Thesis for the degree of Doctor of
Philosophy in Natural Science
with a specialization in Medicinal Chemistry

Toward the synthesis and characterization of bioactive peptides: Engineering of transmembrane peptide conjugates for targeted drug delivery

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Dedicated to all innocent souls
who have been killed in Gaza by the Israeli army genocide
as reported by the United Nations.

If I Must Die
Refaat Alareer

If I must die,
you must live
to tell my story
to sell my things
to buy a piece of cloth
and some strings,
(make it white with a long tail)
so that a child, somewhere in Gaza
while looking heaven in the eye
awaiting his dad who left in a blaze —
and bid no one farewell
not even to his flesh
not even to himself —
sees the kite, my kite you made, flying up above,
and thinks for a moment an angel is there
bringing back love.
If I must die
let it bring hope,
let it be a tale.

Abstract

Over the past years, there has been a growing interest in therapeutic peptides within the pharmaceutical industry with more than 60 peptides have been approved and administrated worldwide in clinics. This is credited to the novel production and analytic techniques that are shifting the existing conditions in the discovery of peptide medications. Peptides have been produced with both chemical and biological approaches, in conjunction with new structural modifications and delivery techniques, that have facilitated overcoming the intrinsic poor pharmacokinetics of peptides and have permitted the constant progress of this therapy area.

This thesis focuses on developing newly modified peptides covering multiple therapeutic areas. The results achieved in paper **I**, report the synthesis and characterization of hydrophobic transmembrane (TM) peptides with a sequence corresponding to the TM domain of the human epidermal growth factor receptor 2 (ErbB2) to inhibit accelerated cancer cell growth. Furthermore, the paper introduces the engineering of two novel surface-active ionic liquid nanocarriers to deliver the TM peptides as a method to improve their anticancer efficacy. This synergic anticancer activity establishes a solid platform for the future design of more efficient therapeutic modalities with reduced toxicity to normal cells.

Paper **II** describes the optimization of an efficient synthetic strategy employing pseudoprolines toward the synthesis of holin peptide variants possessing 44–68 amino acid lengths. The holins are small hydrophobic proteins produced by bacteriophages and aggregate to form holes in the membrane that assist in the cleavage of the bacterial host cell wall.

Site-specific conjugation methods are of great value in drug discovery. Paper **III** introduces a novel ionic liquid-based conjugation approach for the functionalization of thiol-containing compounds. The method utilizes ionic liquid as a solvent and precursor for the conjugation reaction. This study sets the ground for further exploration of the use of active ionic liquids for modifying or conjugating larger and more complex therapeutic models such as antibodies or proteins.

The bacterial transpeptidase Sortase A (SrtA) is a crucial virulence factor, present ubiquitously in many infective Gram-positive bacteria. By targeting virulence factors, efficient remedy can be achieved, without triggering antibiotic resistance. Paper **IV**, reports several promising peptidomimetic inhibitors of SrtA assayed *in vitro* with IC_{50} values below 200 μM. Some of which can advance into further clinical candidates.

Sammanfattning

Under det senaste decenniet har intresset för terapeutiska peptider inom läkemedelsindustrin växt stort. Idag finns det upp åt 60 olika peptider som är godkända och används globalt på kliniker. Detta tack vare nya produktions-, modifierings- och analytiska teknologier som förändrat status-quo i upptäckten av peptidterapier. Peptider har producerats med både kemiska och biologiska metoder, tillsammans med nya strukturella modifieringar och leveransstrategier, som har hjälpt till att förbättra peptiders bristande farmakokinetik och har möjliggjort fortsatta framsteg inom just detta område.

Denna avhandling fokuserar på att utveckla nya modifierade peptider som täcker flera terapeutiska områden. Resultaten som uppnåddes i artikel **I** rapporterar syntes och karakterisering av hydrofoba transmembrana (TM) peptider, med en sekvens som motsvarar TM-domänet för den humana epidermala tillväxtfaktorreceptorn 2 (ErbB2), för att hämma cancercelltillväxt. Dessutom introducerar artikeln konstruktionen av två nya ytaktiva joniska flytande nanobärare för att leverera TM-peptiderna som en metod för att förbättra deras anticanceraktivitet. Denna synergiska anticanceraktiviteten etablerar en solid plattform för framtida design av mer effektiva terapeutiska modaliteter med minskad toxicitet för hälsosamma celler.

Artikel **II** beskriver en effektiv syntetisk strategi som optimerar pseudoproliner för syntes av holinpeptidvarianter som är 44 till 68 aminosyror långa. Holinerna är små hydrofoba proteiner som produceras av bakteriofager. Holinpeptiderna kan aggregera och bilda hål i cellmembranet som hjälper till vid klyvningen av den bakteriella värdcellens cellvägg.

Platsspecifika konjugationsmetoder är av stor betydelse för upptäckten av nya läkemedel. Artikel **III** introducerar så kallade ionic liquids, eller jonvätskor, som bas för konjugeringsmetoder för funktionalisering av tiol-föreningar. Metoden använder jonvätskor som lösningsmedel och reagens för konjugeringsreaktionen. Denna studie lägger grunden för ytterligare undersökningar av användningen av aktiva jonvätskor för att modifiera, märka eller konjugera större och mer komplexa terapeutiska modaliteter såsom proteiner och antikroppar.

Det bakteriella transpeptidaset Sortase A (SrtA) är en kritisk virulensfaktor, som det finns rikt av i grampositiva bakterier, av vilka många är patogena. Genom att rikta in sig på virulensfaktorer kan effektiv behandling uppnås, utan att inducera antibiotikaresistens. Artikel **IV**, rapporterar flera lovande peptidomimetiska hämmare av SrtA som är analyserade in vitro med IC_{50}-värden under 200 μM. Några av dem har potential att gå vidare till ytterligare klinisk avancering.

Contents

Publications

The dissertation is based on the following research papers, which are referred to in the text by their Roman numerals.

Paper I: **Abujubara, H.**; Bharmoria, P.; Alvarez, S. W.; Appiah, E.; Moth-Poulsen, K.; Sayin, V.I.; Tietze, A. A., Transmembrane peptide-loaded ionic liquid carriers synergy for targeting ErbB2-positive cancer. Submitted Manuscript.

Paper II: Weber, W.; Roeder, M.; Probanowski, T.; Yang, J.; **Abujubara, H.**; Koeppl, H.; Tietze, A. A.; Stein, V., Functional Nanopore Screen: A Versatile High-Throughput Assay to Study and Engineer Protein Nanopores in Escherichia coli. *ACS Synth Biol* **2022,** *11* (6), 2070-2079.

Paper III: **Abujubara, H.**; Yang, J.; Birsen Otyakmaz, A.; Noisier, A. F. M.; Czechtizky, W.; Lemurell, M.; Tietze, A. A., Ionic Liquid-Mediated Approach for the Synthesis of Site-Specific Thioether Conjugates. *Chemistry Eur. J.* **2023,** *29* (28), e202203915.

Paper IV: **Abujubara, H.** ‡; Hintzen, J. C. J. ‡; Rahimi, S.; Mijakovic, I.; Tietze, D.; Tietze, A. A., Substrate-derived Sortase A inhibitors: targeting an essential virulence factor of Gram-positive pathogenic bacteria. *Chem Sci* **2023,** *14* (25), 6975-6985.

‡ Equal contribution

Papers that I have contributed to during my PhD but are not included in the thesis:

Paper V: Hintzen, J. C. J.; **Abujubara, H.**; Tietze, D.; Tietze, A. A., The Complete Assessment of Small Molecule and Peptidomimetic Inhibitors of Sortase A Towards Antivirulence Treatment. *Chem. Eur. J.* **2024,** *30* (38), e202401103.

Contribution report

Paper I: Planned, conducted, and analyzed all the experiments except for DSC, ITC, bioinformatic analysis of cancer cells, cancer cells culturing and western blot analysis. The TEM imaging was performed together with the technician at the cell imaging center at GU. DLS and Zp measurements were carried out together with our collaborator in Spain. Interpreted the results and wrote the main part of the manuscript.

Paper II: Performed the chemical synthesis and LC-MS characterization of the holin peptide variants and wrote that part of the manuscript.

Paper III: Planned, conducted, and analyzed all the experiments except for the peptide racemization study, bifunctional linker study for the functionalization of cysteine-free peptide and biotinylation of the cysteine-containing peptide. Supervised the synthesis and conjugation of model peptides. Optimizing the thiolation reaction conditions was performed together with Jie Yang. Interpreted the results and wrote the main part of the manuscript.

Paper IV: Performed the chemical synthesis, analytical characterization and FRET bioactivity testing for the first generation of peptidomimetic inhibitors. Sortase A enzyme expression and purification were performed together with our collaborator at Lunderg Lab. Performed and interpreted the biochemical and kinetic analysis for the expressed enzyme. Planned experiments, interpreted the results and co-wrote the manuscript.

Abbreviations

Å	Angstrom
aa	Amino acid
AAC	Azide-alkyne cycloaddition
Acm	Acetamidomethyl
ACN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
ADC	Antibody-drug conjugates
Ado	Amino-3,6-dioxaoctanoic acid
AMPs	Antimicrobial peptides
BAIL	Biamphiphilic
BBB	Blood-brain barrier
Boc	Tert-butyloxycarbonyl
CAC	Critical aggregation concentrations
Cbz	Benzyloxycarbonyl
CD	Circular dichroism
CLSM	Confocal laser scanning microscopy
C-NDI-C	N-desymmetrized naphthalenediimides
CNS	Central nervous system
CPPs	Cell-penetrating peptides
Cryo-TEM	Cryogenic transition electron microscopy
CTG	Cell Titre Glo
CWA	Cell-wall anchored
Da	Dalton

DBCO	Aza-dibenzocyclooctyne
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
D_H	Hydrodynamic radius
DLS	Dynamic light scattering
DM	Diabetes Mellitus
DMAP	4-Dimethylaminopyridine
DMF	N,N-Dimethylformamide
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
DVB	Divinylbenzene
E. Coli	Escherichia coli
ECD	Extracellular domain
ED_{50}	Median effective dose
EGF	Epidermal growth factor
ELSD	Evaporative light scattering detection
ErbB	Epidermal growth factor receptor
ESI-MS	Electrospray ionization mass spectrometry
FA	Formic acid
FAM	5/6-Carboxyfluorescein
FDA	Food and Drug Administration
Fmoc	9-Fluorenylmethoxycarbonyl
FPLC	Fast protein liquid chromatography
FRET	Fluorescence resonance energy transfer

GLP-1	Glucagon-like peptide 1
Gly5	Pentaglycine
GnRH	Gonadotropin-releasing hormone
GPCRs	G protein-coupled receptors
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
Hmb	2-Hydroxy-4- methoxybenzyl
HMBC	Heteronuclear Multiple Bond Correlation
HOBt	1-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
HSQS	Heteronuclear single quantum coherence
IAAOSu	Iodoacetic acid N-hydroxysuccinimide ester
IL	Ionic liquid
IL1	1-Ethyl-3-methylimidazolium acetate
IMAC	Immobilized metal affinity chromatography
IPOH	Isopropanol
IPs	Interfering peptides
ITC	Isothermal titration calorimetry
JNK	C-Jun N-terminal kinases
LPPS	Liquid phase peptide synthesis
MAIL	Mono-amphiphilic
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen-activated protein kinase
MBS	M-maleimidobenzoyl-N-hydroxysuccinimidyl ester

MeOH	Methanol
MRSA	Methicillin-resistant *S. aureus*
MS	Mass spectrometry
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NCL	Native chemical ligation
NHC	N-heterocyclic carbenes
NHS	N-Hydroxysuccinimide
NMDA	N-methyl-d-aspartate
NMP	N-Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
NRPs	Non-ribosomal peptides
OG	Octyl β-D-glucopyranoside
PDA	Photodiode array
PEG	Polyethylene glycol
PI3K/AKT	Phosphatidyl inositol 3-kinase
PMT	Photomultiplier
PPIs	Protein-protein interactions
PS	Polystyrene
RP	Reversed phase
SAIL	Surface active ionic liquid
Scm	Carbomethoxysulfenyl
Scm-Cl	Methoxycarbonylsulfenyl chloride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SL	Staudinger ligation
SLS	Sodium lauryl sulfate
$SOCl_2$	Thionyl chloride
SPDP	N-succinimidyl 3-(2-pyridyldithio) propionate
SPPS	Solid-phase peptide synthesis
SrtA	Sortase A
TcdB	*Clostridioides difficile* toxin B
TCEP	Tris(2-carboxyethyl)phosphine
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFE	2,2,2-Trifluoroethanol
TfOH	Triflic acid
THF	Tetrahydrofuran
TM	Transmembrane
TMD	Transmembrane domain
TOCSY	Total correlation spectroscopy
tR	Retention time
UPLC	Ultra-performance liquid chromatography
Uv/Vis	Ultraviolet-visible
VEGF	Vascular endothelial growth factor
ZP	Zeta potential

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Chapter 1
1.0 Introduction

1.1 Peptides in drug discovery

The rapid development of molecular biology has led to tremendous progress in finding effective approaches to the drug discovery process.[1] Traditionally, natural products constituted the main source of newly discovered drugs for many life-threatening diseases for which no other treatments were available yet.[2] However, due to the challenges associated with natural products, such as formulation limits, high extraction costs, and poor targeting, the drug discovery process has been shifted toward high throughput synthetic chemistry-based techniques. In the previous years, the drug discovery industry has been centered on the development of small molecule-based drugs.[3] Typically, small molecules are compounds that have a molecular weight < 500 Daltons (Da) which are easier to synthesize and characterize relative to medicinal plants.[4] Thousand of these molecules were screened against different biological targets in the pharmaceutical industry, leading to new drug leads for different human diseases. It is well known that small molecules are of inevitable importance in the management of current diseases. Nevertheless, the side effects associated with small molecule-based drugs, the recent advances in molecular biology and increased concern about targeting "undruggable" targets have ratified an expansion of drug design strategies over the classical small molecules.[5] "Undruggable" refers to target proteins that are challenging to target pharmacologically since they lack defined pockets for ligand interaction with flat functional interfaces.[6] Those limitations make rational drug design very challenging and necessitate innovative approaches and tactics for drug design. Among those approaches, peptides are an exceptional class of pharmaceutical complexes that consist of chains of amino acids with molecular weights in the range of 500-5000 Da.[7]

The significance of peptide therapy was first realized in the early 1920s following the isolation of insulin, a 51 amino acid (aa) peptide, from animal pancreas.[8] This was a remarkable achievement in the field of drug discovery and gained a gigantic public appreciation after the commercialization of the first peptide drug in 1923. During the early 1950s with the commencement of

solution phase peptide synthesis, Vigneaud *et al*. constructed a peptide with an oxytocin-like effect.[9] The solution-phase, or classical strategies for peptide synthesis have been valuable for laboratory applications and large-scale production.[10] However, the tedious synthesis and purification procedures hampered the exploration of more peptide-based drugs. Later on, the invention of solid-phase peptide synthesis (SPPS) in 1963 by Nobel laureate, Robert B. Merrifield, accelerated the synthesis and discovery of therapeutic peptides.[11] Following the advancement in sequence elucidation and chemical synthesis of peptides, more peptide hormones were identified and characterized, in which, synthetic oxytocin and vasopressin were introduced in the clinic.[12] In the meanwhile, the isolation of peptides from animal sources continued for many years for the insulin and adrenocorticotrophic hormone (ACTH) from cattle to treat different endocrine illnesses. Even though, for almost 90 years, animal-derived insulins dominated the insulin market, they could not meet the big market demand later on, and the production was replaced by recombinant DNA technology in 1982.[7] The exploitation of microwave irradiation in solid- and solution-phase synthesis and the implementation of modern synthetic strategies through native chemical ligation (NCL) and Staudinger ligation (SL) has led to further progress in peptide chemistry. This is evidenced by the introduction of Food and Drug Administration (FDA) approval of synthetically derived peptide drugs including, leuprolide (1985) and goserelin (1989).[10]

Recent technologies made substantial progress in peptide therapeutics and accelerated their development, leading to over 50 peptide drugs approved worldwide with another ca. 170 peptides in clinical evaluation.[13] As venoms of cephalopods and arthropods were identified as a natural source of bioactive peptides, new potential therapeutics were identified by the extraction of their natural products.

A recent global industry on peptide drugs showed global sales of more than $70 billion in 2019 with the prediction of a compound annual growth rate of 9.1% from 2016 to 2024.[14] Interestingly, the top-selling peptide drugs in 2019 were glucagon-like peptide 1 (GLP-1) for treating type 2 diabetes mellitus, including liraglutide (Victoza) and Trulicity (dulaglutide) generated sales of at least two billion USD sales annually.[15] Due to the remarkable achievements in peptide drugs worldwide, their advancement has become one of the hottest areas in pharmaceutical research.

1.2 Advantages and limitations of peptide therapeutics

Peptides are a unique category of pharmaceutical complexes, molecularly positioned between small organic molecules and proteins, yet biochemically

distinct from both and have reshaped our modern pharmaceutical industry.[16] Peptides are natural ligands for most endocrine signaling pathways acting as growth factors, hormones, ion channel ligands or neurotransmitters. Typically, these natural ligands can trigger signal transduction pathways inside the cell as well as ion-gated channels. For instance, the peptide is a natural ligand for the G protein-coupled receptors (GPCRs), which are significant drug targets for around 50% of drugs currently on the market because these receptors are involved in the signaling pathways of many diseases.[17] Peptides that are agonists of signal transduction pathways like the previously mentioned insulin and GLP-1 are the most successful peptide drugs. Therefore, peptides are good starting points as a template for new structural leads in drug discovery that can be modified to generate potent compounds with more selective action with fewer side effects.

The pharmacokinetic properties of peptides are suboptimal due to their proteolytic instability in serum which can occur by the action of around 500 different proteases and peptidases.[18] Moreover, the peptides are less known to experience vital hepatic metabolism mediated by cytochrome P450 oxidation and excreted through renal filtration.[19] As a result, peptides have rapid renal clearance with insufficient plasma exposure which could limit their half-life and their tissue accumulation hence the bioavailable concentration. Nevertheless, the peptide metabolites are mainly amino acids with few incidents of drug-drug interactions and lower tissue accumulation making them relatively lower in toxicity than small molecule-based drugs.

Peptides have weak membrane permeability and most of their therapeutic applications are transmembrane and extracellular targets due to their inability to penetrate the intestinal mucosa.[13] The peptide's high molecular weight and polarity that can come from their length and amino acid composition impede them from crossing the cell membrane. This factor in addition to the presence of digestive enzymes leads to poor oral bioavailability for the peptides. Therefore, peptide therapies require subcutaneous or intravenous administration which can decrease patient compliance and convenience, especially for chronic, outpatient therapy.[12] Apart from that, peptides are unable to traverse over the blood-brain barrier (BBB) which is an optimistic point from a toxicological prospect, yet, it precludes central nervous system (CNS) targeting.

In previous years, small molecules, both naturally isolated or chemically synthesized, have been a mainstay in the management of different diseases due to their oral bioavailability, good membrane penetration with their small size, and low production costs. However, their relatively small size implies that it is challenging for them to prevent large surface interactions efficiently, for

instance in the case of protein-protein interactions (PPIs). It is approximated that there are at least 140,000 pairwise PPI that modulate the downstream signaling events in the human body.[20] PPI interfaces have a large surface area of around 1500–3000 $Å^2$ while small molecules can just cover around 300–1000 $Å^2$ of the binding pocket of the protein surface rendering it difficult to effectively modulate the protein function.[21] In contrast, the peptides have unique physiochemical properties due to their larger size with more flexible backbone and additional intermolecular interactions making them more potent inhibitors of PPI than small molecules. As a result, peptide drugs are more specific, leading to more selective and effective consequences with nominal off-target side effects hence lower toxicity than small molecule drugs.

Peptides are more advantageous in diseases such as cancer, neurodegeneration, and chronic inflammation that are driven by abnormal PPIs in which several promising bioactive peptides are under clinical development. Examples include but are not limited to; Brimapitide (XG-102) which is an inflammatory suppressor for the c-Jun N-terminal kinases (JNK)[22], and Motixafortide (BL-8040) which is being developed for the treatment of multiple myeloma.[10] When it comes to Biologics, particularly antibodies or proteins, they can exhibit better pharmacokinetic profiles than peptides, even though, peptides have better tissue penetration with reduced activation of immune responses in the body because of their relatively small size.[23] Regarding the manufacturing cost of peptides, which are considered relatively high in comparison with small molecules, yet easier to synthesize with lower production cost than biologics.[24] Currently, most of the peptides are chemically synthesized via the SPPS technique, yet larger peptides like insulin are produced by recombinant technology.[25] Despite the aforementioned limitations, recently, there have been advances in the synthesis of peptides to modulate their pharmacokinetic properties and overcome synthetic and purification challenges that led to the identification of potential peptide drug candidates.[25, 26]

1.3 Peptide discovery approaches

The first step within the drug discovery procedure is the selection of lead compounds with a profile that will increase their success as a clinical candidate.[12] Traditionally, the choice of peptide leads for the discovery of peptide-based therapeutics has employed general strategies that can be summarized into the following categories: nature-derived, hormone-mimics, phage-displayed peptides and peptidomics. Each category will be discussed in the following sections in more detail.

1.3.1 Natural peptide-based hormones in the human body.

The history of peptide drug discovery began by utilizing peptide-based natural hormones with well-known biological functions as starting points for drug-lead discovery. This strategy has been used for treating disorders caused by hormone deficiencies, such as the decrease of insulin hormone levels necessary to control blood glucose levels in patients with Diabetes Mellitus (DM). Diabetes is generally treated either by enhancing insulin secretion through targeting the GLP-1 receptor or by using insulin injection.[13] Searching for animal-derived natural peptides and hormones, such as insulin, somatostatin, GLP-1 and oxytocin, was the first strategy employed for peptide-based drug discovery and development. Nevertheless, the drawbacks related to these natural peptides including short half-life, lower potency, non-selectivity, limited availability and immunogenicity provoked an interest in improving their natural sequences to find synthetic analogs with more drug-like properties.

1.3.2 Peptides mimicking hormones.

Medicinal chemistry has utilized methods to augment the potency and pharmacokinetic profiles of natural hormone-mimetic peptide drugs. These strategies have been employed to improve their molecular stability by reducing vulnerability to proteolytic cleavage or linking the peptide to lipophilic entities that attenuate the renal clearance of the peptide through binding to serum albumin.[16] A particularly thriving case is the discovery of synthetic equivalents of the hormone GLP-1, this 37 aa natural peptide displays a very rapid circulating half-life in vivo rendering it an implausible drug candidate. Extensive efforts have been made to boost the stability of the peptide hormone while preserving its potency which led to the discovery of the three top-selling peptide drugs for the treatment of DM: Victoza (liraglutide), Ozempic (semaglutide) and Trulicity (dulaglutide).[7]

Many other hormone-derived peptide therapies have been developed using a similar approach including short and long-acting insulin analogs for the treatment of DM,[27]; octreotide, a peptide-based drug mimicking somatostatin for the treatment of some growth hormone-related tumors[28]; atosiban, an oxytocin analog for controlling premature labor[29] and gonadotropin-releasing hormone (GnRH) derived peptide drugs such as degarelix and leuprolide for the treatment of prostate cancer.[30]

1.3.3 Peptides from natural resources.

Several bioactive peptides derived from plants, animals, bacteria, viruses and fungi have some therapeutic benefits including toxins, venoms, cyclotides,

lantipeptides and non-ribosomally synthesized peptides.[16] Typically, venom peptides are considered as vascular endothelial growth factor (VEGF) analogs that target membrane-bound ion channels and receptors.[31] They are mostly disulfide-rich cyclic peptides with up to 80 residues and are derived from snakes, scorpions, and cone snails. Processing of peptides from venoms has led to several prosperous drugs such as ziconotide which is a peptide derived from *Conus magus* venom and is applied to cure chronic neuropathic pain, providing several benefits over the opioid drugs.[32] Another toxin is linaclotide, which is a cysteine-rich 14 aa bicyclic peptide derived from Escherichia coli (*E. coli*) that has been approved for the healing of chronic idiopathic constipation as well as irritable bowel syndrome.[13] Cyclic lanthipeptides bearing a macrocyclic thioether linkage are also isolated mostly from bacterial sources and possess antimicrobial properties. Another attractive family of natural products is the so-called defensins, which are cysteine-rich peptides with a common β-sheet core.[33] Fungal defensins have antimicrobial properties and are currently being evaluated in clinical stages for the management of some chronic inflammatory disorders such as asthma and arthritis. Another interesting example of peptides produced by viral phages is the holin peptides which possess antimicrobial properties and will be discussed in more detail in **Chapter 3**.[34]

Cyclotides are plant-derived peptides possessing three conserved disulfide bridges and a head-tail cyclized backbone forming a cyclic cysteine knot motif. Their structures retain an extraordinary degree of topological flexibility, and oral bioavailability and have insecticidal activity.[16]

Non-ribosomal peptides (NRPs) cover another cluster of peptides derived from natural resources. This class contains non-proteinogenic amino acids with a diversity of cyclization motifs, methylated amino acids as well as other structural characteristics that afford their distinct properties.[7, 13] The most-studied NRPs are predominantly derived from bacterial or fungal resources, such as membrane-permeable cyclosporin that has outstanding oral bioavailability because of its cyclization and N-methylation. Cyclodepsipeptides also belong to NRPs, possessing cyclic structures with ester linkages, usually identified in plants with significant antitumor activity.

Indeed, there are many peptide-based natural products that have been reported wherein we have summarized some of them and yet many to be discovered.

The utilization of peptidomimetics is considered a valuable tool to mimic a peptide by incorporating non-natural modifications into the natural peptide sequence in order to improve peptide pharmacokinetic properties.[24] Before modulating the natural peptide sequence, it is critical to determine the core

motifs required for the biological activity. This is performed mostly by alanine-scan, through replacing each residue with alanine to create a series of peptide analogs and verifying key residues responsible for biological activity. A design of peptidomimetic inhibitors will be demonstrated in **Chapter 5** possessing a sequence similarity to the natural Sortase enzyme substrate present in Gram-positive bacteria.

1.3.4 Targeting G protein-coupled receptors (GPCRs)

GPCRs proteins are the biggest family of cell surface receptors in eukaryotics and embrace eight hundred members identified in the human genome. Aberrant GPCR signaling causes a variety of human diseases including metabolic disorders, mental disorders, cardiovascular disease, neurodegenerative diseases, and cancer.[16] Peptides are the natural substrates for GPCRs, triggering signal transduction processes within the cell, such as insulin, GLP-1, etc. For peptide discovery, the most common strategy to develop a peptide that ultimately produces extremely potent and selective action has been done by modification of an already established naturally occurring signal peptide whose mechanism of action and cellular pathway is known. The vast majority of currently available drugs act as either agonists or antagonists upon this superfamily of receptors. Receptor tyrosine kinases are signaling mediators of GPCRs and Epidermal growth factor receptors (EGFR; ErbB-1 in humans) in which their overexpression is involved in different types of cancer.[35] More details about the ErbB2 receptor will be described in **Chapter 2**, which includes our developments of TM peptide targeting ErbB2 and possessing anticancer activity.

1.3.5 Phage display technique

Phage display is an efficient high-throughput sequencing tool directed to find ligands of biological targets, including antibodies and peptides which was first reported in 1985 by Smith.[13] Basically, the method applies recombinant strategies to obtain target ligands on the surface of the bacteriophage and produce peptides with proteinogenic amino acid chains only. The identified peptide leads are usually subsequently optimized using a medicinal chemistry approach to enhance their drug-like properties (potency, pharmacokinetics, solubility, etc.). The Phage display technique has been broadly exploited to determine novel peptide ligands, for example; to find potential peptide agonists of the GLP-1 receptor derived from proteins, or venoms, as reported by Lerner *et. al*. Heinis *et al.* used this approach to produce chemically modified bis-thioether cyclic peptides. Another novel display approach was developed by Schumacher *et al.* to explore the D-chirality of peptides by mirror-image phage

display. These achievements have led to the assembly of several display libraries for the discovery of novel clinical peptide candidates.[7]

1.3.6 Peptidomics and rational design

In recent years, the peptidomics field has arisen and become a potent method for the discovery of novel peptides with innovative bioactivity. Peptidomics correlates peptide sequence analysis with peptide profiling in a range of fluids and tissues, allowing a systematic record of genetically coded polypeptides. Recent improvements in bioinformatics and mass spectroscopy enabled the sequencing and identification of peptides embedded in the tissue of interest with extraordinary sensitivity. One demonstration of this technology is the discovery of humanin which is a 24 aa peptide synthesized in the cytoplasm with a range of interesting biological activities. Future goals of peptidomics will probably be in the recognition of important peptide biomarkers and the discovery of potential substrates for peptide-based drugs.[16]

Rational design of peptides is another good approach in peptide drug discovery which involves computational analysis and bioinformatics in order to resolve the crystal structure of the target PPIs.[12] This technique enables identification of peptide modulators for PPIs and structure-activity relationship which can be used to enhance the bioactivity of peptide lead compounds. In the course of developing a peptide-based drug, the selection of a promising lead compound is the first step, which is succeeded by either chemical or biological synthesis with some sequence alteration to enhance its pharmaceutical characteristics. In the next chapter, I will summarize the basic methods used for peptide synthesis and modification.

1.4 Peptide synthetic strategies

Peptides can be produced by chemical synthesis, recombinant techniques utilized in biochemical protein expression, isolation from their natural sources, or a combination of these routes.[36] Expression of peptides and proteins by genetically modified microorganisms, mainly by *E. coli*, has lower costs in general but is limited to the generation of proteinogenic amino acids.[37] Nonetheless, the thesis focuses on chemical peptide synthesis, specifically, SPPS. The classical approaches to chemically synthesize peptides are usually performed either through Liquid-Phase Peptide Synthesis (LPPS) or SPPS. The early syntheses of peptides were performed in solutions that require careful selection of protecting groups and many tedious workups which can be challenging for the production of longer and more complex peptides. However, the method remains valid for shorter peptides. Indeed, the seminal work of the

Nobel Laureate Bruce Merrifield by introducing SPPS was the giant step for the evolution of peptide synthesis which simplified the production of large and complex peptides.[38] The SPPS methodology has since been notably progressed over time and plays a key role in modern peptide synthesis.

The concept of SPPS relies on generating a peptide chain that is covalently attached to an insoluble polymeric support (resin). The resin is made of polymeric solid support connected enduringly to a linker (dual functional spacer) that enables the provisional connection of the first amino acid to the support. Linkers are the chemical units used to attach and immobilize the first amino acid of the growing peptide chain to the resin bead. Afterward, the attached peptide is elongated by a series of peptide bond formation steps and cleaved at the end to obtain the desired peptide in solution. The SPPS involves peptide synthesis through the use of orthogonally protected amino acids by two major strategies: The 9-fluorenylmethoxycarbonyl (Fmoc)-SPPS approach, which is the relatively most commonly used method, and the tert-butyloxycarbonyl (Boc)-SPPS.[36]

The scheme in **Figure 1** illustrates the Fmoc-based SPPS strategy which relies on the the serial addition of Fmoc-protected amino acid building blocks to an insoluble resin, resulting in a growing polypeptide chain anchored to the solid support.[39] Initially, the carboxyl group of an Fmoc-protected amino acid is anchored to the resin support using a suitable coupling agent (**Figure 1 i**), after that washing is done using N,N-dimethylformamide (DMF) to remove unreacted residues. After loading the first amino acid, the base-labile Fmoc protecting group of the α-amino is removed usually with a piperidine reagent (**ii**), followed by washing with DMF. Afterwards, another coupling of the

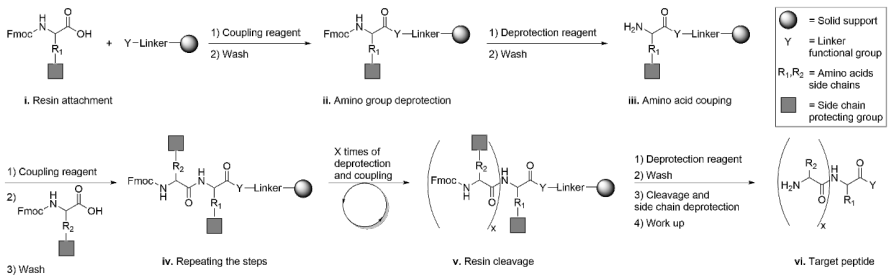


Figure 1. Fmoc-based SPPS protocol.

second Fmoc-protected amino acid is done with the first residue forming a peptide bond and generating an immobilized dipeptide, followed by washing with DMF (**iii**). Subsequently, the deprotection and coupling cycles are performed again to construct the complete chain assembly as an immobilized protected peptide (**iv**). In the end, the Fmoc group is removed from the N-

terminal amino acid using a piperidine reagent (**v**). For the deprotection of the side-chain groups as well as the disconnection of the peptide from the solid support, the resin is usually treated with trifluoroacetic acid (TFA) (**v**). Additional work-up for the fully unprotected peptide in cold ether to remove TFA and side products yielding a C-terminal amide peptide or a C-terminal acid or other functionality based on the type of linker used (**vi**).[40] It is important to mention that the peptide sequence is usually constructed linearly from the C-terminal to the N-terminal by repeated cycles (known as C →N strategy), yet, other immobilization strategies can be applied. The implementation of the orthogonal protecting groups allows directing the synthesis following the C →N pathway and minimizing side reactions. This is achieved by employing a base-labile N-Fmoc protecting group to be used for the α-amino and acid-labile protecting groups to be used for the side chains as well as acid–labile linkers. Different coupling agents can be used for the synthesis meanwhile, the washing is usually performed in DMF to remove soluble byproducts and excess reagents used during the coupling and deprotection steps. DMF is used during the SPPS as the main polar aprotic solvent for coupling and deprotection steps as well as swelling of resin, however, the use of N-Methyl-2-pyrrolidone (NMP) has increased due to the improved solubility of amino acid derivatives in this solvent.[41]

1.5 Fundamentals of modern peptide synthesis

At present, there are numerous types of resins available in the market that can be chosen to synthesize the desired peptide effectively. Polystyrene (PS) is the most common core solid support being used in SPPS. Resins of this type are mostly cross-linked polymers with 1–2% divinylbenzene (DVB) in the form of beads which are insoluble in all common solvents. Normally, the commercially available cross-linked polystyrene resins are spherical beads, small in size with two different size distributions: 200–400 mesh (corresponding to a diameter of 35–75 micrometers) and 100–200 mesh (75–150 micrometers).[42] It has to be noted that resin swelling is the most critical condition for effective chain elongation during SPPS. Cross-linked polystyrenes resins are not soluble in all ordinary solvents, However, aprotic ones such as dichloromethane (DCM), DMF, and tetrahydrofuran (THF) can solvate and swell the resin efficiently.

Linkers that attach the peptide to the solid resin support have considerable impacts on the SPPS outcomes. Modern peptide research has developed a wide variety of linkers devised for polystyrene core resin in the past few years.[43] Linkers can be classified into several categories that include the cleavage

conditions under which the peptide product can be released from the resin, which could be acidic, basic, photolytic, etc.[44] The chemistry used within the specific cleavage conditions will determine the final functionalization at the peptide product terminal. An important parameter to be considered when choosing a resin is the loading capacity. Resins with comparatively high loading capacity (0.5–3 mmoles /gram) result in higher efficiency, however, for the synthesis of larger and more challenging sequences a lower loading capacity is needed (0.1–0.2 mmol/g). It is important to note that the generation of the peptide bond in challenging sequences is not easily reachable, and a coupling reagent is needed to obtain a high coupling rate.[45] Several coupling reagents were developed to obtain better incorporation efficiency with lower side reactions and minimal racemization and have been used in the peptide synthesis industry as well as in academia.[38, 46]

The coupling of the first amino acid constitutes a fundamental step to accomplish effective synthesis and there have been strategies for monitoring the coupling steps during the synthesis. The Kaiser test is one of the most used qualitative tests to evaluate the completeness of the coupling steps relying on the presence of free primary amino groups.[50] Additionally, the Fmoc-group gives the benefit that allows monitoring of coupling through simple UV spectrophotometry analysis.[51] The base-labile Fmoc protection is quantitatively cleaved by treating the resin with 20% piperidine in DMF, producing the dibenzofulvene–piperidine adduct. This adduct displays a distinct UV absorbance maxima that can be used to calculate the moles of the cleaved Fmoc as representative of coupling efficiency or resin loading.

1.6 Specific considerations in SPPS

The utilization of solid support in SPPS has clear advantages, in which the coupling reactions can be driven toward completion by using a large excess of soluble reagents at high concentrations.[50, 51] Additionally, side products and excess reagents can be eliminated from the growing immobilized peptide by simple filtration and washing steps. All the synthetic procedures can be executed in one vessel which led to the design of automatic peptide synthesizers that allow the production of large combinatorial peptide libraries. It also allows the installation of additional chemical modifications to the parent peptide structure which cannot be achieved with the conventional expression techniques.[47]

The use of Fmoc-SPPS is relatively routine for the assembly of peptides of less than 50 residues, however for longer peptides (with more than 50 amino acids) the chemical synthesis is yet difficult, specifically in industrial production. Even though the Fmoc is the preferred strategy, the Boc-SPPS is

more advantageous in the case of the production of long peptides since TFA deprotection between the coupling cycles disrupts the aggregation efficiently during the peptide elongation.[36] Peptide aggregation on the resin is a critical problem that hinders the synthesis and occurs due to intermolecular interactions that lead to the creation of secondary-structural elements on the solid support. Several strategies have been applied to mitigate the issue, including the use of low-substitution resins, microwave heating and pseudoprolines which will be discussed in more detail in **Chapter 3**.[40]

The formation of aspartimides for certain sequences has been a major issue in SPPS. Aspartimide formation is a side reaction that occurs via the cyclization of aspartic acid residues which arises either throughout chain elongation or in the final TFA cleavage step. The reaction might come from repetitive base treatments and its sequence-dependent. The cyclic aspartimide can hydrolyse or interact with piperidine leading to a mixture of side products which decreases the purity of the crude peptide.[40] Peptide chemists found that including 0.1 M of 1-hydroxybenzotriazole (HOBt) in the piperidine solution during deprotection or using the 2-hydroxy-4- methoxybenzyl (Hmb) group to protect Asp side chain could minimize this side reaction.

Generation of diketopiperizine is a regularly arising side reaction in solid-phase synthesis induced via an intramolecular attack of the peptide N-terminal nucleophilic amino group at the amide backbone. This attack resulted in the creation of a truncated peptide molecule at the point of attack and the liberation of a six-membered diketopiperazine.[48] The reaction predominantly occurs during the Fmoc elimination and is facilitated by the presence of a secondary amine at the peptide terminal.

Cys residues have a high degree of racemization that can racemize as a result of repeated piperidine deprotection and can be minimized by using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) base for the deprotection.[38] The steric hindrance of the 2-chlorotrityl resin linker can reduce diketopiperazine and Cys racemization side reactions during Fmoc SPPS chemistry. Importantly, the TFA acidolysis of side-chain protecting groups and anchoring linkages results in the liberation of reactive species which necessitates utilizing effective scavengers to preserve the amino acids integrity. Additionally, many other different factors need to be considered when performing the SPPS such as stirring and heating which proved to improve the synthesis in certain conditions.[49] In the last twenty years, scientists have developed several approaches to enhance the efficiency of SPPS as well as some post-synthetic modifications to improve peptide characteristics.

1.7 Strategies to overcome challenges of difficult peptides synthesis

During chain assembly of the peptide, the deprotection steps and coupling reactions can not proceed toward completion due to the self-association and aggregation of the peptide chain via intramolecular hydrogen bond formation.[13] This leads to incomplete solvation and inaccessibility of the reagents to the N-terminal amino group due to the β-pleated sheet-like structures stabilized through the H-bonds formed on the backbone between the hydrogen amides and the carbonyls groups of the elongating peptide chains.[50] Peptide aggregation phenomena can start from the fifth-coupled residue and it is sequence-dependent, particularly for the one rich in hydrophobic residues. The expression of "difficult peptides" has been denoted to describe peptides that are susceptible to aggregation with a high tendency to present a challenge in every step of their production: synthesis, analysis and characterization.[51 52, 53 50, 51, 54] The aggregation can happen throughout the solid phase synthesis cycles or after the final cleavage due to significant inter- or intra-molecular interactions.[50] These difficult sequences also include homo polymers that consist of only a single type of amino acid within their sequences, amino acids with bulky or sterically hindered side chains, and amphiphilic self-assembling peptides.

Nevertheless, there are several tactics by which the synthesis of difficult peptides can be improved.[36, 39, 50] Using resins with a low loading capacity (degree of substitution) decreases the chance for the growing peptide chains to interact and aggregate. Also, the use of SPPS combined with elevated temperatures or microwave-assistance was found to improve the synthesis efficiency. The engagement of chaotropic salts such as lithium chloride or potassium thiocyanate or solvents like trifluoroethanol or hexafluoroisopropanol during synthesis is described to reduce aggregation.[55, 56] Additionally, the incorporation of amino acid derivatives with the Hmb protecting group every 6–7 residues is a strategy known to disrupt aggregation.[57, 58] Glycosylation and Pegylation are the two most considered chemical modifications to improve non-polar peptides' aqueous solubility.[59-61] Glycosylation entails the conjugation of a peptide to a sugar moiety and Pegylation to a polyethylene glycol (PEG) motif. Both conjugates have been found to enhance the solubility of peptides and reduce aggregation to tackle complex characterization or even to address unachievable purifications. It was found that the attachment of short peptide tags of basic aa as poly-lysine and poly-arginine or the acidic sequences poly-glutamates and poly-aspartates could enhance the solubility of the non-polar peptides. These tags have

specifically addressed the purification and analytical characterization of some difficult sequences. An interesting example of difficult peptide sequence synthesis and characterization that possesses anticancer activity will be further discussed in **Chapter 2**.

Every synthetic process has restrictions, and even in the hands of highly qualified peptide chemists, certain sequences confront difficulties within their preparation. In modern peptide synthesis, High-performance liquid chromatography (HPLC) has obtained significance as an analytical tool for its rapidity, sensitivity and resolving power which facilitates the elimination of several of the low-level side-products that occur throughout peptide chain assembly and after cleavage.[62, 63] Stationary phases typically used in peptides analysis and purification are reversed-phase which is silica-based supports modified by butyl (C4), octyl (C8) or octadecyl (C18) functional groups or diphenyl moieties.[64] Additionally, these supports can be adjusted in various methods concerning their particle size and shape, pore size, as well as surface chemistry to allow effective separation. Routinely, the progress of peptide analysis and purification can be monitored simply by an Ultraviolet-visible (UV-vis) detector, or ion-trap electrospray mass spectrometry (MS).

1.8 Posttranslational modifications of peptides

Recently, there has been significant progress in peptide synthesis strategies which facilitated the assembly of different peptide sequences. Nonetheless, peptides have constitutional physicochemical and poor pharmacokinetic characteristics which make the development of orally administered bioactive analogs challenging as described previously in **section 1.2**. Current research focuses on the development of delivery systems or peptide carriers as well as novel dosage forms to introduce more peptide-based drugs into the market.[10] Additionally, various structural modifications have been used for the modulation of peptide properties such as backbone modification to amend the proteolytic stability of the peptide.

The modifications include substitution of L-amino acids by D-amino acids, replacement of amide bonds with triazole, incorporation of methyl-amino acids, and utilization of peptoids and β-amino acids. The insertion of these non-natural modulations into the peptide sequence is an efficient approach for increasing the plasma half-life of the peptide. Peptide cyclization is another common strategy to increase peptide proteolytic stability and cell-permeability thus improving oral bioavailability. Cyclization can be done in several ways, such as head-to-tail, side chain-to-side chain, and backbone-to-side chain cyclization. Additionally, cyclization can help in the stabilization of

the peptide secondary structure by pre-organizing intramolecular interactions. Stapled peptides represent a recent strategy to form a peptide macrocycle by linking two amino acid side chains covalently, in which the resulting macrocycle has α-helix mimicry and can be used to identify PPIs modulators.[7] Modification of the N- or C-terminus by alkylation and the use of peptide conjugates are also frequently used strategies to modulate peptide bioavailability. Conjugates through pegylation, which was introduced in the previous section, could enhance peptide solubility and lower the immune reactions to peptides hence increasing peptide bioavailability. Currently, there are 15 pegylated peptides or protein-based FDA-approved therapies in the market.

Lipidation is probably one of the most valuable modifications used for the development of peptide therapeutics.[26] Lipids are fully biodegradable and identified to increase the half-life of their peptide conjugates by stabilizing their structure or binding to the cell membrane. Lipidated conjugates have an important feature being able to bind to human serum albumin ensuring prolonged time of circulating peptide featuring longer half-life time. Another significant feature of lipidated peptides is their ability to self-associate into oligomeric macromolecules which renders them appropriate for subcutaneous injection with extended duration of action. Nowadays, anti-diabetic drugs are the most distinguished lipidated peptide drugs on the market which include glucagon-like peptide-1 (GLP-1) receptor agonists: semaglutide (Ozempic®) and liraglutide (Victoza®) and the insulin derivative detemir (Levemir®). Various structural modifications can be accessible due to the recent advances in peptide conjugation strategies. In **Chapter 4** a novel conjugation strategy for Cys-containing peptides based on ionic liquids will be introduced.

1.9 Bioactive peptides mechanism of action

Novel strategies for peptide synthesis and pharmacokinetic modulation allowed the generation of peptide drugs that have accessed the market for a variety of ailments including metabolic disorders (particularly diabetes), cancer, cardiovascular disorders, human immunodeficiency infection and chronic pain.[7] Most peptide-based therapies modulate peripheral extracellular targets due to difficulty in crossing cell membranes, yet can be designed to cross through the cell membrane and internalize inside the cell to different compartments.

Therapeutic peptides can act as neurotransmitters, ligands, growth factors, hormones, anti-infective agents and cytotoxic agents by targeting a wide variety of molecular targets and triggering various effects. Peptide pharmaceuticals can modulate the action of biological receptors by acting as

agonists or antagonists. As agonists, peptide mimicking hormones such as gonadotropin-releasing hormone, insulin, oxytocin and vasopressin are replacement remedies for biological ligands acting on cellular surface receptors. These peptides usually induce intracellular signal transduction pathways and are extremely potent and selective drugs.[13, 65] Peptide antagonists usually are slower to reach the market due to the fact that antagonists have to occupy more than 50% of the target receptor and compete with the native ligand in order to be efficient whereas for receptor activation only 5–20% occupancy is needed. Atosiban is an example of a peptide-based antagonist of oxytocin used to suppress premature labor. An interesting approach to improve potency was reported to utilize warheads by forming covalent interactions with the target protein side chains in the binding site. Nevertheless, some safety challenges regarding their potential immunogenicity and low selectivity hinder the usage of covalent peptide drug candidates.[66]

Outside the basic on/off-switch strategy of agonism and antagonism, alternative and more complex strategies have been investigated to target abnormal GPCR signaling of "orphan" receptors that have unidentified cognate ligands. The complex and irregular GPCR signaling involves seven TM conformational ensemble receptors with various signaling pathways and can be stated as multidirectional allosteric effects embedded in several health disorders. Rapastinel is a peptide-based allosteric modulator of N-methyl-d-aspartate (NMDA) receptor which acts as a weak partial agonist with FDA-approved antidepressant and cognitive enhancing therapy.[67] Pepducins are lipidated peptides under clinical phase development for the inhibition of arterial thrombosis and platelet activation by targeting the intracellular GPCR/G-protein interface.

Peptide inventions developed so-called interfering peptides (IPs), which interfere with pathogenic PPIs, The IPs block the interacting protein surfaces by taking advantage of peptides over small molecules in this area as explained in **section 1.2**. Dysregulated protein interaction networks cause signaling changes that stimulate several diseases in the human body.[65] Currently, several promising IPs are under clinical evaluation for tumor suppression and have shown promising blocking of ubiquitin-ligase MDM2 and p53 proteins interaction and the interaction between CXCR4 and its ligand CXCL12. The previously mentioned Brimapitide is a peptide-based drug of this category under a phase III trial for the treatment of acute sensorineural hearing loss, which blocks the interactions of JNK.

Additionally, many peptides with cytotoxic activity against tumor cells or pathogenic microorganisms have shown activity by inducing membrane disruption, apoptosis or through inhibition of discrete internal targets.[65] These

peptides are described to form channels or pores in the cell membrane, which leads to membrane disruption, cell breakage and necrosis. Additionally, internalization of the peptide can also disrupt the mitochondrial membrane resulting in the release and activation of caspases, and induction of apoptosis.[18] Several complex actions can be triggered by peptides that interfere with cell cycle regulation, DNA repair pathways and immune modulation which found good applications as wound healing, vaccines, anti-inflammation, and anti-angiogenic. Additionally, the progress in the field of cell-penetrating peptides (CPP) and peptide conjugation with therapeutics have extended the intracellular and extracellular domains that can be targeted by peptides.

1.10 Limitations and aims of the thesis

The research outlined here aims to advance the understanding and application of peptide science by exploring the design of peptide-based therapeutics with different biological activities. This includes the development of a novel bioconjugation methodology, which is crucial for drug design and development. Additionally, the thesis covers developing novel synthetic strategies for difficult TM peptides and finally the delivery of peptides in biological systems. Ultimately, the thesis provides solutions with potential real-world applications.

The **first part** of the thesis focuses on the development of a strategy for targeted cancer therapy. Cancer is clearly the most deadly disease in the developed world and most of the existing treatments are not effective enough to provide a full cure for patients.[68, 69] Over-expression of the human ErbB2, has been reported in various cancer types.[70] Conventional small organic molecule-based therapy remains the principal mode of cancer treatment. However, these chemotherapies are not effective due to the adverse effects on normal cells and the development of multi-drug resistance by cancer cells.[71] Clinical trails have demonstrated that breast tumors overexpressing the ErbB2 protein are less amenable to treatments including, methotrexate, cyclophosphamide, epirubicin or 5-fluorouracil.[72, 73]

Although antibodies and antibody-drug conjugates such as trastuzumab, pertuzumab and margetuximab have proven effective in targeting the extracellular domain (ECD) of the ErbB2 receptor, they are poorly distributed in solid tumors owing to their bulky nature, target healthy cells expressing ErbB2 receptor, and cause immunogenic reactions that adversely affect cancer patients, limiting their therapeutic efficacy.[74-76] Moreover, patients develop resistance to these antibody-based therapies. The synthesis of antibodies requires sophisticated manufacturing processes such as antibody engineering, advanced purification, and strict quality control measures, making large-scale

antibody production a highly expensive venture.[75, 77] Taking into account all adverse effects of antibody therapy, there is a need to find alternative ways of targeting ErbB2 receptors in which peptides can be good treatment options.

Even though TM peptides have proven to be effective at targeting the ErbB2 receptor in animal breast cancer models, as well as having combined anticancer and immunostimulatory properties, their hydrophobic nature requires special techniques in peptide synthesis and design.[78-82] The previously reported hydrophobic TMs holding anticancer activity were synthesized by external companies with no detailed explanation of their synthesis and characterization.[83, 84] Moreover, the antiproliferative activity of the TM domains on cancer cell lines in these studies was erratic in which inhibition occurred only at the lower nanomolar concentration while no or very little effect was observed at the higher nanomolar concentrations.[83] In another study, the maximum effect at micromolar concentration on cell lines occurred within 48 hr treatment time with a drop of activity following 72 hr incubation that could indicate transient inhibition.[84] Additionally, previous studies relied on detergent-based vehicles to solubilize and deliver the hydrophobic peptides into the cells, however, their poor compatibility with mass spectrometry as well as their dose-dependent cytotoxicity can limit their usage and necessitate finding alternative delivery strategies.[85, 86] Herein, the work done in Paper **I**, aims to address these challenges by optimizing the synthesis and delivery of TM domains possessing anticancer activity. It is important to highlight that the synthesis of TM fragments is quite challenging and Paper **II** demonstrates the chemical synthesis of the holin TM peptide variants by implying the SPPS strategy with the inclusion of pseudoprolines.

The **second part** of this work focuses on the development of a thioether-based conjugation technique. In recent years, the conjugation strategy of selective labeling of thiol-containing biomolecules has shown an increased interest in the need to develop novel peptide-drug conjugates.[87] Peptide conjugation with other modalities can enhance their therapeutic index as well as improve the peptide's physiochemical properties. Though efficient and selective conjugation belongs to the limiting steps in the synthesis of amino acids containing conjugates. Enzymatic, chemical or photo-conjugation techniques could enable post-synthetic reactions by the inclusion of non-proteinogenic amino acids and fluorescent labels into the biomolecules.[88, 89] Recently, there have been many reported enzyme-mediated site-specific conjugations of thiol-containing biomolecules in which some of these drugs containing thioether conjugates were FDA-approved.[90] Usually, in the field of organic chemistry, thioether bonds are introduced under strong basic conditions via the condensation of organic halides with a thiol.[91, 92] This is

primarily due to the absence of mild processes with less harsh conditions to afford thioether conjugated biomolecules. Through the high toxicity of alkyl halides, the introduction of novel and milder methods to form conjugates is desirable as in the case of involvement of ionic liquids (ILs) which will be discussed in Paper **III**. Additionally, the superior solvation and stabilization properties that ILs possess render them a suitable medium for biomolecules and can address the issues of protein misfolding, aggregation and insolubility which can arise when using conventional solvents.[167]

The **third part** of this work explores the rise of bacterial resistance to conventional antibiotics which has gained considerable attention worldwide making infections life-threatening and harder to treat.[7] Due to this, antivirulence compounds have gained prevalence as an alternative strategy to treat resistant bacterial infections while avoiding the development of resistance. The search for antivirulent inhibitors has led to the discovery of promising anti-virulence drug candidates.[93] In the scope of paper **IV**, the anti-virulence activity of a set of peptide-based compounds is investigated to target the SrtA enzyme aiming to contribute to the development of novel anti-virulence treatments. Attractively, none of the SrtA-targeting molecules have reached the clinics and most of the previously reported peptidomimetic inhibitors possess low potency with high micromolar inhibition. Nonetheless, mostly covalent inhibitors are among the ones evaluated for clinical applications, in which these inhibitors can interact with other cysteine proteases and lead to several side effects in the body.[94]

Chapter 2

2.0 Targeting ErbB2-positive cancers by transmembrane peptide-loaded ionic liquid nanocarriers (Paper I)

2.1 The ErbB2 protein in cancer

Cancer is one of the most lethal human illnesses accounting for about 10 million deaths globally in 2020.[68, 69] Cancer is a disorder that results from genetic shifts in the body cells leading to unusual cellular growth that may be allocated to other body parts by a process called metastasis.[95] The abnormal growth of cells in a group called a tumor, in which these tumor cells have the capacity to fuse with each other and with non-tumor cells through an ambiguous mechanism. Among the unregulated genes, the ErbB2 gene exhibits a significant role in body tumors.[70] Over-expression of ErbB2 receptor has been reported in various cancer types, including but not limited to breast,[96] gastric,[97] ovarian,[98] and lung cancer, [99] is closely associated with aggressive tumor types and poor prognosis in cancer patients. Its activation occurs through dimerization with itself or other members of the EGFR family (ErbB1, ErbB3, or ErbB4), leading to the activation of cascades of downstream signaling, including the phosphatidyl inositol 3-kinase (PI3K)/AKT and the mitogen-activated protein kinase (MAPK) pathways, and corresponding phosphorylation, which promotes cell proliferation and survival.[100, 101]

Among the receptor subtypes, ErbB2 is unique and is considered an atypical member of the receptor family. It is a 185 kDa transmembrane protein consisting of 1255 amino acids, which does not have an endogenous ligand.[102, 103] ErbB2 protein structure can be divided into three major parts: ECD, a single TM domain and an intracellular cytoplasmic tyrosine kinase domain.[102] The ECD of the monomeric ErbB2 is divided into four domains (I-IV) which encompass the dimerization loop in the domain II. While the cytoplasmic part consists of a juxtamembrane linker segment, the kinase domain components and most of the adapter binding sites are carried through its carboxyl-terminal tail.[104]

In spite of the progress in verifying the contribution of ErbB2 overexpression in cancer metastasis, the exact mechanistic pathways of cancer metastasis mediated by ErbB2 are largely unknown and necessitate the exploration of new treatments for ErbB2-overexpressing cancers and anti-metastasis.[70]

2.2 TM peptides as targeted anticancer therapy

As introduced in the first chapter peptides have a superior advantage in treating a wide variety of cancers because they have high receptor binding specificity, and do not cause many immunogenic reactions with less adverse side effects. Moreover, Peptide synthesis is less expensive and multiple functional group modifications can be carried out to attach other bio- or small molecules to the peptide.[105-107] Peptide sequences corresponding to the TM region of the receptor have been also evaluated in a few studies for their anticancer effect.[108] The TM peptides consist of hydrophobic amino acid moieties that span and mimic the TM region of the receptor. These TM domains could act as competitors by sterically hindering the receptor dimerization process thereby inhibiting the intracellular signaling cascade.

Lofts *et al*. reported the expression of a short TM sequence of the rat ErbB2 receptor and showed for the first time that the constructed pentapeptide could inhibit the growth of genetically mutated cells, suggesting that the TM domain is involved in the protein dimerization.[109] Further studies report hydrophobic TM domain of native sequences of the human ErbB1 (SIATGMVGALLLLLVVALGIGLFM) and ErbB2 (SIISAVVGILLVVVLGVVFGILI) and their mutants flanked by extracellular, intracellular and signal sequences.[83] The transfected expressed peptides blocked the dimerization and subsequently inhibited the activation of overexpressed ErbB1 and ErbB2 receptors in epidermal and ovarian cancer cells, respectively.[83] Their findings consolidate the notion that TM domains are involved in ErbB receptor activation.[110]

Later, Arpel *et al.* reported a 27 mer TM peptide (VTFIIATVEGVLLFLILVVVVGILIKRR, NeuNT) mimicking the TM domain of the mutant murine ErbB2) with a V664E substitution.[84] The substitution is an oncogenic mutation that leads to carcinogenesis and constitutive activation of the receptor in a dimeric form.[111] The membrane-mimicking peptide showed significant interaction with murine analogs of ErbB1, ErbB3, and ErbB4 with no interactions with other proteins transmembrane domains through bacterial protein expression analysis, supporting the fact the ErbB2 TMD can form stable homo- and heterodimers.

At micromolar dosages, this peptide suppressed metastasis as well as breast tumor growth in a mouse model with genetically induced breast tumors.

The short peptide GP2 (IISAVVGIL, aa 366–379) derived from the TM region of the ErbB2 protein, has been evaluated as a peptide-based vaccine in breast cancer clinical trials highlighting the potential application of TM peptides for promoting immune-targeting of cancer cells.[112-114] The GP2 peptide induced cytotoxicity, even with higher immunogenic potential, relative to the peptide E75 (KIFGSLAFL, aa 366–379), which is derived from the extracellular domain III.[115] Its immunogenic activity was attributed to the ability to stimulate cytotoxic T lymphocytes to recognize and kill ErbB2-expressing cancer cells. Some other previous studies reported the expression or the chemical synthesis of the hydrophobic TM sequence of the ErbB2 receptor and showed their ability to stop the growth of genetically mutated cells.[83, 84, 109-115] Their findings consolidate the notion that TM domains are involved in ErbB receptor activation and highlight the potential application of TM peptides for the suppression of tumor growth and metastasis.

2.3 Development of novel TM peptides for targeted cancer therapy

In the scope of the thesis, for the first time, novel synthesis and characterization procedures are developed for peptides derived from the TM domain of the human ErbB2 receptor. Additionally, two novel IL-based nanocarriers were designed to act as a shuttle and deliver the peptides into ErbB2-positive cancer cell lines (**Figure 2**). Herein, the TM domain is expected to act as a competitor for the receptor dimerization process thereby inhibiting the intracellular downstream signaling cascade. In turn, the ionic liquids were previously reported to possess surface-active properties, named surface-active ionic liquids (SAILs). They are described to pre-organize into micelles or bi-layers (liposomes) and induce rigidification/fluidization or rupture of the cell membranes depending on their concentrations.[116, 117] Previous reports identified IL as a perfect system for the solubilization of highly hydrophobic peptides,[78-81] performing bioconjugations and other modifications. Moreover, recent studies report the use of ILs as effective nanocarriers due to their easy tunability by introducing long alkyl chains of varying lengths in their structures to generate so-called SAILs.[118, 119] The SAILs can form colloidal structures like micelles/vesicles in water in a similar fashion to the conventional ionic surfactants, though at low concentrations due to their high surface activity.[120] This study was focused on exploring the synergistic effect of the ionic liquid nanocarriers loaded with TM peptide on a set of ErbB2-positive cancer cell lines, including lung, breast, and pancreatic.

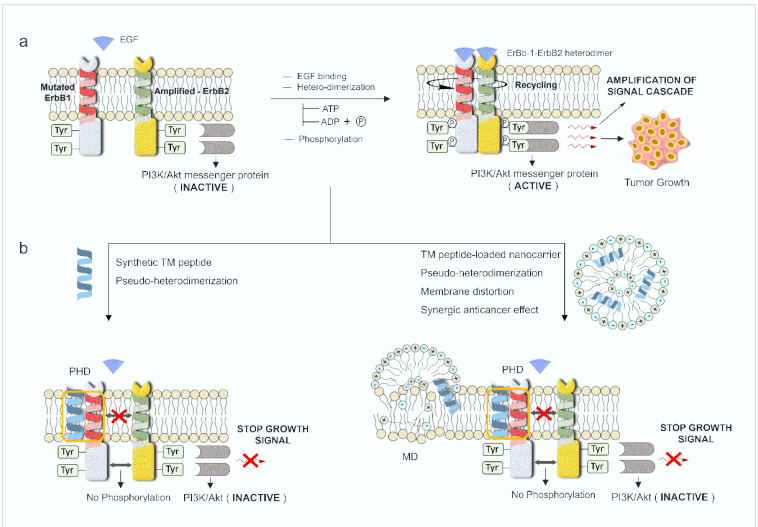


Figure 2. Conceptual design of TM peptide-loaded ionic liquid nanocarriers. The proposed mechanism of inhibition of the dimerization for transmembrane domains of the ErBb2 receptor. (**a**) Epidermal growth factor (EGF) Ligand binding to the ErBb2 receptor induces dimerization and phosphorylation of PI3K-Akt intracellular signal transduction pathway leading to receptor activation. In the event of irregular signal amplification, the dimerization process is correlated with pathological conditions such as tumor growth. (**b**) Inhibition of receptor dimerization by either introducing a synthetic TM peptide that mimics the TM domain of the ErBb2 membrane protein (left) or by delivering the TM peptides using biamhphiphilic nanocarrier (Right). Both routes will stop the phosphorylation and signal transduction pathway leading to the inactivation of the overexpressed ErBb2 receptor and stopping tumor growth. The synthetic TM peptide can act as a competitive inhibitor forming a Pseudo-heterodimer while the nanocarrier will have an additional membrane fusion effect that can distort and alter the cancer cell membrane.

2.4 SPPS and chromatography of TM peptides

TM peptides belong to “difficult peptides” and their synthesis, analysis and purification have been described to be quite challenging.[51] Considering peptide’s hydrophobic nature, structural modifications were introduced to the TM peptide to improve its physicochemical properties. Therefore, three TM peptide variants **V1-V3** were synthesized (**Table 1**). The C-termini of all three variants (**V1-V3**) were modified with a poly-lysine tag followed by a miniPEG linker (Ado, amino-3,6-dioxaoctanoic acid). Additionally, a fluorescence tag 5/6-carboxyfluorescein (FAM) was introduced at the N-termini of peptide variants **V2** and **V3** enabling tracking of this peptide by microscopy through fluorescence detection. The FAM moiety was coupled directly to the TM peptide affording **V2,** meanwhile, a PEG_4 spacer was installed for **V3.** The incorporation of short ionic peptide-solubilizing tags and PEGlyation has been reported to improve the solubility of some hydrophobic peptides, reduce the

tendency to aggregation and lead to better yields as well as easier charecterisation.[78, 121, 122]

Table 1. The synthesized TM-peptides sequence using SPPS. The ErbB2 protein TM fragment 653-675 aa of the whole protein.

Peptide variant	Amino acids sequence	Calculated Mw [g/mole]	Experimental Mw[(d)] [g/mole]	ppm	tR[(e)] [min]
ErbB2_TM	SIISAVVGILLVVVLGVVFGILI	-	-	-	-
V1	SIISAVVGILLVVVLGVVFGILI-Ado-KKKK[(a)]	984.3191	984.3067	12.6	4.7
V2	FAM-SIISAVVGILLVVVLGVVFGILI-Ado-KKKK[(b)]	1103.6683	1103.6897	19.4	4.6
V3	FAM-PEG4-SIISAVVGILLVVVLGVVFGILI-Ado-KKKK[(c)]	1186.0490	1186.6244	485	4.8

(**a**) Ado: Amino -3,6-dioxaoctanoic acid. (**b**) FAM: 5(6)-Carboxyfluorescein. (**c**) PEG(4): Amino-4,7,10,13-tetraoxapentadecanoic acid. (**d**) Mass peaks detected as [M+3H]3+. (**e**) Retention time (tR) based on LC-UV 214 nm detection.

The TM core sequence was synthesized by an automated *Intavis*® MultiPep CF peptide synthesizer with real-time UV monitoring to evaluate the efficiency of each coupling cycle. The synthesizer monitors the coupling cycle efficiency by indirect UV-based analysis of the deprotection step via complex calculations. (**Figure 3**).

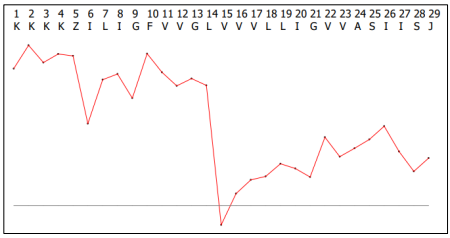


Figure 3. UV trace generated by the peptide synthesizer that shows the efficiency of each Fmoc-deprotection step. The z-letter code denotes the Ado group.

The solubility belongs to a very important property, which allows not only functional groups to react properly during the synthesis to form amide bonds. In turn, peptide insolubility can hinder their analytical characterization and prevent their purification.[50] In the current study three variants **V1-V3** were tested on their solubility by optical observation of the peptide-solvent mixture for the occurrence of any precipitate, gel formation, air bubbles, turbid mixture, or phase separation (**Table 2**). The tests were connected with preliminary analytical HPLC runs that indicate the presence of the target

peptide in the tested solvent to detect a peak at 445 nm absorption wavelength corresponding to the **V2** peptide. Based on previous studies, the use of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) increases the solvation potential for hydrophobic peptides since it mimics the cell membrane natural environment and stabilizes the α-helical structure of the TM region through intramolecular H-bonds.[123, 124] The investigations showed that using the ternary mixture of (20%HFIP:40%MeOH:40%H_2O+0.1%TFA) afforded a homogenous yellow solution when dissolving both **V2** and **V3** peptides.

Table 2. Solubility profile of ErBb2 **V2** peptide.

Solvent Mixture[a]	Solubility[e]	HPLC detection[h]
100% ACN	precipitate	N.d[i]
100% H_2O	precipitate	N.d
ACN:H_2O	soluble (>33% ACN)[f]	No peak
100% HFIP	soluble	N.d
20% HFIP: (80%H2O+0.1%TFA)	soluble	No peak
20% HFIP: 70% H_2O: 10% ACN[b]	phase separation with gel formation	N.d
10% ACN: 70% H_2O: 20% HFIP[b]	phase separation with gel formation	N.d
40% ACN: 40% H_2O: 20% HFIP[b]	phase separation with gel formation	N.d
16% HFIP: 66% H_2O: 18% IPOH[c]	phase separation	N.d
14% HFIP: 56% H_2O: 30% IPOH[c]	phase separation	N.d
12% HFIP: 48% H_2O: 40% IPOH[c]	turbid mixture	N.d
16% HFIP: 66% H_2O: 18% MeOH[d]	soluble	Found peak
14% HFIP: 56% H_2O: 30% MeOH[d]	soluble	Found peak
12% HFIP: 48% H_2O: 40% MeOH[d]	soluble	Found peak
100% MeOH	soluble	No peak
MeOH:H_2O	soluble (<85% H_2O)[g]	Inconsistent[j]

(**a**) Solvent mixtures contained either mono, binary, or trinary solvents. The mono and binary mixtures were prepared first and then added to the peptide all at once. (**b**) The trinary mixture of HFIP:H_2O:ACN was added in sequential order as H_2O-HFIP mixture first then ACN or reversed. (**c**) In this trinary mixture, the IPOH was added gradually to a peptide dissolved in a 20% HFIP: (80%H_2O+0.1%TFA). (**d**) In this trinary mixture, the MeOH was added gradually to a peptide dissolved in a 20% HFIP: (80%H_2O+0.1%TFA). (**e**) The solubility tests were made by dissolving 0.5 mg of V2 peptide in 0.5 mL of solvent (300 μM concentration) through visual inspection of the peptide-solvent mixture. (**f**) Formation of precipitates when using < the ACN% threshold. (**g**) Air bubble generation when using > the H_2O% threshold. (**h**) HPLC detection by the ability to see a peak at 445 nm absorption wavelength corresponding to the analyzed peptide during a run of in C4 functionalized silica column with a gradient method starting at 40% to 90% of ACN over H_2O + 0.1% TFA as additive. (**i**) N.d indicates that the run was not performed to avoid damaging the column due to expected blockage or high acidity. (**j**) Different analytical runs were made here using various H_2O:MeOH as mobile phase eluent systems at various strengths, however, peak appearance was inconsistent when repeating the analysis.

After addressing the solubility issues for the ErBb2 peptides, the analytical characterization was performed using a C4 functionalized column in the HPLC

system as a better choice for hydrophobic peptides than a C18 column since it has a reasonable degree of hydrophobicity and can lead to effective separation.[54] After many attempts of using different eluents at various gradients with novel eluent additives, the variants were successfully characterized by employing a lower concentration of ACN to accomplish separation and peak detection. This unique behavior of the peptides is a feature of "difficult sequences" that were successfully overcome after numerous analytical experiments.[50, 51] Noteworthy, the molecular weights measured by Electrospray Ionisation Liquid Mass Spectrometry (ESI-MS) were consistent with the theoretical molecular weights calculated for each peptide variant.

2.5 Fluorescence and secondary structure analysis

Peptide-based fluorescent probes are beneficial to understanding the molecular interactions through direct monitoring and detection of the biomolecules in live cells.[125] Conjugation of the fluorophore, FAM moiety, to the peptide was confirmed by UV-Vis and fluorescence spectroscopy. The **V2** and **V3** fluorescence spectra showed excitation and emission maxima at 499 nm and 520 nm respectively, which aligned with the reference UV/Vis spectra of FAM in the literature.[126, 127]

The secondary structure of peptides plays a significant role in receptor recognition and biological activity.[107] Thus, the structural conformation of the peptide variants **V1**, **V2** and **V3** was evaluated using Circular dichroism (CD) spectroscopy. CD is an optical spectroscopic technique that has been widely used to study the secondary structure of proteins and polypeptides in solution.[128] This technique detects different absorption of right- and left-circularly polarised light by the amide and carbonyl groups of the polypeptide backbones to elucidate structural details about peptide or protein conformations.[129] This is based on the fact that ordered (α-helix or β-sheet) and unordered (random coil) conformations exhibit distinctly different CD spectra.[130] Generally, the spectra of peptides are dominant by their amide groups that experience varying degrees of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions as a result of absorbing circularly polarized light from the far UV region (190 to 230 nm).[131]

In this work, the three ErBb2 variants were subjected to CD analysis to check for their proper folding. CD measurements were performed in two different media: 2,2,2-trifluoroethanol (TFE) as a membrane-mimicking solvent and aqueous octyl β-D-glucopyranoside (OG) which is the dissolvation media used later for the biological activity testing. All peptides (**V1** to **V3**) dissolved in TFE showed a characteristic α-helix (46-51%) represented by two negative bands at 208 and 222 nm and a positive band at 195 nm. On the other

hand, the detergent OG contributed to the conformational transition to a disordered random coil (43-46%) with a negative band at 192 nm. The conformational transition to a predominant random coil structure in OG can be attributed to the hydrophobic interactions of the alkyl chain of OG with the peptides, which induces unfolding.[132,133, 134] However, the secondary structure of TM peptides in TFE is in line with the fact that the transmembrane segment of the human receptor tyrosine-protein kinase ErBb2 (Gene ERBB2) adopts a predominantly α-helical conformation, as reported by the sequence analysis in the UniProt database (P04626).

After the successful synthesis and characterization of the peptides and with the fact that the peptide has poor pharmacokinetic properties, SAILs as nanocarriers were designed and synthesized. This is to increase the peptide permeability through the cancer cells for enhanced anticancer activity.[135]

2.6 Synthesis of nanocarriers

In order to transport the TM peptides from the site of administration to the site of action as well as increase the peptides' permeability through the cancer cellular matrix for enhanced anticancer activity, we designed and synthesized SAILs as nanocarriers.[135] The SAIL was formulated through the esterification of L-proline followed by metathesis of the cation with sodium lauryl sulfate (SLS) anion in two-step reactions (**Figure 4**).[136, 137] The synthetic scheme afforded both mono-amphiphilic (MAIL) carrier represented as [Pro][Cl] and Biamphiphilic (BAIL) carrier represented as [Pro][LS].

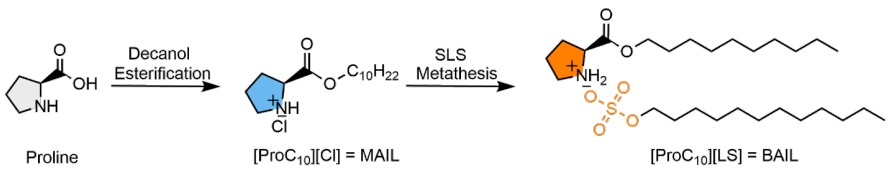


Figure 4. Synthesis scheme for the formation of [Pro][LS]. The esterification reaction conditions: 1.0 equiv. of Proline (1.1 M), 4.4 equiv. of decanol (4.8 M), 1.2 M of $SOCl_2$, 5.2 mM of DMAP and decanol as a solvent at 60 °C for 5 hrs. The metathesis reaction conditions: 1.0 equiv. of both the ester and SLS (4.8 μM) in 0.15 M aqueous HCl as a solvent at 60 °C for 5 h.

2.7 Physicochemical characterization of SAILs

Evaporative light scattering detection (ELSD) is a powerful tool for determining non-volatile compounds lacking a chromophore with no UV absorption such as in the case of the synthesized SAILs.[138] The structure of both carriers was confirmed by H^1 and C^{13} NMR and ELSD with high-resolution mass spectrometry (HR-MS).

Sufficient characterization of Nanoparticles (NPs) is of vital significance to create well-defined nanoformulations of certain therapeutic benefits.[139] This is important due to the safety issues that are usually promoted by some physical properties of NPs such as surface charge, particle size and shape in addition to other factors.[140] Among these factors, particle size and surface charge are regularly studied since they account for vital properties of NPs including their dissolution, cellular uptake and toxicity.[141] Dynamic light scattering (DLS) and zeta potential (ZP) studies have gained popularity due to their simplicity, reproducibility, affordability and non-invasive action to determine particle size and surface charge.

In DLS instruments, the dispersed NPs interact with light and scatter the incident light with an intensity proportional to the 6^{th} power of the particles' radii.[142] The light scattering from the continuously mobile Brownian-motion of the particles causes fluctuations of the photon count over time collected by the detector. Then the D_H (hydrodynamic radius) of the particles is calculated using complex mathematical functions.[140] The D_H (hydrodynamic radius) is a hypothetical measurement of the hard sphere radius that diffuses at equal velocity as the molecule being measured.[143] The ZP, is the shear plane potential for colloid particles traveling under the influence of an applied electric field. This is measured by complex mathematical calculations for the electrophoretic mobility of the colloidal particles that need to be reached in order to drive a unit positive charge from infinity to the colloidal surface.[144]

In aqueous solutions, SAILs spontaneously self-arrange and form micellar-like assemblies.[145] To gain an insight into the size, shape and morphology of MAIL and BAIL self-assembled micellar structures, DLS and cryogenic transition electron microscopy (Cryo-TEM) were performed. The MAIL formed vesicle-like micelles with a diameter below 200 nm with aggregated micellar structures displaying ribbon-like structures (**Figure 5a**).[146] As expected, the BAIL nanocarrier formed spherical micelles with a distinct bilayer with a diameter below 100 nm **(Figure 5b**). We accessed not only the shape and size of vesicle-like micelles formed by the SAILs, but also their quality, by measuring bilayer capacitance using electropermeability measurements.[147]

Additionally, differential scanning calorimetry (DSC) and Isothermal titration calorimetry (ITC) were performed to define the physical and chemical properties of the synthesized SAILs. Calorimetry is a primary technique to measure the thermodynamic properties of materials to establish an association between definite physical properties of the measured substance and temperature.[138, 148] Amongst different sorts of calorimeters, DSC is a common thermal analysis technique that measures the changes of sample physical

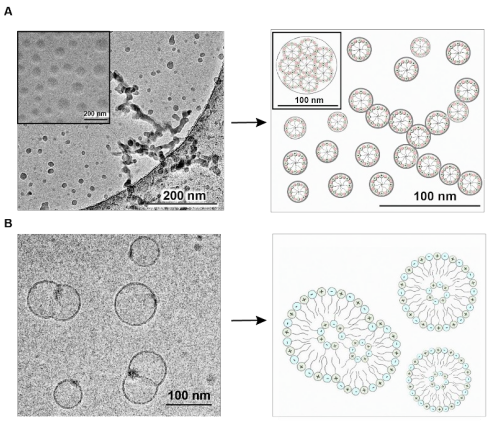


Figure 5. (**a**) Cryo-TEM image showing the structure of [ProC$_{10}$][Cl] vesicles in phosphate buffer solution with a graphical representation of the mono-amphiphilic carriers. (**b**) Cryo-TEM image of [ProC10][LS] aggregates in PBS confirming the aggregates to be bilayer vesicles with a graphical representation of the vesicular (single and fused) structures observed from Cryo-TEM imaging.

properties over time, relative to temperature. ITC is a general technique that is used in colloidal mixtures and can allow precise thermodynamic elucidation of association processes occurring in complex systems with high sensitivity.[149] The technique is one of the most used methods for studying surfactant aggregation in the solution phase.[150] The Titration procedure stands for varying the colloid composition by adding one or more components while monitoring heat exchange during the process. Moreover, these studies are usually executed under isothermal conditions, therefore it is referred to as Isothermal Titration Calorimetry.

2.8 Bioactivity testing

Since ErbB2 receptor protein is overexpressed in many cancer types,[83, 84] we investigated the selectivity and antiproliferative effect of the TM peptide variants and peptide-loaded nanocarriers *in vitro* on cancer cell lines with varying ErbB2 protein expression levels using the Cell Titre Glo (CTG) assay.[151] A panel of four cancer cell lines was used in this study, which included BT474 and AU565 human breast cancer cells, A549 human lung cancer cells, CFPAC-1 human pancreatic cancer cells. Considering the hydrophobic nature of the TM peptide variants (**V1-V3**), the ionic detergent OG was used to solubilize the peptides by rehydration of the peptide films before *in vitro* studies.[152] Indeed, no significant reduction in viability was observed in all cancer cells after incubation with the peptide variants for 24 h. However, a pronounced reduction in cell viability amongst all cell lines except A549 cells when the incubation period was extended to 72 h. The peptide

variants showed a dose-dependent decrease in cell viability at low micromolar concentrations (0.04 -1.25 μM). Among the variants, the **V3** peptide showed the most intense effect and the growth inhibition was the highest on the AU565 cell lines which have high RNA ErbB2 expression levels.

The micelles of different SAILs were previously reported to act as anticancer agents through the induction of cellular membrane rupture.[153] They were reported to induce rigidification/fluidization or disruption of the lipid membranes in a concentration-dependent manner.[116, 117] To the best of our knowledge, the only work reported in this direction reports azolium cation-based SAILs against C6 glioma tumor cells, but with poor selectivity.[153] Therefore, the cytotoxic action of BAIL and MAIL nanocarriers were evaluated against the selected cancer cell lines and showed good antiproliferative effect within 48 h incubation time when used at their critical aggregation concentration (cac).

The synergistic anticancer activity of TM peptides in combination with MAIL or BAIL nanocarrier was further investigated. In a comparative study in which the peptide incorporated SAILs prepared via reverse evaporation technique,[152] TM peptide-loaded nanocarrier treatment caused a substantial reduction in viablities in all cells with a significant decrease observed in BT474 cells (**Figure 6**). Interestingly, the combination of nanocarriers with TM-peptides displayed a synergistic cytotoxic activity in all cancer cell lines in a dose-dependent manner. The loaded **V3** peptide in the carrier led to complete cancer cell death in most of the cell lines especially when using a higher concentration of the carrier than its cac.

Both nanocarriers could have a positive impact in augmenting the activity of the TM peptide by changing the membrane lipid distribution as well as damaging the cell membrane,[154] allowing the TM peptide acting as a pseudo-dimerization partner to be inserted into the cell membrane, and thus block the dimerization of the ErbB2 receptor (**Figure 2**). Furthermore, by inhibiting ErbB2 dimerization, the **V3**-nanocarrier combination could prevent receptor autophosphorylation and downregulate downstream MAPK and PI3K/Akt signaling leading to cell death which was confirmed by later western blot analysis.

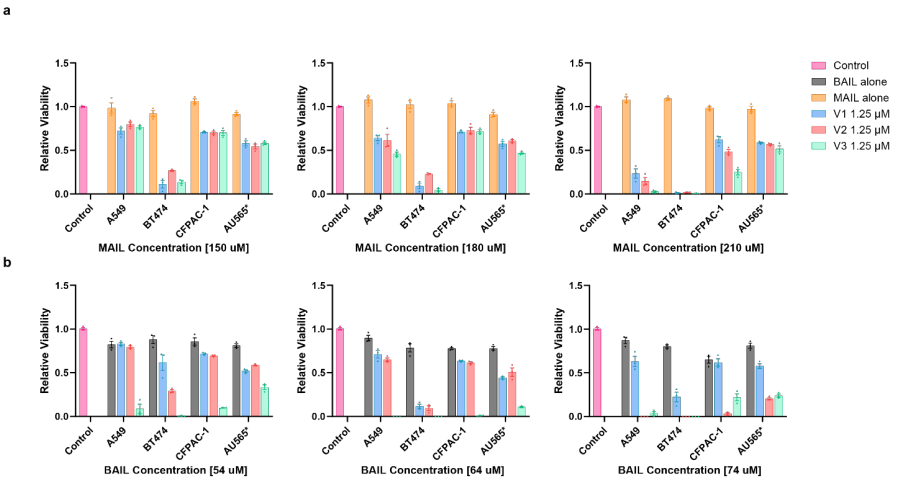


Figure 6. Cell viability analysis for the anticancer activity of the peptide-nanocarrier formulations. Cell viability of cancer cell lines following 24 h incubation with (**a**) Peptide-MAIL formulation and (**b**) Peptide-BAIL formulation except for AU565 cell lines which were incubated for 48 h and marked by an asterisk. The carrier was incorporated into the peptide at different concentrations as indicated by the x-axis.

2.9 Peptide localization studies

To better comprehend the mechanism of the TM peptide, confocal laser scanning microscopy (CLSM) was utilized to visualize the fluorescently labeled **V3** peptide in BT474 breast cancer cells (**Figure 7a**). CLSM uses a laser source to excite fluorescent molecules in which the laser is scanned in X,Y directions and the emitted light is collected by a PMT (photomultiplier) detector to generate an image.[155] The technology increases the resolution of the captured image by suppressing the out-of-focus lights to pass to the detector.[156] After staining the cell membrane of BT474 cell lines, the **V3** peptide variant (green) localized mainly within the cell membrane without any noticeable intracellular penetration following 24 h incubation period. This verifies our assumption that the TM peptide exerts its action as a pseudo-dimerization partner within the membrane part of the ErbB2 receptor. The **V3** combined SAILs imaging (**Figure 7b,c**) showed a deformed membrane structure with disrupted stain in relative to the untreated cells (**Figure 7d**) indicating the ability of the combination to induce membrane rupture and kill the cancer cells.

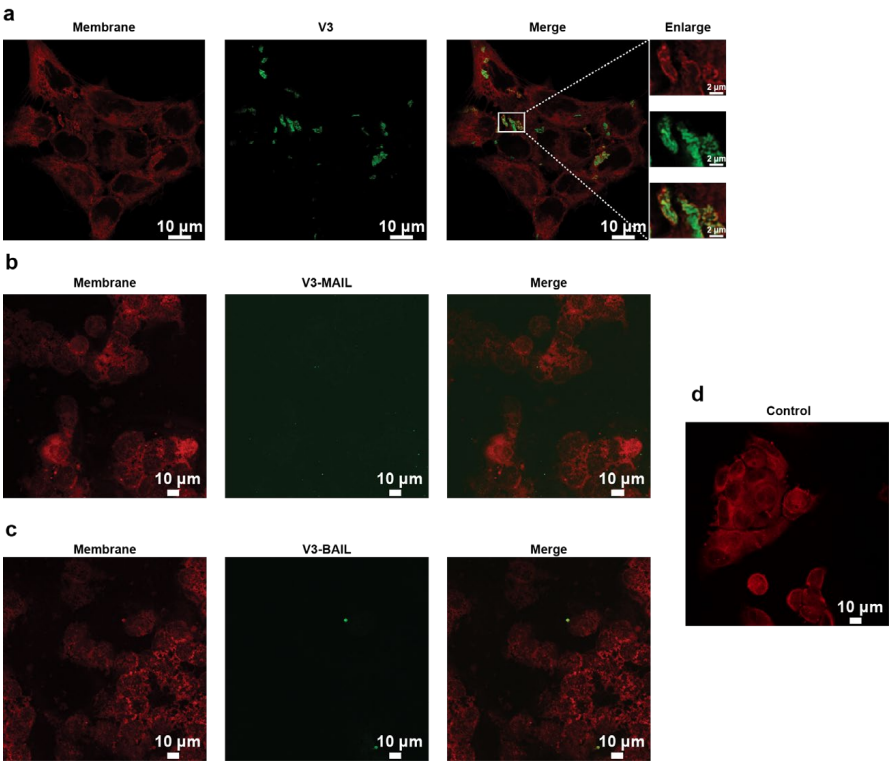


Figure 7. (**a**) CLSM images of the BT474 cell line following 24 h post-incubation with **V3** peptide (red: membrane dye Cell Mask, green: carboxyfluorescein peptide, merged and enlarged section showing localization of peptide in the membrane). The confocal imaging shows the localization of the TM peptide following 2 h incubation of the **V3** peptide-loaded with MAIL (**b**) or BAIL (**c**) nanocarriers. (**d**) Imaging of untreated BT474 cells cultured for 2 h on an RPMI growth media and stained with Deep-red Cell Mask membrane stain. Scale bar = 10 µM, magnification power = 63x in (**a**) and 20x in (**b**, **c** and **d**).

2.10 Summary of paper I

It is well known that preparing synthetic transmembrane peptides is still not simple and the need for a consistently reliable method for the characterization of small hydrophobic peptides has been challenging over the years. In summary, we reported a novel synthesis and characterization technique for three variants of ErbB2 TM-derived hydrophobic peptides. The peptides displayed α-helical structures similar to the TM segment of the receptor when tested in a membrane-mimicking environment. Modification of TM peptide by PEGylation and polylysine tagging improved peptide physiochemical properties which can be applied to other hydrophobic TM domains. Also, the developed method here was found to be reproducible, efficient, and simple in

the fact that it was performed via a general Fmoc-based solid-phase peptide synthesis protocol, solubilized and purified via a distinctive mobile phase system.

To transport TM peptides to the desired physiological target, two novel SAILs were designed, namely BAIL and MAIL which self-assembled into vesicle-like and micellar nanocarriers, respectively, and with hundreds of nanoscale hydrodynamic sizes. The TM peptide showed toxicity to ErbB2-positive cancer cells within the micromolar concentration range through inhibiting receptor dimerization. The SAILs nanocarriers displayed concentration-dependent toxicity against these cancer cells. Interestingly, the combination of TM peptides with IL carriers resulted in synergistic anticancer effects with remarkable cell toxicity, in which the IL nanocarriers enhanced the activity of TM-peptides by disrupting and damaging the cell membrane, allowing TM peptide to exert its action within a shorter period. Thus, the combination produced a higher level of efficacy against ErbB2-positive cancer cells and subsequently suppressed ErbB2 signaling, particularly the MAPK and PI3K-Akt pathways. The proposed mechanism of action of these combinations was supported by CLSM imaging by showing their fusion with the cell membrane compartments of the treated cells. This implies that this nano-formulation is a promising drug candidate for treating breast cancer, lung cancer and pancreatic cancer.

The molecular characterization of the TM segment within the phospholipid bilayer is vital for a better understanding of the biological mechanisms that control membrane protein functions. For their proper characterization, the synthesis of these TM parts should be applicable and efficient to generate good native mimics. In the next chapter, the synthesis of some holine TM peptides will be explained.

Chapter 3

3.0 Synthesis and characterization of TM holin peptide variants (Paper II)

3.1 The Holin peptides

Holins are a group of small hydrophobic proteins produced by bacteriophages and accumulate as homodimers within the bacterial cell's inner membrane.[34] Once reaching a critical concentration, they aggregate to form holes for the release of the endolysins hydrolytic enzymes to cleave the bacterial host cell wall by degrading the peptidoglycan layer.[157] Because of their ability to damage the bacterial cell membrane, there have been recent studies for developing new antibacterial agents.[158]

Holins consist of short polypeptides, with up to 130 aa length, and a positively charged hydrophilic C-terminal domain as well as possess one to three hydrophobic transmembrane domains (TMDs).[159] Based on the number of TMDs, holins are categorized into three topology classes, in which class I possesses three TMDs, class II with two TMDs and class III holins have only one TMD. Class II holins are usually shorter than other classes, possessing 65–95 aa length, retaining two TMDs and N-in C-in topology (both terminals are inside the cytoplasm). Pinholins belong to this class and work slightly differently from the other typical holins due to their size.[160, 161] These lytic proteins form pinholes with <2 nm in diameter in the cell inner membrane of the host bacteria, therefore, they are too small to transport endolysin but allow the passage of ions that leads to membrane depolarization.

Among the most studied pinholins, is the $S^{21}68$ protein encoded by the lambdoid bacteriophage *S21* gene with the size of 68 aa residues and two TMDs (**Figure 8**).[160] After exceeding a critical concentration, the TMDs aggregate to form oligomers which lead to the formation of pinholes and activate the membrane-anchored endolysin release.[162]

In the scope of this thesis, the chemical synthesis of the $S^{21}68$ pinholin peptide with other four variants was achieved by implying the SPPS strategy with the inclusion of pseudoprolines dipeptides (**Figure 8**). The synthesized holins were evaluated for their assembly and stability to form nanopores by our collaborators through functional nano-screen techniques.[82] In **Figure 8**, the four variants are indicated with the number of amino acids in each variant, so

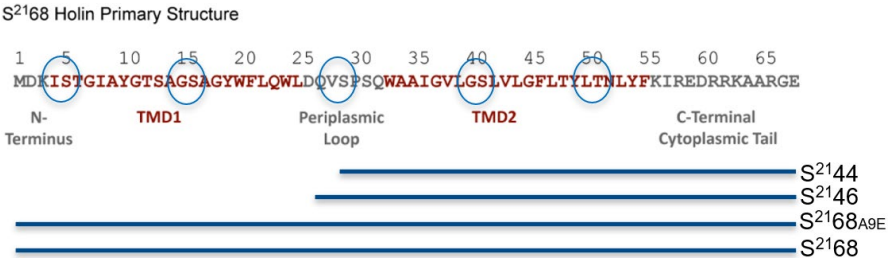


Figure 8. The primary structure of the holine peptide S^{21} with its 68 amino acid components. Below the structure, are the four chemically synthesized peptide variants with different amino acid lengths. The circles indicate the positions where the pseudoprolines were added to enhance the synthesis.

the $S^{21}44$ variant contains 44 aa starting from the C-terminal and so on. The variant $S^{21}68^{A9E}$ contains glutamate instead of alanine at position 9. For the $S^{21}68$ and $S^{21}68^{A9E}$ peptide variants, pseudoprolines were inserted into the five circled positions to allow the successful synthesis. The synthesis, purification, and characterization of the four holin peptides are explained in the next section.

3.2 Pseudoprolines assisted synthesis of holin peptides

With the substantial growth of peptide-based treatments, more effective protocols are needed to be developed for peptide synthesis. Since the advent of SPPS by Professor Merrifield in 1963,[11] several synthetic innovations have been created to improve functionalized solid supports,[163] activation methods,[45] specific protecting groups [164] and the automation of protocols. Yet still, the SPPS allows the preparation of different peptide lengths at various scales. Nonetheless, the synthesis of complex peptides is still hindered by intrinsic difficulties including inadequate solvation of the growing peptide chain throughout the SPPS which often leads to incomplete coupling cycles.[165] These unfavorable challenges result mainly due to the aggregation of the peptide chains that can form β-sheets secondary structures.[166]

In order to tackle these difficulties, many techniques were applied to suppress the degenerative effect of aggregation by involving elevated reaction temperature,[167] use solubilizing protecting groups [168], chaotropic salts or pseudoprolines dipeptides.[169] The pseudoproline derivative is a dipeptide that consists of oxazolidine/thiazolidine derivatives with proline-like ring structures that act as a temporary protection group for serine, threonine and cysteine side chains (**Figure 9**).[170] Pseudoprolines are a powerful tool to enhance the quality and yield of the chemically synthesized peptides enabling in many cases the assembly of difficult peptides that otherwise could not be made. [171] This is due to inducing a kink" conformation in the peptide backbone,

as a result of their preferences for the *cis*-amide conformation which disrupts the peptide self-aggregation and β-structure formation. [172] Mechanistically, the *cis* amide bond formation and the absence of hydrogen atom on the α-amino group interrupt the hydrogen bonding continuity within the backbone which is critical for peptide aggregation. Thus, improving coupling efficiency and solvation of the growing peptide chain considerably throughout the synthesis. Additionally, the kink that pseudoprolines creat proved to accelerate the cyclization reaction in cyclized peptides as well as increase their yield.

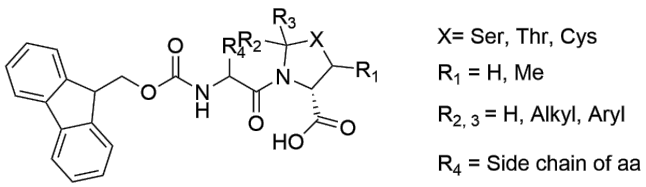


Figure 9. The general structure of pseudoprolines derivatives.

In recent years many dipeptide structures have been produced and incorporated as building blocks in the Fmoc-based SPPS of serine, threonine and cysteine-containing peptides. The dipeptide pseudoprolines are stable under the conditions used for Fmco removal and they undergo ring opening upon exposure to acidic conditions to generate the parent amino acid sequence (**Figure 10**).[169] Typically, the oxazolidine ring can be opened within several hours of treatment with TFA-based cleavage cocktails. On the other hand, thiazolidine derivatives have higher stability to TFA and need strong acids, such as neat Triflic acid (TfOH), to achieve quantitative deprotection in a reasonable time frame.[173, 174] Moreover, the substitution of the C2 position (R_2 and R_3 in **Figure 9**) results in different chemical stability for acidolysis. Dimethylated pseudoprolines can be deprotected with TFA in DCM or other aprotic solvents, However, they are stable to the mild acidic conditions used for cleavage of 2-chlorotrityl resin and Saiber amide resin. Unsubstituted oxazolidines have higher acidic stability and require exposure to TfOH for their ring opening. The acidic cleavage of the oxazolidine ring following exposure to an aqueous acid (like TFA) leads to C–O bond activation via coordination of the oxygen to the $(H_3O)^+$ Lewis acid (**Figure 10 i**). Followed by the cleavage of the intracyclic C–O bond (**ii**) forming an iminium intermediate (**iii**). Then the iminium ion undergoes 1,2-addition by water followed by proton transfer steps (**iv** and **v**). Then 1,2 elimination (**vi)** of the amine afforded the parent aa in the peptide sequence with the oxonium ion **(vii**) which is then deprotonated to give the neutral Aldehyde or ketone **(viii**) based on the substation of the C2 of oxazolidine.[175, 176] The regioselectivity of ring-

opening in the oxazolidine is controlled via the formation of stable acyclic iminium intermediate which results from the C–O bond cleavage. The parent peptide could be Cys in the case of using thiazolidine derivatives or Ser and Thr when using an oxazolidine ring (in which the substitution of R_1 is H in the case of Ser or Me in the case of Thr).

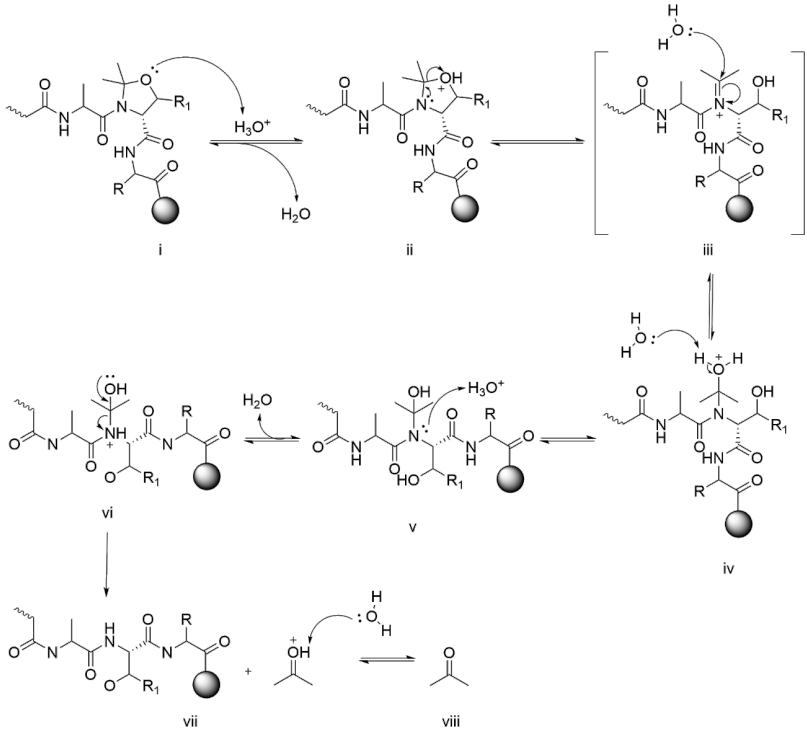


Figure 10. The general mechanism of acid-catalyzed ring opening of pseudoprolines derivatives.

The S^{21} holin variants peptides were synthesized with a standard Fmoc-SPPS protocol and characterized with HPLC (**Figure 11**). For the $S^{21}68$ and $S^{21}68^{A9E}$ peptides, pseudoprolines were inserted and coupled to assist the synthesis. Within the sequence the following pseudoprolines were inserted: Ile-Ser(Psi(Me,Me)pro)-OH at position 4-5, Gly-Ser(Psi(Me,Me)pro)-OH at positions 15-16 and 40-41, Val-Ser(Psi(Me,Me)pro)-OH at position 28-29, and Leu-Thr(Psi(Me,Me)pro)-OH at position 50-51 (**Figure 8**).

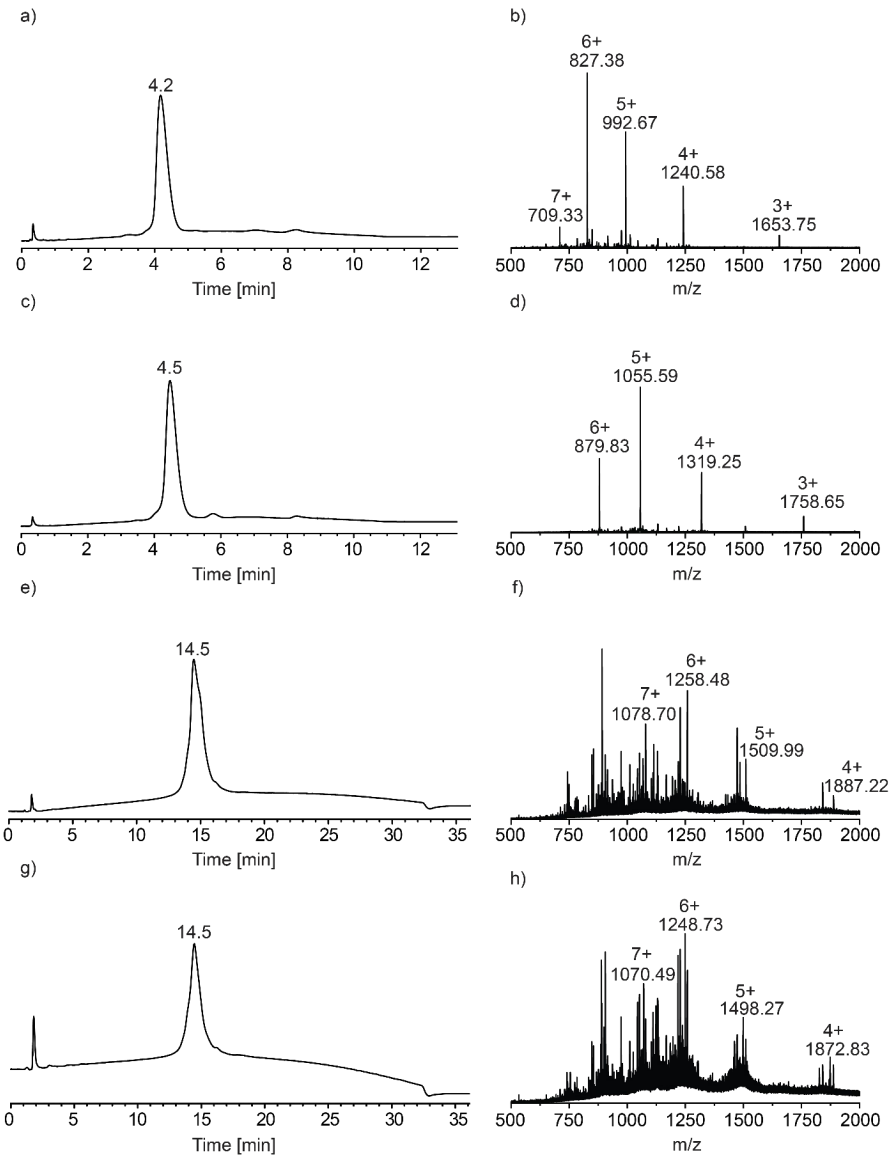


Figure 11. RP-HPLC and ESI-MS characterization of purified synthetic peptides (**a-b**) $S^{21}44$, (**c-d**) $S^{21}46$, (**e-f**) $S^{21}68^{A9E}$ and (**g-h**) $S^{21}68$. Mobile phase A was 0.1%TFA in H_2O, Mobile phase B was 0.1%TFA in ACN. The gradient method for (**a** and **c**) was 50–90% eluent B in 10 min, the detection was at 214 nm at a flow rate of 2 mL/min, C18 column; for (**e** and **g**) was 40−90% eluent B over 30 min detection was at 214 nm at a flow rate of 1 mL/min, C4 column. ESI-MS spectra of (**b**) $S^{21}44$ with sum formula of $C_{226}N_{63}O_{62}H_{369}$ and calculated mass peak for $[M+6H]^{6+}$ 827.30; (**d**) $S^{21}46$ with sum formula of $C_{242}N_{67}O_{65}H_{388}$ and calculated mass peak for $[M+5H]^{5+}$ 1055.58; (**f**) $S^{21}68^{A9E}$ with sum formula of $C_{346}H_{529}N_{90}O_{98}S$ and calculated mass peak for $[M+6H]^{6+}$ 1258.48; (**h**) $S^{21}68$ with sum formula of $C_{344}H_{526}N_{90}O_{96}S$ and calculated mass peak for $[M+6H]^{6+}$ 1248.65.

3.3 Summary of paper II

As a summary, four holin peptides were synthesized and characterized to be further used in functional studies of their formed nanopores. It is important to mention that the synthesis of $S^{21}68$ and $S^{21}68^{A9E}$ holins was not successful when performed without the incorporation of pseudoprolines derivatives, due to their ability to reduce peptide aggregation and improve their solubility during the SPPS.[172] Solubility is an important factor that causes a range of chemical and biological phenomena in such aggregation of proteins and peptides due to the collapse of the polypeptide chain.[177] In this context, ILs have been reported to be excellent solvents for wide ranges of peptides and proteins.[178] The next chapter demonstrates an ionic liquid-mediated conjugation strategy that was developed within the thesis work to conjugate peptides and functionalize them.

Chapter 4

4.0 Ionic Liquid-Mediated Approach for the Synthesis of Site-Specific Thioether Conjugates (Paper III)

4.1 Conjugation of thiol-containing biomolecules

In the last few years, there has been an increased interest in the discovery of innovative drug conjugation techniques.[87] Conjugation was originally devoted to cancer therapy to enhance the chemotherapeutic index of these drugs, However, currently, it has been used within various therapeutic areas to deliver potent molecules.[179] The creation of physically defined connecting points between the molecules to be conjugated is a vital step during the development of efficient and safe conjugates. Generally, these connection sites rely on some reactive functional groups present in amino acids, for instance, lysine or cysteine. Recently, there have been many reports of enzyme-mediated site-specific conjugation of thiol-containing biomolecules, that showed significant therapeutic benefits through the inclusion of antibody-small molecule drug conjugates (ADC).[180, 181]

Remarkably, thiol-maleimide ADCs represent the majority among the FDA-approved medications.[90] Thioether bonds in proteins are naturally formed by treatment with a base which leads to the elimination of one of the sulfur atoms from a disulfide bridge, creating a carbon-sulfur-carbon bond.[182] Nevertheless, several natural proteins possess disulfide bonds as in lactalbumin, animal feathers, wool and human hair.[183, 184] Moving further from the traditional ADC, conjugates with other biomolecules like nucleotides and peptides are becoming more common.[3, 88, 185]

Peptides have shown potential bioactivity in many therapeutic fields, rendering them good candidates for bioconjugation with other modalities. Additionally, the conjugation of peptides with certain moieties can enhance their physiochemical and bioactivity properties. Among the most commonly used reagents to conjugate peptides are iodoacetic acid N-hydroxysuccinimide ester (IAAOSu) (**Figure 12 i**)[186], m-maleimidobenzoyl-N-hydroxysuccinimidyl ester (MBS) (**Figure 12 ii**)[187] and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP)[188] (**Figure 12 iii**). These reagents need a Cys

fragment to be present within the peptide structure, otherwise, conjugation might occur at the N-terminal during SPPS. The conjugation to peptides proceeds in two steps, in which in the first step, the molecule of interest to be conjugated to the peptide (which include protein, small organic molecule or etc,) attack the carbonyl carbon and expell N-Hydroxysuccinimide (NHS) as a good leaving group (**Figure 12 iv**). In the next step, the added cysteine peptide

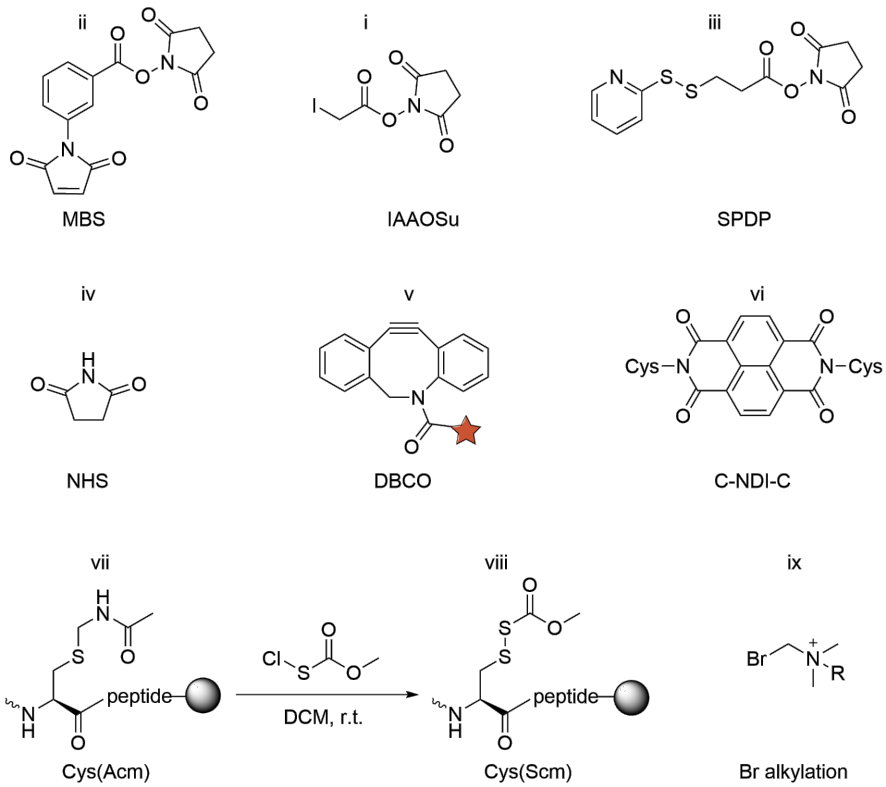


Figure 12. Common reagents used for the formation of peptide conjugates.

reacts with either the alkyliodide of IAAOSu, m-maleimidobenzoyl group of the MBS, or the 2-pyridyldisulfide group of SPDP forming a covalent bond between the conjugate and peptide. Houen *et al* demonstrated the conjugation of numerous peptides to ovalbumin protein by employing IAAOSu as a coupling intermediate.[189] Additionally, Carlsson and coworkers were the first to describe the preparation of disulfide-linked protein-protein conjugates based on the SPDP reagent in a reversible approach.[188] The maleimide involvement in the conjugation of peptides has been studied extensively in different applications, in one interesting article, it was reported to produce peptide-conjugate vaccines against group A streptococcus bacteria.[190] Additionally, the

covalent attachment of peptides to porphyrins and related macrocycles was investigated using thiol/maleimide or thiol/haloacetamide ligation in order to enhance the selectivity and efficacy of photodynamic cancer treatment.[191]

Recently, there have been different approaches for the site-specific conjugation of thiol-containing peptides so we can mention a few of them briefly.[192] In one recent study, a site-selective conjugation for the cysteine residue was developed in a seven-residue peptide by reacting with aza-dibenzocyclooctyne moiety (DBCO), in which the DBCO-tag found to have increased reaction rate to form thiol-yne conjugate, enabling selective conjugation of DBCO-linked fluorescent probes or drug molecules to the peptide (**Figure 12 v**).[193] Additionally, Thiol-bearing N-desymmetrized naphthalenediimides (C-NDI-C) have been linked to Cystien-containing peptides through dynamic disulfide bonds to enhance their proteolytic stability (**Figure 12 vi**).[194]

By taking advantage of different orthogonally protected Cystein groups, Gunno *et al*, developed a strategy to conjugate proteins bearing exposed Cys residue to peptides containing Cys with a methoxycarbonylsulfenyl (Scm) group.[195] Three different cysteine-containing proteins were conjugated with excess equivalents of the targeted peptides to form disulfide conjugates in a site-selective way. The model peptides used in the study were prepared on resin by standard SPPS procedures using the commercially available Fmoc-Cys with acetamidomethyl (Acm) group which was converted on resin into Cys(Scm) prior to conjugation (**Figure 12 vii**, **viii**). The cleaved Scm-containing peptides from the resin were functionalized with different fluorophores, non-peptidic entities and cell-penetrating motifs.

In a recent study, a conjugation of bromide alkylation reagents containing trimethyllysine analogs was performed selectively to the Cys of the histone H3 protein (**Figure 12 ix**).[92] Among various antimicrobial agents; such as lantibiotics (peptide antibiotics);[196] the thioether bond is common.[197] Prenylated protein structures encompass thioether bonds connecting Cys residues with long hydrocarbon entities which aid in protein localization in the plasma membrane.[197] Due to the vast importance of conjugation and its applications, the involvement of novel and milder methods was considered as in the case of ionic liquids.

4.2 Ionic liquid and their applications

Ionic Liquids are purely ionic salts consisting of a non-coordinated organic cation and either organic or inorganic anion that are liquid below 100°C and most commonly have melting points below room temperature.[198] This unique behavior of IL that makes it different than ordinary liquids and solid salts is

due to the lower symmetry of ion distribution in the ILs.[199] Additionally, charges of both anions and cations are resonating over a larger volume of the molecule. Strong Coulomb (ionic) interactions within ILs lead to many advantageous physicochemical characteristics including high thermal stability, electrical conductivity, inflammability, and negligible vapor pressure.[200]

The selection of the ionic components of the IL has a strong influence on their physicochemical properties and there have been almost 1,000 ionic liquids described in the literature within the previous years.[178, 201, 202] Standard structures consist of organic-based cations with inorganic or organic anions and can include but are not limited to the structures summarized in **Figure 13**. In general, ILs have very appealing solvent properties in a wide range of biomolecules with solubility that are difficult to achieve in ordinary molecular solvents.[203] IL possesses a dual character of both an anion and a cation, in comparison to conventional solvents, making them more a suitable medium for biomolecules. Some ILs have been reported to be effective platforms for protein stability and folding, especially for some cysteine-rich peptides, when used in combination with chaotropic additives due to their ability to disrupt aggregation during protein refolding.[203, 204] The nucleophilic nature of the IL increases their potential to break both the intramolecular hydrogen bonds network within the protein as well as the intermolecular bonding, hence increasing their solvation effect and reducing protein aggregation and misfolding.[178] The solvent properties of ILs can be modified by choosing an appropriate selection of component ions and optimizing the IL concentration. Moreover, the vesicle-forming ability of biamphiphilic IL upon reaching a critical aggregation concentration through crosslinking of their chains via electrostatic interactions and hydrophobic interactions has been illustrated in **Chapter 2**.

ILs have gained more popularity in different fields of chemistry in view of environmentally friendly alternative solvents for biomolecules, yet not adequate studies on their potential toxicity. [205] The type of anion/cation in ILs, their interactions, the length of alkyl chains and their biodegradability have a significant role in their toxicity.[206] Due to the impressive characteristics that ILs possess, they have so many applications within the field of peptide chemistry synthesis,[207] characterization[208] as well as modifications.[209] With this regard, Imidazolium ionic liquids have been extensively used for enzymatic/chemical peptide synthesis, radiolabeling of peptides, native chemical ligation and oxidative folding reactions, mobile phase additives in RP-HPLC and matrices additive for Matrix-assisted laser desorption/ionization (MALDI).[178]

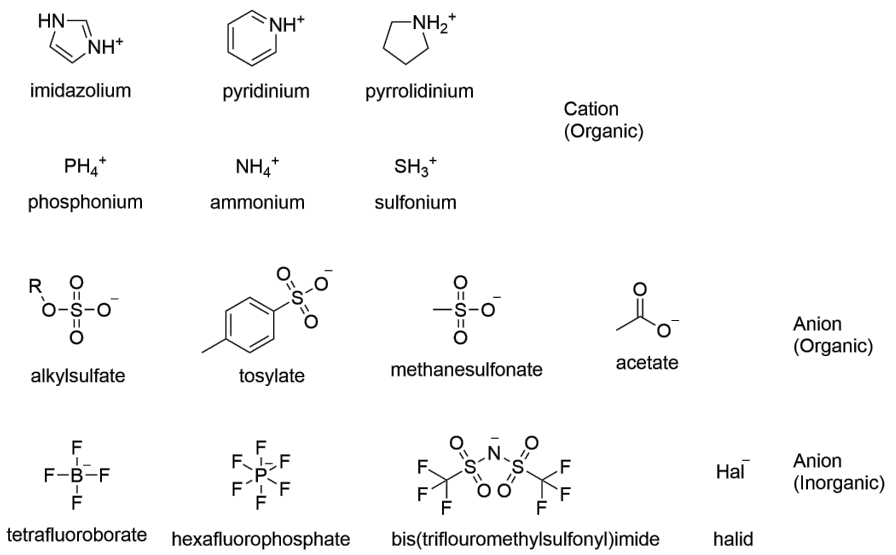


Figure 13.Chemical structure of commonly reported IL components in the literature. Notice that these structures are general and some ionic components were made with alkyl modifications.

Their good solvation properties and as precursors of N-heterocyclic carbenes (NHC) make them very attractive to be studied in different chemical applications.[210] The 1-Ethyl-3-methylimidazolium acetate [C_2mim][OAc] is among the most studied ILs in the literature that possess NHC. The imidazolium ring of the IL can undergo deprotonation on the C2 position and generates an imidazol-2-ylidene which forms singlet carbene, with nucleophilic/basic properties (**Figure 14a**). This process is usually done by strong bases,[211] However, the incorporation of a weak base as a counter-ion into the imidazolium complex such as an acetate ion, was found to induce the formation of carbene (**Figure 14b**).[199] Nevertheless, the actual existence of NHC in imidazolium acetate ILs is still controversial.[210] On one hand, theoretical studies appear to rule out the potential of NHC occurrence in imidazolium acetate because the deprotonation process is unfavored within the polar IL environment. Since the polar environment can make charge stabilization and the proton transfer proccess changes the charged species into neutral molecules. On the other hand, experimental findings support the possibility of NHC existence in imidazolium acetates, by its ability to produce compounds that involve the presence of carbene.[199, 212-214] Nonetheless, it is well known that imidazolium acetate ILs are reactive species and have been used in many fields of chemistry.

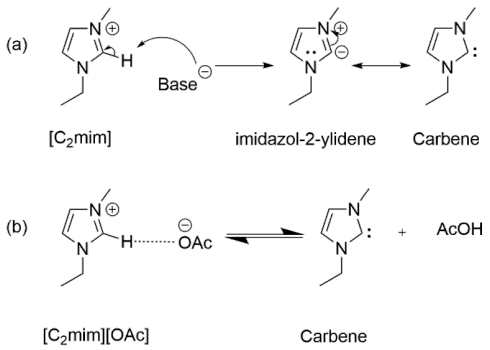


Figure 14. Representation of carbene formation when using a strong base (**a**) and the possible structure of carbene present in imidazolium acetate ionic liquid (**b**).

In previous research work in our lab, it was reported that [C_2mim][OAc] namely (**IL1**), could generate carbene and react with biomolecules containing cysteines selectively.[79] In neat **IL1**, the protonated and deprotonated forms are in equilibrium as depicted in **Figure 14**. As a result, the NHC could be employed in organic syntheses, such as Diels-Alder reactions, Stetter reaction, benzoin condensation and other reactions.[215] Interestingly, the addition of water can quench the NHC in IL rendering them as a good solvent media for oxidative folding or native chemical ligation.[215-218]

In the scope of this thesis, I will demonstrate the development of an **IL1**-mediated conjugation approach for cysteine-containing compounds. To my knowledge, no other findings have been reported for the application of **IL1** in bioconjugation.

4.3 Ionic liquid-mediated thioether conjugates

The formation of thiol-conjugates was the method that caught our attention and has been used for the synthesis of thioether building blocks within antibacterial agents, isotopic labeling, post-translational modifications of proteins and antioxidants in polymers.[88, 195, 219-221] In this work, we showed that the Ionic-liquid, namely **IL1**, can be employed for thiol-conjugation in which a toolbox of active IL salts possessing diverse functional groups was built to functionalize thiol-containing peptides under mild reaction conditions through thioether bond formation (**Figure 15**). Noteworthy, this methodology can be expanded to conjugate other thiol-containing large complexes such as proteins and antibodies. Additionally, this approach could be applicable to various post-synthetic modifications.

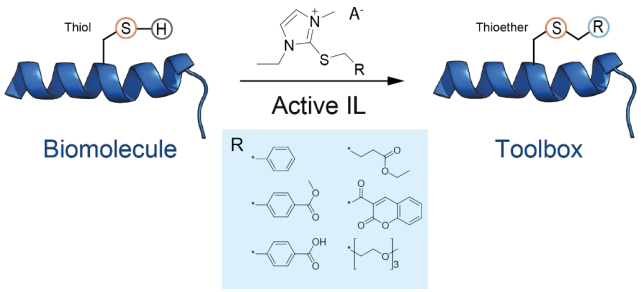


Figure 15. Conjugation of thiol-containing biomolecules using ionic liquid system. The process relies on the creation of a toolbox of "active ILs" with diverse functionalities (R) in which they form thioethers upon reacting with a biomolecule containing thiol moiety. Basically, the thiol-containing biomolecule represented as a blue helix might be a protein, an antibody, a peptide, or even an oligonucleotide. The letter A refers to the anion counterpart of the active IL, which can be either bromide or iodide.

4.4 The use of IL1 to generate active ILs

The conjugation method starts with the creation of called thereafter "active IL", and their synthesis scheme is illustrated in **Figure 16**. Based on some preliminary analysis performed by former lab members, the **IL1** can react with dibenzyl disulfide and afford benzyl-imidazolium structure.[79] Nevertheless, when we executed this reaction under aerobic conditions, 62% of thione **1** was afforded and 9% of benzyl-imidazolium in addition to 29% dibenzyl thioether (**Figure 16 a**). On the other hand, when the reaction was performed under an inert nitrogen atmosphere and upon heating to 50 ºC, thione **1** was afforded with higher conversion yield. Synthetic and structural studies on thione regioselective reactions with alkyl halides previously reported by Lamp *et al*. inspired us to use our successfully synthesized thione **1** as a basic intermediate to synthesize the active ILs toolbox (**2-7**) (**Figure 16 b**).[222] Substituents bearing different groups with various physicochemical properties were included in the generated active ILs.

4.5 Small molecule-based conjugation

In the next step, active ILs were conjugated to a thiol-containing small organic compound. The L-cysteine 2 -naphthylamide (**8a**) and its acetylated version N-acetyl-L-cysteine 2-naphthylamide (**8b**) were used as model compounds to optimize the reaction conditions (**Figure 17**). Considering the fact that NHC presence in IL may affect the reaction outcomes,[210] as well as the NHC quenching by some water addition render them unreactive solvent media, the effect of water addition to **IL1** was tested on the reaction outcomes as a part of conditions optimization.[215-218] The conjugation reaction was found to proceed

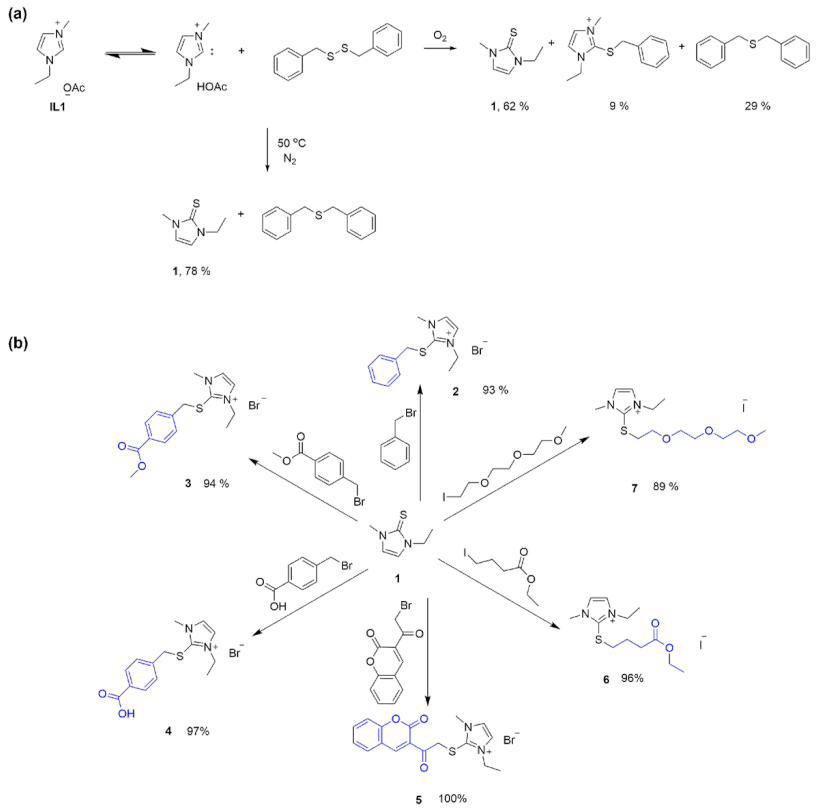
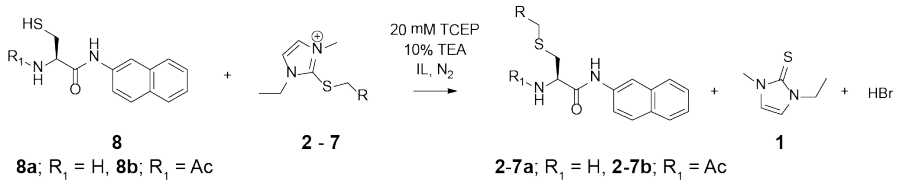


Figure 16. Active ILs toolbox formation following two-step reaction. (**a**) Generation of thione (**1**) under either aerobic or inert nitrogen atmosphere by using [C_2mim][OAc] (**IL1**) as a starting material. (**b**) Thione (**1**) reaction with different alkyl halides to afford the active ILs (**2-7**). The complete reaction conditions are explained in the attached paper **III**.

well using neat **IL1** as a solvent due to its reported superior solubilizing and stabilizing properties.[210] It is important to highlight the fact that Cysteine thiol can exist in distinct oxidation states of sulfenic, sulfinic, sulfonic and disulfide groups that reduce the cysteine conjugation reactivity.[223] Additionally, the conjugation rate can be tuned by changing the pH and the reaction temperature in which can alter the rate of nucleophilic attack made by the cysteine sulfhydryl group of the molecule to be conjugated.[224] Based on the previous information, the reactivity of model substrates was tested with active IL **2** at different conditions for optimization. Both the amount of triethylamine (TEA) as a base and the tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent were adjusted as part of the optimization proccess.



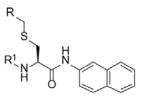
8 **2 - 7** **1**

8a; R_1 = H, **8b**; R_1 = Ac **2-7a**; R_1 = H, **2-7b**; R_1 = Ac

Figure 17. A model reaction of cysteine analogue (**8**) functionalization with active ILs (**2-7**) at optimized reaction conditions. The conditions are further explained in the attached paper **III**.

After optimizing the conjugation parameters, the scope of the reaction was tested on the previously prepared active ILs toolbox (**Table 3**).

Table 3. Conjugation yields of the L-cysteine 2-naphthylamide analogues **8** with active ILs **2-7** at optimized reaction conditions.



R	R^1	Entry	Yield (%)[a]	R	R^1	Entry	Yield (%)[a]
	H	2a	70		H	5a	0
	Ac	2b	79		Ac	5b	n.d.[b]
	H	3a	37		H	6a	0
	Ac	3b	53		Ac	6b	71
	H	4a	24		H	7a	0
	Ac	4b	30		Ac	7b	32

[a] All yields are of isolated products
[b] n.d. = not determined

4.6 Conjugation of peptides

After evaluating the conjugation with a cysteine analogue of a small molecule, the scope was expanded to include the conjugation of cysteine-containing peptides. For this purpose, small heptapeptides with the sequence of H_2N-XCGFPYF-$CONH_2$, where X = K, R , L, W, G, N, E, T (**10-29**) were designed to check the effect of the unprotected chemical entities of different side-chains on the conjugation outcomes when benzylated with the active IL **2** (**Table 4**). The heptapeptides core sequence is derived from the analogue of the longicalycinin A , which is a cytotoxic peptide with anticancer activity against some liver and colorectal adenocarcinoma cancer cell lines.[225] Conjugation of the longicalycinin A derived peptides (**10-29**) was done with the addition of TCEP buffer to inhibit oxidative side products and the reaction was monitored by Ultra-performance liquid chromatography mass spectrometry (UPLC-MS).

Table 4. Benzylation yields of the model peptides conjugation with active IL **2** at optimized reaction conditions explained in the attached paper **III**.

Peptide code	Sequence	Benzylation isolated yield (%)[a]	Peptide code	Sequence	Benzylation isolated yield (%)[a]
10	KCGFPYF	27	**22**	NCGFPYF	13
11	Ac-KCGFPYF	9	**23**	Ac-NCGFPYF	14
12	RCGFPYF	24	**24**	TCGFPYF	30
13	Ac-RCGFPYF	18	**25**	Ac-TCGFPYF	22
14	LCGFPYF	4	**26**	ECGFPYF	20
15	Ac-LCGFPYF	18	**27**	Ac-ECGFPYF	19
16	GCGFPYF	16	**28**	CGFPYF	11
17	Ac-GCGFPYF	30	**29**	Ac-CGFPYF	23
18	WCGFPYF	4	**30**	GPenFPYF	8
19	Ac-WCGFPYF	21	**31**	GGFPYF	-[b]
20	WC*GFPYF	27	**32**	GMFPYF	-
21	Ac-WC*GFPYF	4	**33**	GSFPYF	-

[a] All yields are of isolated products.
[b] - = no product

4.7 Mechanistic Investigations on the conjugation method

In order to confirm that the conjugation process is site-specific toward cysteine, the conjugated peptide **10**, which bears a nucleophilic Lys, was ascertained by Nuclear Magnetic Resonance (NMR) spectroscopic analysis. The thiol moiety seems to be the benzylated group in the peptide since the beta carbon of cysteine displayed a chemical shift at 36 ppm following a quick assessment of the 2D spectra. Additional control studies were performed by subjecting peptides free Cys (**31, 32** and **33** in **Table 4**) to the conjugation conditions in order to check whether the peptide would undergo non-Cys conjugation or whether the amino terminal group would function as a secondary reaction site. As expected, no benzylation occurred even in peptides containing Met (**32**) or nucleophilic Ser (**33**) and the conjugation was specific towards the Cys fragment. The steric hindrance effect on the reactivity of the Cys moiety was checked by benzylating peptide **30**, in which Cys was replaced with penicillamine. It was found that the conjugation of the penicillamine still could take place, however, with a low isolation yield of 8%. It is important to note that the conjugation protocol preserves the stereochemistry of the cysteine stereocenter, additional analyses were performed by RP-HPLC and confirmed that the benzylation reaction proceeded with no racemization of the cysteine stereocenter.

As mentioned in the introduction to this chapter, ILs have appealing solvent properties for chemical reactions relevant to ordinary molecular solvents.[203] Performing the conjugation in an ionic liquid solvent, namely **IL1**, exhibited favorable solubilizing properties for all the reaction components. Notably, all

the tested peptides, active IL **2** benzylating agent, as well as the reducing buffer showed complete solubility within the media. On the other hand, the model peptides were insoluble in other tested solvents such as acetone or acetonitrile. Moreover, the immiscibility of the TCEP buffer in DCM hindered its implementation as a solvent for the conjugation protocol. In one exception, the DMF was found to dissolve all the reaction components efficiently, however, conjugation of peptide **29** in DMF resulted in a lower yield of 12% compared to 23% when using **IL1** as the solvent. Herein, this finding supports the fact that the dual anionic and cationic character of IL could stabilize the reaction transition states more than conventional organic solvents. [174, 175]

In recent conjugation studies, organic halides have been used to form thioether conjugates in biomolecules,[211, 217] therefore, a study was designed to confirm the advantage of using active IL **2** as a benzylating agent over the commonly used benzyl bromide. Peptide **28** was used to check and was treated with both benzylating agents in the same previously optimized reaction conditions. The conjugation using benzyl bromide displayed two benzylated products in UPLC-MS analysis with a lower yield of isolation than the active IL **2** monobenzylation yield.

4.8 Proposed mechanism of conjugation

The previous findings led to the proposed reaction mechanism for conjugation as represented in **Figure 18**. The process could proceed with an SN_2 type reaction in which the nucleophilic thiol of the peptide attacks the active IL **2** coupling agent and expels thione **1** as a leaving group. The resulting thione (**1**) can be recovered from the product mixture by purification and employed again to prepare the active ILs. In general, the reaction isolated yields were decent but still not high enough and indicate the incomplete conversion of the starting material which drove us to perform an in-depth characterization with LCMS analysis of the reaction progress over time. An example of LCMS monitoring for the crude reaction mixture of peptide **3** conjugation is illustrated in **Figure 19** showing the UV traces chromatograms. The chromatogram shows peak

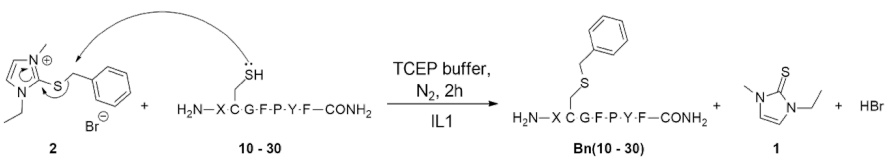


Figure 18. The potential reaction mechanism of model peptides benzylation.

retention times as well as peak area calculated via peak integration. Mass analysis of the found charged fragments in MS was performed for each peak,

and some possible compounds were elucidated in which their theoretical masses match the experimental mass data. Based on the analyzed mass, some of the generated side products could include the peptide with an extra 107.9 D

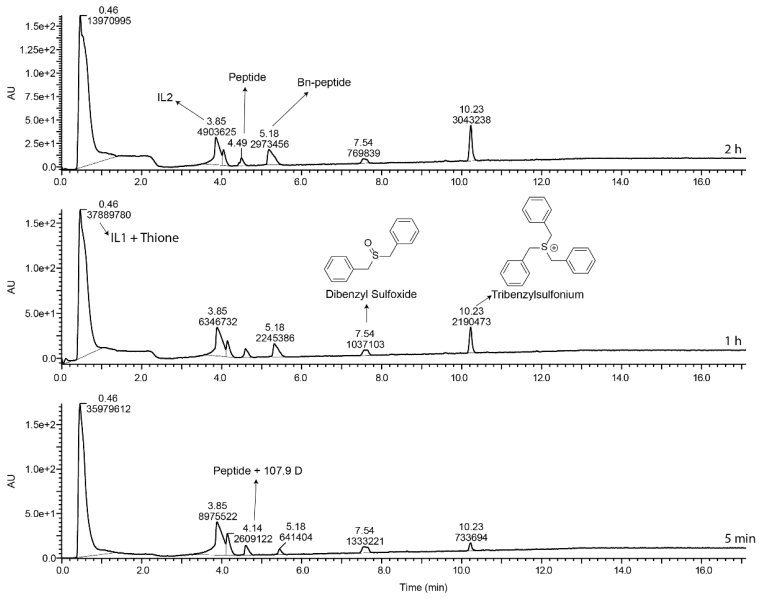


Figure 19. LCMS chromatograms for the analysis of the crude conjugation mixture of peptide **3** over time. Each chromatogram indicates the time point of analysis and they are ordered as follows: The bottom chromatogram stands for the analysis at 5 min, the middle one stands for 1 h and the top one stands for 2 h time point. The chromatograms show peak retention times and area. The LC method used for the analysis is explained in the attached paper **III**.

which might be an imidazolium adduct attached. One possible explanation is the TCEP-mediated desulfurization of Cysteine thiol to generate dehydroalanine which attacks the electrophilic NHC in the bulk solvent of **IL1**.[226] The generated thiol in the media can then attack the active IL **2** to form dibenzyl sulfoxide (7.54 min) and Tribenzylsulfonium (10.23 min) which were present in all conjugation chromatograms of the crude peptides. It is noticed that the peak area of the side product Tribenzylsulfonium increases with time which could account for the lower conjugation yield. Thione was produced and detected at an earlier retention time due to its high polarity and supported the possible SN_2 conjugation reaction mechanism (**Figure 18**). Unfortunately, due to the smearing of the **IL1** solvent peak in the C18 functionalized column, the integration of the thione peak area was not possible. The % of decrease of peak areas for peptide **28** and active IL **2** peaks over time relative to 5 min starting point were estimated by brief statistical analysis. The calculations showed that

31% of the peptide starting material and 45% of the alkylating agent were consumed over time, indicating that the reactants were not fully consumed and this could account for the lower conjugation yield. Nevertheless, the conjugation seems to occur rapidly and the benzylated product peak area increased by 78% over time. It is important to mention that running the reaction beyond 2 h makes the chromatogram less clean with more side products and no changes in the product peak area. The final benzylated product **Bn28** was obtained with a 24% isolated yield with > 95% purity based on the integration of the HPLC chromatogram peaks of the pure benzylated peptide.

Many factors could hamper the full conversion to the conjugate that can arise from some loss during work-up, and purification, as well as the quality of the used solvent and the starting materials. Indeed, the novel conjugation strategy seems to work selectively on Cys-containing peptides, in which it was extended to cover more applications as we will see in the next section.

4.9 Applications of the developed strategy

To demonstrate the usefulness of the conjugation, the functionalization was applied to some cyclic as well as linear bioactive peptides and afforded a decent yield (10-25%). Peptide-free cysteine was also conjugated with a thiol-containing molecule by utilizing the active IL **4** as a bifunctional linker. Moreover, the bifunctional linker was used for biotinylation of peptide **24** by a simple two-step reaction.

4.10 Summary of paper III

In summary, a novel approach was developed for the conjugation of Cys-containing compounds by the use of [C_2mim][OAc] (**IL1**). The **IL1** was used both as a solvent and a reactive precursor to produce activated IL fragments. The ability of **IL1** to act as a reactive precursor is driven by its endogenously formed NHC that can interact with dibenzyl disulfide to produce the thione **1**. The thione was a key component in performing nucleophilic substitution of selected alkyl halides to afford the active ILs toolbox. These active ILs possess a variety of functional groups such as; benzyl, aliphatic or aromatic esters, coumarin, carboxylic acid and PEG. The conditions of the conjugation protocol were optimized with a cysteine analogue of a small molecule. Revealed that 10% of TEA as a base, 20 mM (0.34 equivalents) of TCEP, neat **IL1** solvent and an inert nitrogen atmosphere are the optimum conditions to functionalize the tested thiol-containing small molecules. Interestingly, the acetylation of the naphthylamide cysteine model affected the outcome of conjugation, which led to a successful conjugation with both benzylic and aliphatic substituted active ILs. On the other hand, the non-acetylated

naphthylamide model was less reactive and conjugated only with benzylic active ILs. Unfortunately, the active IL **5** did not proceed for conjugation with any of the naphthylamide cysteine models due to its bulkiness and steric hindrance.

The scope of the reaction was extended to peptide complexes with a slightly tailored reaction conditions. The benzylation of the tested model peptides was found to be successful when TCEP was prepared as a buffer which suppressed oxidative side products as well as the TCEP-peptide adduct. The conjugation conditions were compatible with the relevant side chain groups in unprotected peptides and did not react with any within the set of the tested peptides. Additionally, the conjugation reaction was applied successfully to some cyclic and linear bioactive peptides. Finally, an active IL-based bifunctional linker was employed to conjugate Cys-free peptides.

Antibacterial resistance is a global challenge that necessitates the development of novel antibacterial agents to combat this issue. The next chapter covers the synthesis and bioactivity of naturally derived antibacterial peptides with antivirulence activity.

Chapter 5

5.0 Substrate-derived Sortase A inhibitors: targeting an essential virulence factor of Gram-positive pathogenic bacteria (Paper IV)

5.1 The global problem of bacterial resistance

The occurrence of bacterial resistance to traditional antibiotics has gained paramount attention in the medical world due to its negative influence on the therapeutic efficacy of current medications.[7] Infections are becoming harder to treat due to the increased resistance observed in many pathological bacteria, accelerated by the global misuse, and overprescription of antibiotics in clinics, private use, or the farming industry. Additionally, antibiotic resistance can emerge through mutations in genes or exchange of resistance genes among different bacterial strains, being therefore a naturally developing process.[227]

Most of the conventional antibiotics exert bacteriostatic action by suppressing bacterial growth through inhibition of vital processes in bacteria such as nucleic acid and protein synthesis, or they can act as bactericidal and induce bacterial cell death by targeting the integrity of the bacterial cell wall.[228] Indeed, these approaches were found to exert high selective pressure and significant stress on the bacteria, by threatening its viability, leading to the development of resistance against these therapeutics and rendering antibiotics ineffective.[229-231]

During the last decades, antibiotic resistance developed against most, if not all, natural product-based antibiotics, and has threatened to leave certain diseases untreatable such as endocarditis, pneumonia, and bacteremia.[232] Recently, several reports admit the importance of the development of new antibiotics with novel mechanisms of action such as antivirulence drugs.

5.2 The concept of virulence and virulence factors

It is believed that the origin of the virulence concept arose from understanding why some bacteria harm their host while most others do not.[233] Several theories on the description of virulence have been proposed, current microbiologists define virulence as the ability of a microbe to infect the host and cause damage

as a result of pathogen-host interactions.[234] The extent of virulence can be estimated by measuring the median effective dose (ED_{50}), which is the effective dose producing a disease-related symptom in 50% of a population of animals.[234] Virulence factors are components that allow bacteria to infect and colonize the host, in addition to increasing bacterial ability to harm the host tissues.[235] These factors in bacteria are involved as well in immunoevasion, immunosuppression, gathering of nutrition and adherence to the host cell.[236]

Various kinds of virulence factors have been reported in the literature, including toxins, hydrolytic enzymes and surface proteins.[235, 237] These factors can exist originally within the bacterial genome or acquired from other bacterial species. Bacterial toxins and hydrolytic enzymes include several types of protein and lipopolysaccharide which severely dysregulate critical cellular functions in the host organism.[237] Many bacteria are surrounded by capsules and protective coats, which are mostly composed of sugars, that interfere with macrophages' defense capacity and phagocytosis.[235] Cell surface proteins have a pivotal role in virulence as they mediate bacterial adherence to the host tissues.[238]

Generally, the virulence factors assist bacteria to replicate and spread within the host environment, and crucially, they are not essential for bacterial survival.[239] As an alternative to conventional antibiotics, targeting the virulence factor is a different approach, in which anti-virulence agents have neither bactericidal nor bacteriostatic activity and mostly affect the bacterial capacity to initiate the infection.[230] Indeed, antivirulence therapies disarm bacteria instead of killing them without threatening their viability, consequently, exerting low selective pressure and stress on bacteria to induce the evolution of antibiotic resistance.[229, 240, 241] To demonstrate the validity of targeting virulence factors,[242] the first FDA-approved drug targeting a virulence factor is bezlotoxumab which targets *Clostridioides difficile* toxin B (TcdB). The drug inhibits the action of a cytotoxin produced by *C. difficile* and has been used for the treatment of recurring infections of this bacterium.[243] Another antivirulent approach to tackle bacterial infections has been explored by targeting the Lipid II molecule using teixobactin.[244] Interestingly, teixobactin has been proven to kill bacteria, apparently without the emergence of resistance. These examples prove that targeting antivirulence can be a compelling approach to antibiotic therapy and a good strategy to tackle bacterial infections.

5.3 Bacterial adhesion in virulence

The first key step for bacterial infection and colonization is the adhesion of bacteria to the host tissues,[238] which is an essential process in biofilm formation

that saves the pathogen from the persistent sheer force of the host fluids and provides protection from the host immune system.[245] Additionally, the adhesion is essential for the release of toxins that produce different damages throughout the progression of the infection. Targeting bacterial adhesion is considered a good anti-virulence strategy.[246] Adhesion in both Gram-positive and Gram-negative bacteria involves a multitude of surface protein complexes that are anchored to their cell walls. The cell-wall anchored (CWA) proteins in Gram-negative bacteria are composed of several small subunits of filamentous organelles, hair-like known as pili.[238] On the other hand, Gram-positive bacteria pili consist of structural protein fragments attached covalently to the peptidoglycan layer of the membrane.[247]

The CWA proteins have an essential role in the adhesion between bacteria and the extracellular matrix of the host's proteins like collagen, fibronectin and fibrinogen. Among the most studied subfamilies of CWAs in Gram-positive bacteria are the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules).[247] These proteins are present at the surface of the peptidoglycans layer by the action of Sortases enzymes which are cysteine transpeptidases. Sortases are abundant in Gram-positive strains and contribute significantly to bacterial virulence, among which SrtA is the housekeeping subtype with a potential antivirulence target.[248]

5.4 Sortase A – biological role

Sortase enzymes are responsible for positioning proteins involved in bacterial virulence on the bacterial cell surface in Gram-positive bacteria.[249] Based on their primary sequence and biological function, they can be further divided and classified as Sortase A to F. For instance, Sortase C enzymes are retained in the construction of bacterial pili, which are necessary for locomotion or purpose as a means of communication between bacteria,[250] while the SrtA is decisive for bacterial adhesion to host cells.[251] Considerably, all sortase enzymes are cysteine transpeptidases and have a unique recognition sequence on their target proteins, presenting an opportunity to construct selective inhibitors that target one sortase over the other. The most well-studied sortase is the membrane-bound peptidase SrtA, which is found ubiquitously in Gram-positive bacterial species and has received a lot of consideration as a target for antivirulence treatment with an acknowledged potential as a clinical target.[230, 231, 252]

SrtA enzyme is a membrane-bound transpeptidase with 206 amino acid residues and consists of a transmembrane region at the *N*-terminal along with a catalytic region at the *C*-terminal.[248] It is utilized by gram-positive bacteria to anchor surface proteins, including MSCRAMMs, to the peptidoglycan layer

of the cell wall as explained in **Figure 20**.[253] The MSCRAMMs surface proteins which are destined for anchoring to the cell wall consist of a cytosolic positively charged tail at the C-terminal, a hydrophobic transmembrane region, and a sorting signal containing LPXTG residues, where X symbolize for any amino acid moiety.[248] The anchoring process starts with a recognition of the

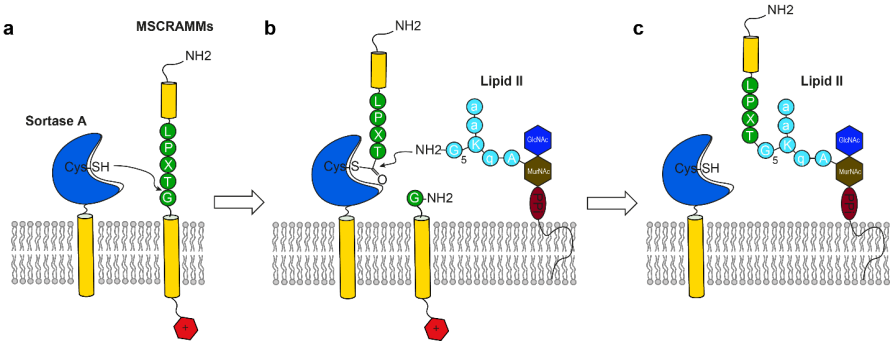


Figure 20. Mechanism of SrtA catalyzed surface protein anchoring: (**a**) recognition of MSCRAMMs through the LPXTG sorting signal, (**b**) cleavage of the scissile bond and transthioesterification, (**c**) transfer and anchoring of the surface protein at the lipid II complex.

conserved sorting signal of MSCRAMMs by SrtA (**Figure 20 a**).[248] Transthioesterification reaction follows afterward, through the cleavage of the peptide bond connecting threonine and glycine residues in the sorting motif (**Figure 20 b**) which generates a thioester acyl-enzyme intermediate. Then, the SrtA mediates a transpeptidation reaction between a pendant pentaglycine (Gly5) motif of the cell wall lipid-II molecule and the thioester intermediate, forming an amide bond connecting the *C*-terminal threonine and lipid II (**Figure 20 c**). The anchored product to the cell wall represents the cell surface proteins that are crucial for forming pathogenic biofilms which enable bacterial adherence to the host and promote virulence.[247]

5.5 Sortase A structure

Recently, several Sortase enzyme structures have been reported in the literature from either NMR spectroscopy or X-ray crystallography analysis. The analysis revealed that sortase enzymes adopt an overall structure consisting of eight strands of β-barrel folds, linked via random coil loops.[254-258] The enzyme has a grove-like structure in which certain loops construct the groove wall, whereas strands β4 and β7 assemble the groove floor. The enzyme active site entails a catalytic triad of highly conserved residues His120, Cys184, and Arg197 present at the end of the groove and functions together to

catalyze the formation of a thiolate nucleophile that attacks the enzyme substrate.

Generally, the core region of the substrate binding pocket is hydrophobic and embedded in a structurally varying environment among the different sortase classes.[259] Whereas the sorting signal for most class A sortases is comprised of the LPXTG motif, it exhibits particular variation for the other sortase classes. Structural studies of SrtA found that the LPXTG sorting signal recognition is done throughout the large groove that delves into the active site at the end of the groove.[260, 261] In the absence of a ligand, the β6/β7 loop was found to be highly flexible which upon ligand binding to the enzyme, the loop will transit from an open "disordered" conformation into a closed "ordered" conformation with a 3_{10} helix. These structural changes will push the substrate deeper into the groove toward the catalytic traid to initiate the transpeptidation reaction. This is just a simple overview of Sortase structure and there are several published reports that provide more in-depth knowledge about structural features of SrtA enzyme. The next section will highlight the potential of SrtA enzyme as a virulence factor.[254-258 277]

5.6 SrtA as a potential antivirulence target

Biofilms as discussed in the previous section, function as a protective mechanism that allows cells to survive and colonize hostile environments, causing serious inflammatory responses.[262] They are considered a mode of growth for the bacteria where they incorporate in a matrix-enclosed environment and adhere to biological or non-biological surfaces.[263] Notably, the matrix-enclosed structure of biofilms, makes the bacteria living within more resistant to antibiotics, rendering their treatment more problematic clinically.[264] It has been well recognized that SrtA as well as other sortase subfamilies have a central role in the initial formation of streptococcal and staphylococcal biofilms throughout bacterial adhesion to the host surface, making sortase inhibition an attractive target when designing antibiofilm treatments.[251, 265, 266]

One particular example is the multidrug-resistant *Staphylococcus epidermidis*, this antibiotic-resistant bacteria cause severe issues in medical settings when forming pathogenic biofilms on catheters and medical devices.[267] Additionally, biofilms of *Streptococcus mutans* have an essential role in the onset of dental caries, in which SrtA has been shown to be involved in this process.[268, 269] Septic arthritis infections affecting synovial fluid and joint tissues caused by other bacteria, fungi or viruses have been directly associated to the action of SrtA.[270] A knockout study of SrtA and SrtB from mice infected with *S. aureus* strains revealed the significance of SrtA, in which

the mice with no SrtA expression showed increased survival rates with less severe symptoms as well as lower weight loss.

As we can conclude SrtA has a pivotal role in bacterial adhesion and suppression of SrtA activity found to reduce biofilm formation and colonization in different bacterial species, with consequent reduction of bacterial virulence.[271] Particularly in combatting multidrug-resistant bacteria such as the methicillin-resistant *S. aureus* (MRSA) which cause severe infections with high morbidity and mortality rates. [280] [272] The exceptional ability of MRSA's to develop resistance against antibiotics necessitates the need for alternative treatments.

Aside from this, SrtA has many features which makes it a promising drug to prevent bacterial virulence. Firstly, SrtA is a characteristic antivirulence target in which it is not crucial for bacterial survival, rendering its targeting with less stress on bacteria and decreased risk of resistance emergence. Also, no eukaryotic SrtA homologues have been identified which implies that selective inhibition would be more feasible with lower side effects.[230] Finally, because SrtA is located on the external surface of the bacteria, it is an accessible target, with no need for the inhibitors to pass through the outer membrane of the bacteria.

To date, numerous SrtA inhibitors have been identified and reported, derived from different types of natural resources, peptides, and small organic compounds. Lately, we have published a review article that highlighted the recent developments in molecules inhibiting SrtA and covered a comprehensive overview of the inhibitors of peptidomimetic type.[94] In the next section, I will focus on some of the previously published peptide-based compounds.

5.7 Peptidomimetic SrtA inhibitors

Antimicrobial peptides (AMPs) comprise an alternative to traditional small molecules-based antibiotics for tackling the issue of bacterial resistance due to their unique mode of action.[273] In compliance with the review of Rima *et al*, there are more than 3257 AMPs that have been reported in the antimicrobial peptide database up to the 1st of July 2021.[274] Usually, AMPs consist of 10–50 amino acids and can fold into different secondary structural patterns as α-helices, β-sheets and loops.[252] The majority are polycationic and access the cytoplasmic membrane by interacting with the negatively charged fragments in the lipid bilayers which cause disruption of bacterial cell membrane.[275]

To date, several peptide-based molecules have been identified to inhibit the SrtA activity with modest efficacy.[230] Most of these peptidomimetics are analogs of the LPXTG sorting motif to facilitate recognition and binding to the

SrtA catalytic site but replace the Thr-Gly scissile bond using a diversity of functional moieties. These inhibitors are either covalent inhibitors of the enzyme Cys184 moiety or non-covalent mimetics of some transition state intermediates. [94]

Herein, I will summarize the non-covalent SrtA reversible inhibitors that are relevant to the peptidomimetic inhibitors developed during my PhD work. For easier structural recognition of peptides, each structure drawing was given a Latin number. The first reported inhibitor was created by McCafferty *et al*. as a phosphinate octapeptide, NH_2-ALPEAψ(PO_2HCH_2)GEE-OH, in which ψ represents a pseudopeptide bond (**Figure 21 I**).[276] In this octapeptide, the Thr-Gly scissile bond in the natural substrate was replaced by a non-hydrolyzable phosphonic isostere Alaψ[-P(0)OH-CH_2-]Gly as a transition state mimic. The Thr moiety was replaced with an Ala residue to facilitate the synthesis. The peptidomimetic displayed poor inhibition with a dissociation constant (*Ki*) equal to 2.5 mM, indicating that the thioester transition state might not be precisely mimicked within the designed inhibitor.

The same research group developed another related phosphinic inhibitor, NH_2-YALPEAψ(PO_2HCH_2)GEE-NH_2 (**Figure 21 II**) carrying an amine functional group at the C-terminus and an extra Tyr residue at the N-terminus.[276] Similarly, the design of this reversible inhibitor was aimed at imitating the structure of the enzyme transition state. However, the peptide showed poor inhibitory activity with a K_i equal to 11.4 mM and an IC_{50} of 10 mM. Again, the low inhibitory activity within the millimolar range indicates that the nonhydrolyzable phopshpnic ester bonds do not serve as an accurate transition state analogue. Within recent years, Rebollo *et al*. constructed thirteen bicyclic peptides that possess LPP motifs that resemble the natural sorting signal (LPXTG), implying that they could bind reversibly to the enzyme's active site (**Figure 21 III**).[229] The peptides' bicyclic structures contain the "PQLPP" residue within the first ring whereas the second ring did not have a specific sequence (**Figure 21 III** where PQLPP sequence is highlighted in blue). The cyclic peptides showed high affinities to the SrtA enzyme with K_i values in the lower micromolar range close to 1 μM with high target selectivity against a panel of proteins. The macrocyclic peptide (**Figure 21 IV**) with the sequence ACHSRCPQLPPCG afforded the strongest inhibition among the other inhibitors with a K_i value of 1.1 ± 0.18 μM. Noteworthy, the bicyclic peptide (**IV**) showed an *in-vitro* inhibition of the SrtA of *S. aureus* at a relatively high IC_{50} value of 167 μM, which might be due to ineffective distribution of the large macrocyclic peptide through the cell wall of the bacteria.

Lately, Wang *et al* reported an oligopeptide inhibitor with the sequence of LPRDA assembled with an N-terminal PEG_{2000} linker and a C-terminal amide to improve its pharmacokinetics and therapeutic effect (**Figure 21 V**).[277] The oligopeptide showed potent anti-SrtA activity with an IC_{50} of 10.61 μM which, to the best of my knowledge, has the highest inhibitory effect among other reversible peptidomimetic inhibitors. The study showed that the biofilm formation, bacterial adhesion to fibronectin, and invasion into the host tissues were all hindered following peptide incubation with S. aureus. Additionally, the display of surface protein A mediated by SrtA was decreased with no bacteriostatic activity against *S. aureus*, indicating that the oligopeptide acts as an antivirulence. Interestingly, *in vivo,* testing showed a reduction of *S. aureus* induced mastitis in a mouse model upon oligopeptide treatment. It is important to note that this is the only in-vivo study made for a peptide with anti-SrtA activity.

Kumari *et al.* developed a substrate-derived peptide His_6-LPETG, which was confirmed to be successfully incorporated into the bacterial cell wall and exhibited biofilm inhibition of the tested *S. aureus* SrtA at 100 μM concentration (**Figure 21 VI**).[266, 278] Additionally, peptides carrying the sequence FLAG-LPETG were tested and showed about 70% biofilm inhibition at 250 μM dose. It is noteworthy to mention that the 6His-LPETG peptide demonstrated no visible effect on the viability of *S. aureus* within the range of the tested peptide concentrations, suggesting it as a good anti-virulence candidate for clinical applications. There has been increasing interest in lowering *S. aureus* virulence with the LPRDA pentapeptide. The peptide was tested by *Bozhkova et al.* on methicillin-susceptible Staphylococcus aureus (MSSA) and displayed a 44% reduction in biofilm formation at a concentration of 100 μmol/L while a 12% decrease was observed with MRSA.[279] The LPRDA peptide was non-poisonous with the tested eukaryotic or bacterial cells at the working concentration which points out its anti-virulence characteristics. Further *in silico* studies performed by *Shulga et al.* on the LPRDA peptide binding interactions found that the most favorable binding mode of the peptide was in a "reverse order" sequence within the binding pocket.[280] Additionally, the LPRDA fragment worked as a "plasticizer" for the β6/β7 and β7/β8 loops and enabled faster sampling of the LPRDA–SrtA complex conformations.

Besides the reported reversible inhibitors, several potential peptide-based covalent inhibitors were also developed that react irreversibly with the Cys184 thiol at the active site of SrtA leading to enzyme inactivation.[94] Within these irreversible analogues, the Thr-Gly scissile bond was replaced with warheads to alkylate the Cys184 thiol group within the active site. Peptidomimetics

possessing vinyl sulfones, diazo ketone, cyanoalkene or other warheads were reported. In general, these peptidomimetics showed high affinity to the SrtA enzyme with inhibitor constants values within the micromolar range. Herin, we have developed some potent peptide-based antivirulence structures as competitive SrtA inhibitors which will be discussed in the next section.

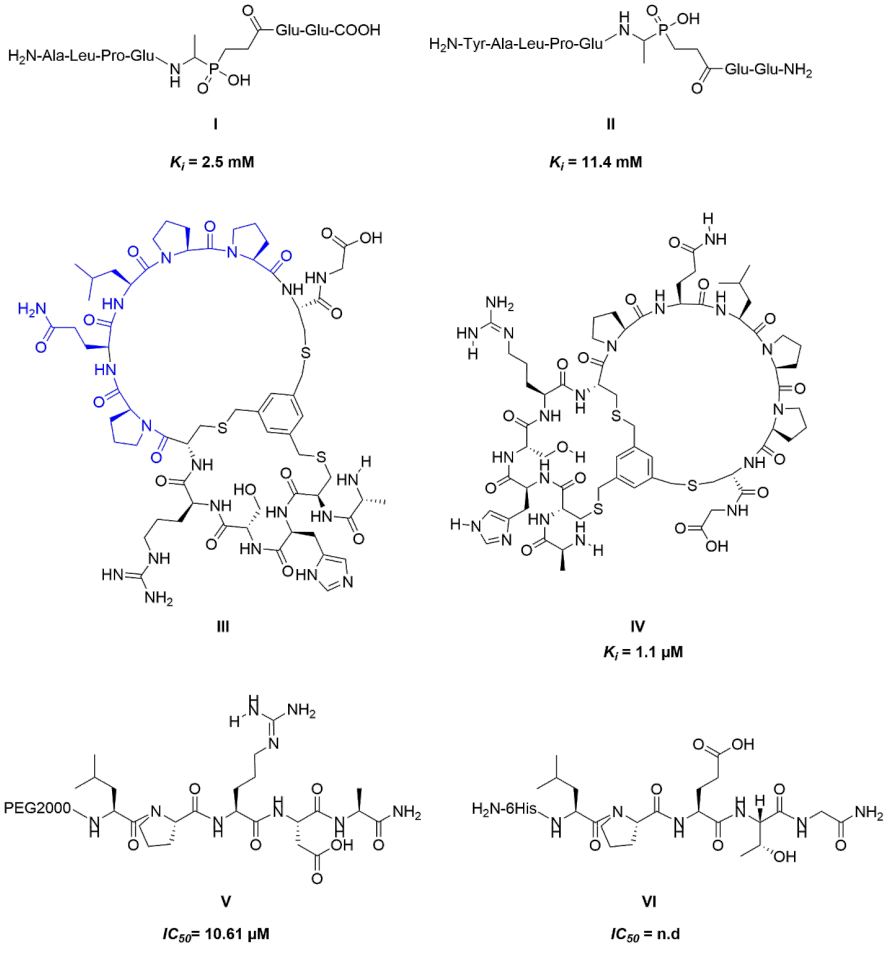


Figure 21. Non-covalent peptide-based inhibitors of SrtA. Phosphonite octapeptide (**I**), and the other derivative (**II**); bicyclic peptides structure (**III**); and the strongest bicyclic inhibitor (**IV**); oligopeptide LPRDA (**V**) and His6-LPETG peptide (**VI**).

5.8 Design of substrate-derived peptidomimetic SrtA Inhibitors

In the scoop of the thesis, a number of peptidomimetic inhibitors were synthesized and assessed for their anti-SrtA effect. The peptides were decorated with various structural modifications on peptide terminals, in which changes at the carboxy-terminal are highlighted in red and at the amino-terminal in blue (**Figure 22**). The design of the first group (**1**-**5**) of peptidomimetic inhibitors was based on the PEG_{2000}-LPRDA-NH_2 structure, the strongest peptidomimetic SrtA inhibitor reported so far with an IC_{50} of 10.61 mM.[277] The second group (**6**-**9**) of peptidomimetic inhibitors was designed based on the sorting motif LPETG sequence which contains the T–G scissile bond.[281] Based on previously published studies of peptidomimetic SrtA inhibitors,[282-285] where most of the reported structures incorporate a benzyloxycarbonyl (Cbz) group at the amino-terminal, the impact of introducing steric bulky groups at the amino-terminal was systematically investigated by developing a third group of peptidomimetic inhibitors (**10**–**14**).

The chemo- and regioselectivities of click chemistry reactions have made it an honored methodology to medicinal chemists and offer significant advantages in the drug discovery box of small molecules and bioactive peptides.[286] Compounds involving the triazole motif, produced via metal-catalyzed azide-alkyne cycloaddition (AAC) click synthesis, have some clinical applications in approved medications with various forms of bioactivity.[287] Therefore, the fourth group of inhibitors, peptides **16** and **17**, were designed to bear triazole entities. This was done also to investigate the influence of changing the amide bond orientation between the leucine and proline residues at the amino-terminal. Naturally, the peptide bond can adopt either a cis- or trans conformation, with the trans-conformation typically being energetically favored due to its relatively less steric hindrance than the cis structure, Nevertheless, the cis-conformation can be of vital importance to bioactivity.[288] [289]

Based on some in-silico analysis, it has been suggested, that restraining the conformational freedom of the peptide bond between Leu and Pro in the substrate-derived inhibitor could have a beneficial effect on their activity. Therefore, this conformational switch could be locked by replacing the amide with the 1,2,3-triazole moiety. As demonstrated in previous literature, 1,2,3-triazoles can serve as substitutes for the amide-bond, in which the 1,4-disubstituted triazole can mimic the amide bond trans-conformation while the 1,5-disubstituted triazole can mimic the cis-conformation.[288, 290] Additionally, compounds involving triazoles has shown to offer greater stability against proteolytic degradation and found to have good clinical applications with a broad range of activity.[291] [287] Both the 1,4-disubstituted and 1,5-disubstituted

triazoles were readily clicked via metal-catalyzed AAC reaction employing Cu and Ru catalysists, respectively.[292, 293]

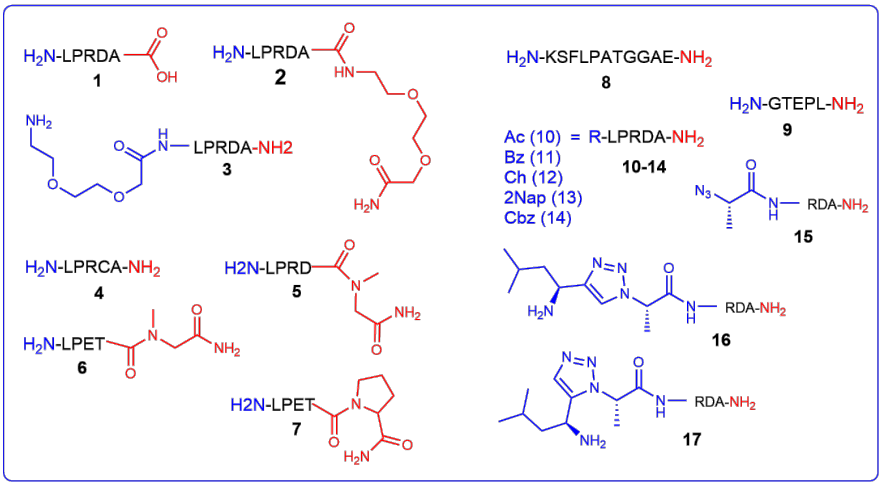


Figure 22. Overview of the chemically synthesized peptidomimetic inhibitors. Ac= Acetyl group, Bz = benzoyl group, Ch = Cyclohexanoyl, 2Nap = 2-Naphthoyl, Cbz = benzyloxycarbonyl.

5.9 Synthesis of peptidomimetic precursors

Peptides (**2-9**) were synthesized in parallel by automated Fmoc-based SPPS protocol. Commercially available Fmoc-protected amino acid derivatives were used for the synthesis. However, for the synthesis of peptide **5**, the Fmoc-protected building block was prepared using Fmoc-Osu under basic conditions according to a previously published protocol (**Figure 23**).[294] On the other hand, the synthesis of peptides **10-17** was done manually and N-terminal alkylation of the peptidomimetic inhibitors (**10**–**14**) was done on resin by treating the solid support excess of the corresponding alkylating agent and base. The completion of the N-terminal functionalization was assessed qualitatively by the Kaiser test.

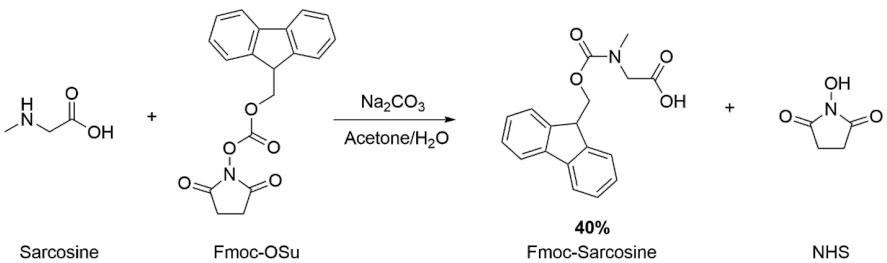


Figure 23. Preparation of the Fmoc-Sarcosine amino acid derivative.

To generate peptide-bearing triazoles, the building blocks derivatives of Leu-Alkyne and L-alanine amino acids were prepared in solution. Fmoc-Leu-alkyne was synthesized through multiple reaction steps without purification of the intermediates (**Figure 24**) following a convenient protocol of synthesizing terminal alkynes in good yield through a one-pot conversion.[295] The Bestmann-

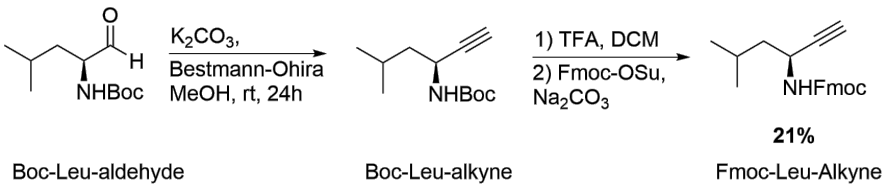


Figure 24. The synthetic protocol for Fmoc-Leu-Alkyne by conversion of commercial Boc-Leu-aldehyde to Boc-Leu-alkyne, followed by deprotection and Fmoc-protection resulting in the target compound.

Ohira reagent is highly convenient for the homologation of aldehydes to terminal alkynes at room temperature under mild conditions with a weak base and complete preservation of stereochemistry. [295, 296] To afford the desired building block, the acid-mediated Boc-deprotection and Fmoc-Osu protection of the alkyne were carried out directly. The synthesis of (S)-2-Azidopropanoic acid building block was completed through a two-step reaction shown in (**Figure 25**) without purification of the reaction intermediates.[297] The first reaction prepared a triflyl azide by treating sodium azide with Triflic anhydride

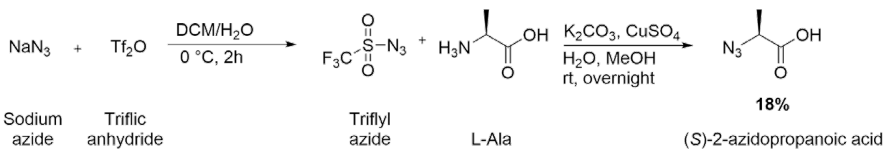


Figure 25. The synthetic root for (S)-2-Azidopropanoic acid. The first reaction results in triflyl azide, which then is converted to (S)-2-Azidopropanoic acid through a cupper-catalyzed overnight reaction.

for 2 h. Next, a copper(II)-catalyzed diazo transfer method of the L-Ala afforded the (S)-2-Azidopropanoic acid with aa decent yield and no need for further purification. After the successful synthesis of the alkyne and azide building blocks, the triazole-based SrtA inhibitors **16** and **17** were synthesized with Fmoc-based SPPS (**Figure 26**). After the coupling of the first three amino acids, (2S)-2-azidopropanoic acid was introduced, resulting in an azide-containing peptide resin. Thereafter, Fmoc-Leu-alkyne was coupled to the azide-bearing peptide resin with either CuAAC or RuAAC to form a 1,4-disubstituted 1,2,3-triazole (**16**) or 1,5-disubstituted 1,2,3-triazole (**17**).[288] Both

click reactions were performed while the peptide chain was still anchored to the resin under microwave irradiation and subsequent Fmoc deprotection and cleavage afforded the peptide triazoles successfully.

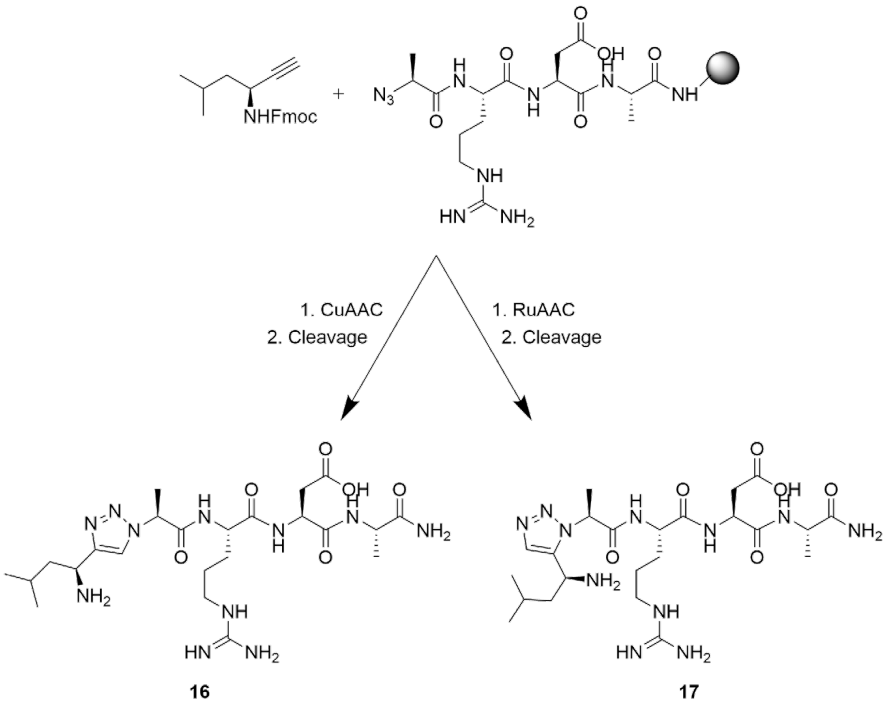


Figure 26. Schematic overview of the synthetic route attempted for the peptide synthesis of **16** and **17** from the azide-containing peptide-resin and Fmoc-Leu-Alkyne via metal-catalyzed azide-alkyne cycloaddition.

Subsequent to the purification, peptides were obtained with at least 95 % purity and were further characterized by HR-MS that showed deviation from their theoretical calculated mass of less than 5 ppm (**Table 5**). Following the preparation of the peptidomimetic inhibitors, the StA enzyme was expressed to be used for the upcoming activity testing.

5.10 Sortase A activity

To study the sortase enzyme activity and peptide mediated inhibition, a truncated wild-type SrtA protein was expressed and purified to homogeneity via immobilized metal affinity chromatography (IMAC) and Fast protein liquid chromatography (FPLC). The enzyme was verified by ESI-MS and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, the enzyme turnover rate was determined by an HPLC-based

Table 5. Peptides pure yields and analytical data.

Peptide code	Sequence	HR-MS (ppm)	tR (min)	Yield (%)
1	LPDRA-OH	4.9	5.4	6.53
2	LPRDAAdo	4.1	15.6	7.12
3	AdoLPRDA	5.9	16.0	8.92
4	LPRCA	4.1	9.7	4.12
5	LPRDSar	3.9	10.5	4.69
6	LPETSar	5.3	9.3	3.57
7	LPETP	5.2	11.8	2.35
8	KSFLPATGGAE	3.3	35.3	5.32
9	GTEPL	0.6	13.2	3.48
10	AcLPRDA	1.1	10.2	3.04
11	BzLPRDSar	0.2	9.4	5.27
12	ChLPRDA	0.4	9.8	5.62
13	2NapLPRDA	5.7	7.6	6.89
14	CbzLPRDA	1.1	6.9	4.06
15	AZARDA	0	5.7	1.64
16	LtARDA	3.0	4.0	2.14
17	LcARDA	3.8	3.8	2.16

kinetic study.[298, 299] For the kinetic analysis, the Abz-LPETGK(Dnp)- NH_2 substrate was employed to assess the expressed SrtA transpeptidation activity, in which the enzyme cleaves Thr–Gly scissile bond with subsequent transpeptidation using H-Gly_3-OH, to produce Abz-LPETGGG-OH and GK(Dnp)-NH_2. This reaction can be monitored easily using RP-HPLC through the analysis of the substrate peak decline over time or the analysis of the poduct GK(Dnp)-NH_2 peak incline over time (**Figure 27 a**). The Michaelis–Menten equation was used to calculate the enzyme kinetic parameters (**Figure 27 b**). From this assay, the enzyme parameters were extracted as the following; *Km* of 834.4 ± 67.8 μM and a *kcat* of 0.066 ± 0.012 s−1, yielding a *kcat/Km* of 78.8 M−1 s−1. Because of the similarity of the synthetic peptides to the SrtA natural substrate, their proteolytic stability toward SrtA cleavage was assessed. Interestingly, the peptides were found to be stable and could therefore be evaluated as potential enzyme inhibitors.

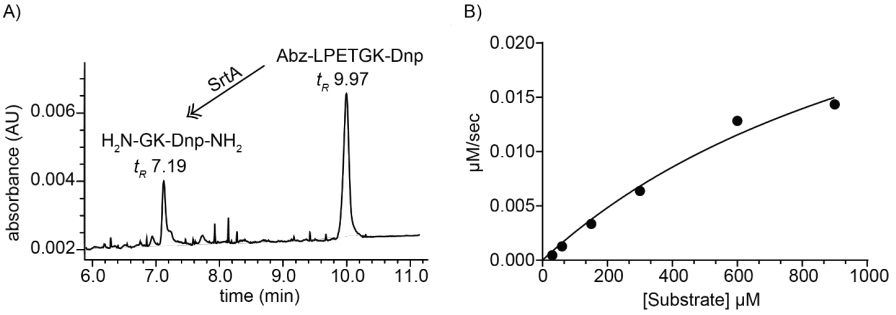


Figure 27. Transpeptidation reaction monitoring in RP-HPLC and Michaelis Menten analysis of SrtA activity. (**A**) SrtA catalyzed cleavage of the substrate Abz-LPETGK-Dnp with the detection of the substrate and GK-Dnp product using RP-HPLC **ISERA®** C18 column. The compounds were separated using a linear gradient from 0 to 40% of acetonitrile containing 0.1% trifluoroacetic acid (TFA) over 10 min and detection wavelength at 355 nm. (**B**) Michaelis Menten plot to calculate the enzyme kinetics. The change of the peak area over time was used to calculate the reaction velocities. The complete experimental details are described in paper **IV**. Estimates of the kinetic parameters (K_M = 834.4 ± 67.8 µM, V_{max} = 15 ±1.81 nM.s^{-1} and *k*cat = 0.066 ±1.81 s^{-1}) were determined.

5.11 Screening for SrtA inhibitors

In order to monitor the activity of SrtA inhibitors, a fluorescence resonance energy transfer (FRET) based assay was employed using the Abz-LPETGK(Dnp)-NH_2 fluorogenic substrate which is internally quenched.[231] The synthetic series of peptidomimetic (**1**-**17**) were evaluated for SrtA inhibition and showed potential competitive inhibitions at various rates within the range of 15% up to 76% (**Table 6**). A previously published small organic compound with confirmed SrtA activity was employed as a reference for the assay.[300] Subsequently, IC_{50} values were estimated by testing different concentrations for the inhibitors with the highest FRET activity; peptides **5** and **7** (**Table 6**). The tested peptides showed an IC_{50} value below 200 µM concentration, in which peptide (**5**), exhibited an IC_{50} of 18.9 µM, rendering it a promising sequence for later *in vitro* studies of SrtA enzyme in *S. aureus*.

The initial series of peptidomimetic SrtA inhibitors showed promising results, and it was the base for the preparation of a second generation of peptides to target SrtA more efficiently. Among these, peptide **18** with the sequence of BZLPRDSar representing the benzoylated version of **5** displayed the most potent inhibitor of this generation with an IC_{50} value of 57.4 ± 1.1 µM. The second generation was synthesized and tested for its bioactivity by a postdoctoral colleague in the lab.

Table 6. The tested peptidomimetic SrtA inhibitors activity data; FRET inhibition and IC_{50} results.*

COMPOUND	FRET INHIBITION [%]	IC_{50} [μM]
REFERENCE (**0**)	77.1 ± 4.1	-
LPDRA-OH (**1**)	29.2 ± 2.6	-
LPRDAADO (**2**)	30.4 ± 2.8	-
ADOLPRDA (**3**)	22.6 ± 2.8	-
LPRCA (**4**)	14.8 ± 2.9	-
LPRDSAR (**5**)	68.5 ± 2.6	18.9 ± 1.2
LPETSAR (**6**)	19.4 ± 2.6	-
LPETP (**7**)	76.0 ± 8.4	136.3 ± 1.2
KSFLPATGGAE (**8**)	18.1 ± 2.9	-
GTEPL (**9**)	37.7 ± 3.1	-
ACLPRDA (**10**)	20.7 ± 2.9	-
BZLPRDA (**11**)	37.2 ± 3.1	-
CHLPRDA (**12**)	21.6 ± 4.2	-
2NAPLPRDA (**13**)	47.4 ± 1.1 (at 10 μM)	-
CBZLPRDA (**14**)	17.0 ± 2.8	-
AZARDA (**15**)	23.9 ± 3.0	-
LTARDA (**16**)	33.0 ± 3.4	-
LCARDA (**17**)	23.3 ± 2.9	-

*Peptides were measured at 200μM concentration, with the exception of 2NapLPRDA, using 200 nM of SrtA and 5-((C4-nitrobenzyl)thio)-1,3,4-thiadiazol-2-amine as a reference inhibitor. The complete experimental details are explained in paper **IV**.

5.12 Additional in-vitro testing

The series of peptidomimetic SrtA inhibitors seems to be among the most active peptidomimetic compounds of this category, as a result, the compounds were further investigated in growth inhibition studies against pathogenic *S. aureus*. As mentioned earlier SrtA is a characteristic antivirulent target in which it's not essential for bacterial survival, therefore the peptidomimetic inhibitors were assessed for their potential to impede bacterial growth.[271] Interestingly, the results indicate that the bacteria were not killed when treated with some of the second-generation peptidomimetic inhibitors, but their growth was hindered marking them as promising antivirulence compounds. Among the tested compounds, **18** displayed the strongest growth inhibition effect at 128 μg/ml concentration.

Additionally, peptide **18**, was checked for its antibiofilm activity in *S. aureus* and found to inhibit biofilm formation effectively at the same concentration used for growth inhibition.[283, 301] Interestingly, bacteria incubated with 128 μg/ml of **18** demonstrated sparsely distributed colonies under the fluorescence microscope relative to control samples. All these results support the fact that the initial growth and bacterial viability were not affected, suggesting selective inhibition against the SrtA enzyme.

5.13 Summary of Paper IV

To summarize, several structures of peptidomimetic SrtA inhibitors were synthesized of which some were highly efficient competitive inhibitors. Among all the synthesized peptide generations, six peptides exhibited an IC_{50} value less than 200 μM, with the LPRDSar peptide showing the lowest IC_{50} (18.9 μM). Peptidomimetics structures of the LPRDA sequence appeared to be the most efficient, with the inclusion of either some degree of aromatic bulk groups or a C-terminal non-scissile motif, the inhibitory potential was further improved.

Based on these findings, the developed structures are among the most potent *in vitro* peptidomimetic inhibitors published to date. Moreover, the six most effective peptides were checked against *S. aureus* bacteria as growth inhibitors. At this point, the peptides possessing a phenyl ring structure, incorporated as N-terminal benzoylation or as a phenylalanine moiety, demonstrated an antivirulence action. Intriguingly, peptidomimetics missing this moiety displayed no growth inhibition against the bacteria. This effect could be due to the enhanced hydrophobic interactions within the binding pocket when benzoylating the N-terminal or adding a terminal phenylalanine. It is noteworthy to mention that the LPRDSar structure demonstrated the strongest FRET inhibition of the isolated enzyme, whereas its benzoylated version BzLPRDSar displayed the strongest impact on bacterial growth. Additionally, the BzLPRDSar showed strong biofilm inhibition at low concentrations which makes it a potential lead molecule.

Chapter 6

6.0 Concluding Remarks and Future Outlooks

New treatment strategies that target the TM domain to suppress receptor dimerization and activation is a barely explored field that can be more efficient and have less toxic side effects than the current antibody therapies.[108] Though in paper **I**, our main focus was to successfully synthesize and characterize transmembrane peptides since the chemical peptide synthesis allows site-specific labeling and modification.[51] Intriguingly, we reported for the first time a novel strategy for targeting ErbB2 receptors in cancer by combining TM peptide and IL nanocarriers. The anticancer action and the pharmacokinetics properties of TM peptide-IL carriers will be further evaluated in *in-vivo* studies in the future. This receptor targeting strategy can be utilized not only for ErbB2 proteins but for different cell surface receptor proteins. By leveraging on the biological activities of TM peptide variants and the IL-carriers (BAIL or MAIL), we also demonstrate a new approach to design transmembrane-peptide decorated IL-nanocarriers (TM-IL nano-formulation) which can be applied as potential modular therapeutic nanomedicine and as nanodrug delivery systems of anticancer drugs and immunomodulators to ErbB2-positive tumors. Importantly, BAIL's cytotoxic action could be used to enhance the anticancer activity of existing therapies. Furthermore, the findings presented here could serve as a starting point for building more Biamphphlic-based nanocarriers that could be synthesized with room to optimize their delivery and trackability to be used as treatment and diagnostics tools.

Holin peptides serve a diversity of potential and established functions in bacteria other than their ability to disrupt the bacterial cell membrane.[302] Additionally, they have been involved in the transferring process of genetic material from one bacterium to another, contributing to the biofilm formation and oxidative stress responses.

Holin homologues have also been detected in plant chloroplasts, animal apoptosis-promoting proteins as well as in archaea implying that these proteins are abundant among the three domains of life. Currently, there are 58 identified families of holins but many more families have yet to be discovered. Lately, Phage holin techniques are being investigated as novel potential biomedical tools to overcome the issue of bacterial resistance,[303, 304] with other potential

applications as drug delivery systems into eukaryotic cells and diagnostic methods based on genetically engineered phage. It is expected that future investigations on holins will lead to the revelation of other functions and the discovery of innovative applications.

Holin peptides like many other natural peptides are valuable sources of new drugs and have significant applications yet to be discovered due to the inability to synthesize them or the limited amount of their extraction.[2] The pseudoprolines employed in the work of Paper **II** allowed the successful synthesis of the Holin variants. These pseudoprolines are easy to synthesize, commercially available, simple to use and have been used for the successful synthesis of many sparingly soluble peptides.[171]

It is essential to acknowledge the inherent constraints encountered during the synthesis of the previously mentioned TM peptides. These include the complexity of the synthesized peptide structures and their high tendency to aggregate which results in low yields.[50] Some of the primary challenges included tedious and time-consuming purifications required to achieve the desired purity due to the smearing effect of these peptides in RP-HPLC systems. Additional washing routines are essential before and after the characterization of these peptides to keep the apparatus used running without any blockage problems due to frequent precipitation. The nonspecific aggregation behavior of these peptides might limit the generalizability of the findings on alternative peptide sequences where further optimization might be necessary. Additionally, the organic synthesis of the novel BAILs presents several challenges, particularly in achieving the desired structure with good yield. The target structure was obtained after attempting five different synthetic routes.

To my knowledge, no other studies have identified the utilization of **IL1** in the conjugation of biomolecules, of which only a few ILs are described to generate carbenes, namely the methylthiazolium based ILs.[305] However, these aforementioned ILs were not considered yet for the modifications of biomolecules. In paper **III**, for the first time, [C_2mim][OAc] IL is employed for the site-specific conjugation of Cys-containing peptides. This methodology could facilitate the synthesis of molecular probes (like drug-peptide conjugates), and biological tools (through labeling and localization of drug receptors in animal models) as well as improve the physicochemical properties of the conjugated peptides. Indeed, [C_2mim][OAc] has a lower toxicity than conventional organic solvents with more appealing solubilizing properties and it's the ionic liquid of choice for dissolving some of the previously reported reasonably hydrophobic peptides.[218]

There are many thiol conjugation techniques reported as mentioned in section **4.1**. However, this developed strategy proceeds with the preservation of substrate stereochemistry and uses mild reaction conditions. Although the yields of the isolated peptide conjugates were low (around 30%), the major goal was to alkylate and isolate the products successfully. Therefore, this methodology is considered a promising starting point for the conjugation of larger biomolecular complexes like proteins and antibodies with a possibility for optimization to improve the product yield. Additionally, selenocysteine-peptide was synthesized and the benzylation protocol was tested and occurred at a very low rate. The TCEP induced deselenization of the selenocysteine peptide and formed side products which indicates that the reaction conditions developed here are not optimal for Selenide chemistry, yet to be improved in future studies.

This methodology is a good contribution to the field of ionic liquids-mediated chemical reactions, due to their good solubilizing properties and structure-stabilizing abilities.[178, 210] Different sorts of ionic liquids will be studied in our future work with enhanced solubilizing properties and more stable carbenes.

With the emergence of the global challenge of bacterial resistance, developing anti-virulence treatments, instead of conventional antibacterial therapies has gained substantial interest. When applying antivirulence therapy, the pathogens become incapable of creating a risk, without killing them, hence rendering them less stressful to emerge resistance toward the antivirulence therapy. Among different virulence factors, SrtA enzyme is a very potential target since the enzyme is a membrane-bound accessible target, with a vital role in bacterial adhesion to host cells. Previous reports showed that the suppression of SrtA activity in *S. aureus* is an efficient method that inhibits biofilm formation. Additionally, MRSA and septic arthritis infections have been directly correlated to the SrtA action, confirming the clinical necessity for SrtA inhibition to produce effective medications.[306, 307] Nevertheless, most of the previously reported SrtA inhibitors were based on a covalent mode of inhibition, which could to a lead to side effects when interacting with other cysteine-proteases in the body. Notably, few of them are efficient peptidomimetic-based inhibitors and herein within the work published in paper **IV**, several novel non-covalent SrtA inhibitors were developed with sequences based on either the enzyme natural substrate sorting motif (LPETG) or a previously reported inhibitor (LPRDA).[277]

The work is a valuable contribution towards targeting *S. aureus* SrtA as a basic virulence factor present ubiquitously in Gram-positive bacterial strains, with potential future application in septic arthritis treatments. I beleive that

with further structural modulation and *in vivo* analysis, the peptidomimetic SrtA inhibitors could be good clinical candidates, and act as potential lead compounds. The peptidmomiteics developed her possess intrinsic simple structure with low molecular weight and good water solubility rendering them outstanding lead compounds for furthere drug development. Moreover, the incorporation of non-scissile bonds, like sarcosine, provides protection against host proteolytic enzymes, improving their probability of reaching their targets. Furthermore, the peptidomimetics SrtA inhibitors could potentially be used in combination with other antivirulence factors or conventional antibiotics and increase their clinical applications. Taken together, with further *in vivo* tests and structure-activity relationship studies, these inhibitors could be a good starting point to develop remedies hindering the rapid increase of bacterial resistance to current medications.

It is noteworthy to mention that, the pentapeptide Leu-Pro-Arg-Asp-Ala (LPRDA) is one of the most investigated peptides acting against *S. aureus* SrtA enzyme. The sequence might be a good lead for the development of novel antivirulence agents. We think that the pentapeptide sequence LPRDA deserves extra studies via *in vivo* models to prove its potential as an antivirulence agent.

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