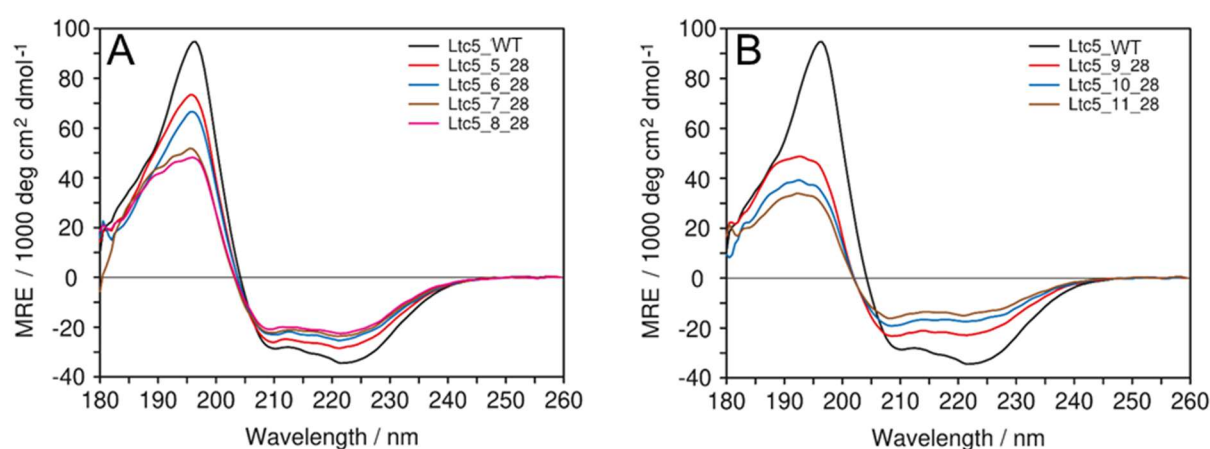


Figure 36: CD-spectra of the truncated Ltc5 analogues measured in SUVs.

SUVs were composed of DMPC/DMPG (7:3) at a P/L of 1:50. The measurements were conducted at 30 °C. A) CD spectra of Ltc5 5-28 to Ltc5 8-28. B) CD spectra of Ltc5 9-28 to Ltc5 11-28.

In the spectra measured in the presence of SUVs, two main observations can be made:

- 1) There is a length-dependent decrease in the intensity of the bands associated with α -helical secondary-structure (positive band at ~197 nm and negative double minima at 208 nm and 222 nm).
- 2) The relative intensity between the band at 208 nm and 222 nm is decreased depending on the length of the observed peptide. Spectra of shorter peptides show a decrease of the negative band at 222 nm compared to the band at 208 nm. Deconvolution using the web tool and database of DichroWeb^{58,59} (**Table 18**) revealed that both effects can be associated with a decline in α -helicity dependent on peptide length of the NTAs. The Ltc5 WT is 89 % helical in contact with lipid bilayers. The NTAs can be divided into two groups. Ltc5 5-28 and Ltc5 9-28 form

the first group. They show little to no change in helicity with 88 % to 80 % α -helical content, respectively. The second group includes Ltc5 10-28 and Ltc5 11-28. These two peptides show a significant reduction in α -helical content compared to the Ltc5 WT with 68 % for Ltc5 10-28 and 61 % for Ltc5 11-28.

Throughout both groups, one general trend can be observed. More extensively truncated N-termini seem to correlate with less α -helical content. The outlier is Ltc5 9-28, which shows a similar α -helical content to the longer NTA Ltc5 7-28. And truncations exceeding position nine have a significant impact with regard to secondary structure.

Table 18: Deconvolution of the CD spectra of Ltc5 WT and the NTAs.

The results shown correspond to the spectra measured in either in 10 mM PB measured at 25°C (top) or 10 mM PB in the presence of SUVs made from DMPC/DMPG 7:3 at a P/L of 1:50 measured at 30°C (bottom). Deconvolution includes the secondary-structure motifs helix, strand, turn and unordered.

PB	Secondary structure motif [%]			
	Helix	Strand	Turn	Unordered
Ltc5 WT	16	22	25	36
Ltc5 5-28	4	22	14	60
Ltc5 6-28	3	25	14	57
Ltc5 7-28	3	27	14	55
Ltc5 8-28	2	28	14	55
Ltc5 9-28	2	28	13	55
Ltc5 10-28	3	25	13	58
Ltc5 11-28	2	26	14	57

SUVs	Secondary structure motif [%]			
	Helix	Strand	Turn	Unordered
Ltc5 WT	89	6	3	3
Ltc5 5-28	88	6	4	3
Ltc5 6-28	86	6	4	4
Ltc5 7-28	82	3	5	9
Ltc5 8-28	80	5	4	10
Ltc5 9-28	83	1	5	9
Ltc5 10-28	68	6	8	19
Ltc5 11-28	61	8	10	20

The orientation of the truncated Ltc5 analogues in lipid bilayers was next analysed using OCD spectroscopy. Experimental conditions were kept identical to those of the previous OCD measurements to ensure comparability. Lipid bilayers composed of DMPC/DMPG (7:3) were reconstituted with peptide at a P/L of 1:100, and measurements were carried out above the T_m of the lipids at 30°C as previously

described (see methods, Chapter 12). The resulting OCD spectra are shown in **Figure 37**. Measurements were performed at least in duplicate.

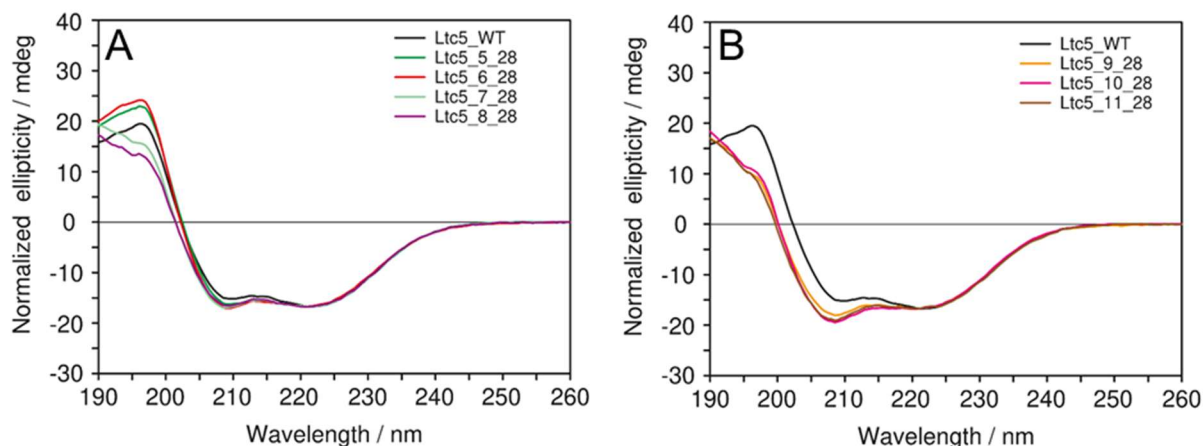


Figure 37: Normalized OCD spectra of the NTAs and the Ltc5 WT.

Measurements were conducted in DMPC/PG (7:3), P/L 1:100, hydration rate 97 %, 30°C. Panel A) shows the spectra of Ltc5 5-28 until Ltc5 8-28. Panel B) contains the spectra of Ltc5 9-28 until Ltc5 11-28.

The OCD spectra of the truncated Ltc5 peptides display the same artefact caused by the high number of aromatic residues, as previously described.⁴⁷ The intensity of the negative band at 208 nm increases with each removal of an aromatic residue in the NTAs. Thus, the reduction in signal at 208 nm is expected to reflect not only changes in peptide orientation within the lipid bilayer, but also the number of aromatic residues present. Ltc5 WT, which contains all seven aromatic residues, exhibits the lowest negative signal intensity at 208 nm after normalisation. The analogues Ltc5 5-28, 6-28, 7-28, and 8-28 (panel A, **Figure 37**) all show similar signal intensities, consistent with the fact that each peptide contains five aromatic residues. Ltc5 9-28, which likewise has five aromatic residues, shows a slightly decreased negative signal at 208 nm compared to the other peptides containing five aromatic residues. This difference may be attributed to subtle changes in peptide orientation within the lipid bilayer rather than solely to the described artefact. The highest negative signal intensities at 208 nm are observed for Ltc5 10-28 and Ltc5 11-28, which contain four and three aromatic residues, respectively. The nearly identical intensity of the 208 nm band for these two analogues, despite Ltc5 10-28 having one additional aromatic residue, suggests that

Ltc5 11-28 may be slightly more tilted within the lipid bilayer than Ltc5 10-28. The difference lies within the margin of error of the method. Overall, the OCD spectra of all truncated Ltc5 analogues are consistent with α -helices lying flat on the lipid bilayer in an S-state under the applied testing conditions. None of the NTAs show signs of aggregation or oligomerisation.

8.1.3 Antimicrobial activity

The NTAs of Ltc5 are all amphipathic (see **Figure 34**), α -helical (CD) and oriented in an S-state in lipid bilayers (OCD). Overall, they show a high degree of structural similarities to the Ltc5 WT. Thus, the NTAs are expected to show behaviour similar to the Ltc5 WT with regard to their antimicrobial activity. Potential outliers could be Ltc5 10-28 and Ltc5 11-28. These two analogues showed significantly reduced α -helical content compared to the Ltc5 WT peptide. The antimicrobial activity of the NTAs was analysed using a MIC assay as previously described (see methods, Chapter 12). The MIC results are summarised in **Table 19**. The assay was conducted twice to ensure reproducibility.

Table 19: MIC assay conducted on the Ltc5 WT and all NTAs.

The assay was conducted on two Gram-negative strains, *E. coli* and *Acinetobacter sp.*, and two Gram-positive strains, *B. spizizenii* and *S. xylosus*. The MIC assay was performed in a two-fold peptide serial dilution in a range of 256 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$.

Bacteria strain	MIC [$\mu\text{g/mL}$]							
	WT	5-28	6-28	7-28	8-28	9-28	10-28	11-28
<i>E. coli</i>	4	8	16	16	64	64	128	128
<i>Acinetobacter sp.</i>	4	8	8	16	32	64	256	256
<i>B. spizizenii</i>	4	4	8	4	8	8	8	8
<i>S. xylosus</i>	2	2	2	4	4	2	4	4

The MIC assay revealed clear, strain-dependent antimicrobial activity for the NTAs, which became more pronounced with increasing truncation of the analogues. The activity of the NTAs against the Gram-positive strains *S. xylosus* and *B. spizizenii* remains within one dilution factor of the Ltc5 WT. Even the shortest NTA, namely Ltc5 11-28, is highly active against both strains. The activity of the NTAs against Gram-negative strains, on the other hand, decreases with

further truncation of the N-terminus. Ltc5 5-28 is still highly antimicrobially active with a MIC of 8 µg/mL against both Gram-negative strains. The shorter Ltc5 6-28 and Ltc5 7-28 analogues already show a twofold dilution step increase in MIC against the Gram-negative strains compared with Ltc5 WT and, importantly, also relative to their activity against the Gram-positive strains. The first significant strain dependent difference in activity can be observed for Ltc5 8-28. For this analogue, an increased MIC of 32 µg/mL to 64 µg/mL was observed against Gram-negative strains, while retaining a low MIC of 4 µg/mL to 8 µg/mL against the Gram-positive strains. The further truncated NTAs Ltc5 9-28, Ltc5 10-28 and Ltc5 11-28 continue this trend with further increased MIC values against Gram-negative strains while retaining low MIC values against the Gram-positive strains. Ltc5 10-28 and Ltc5 11-28 combine the highest MIC values observed against Gram-negative bacteria, with values of 128 µg/mL against *E. coli*, 256 µg/mL against *Acinetobacter sp.* while retaining low MIC values against Gram-positive strains. Namely, 4 µg/mL against *S. xylosus* and 8 µg/mL against *B. spizizenii*. These two analogues exert the highest strain specific selectivity observed.

8.1.4 Hemolytic activity

The altered structural properties of the N-terminally truncated Ltc5 analogues were shown to impact the antimicrobial activity. Previous truncation experiments on the N-terminus of Ltc5 by Polyansky et al.⁷⁸ demonstrated a decrease in activity against erythrocyte membranes for an analogue lacking the first three amino acids. To investigate the effects of further N-terminal truncations on hemolytic activity, a hemolysis assay was performed as previously described. As the removal of three amino acids had already resulted in reduced hemolytic activity in the work by Polyansky et al.⁷⁸, the more extensively truncated Ltc5 analogues were likewise expected to exhibit decreased hemolytic activity compared to Ltc5 WT.

The hemolysis assay was conducted as a two-fold peptide serial dilution from 256 $\mu\text{g/mL}$ down to 1 $\mu\text{g/mL}$ as previously described (see methods, Chapter 12). The results of the assay are shown in **Figure 38**. Results are presented as the mean of three separate measurements.

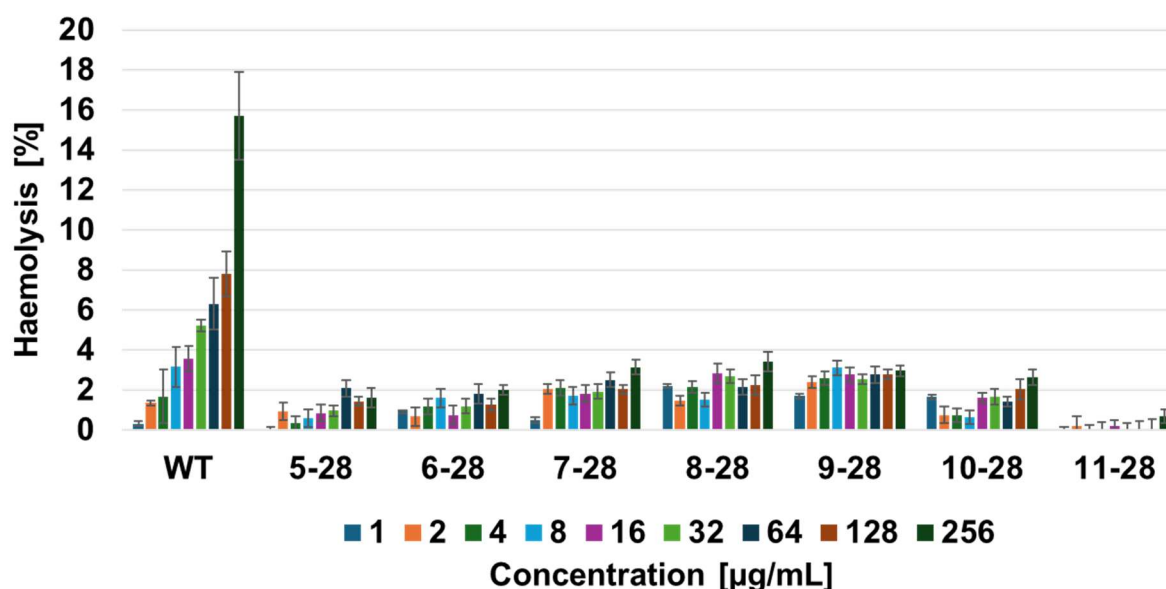


Figure 38: Concentration dependent hemolysis assay performed with Ltc5 WT and all NTAs.

The assay was conducted in a two-fold peptide serial dilution in the concentration range of 256 $\mu\text{g/mL}$ to 1 $\mu\text{g/mL}$.

The hemolysis assay revealed a substantial reduction in hemolytic activity for all NTAs. They all caused well below 5 % hemolysis at all tested concentrations. The data show that the longest truncated peptide, Ltc5 5-28, has very low hemolytic activity (<2 % at 256 $\mu\text{g/mL}$), which increases slightly for the further truncated NTAs and then decreases again for Ltc5 10-28 (2.3 % at 256 $\mu\text{g/mL}$) and Ltc5 11-28 (<1 % at 256 $\mu\text{g/mL}$). Despite the small values and some outliers in the data points, the trend is clearly evident. For Ltc5 11-28, an almost complete loss of hemolytic activity is observed at all concentrations, with a value of 0.6 % at the highest tested peptide concentration of 256 $\mu\text{g/mL}$.

8.1.5 Vesicle leakage

Another method used to assess potential damage caused by the truncated Ltc5 analogues to lipid bilayers was the vesicle leakage assay. LUVs composed of POPC/POPG (1:1) were added to the peptide solutions, and measurements were performed at a P/L of 1:25 at 30°C as previously described (see methods, Chapter 12). The resulting data are presented in **Figure 39**.

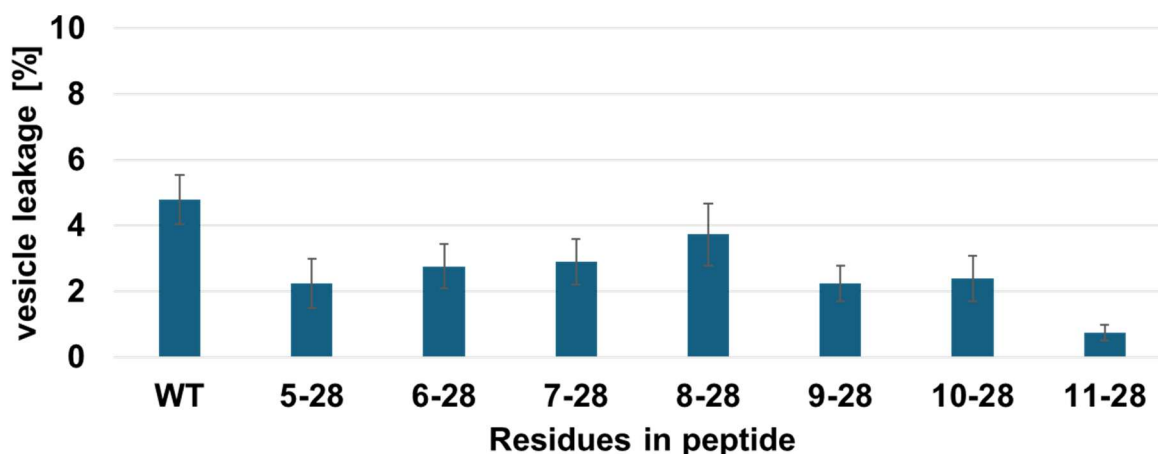


Figure 39: Vesicle leakage assay performed on all NTAs and the Ltc5 WT.

The assay was conducted in LUVs made from POPC/POPG (1:1) at a P/L of 1:25 at 30°C. Ltc5 WT was used as a reference. The x-axis shows vesicle leakage while the y-axis shows the tested analogues.

All tested NTAs show very low vesicle leakage below 4 %. A trend similar to that observed in the hemolysis data can be seen in the vesicle leakage data. Ltc5 5-28 shows a drop in leakage compared to Ltc5 WT. Upon further truncation, the leakage increases up to Ltc5 8-28. Ltc5 9-28 represents the outlier relative to the hemolysis data. It displays reduced vesicle leakage, whereas a slight increase in hemolytic activity was observed compared to Ltc5 8-28. The remaining truncated analogues, Ltc5 10-28 and Ltc5 11-28, show decreased vesicle leakage. Overall, none of the NTAs show any significant vesicle leakage.

8.2 Discussion

The aim of this chapter was to find truncated analogues of Ltc5 with an improved therapeutic index. In previous studies by Polyansky et al.⁷⁸ truncating the N-terminus by three amino acids led to a decrease in hemolytic and cytotoxic side effects for the truncated analogue while retaining high antimicrobial activity. In this chapter, the N-terminus of Ltc5 was systematically truncated up to position 11. The respective analogues were then analysed with regard to their biophysical properties and biological activity.

To monitor changes in the secondary structure induced by the N-terminal truncation and to check for possible aggregation or oligomerisation, all N-terminal truncated analogues (NTAs) were examined using CD spectroscopy. Measurements were conducted either in 10 mM PB or in 10 mM PB in the presence of SUVs composed of DMPC/DMPG (7:3) at a P/L of 1:50. The CD spectra indicate that all NTAs of Ltc5 remain predominantly disordered in PB, similar to the Ltc5 WT peptide. These results suggest that none of the truncated analogues undergo significant aggregation or oligomerisation in solution (**Figure 35**). The secondary-structure deconvolution of the CD spectra in 10 mM PB supports the observations made. It showed that all NTAs are more than 50 % unordered in solution (see **Table 18**). This is an important control, as any pre-formed structure could have implied peptide-peptide assembly. Instead, the peptides appear to be monomeric and unstructured prior to membrane binding. In membrane-mimicking environments, however, clear differences emerge (**Figure 36**). Here the CD spectra show characteristic α -helical features with a negative minimum at 208 nm and 222 nm and a strong positive signal at roughly 195 nm for all NTAs (**Figure 36**). The signal intensity diminishes stepwise as more N-terminal residues are removed.

Deconvolution of the CD spectra confirmed a stepwise decline in helical content with each truncation of a hydrophobic residue, with a notable drop once the truncation extends beyond Tyr9. The reduced α -helicity observed for the truncated Ltc5 peptides could have a variety of causes. The two most likely causes are: 1) reduced binding to the membrane, leaving a larger fraction of peptide in its disordered solution state. 2) the peptides might bind to the membrane but adopt a less helical conformation. The results favour the first explanation. The overall loss of helical signal correlates with the

loss of hydrophobic residues and hence weaker expected membrane association, rather than an intrinsic inability to form helices. In fact, the shorter analogues are still capable of adopting helices (over 70-80 % α -helical content, see **Table 18**) as they retain their amphipathic properties (see helical wheel **Figure 34**).

This interpretation is supported by the systematically decreasing HPLC RT of the truncated peptides (Table 17). The less hydrophobic the respective NTA, the faster it elutes. Ltc5 11-28 (the most truncated peptide) elutes much earlier than the full-length Ltc5 WT peptide, reflecting a significantly reduced overall hydrophobicity. In general, the trend in RT and its correlation with hydrophobicity aligns with the trend in observed helicity in the NTAs. This supports the hypothesis that the highly hydrophobic N-terminal region is critical for strong membrane binding and consecutive folding in Ltc5 WT. All analogues up to Ltc5 9-28 retain over 80 % α -helical content in the presence of SUVs. A sharp decrease in helicity (down by 21 % and 28 % relative to Ltc5 WT) occurs in the shortest NTAs Ltc5 10-28 and Ltc5 11-28. After removing Tyr9, a significant decrease in helicity is observed as mentioned previously. This suggests that Tyr9 is important for membrane binding. The Ltc5 10-28 and Ltc5 11-28 have one hydrophobic (Phe10) or no hydrophobic residue, respectively, at the outer position of the N-terminus. Both show a strong decrease in helicity and, therefore, likely reduces membrane binding. This might suggest that one hydrophobic residue at the N-terminus on its own does not provide as much membrane association as the combination of two hydrophobic residues (Tyr9, Phe10) positioned next to one another. In addition, the two hydrophobic residues are also aromatic. Aromatic amino acids are known to anchor peptides strongly at the lipid water interface of membranes, further adding to this effect.^{94,95} Tyr9 and Phe10 could provide a hydrophobic nucleation site that helps the peptide latch onto the membrane and fold. When this site is removed, the peptide either does not bind as readily or cannot nucleate a stable helical conformation upon binding.

OCD spectroscopy was used to determine how the NTAs orient relative to the lipid bilayer. Notably, all Ltc5 variants tested in this chapter, including the Ltc5 WT and the shortest NTA, exhibit OCD spectra consistent with a surface bound S-state. No emergence of an I-state (inserted orientation) was detected for any of the peptides. The OCD spectra also revealed the previously mentioned artefact associated with aromatic residues. Peptides with a high number of aromatic residues such as Phe or

Tyr can show an decreased negative band near 208 nm after normalization even though they are aligned parallel on the membrane, as reported previously.⁴⁷ In our data, this 208 nm band intensity diminishes when the N-terminal aromatic residues (particularly Tyr9 and Phe10) are removed. Truncations that eliminated aromatic side chains yielded the expected decrease in the OCD signal at 208 nm.

The combined CD and OCD data further support the interpretation that the reduced helicity observed by CD spectroscopy reflects diminished membrane binding rather than an impaired intrinsic ability of the NTAs to form α -helices. In CD measurements, the lower helicity was attributed to less efficient bilayer association of the NTAs as a consequence of their reduced hydrophobicity. Under OCD conditions, however, peptide sample preparation effectively enforces a membrane-bound state due to the limited amount of surrounding bulk water, thereby prohibiting an unbound state. Accordingly, if a peptide exhibits reduced helicity in CD but its OCD spectrum closely resembles that of a highly helical reference peptide, here Ltc5 WT, the decrease in helicity during CD is more plausibly explained by reduced binding efficiency than by a failure to adopt a helical structure. As mentioned previously, all NTAs are still highly amphipathic in their α -helical conformation, lowering the ΔG for α -helix formation. This pattern is observed particularly for the least helical NTAs, Ltc5 10-28 and Ltc5 11-28. Although these peptides show reduced helicity in CD measurements (**Table 18**), their OCD spectra are highly similar to that of Ltc5 WT (**Figure 37**).

The key conclusion from OCD is that all truncated Ltc5 analogues are oriented parallel to the bilayer, just like Ltc5 WT, and that, in combination with the CD data, reduced N-terminal hydrophobicity decreases the binding ability of the NTAs.

The fact that even full-length Ltc5 stays on the membrane surface, and does not tilt into the membrane, suggests that Ltc5 WT likely operates via a mechanism which does not require a tilt into the membrane. Many amphipathic α -helical AMPs, such as magainins or cecropins, similarly remain aligned on the membrane surface where they cover and disrupt the lipid bilayer without full insertion.^{111,112} In such mechanisms, initial binding to the membrane surface is crucial. The N-terminus of Ltc5 could be evolutionarily optimized for membrane binding. The N-terminal helix part of Ltc5 WT contains an unusually high density of hydrophobic residues, including four aromatic ones (Phe2, Phe3, Tyr9, Phe10) within the first 10 positions. Aromatic residues in

peptides are well-known to serve as interfacial anchors: their side chains preferentially partition into the membrane interface region, where the polar/apolar boundary provides an energetically favourable site.¹¹³ In Ltc5 WT, the two Phe residues at positions two and three likely act as the primary anchors that initially penetrate the lipid headgroup region. This then facilitates insertion of the nearby Met6 followed by the aromatic pair Tyr9 and Phe10. The hypothesis is that binding and folding occur sequentially. First, electrostatic attraction brings the positively charged peptide to the negatively charged membrane surface. Then, the N-terminal hydrophobic patch (especially Phe2 and Phe3) embeds into the outer leaflet, nucleating the first turn of the α -helix. From this nucleus, the helix can propagate. Additional hydrophobic residues along the peptide (Leu, Phe, Tyr in the core and C-terminal helix) insert into the membrane as the peptide folds into its full α -helical secondary-structure across the membrane interface.

This model, depicted schematically in **Figure 40**, explains why the Ltc5 WT so effectively attains a helical structure upon membrane contact. The highly hydrophobic N-terminus anchors the peptide into the membrane, and folding follows cooperatively.

N-terminal truncation disrupts this mechanism. Reducing the hydrophobicity at the N-terminus leads to the peptide having a weaker initial foothold on the membrane. Indeed, all truncated analogues still adopt an amphipathic helix when forced to bind (OCD), but they are observed to bind less (CD), as previously explained. The Gibbs free energy during the binding and folding process is governed by three effects.¹¹⁴ First, the initial electrostatic attraction between a cationic peptide and the anionic membrane. Second, hydrophobic embedding of the peptide in the membrane. Third, hydrogen bonding during the transition from random coil to α helix formation. In a paper by J. Seelig¹¹⁴ the decrease of ΔG observed during binding due to hydrophobic effects and the decrease in ΔG observed for the subsequent folding (hydrogen bond formation in the peptide backbone per α -helical residue) are described as being about equal for the 23 residue long AMP magainin 2a. The truncation of the N-terminus of Ltc5 reduces the NTA's hydrophobicity and length. Thus, the overall reduction in Gibbs free energy due to binding and folding should also be reduced for the NTAs compared to the Ltc5 WT. This suggests a reduction in membrane binding, as described by the combined CD and OCD results.

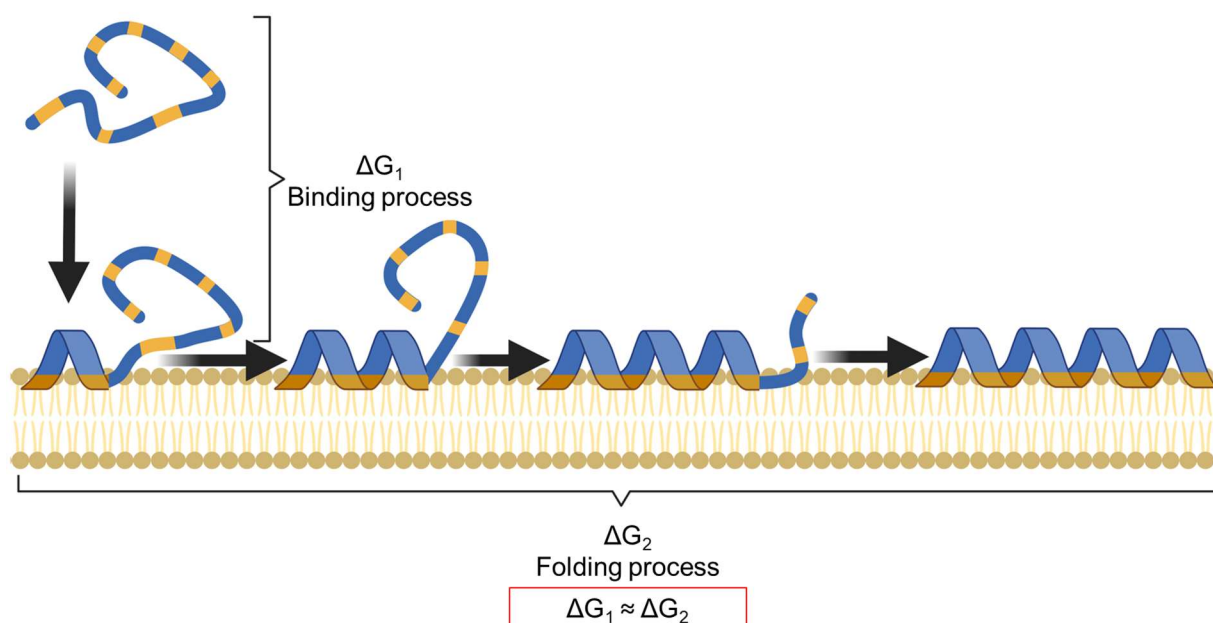


Figure 40: Cartoon depiction of the membrane-binding and folding mechanism of amphipathic, α -helical peptides.

The initially disordered peptide first makes contact with the lipid bilayer and consecutively begins to fold into its α helical secondary structure. Seelig et al.¹¹⁴ showed, using calorimetric methods on the AMP magainin 2a, that the reduction in free energy associated with membrane binding through hydrophobic effects alone (excluding electrostatics¹¹⁴) is similar to the reduction in free energy accompanying the subsequent folding process ($\Delta G_1 \approx \Delta G_2$). A similar distribution could be possible for Ltc5. Created in <https://BioRender.com>

Polyansky et al.⁷⁸ found that deleting the first three residues of Ltc5, removing two out of the five N-terminal hydrophobic/aromatic residues in the process, caused a pronounced drop in its membrane-disruptive activity. Our stepwise truncation study extends this insight by pinpointing Tyr9 and Phe10 as an essential secondary membrane anchoring point in addition to Phe2 and Phe3. Tyr9 and Phe10 could be responsible for the membrane engagement in the truncated Ltc5 analogues after the initial Phe2/Phe3 anchors are removed. This is supported by the Ltc5 10-28 analogue (lacking Tyr9) binding significantly less efficiently (CD, OCD) than the Ltc5 9-28 analogue (which retains Tyr9). Despite both having an aromatic residue right at the N-terminus, Phe10 in Ltc5 10-28 and Tyr9 in Ltc5 9-28, only the NTA with two adjacent hydrophobic residues remains highly helical/bound. Having two adjacent hydrophobic residues (Tyr9 and Phe10) at the membrane interface seems to increase the membrane affinity compared to just one. This pair might create a strong hydrophobic

anchor. While removal of one of the two double-hydrophobic motifs (here Phe2 and Phe3) appears to be tolerated with respect to initial binding and subsequent folding, disrupting the second double-hydrophobic motif (Tyr9 and Phe10) as well results in significantly less effective membrane binding. The Ala-scan in Chapter 5 showed that the removal of a single residue within either of these two double hydrophobic anchoring points is well tolerated.

The functional consequences of N-terminal truncation are clearly reflected in the NTAs' antimicrobial activities. Full-length Ltc5 WT is a broad-spectrum antimicrobial peptide, active against both Gram-positive and Gram-negative bacteria.^{47,106} Truncating the N-terminus leads to a decrease of antimicrobial activity against Gram-negative bacteria as a function of the N-terminal hydrophobicity. The data show that with progressive truncation of the N-terminus, the Gram-selective behaviour becomes more pronounced. Ltc5 7-28 shows MIC values slightly elevated against Gram-negative bacterial strains (8 µg/mL-16 µg/mL) and low against Gram-positive bacterial strains (2 mg/mL-4 µg/mL). For Ltc5 9-28, the Gram-negative MIC values have jumped to 64 µg/mL-128 µg/mL for Gram-negative strains, well beyond anything considered highly antimicrobial, while the antimicrobial activity observed for the Gram-positive bacteria strains remains high with 2 µg/mL to 8 µg/mL. Truncating beyond position nine, as in Ltc5 10-28, increases the Gram-negative MIC values to 128 µg/mL-256 µg/mL. The respective analogues are essentially inactive against Gram-negative bacteria, while the MIC values against Gram-positive bacteria remain in the single-digit area. Clearly, a threshold amount of N-terminal hydrophobicity is required for activity against Gram-negative bacterial strains.

The most plausible explanation for the Gram-selectivity is, that Gram-negative bacteria present a much tougher barrier for peptide entry and the truncated peptides lack the necessary attributes to overcome it. Unlike Gram-positive bacteria, which have a single lipid membrane surrounded by a porous peptidoglycan layer, Gram-negative bacteria possess two lipid membranes.^{23,24} They possess an outer membrane in addition to the inner cytoplasmic membrane, with the two separated by a peptidoglycan-containing periplasm. This dual-membrane envelope can span ~20 nm-80 nm in thickness^{23,24}, significantly more than the ~15 nm-20 nm a peptide would have to traverse to penetrate a Gram-positive bacterial cell.^{23,24} The outer membrane of Gram-negative bacteria, rich in lipopolysaccharide (LPS), is known to act as a formidable permeability

barrier that can repel or slow down the entry of antimicrobial peptides. A peptide needs sufficient hydrophobic and electrostatic drive to insert into or disrupt the outer membrane before it can reach the inner membrane. In our series, full-length Ltc5 with its hydrophobic N-terminus, including the two double motifs of Phe2/Phe3 and Tyr9/Phe10, is most likely capable of traversing or disturbing the outer membrane with ease. The truncated analogues, with progressively lower hydrophobicity, might be less adept at this. The decreased hydrophobicity of analogues like Ltc5 9-28 and Ltc5 10-28 likely translates to weaker membrane interactions and an inability to partition into the outer membrane leaflet. Consequently, these shorter peptides either do not accumulate at the bacterial surface to sufficient concentrations to exert their mechanism of action or cannot insert deep enough to cause lethal membrane perturbations in Gram-negative bacterial membranes. By contrast, Gram-positive bacteria lack an outer membrane, so even a less hydrophobic peptide can more readily access the single lipid bilayer and exert its effects. This explains why Ltc5 10-28, for example, still killed *B. spizizenii* and *S. xylosus* at low micromolar levels but was essentially inactive against *E. coli*. In summary, truncation converts Ltc5 from a broad-spectrum AMP into a Gram-positive-selective AMP, possibly by lowering its ability to negotiate the outer membrane of Gram-negative bacteria.

From a mechanistic standpoint, our findings highlight the importance of the hydrophobic patch at the N-terminus in enabling Ltc5 to target a wide range of bacteria. The Ltc5 WT, with its pair of aromatic Phe residues at positions two and three (and additional aromatics within the first ten residues), is optimally equipped to engage bacterial membranes, whereas truncating those features tilts the balance toward lower membrane affinity and loss of activity, especially against Gram-negative bacteria. In practical terms, the N-terminus of Ltc5 can be seen as a determinant of broad activity. It provides the extra hydrophobic “punch” needed to penetrate more robust defences like the outer membrane of Gram-negative bacteria. Without it, the peptide’s antimicrobial spectrum narrows significantly. This could have implications for therapeutic design. Enhancing the hydrophobic character of a peptide’s N-terminus might boost activity against Gram-negative pathogens¹⁰⁶, whereas trimming or removing hydrophobic residues can confine activity to less complex targets like Gram-positive bacterial membranes or membranes lacking strong outer barriers.

To monitor the development of hemolytic side effects for the NTAs, a hemolysis assay was conducted, using human red blood cells. As expected, reducing the hydrophobicity at the N-terminus of Ltc5 markedly decreased its hemolytic potency in every NTA compared to the Ltc5 WT. This is in line with the decreased HPLC-RT observed. This effect was already described previously by Polyansky et al.⁷⁸ for a three residue truncated analogue of Ltc5. Ltc5 10-28 and Ltc5 11-28 again showed the least activity, which can be related to the disruption of the Tyr9/Phe10 double motif. Less hydrophobicity leads to weaker membrane interaction (CD, OCD) and therefore even further reduced hemolytic activity. Within the NTAs only small trends can be observed, which can be considered to fall within experimental error. This correlation between hydrophobicity and hemolysis is well documented for AMPs. Generally, increasing a peptide's overall hydrophobicity tends to increase its lytic activity against mammalian membranes.¹¹⁵ Aromatic residues, in particular, are expected to be able to insert into the lipid raft regions of erythrocyte membranes (which are rich in cholesterol and neutral lipids), disrupting them and causing cell lysis.⁷⁷ Therefore, it makes sense that truncating Ltc5, effectively dialling down its hydrophobicity on the N-terminus, renders it less damaging to RBCs. From an application perspective, this reduced hemolysis is beneficial, as it indicates improved selectivity. A patient with a Gram-positive bacterial infection could be treated with a Gram-positive-selective AMP like Ltc5 11-28, with minimal adverse effects, rather than administering a nonselective AMP, associated with greater side effects. Indeed, our truncations achieved the intended effect of substantially lowering hemolytic activity, which aligns with earlier reports where N-terminal modifications to AMPs reduced their cytotoxicity.⁷⁸

Membrane vesicle leakage experiments further illustrate these points. In an ANTS/DPX-release assay using large unilamellar vesicles (LUVs) composed of POPC/POPG (1:1), the Ltc5 WT does not induce significant leakage of vesicle contents at the P/L tested. A similar behaviour was also expected for the NTAs. Indeed, all NTAs caused low levels of dye leakage (<5 %), consistently lower than the Ltc5 WT. The reduced hydrophobicity of the NTAs, leading to reduced membrane affinity, can once more explain this observation. Ltc5 11-28 showed the lowest activity in this assay, similar to the hemolysis assay. The differences observed compared to the other NTAs are small, but present. This overall low membranolytic activity in hemolysis and vesicle leakage assays suggests that the mechanism of action of Ltc5 and the NTAs might

involve more subtle membrane disruptions than the wholesale formation of large pores, as already discussed in the previous chapters. A disruption of the membrane function rather than its integrity might seem plausible. Another explanation could involve the selective targeting of intracellular components.

In conclusion, the results demonstrate that the N-terminal hydrophobicity of Ltc5 is required for its broad-spectrum antimicrobial activity. Removing even a few residues from the N-terminus can tip the balance, converting Ltc5 from a highly potent, broad-spectrum bacterial toxin into a much milder, Gram-positive-selective peptide with reduced cytotoxicity. The N-terminal hydrophobic double motifs, namely Phe2/Phe3 and Tyr9/Phe10, might act as a membrane anchor, initiating peptide binding and folding at the lipid interface. When both motifs are intact as in the Ltc5 WT, the peptide readily binds to bacterial membranes, folds into a stable amphipathic helix, and can exert its mechanism of action (**Figure 40**). While the presence of one of the double motifs (Tyr9/Phe10) seems to be sufficient for membrane binding, only the presence of both double aromatic motifs enables the optimised membrane interaction of Ltc5 WT. These findings would support a model in which membrane binding and folding are tightly coupled to the N-terminal properties for Ltc5 WT. Strategic placement of hydrophobic residues at the N-terminus of Ltc5 most likely governs the delicate balance between efficacy and selectivity. NTA Ltc5 11-28 represents an optimised antimicrobial agent with pronounced selectivity against Gram-positive bacterial strains and very low hemolytic side effects. It illustrates how a deeper understanding of structure-function relationships can guide the design of new analogues with improved selectivity and therapeutic indices.

9 Unified Mechanism of the Antimicrobial Peptide Latacin 5

Ltc5 is a linear, highly α -helical and amphipathic AMP with a high net positive charge of ten and an unusually high number of aromatic residues in its primary sequence. Ltc5 has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria with MIC values between 2 $\mu\text{g/mL}$ and 4 $\mu\text{g/mL}$, while exerting only minor side effects in the form of hemolysis against human erythrocytes. In this work, it was established, that Ltc5 lies flat on the membrane surface in an S-state by OCD and $\text{ss}^2\text{H-NMR}$ analysis (Chapter 5-8) and shows only little rotational or tilting motion within the membrane. This membrane bound S-state in combination with low membrane disrupting activity (hemolysis, vesicle leakage) led to the conclusion that Ltc5 most likely acts through a non-membranolytic mechanism of action by disrupting the membrane function rather than the structural membrane integrity. The possibility of intracellular targets is also given.

Even though these insights narrow down the possible ways in which Ltc5 exerts its activity, there is still a broad range of mechanisms that could apply. In this chapter, an attempt will be made to gain further insights into effects underlying the mechanism of action of Ltc5. The aim of this chapter is to formulate a unified mechanism of action for Ltc5.

First, the nature of the mechanism will be further investigated. The previously performed MIC assay only allows the determination of bacteriostatic, but not bactericidal, activity. Therefore, a minimal bactericidal concentration (MBC) assay will be conducted to determine whether the mechanism of action under investigation is lethal to bacteria. This chapter will address possible membrane-focused mechanisms of action that fulfil all of the previously described requirements. One example of a non-membranolytic mechanism of action is membrane depolarisation. As prokaryotes do not have intracellular compartments like mitochondria, they solely rely on their outer membrane for energy metabolism. Thus, depolarisation of the bacterial membrane interrupts a fundamental process and can ultimately reduce bacterial growth or cause cell death. In **Figure 41** (panel A), the principle of membrane depolarisation is shown.³⁷ Non-lytic membrane depolarisation is the effect investigated in this chapter. It is used as an umbrella term for the observed phenotype of depolarised membranes. One

possible explanation for this phenotype is the action of anion carriers. In this case, the electrostatic attraction of the peptide pulls anions through the membrane, leading to a reduction or collapse of membrane potential (Panel A, **Figure 41**).³⁷ The exact mechanisms underlying membrane depolarisation without membrane disruption are not yet fully understood, but this phenomenon is regularly observed for AMPs.^{116,117}

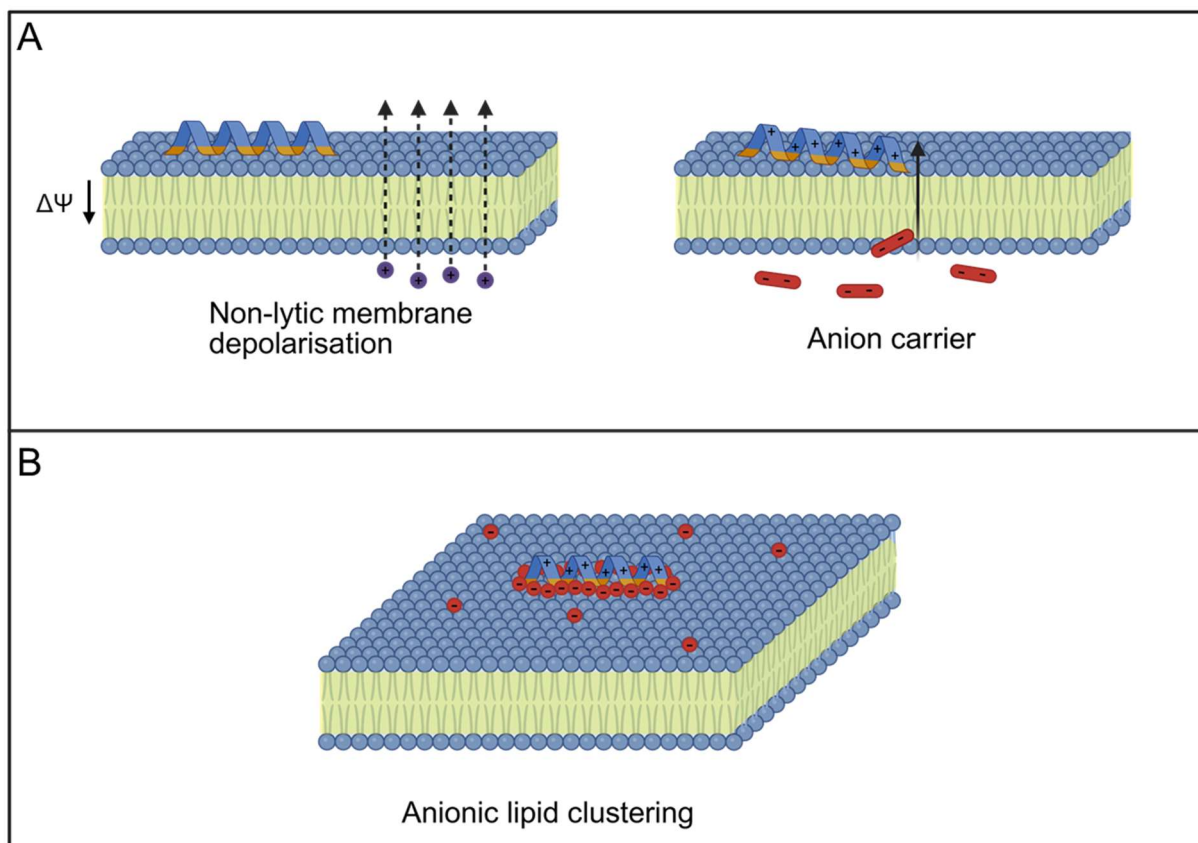


Figure 41: Selection of possible non-membranolytic mechanisms of action of AMPs acting on the membrane itself.

A) mechanisms that lead to depolarisation of the membrane. $\Delta\Psi$ describes the proton motive force. B) anionic lipid clustering. Modified after source 37. Created in <https://BioRender.com>

One premise of this work is that membrane depolarisation caused by Ltc5 happens without major membrane disruption. Thus far, membrane disruption has only been assessed in vitro on membrane mimicking vesicles or in vivo on eukaryotic cells (hemolysis assay). Using a propidium iodide (PI) uptake assay, this scope will be expanded to membrane damage caused to bacteria in vivo. Depolarisation alone could, in principle, also result from pore formation by Ltc5 occurring selectively in bacterial membranes. The PI uptake addresses this issue and provides the needed certainty with regard to membrane disruption in bacteria in vivo, caused by Ltc5.

As mentioned, membrane depolarisation is only an observed phenotype and does not describe a specific mechanism of action. A more direct mechanistic observation that can lead to depolarisation is the clustering of anionic lipids. The possibility of anionic lipid clustering will be investigated using differential scanning calorimetry (DSC). Cationic AMPs have shown the ability to cluster lipids with anionic head groups.^{37,118} This cluster formation can also lead to lipid packing defects that can cause an increased ion permeability. Furthermore, such a mechanism can interfere with the lateral arrangement of lipid bilayers and prohibit the formation of naturally occurring lipid domains.¹¹⁹

The aim of this chapter is to start elucidating the mechanism of action of Ltc5. First, its antimicrobial activity is examined to determine whether Ltc5 exerts a purely bacteriostatic effect or also displays bactericidal properties. Here, a minimum bactericidal concentration (MBC) assay is applied. Next, assays are conducted which align with the previous findings on the biophysical and bioactivity profile of Ltc5. The ability to depolarise bacterial membranes without causing major membrane disruption *in vivo* will be addressed using a combination of a membrane depolarisation assay (DiSC₃(5)) and a PI uptake assay. Anionic lipid clustering could explain membrane depolarisation and is highly plausible given the high positive net charge of ten in Ltc5. This mechanism will be investigated using DSC on model membranes. Although intracellular targets are a possible component of the mechanism of action of Ltc5, the focus in this work is placed on membrane-based mechanisms.

9.1 Results

9.1.1 Minimal bactericidal concentration of Latacin 5

Distinguishing between bacteriostatic and bactericidal activity will help clarify the nature of the mechanism of action of Ltc5. Answering this question is essential before selecting which membrane-based mechanisms to investigate next, as it determines whether the analysis should focus on non-lethal or lethal modes of action. Thus far, the antimicrobial activity of Ltc5 has been assessed solely by its MIC. Although this approach is widely used,^{2,47,64} it has important limitations. In a typical MIC experiment, a relatively small inoculum ($1-2 \times 10^8$ CFU/mL) is exposed to a range of peptide concentrations. If the peptide is bacteriostatic at a given concentration, cells will not

proliferate during the overnight incubation but may remain metabolically active, such that the total number of cells remains essentially unchanged. Given the low absolute number of cells present initially, residual metabolic activity may be insufficient to reduce resazurin added at the end of the MIC assay. Consequently, the colorimetric readout does not reliably discriminate between bacterial killing and bacteriostatic activity. The latter refers to the persistence of a small, non-growing yet viable population that could resume growth after transfer to a fresh agar plate. The concentration that leads to the death of 99.9 % of the bacteria, as determined by CFU count on the agar plates, is considered the MBC.

The MBC assay was carried out with slight adaptations as previously described (see methods, Chapter 12).¹²⁰ The results of the MBC assay are summarised in **Table 20**. The assay was performed twice in triplicate to ensure reproducibility.

Table 20: Results of the MBC assay of Ltc5.

The assay was performed on the same strains as the MIC assay in Chapter 5. The table contains the respective strain in the left column, the MIC value of the Ltc5 WT in the central column and the MBC value of the Ltc5 WT in the right column.

Bacteria strains	MIC/MBC [$\mu\text{g/mL}$]	
	MIC	MBC
<i>E. coli</i>	4	4
<i>Acinetobacter sp.</i>	4	4
<i>B. spizizenii</i>	4	4
<i>S. xylosus</i>	2	4

The MBC assay shows that Ltc5 is bactericidal at its MIC for all tested strains. Only for *S. xylosus* is the MBC one dilution step higher than the MIC (2 $\mu\text{g/mL}$ MIC vs. 4 $\mu\text{g/mL}$ MBC), which can be considered within experimental error. Consequently, membrane depolarisation was selected for further investigations of the mechanism of action of Ltc5. Membrane depolarisation can induce bacterial death without the need for membrane disruption.¹²¹

9.1.2 Membrane depolarisation induced by Latacin 5

The MBC of Ltc5 was determined to be the same as its MIC. Therefore, the mechanism of action of Ltc5 must account for bactericidal activity induced by a surface bound peptide. One known mechanism of action that would fulfil all requirements is membrane depolarisation.³⁷ Thus, the ability of Ltc5 to depolarise bacterial membranes was investigated using the cationic dye 3,3-dipropylthiadicarbocyanine iodide (DiSC₃(5)) in a membrane depolarisation assay. DiSC₃(5) is a fluorescent dye with an excitation maximum at 622 nm and an emission maximum at 670 nm.¹²¹ It accumulates in polarised membranes, where its fluorescence signal is self-quenched. Upon membrane depolarisation, the dye is released back into the medium, resulting in an increase in fluorescence intensity. This makes DiSC₃(5) a convenient and cost-effective probe for monitoring changes in membrane potential.

The membrane depolarisation assay protocol used in this work was adapted from Derk te Winkel et al. (2016).¹²¹ Bacteria (namely *E. coli*) were washed twice in PBS containing 0.2% glucose as carbon source, as the standard growth medium (Mueller–Hinton (MH) broth) interfered with the fluorescence signal. DiSC₃(5) is added, and its accumulation in the bacterial membrane is monitored by measuring the fluorescence emission at 670 nm. As the dye accumulates in the membrane, fluorescence is progressively self-quenched, leading to a decrease in signal. Once a stable minimum is reached, the assay is initiated by adding the AMP of interest, here Ltc5, to the bacterial suspension. **Figure 42** shows representative data acquired during the DiSC₃(5) accumulation in the membrane and subsequent self-quenching of the fluorescence signal. A stable baseline is typically reached after approximately 20 minutes.

Ltc5 was added to the prepared *E. coli* (OD₆₀₀ 0.2) emulsion using a two-fold dilution series ranging from 256 µg/mL to 2 µg/mL. The results of the depolarisation assay with Ltc5 on *E. coli* are presented in **Figure 43**. The assay was conducted three times to ensure reproducibility.

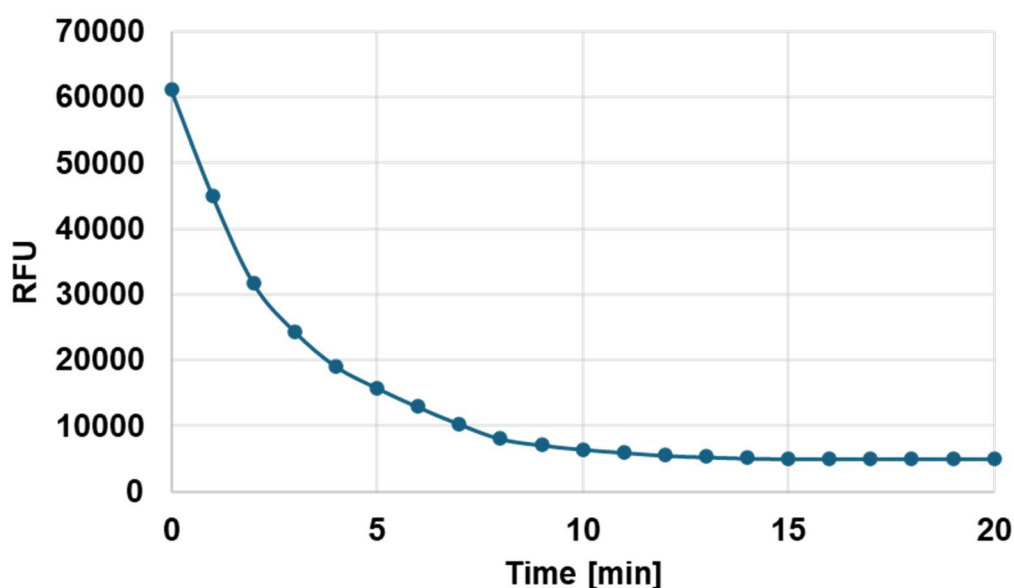


Figure 42: DiSC₃(5) uptake after adding the dye to the washed and rested *E. coli* emulsion.

The signal reduction (Excitation 622 nm, emission 670 nm) correlates to the DiSC₃(5) accumulating in the bacterial membrane leading to a self-quenching of the fluorescence.

A concentration-dependent depolarisation induced by Ltc5 was observed. At low peptide concentrations of 8 µg/mL and below (**Figure 43**, A), only minor, transient depolarisation was detected, which was subsequently reversed as the fluorescence signal returned close to the baseline level. A similar effect was observed at 16 µg/mL, 32 µg/mL, and, to a lesser extent, at 64 µg/mL. However, at these concentrations, the membrane potential was not fully restored. The lowest concentration at which an irreversible depolarisation was observed was 16 µg/mL (panel B, **Figure 43**). Increasing the peptide concentration from 128 µg/mL to 256 µg/mL resulted only in a modest increase in fluorescence, suggesting that the membrane is already close to complete depolarisation at 128 µg/mL.

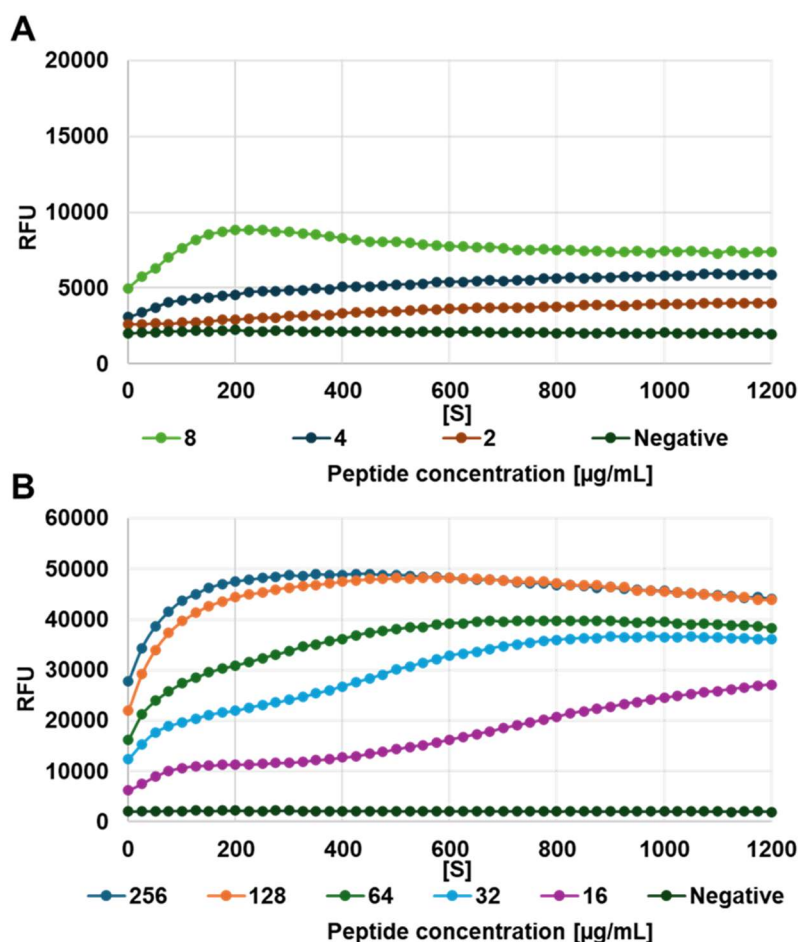


Figure 43: Membrane depolarisation assay using the cationic dye DiSC₃(5).

Ltc5 was added in a two-fold dilution series from 256 µg/mL to 2 µg/mL. A) Results at peptide concentrations of 2 µg/mL to 8 µg/mL. B) shows the data of 16 µg/mL peptide till 256 µg/mL. The negative control does not contain any peptide. The x-axis shows the fluorescence intensity in RFU. The y-axis shows the duration of the assay in seconds. The assay was carried out with *E. coli* grown to the exponential phase and washed twice in PBS containing glucose. The assay was performed at an OD₆₀₀ of 0.2 at 37°C.

9.1.3 In vivo membrane disruption induced by Latacin 5

The current working hypothesis for Ltc5 is that it induces membrane depolarisation without causing substantial membrane disruption. Previous assays in this study examined membrane-disruptive effects in SUVs and in human eukaryotic cells. The PI uptake assay extends this analysis by assessing membrane disruption in bacterial cells in vivo and therefore provides an important complement to the depolarisation assay. A pronounced increase in PI uptake following treatment with Ltc5 would indicate

membrane disruption or pore formation in bacterial membranes. The assay conditions of the PI uptake assay closely resemble those used in the depolarisation assay, thereby ensuring comparability between the datasets. The PI uptake assay provides an elegant way to assess selective membrane disruptive activity of Ltc5 against bacterial membranes.

In this assay, the membrane-impermeant dye PI is added to a bacterial suspension (see Chapter 12). If the peptide compromises the membrane sufficiently to allow PI entry, the dye crosses the membrane and intercalates into the DNA of targeted bacteria. DNA intercalation of PI increases its fluorescence intensity by approximately 20-30-fold.¹²² Therefore, an increase in fluorescence emission is interpreted as an indication of severe membrane disruption or pore formation. DNA-bound PI is excited at 535 nm and emits at 617 nm.⁴¹ The PI uptake assay was performed with minor adaptations as described by Ye et al. (see methods, Chapter 12).⁴¹ The PI uptake assay was conducted with Ltc5 on *E. coli*. The resulting spectra are shown in **Figure 44**. Measurements were repeated three times.

For Ltc5, only minimal PI uptake was observed compared to the negative control without peptide. The highest fluorescence was detected at a peptide concentration of 16 µg/mL, where the signal increased from ~20,000 RFU (negative control) to ~42,000 RFU, corresponding roughly to a two-fold increase. Repeated experiments showed that the maximal RFU was not obtained at the highest peptide concentration tested, but consistently at around a peptide concentration of 16 µg/mL. The magnitude of PI uptake appears to remain small. Thus, under the tested conditions, Ltc5 does not appear to induce substantial PI entry, even at high concentrations.

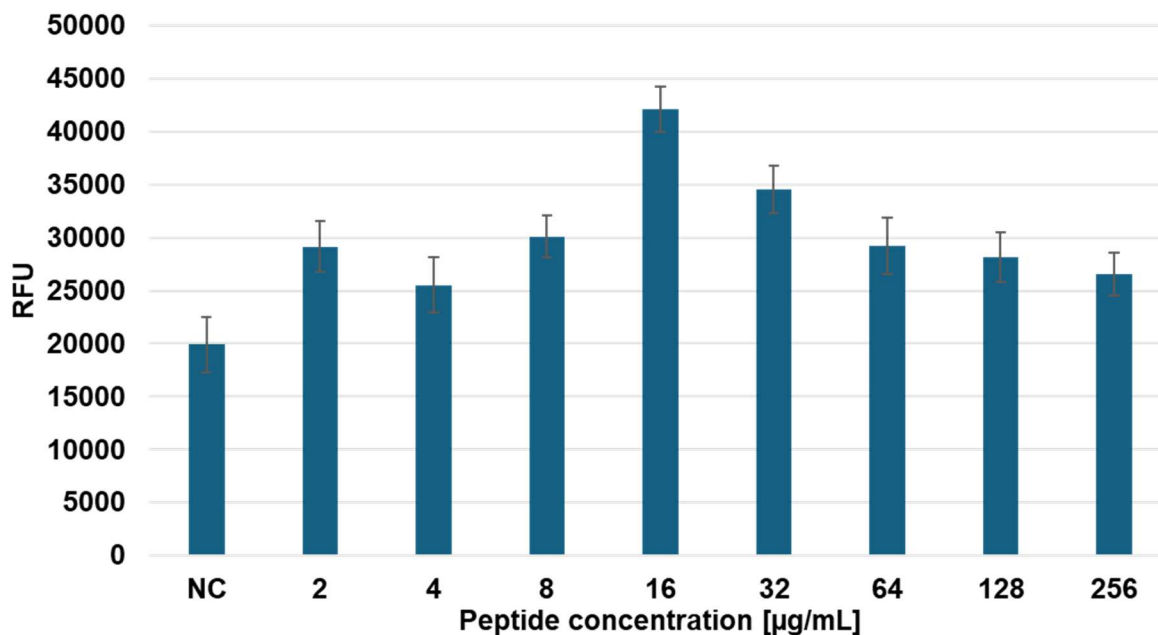


Figure 44: Membrane disruption assay, using PI, with Ltc5 WT.

The assay was carried out with *E. coli* grown to the exponential phase and washed twice in PBS containing 0.2 % glucose. After washing, the cells were diluted to an OD₆₀₀ of 0.1 which is the final OD used for the assay.

9.1.4 Anionic lipid clustering caused by Latarcin 5

As Ltc5 was shown to induce depolarisation of bacterial membranes, an anionic lipid clustering assay was subsequently performed using differential scanning calorimetry (DSC). As discussed previously, membrane depolarisation should be regarded as a phenotypic outcome rather than a mechanism of action in itself. In contrast, anionic lipid clustering represents a more specific mechanistic process that may account for the observed membrane depolarisation in the absence of detectable membrane disruption. The lipid clustering experiment was conducted according to Wadhwani et al., with modifications.¹¹⁸ POPE was mixed with the anionic cardiolipin analogue TOCL at a molar ratio of 75:25. The lipid mixture was co-solubilised in MeOH/CHCl₃ (1:1) together with Ltc5 WT at a P/L of 1:20. After evaporation of the organic solvent under a nitrogen stream and subsequent drying in vacuo, the lipid-peptide film was rehydrated in PIPES buffer to form MLVs. With this sample preparation, the peptide is

expected to be evenly distributed across all lamellae of the MLVs. In addition, a peptide-free lipid sample was prepared as a control (see methods, Chapter 12).

For the pure lipid sample, one ideally expects a single cooperative transition (single T_m) corresponding to a well-mixed POPE/TOCL system undergoing a phase transition at one temperature. If lipid segregation occurs, the transition peak is expected to split into at least two peaks, which shift toward the individual T_m values of POPE and TOCL, respectively. During all DSC measurements, each sample underwent three heating and three cooling scans ranging from 3°C to 40°C with a ΔT of 1°C/min. The gel-to-liquid crystalline phase transition temperature of TOCL is well below 0°C at -15°C, and that of POPE is around 24°C.¹¹⁸ Thus, the mixed system is expected to exhibit a T_m peak in between these temperatures. Measuring below freezing temperature carries the risk of damaging the DSC unit. Therefore, all measurements were conducted at 3°C and higher.

The DSC curves were reproducible across the three heating and cooling scans. **Figure 45** shows three heating cycles of a pure lipid mixture (grey) and a lipid-peptide mixture (red). Measurements were repeated twice to ensure reproducibility.

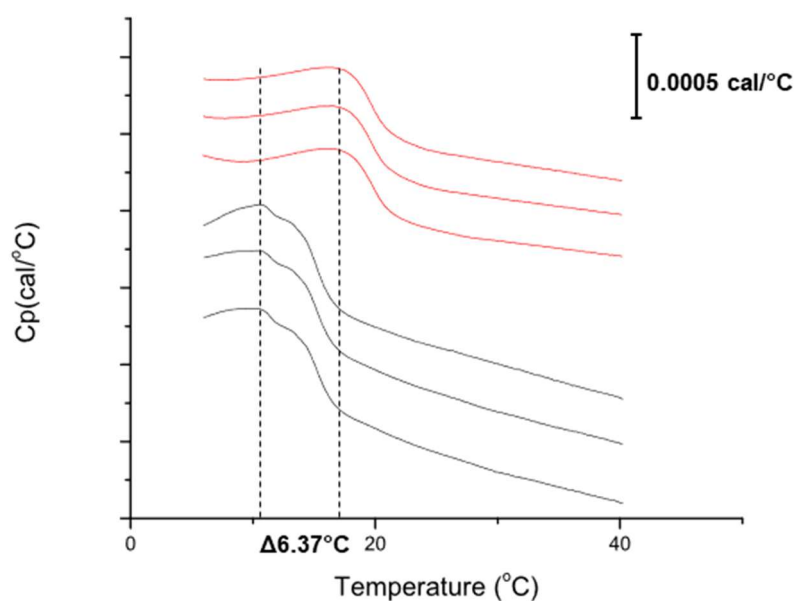


Figure 45: Anionic lipid clustering monitored using DSC measurements.

Lipids used: POPE/TOCL (75:25). Temperature range 3°C to 40°C with a ΔT of 1°C per minute during the heating cycle. Red curves: Lipids with peptide (P/L 1:20). Grey curves: lipids only. A temperature differences in T_m of 6.37°C is observed, indicating lipid clustering caused by Ltc5.

Two transition peaks are observed at approximately 11°C and 13°C for the pure lipid mixture. These signals are consistently observed in all heating cycles of the pure lipid mixture. These two peaks likely reflect the presence of slightly POPE- or TOCL-enriched microdomains within an overall mixed system. Nonetheless, the observed transitions are compatible with the expected T_m range for the POPE/TOCL mixture. The red curve in **Figure 45** represents the sample containing the same lipid mixture with added Ltc5 at a P/L of 1:20. In this case, a single, more cooperative transition peak is observed at ~19°C. This shift toward higher temperatures and apparent merger into a single peak indicate the formation of more compositionally homogeneous lipid domains, enriched in POPE. The observed effect is therefore consistent with anionic lipid clustering driven by Ltc5, where the anionic TOCL preferentially associates with the cationic peptide, leaving POPE-enriched regions that exhibit a distinct phase transition behaviour. A second peak below the measured temperature range would be expected to appear for TOCL rich domains. Due to the temperature limitations of the method, this peak could not be observed.

9.2 Discussion

Ltc5 is a potent, broad-spectrum antimicrobial peptide, inhibiting both Gram-negative and Gram-positive bacterial growth at low micromolar concentrations (MIC). Throughout this work, Ltc5 remained surface-bound in an S-state on lipid bilayers of various compositions (OCD, ssNMR) and caused minimal membrane disturbance in vesicle leakage and hemolysis assays. Ltc5 therefore consistently showed little to no indications of stable pore formation as part of its mechanism of action. These observations largely exclude common barrel-stave or toroidal-pore formation and instead point toward a more subtle mechanism of action. Ltc5 seems to operate via a non-membranolytic mechanism of action. The aim of this chapter is to further improve the understanding of the antimicrobial mechanism of action used by Ltc5. In an effort to narrow down the possible modes of action, an MBC assay was conducted to guide the experimental path and decrease the number of possible mechanisms. The MBC of Ltc5 was determined to be 4 µg/mL for all of the tested bacterial strains. The MBC of Ltc5 is therefore equal to its MIC. Only for *S. xylosus* was an increase in MBC (4 µg/mL) compared to the MIC (2 µg/mL) observed. A difference of one dilution is, however, within the error of the method and should therefore not be considered significant. Based on this result, the mechanism of action of Ltc5 is not only bacteriostatic but also bactericidal. If the main efficacy of the mechanism of action of Ltc5 is exerted on the membrane, it therefore has to have potent effect that can lead to bacterial death.

Such a mechanism could result in the depolarisation of bacterial membranes. Using a depolarisation assay based on the fluorescent dye DiSC₃(5), it was shown that Ltc5 can disturb the membrane potential of *E. coli* at a concentration of 16 µg/mL and above (**Figure 43**). At concentrations of 4 µg/mL to 8 µg/mL, Ltc5 showed minor, reversible depolarisation events (**Figure 43**). Membrane depolarisation indicates that ions are able to cross the cytoplasmic membrane, thereby collapsing the proton-motive force (PMF). Importantly, this loss of PMF occurs in the absence of gross membrane leakage, as proven by a PI uptake assay. PI is a DNA-binding dye that only enters cells with compromised membranes. The PI uptake was minimal for Ltc5 (**Figure 44**) on *E. coli*, indicating an intact membrane. This implies that Ltc5 enables the passage of small ions (H⁺, K⁺, Na⁺, Cl⁻ and others) across the bilayer without forming long-lived pores large enough for bulky molecules to pass. Ltc5 likely creates transient or

nanoscopic membrane defects that increase ion permeability but do not fully rupture the membrane. Similar phenomena have been described in the literature. The Trp rich antimicrobial peptide indolicidin dissipates ion-gradients while in an S-state and showing no indications of major membrane disruption.¹²³ Although it shows dye release in an ANTS/DPX dye release assay, it is still considered non membrane disruptive. The observed dye release was attributed to a carrier-like mechanism of the peptide.¹²³ In a more recent work, indolicidin is proposed to depolarise membranes by forming short-lived voltage-spanning water channels or to induce membrane thinning, rather than stable pores.¹²⁴ Though indolicidin is not α -helical, Ltc5 might adopt a similar mechanism of action. The functional consequences of membrane depolarisation are drastic. By disrupting the membrane potential, Ltc5 can effectively starve bacteria of energy. The proton gradient across the inner membrane drives ATP synthesis and powers multiple transport processes. Even a partial and reversible depolarisation, as observed for Ltc5 at concentrations below 8 $\mu\text{g/mL}$, could force the cell to burn ATP in an attempt to restore the gradient via active ion transport. The ability of Ltc5 to cause even moderate proton leakage could thus be a severe energetic burden on bacteria, explaining the observed MIC and MBC of 2 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$. The observed depolarisation has to be viewed as a phenotype. The reason behind the depolarisation is not yet resolved.

The results of the depolarisation and PI uptake assays are in line with previously made assumptions. Ltc5 permeabilises membranes to ions but not to larger molecules like PI or ANTS/DPX (vesicle leakage assay). The mechanism of action is therefore not expected to form long-lived channels or transmembrane pores. Instead, Ltc5 might disturb the lateral membrane organization, for example by thinning the bilayer or inducing lipid packing artefacts in ways that increase permeability for ions without catastrophic membrane disruption.

The formation of anionic lipid clusters caused by Ltc5 could explain all the observed effects. Ltc5 has a strong cationic character (plus ten net charge). This enables interactions with negatively charged lipid head groups in membranes while surface bound in an S-state. Indeed, the high charge density of Ltc5 should favour binding to anionic phospholipids like phosphatidylglycerol (PG) and cardiolipin (CL), which are abundant in bacterial inner membranes. If this effect is strong enough to alter the lateral organisation of lipids, it could also be responsible for the observed activity of Ltc5 in

MIC, MBC, hemolysis, vesicle leakage, PI uptake and most importantly membrane depolarisation. An anionic lipid clustering experiment was conducted to investigate this theory. Using differential scanning calorimetry (DSC) on model membranes, we found evidence that Ltc5 reorganises lipid domains by clustering anionic lipids. A 3:1 mixture of zwitterionic POPE and anionic cardiolipin (TOCL) was used to mimic the inner membrane composition of *E. coli*.¹¹⁸ In the presence of Ltc5, the DSC thermogram of this lipid mixture induced an altered phase transition shifted by $\sim 6.4^{\circ}\text{C}$ relative to the pure lipid control (**Figure 45**). Such changes in T_m indicate that the peptide induces a significant degree of lipid segregation or even domain formation. This lateral segregation of lipid species is a solid indication of the anionic lipid clustering effect.

The formation of such clusters or domains can severely perturb membrane function on multiple levels. Most importantly for the context of this work, the formation of anionic lipid clusters by AMPs can cause lipid packing defects which can increase membrane permeability at the boundaries of these domains.¹¹⁸ It could therefore be a main cause of the observed membrane depolarisation without the need for full out membrane disruption. Additionally, clustering of key lipids like cardiolipin can inactivate membrane-associated proteins that require those lipids for proper localisation or activity. For example, cardiolipin is known to organize into microdomains at the cell poles and division septum, serving as a platform for proteins involved in cell division and cell envelope synthesis.¹¹⁹ If Ltc5 segregates cardiolipin into lipid clusters, it could delocalize transmembrane proteins such as MinD or cell wall synthesis enzymes that normally partition into CL-rich zones. This concept parallels the mechanism of action of certain membrane-targeting antibiotics.^{18,19} The amphipathic drug 3',6-dinonyl neamine (diNn) was recently shown to target cardiolipin microdomains, disassembling them into diffuse clusters and thereby preventing multiple membrane based processes.¹²⁵ In *P. aeruginosa*, diNn caused CL at the poles to disperse, which was accompanied by a dramatic loss of rod shape and impairment of respiration and ATP synthesis.¹²⁵ In essence, disturbing CL domains led to a cascade of fatal cellular effects. This resulted in loss of proper cell morphogenesis, inhibited energy production, and a slowed growth rate. Although Ltc5 is a peptide and diNn is a modified aminoglycoside, a similar mechanism of action seems plausible. Both are cationic amphiphiles that relocate critical lipids.

A summary of the insights gained on the mechanism of action of Ltc5 is shown in **Figure 46**. Combining the results of this work, we propose a unified mechanism for Ltc5 that highlights its non-membranolytic yet membrane-centric mechanism of action. Ltc5 is electrostatically drawn to the negatively charged membrane surface of bacteria (rich in PG/CL). The hydrophobic N-terminal region, especially residues Phe2/Phe3 and Tyr9/Phe10, inserts into the outer membrane leaflet interface, anchoring the peptide. Ltc5 then folds into an amphipathic α -helix (CD) lying parallel to the bilayer normal in an S-state (OCD, ss²H-NMR). In this orientation, Ltc5 can exert its lethal antimicrobial activity (MBC). The array of hydrophobic and aromatic residues in the hydrophobic sector of Ltc5 embeds at the membrane interface, while cationic side chains engage with anionic lipid headgroups. The membrane orientation therefore supports the formation of anionic lipid clusters (DSC) which might lead to lipid packing defects at their borders. These defects can increase the local membrane permeability to ions (Depolarisation assay) while the membrane still forms a stable barrier for larger molecules like PI or ANTS/DPX. Resulting depolarisation then undermines the energy metabolism of targeted cells, thereby explaining the high antimicrobial activity. Lipid clustering could also lead to the dispersion of membrane domains, thus interfering with the activity of transmembrane proteins. Recent developments in antimicrobial research suggest that antimicrobial peptides (AMPs) often exert their activity through multiple mechanisms of action.³² This concept may also be applicable to Ltc5. Importantly, none of the results obtained in the present study exclude the possibility of additional intracellular targets that Ltc5 might act on.

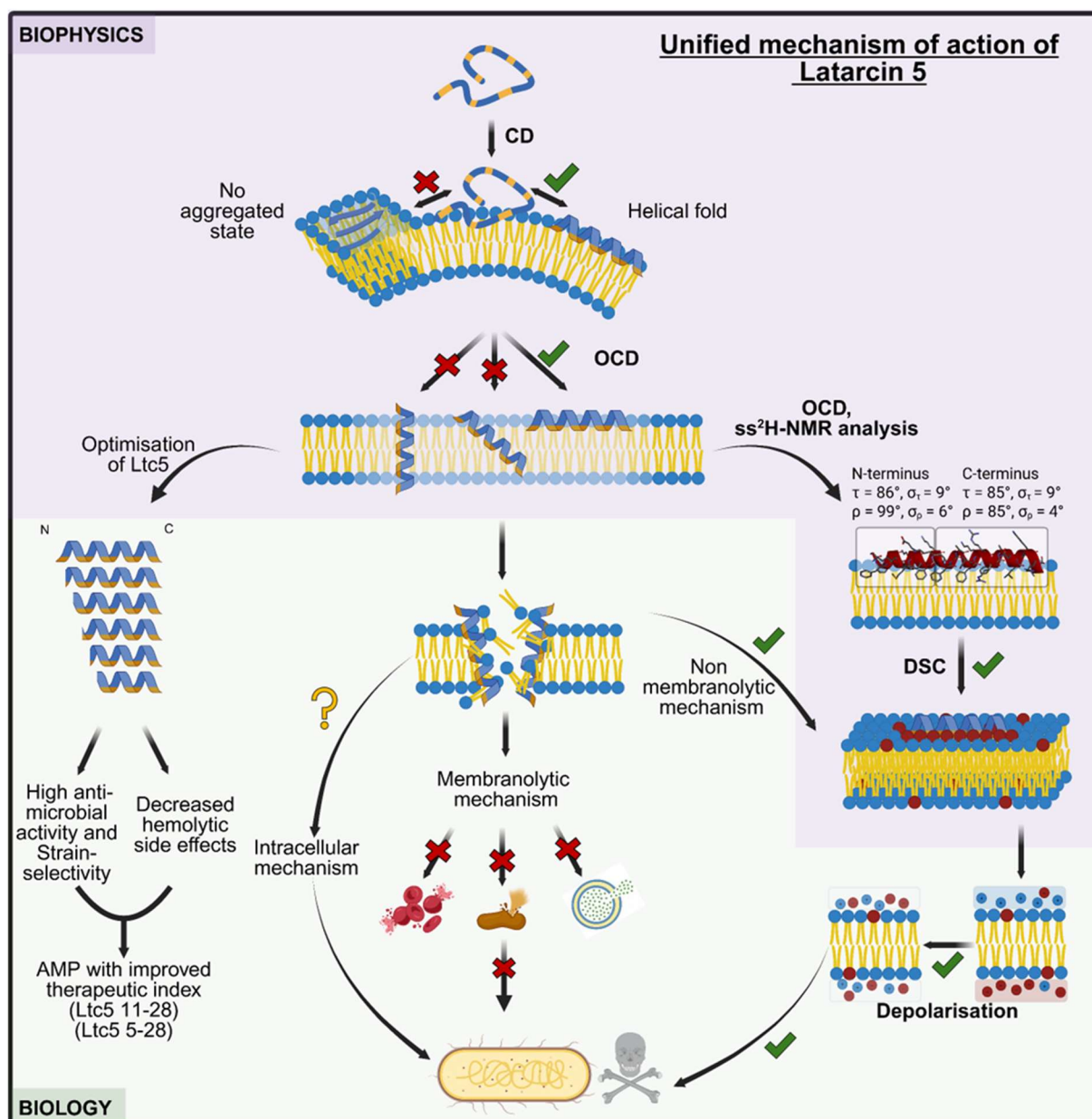


Figure 46: Unified mechanism of action of Ltc5.

This figure presents a unified model of Ltc5's mechanism of action, based on the data accumulated in this work, and highlights the experimental methods used to derive these findings. Ltc5 displays antimicrobial activity by impairing membrane function without compromising overall membrane integrity. In addition to this membrane-associated effect, intracellular targets may also contribute to its activity. Purple background: structure investigation. Green background: investigation of the mechanism of action. Created in <https://BioRender.com>

10 Conclusion and Outlook

Ltc5 is a linear, α -helical, amphipathic, membrane-active peptide isolated from the venom of the spider *Lachesana tarabaevi*. Ltc5 exhibits broad-spectrum antimicrobial activity with low hemolytic activity, but its membrane-bound structure remains unknown. Determining the structure of Ltc5 in the membrane may therefore help clarify its mode of action and support the development of novel antimicrobial agents inspired by natural compounds. To this end, an alanine scan was performed on Ltc5 using Ala-d₃ in Chapter 5 to identify the role of individual amino acids in structure and function. The results of this chapter demonstrate that replacement of any residue in Ltc5 by alanine is well tolerated and does not influence structure, orientation, antimicrobial activity, hemolytic activity, or vesicle leakage. Thus, each Ala-d₃ analogue can be utilized for structural analysis of Ltc5 using ss²H-NMR spectroscopy. Structural analysis shows that, upon interaction with lipid bilayers, Ltc5 adopts an almost perfectly straight α -helical conformation, with N- and C-terminal segments displaying nearly identical tilt angles (86° at the N-terminus and 85° at the C-terminus) but clearly distinct azimuthal rotation angles (99° and 85°, respectively). The α -helix is oriented parallel to the membrane surface, with the hydrophobic sector of Ltc5 facing the hydrophobic core of the bilayer. This chapter also highlights that membrane damage is not the primary mechanism of action for Ltc5. This suggests that Ltc5 employs either a milder mechanism in which the helix remains on the membrane surface or targets the intracellular compartment of the bacteria, as previously hypothesized.⁴⁷ Both possibilities were evaluated in the next chapters.

To enter the bacterial cell, Ltc5 must cross the hydrophobic core of the bilayer. For an amphipathic peptide, this usually requires a tilt of the helix in the membrane followed by helix assembly or crossing of the hydrophobic barrier. Therefore, in order to trap the active state of Ltc5 in the membrane, two parameters were varied, namely membrane thickness and peptide concentration. The results presented in Chapter 6 demonstrate that Ltc5 does not reorient from a surface-bound state; however, in thin bilayers at high peptide concentrations, it remodels the membrane and induces transient lipid phases, which may either temporarily disrupt the bilayer and cause loss of membrane potential or facilitate peptide uptake. This lead was pursued further in the last chapter.

In the next two chapters, the influence of aromaticity and hydrophobicity on the structure and function of Ltc5 was examined to pursue aims 3 and 4. Ltc5 is rich in aromatic residues on the hydrophobic face of the surface-bound helix. In Chapter 7, aromatic residues were systematically replaced with alanine, leucine, or cyclohexylalanine, and the resulting effects on structure and function were analysed. The results of this investigation revealed that a certain degree of hydrophobicity is critical throughout the helix. If this is supplied by aromatic residues, it is even better. This explains why evolution chose aromatic residues over other available hydrophobic ones, as these residues integrate efficiently into the membrane and may even assist Ltc5 in accessing intracellular targets (if any). For aim 4 (Chapter 8), the influence of a high abundance of hydrophobic residues at the N-terminus of Ltc5 was evaluated. For this aim, N-terminal residues were systematically truncated with the expectation of further reducing the hemolytic and cytotoxic side effects and obtaining a therapeutic analogue of Ltc5. Surprisingly, this N-terminal truncation resulted in strain selectivity for Gram-positive bacteria. The truncated peptides obtained showed almost no hemolysis (Ltc5 11-28). The main aim was also fulfilled, namely, to identify a broad-spectrum therapeutic analogue of Ltc5 with low hemolytic side effects (Ltc5 5-28).

In the last chapter, in light of all the above results and to evaluate the non-membranolytic mechanism of Ltc5, clustering of anionic lipids and membrane depolarization abilities of Ltc5 were evaluated. The results in this chapter demonstrate that the high charge of Ltc5 is indeed capable of promoting clustering of anionic lipids. Furthermore, it was demonstrated that Ltc5 is able to induce membrane depolarisation in *E. coli*. Thus, Ltc5 appears to act on several levels, compromising membrane function by disturbing membrane homogeneity and inducing depolarisation, in addition to acting on suspected intracellular targets that remain to be identified and were beyond the scope of this study.

Future studies should focus on further elucidating the membrane-depolarizing and lipid-clustering effects of Ltc5, as well as identifying potential targets at the membrane surface. In particular, it will be important to determine the peptide concentrations at which specific mechanisms become operative and to assess whether Ltc5 employs a common mechanism of action across different bacterial strains. As reported for several antimicrobial peptides, the presence of intracellular targets should also be considered and systematically evaluated for Ltc5.

11 Appendix

11.1 Instrumentation and Materials

Devices and tools

CD spectrometer	Jasco J-815 CD spectrometer equipped with Jasco Julabo FP35 refrigerated/heating circulation
OCD spectrometer	Jasco J-810 spectropolarimeter equipped with Jasco Julabo FS18 refrigerated/heating circulation
Vacuum pumps	Leybold Heraeus minni A, 1.0116 and Divac 2.2L
NMR Spectrometer	Bruker Ultrashield 500 MHz cryomagnet, Bruker Avance III console
Probeheads	³¹ P: modified Bruker HFP flatcoil probehead ² H: home-built goniometer probehead
Heat cabinet	Heraeus Kelvitron t, UT 6120
Fluorescence spectrometer	Horiba Jobin Yvon Fluorolog FL-1065 with Huber Ministat CC Compatible Control refrigerated and heating circulator
High intensity ultrasonic bath	Hielscher UTR200 ultrasonic processor
Heater for ultrasonic bath	Bandelin Sonorex digital 10P
2 Vortex mixers	IKA Vortex genius 2 / 3 and BioVortex V1
Fine balance	Satorius ME-5 micro balance, 5.1 g x 0.001 mg
Stir plate	Magnetic hot plate stirrer IKA RH basic 2
pH meter	Laboratory benchtop meter PCE-BPH 1

Spectrophotometer	Bio-Rad SmartSpec Plus Spectrophotometer
Safety Cabinet	ThermoScientific HeraSafe KS12 Class 2 Safety Cabinet
Autoclave	HP Labortechnik GmbH Varioklav class 100
Incubator	Heraeus Function line Incubator
2 Centrifuges	Heraeus fresco 17 and Sigma 2-6
Shaker	Eppendorf ThermoMixer C
	Heidolph Unimax 1010 + incubator 1000
Heating bath	Köttermann Typ 3042

Materials

Extruder	Avanti Polar lipids mini-extruder with holder and Hamilton syringes
Polycarbonate membrane	Whatman nucleopore track-etched membranes, 0.1 μ M
Membrane stabilization disc	Whatman drain disc 10 mm PE
Pipettes	Eppendorf reference series for different volumes
Pipette tips	Sarstedt pipette tips for different volumes
Centrifuge tubes	Sarstedt screwcap tube different volumes
Reaction tubes	Sarstedt safeseal reaction tube different volumes
Quartz glass plates (OCD)	Hellma Analytics, Substrate from SQ1, 20 mm * 1.6 mm, polished (P3), PB: 90 %, 3/10 ur at 632.8 nm, 5/4x0.16, SFs 0.2/45°, uncoated; 002963
Glass plates (NMR)	Paul Marienfeld microscope cover glasses, 7.5 mm x 12 mm

Laboratory tape	Parafilm Laboratory Film PM-992 and 996
Quartz cuvette	Hellma Analytics 101 - Macro Cell, quartz glass, 10x10 mm
Disposable cuvette	Disposable Sarstedt Cuvettes, 4.2 mL, PMMA
Microtiter Plate Reader	Biotek Synergy HTX multi-mode reader
Differential Scanning Calorimeter	MicroCal VP-DSC microcalorimeter (Malvern, UK)
Chemicals	
Chloroform	Merck Uvasol, for spectroscopy, K57136347 549
Methanol	Fischer, Analytical reagent grade, 2498069
Acetone	Thermo scientific, 2532854
Ethanol	Fischer, 96 %, 2543333
MQ-Water	Purified with Millipore direct-Q5
Phosphate buffer	Merck 1.09887.0001, Titrisol phosphate buffer concentrate
DMPC	COATSOME MC-4040, 1,2-Dimyristoyl-sn-glycero-3-phosphocholine; NOF Corporation
DMPG	COATSOME MG-4040LS, 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol, sodium salt; NOF Corporation,
POPC	COATSOME MC-6081, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; NOF Corporation
POPG	COATSOME MG-6081LS, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol, sodium salt; NOF Corporation

ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid, sodium salt; ThermoFisher Scientific, 0131-11
DPX	p-xylene-bis-pyridinium bromide; ThermoFisher Scientific, X1525
NaCl	Sodium chloride; Merck, 040110850
HEPES	Alfa Aesar, HEPES 99% dry basis, 179336A
PIPES	Acros organics, PIPES, disodium salt, 99%, 344085
Triton X-100	t-Octylphenoxypolyethoxyethanol; Merck, T9284
Rho-PE	Avanti Polar Lipids, 18:1 Liss Rhod PE, 810150C
Sephacryl HR-100	Sigma, S100HR, SLCR5204
LB-Broth	Carl Roth, X986.1
LB-Agar	Carl Roth, 412190794
MH-Broth	BD, BBL Mueller Hinton II Broth, Cation adjusted, 4102223
Propidium Iodide (PI)	Fluka, Propidium Iodide, BCBM1455V
DiSC ₃ (5)	Ata Aesar, 3,3'-Di-n-propylthiadicarbocyanine Iodide, 10168779
Bacterial strains, Gram positive	Bacillus spizizenii (DSM 347), Gram-positive Staphylococcus xylosus (DSM 20267), Gram-positive
Bacterial strains, Gram negative	Acinetobacter sp. (DSM 586), Gram-negative Escherichia coli (DSM 1116), Gram-negative

Software and Programs

Polar manager	Spectrometer control software Self-written by Siegmur Roth, IBG-2, KIT
ProCD12	CD data analysis Self-written by Siegmur Roth, IBG-2, KIT
DichroWeb	CD spectrum deconvolution B.A. Wallace and L. Whitmore ^{58,59}
Topspin software	Bruker Biospin; for spectrum acquisition and spectrometer control
ocd2psnorm1	Plotting and automated normalisation of OCD spectra. Program written by Dr. Erik Strandberg, IBG-2 KIT
dx2psM5a	Automated plotting of NMR spectra. Program written by Dr. Erik Strandberg
Oridyn	Fitting program used for the structural fits of ^2H -NMR data.
BioVia Studio Discovery 2025 https://BioRender.com	3D modelling of peptide α -helices Platform for creating biological figures

11.2 Buffers and solutions

LB-Agar (MIC)	8.0 g dissolved in 200 mL dd H ₂ O, pH = 7.4 at RT, autoclaved
MH-medium (Müller-Hinton-Bouillon) (MIC)	8.8 g dissolved in 400 mL dd H ₂ O, pH = 7.3 at RT, autoclaved
Tris buffer (Hemolysis)	20.8 g dissolved in 1000 mL dd H ₂ O pH 7.6 at RT for washing steps, pH 7.6 at 37°C for reaction steps
Internal Buffer (Vesicle leakage)	ANTS (12.5 mM, 53.4 mg) DPX (45 mM, 190 mg)

	HEPES (10 mM 23.8 mg)
	NaCl (50 mM, 29.2 mg)
	Dissolve in 10 mL dd H ₂ O, pH = 7.5 at RT, store at 4°C
External Buffer (Vesicle leakage)	HEPES (10 mM, 953 mg)
	NaCl (150 mM, 3.51 g)
	Dissolved in 400 mL dd H ₂ O, pH = 7.4
Mobile phase A (HPLC)	dd H ₂ O (90%, 4475 mL)
	Acetonitrile (10 %, 500 mL)
	TFA (1 M, 25 mL)
Mobile phase B (HPLC)	dd H ₂ O (10%, 475 mL)
	Acetonitrile (90 %, 4500 mL)
	TFA (1 M, 25 mL)
0.5 PBS (Washing Bacteria)	NaCl (68.4 mM, 4g)
	KCl (1.3 mM, 100 mg)
	Na ₂ HPO ₄ (5.1 mM, 0.72 g)
	KH ₂ PO ₄ (0.9 mM, 122.5 mg)
	Dissolve in 800 mL dd H ₂ O, pH 7.4
	Autoclave, Add sterile filtered sugar solution to get 0.2% (w/v) glucose after autoclaving.

12 Methods

12.1 Peptide synthesis

Peptides used in this thesis were synthesized in-house by the author as well as by Kerstin Scheubeck and Andrea Eisele using a multi-synthesizer (Syro II, Biotage) with adaptation according to Merrifield et al.¹²⁶ Peptide assembly was performed on rink amide 4-methylbenzhydrylamine (MBHA) resin resin (loading 0.78 mmol/g) on a 100 μ mol scale. N,N-dimethylformamide (DMF) was used as solvent for all amino acids except tryptophan, for which N-methyl-2-pyrrolidone (NMP) was used. Amino acid stock solutions were prepared at 0.57 M. Couplings were carried out as double-coupling steps using HOBt and HBTU (0.49 M) as coupling reagents, with a reaction time of 30 min per coupling step. Following chain assembly, peptide-resins were washed three times with methanol and dried under reduced pressure.

Reductive cleavage and peptide precipitation

Fully assembled peptides were cleaved from the resin using trifluoroacetic acid (TFA). To minimize oxidation of methionine, cleavage was performed under reducing conditions using a two-solution system. Cleavage solution 1 consisted of 80% (v/v) TFA, 2.2% (v/v) ethanedithiol, 3.2% (v/v) thioanisole, 5.1% (v/v) dimethyl sulfide, 3.6% (v/v) triisopropylsilane, and 5.9% (w/v) phenol. Cleavage solution 2 was prepared by dissolving 2.5 g ammonium iodide in 2.5 mL H₂O. Solution 2 was added to solution 1, and 5 mL of the resulting mixture were used to cleave 500 mg resin. Cleavage was performed on ice (0°C) for 4 h. The resin was removed by filtration, and the filtrate was concentrated under a stream of nitrogen. Crude peptides were precipitated by addition of 25 mL cold diethyl ether, followed by centrifugation. The ether supernatant was decanted, and the pellet was dissolved in 30 mL acetonitrile/H₂O (1:1, v/v). The solution was frozen at -80°C for 16 h and subsequently lyophilized.

Purification by reversed-phase HPLC

Peptides were purified by reversed-phase chromatography using a C18 column (Vydac) on an HPLC system (Jasco). Separations were performed using gradient elution with two mobile phases: eluent A (90 % H₂O, 10 % Acetonitrile, 1 M TFA) and eluent B (10 % H₂O, 90 % Acetonitrile, 1 M TFA). The gradient was adjusted

depending on the peptide, typically, the composition was increased from 0-58% B to 30-58% B over 16 min. Purification runs were conducted at a constant flow rate of 7 mL/min with a total cycle time of 20 min. The column temperature was maintained at 35°C, and 5 mg peptide were injected per run.

12.2 Solid-state NMR spectroscopy

Sample preparation

Microscope cover glasses (7.5 mm × 12 mm, No. 0; Paul Marienfeld GmbH & Co. KG) were cleaned by sequential washing twice with chloroform, water, methanol, and chloroform (in this order). Cleaned glass plates were placed on filter paper in a closed Petri dish to minimize airborne particulate contamination. Defined amounts of lipid and peptide were weighed into separate 0.5 mL microcentrifuge tubes. Peptides were dissolved in H₂O/methanol (4:6, v/v) by vortex mixing until a clear solution was obtained. Lipids were dissolved in methanol/chloroform (4:6, v/v) by vortex mixing until fully solubilized. The lipid solution was then added to the peptide solution. The lipid tube was rinsed twice with 10 µL chloroform/methanol (1:1, v/v), and the rinses were combined with the peptide-lipid mixture, followed by vortex mixing. The resulting peptide-lipid solution was spotted onto the centre of each glass plate. Subsequently, the peptide-lipid tube was rinsed twice with 10 µL chloroform/methanol (1:1, v/v), and the combined 20 µL rinse was spotted onto the same glass plate. Spotted samples were dried in a fume hood for at least 30 min and then placed in a desiccator under reduced pressure overnight. On the following day, glass plates were stacked and transferred to a hydration chamber at 48°C and 96 % relative humidity for 20-24 h. After hydration, stacks were sealed by wrapping in Parafilm and cling film to prevent drying during the measurements.

Measurements

All solid phase NMR experiments were performed on a 500 MHz cryomagnetic by the manufacturer Bruker.

Solid-state ^{31}P experiments

To assess lipid orientation, ^{31}P - NMR spectra were acquired immediately before and after each ^2H experiment. For each sample, a ^1H spectrum was recorded to provide a reference for the corresponding ^{31}P spectrum. All ^{31}P and ^1H measurements were conducted at 308 K (above T_m of the used lipids) using a flat-coil dual-resonance probe ($^1\text{H}/\text{X}$). The ^{31}P experiments employed a Hahn-echo pulse sequence¹²⁷ with a 5 μs 90° pulse, an echo time of 30 μs and 2 s relaxation delay time. During acquisition, ^1H decoupling was applied using a dedicated decoupling sequence¹²⁸ to suppress ^{31}P - ^1H scalar coupling. Each ^{31}P spectrum was obtained by accumulating 256 scans.

Solid-state ^2H experiments and structural analysis

The deuterium spectra were acquired using a ^2H goniometer probe head. Each sample was measured twice, with the sample oriented at 0° and 90° relative to the bilayer normal. Spectra were recorded using a 90°-90° solid-echo pulse sequence with a pulse length of 4.45 μs , an echo time of 70 μs , 100 ms relaxation delay time and 300,000 to 1,000,000 accumulated scans. The observed quadrupolar splittings were used to infer the spatial orientation of the labelled residues, determined by the two angles τ (tau) and ρ (rho). The tilt angle, τ , defines the angle between the peptide long axis and the lipid bilayer normal, whereas the azimuthal rotation angle, ρ , describes rotation about the peptide long axis. Peptide orientation was estimated by fitting the experimental quadrupolar splittings to calculated splittings derived from previously determined structural models, using a best-fit procedure discussed in section 13.3.

12.3 Circular dichroism and oriented circular dichroism

Preparation of SUVs for circular dichroism spectroscopy

For vesicle preparation, stock solutions of DMPC and DMPG (5 mg/mL each) were prepared in chloroform/methanol (1:1, v/v). Aliquots were combined to obtain a DMPC/DMPG molar ratio of 7:3. The solvent was removed under a stream of nitrogen, and residual solvent was eliminated by placing the lipid film under vacuum for 3 h. The

dried lipids were then hydrated by addition of 1 mL distilled water in a so-called swelling step, followed by vortexing for 5 min. The resulting lipid suspension was subjected to ten freeze-thaw cycles to promote homogeneous hydration of the headgroups and to form multilamellar vesicles (MLVs). Finally, the MLV dispersion was ultrasonicated for 16 min right before use to obtain small unilamellar vesicles (SUVs).

Circular dichroism

Circular dichroism (CD) spectroscopy is a well-established method to determine the secondary-structure of peptide under different conditions. For CD measurements, peptide stock solutions were prepared in distilled water at 0.5 mg/mL. Stock concentrations were determined by UV spectroscopy using the tyrosine absorbance (see subsequent chapter for details). For CD acquisition, peptides were diluted to 0.1 mg/mL for measurements without lipids present. Each peptide was measured in either H₂O, 10 mM PB or in 10 mM PB in the presence of small unilamellar vesicles (SUVs) composed of DMPC/DMPG (7:3 molar ratio). SUV preparation is described in the following chapter.

For lipid containing samples, 300 μ L solutions were prepared in 10 mM PB with SUVs at 1 mg/mL and a peptide-to-lipid ratio (P/L) of 1:50. Samples were transferred to a 0.1 cm pathlength quartz cuvette (Hellma, Müllheim, type 110-QS). CD spectra were recorded on a Jasco J-815 spectropolarimeter or a Jasco J-1500 spectropolarimeter using the acquisition parameters listed in **Table 21**. Prior to sample measurements, a reference spectrum was collected and subsequently used for baseline subtraction via the instrument control software. The corrected spectra were smoothened, and the baseline at 260 nm was set to zero. Ellipticity values were then converted to mean residue ellipticity (MRE) per peptide bond.

Table 21: Parameters applied during CD-measurements

Wavelength	180-260 nm
Temperature	Lipids 30°C, No Lipids 25°C
Atmosphere	Low oxygen (constant nitrogen flow)
Data Pitch	0.5
Scanning Method	continuous
Scan speed	10 nm/min
Response time	8 sec
Band width	1 nm
Accumulations per sample	3

Oriented circular dichroism

Oriented circular dichroism (OCD) is a well-established method for probing the orientation of α -helical segments in peptides and proteins within lipid bilayers.⁴⁷ OCD typically distinguishes three limiting states: the surface state (S-state), in which the helix is oriented approximately parallel to the membrane plane, the inserted state (I-state), in which the helix is oriented approximately perpendicular to the membrane plane and the tilted state (T-state), which represents an intermediate orientation between the S- and I-states, with the helix adopting a finite tilt within the bilayer.

Peptide stock solutions were prepared at 0.2 mg/mL. The lipid system DMPC/DMPG (7:3 molar ratio) was used, and lipid stock solutions were prepared at 5 mg/mL. All stock solutions were prepared in chloroform/methanol (1:1, v/v). Samples contained a constant peptide amount of approximately 10 μ g, while the lipid amount was adjusted according to the desired peptide-to-lipid ratio (P/L). Based on preliminary measurements, a molar P/L of 1:100 was selected for subsequent experiments. Hamilton syringes were used to minimize volumetric error when handling chloroform-containing solutions.

After mixing and vortexing, samples were spread onto quartz plates (20 mm diameter) and dried for at least 30 min in a fume hood. The plates were then placed under reduced pressure and dried for an additional 2 h to remove residual organic solvents. Subsequently, samples were transferred to hydration chambers and rehydrated overnight at 30°C under an atmosphere maintained at 97% relative humidity. On the following day, plates were mounted in a measurement cell developed by the Ulrich group at IBG-2 (KIT) and spectra were recorded at 30°C. Humidity was determined

right before the measurement to ensure full hydration of the samples in the measurement cell.

In the measurement cell, samples were oriented such that the plate normal was perpendicular to the incident light beam. For each sample, spectra were acquired at eight rotational positions to account for potential inhomogeneities arising from sample preparation. Successive spectra were recorded after rotating the plate by 45° about the axis normal to the light beam, and two measurements were collected at each rotational position. Acquisition parameters are summarised in **Table 22**.

For data processing, the eight spectra obtained per sample were baseline corrected by setting the signal to 0 mdeg at 260 nm. The spectra were then averaged using Polar Manager. Finally, spectra were normalized to facilitate comparison among different peptides.

Table 22: Parameters applied during OCD measurements

Wavelength	180-260 nm
Temperature	30°C
Atmosphere	Low oxygen (constant nitrogen flow)
Data Pitch	0.5
Scanning Method	continuous
Scan speed	20 nm/min
Response time	4 sec
Band width	1 nm
Accumulations per angle	2
Angles	8

12.4 Antimicrobial activity assays

Minimal inhibitory concentration

The minimum inhibitory concentration (MIC) was determined for four bacterial strains: the Gram-negative strains *Escherichia coli* (DSM 1116) and *Acinetobacter sp.* (DSM 586), and the Gram-positive strains *Bacillus spizizenii* (DSM 347) and *Staphylococcus xylosus* (DSM 20267). The assay was performed with minor adaptations as previously described by Amsterdam.¹²⁹ For each strain, an overnight culture was set up from a cry stock by inoculating 10 mL Mueller-Hinton (MH) broth (Subsequently referred to as

medium) and incubating at 37°C with shaking at 220 rpm for 20-22 h. The following day, cultures were streaked onto LB agar plates, incubated overnight at 37°C, and stored at 4°C until use. For MIC determination, a second set of overnight cultures was prepared as described above, using single colonies taken from the corresponding LB agar plates as inoculum. To estimate cell density, a 1:10 dilution of each overnight culture was prepared in a disposable cuvette and the optical density was measured at 550 nm (OD₅₅₀). Based on these measurements, the volume of overnight culture required to obtain 10 mL of a bacterial suspension at OD₅₅₀ of 0.2 was calculated. This suspension was incubated for 2-3 h at 37°C with shaking at 220 rpm. After incubation, a 1:1 mixture of culture and medium was measured again at OD₅₅₀ to calculate the volume required to prepare a 1 mL bacteria/medium stock solution at OD₅₅₀ = 0.2. From this OD₅₅₀ = 0.2 stock, 20 mL dilutions of 1:100 (Gram-positive strains) and 1:1000 (Gram-negative strains) were prepared. The inoculum of the MIC was set at ~ 5x10⁵ CFU/mL. MIC assays were performed in 96-well microtiter plates, with three peptides tested per plate together with positive and negative controls (Table 21). For each peptide, a stock solution of 1.024 mg/mL was prepared in sterile water. In the assay plate, 50 µL of double-concentrated medium was dispensed into row A, and 50 µL of standard medium into rows B-H. For the control wells, an additional 50 µL medium was added per well. Subsequently, 50 µL of the respective peptide stock solution was added in triplicate to row A (e.g., columns 1-3 for peptide 1, etc.), while columns 10 and 11 contained the positive and negative controls (PK and NK). A two-fold serial dilution series was generated down the plate using a 12-channel multichannel pipette. Wells in row A (columns 1-10) were mixed by repeated aspiration/dispensing, and 50 µL was transferred from row A to row B. Row B was mixed and 50 µL was transferred to row C, continuing sequentially to row H. After mixing row H, 50 µL was discarded to maintain constant volume. This procedure yielded a final peptide concentration of 256 µg/mL in row A and a dilution series down to 2 µg/mL in row H. Subsequently, 50 µL of the appropriate bacterial dilution was added to all wells, except the negative control, to reach an inoculum of 5 x 10⁵ in each well. Plates were incubated overnight at 37 °C. On the following day, 20 µL resazurin solution (0.2 mg/mL) was added to each well, followed by incubation at 37°C for 2 h. Plates were then documented by photography.

Minimal bactericidal concentration assay

Minimum bactericidal concentration (MBC) assay. The minimum bactericidal concentration (MBC) assay was performed with modifications based on the procedure described previously by Yang et al.¹³⁰ and was conducted consecutively following the MIC assay. After the final overnight incubation of the MIC assay, 50 μ L of the unstained bacterial suspension was spread onto LB agar plates. For each sample, three concentrations, one dilution below the MIC, the MIC itself, and one dilution above the MIC, were plated. The agar plates were incubated overnight for 20-22 h, after which the number of colony-forming units (CFUs) was determined. The CFU/mL value corresponding to the plated 50 μ L volume was calculated from the inoculum used in the MIC assay. The MBC was defined as the lowest antimicrobial concentration resulting in a 99.9% reduction in viable bacteria relative to the initial inoculum.

12.5 Hemolysis assay

To assess peptide toxicity toward human cells, hemolytic activity was evaluated using erythrocytes of the blood type A, Rh(D) positive. The blood was obtained from the blood bank of Städtisches Klinikum Karlsruhe gGmbH and, after opening, was stored at 5°C and used within two weeks. For the assay, 5 mL of erythrocytes were washed in 40 mL wash Tris-buffer (172 mM, pH 7.6) and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the wash step was repeated twice. Subsequently, 1 mL of the erythrocyte pellet was diluted with 9 mL Tris-buffer to yield a 1:10 suspension. From this suspension, 500 μ L were further diluted in 9.5 mL Tris-buffer. The resulting erythrocyte suspension was equilibrated to 37°C prior to use. Peptide stock solutions were prepared in water at 1.024 mg/mL. For each peptide, a two-fold serial dilution series was prepared in nine micro reaction tubes to obtain final peptide concentrations from 256 μ g/mL to 1 μ g/mL. Briefly, 200 μ L RB were dispensed into each tube. Then, 200 μ L peptide stock solution was added to the tube corresponding to 256 μ g/mL and mixed thoroughly by repeated pipetting. Next, 200 μ L were transferred from the first tube to the second, mixed, and serially transferred in the same manner through the ninth tube, from which 200 μ L were discarded to maintain constant volume. Positive and negative controls were prepared in triplicate. For positive

controls, 200 μ L of 0.2% (v/v) Triton X 100 in RB were used, whereas negative controls contained 200 μ L RB only. Subsequently, 200 μ L of the pre-warmed erythrocyte suspension was added to each tube, including controls. Samples were incubated at 37°C for 30 min with shaking, followed by centrifugation at 13,000 rpm for 10 min. After centrifugation, 350 μ L of the supernatant were transferred to a disposable cuvette and absorbance was measured at 540 nm. Each measurement was done in triplicates.

12.6 Vesicle leakage assay

This assay is highly sensitive to the P/L. Therefore, peptide stock concentrations (in water) were determined by UV spectroscopy. Absorbance at 280 nm (tyrosine absorption maximum) was measured and concentrations were calculated using the Lambert-Beer law. From the stock solutions, aqueous peptide working solutions of 75 μ M and 150 μ M were prepared. Lipid vesicles were prepared one day prior to the measurements. POPC and POPG were mixed at a 1:1 molar ratio to yield a total lipid concentration of 100 μ M in the assay. This composition is widely used as a model for bacterial membranes. Lipids were dissolved in chloroform/methanol (1:1, v/v) and vortexed for at least 60 s. To enable quantification of lipid loss during preparation, 0.1 mol% Rho-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) was added as a fluorescent marker, followed by an additional vortexing step. Organic solvents were removed under a stream of nitrogen and by subsequent drying under reduced pressure for 2 h. The resulting lipid film was rehydrated in 10 mL internal buffer (pH 7.5) containing ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, 12.5 mM), DPX (p-xylene-bis-pyridinium bromide, 45 mM), HEPES (10 mM), and NaCl (50 mM) in Milli-Q water. Ten freeze-thaw cycles (liquid nitrogen and a 50°C water bath) were performed to generate multilamellar vesicles (MLVs) with fully hydrated lipids. Large unilamellar vesicles (LUVs) were obtained by extrusion of the MLV suspension 41 times through a polycarbonate membrane with a pore size of 100 nm. Vesicles were stored at room temperature and used the following day. To remove external ANTS/DPX, the vesicle suspension was passed through a size-exclusion column packed with Sephacryl S-100 (fractionation range approximately 1,000-100,000 Da). This procedure yields LUVs encapsulating ANTS and DPX while removing unencapsulated dye and quencher from

the external medium. Upon peptide-induced membrane permeabilization, ANTS and DPX are released and diluted into the external buffer, resulting in dequenching and an increase in ANTS fluorescence that can be monitored over time. Prior to the leakage assay, the effective lipid concentration of each LUV preparation was determined from the rhodamine fluorescence signal, as lipid losses during extrusion and gel filtration can alter the final concentration. For leakage measurements, the cuvette holder was equilibrated to 30°C. Peptide was added to a quartz cuvette (10 mm × 10 mm) containing external buffer to reach 4 µM under continuous stirring, and fluorescence emission at 515 nm was recorded upon excitation at 355 nm. After 100 seconds, LUVs were added to a final lipid concentration of 100 µM (total volume 1.5 mL), the baseline intensity was noted after the initial injection peak (I_{100}). Fluorescence was measured over time and the value after 700 seconds (I_{700}) was used to calculate leakage. Total leakage was then induced by addition of 20 µL of 20 % Triton X-100, and the maximal fluorescence intensity (I_{max}) was recorded. The dilution introduced by detergent addition was accounted for in the leakage calculation.

Leakage was calculated according to:

$$L = \frac{I_{700} - I_{100}}{1.01 * I_{max} - I_{100}} \quad (1)$$

where the factor 1.01 corrects for dilution upon addition of 20 µL detergent to a total volume of 1.5 mL. To account for intrinsic vesicle instability, each LUV batch was also measured in the absence of peptide, and the resulting self-leakage was subtracted from peptide-induced leakage values.

12.7 Membrane depolarisation assay

The membrane depolarisation assay was performed with modifications to the method described by Te Winkel et al.¹²¹ Bacteria were cultured as described for the MIC assay (Chapter 12). Prior to use, cells from day cultures were washed twice with 0.5 x PBS supplemented with 0.2% (w/v) glucose, because residual MH medium caused signal loss during the assay. Glucose was included as a carbon source to support bacterial metabolism. The optical density of the washed bacterial suspension was measured at 600 nm and adjusted to $OD_{600} = 0.2$. To allow recovery from washing-induced stress

and reestablishment of membrane integrity, the suspension was rested for at least 1 h before use.

A two-fold peptide dilution series (final concentrations 256 $\mu\text{g/mL}$ -2 $\mu\text{g/mL}$) was prepared in PBS containing 0.2% glucose. Aliquots (50 μL) were dispensed into a 96-well plate, followed by 50 μL of DiSC₃(5) to yield a final dye concentration of 50 $\mu\text{g/mL}$ per well. Subsequently, 100 μL of the bacterial suspension were added to obtain a final OD₆₀₀ of 0.1 (CFU/mL $\sim 5 \times 10^7$) and a total volume of 200 μL per well. Fluorescence was recorded at 37°C using an Agilent Synergy HTX microplate reader in kinetic mode with measurements taken every 2 min and shaking between reads. DiSC₃(5) fluorescence was measured with excitation at 622 nm and emission at 670 nm.

12.8 Membrane disruption assay

The membrane disruption assay was performed with modifications to the protocol described by Ye et al.⁴¹ Bacteria were cultured as described for the MIC assay (Chapter 12). Prior to use, cells from day cultures were washed twice with PBS supplemented with 0.2% (w/v) glucose, because the presence of MH medium resulted in signal loss during the assay. Glucose was included as a carbon source to sustain bacterial metabolism. The optical density of the bacterial suspension after washing was measured at 600 nm and adjusted to OD₆₀₀ = 0.2. Because the washing procedure can transiently impair membrane integrity, the washed cells were allowed to rest for at least 1 h to re-establish membrane integrity before starting the assay.

A two-fold peptide dilution series (final concentrations 256–2 $\mu\text{g/mL}$) was prepared in PBS containing 0.2% glucose. Aliquots (50 μL) were dispensed into a 96-well plate, followed by 50 μL propidium iodide (PI) to yield a final PI concentration of 10 μM per well. Subsequently, 100 μL of the bacterial suspension were added to obtain a final OD₆₀₀ of 0.1 (CFU/mL $\sim 5 \times 10^7$) and a total assay volume of 200 μL per well. Negative controls lacking peptide were included. Fluorescence measurements were performed at 37°C using an Agilent Synergy HTX microplate reader, recording fluorescence at 2 min intervals with shaking between reads. PI fluorescence was measured with excitation at 535 nm and emission at 590 nm.

12.9 Anionic lipid clustering assay

Measurements were performed with a MicroCal VP-DSC microcalorimeter (Malvern, UK) as previously described.¹¹⁸ Lipid films were prepared with POPE/TOCL (75:25) with and without peptide. Peptide was added at a P/L of 1:20 while the samples without peptide served as reference for subsequent measurements. The samples were rehydrated in buffer containing 20 mM PIPES, 140 mM NaCl, 1 mM EDTA, pH 7.4 to generate MLVs and underwent 10 freeze thaw cycles to ensure full hydration of the lipids.

The samples were then brought to 3°C and scanned from 3 to 40°C with three cycles of heating and cooling per measurement. The reference cell was filled with degassed PIPES buffer. Thermograms were collected in consecutive heating and cooling cycles with a scanning rate of 1°/min. Only the heating scans were analysed. The cell volume was 0.51 mL. The total lipid concentration in the calorimeter cell was 2.5 mg/mL. Calorimeter data were plotted with Origin 13.6.

13 Theory

In this chapter, the fundamental principles of NMR spectroscopy, CD and OCD spectroscopy and fluorescence are described in detail, as these techniques constitute the core bioanalytical methods employed in this work.

13.1 NMR-Spectroscopy

In nuclear magnetic resonance (NMR) spectroscopy, nuclei with a non-zero spin are placed in a strong external static magnetic field B_0 . In this field, the nuclear spin states split into different energy levels. By applying a radiofrequency pulse at the resonance frequency of the nucleus, transitions between these energy levels can be induced. The resulting signal contains information about the chemical environment, structure and dynamics of the investigated sample. Nuclear spin is a quantized angular momentum and can only adopt discrete values. It can be described with the following formula:^{131,132}

$$P = \sqrt{I(I + 1)}\hbar$$

$\hbar$ is the reduced Planck's quantum of action and can be described as $\hbar = h/2\pi$. I is the angular momentum quantum number or nuclear spin quantum number. I can take values from 0, 1/2, 1, 3/2, 2, (...) to 6, depending on the type of atomic nucleus. The value of I depends on the composition of the nucleus, i.e. on the number and arrangement of protons and neutrons. For ^1H nuclei for example, a value of $I=1/2$ is observed.^{131,132}

Adding to the spin every nucleus has a charge. Charge and spin combined result in a magnetic moment μ . μ is proportional to P and can be described by the following formula.^{131,132}

$$\mu = \gamma P$$

γ is a nucleus-specific constant of proportionality. The value of γ varies from element to element and is known as the gyromagnetic ratio. The larger the γ -value for an element, the more sensitive the nuclei are to NMR. By substituting the formula for P into the formula for μ , this generally valid formula follows:^{131,132}

$$\mu = \gamma \sqrt{I(I+1)} \hbar$$

Nuclei for which the nuclear spin I is equal to 0 do not generate a nuclear magnetic moment and therefore generate no detectable signal for NMR-spectroscopic methods.^{131,132} The vectors of nuclear angular momentum P as well as the vector of the magnetic moment μ point in the same direction for most nuclei. To measure a sample, it gets placed in a static magnetic field B_0 .^{131,132} In the external magnetic field B_0 , the nuclear spin states become quantized with respect to the field direction. For a nucleus with spin I , $2I+1$ magnetic quantum states are possible. The thus resulting angular momentum P_z only adopts values which are an integer or half-integer multiples of $\hbar$. $P_z = m\hbar$ with m being the magnetic quantum number that takes values of $m = I, I-1, (...), -I$. This allows for $2I+1$ different value for m . This is also called directional quantization and is crucial for NMR spectroscopy. E.g., this property results in the values $m=-1/2$ and $m=+1/2$ for nuclei with a spin of $1/2$ as in ^1H .^{131–133}

For the magnetic moment μ_z , which is also aligned in the z-direction, resulting in the following formula:

$$\mu_z = m\gamma\hbar$$

The splitting of nuclear spins in a static magnetic field B_0 is called Zeeman splitting or Zeeman effect.^{131–133}

The resulting core dipoles precess around the z-axis parallel to the direction of B_0 . This motion can be described with the Larmor frequency ν_L . It is proportional to the magnetic flux density of B_0 and corresponds to the frequency of dipole processing around the z-axis. Due to the directional quantization only fixed angles are allowed for these precessing dipoles.^{131–133} NMR excitation occurs when the frequency of the applied radiofrequency field matches the Larmor frequency of the nucleus. Under this resonance condition, energy can be absorbed and transitions between the spin states can be induced. The exact resonance frequency depends on both the magnetic field strength and the nuclear environment.

$$\nu_L = \left| \frac{\gamma}{2\pi} \right| B_0$$

Considering the energy E of these magnetic dipoles, the following relationship is obtained : ^{131–133}:

$$E = -\mu_z B_0 = -m\gamma\hbar B_0$$

For nuclei with $I = 1/2$ such as ^1H , two spin states are possible in the magnetic field. For nuclei with a positive gyromagnetic ratio, the lower-energy α -state is aligned parallel to B_0 , whereas the higher-energy β -state is aligned antiparallel to B_0 .^{131–133}

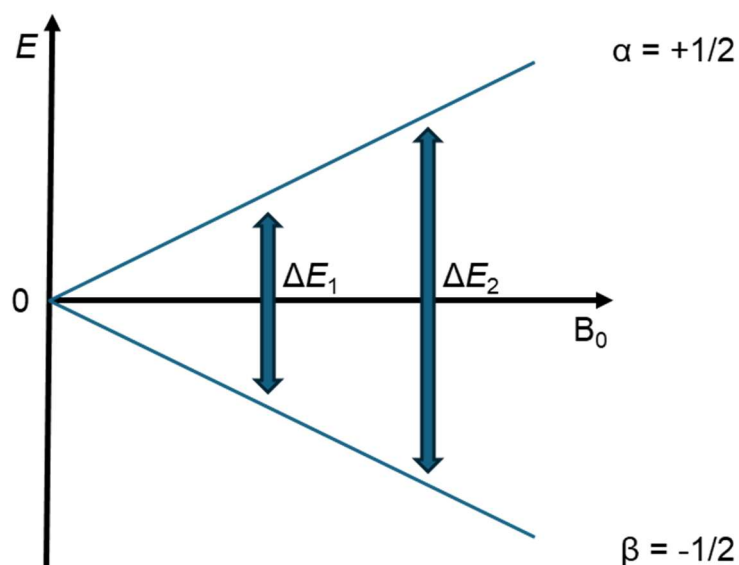


Figure 47: Representation of the energy levels and their proportional dependence on B_0 for ^1H .

Modified after 131.

ΔE , the energy difference between two neighbouring states, can be described with the following formula:^{131,132}

$$\Delta E = \gamma \hbar B_0$$

To describe the distribution of nuclei to the different energy levels, the following formula is suitable:^{131,132}

$$\frac{N_\beta}{N_\alpha} = e^{-\frac{\Delta E}{kT}} = e^{-\frac{\gamma \hbar B}{kT}}$$

Here N_β describes the number of nuclei in a higher energy level and N_α those that are in the lower energy level. k is the Boltzmann constant ($1.3805 \cdot 10^{-23} \text{ J} \cdot \text{K}^{-1}$) and T is the absolute temperature. The basis of this formula is the Boltzmann statistics of quantum mechanics. Since the energy difference between the spin states is small compared with the thermal energy, the populations of the two states differ only slightly.

Nevertheless, this small population difference is sufficient to produce a measurable net magnetization along the direction of the external magnetic field.^{131,132}

The sum of all magnetic components oriented in field direction z , which are located within the sample, results in the macroscopic magnetization M_0 . Since N_α is larger than N_β , this results in a net macroscopic magnetization in the field direction.^{131,132}

After excitation by a radiofrequency pulse, the transverse magnetization precesses in the x - y plane and induces a time-dependent voltage in the receiver coil. This signal is called the free induction decay (FID). By Fourier transformation of the FID, the time-domain signal is converted into the frequency-domain NMR spectrum.

The resonance frequency of a nucleus is influenced by its local electronic environment. Electrons partially shield the external magnetic field, so nuclei in different chemical environments resonate at slightly different frequencies. These frequency differences are reported as the chemical shift δ , usually in parts per million (ppm), and form the basis for the structural interpretation of NMR spectra.

To detect an NMR signal, a radiofrequency field B_1 is applied perpendicular to the static field B_0 . If the frequency of B_1 matches the Larmor frequency, the net magnetization can be tilted away from the z -axis. A pulse that rotates the magnetization by 90° into the transverse plane is referred to as a 90° pulse.^{131,132}

After a 90° pulse, the net magnetization is located in the transverse plane and precesses around B_0 at the Larmor frequency. This precessing transverse magnetization induces a current in the detection coil, which is recorded as the NMR signal. Over time, the signal decays due to relaxation processes. The return of the magnetization to thermal equilibrium is called relaxation. Two relaxation processes are distinguished. Longitudinal relaxation, characterized by the time constant T_1 , describes the recovery of the magnetization along the z -axis. Transverse relaxation, characterized by T_2 , describes the loss of phase coherence in the x - y plane. In practice, the recycle delay between scans must be chosen long enough to allow sufficient T_1 relaxation. Short T_2 values lead to rapid signal decay and therefore to broader spectral lines.^{131,132}

In summary, the most important NMR observables are the resonance frequency, expressed as chemical shift, and the relaxation behaviour of the nuclei, both of which provide information on molecular structure and dynamics.

13.2 Solid-state NMR spectroscopy

Solid-state NMR spectroscopy is used to investigate the structure, orientation and dynamics of molecules in non-isotropic systems such as membranes, solids or aligned lipid bilayers. In contrast to solution NMR, anisotropic interactions are not fully averaged by molecular tumbling and therefore remain observable in the spectra. These interactions contain valuable structural information, for example on molecular orientation and mobility. In membrane-oriented solid-state NMR, the most relevant anisotropic interactions are the chemical shift anisotropy and, depending on the observed nucleus, dipolar or quadrupolar interactions. In the present work, oriented lipid bilayers were prepared on glass plates. After drying and rehydration, the bilayers adopt a defined orientation relative to the magnetic field, which allows structural information on both the membrane and the incorporated peptides to be obtained.^{133,134}

A particularly useful nucleus for studying lipid membranes is ^{31}P , because phospholipid headgroups contain phosphate moieties. Investigations of lipid bilayers can be performed by solid-state ^{31}P - NMR experiments. Since many membrane lipids contain phosphate groups in their polar headgroups, ^{31}P can often be observed without isotopic labelling. In addition, ^{31}P has spin 1/2, which makes it well suited for NMR spectroscopy. In solid-state ^{31}P - NMR, the spectral position and line shape are sensitive to the orientation and order of the phospholipid headgroups. Well-oriented lamellar bilayers typically give rise to characteristic narrow resonances, whereas unoriented or highly disordered membrane systems produce broader signals or powder-type line shapes. Thus, ^{31}P -NMR provides information on membrane alignment, phase state and perturbations induced by peptides.^{133,134}

In addition to ^{31}P -NMR of the lipid headgroups, oriented ^2H NMR can be used to analyse the orientation and dynamics of deuterium-labelled molecules in the membrane.

Solid-state ^2H NMR is a powerful method for studying the orientation and dynamics of deuterium-labelled molecules in lipid bilayers. Because ^2H is a quadrupolar nucleus with spin 1, its NMR spectrum is strongly influenced by molecular orientation and motion. For this reason, specific deuterium labels are introduced at defined molecular positions. The resulting quadrupolar splittings reflect the orientation of the labelled C-D bonds with respect to the magnetic field and are therefore highly useful for analysing peptide alignment, segmental mobility and membrane order.

13.3 Structural fitting

The deuterium quadrupolar splitting $\Delta\nu_Q$ (often denoted D) is the main experimental observable in oriented solid-state ^2H -NMR. $\Delta\nu_Q$ of a deuterium nucleus arises from the interaction of the nuclear quadrupole moment with the electric field gradient at the nucleus and depends on the orientation of the corresponding interaction tensor relative to B_0 .⁵⁴ Each position k in a peptides primary sequence that has a deuterium label (here Ala- d_3) yields a quadrupolar splitting (D_{exp} at position k) that can be used in the fitting process.⁵⁴

The fitting approach used in this work follows the so-called GALA method (Geometric Analysis of Labeled Alanines). In this method, the set of measured splittings is interpreted using a geometric helix model that assumes an α -helix with a rigid body positioned in an aligned lipid bilayer.⁵⁴ The helix orientation is described by the tilt angle (τ) relative to the membrane-normal and the azimuthal rotational angle (ρ) about the helix axis itself, starting at residue 12. As side chains advance systematically around the helix with rising residue number, the orientation of the labelled Ala- d_3 tensors vary periodically according to their position in the helix. Therefore, the measured $\Delta\nu_Q$ values plotted against the respective residue position corresponds to a distinct quadrupolar wave. This wave can be fitted by varying τ and ρ values, minimizing the root-mean-square deviation (RMSD) from the experimental values. To do so, the fitting program assumes a defined local geometry for the alanine labels. In the GALA formalism, the orientation relevant for the ^2H splittings of Ala- d_3 is parameterized via the $\text{C}\alpha$ - $\text{C}\beta$ direction in the helix frame, described by fixed angles $\varepsilon_{\parallel} = 121.1^\circ$ and $\varepsilon_{\perp} = 53.2$ using WALP conventions.^{54,87} In addition, a quadrupolar constant is chosen so that an immobilized Ala- d_3 would yield a quadrupolar splitting of 84 kHz when $\text{C}\alpha$ - $\text{C}\beta$

is aligned with B_0 and is reduced in relation to the rigid-limit coupling due to internal methyl dynamics. During the fitting process computed splittings D_{cal} are calculated for every labelled position k . The best-fit parameters are then achieved by minimizing the RMSD between experiment and calculation using the following formula:

$$RMSD_{GALA} = \sqrt{\frac{1}{N} \sum_{k=1}^N (D_{exp,k} - D_{cal,k})^2}$$

where N is the number of labels.

In addition to τ and ρ angles, $\Delta\nu_Q$ also depends on molecular motions. The simplest model of dynamics is to introduce a scaling factor or molecular parameter S_{mol} , which accounts for a reduction in the splittings due to motion. $D(\tau, \rho, S_{mol})$

Using this method, an uncertainty of the tilt angle may occur, even though the experimental splittings appear to fit very well. The reason is that both the orientation and motional averaging affect the quadrupolar splittings.^{54,87}

To avoid this uncertainty, a $_{dyn}GALA$ model was developed by Strandberg et al.⁸⁷ $_{dyn}GALA$ characterizes the helix by a distribution of tilt and rotational angles and assumes the angles as a Gaussian distribution as follows.⁵⁴

$$q \sim N(q_0, \sigma_q^2)$$

where q_0 is the mean angle and σ_q defines the width of the distribution (the standard deviation of the fluctuations).

The predicted quadrupolar splittings are then calculated by averaging over τ and ρ distribution: splittings are calculated for a set of q values gained from the Gaussian centred at q_0 with width r_q and the resulting $D_{cal,k}$ values are averaged. The resulting prediction is therefore dynamically averaged. The dynamically averaged $D_{cal,k}$ is then used to calculate the minimum RMSD as described above.^{54,87} $D_{cal}(\tau, \rho, \sigma_\tau, \sigma_\rho)$.

13.4 Circular dichroism

Circular dichroism (CD) spectroscopy is based on the different absorption of left- and right-circularly polarized light by chiral molecules. Linearly polarized light can be described as the superposition of left- and right-circularly polarized components of equal amplitude. When such light passes through a chiral sample, the two components are absorbed to different extents, which results in elliptically polarized light after transmission. This differential absorption is referred to as circular dichroism and forms the basis of CD spectroscopy (**Figure 48**).^{135,136} Chiral molecules are considered optically active. If the molecule is not chiral, both components get absorbed to the same degree, resulting in linear polarized light after passing through the sample. Such molecules are considered optically inactive. Racemic mixtures are optically inactive in CD spectroscopy because the two enantiomers contribute equal but opposite CD signals, which cancel each other. Thus, CD spectroscopy measures the differential absorption of left- and right-circularly polarized light as a function of wavelength.^{135–137}

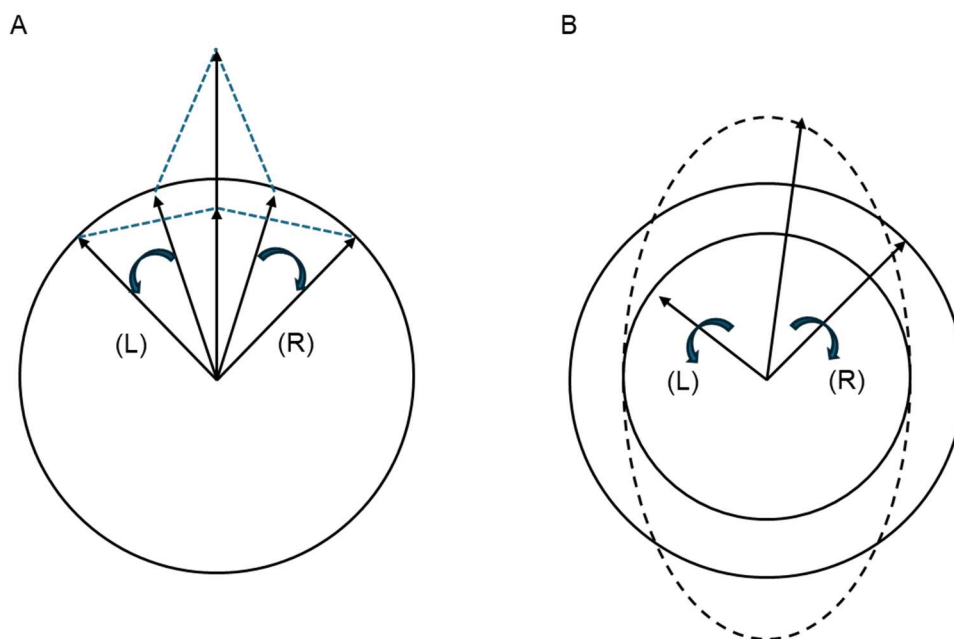


Figure 48: Explanation of the CD effect.

A) If both left and right polarized light are absorbed equally the light is linear polarized after passing through a substance, meaning that the amount of left and right polarized light is equal. B) If one component is absorbed to a higher degree than the other, the components of the light show different amplitudes resulting in elliptically polarized light. Modified after ref. 135 and 137.

n CD spectroscopy, the difference in absorption of left- and right-circularly polarized light is expressed as the differential extinction, $\Delta\epsilon$. Experimentally, this difference is commonly reported as the ellipticity θ , which is measured as a function of wavelength. The observed ellipticity depends on the concentration of the sample and the optical path length.^{137,138}

$$\Delta\epsilon = (\epsilon_L - \epsilon_R)$$

$$\theta = 32.98 * \Delta\epsilon$$

$$\theta(\lambda) = 32.98 * (\epsilon_L - \epsilon_R) * c * d$$

c being the concentration and d the path length of the cuvette used

From the ellipticity $\theta(\lambda)$, the mean residue ellipticity (MRE) can be calculated. MRE represents the ellipticity normalized to one amino acid residue and therefore allows comparison of peptides or proteins of different lengths. For this reason, MRE is commonly used in peptide and protein CD spectroscopy.^{137,138}

$$\theta_{MRW}(\lambda) = MRE = \frac{MRW * \theta(\lambda)}{10 * d * c} = \frac{M}{(N - 1)} * \frac{\theta(\lambda)}{10 * d * c}$$

Here, M denotes the mean residue weight, c the concentration, and d the optical path length.

CD spectroscopy is a well-established method for the structural analysis of peptides and proteins. In particular, it provides information on the overall secondary-structure content and on conformational changes induced by ligand binding, denaturation, or membrane interaction. Since the peptide bond acts as the relevant chromophore in the far-UV region, CD spectra are commonly recorded between 180 and 260 nm. However, CD spectroscopy yields mainly ensemble-averaged structural information rather than high-resolution structural details. Aggregation or light scattering can distort the spectra and should therefore be minimized during sample preparation.^{135,137} In the far-UV region, CD spectroscopy is particularly useful for the analysis of peptide and protein secondary structure. α -Helices typically show two negative bands near 208 and 222 nm and a positive band around 190 nm. β -Sheet structures and unordered conformations show different characteristic spectral shapes. CD spectroscopy therefore allows a rapid assessment of conformational changes and secondary-structure content.^{62,135–137}

In the far-UV region, CD spectroscopy is particularly useful for the analysis of peptide and protein secondary structure. α -Helical conformations typically exhibit two negative bands at approximately 208 and 222 nm and a positive band near 190 nm. In contrast, β -sheet structures show a negative band around 218 nm and a positive band near 195 nm, whereas unordered or random-coil conformations are characterized by a strong negative band near 198 nm and only weak ellipticity at higher wavelengths. These characteristic spectral features allow CD spectroscopy to be used for the rapid assessment of secondary-structure content and conformational changes.^{137,138}

13.5 Oriented circular dichroism

Oriented circular dichroism (OCD) is used to obtain information on the orientation of peptides in aligned lipid bilayers. For this purpose, the sample is deposited on a quartz plate and kept under hydrated conditions during the measurement so that the bilayer retains its structural integrity and a sufficient degree of molecular mobility. A common limitation of this sample preparation is the occurrence of artefacts caused by inhomogeneous sample distribution or imperfect alignment. To reduce such artefacts, spectra are recorded at different rotation angles of the sample around the beam axis and subsequently averaged.^{62,137,138}

OCD of α -helical peptides is based on the anisotropic orientation of the electronic transition dipole moments of the peptide bonds relative to the helix axis. In an oriented membrane sample, the measured CD spectrum therefore depends not only on secondary structure but also on the orientation of the helix with respect to the membrane normal and the incident light. As a result, OCD can be used to distinguish between surface-bound, tilted, and inserted helical states in lipid bilayers.^{62,138} In this description, $\theta_{\perp}$ and $\theta_{\parallel}$ denote the contributions of transitions polarized perpendicular and parallel to the helix axis, respectively, whereas θ_v represents the ellipticity of the corresponding isotropic sample.^{137,138}

$$\theta_v = \frac{(\theta_{\perp} - \theta_{\parallel})}{3}$$

T

The resulting OCD spectra allow analysis of the orientation of an α -helical peptide in the lipid bilayer. In particular, the ellipticity near 208 nm is highly informative. A

pronounced negative band at 208 nm is characteristic of a surface-bound helix (surface state, S-state). If the ellipticity at 208 nm approaches zero or becomes positive, this indicates an inserted state (I-state), in which the helix is oriented approximately perpendicular to the membrane plane. Intermediate values are commonly interpreted as a tilted state (T-state), in which the helix adopts an orientation between the S- and I-states.^{62,137,138} By analysing θ_v at this wavelength the peptides helix can be assigned into three different states. If a distinct negative ellipticity is observed it is a strong indication that the α -helix is laying on the bilayers surface in a so-called Surface state (S-state).^{62,63} If the ellipticity at 208 nm goes towards the zero line or above, this indicates that the α -helix is positioned in an Inserted state (I-state), meaning it is inserted into the bilayer at close to 90° respective to the plane of the bilayers. When the negativity is somewhere in between the two describes cases, the α -helix is considered a Tilted-state (T-state), meaning it is somewhere in between the angles of an S- and an I-state and most likely tilted in the bilayer. However, the last case can be an artefact of the mobility of the peptide. If the peptide switches between an S- and an I-state the spectrum can look like a T-state.⁶² However, such an intermediate spectrum does not necessarily prove a uniquely tilted helix, since it may also arise from dynamic averaging or from a mixture of surface-bound and inserted peptide populations.

The orientation adopted by an α -helical peptide depends on several experimental parameters, including hydration level, lipid composition, temperature, and peptide-to-lipid ratio (P/L). Many peptides are predominantly found in the surface state at low concentration, whereas increasing peptide concentration can promote tilting or insertion into the bilayer.^{62,63}

For oriented α -helical peptides in lipid bilayers, the OCD spectrum is interpreted mainly on the basis of the band near 208 nm, since this signal is highly sensitive to helix orientation. A pronounced negative band at 208 nm is characteristic of a surface-bound helix lying parallel to the membrane surface. If the 208 nm band decreases strongly, approaches zero, or becomes positive, this indicates a transmembrane orientation with the helix aligned approximately parallel to the membrane normal. Intermediate intensities are commonly assigned to tilted states, in which the helix adopts an orientation between these two limiting cases.^{62,63}

13.6 Fluorescence

Fluorescence is a physical process that describes an energy relaxation of an excited molecule (fluorophore) into a ground state while emitting light.¹³⁹ Fluorescence is a photoluminescent process during which the fluorophore absorbs light and is promoted from a singlet ground state S_0 into an excited singlet state S_n , where n depends on the amount of absorbed energy. From the S_n state the molecule can return into its ground state by rapid vibrational relaxation and internal conversion into the lowest excited singlet state S_1 . This relaxation occurs without light emission. The energy difference from S_n to S_1 is typically transformed into heat. Relaxation processes emitting fluorescence usually occur from the S_1 state into the S_0 state. As some energy is transformed non-radiatively during this whole process, fluorescence is always accompanied by a so-called Stokes shift. The Stokes shift describes the energy discrepancy between the excitation wavelength and the emitted wavelength.

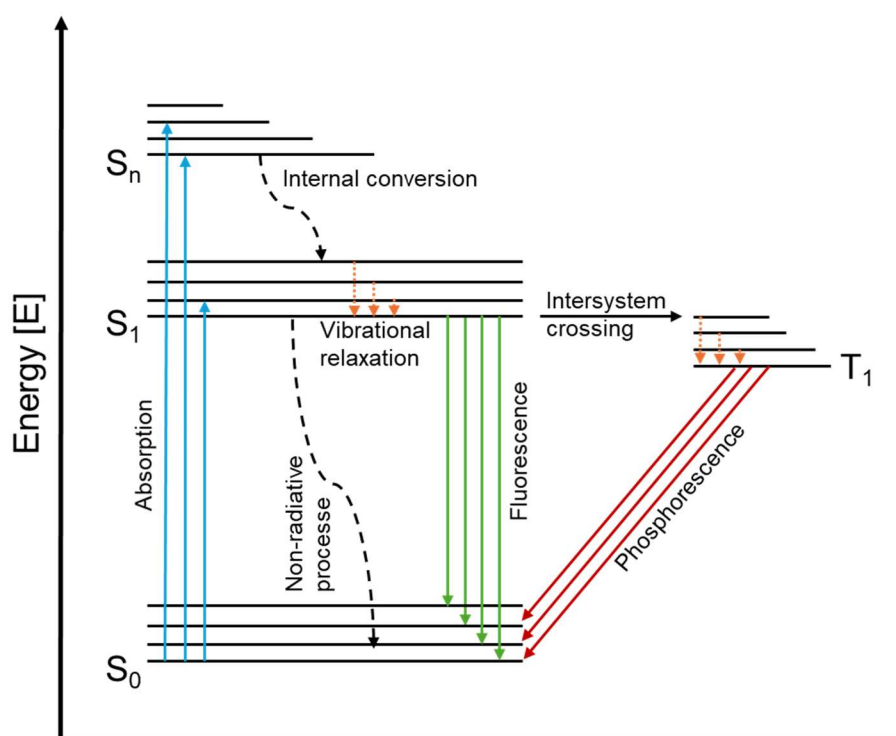


Figure 49: Jablonski diagram describing the schematic photo physics of a fluorophore.

Vertical axis shows energy [E]. The diagram includes the singlet state S_0 , S_1 and S_n as well as the triplet state T_1 and the different excitation and relaxation processes involved. This figure was adapted from ref. 139.

The different types of energy conversion that can occur are summarised in **Figure 49** in a so-called Jablonski diagram.¹³⁹

The intersystem crossing also describes a non-radiant energy conversion from the S_1 into a T_1 state. From T_1 the most common relaxation occurs through phosphorescence.

The fluorescence intensity depends on the concentration of the fluorophore, the excitation intensity and the quantum yield $[\Phi]$ of the fluorophore. Φ is governed by the competition between radiative decay (k_f) and non-radiative (k_{nr}) decay. During a quenching process, the pathways of relaxation are altered due to the presence of another molecule (quencher). Dynamic quenching, for example, describes the collision of a fluorophore and a quencher in an excited state. This leads to reduced fluorescence lifetime or the deactivation of the fluorescence relaxation pathway. The process follows the Stern-Volmer relation:

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{SV}[Q]$$

With $K_{SV} = k_q\tau_0$. I_0 = fluorescence intensity without quencher. I = fluorescence intensity with quencher. τ_0 = fluorescence lifetime without quencher. τ = fluorescence lifetime with quencher. Q = concentration of the quencher.

In static quenching a non-fluorescent ground-state complex forms between fluorophore and quencher before excitation events. The fluorophores remaining free of a quencher have an unchanged fluorescence lifetime but the overall observed intensity drops due to the decrease of free fluorophores.

14 Appendix

Table A 1: List of all 28 Ala-d₃ analogues and the Ltc5 WT.

Kind of mutation, name of the analogue, primary sequence, Mw in Da, net charge and retention time of each peptide is included in the table.

Ltc5 analogues	Name	Primary sequence	Mw [Da]	Net Charge	Ret. Time
Wildtype	WT	GFFGKMKEYFKKFGASFKRRFANLKKRL-amide	3430.18	10	12.3
G1 Ala-d ₃	G1	(Ala-d ₃) FFGKMKEYFKKFGASFKRRFANLKKRL-amide	3447.21	10	11.7
F2 Ala-d ₃	F2	G (Ala-d ₃) FGKMKEYFKKFGASFKRRFANLKKRL-amide	3357.17	10	11.4
F3 Ala-d ₃	F3	GF (Ala-d ₃) GKMYFKKFGASFKRRFANLKKRL-amide	3357.17	10	11.3
G4 Ala-d ₃	G4	GFF (Ala-d ₃) KMKEYFKKFGASFKRRFANLKKRL-amide	3447.21	10	12.4
K5 Ala-d ₃	K5	GFFG (Ala-d ₃) MKEYFKKFGASFKRRFANLKKRL-amide	3376.08	9	12.5
M6 Ala-d ₃	M6	GFFGK (Ala-d ₃) KEYFKKFGASFKRRFANLKKRL-amide	3373.06	10	11.5
K7 Ala-d ₃	K7	GFFGKM (Ala-d ₃) EYFKKFGASFKRRFANLKKRL-amide	3376.10	9	12.7
E8 Ala-d ₃	E8	GFFGKMK (Ala-d ₃) YFKKFGASFKRRFANLKKRL-amide	3375.14	11	12.1
Y9 Ala-d ₃	Y9	GFFGKMKE (Ala-d ₃) FKKFGASFKRRFANLKKRL-amide	3341.18	10	12.1
F10 Ala-d ₃	F10	GFFGKMKEY (Ala-d ₃) KFKGASFKRRFANLKKRL-amide	3357.17	10	11.5
K11 Ala-d ₃	K11	GFFGKMKEYF (Ala-d ₃) KFGASFKRRFANLKKRL-amide	3376.08	9	12.4
K12 Ala-d ₃	K12	GFFGKMKEYFK (Ala-d ₃) FGASFKRRFANLKKRL-amide	3376.08	9	12.5
F13 Ala-d ₃	F13	GFFGKMKEYFKK (Ala-d ₃) GASFKRRFANLKKRL-amide	3357.17	10	11.6
G14 Ala-d ₃	G14	GFFGKMKEYFKKF (Ala-d ₃) ASFKRRFANLKKRL-amide	3447.21	10	12.9
A15 Ala-d ₃	A15	GFFGKMKEYFKKFG (Ala-d ₃) SFKRRFANLKKRL-amide	3433.18	10	12.3
S16 Ala-d ₃	S16	GFFGKMKEYFKKFGA (Ala-d ₃) FKRRFANLKKRL-amide	3417.18	10	12.4
F17 Ala-d ₃	F17	GFFGKMKEYFKKFGAS (Ala-d ₃) KRRFANLKKRL-amide	3357.17	10	11.4
K18 Ala-d ₃	K18	GFFGKMKEYFKKFGASF (Ala-d ₃) RRFANLKKRL-amide	3376.08	9	12.6
R19 Ala-d ₃	R19	GFFGKMKEYFKKFGASFK (Ala-d ₃) RFANLKKRL-amide	3348.07	9	12.7
R20 Ala-d ₃	R20	GFFGKMKEYFKKFGASFKR (Ala-d ₃) FANLKKRL-amide	3348.07	9	13.2
F21 Ala-d ₃	F21	GFFGKMKEYFKKFGASFKRR (Ala-d ₃) ANLKKRL-amide	3357.17	10	11.5
A22 Ala-d ₃	A22	GFFGKMKEYFKKFGASFKRRF (Ala-d ₃) NLKKRL-amide	3433.18	10	12.1
N23 Ala-d ₃	N23	GFFGKMKEYFKKFGASFKRRFA (Ala-d ₃) LKKRL-amide	3390.15	10	12.3
L24 Ala-d ₃	L24	GFFGKMKEYFKKFGASFKRRFAN (Ala-d ₃) KKRL-amide	3391.10	10	11.5
K25 Ala-d ₃	K25	GFFGKMKEYFKKFGASFKRRFANL (Ala-d ₃) KRL-amide	3376.08	9	12.7
K26 Ala-d ₃	K26	GFFGKMKEYFKKFGASFKRRFANLK (Ala-d ₃) RL-amide	3376.08	9	12.2
R27 Ala-d ₃	R27	GFFGKMKEYFKKFGASFKRRFANLKK (Ala-d ₃) L-amide	3348.07	9	12.4
L28 Ala-d ₃	L28	GFFGKMKEYFKKFGASFKRRFANLKKR (Ala-d ₃) -amide	3391.10	10	11.5

Table A 2: Deconvolution results of CD of all measured Ltc5 Ala-d₃ analogues spectra measured in 10 mM PB.

Measurements were conducted at 25 °C. The table includes the distribution of secondary structures in percent. Included secondary-structures are helix, strand, turn and unordered.

Ltc5 WT	Helix [%]	Strand [%]	Turn [%]	Unordered [%]
Ltc5 G1	16	22	25	36
Ltc5 F2	5	30	25	36
Ltc5 F3	7	21	15	57
Ltc5 G4	11	21	15	52
Ltc5 K5	11	28	20	41
Ltc5 M6	11	25	17	47
Ltc5 K7	3	40	20	38
Ltc5 E8	6	37	18	40
Ltc5 Y9	5	26	17	52
Ltc5 F10	3	38	18	41
Ltc5 K11	3	38	19	40
Ltc5 K12	5	35	20	40
Ltc5 F13	5	30	20	45
Ltc5 G14	3	38	18	40
Ltc5 S16	4	35	21	39
Ltc5 F17	11	34	17	48
Ltc5 K18	3	37	19	41
Ltc5 R19	17	24	16	43
Ltc5 R20	18	22	17	44
Ltc5 F21	3	40	20	37
Ltc5 N23	3	38	19	40
Ltc5 L24	12	24	16	48
Ltc5 K25	3	40	19	38
Ltc5 K26	16	23	20	41
Ltc5 R27	11	26	16	47
Ltc5 L28	11	26	18	46
Ltc5 WT	6	33	21	40

Table A 3: Deconvolution results of CD spectra of Ltc5 Ala-d₃ analogues in SUVs.

The SUVs were composed from DMPC/DMPG 7:3 at a P/L of 1:50. The table includes the distribution of secondary structures in percent. Included secondary structures are helix, strand, turn and unordered.

Name	Helix [%]	Strand [%]	Turn [%]	Unordered [%]
Ltc5 WT	89	6	3	3
Ltc5 G1	88	3	4	4
Ltc5 F2	91	3	4	2
Ltc5 F3	90	2	4	3
Ltc5 G4	84	6	5	5
Ltc5 K5	83	7	5	6
Ltc5 M6	88	5	3	4
Ltc5 K7	87	4	4	5
Ltc5 E8	87	4	3	5
Ltc5 Y9	86	6	5	3
Ltc5 F10	89	6	3	4
Ltc5 K11	88	4	5	3
Ltc5 K12	86	5	6	3
Ltc5 F13	92	3	2	5
Ltc5 G14	90	3	1	7
Ltc5 S16	84	6	5	5
Ltc5 F17	87	5	3	5
Ltc5 K18	85	6	4	5
Ltc5 R19	85	6	5	4
Ltc5 R20	89	4	4	3
Ltc5 F21	88	5	3	3
Ltc5 N23	88	4	4	4
Ltc5 L24	87	5	3	4
Ltc5 K25	83	7	5	6
Ltc5 K26	88	4	3	4
Ltc5 R27	85	7	5	4
Ltc5 L28	81	6	7	5

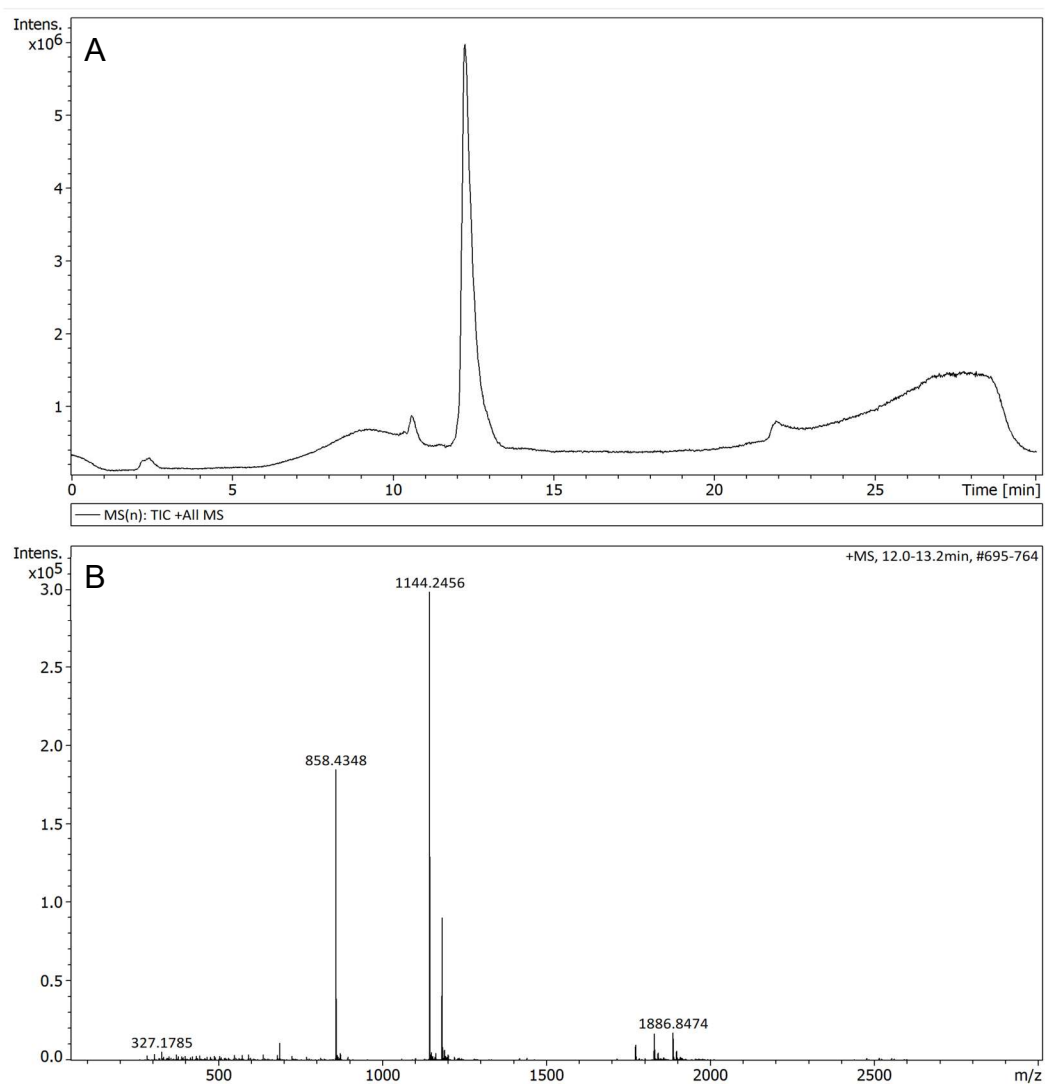


Figure A 1: LC-MS spectra of the Ltc5 WT.

A) HPLC chromatogram. B) ESI-MS corresponding to the HPLC chromatogram in A. The observed masses correspond to the peptide mass plus TFA counterions (~114 Da) from peptide synthesis.

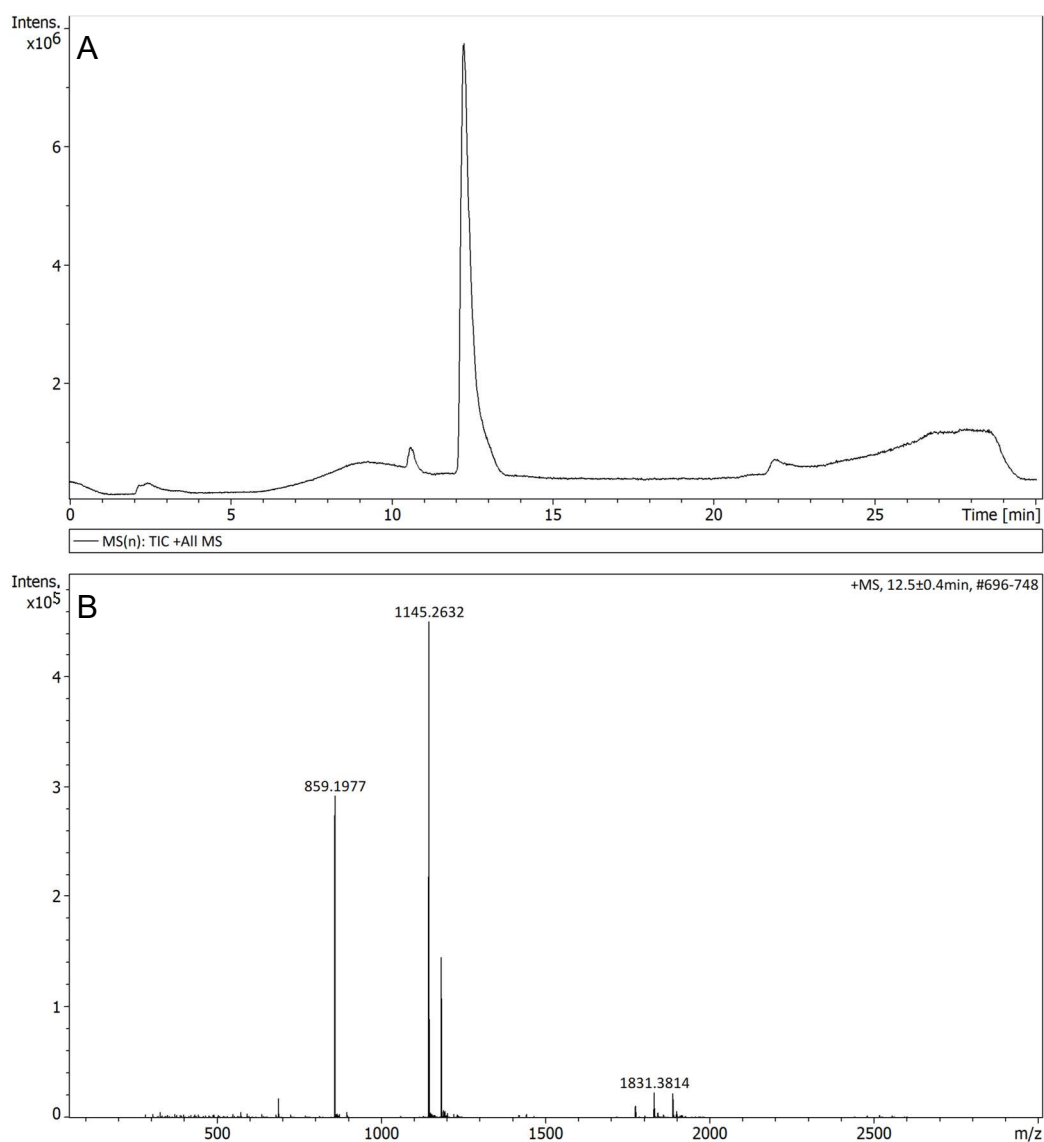


Figure A 2: LC-MS spectra of the Ltc5 A15.

A) HPLC chromatogram. B) ESI-MS corresponding to the HPLC chromatogram in A. The observed masses correspond to the peptide mass plus TFA counterions (~114 Da) from peptide synthesis.

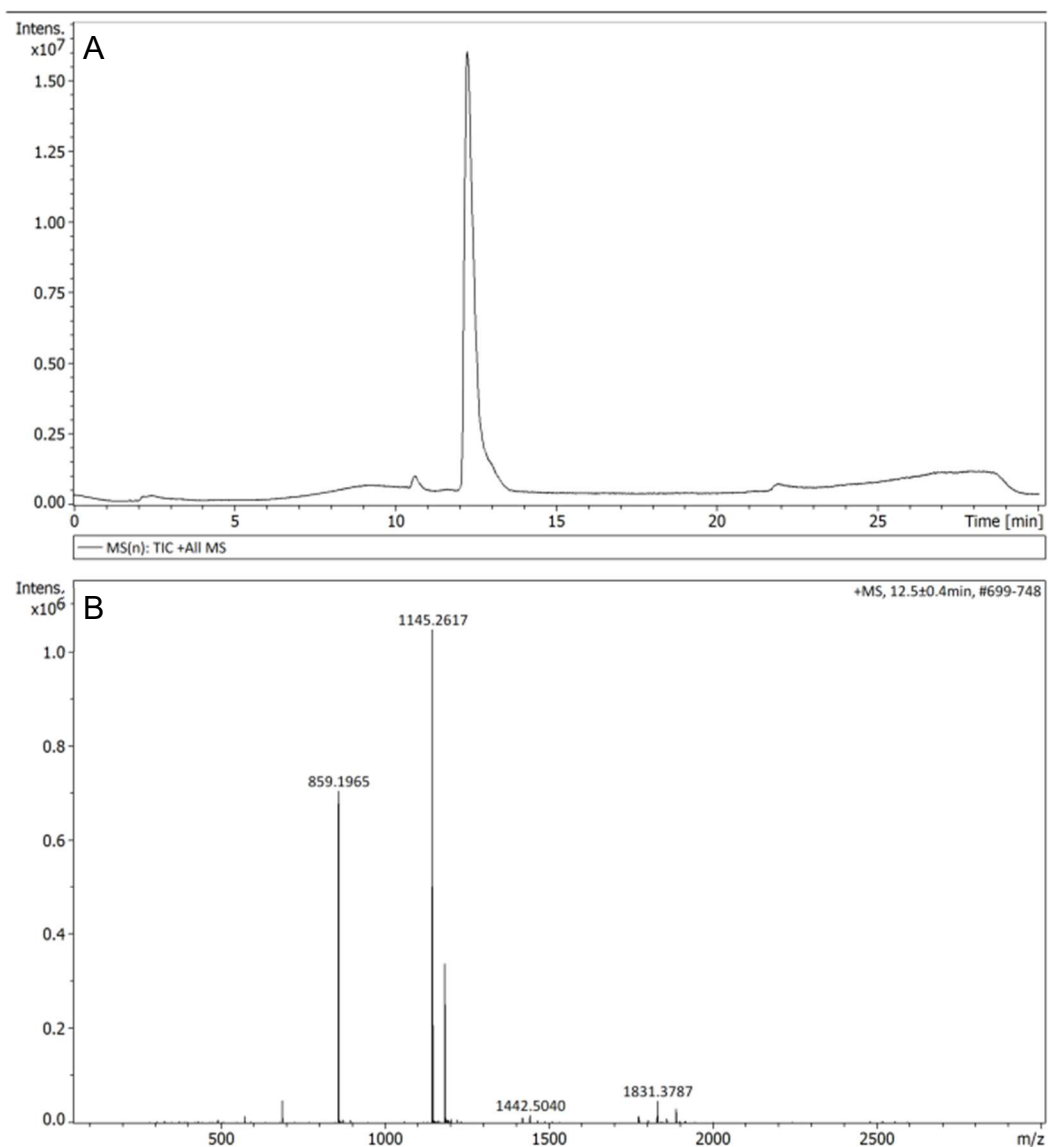


Figure A 3: LC-MS spectra of the Ltc5 A22.

A) HPLC chromatogram. B) ESI-MS corresponding to the HPLC chromatogram in A. The observed masses correspond to the peptide mass plus TFA counterions (~ 114 Da) from peptide synthesis.

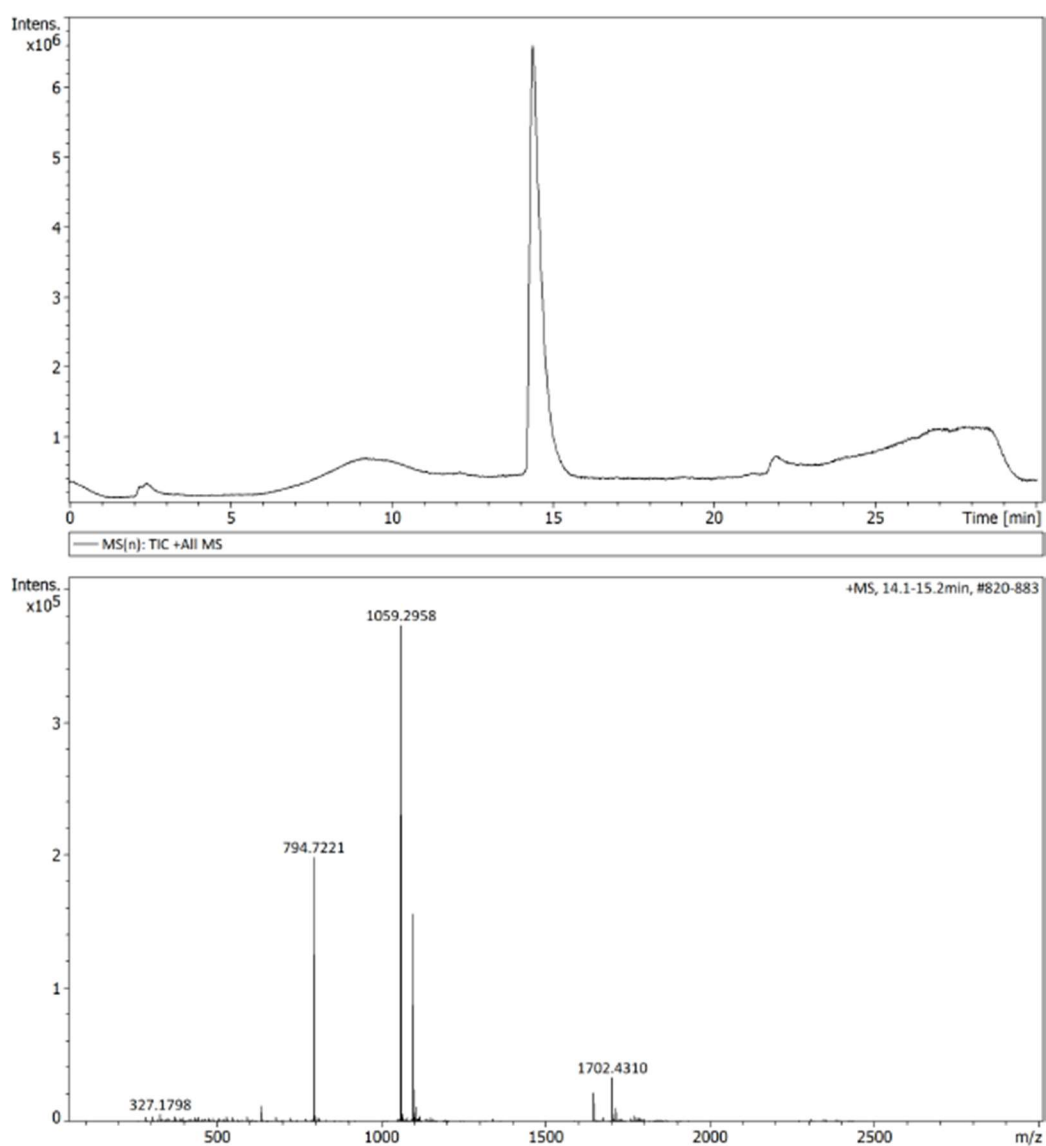


Figure A 4: LC-MS spectra of the Ltc5 ChA.

A) HPLC chromatogram. B) ESI-MS corresponding to the HPLC chromatogram in A. The observed masses correspond to the peptide mass plus TFA counterions (~114 Da) from peptide synthesis.

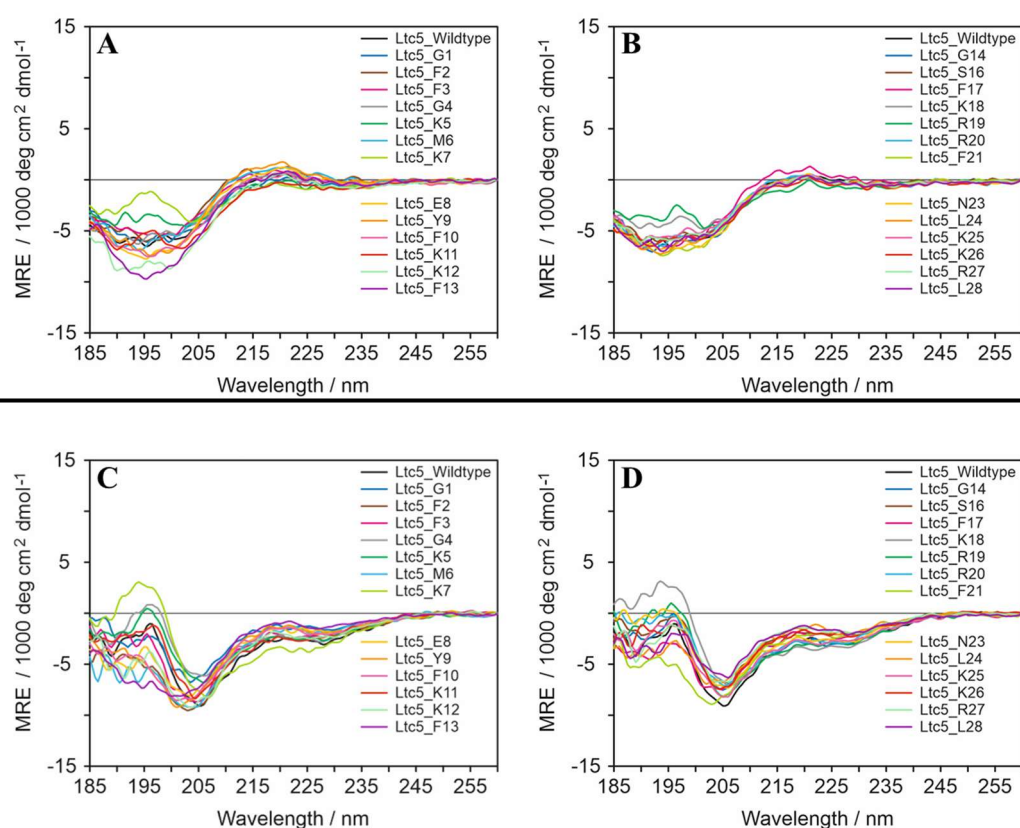


Figure A 5: CD data of all Ala-d₃ analogues measured.

Measurements were conducted A) and B) in H₂O or C) and D) in 10 mM Phosphate buffer 25 °C. Under both conditions all tested peptides remain mostly unordered. No phosphate dependent development of any secondary-structure could be observed.

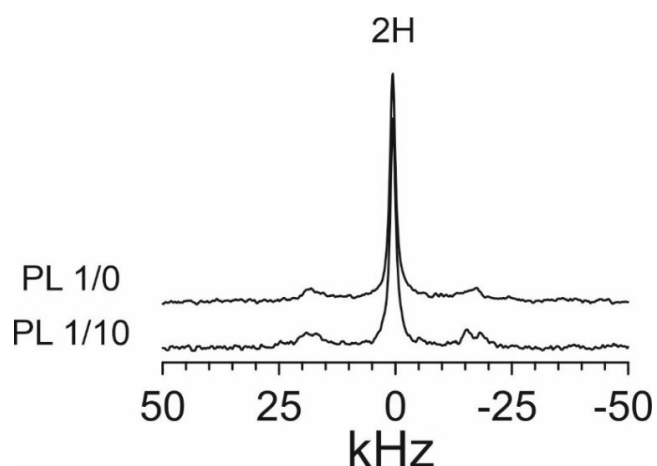


Figure A 6: ²H-NMR spectra of Ltc5 A15 Ala-d₃.

Upper spectrum: Sample with only peptide (P/L 1:0). Lower spectrum: Ltc5 A15 measured in DMPC/DMPG (7:3) at a P/L of 1:10.

15 List of Tables

<i>Table 1: List of clinically approved bioactive peptides derived from animal venom of different species.</i>	<i>7</i>
<i>Table 2: List of exemplary AMPs from diverse origins.</i>	<i>9</i>
<i>Table 3: List of AMPs derived from spider venom.</i>	<i>11</i>
<i>Table 4: Latarecins, seven AMPs derived from spider venom.</i>	<i>14</i>
<i>Table 5: Analogues used for the Ala-scan of Ltc5.</i>	<i>21</i>
<i>Table 6: MIC assay performed in a two-fold peptide dilution series.</i>	<i>28</i>
<i>Table 7: Values of quadrupolar couplings of all Ala-d₃ analogues.</i>	<i>38</i>
<i>Table 8: Calculated values based on the quadrupolar splittings from the Ala-d₃ analogues of Ltc5.</i>	<i>40</i>
<i>Table 9: Phospholipids of varying length used to test the behaviour of Ltc5 in different bilayer thicknesses.</i>	<i>58</i>
<i>Table 10: Ala to Ala-d₃ labelled analogues of Ltc5.</i>	<i>60</i>
<i>Table 11: Deconvolution of CD spectra in three different lipid systems.</i>	<i>63</i>
<i>Table 12: Quadrupolar splittings ($\Delta\nu_Q$) of Ltc5 A15 and Ltc5 A22 measured in three different lipid compositions.</i>	<i>78</i>
<i>Table 13: List of peptides with aromatic residues replaced.</i>	<i>82</i>
<i>Table 14: Deconvolution of the CD spectra of Ltc5 WT and the Ltc5 analogues with replaced aromatic residues.</i>	<i>86</i>
<i>Table 15: MIC assay performed with the Ltc5 analogues depleted of aromaticity.</i>	<i>89</i>
<i>Table 16: Summary of the results of all Ltc5 analogues with depleted aromaticity.</i>	<i>100</i>
<i>Table 17: List of Ltc5 analogues with a truncated N-terminus.</i>	<i>104</i>
<i>Table 18: Deconvolution of the CD spectra of Ltc5 WT and the NTAs.</i>	<i>109</i>
<i>Table 19: MIC assay conducted on the Ltc5 WT and all NTAs.</i>	<i>111</i>
<i>Table 20: Results of the MBC assay of Ltc5.</i>	<i>127</i>
<i>Table 21: Parameters applied during CD-measurements</i>	<i>152</i>
<i>Table 22: Parameters applied during OCD measurements</i>	<i>153</i>

16 List of Figures

<i>Figure 1: Scheme of drug development pipeline based on animal venoms.</i>	<i>6</i>
<i>Figure 2: Helical wheel (left) and helical mesh (right) representations of Ltc5.</i>	<i>19</i>
<i>Figure 3: CD spectra of all Ala-d₃ analogues measured in small unilamellar vesicles (SUVs)</i>	<i>24</i>
<i>Figure 4: Normalized OCD spectra of the Ltc5 WT and all Ala-d₃ analogues.</i>	<i>26</i>
<i>Figure 5: Concentration dependent hemolysis of all Ala-d₃ analogues.</i>	<i>29</i>
<i>Figure 6: Concentration-dependent Vesicle leakage of Ltc5 WT.</i>	<i>31</i>
<i>Figure 7: Results of the vesicle leakage assay performed on all Ala-d₃ analogues of Ltc5.</i>	<i>32</i>
<i>Figure 8: Solid-state NMR spectra of Ala-d₃ labelled Ltc5 at positions one to fourteen.</i>	<i>35</i>
<i>Figure 9: Solid-state NMR spectra of Ala-d₃ labelled Ltc5 at positions 15 to 28.</i>	<i>36</i>

Figure 10: Definition of the helical orientation angles τ and ρ used for peptide helices.	40
Figure 11: Fit of the quadrupolar splitting including positions 2-11 without position 8.	42
Figure 12: Fit of the quadrupolar splitting including positions 12-27 without positions 16 and 20.	43
Figure 13: Helical mesh of Ltc5 with separate N- and C-terminal part.	50
Figure 14: Depiction of Ltc5 aligned according to the centres of hydrophobicity.	51
Figure 15: Helical meshes of the Ltc5 WT showing the artefacts found for Ltc5 S16 and Ltc5 R20.	51
Figure 16: Comparison N- and C-terminal fitting curve.	52
Figure 17: Depiction of a peptide α-helix.	53
Figure 18: 3D-cartoon depiction of the α-helix of Ltc5.	54
Figure 19: Cartoon representation of Ltc5 from three different perspective.	55
Figure 20: CD spectra of Ltc5 A15, Ltc5 A22 and Ltc5 WT in three different lipid systems.	62
Figure 21: Normalized OCD spectra of Ltc5 A15, A22 and Ltc5 WT measured in three different lipid systems.	64
Figure 22: Vesicle leakage of Ltc5 A15, Ltc5 A22 and Ltc5 WT measured in three different lipid systems.	67
Figure 23: Solid-state ^{31}P- and ^2H-NMR spectra of 1) Ltc5 A15 and 2) Ltc5 A22 in DLPC/DLPG (7:3).	69
Figure 24: Solid-state ^{31}P- and ^2H-NMR spectra of 1) Ltc5 A15 and 2) Ltc5 A22 in DMPC/DMPG (7:3).	72
Figure 25: Solid-state ^{31}P- and ^2H-NMR spectra of 1) Ltc5 A15 and 2) Ltc5 A22 in DErPC/DErPG (7:3).	74
Figure 26: Cartoon depiction of the two lipid phases hexagonal (H_i) and lamellar (L_a).	79
Figure 27: Helical wheel and horizontal mesh of Ltc5 with all aromatic residues marked by an orange circle.	82
Figure 28: CD spectra of Ltc5 analogues with all aromatic residues replaced.	84
Figure 29: CD spectra of Ltc5 analogues with all aromatic residues replaced.	85
Figure 30: Normalized OCD spectra of the Ltc5 analogues without aromatic residues.	87
Figure 31: Concentration dependent hemolysis assay performed on Ltc5 analogues depleted of Aromaticity.	90
Figure 32: Vesicle leakage assay performed on the Ltc5 analogues depleted of aromaticity.	91
Figure 33: Helical wheel and horizontal helical mesh of Ltc5 with adjusted N- and C-terminal helix part.	102
Figure 34: Helical wheels of all seven truncated analogues of Ltc5.	105
Figure 35: CD spectra of Ltc5 analogues with a truncated N-terminus.	107
Figure 36: CD-spectra of the truncated Ltc5 analogues measured in SUVs.	108
Figure 37: Normalized OCD spectra of the NTAs and the Ltc5 WT.	110
Figure 38: Concentration dependent hemolysis assay performed with Ltc5 WT and all NTAs.	113
Figure 39: Vesicle leakage assay performed on all NTAs and the Ltc5 WT.	114
Figure 40: Cartoon depiction of the membrane-binding and folding mechanism of amphipathic, α-helical peptides.	119
Figure 41: Selection of possible non-membranolytic mechanisms of action of AMPs acting on the membrane itself.	125
Figure 42: DiSC₃(5) uptake after adding the dye to the washed and rested <i>E. coli</i> emulsion.	129
Figure 43: Membrane depolarisation assay using the cationic dye DiSC₃(5).	130
Figure 44: Membrane disruption assay, using PI, with Ltc5 WT.	132

Figure 45: Anionic lipid clustering monitored using DSC measurements.	133
Figure 46: Unified mechanism of action of Ltc5.	139
Figure 47: Representation of the energy levels and their proportional dependence on B_0 for 1H.	162
Figure 48: Explanation of the CD effect.	167
Figure 49: Jablonski diagram describing the schematic photo physics of a fluorophore.	171

17 List of References

1. Ageitos, L., Torres, M. D. T. & De La Fuente-Nunez, C. Biologically Active Peptides from Venoms: Applications in Antibiotic Resistance, Cancer, and Beyond. *IJMS* **23**, 15437 (2022).
2. Kozlov, S. A. *et al.* Latarecins, Antimicrobial and Cytolytic Peptides from the Venom of the Spider *Lachesana tarabaei* (Zodariidae) That Exemplify Biomolecular Diversity. *Journal of Biological Chemistry* **281**, 20983–20992 (2006).
3. Kim, E. *et al.* Animal Venom in Modern Medicine: A Review of Therapeutic Applications. *Toxins* **17**, 371 (2025).
4. Shi, Z. *et al.* A Comprehensive Overview of the Antibiotics Approved in the Last Two Decades: Retrospects and Prospects. *Molecules* **28**, 1762 (2023).
5. Lee, S. W., Foley, E. J. & Epstein, J. A. Mode of Action of Penicillin: I. Bacterial Growth and Penicillin Activity— *Staphylococcus aureus* FDA. *J Bacteriol* **48**, 393–399 (1944).
6. Lavergne, V. *et al.* Optimized deep-targeted proteotranscriptomic profiling reveals unexplored *Conus* toxin diversity and novel cysteine frameworks. *Proc. Natl. Acad. Sci. U.S.A.* **112**, (2015).
7. Cardoso, F. C., Walker, A. A., King, G. F. & Gomez, M. V. Holistic profiling of the venom from the Brazilian wandering spider *Phoneutria nigriventer* by combining high-throughput ion channel screens with venomomics. *Front. Mol. Biosci.* **10**, 1069764 (2023).

8. Vidya, V. *et al.* Venom peptides – A comprehensive translational perspective in pain management. *Current Research in Toxicology* **2**, 329–340 (2021).
9. Lin, J., Chen, S., Butt, U. D., Yan, M. & Wu, B. A comprehensive review on ziconotide. *Heliyon* **10**, e31105 (2024).
10. Andreozzi, F. *et al.* The GLP-1 receptor agonists exenatide and liraglutide activate Glucose transport by an AMPK-dependent mechanism. *J Transl Med* **14**, 229 (2016).
11. Werner, U., Haschke, G., Herling, A. W. & Kramer, W. Pharmacological profile of lixisenatide: A new GLP-1 receptor agonist for the treatment of type 2 diabetes. *Regulatory Peptides* **164**, 58–64 (2010).
12. Wang, R., Yi, S. & Liang, S. Mechanism of action of two insect toxins huwentoxin-III and hainantoxin-VI on voltage-gated sodium channels. *J. Zhejiang Univ. Sci. B* **11**, 451–457 (2010).
13. Yu, X. *et al.* Melittin-lipid nanoparticles target to lymph nodes and elicit a systemic anti-tumor immune response. *Nat Commun* **11**, 1110 (2020).
14. Lee, G. & Bae, H. Anti-Inflammatory Applications of Melittin, a Major Component of Bee Venom: Detailed Mechanism of Action and Adverse Effects. *Molecules* **21**, 616 (2016).
15. Tonin, G. & Klen, J. Eptifibatide, an Older Therapeutic Peptide with New Indications: From Clinical Pharmacology to Everyday Clinical Practice. *IJMS* **24**, 5446 (2023).
16. Choi, J. H. *et al.* Melittin, a honeybee venom-derived antimicrobial peptide, may target methicillin-resistant *Staphylococcus aureus*. *Molecular Medicine Reports* **12**, 6483–6490 (2015).

17. Gargate, N., Laws, M. & Rahman, K. M. Current economic and regulatory challenges in developing antibiotics for Gram-negative bacteria. *npj Antimicrob Resist* **3**, 50 (2025).
18. Cardona, S. T., Rahman, A. S. M. Z. & Novomisky Nechcoff, J. Innovative perspectives on the discovery of small molecule antibiotics. *npj Antimicrob Resist* **3**, (2025).
19. Butler, M. S., Henderson, I. R., Capon, R. J. & Blaskovich, M. A. T. Antibiotics in the clinical pipeline as of December 2022. *J Antibiot* **76**, 431–473 (2023).
20. Davies, J. & Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev* **74**, 417–433 (2010).
21. Salam, Md. A. *et al.* Antimicrobial Resistance: A Growing Serious Threat for Global Public Health. *Healthcare* **11**, 1946 (2023).
22. Li, J. *et al.* Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. *Front. Neurosci.* <https://doi.org/10.3389/fnins.2017.00073> (2017) doi:10.3389/fnins.2017.00073.
23. Berg, J. M., Tymoczko, J. L., Gatto, G. J. & Stryer, L. *Stryer Biochemie*. (Springer Berlin Heidelberg, Berlin, Heidelberg, 2018). doi:10.1007/978-3-662-54620-8.
24. Müller-Esterl, W. *Biochemie*. (Springer Berlin Heidelberg, Berlin, Heidelberg, 2018). doi:10.1007/978-3-662-54851-6.
25. Chen, C. H. & Lu, T. K. Development and Challenges of Antimicrobial Peptides for Therapeutic Applications. *Antibiotics* **9**, 24 (2020).
26. Moretta, A. *et al.* Antimicrobial Peptides: A New Hope in Biomedical and Pharmaceutical Fields. *Front. Cell. Infect. Microbiol.* **11**, 668632 (2021).
27. Oliveira, M. *et al.* An Overview of the Recent Advances in Antimicrobial Resistance. *Microorganisms* **12**, 1920 (2024).

28. Singh, B., Bhat, A. & Ravi, K. Antibiotics Misuse and Antimicrobial Resistance Development in Agriculture: A Global Challenge. *Environ. Health* **2**, 618–622 (2024).
29. Vila-Farrés, X. *et al.* Sequence-activity relationship, and mechanism of action of mastoparan analogues against extended-drug resistant *Acinetobacter baumannii*. *European Journal of Medicinal Chemistry* **101**, 34–40 (2015).
30. Wanandy, T., Gueven, N., Davies, N. W., Brown, S. G. A. & Wiese, M. D. Pilonins: A review of the structure and mode of action of venom peptides from an Australian ant *Myrmecia pilosula*. *Toxicon* **98**, 54–61 (2015).
31. Costa, B. A. *et al.* Interaction of the Rattlesnake Toxin Crotamine with Model Membranes. *J. Phys. Chem. B* **118**, 5471–5479 (2014).
32. Browne, K. *et al.* A New Era of Antibiotics: The Clinical Potential of Antimicrobial Peptides. *IJMS* **21**, 7047 (2020).
33. Seyfi, R. *et al.* Antimicrobial Peptides (AMPs): Roles, Functions and Mechanism of Action. *Int J Pept Res Ther* **26**, 1451–1463 (2020).
34. Zasloff, M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. USA* (1987).
35. Imura, Y., Choda, N. & Matsuzaki, K. Magainin 2 in Action: Distinct Modes of Membrane Permeabilization in Living Bacterial and Mammalian Cells. *Biophysical Journal* **95**, 5757–5765 (2008).
36. Langenegger, N., Nentwig, W. & Kuhn-Nentwig, L. Spider Venom: Components, Modes of Action, and Novel Strategies in Transcriptomic and Proteomic Analyses. *Toxins* **11**, 611 (2019).

37. Nguyen, L. T., Haney, E. F. & Vogel, H. J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends in Biotechnology* **29**, 464–472 (2011).
38. Nguyen, K. T., Le Clair, S. V., Ye, S. & Chen, Z. Molecular Interactions between Magainin 2 and Model Membranes in Situ. *J. Phys. Chem. B* **113**, 12358–12363 (2009).
39. Lauer, S. M. *et al.* The proline-rich antimicrobial peptide Api137 disrupts large ribosomal subunit assembly and induces misfolding. *Nat Commun* **16**, 567 (2025).
40. Lüddecke, T., Herzig, V., Von Reumont, B. M. & Vilcinskas, A. The biology and evolution of spider venoms. *Biological Reviews* **97**, 163–178 (2022).
41. Ye, Z. *et al.* Synergistic collaboration between AMPs and non-direct antimicrobial cationic peptides. *Nat Commun* **15**, 7319 (2024).
42. Yan, L. & Adams, M. E. Lycotoxins, Antimicrobial Peptides from Venom of the Wolf Spider *Lycosa carolinensis*. *Journal of Biological Chemistry* **273**, 2059–2066 (1998).
43. Azim, S. *et al.* Venom peptides cathelicidin and lycotoxin cause strong inhibition of *Escherichia coli* ATP synthase. *International Journal of Biological Macromolecules* **87**, 246–251 (2016).
44. Kuhn-Nentwig, L. *et al.* Cupiennin 1, a New Family of Highly Basic Antimicrobial Peptides in the Venom of the Spider *Cupiennius salei* (Ctenidae). *Journal of Biological Chemistry* **277**, 11208–11216 (2002).
45. Pukala, T. L. *et al.* Cupiennin 1a, an antimicrobial peptide from the venom of the neotropical wandering spider *Cupiennius salei*, also inhibits the formation of nitric oxide by neuronal nitric oxide synthase. *The FEBS Journal* **274**, 1778–1784 (2007).

46. Santos, D. M. *et al.* LyeTx I, a potent antimicrobial peptide from the venom of the spider *Lycosa erythrognatha*. *Amino Acids* **39**, 135–144 (2010).
47. Wadhwani, P. *et al.* Membrane Interactions of Latarcins: Antimicrobial Peptides from Spider Venom. *IJMS* **22**, 10156 (2021).
48. Lima, W. G. *et al.* Highlighting the Potential of LyeTx I, a Peptide Derived from the Venom of the Spider *Lycosa erythrognatha*, as a Potential Prototype for the Development of a New Antimicrobial Against Carbapenem-Resistant *Klebsiella pneumoniae*. *Pharmaceuticals* **18**, 679 (2025).
49. Lima, W. G. *et al.* A short synthetic peptide, based on LyeTx I from *Lycosa erythrognatha* venom, shows potential to treat pneumonia caused by carbapenem-resistant *Acinetobacter baumannii* without detectable resistance. *J Antibiot* **74**, 425–434 (2021).
50. Fuscaldi, L. L. *et al.* Shortened derivatives from native antimicrobial peptide LyeTx I: *In vitro* and *in vivo* biological activity assessment. *Exp Biol Med (Maywood)* **246**, 414–425 (2021).
51. Tamargo, J., Le Heuzey, J.-Y. & Mabo, P. Narrow therapeutic index drugs: a clinical pharmacological consideration to flecainide. *Eur J Clin Pharmacol* **71**, 549–567 (2015).
52. Hatton, I. A. *et al.* The human cell count and size distribution. *Proc. Natl. Acad. Sci. U.S.A.* **120**, e2303077120 (2023).
53. Reißer, S., Prock, S., Heinzmann, H. & Ulrich, A. S. Protein ORIGAMI: A program for the creation of 3D paper models of folded peptides. *Biochem Molecular Bio Educ* **46**, 403–409 (2018).

54. Grage, S. L. *et al.* Comparative analysis of the orientation of transmembrane peptides using solid-state ^2H - and ^{15}N -NMR: mobility matters. *Eur Biophys J* **41**, 475–482 (2012).
55. Tremouilhac, P., Strandberg, E., Wadhwani, P. & Ulrich, A. S. Conditions affecting the re-alignment of the antimicrobial peptide PGLa in membranes as monitored by solid state ^2H -NMR. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1758**, 1330–1342 (2006).
56. Glaser, R. W., Sachse, C., Dürr, U. H. N., Wadhwani, P. & Ulrich, A. S. Orientation of the antimicrobial peptide PGLa in lipid membranes determined from ^{19}F -NMR dipolar couplings of 4- CF_3 -phenylglycine labels. *Journal of Magnetic Resonance* **168**, 153–163 (2004).
57. Laqua, K. Structure-Function Analysis of Latacin5: an Antimicrobial Peptide from Spider Venom. (KIT, Karlsruhe, 2023).
58. Miles, A. J., Ramalli, S. G. & Wallace, B. A. Dichroweb, a website for calculating protein secondary structure from circular dichroism spectroscopic data. *Protein Science* **31**, 37–46 (2022).
59. Whitmore, L. & Wallace, B. A. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Research* **32**, W668–W673 (2004).
60. Miles, A. J. & Wallace, B. A. Circular dichroism spectroscopy of membrane proteins. *Chem. Soc. Rev.* **45**, 4859–4872 (2016).
61. Miles, A. J., Janes, R. W. & Wallace, B. A. Tools and methods for circular dichroism spectroscopy of proteins: a tutorial review. *Chem. Soc. Rev.* **50**, 8400–8413 (2021).

62. Bürck, J. *et al.* Conformation and Membrane Orientation of Amphiphilic Helical Peptides by Oriented Circular Dichroism. *Biophysical Journal* **95**, 3872–3881 (2008).
63. Bürck, J., Wadhwani, P., Fanghänel, S. & Ulrich, A. S. Oriented Circular Dichroism: A Method to Characterize Membrane-Active Peptides in Oriented Lipid Bilayers. *Acc. Chem. Res.* **49**, 184–192 (2016).
64. Dubovskii, P. V. *et al.* Latarcins: versatile spider venom peptides. *Cell. Mol. Life Sci.* **72**, 4501–4522 (2015).
65. Wadhwani, P., Afonin, S., Ieronimo, M., Buerck, J. & Ulrich, A. S. Optimized Protocol for Synthesis of Cyclic Gramicidin S: Starting Amino Acid Is Key to High Yield. *J. Org. Chem.* **71**, 55–61 (2006).
66. Strandberg, E. *et al.* Influence of C-terminal amidation on the antimicrobial and hemolytic activities of cationic α -helical peptides. *Pure and Applied Chemistry* **79**, 717–728 (2007).
67. Chen, Y. *et al.* Role of Peptide Hydrophobicity in the Mechanism of Action of α -Helical Antimicrobial Peptides. *Antimicrob Agents Chemother* **51**, 1398–1406 (2007).
68. Ellens, H., Bentz, J. & Szoka, F. C. Proton- and calcium-induced fusion and destabilization of liposomes. *Biochemistry* **24**, 3099–3106 (1985).
69. Steger, L. M. E. *et al.* Structural and functional characterization of the pore-forming domain of pinholin S ²¹
68. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 29637–29646 (2020).
70. Strandberg, E., Zerweck, J., Wadhwani, P. & Ulrich, A. S. Synergistic Insertion of Antimicrobial Magainin-Family Peptides in Membranes Depends on the Lipid Spontaneous Curvature. *Biophysical Journal* **104**, L9–L11 (2013).

71. Rance, M. & Byrd, R. A. Obtaining high-fidelity spin-1/2 powder spectra in anisotropic media: phase-cycled Hahn echo spectroscopy. *Journal of Magnetic Resonance* 221–240 (1982).
72. Davis, J. H., Jeffrey, K. R., Bloom, M. & Valic, M. I. Quadrupolar Echo Deuteron Magnetic Resonance Spectroscopy In Ordered Hydrocarbon Chains. *Chemical Physics Letters* **42**, 390–394 (1976).
73. Strandberg, E., Tiltak, D., Ehni, S., Wadhwani, P. & Ulrich, A. S. Lipid shape is a key factor for membrane interactions of amphipathic helical peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1818**, 1764–1776 (2012).
74. Serrano, L. & Fersht, A. R. Capping and α -helix stability. *Nature* **342**, 296–299 (1989).
75. Fernández-Vidal, M., Jayasinghe, S., Ladokhin, A. S. & White, S. H. Folding Amphipathic Helices Into Membranes: Amphiphilicity Trumps Hydrophobicity. *Journal of Molecular Biology* **370**, 459–470 (2007).
76. Grau-Campistany, A. *et al.* Hydrophobic mismatch demonstrated for membranolytic peptides and their use as molecular rulers to measure bilayer thickness in native cells. *Sci Rep* **5**, 9388 (2015).
77. Strandberg, E. *et al.* Trp residues near peptide termini enhance the membranolytic activity of cationic amphipathic α -helices. *Biophysical Chemistry* **318**, 107365 (2025).
78. Polyansky, A. A. *et al.* N-terminal amphipathic helix as a trigger of hemolytic activity in antimicrobial peptides: A case study in laticins. *FEBS Letters* **583**, 2425–2428 (2009).

-
79. Resende, J. M. *et al.* Membrane structure and conformational changes of the antibiotic heterodimeric peptide distinctin by solid-state NMR spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 16639–16644 (2009).
80. Khandelia, H. & Kaznessis, Y. N. Molecular Dynamics Simulations of the Helical Antimicrobial Peptide Ovispirin-1 in a Zwitterionic Dodecylphosphocholine Micelle: Insights into Host-Cell Toxicity. *J. Phys. Chem. B* **109**, 12990–12996 (2005).
81. Strandberg, E. *et al.* Temperature-Dependent Re-alignment of the Short Multifunctional Peptide BP100 in Membranes Revealed by Solid-State NMR Spectroscopy and Molecular Dynamics Simulations. *ChemBioChem* **24**, e202200602 (2023).
82. Afonin, S., Grage, S. L., Ieronimo, M., Wadhwani, P. & Ulrich, A. S. Temperature-Dependent Transmembrane Insertion of the Amphiphilic Peptide PGLa in Lipid Bilayers Observed by Solid State¹⁹F NMR Spectroscopy. *J. Am. Chem. Soc.* **130**, 16512–16514 (2008).
83. Matsuzaki, K. Control of cell selectivity of antimicrobial peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1788**, 1687–1692 (2009).
84. Pan, J. *et al.* Molecular structures of fluid phase phosphatidylglycerol bilayers as determined by small angle neutron and X-ray scattering. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1818**, 2135–2148 (2012).
85. Kučerka, N. *et al.* Structure of Fully Hydrated Fluid Phase DMPC and DLPC Lipid Bilayers Using X-Ray Scattering from Oriented Multilamellar Arrays and from Unilamellar Vesicles. *Biophysical Journal* **88**, 2626–2637 (2005).
86. Grau, B. *et al.* The role of hydrophobic matching on transmembrane helix packing in cells. *CST* **1**, 90–106 (2017).

87. Strandberg, E., Esteban-Martín, S., Salgado, J. & Ulrich, A. S. Orientation and Dynamics of Peptides in Membranes Calculated from ^2H -NMR Data. *Biophysical Journal* **96**, 3223–3232 (2009).
88. Strandberg, E., Wadhwani, P., Tremouilhac, P., Dürr, U. H. N. & Ulrich, A. S. Solid-State NMR Analysis of the PGLa Peptide Orientation in DMPC Bilayers: Structural Fidelity of ^2H -Labels versus High Sensitivity of ^{19}F -NMR. *Biophysical Journal* **90**, 1676–1686 (2006).
89. Glaser, R. W. *et al.* Concentration-Dependent Realignment of the Antimicrobial Peptide PGLa in Lipid Membranes Observed by Solid-State ^{19}F -NMR. *Biophysical Journal* **88**, 3392–3397 (2005).
90. Kučerka, N., Nieh, M.-P. & Katsaras, J. Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidylcholines as a function of temperature. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1808**, 2761–2771 (2011).
91. Nagle, J. F. & Tristram-Nagle, S. Structure of lipid bilayers. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes* **1469**, 159–195 (2000).
92. Kyte, J. & Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132 (1982).
93. Whitmore, L. & Wallace, B. A. Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers* **89**, 392–400 (2008).
94. Shahmiri, M., Enciso, M. & Mechler, A. Controls and constraints of the membrane disrupting action of Aurein 1.2. *Sci Rep* **5**, (2015).
95. Perrin, B. S. *et al.* High-Resolution Structures and Orientations of Antimicrobial Peptides Piscidin 1 and Piscidin 3 in Fluid Bilayers Reveal Tilting, Kinking, and Bilayer Immersion. *J. Am. Chem. Soc.* **136**, 3491–3504 (2014).

96. Miles, A. J. & Wallace, B. A. Circular dichroism spectroscopy of membrane proteins. *Chem. Soc. Rev.* **45**, 4859–4872 (2016).
97. Amorim-Carmo, B. *et al.* Antimicrobial Peptide Analogs From Scorpions: Modifications and Structure-Activity. *Front. Mol. Biosci.* **9**, 887763 (2022).
98. Juhász, T. *et al.* Interplay between membrane active host defense peptides and heme modulates their assemblies and in vitro activity. *Sci Rep* **11**, 18328 (2021).
99. Zeth, K. & Sancho-Vaello, E. Structural Plasticity of LL-37 Indicates Elaborate Functional Adaptation Mechanisms to Bacterial Target Structures. *IJMS* **22**, 5200 (2021).
100. M. Alhattab, D., Khan, Z., Alshehri, S., H. Susapto, H. & A. E. Hauser, C. 3D bioprinting of ultrashort self-assembling peptides to engineer scaffolds with different matrix stiffness for chondrogenesis. *IJB* **9**, 719 (2024).
101. Neubauer, D. *et al.* Retro analog concept: comparative study on physico-chemical and biological properties of selected antimicrobial peptides. *Amino Acids* **49**, 1755–1771 (2017).
102. Tsai, C.-Y. *et al.* Helical structure motifs made searchable for functional peptide design. *Nat Commun* **13**, 102 (2022).
103. Shin, G. *et al.* Cyclohexylalanine-Containing α -Helical Amphipathic Peptide Targets Cardiolipin, Rescuing Mitochondrial Dysfunction in Kidney Injury. *J. Med. Chem.* **67**, 3385–3399 (2024).
104. Maturana, P. *et al.* Lipid selectivity in novel antimicrobial peptides: Implication on antimicrobial and hemolytic activity. *Colloids and Surfaces B: Biointerfaces* **153**, 152–159 (2017).

105. Vahedi, A., Bigdelou, P. & Farnoud, A. M. Quantitative analysis of red blood cell membrane phospholipids and modulation of cell-macrophage interactions using cyclodextrins. *Sci Rep* **10**, 15111 (2020).
106. Mironov, P. A. *et al.* NMR Unveils Activity Mechanism of Linear Spider Venom Peptide Fragments Selected by Neural Networks Against Staphylococci Including MRSA. *Pharmaceutics* **17**, 1526 (2025).
107. Raghunathan, K. & Kenworthy, A. K. Dynamic pattern generation in cell membranes: Current insights into membrane organization. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1860**, 2018–2031 (2018).
108. MacCallum, J. L., Bennett, W. F. D. & Tieleman, D. P. Distribution of Amino Acids in a Lipid Bilayer from Computer Simulations. *Biophysical Journal* **94**, 3393–3404 (2008).
109. Ilieva, Y. *et al.* Cytotoxicity and Microbicidal Activity of Commonly Used Organic Solvents: A Comparative Study and Application to a Standardized Extract from *Vaccinium macrocarpon*. *Toxics* **9**, 92 (2021).
110. Gan, B. H., Gaynord, J., Rowe, S. M., Deingruber, T. & Spring, D. R. The multifaceted nature of antimicrobial peptides: current synthetic chemistry approaches and future directions. *Chem. Soc. Rev.* **50**, 7820–7880 (2021).
111. Roy, A., Sarangi, N. K., Ghosh, S., Prabhakaran, A. & Keyes, T. E. Leaflet by Leaflet Synergistic Effects of Antimicrobial Peptides on Bacterial and Mammalian Membrane Models. *J. Phys. Chem. Lett.* **14**, 3920–3928 (2023).
112. Carrer, M. *et al.* Accelerating Lipid Flip-Flop at Low Concentrations: A General Mechanism for Membrane Binding Peptides. *J. Phys. Chem. Lett.* **14**, 7014–7019 (2023).

113. Salnikov, E. S. *et al.* Membrane topologies of the PGLa antimicrobial peptide and a transmembrane anchor sequence by Dynamic Nuclear Polarization/solid-state NMR spectroscopy. *Sci Rep* **6**, 20895 (2016).
114. Seelig, J. Thermodynamics of lipid–peptide interactions. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1666**, 40–50 (2004).
115. Gan, B. H., Gaynord, J., Rowe, S. M., Deingruber, T. & Spring, D. R. The multifaceted nature of antimicrobial peptides: current synthetic chemistry approaches and future directions. *Chem. Soc. Rev.* **50**, 7820–7880 (2021).
116. Koynarev, V. R. *et al.* Structural pores not required: Antimicrobial peptides induce ion permeabilization of lipid membranes through transient water channels. *Proc. Natl. Acad. Sci. U.S.A.* **122**, e2517944122 (2025).
117. Ön, A. *et al.* Bactericidal Activity to Escherichia coli: Different Modes of Action of Two 24-Mer Peptides SAAP-148 and OP-145, Both Derived from Human Cathelicidine LL-37. *Antibiotics* **12**, 1163 (2023).
118. Wadhwani, P. *et al.* Membrane-Active Peptides and the Clustering of Anionic Lipids. *Biophysical Journal* **103**, 265–274 (2012).
119. Zweytick, D. *et al.* N-acylated Peptides Derived from Human Lactoferricin Perturb Organization of Cardiolipin and Phosphatidylethanolamine in Cell Membranes and Induce Defects in Escherichia coli Cell Division. *PLoS ONE* **9**, e90228 (2014).
120. Wijesundara, N. M., Lee, S. F., Cheng, Z., Davidson, R. & Rupasinghe, H. P. V. Carvacrol exhibits rapid bactericidal activity against Streptococcus pyogenes through cell membrane damage. *Sci Rep* **11**, 1487 (2021).

121. Te Winkel, J. D., Gray, D. A., Seistrup, K. H., Hamoen, L. W. & Strahl, H. Analysis of Antimicrobial-Triggered Membrane Depolarization Using Voltage Sensitive Dyes. *Front. Cell Dev. Biol.* **4**, 1–10 (2016).
122. Ma, B. *et al.* Contemporaneous Measurement of Outer and Inner Membrane Permeability in Gram-negative Bacteria. *BIO-PROTOCOL* **10**, (2020).
123. Ladokhin, A. S., Selsted, M. E. & White, S. H. Bilayer Interactions of Indolicidin, a Small Antimicrobial Peptide Rich in Tryptophan, Proline, and Basic Amino Acids. *Biophysical Journal* **72**, 794–805 (1997).
124. Koynarev, V. R. *et al.* Structural pores not required: Antimicrobial peptides induce ion permeabilization of lipid membranes through transient water channels. *Proc. Natl. Acad. Sci. U.S.A.* **122**, e2517944122 (2025).
125. El Khoury, M. *et al.* Targeting Bacterial Cardiolipin Enriched Microdomains: An Antimicrobial Strategy Used by Amphiphilic Aminoglycoside Antibiotics. *Sci Rep* **7**, 10697 (2017).
126. Merrifield, R. B. Automated Synthesis of Peptides. *Science* **150**, 178–185 (1965).
127. Hahn, E. L. Spin Echoes. *Phys. Rev.* **80**, 580–594 (1950).
128. Bloom, A. L. & Shoolery, J. N. Effects of Perturbing Radiofrequency Fields on Nuclear Spin Coupling. *Phys. Rev.* **97**, 1261–1265 (1955).
129. Lorian, V. *Antibiotics in Laboratory Medicine*. (Williams and Wilkins, Baltimore, 1996).
130. Yang, K. B. *et al.* High-resolution landscape of an antibiotic binding site. *Nature* **622**, 180–187 (2023).
131. Keeler, J. *Understanding NMR Spectroscopy*. (Wiley, West Sussex, 2010).

132. Claridge, D. W. *High-Resolution NMR Techniques in Organic Chemistry*. (Elsevier, Amsterdam, 2016).
133. Strandberg, E. & Ulrich, A. S. NMR methods for studying membrane-active antimicrobial peptides. *Concepts Magnetic Resonance* **23A**, 89–120 (2004).
134. Separovic, F. & Sani, M.-A. *Solid-State NMR, Applications in Biomembrane Structure*. (IOP Publishing, Bristol UK, 2020).
135. Kelly, S. M. & Price, N. C. Circular Dichroism: Studies of Proteins. in *Encyclopedia of Life Sciences* (Wiley, 2009). doi:10.1002/9780470015902.a0003043.pub2.
136. Greenfield, N. J. Applications of circular dichroism in protein and peptide analysis. *TrAC Trends in Analytical Chemistry* **18**, 236–244 (1999).
137. Berova, N., Nakanishi, K. & Woody, R. W. *Circular Dichroism: Principles and Applications*. (Wiley-VCH, New York USA, 2000).
138. Nordén, B., Rodger, A. & Dafforn, T. *Linear Dichroism and Circular Dichroism: A Textbook on Polarized-Light Spectroscopy*. (RSC Publishing, Cambridge UK, 2019).
139. Valeur, B. & Berberan-Santos, M. N. *Molecular Fluorescence: Principles and Applications*. (Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim De, 2012).

18 List of publications

Structure and Function Analysis of Latarcin 5 (in Bearbeitung)

K. Laqua-Acosta, P. Wadhwani, E. Strandberg and A. S. Ulrich

Structure-Activity relationship of Latarcin 5: Role of aromatic and hydrophobic residues on biological function. (In Bearbeitung)

K. Laqua-Acosta, P. Wadhwani, E. Strandberg and A. S. Ulrich