Arginine and Tryptophan rich Antimicrobial Peptides (AMPs)

Modifications, Application and Mode of action

Dissertation

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à Julien pour son amour et soutien
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Abstract

«Arginine and Tryptophan rich Antimicrobial Peptides (AMPs). Modifications, Application and Mode of action»

1st Referee: Prof. Dr. N. Metzler-Nolte
2nd Referee: Jun. Prof. J. E. Bandow

Multi-resistant bacteria occur more and more widespread. It is therefore necessary to find new compounds able to overcome this problem. Such a novel class of compounds are the cationic antimicrobial peptides (AMPs). This work presents the synthesis of those AMPs as well as the metalloocene modified one. We wanted to explore further this area and we used additional biological experiments to achieve this aim.

Peptides and the subsequent labelling with ferrocene and ruthenocene carboxylic acids were carried out using solid phase peptide synthesis (SPPS). The metalloocene markers were generally introduced to the N-terminus. Diverse linkers, resins and protecting groups were used for the successful peptide synthesis.

Another synthetic perspective in this work was the synthesis of branched AMPs motivated by the dendrimers chemistry. Large number of alkynes and azides were successfully synthesised and then applied in «click» reactions. This strategy provides numerous model molecules, but was not successful in the case of more complex molecules as peptides.

In the last part of this work we present the biological techniques and the results. All modified compounds have shown antimicrobial activity in range of 7 to 0.9 µg/mL. Proteomic approach (isolation and characterisation of proteins) as well as MudPIT (protein mass analysis) were used to study the mode of action of the studied AMPs.

The protein profile of B. subtilis, after treatment with AMPs was highly similar to that of the detergent triton X-100 (detergent). Moreover, triton X-100 has also shared proteins markers with valinomycin (dodecadepsipeptide) and bacitracin (antibiotic) which inhibit cell wall biosynthesis at a membrane-bound step. Together these results suggest that those AMPs, as triton X-100 and bacitracin, target the cytoplasmic membrane.
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Abreviations

AA – Amino Acid
Acm – Acetamidometyl
AcOH – Acetic acid
AMPs – Antimicrobial Peptides
Arg (R) – Arginine
AU – Absorbance units
aq. – Aqueous
Boc – tert-Butoxy carbonyl
(Boc)₂O – Boc anhydride or Di-tert-butyl pyrocarbonate
°C – Degree Celsius
(CF₃CO)₂O – Trifluoroacetic anhydride
Cp – Cyclopentadienyl
Cys (C) – Cysteine
DIC – N,N’-Diisopropylcarbodiimide
DIPEA – Diisopropylethylamin
DMAP – 4-(Dimethylamino) pyridine
DMF – Dimethylformamid
DMSO – Dimethyl sulfoxide
DTNB – 5,5’-Dithiobis(2-nitrobenzoic acid)
DTT – Dithiothreitol
EDAC – N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
ESI – Electrospray ionization
e⁻ – Electron
eq. – Equivalent
FAB – Fast Atom Bombardment
FCS – Fetal calf serum
Fmoc – Fluorenlymethoxycarbonyl
GC-MS – Gas chromatography-mass spectrometry
Gly (G) – Glycine
g – Gram
h – Hour
HATU – 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanamininium
HCl – Hydrochloric acid
HeLa – Cervical cancer cell line
HepG2 – Hepatocellular carcinoma cell line
HMPA – Tris(dimethylamino)phosphine oxide
HOBut – 1-Hydroxy-1H-benzotriazole
HPLC – High Performance Liquid Chromatography
HT-29 – Human colon adenocarcinoma cell line
IBX – 2-Iodoxybenzoic acid
J – Coupling constant in Hz
LC-MS – Liquid chromatography-mass spectrometry
Leu (L) – Leucine
MALDI – TOF - Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MIC – Minimum Inhibitory Concentration
min – Minutes
mg – Milligram
µM – Microlitres
mL – Millilitres
mm – Millimetres
m/z – Mass-to-charge ratio
MOA – Mode of action
MTP – Microtiterplates
Na – Sodium
nm – Nanometre
N – Nitrogen
NMR – Nuclear magnetic resonance
o.n. – Over night
PAMAM – Polyamidoamine
Pbf – 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyle
PDA – Photodiode Array
PG – Protecting group
Phe (F) – Phenylalanine
PPI – Polyethyleneimine
**PyBOP** – Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate

**PyBroP** – Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate

**Rf** – Frontal report

**RP** – Reverse Phase

**r.t.** – Room temperature

**sat.** – Saturated

**SMH** – Shai-Matsuzaki-Huang

**SPPS** – Solid Phase Peptide Synthesis

**T** – Tryptamine

**TBTU** – O-(benzotriazole-1-yl)-N,N,N’’,N’’-tetramethylurionium tetrafluoroborate

**TFA** – Trifluoro acetic acid

**THF** – Tetrahydrofuran

**TIS** – Triisopropylsilan

**TLC** – Thin Layer Chromatography

**TNBS** – 2,4,6-Trinitrobenzene sulfonic acid

**Trp (W)** – Tryptophan

**v/v** – Volume in volume

**Z** – Benzoyloxycarbonyl

**λ** – Wavelength
Chapter I. Introduction

1. Antibiotics. Historical overview

Antibiotics or originally called «antiobiosis» (greek, αντι – anti, against and βιος – bios, life), are drugs with bacteriostatic or bactericide action. This term was introduced by the French bacteriologist Jean Paul Vuillemin (1861-1932) \[1\] and renamed antibiotics by Selman Waksman, an American microbiologist in 1942 \[2\].

First description of this phenomenon was reported by Louis Pasteur and Robert Koch in earlier 1877, when an airborne bacillus could inhibit the growth of Bacillus anthracis \[3\].

Three years later in 1880s Paul Ehrlich, a German medical scientist, start the research on synthetic antibiotic chemotherapy and started the beginning of a new science. In 1906, he discovered the structural formula of Atoxyl, a chemical compound active in cases of sleeping sickness and won the Nobel Prize for Medicine, together with Ilya Ilyich Mechnikov in 1908.

In 1909, Ehrlich and his student Sahachiro Hata synthesised the man-made antibiotic Salvarsan \[4\] effective against Syphilis and developed the theory of the so called "magic bullet" (drug who selectively will bind and kill bacteria without harming the human host).

However, Salvarsan was never commercialised as an antibiotic, because of side effect and the later discovery of the more promising Penicillin.
The work of Ehrlich was followed by the discovery of Prontosil by Domagk (Nobel Prize for Medicine, 1939). Prontosil was the first commercially available antibacterial antibiotic developed by Domagk and his research team in Bayer Laboratories, Germany. This first sulfonamide drug with relatively broad effect against Gram-positive cocci, but not against enterobacteria, opens the era of the antibiotics.

Without doubt the discovery of natural antibiotics, produced by microorganisms, started with the earlier observation of Louis Pasteur of this phenomenon, when he wrote «if we could intervene in the antagonism observed between some bacteria, it would offer perhaps the greatest hopes for therapeutics».

In 1875, John Tyndall report for first time that species, called Penicillium spp (Fig. 1), caused the dead of bacterial colonies. Later the potential of that observation will confirm Pasteur’s theory. However, his work went by without much notice from the scientific community until Alexander Fleming's discovery of Penicillin in 1928. Even then the therapeutic potential of Penicillin was not fully established.

![Penicillium spp.](Image)

**Fig. 1 Penicillium spp.** (Submitted by J.M. Miller, Ph.D., CDC, Atlanta, GA www.asm.org)

More than ten years later, Ernst Chain and Howard Florey became interested in Fleming's work, and came up with the purified form of Penicillin. The purified antibiotic displayed antibacterial activity against a wide range of bacteria. At that time, no one had discovered a compound with such an activity and this fact stimulates the search for antibiotic compounds with similar capacity. The Penicillin discovery brought Ernst Chain, Howard Florey and Alexander Fleming the 1945 Nobel Prize in Medicine.
Rene Dubos \[5\] showed another example for bacterial antagonisms in 1939 when he isolated **Gramicidin**. This antibiotic was commercially manufactured, during the Second World War, with effect on wounds and ulcers.

2. Antibiotic resistance \[^{6, 7}\]

The biggest mistake in the Ehrlich theory of the "magic bullet" seemed to be persistent even in our days and that is the unexpected effect of bacterial resistance. Since their introduction into human medicine, the antibiotics show an enormous impact on treatment of infectious diseases and the success of medical procedures, such as surgery and chemotherapy. Hence, the rise of bacterial resistance seems to put on question the gains from an antibiotic treatment.

Another reason, which can not be underestimate, is the low number of new drugs. After the introduction of **Quinolones** in the early 1960s, followed more than 30 years later by the release of the streptogramins **Synercid** and the oxazolidinone **Linezolid**, there were no new antibiotic chemical structures (Fig. 2).

---

**Fig. 2** The frequency of MRSA (Methicillin-resistant Staphylococcus aureus) among blood cultures with *Staphylococcus aureus* in England and Wales 1992-2002 (from http://www.who.int/drugresistance/en/) (A) new commercial antibiotics for 1910 to 2005 (B).
Conclusions

The infectious diseases are one of the major causes of death worldwide. Unfortunately, the newly synthesised antibiotics are niche drugs developed for antibiotic-resistant pathogens, and their restricted activity somewhat limit their impact. Thus, the development of novel antibacterial classes has crucial importance in the way of overcoming the remarkable adaptability of the bacteria. Such a promising new class of antibacterial drugs are the antimicrobial peptides (AMPs)\textsuperscript{[8-13]}. In the next part would demonstrate the characteristics of the AMPs as well as our interest to work with it.
3. Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are a diverse group of molecules, isolated from many tissues and cell types in a variety of invertebrate, plant and animal species (Tab.1). Since the beginning of the 20th century this new class of drugs attracts the researcher’s attention and an important number of active compounds were recognised and isolated between 1920 and 1950. They were described as being able to kill or slow the growth of invading infecting microorganisms and to help natural mechanisms of adaptive immunity. Thus the field of antimicrobial peptide research was born.

Shortly afterwards, antimicrobial substances were purified by Hirsch and correlated with the presence of low-molecular-mass cationic compounds.

The natural cationic peptides are generally 12–50 aminoacids in length, have a net positive charge (excess of basic lysine and/or arginine residues), and contain around 50% hydrophobic aminoacids. Often due to the presence of disulphide bridges, or contact with membranes, they fold into three-dimensional amphiphilic (possessing both hydrophilic and lipophilic properties) structures in which the positively charged and hydrophilic domain(s) are well separated from the hydrophobic domain(s). Such a combination is well suited to interacting with membranes, especially bacterial membranes with their negatively charged and hydrophilic head groups and hydrophobic cores.

The positive charges also influence specificity of the peptide toward the target membrane; variation in only one charge can lead to dramatic differences in haemolytic and antibacterial properties as in the case of Pardaxin and Indolicidin analogues.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-helical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecropin A</td>
<td>KWKLFKIEKVGQNIIRDGIKAGPAVAVVQATQIKAa</td>
<td>Silk moth</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>GIKFLHSAKKFGKAFLVGEIMNS</td>
<td>Frog</td>
</tr>
<tr>
<td>Pexiganan</td>
<td>GIKFLKAKKFGKAFLKILKKA</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Dermaaseptin</td>
<td>ALWKTMLKLGLMGHAALGAANTAISGQT</td>
<td>Arboreal frog</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>frog</td>
</tr>
<tr>
<td>LL-37</td>
<td>LLGDFFRKSKEKIGKEFKRIVQRKDFLRNLVPRTES</td>
<td>Man</td>
</tr>
<tr>
<td>Buforin II</td>
<td></td>
<td>Vertebrate</td>
</tr>
<tr>
<td><strong>One disulphide bond</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bactenecin 1</td>
<td></td>
<td>Cow</td>
</tr>
<tr>
<td>Thanatin</td>
<td></td>
<td>Insect</td>
</tr>
<tr>
<td>Brevinin 1T</td>
<td></td>
<td>Rana frogs</td>
</tr>
<tr>
<td>Ranalexin</td>
<td></td>
<td>Rana frogs</td>
</tr>
<tr>
<td>Ranateurin 1</td>
<td></td>
<td>Rana frogs</td>
</tr>
<tr>
<td>Esculentin 1</td>
<td>GFSKLGRRKLKNLISGLKNVEGMDVTRGIDIAGCKKGECA</td>
<td>Rana frogs</td>
</tr>
<tr>
<td><strong>Two disulphide bonds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tachypleisin</td>
<td></td>
<td>Horseshoe crab</td>
</tr>
<tr>
<td>Androctonin</td>
<td></td>
<td>Scorpion</td>
</tr>
<tr>
<td>Protegrin 1</td>
<td></td>
<td>Pig</td>
</tr>
<tr>
<td><strong>Three disulphide bonds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-defensin (HNP3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-defensin (TAP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>θ-defensin (sapecinA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defensin</td>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>Defensin</td>
<td></td>
<td>Cow</td>
</tr>
<tr>
<td>Defensin</td>
<td></td>
<td>Monkey</td>
</tr>
<tr>
<td>Defensin</td>
<td></td>
<td>Insect</td>
</tr>
<tr>
<td>Thionin (crambin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Four disulphide bonds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defensin</td>
<td>QKLC1QRPSGTSGVC2GNNAC3KNQC4IRLEKARHGSC2NYVFAHCAC1IC2YFPC1</td>
<td>Radish Seeds</td>
</tr>
<tr>
<td>Drosomycin</td>
<td>DC1LSGRYKGPC2AVVNDNCT2RRVC2KEEGRSSGHC2PSLKC3WC2EGC1</td>
<td>Drosophila</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>DTHFPC1IFC2GC3HC2HRSKC2 GMC2 C,4KT</td>
<td>Human</td>
</tr>
<tr>
<td><strong>Linear, not α-helical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bac 5</td>
<td>RFRPPRRPPFIRPFPFYPFRFPFPFRPPFPFRPPFLPRPPFPPFRPPFLGPFPFa</td>
<td>Cow</td>
</tr>
<tr>
<td>PR-39</td>
<td>RRRPRPPYLRPRPPFPFPPFPFLPRPPFPPFPFRPPFPPFRPPFa</td>
<td>Pig</td>
</tr>
<tr>
<td>Indolicidin</td>
<td>ILPKWKPWPPWRa</td>
<td>Cow</td>
</tr>
<tr>
<td>Apidaecin</td>
<td>GNNRPVYIPOPRPPHRPI</td>
<td>Honeybee</td>
</tr>
<tr>
<td>Pyrrhocoricin</td>
<td>VDKGSLPRPPPPFRPPYPHRPN</td>
<td>Insect</td>
</tr>
<tr>
<td>Histatin 5</td>
<td>DSHAKRHGHYKRFKHEKHHSHRY</td>
<td>Saliva</td>
</tr>
</tbody>
</table>

Cysteines paired in disulphide linkages are noted by common numerical subscripts. C-terminal amides are noted by a. In θ-defensin, the first and last residues are joined in a peptide bond.
While most antibiotic peptides described in the literature are strongly cationic, it should be mentioned that few examples of anionic peptides are known. Examples include poly Asp-containing peptides [20], or Glu-rich enkelytin [21] active against Gram-positive organisms. They have minimal inhibitory concentrations (MICs) against common bacterial strains higher than those of typical cationic peptides. Electron micrographs of bacteria exposed to this peptide do not show the typical alterations observed for cationic peptides, suggesting a different mode of action in comparison with the cationic.

The field expanded further when Hans Boman, Michael Zasloff and Robert Lehrer independently isolated and purified insect cecropins, amphibian magainins and mammalian defensins, respectively [22, 23].

Now, more than 880 different antimicrobial peptides have been identified or predicted from nucleic acid sequences and can be consulted on http://www.aps.unmc.edu/AP/main.html and http://www.bbcm.univ.trieste.it/~tossi/antimic.html. The data bases contain antimicrobial peptides produced in many tissues and cell types of a variety of invertebrate, plant and animal species [24-28], certain cytokines and chemokines [29, 30], selected neuropeptides and peptide hormones [31, 32], and fragments of larger proteins [33, 34].

The main difficulty for the development of AMPs, as systemic therapy, is the very high doses often close to the toxic doses of the peptide (such as magainin) [35], needed for an antibacterial effect. Antimicrobial peptides in pharmaceutical development are shown in Table 2.
Tab. 2 Commercial development of antimicrobial peptides of animal origin \(^{(28)}\)

<table>
<thead>
<tr>
<th>Use</th>
<th>Peptide</th>
<th>Company</th>
<th>Application</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topical</td>
<td>Pexiganan (MSI-78)</td>
<td>Magainin (Genaera)</td>
<td>Infected diabetic foot ulcers</td>
<td>Completed Phase III</td>
</tr>
<tr>
<td>Topical</td>
<td>MBI-226</td>
<td>Micrologix</td>
<td>Catheter infection</td>
<td>Phase III</td>
</tr>
<tr>
<td>Topical</td>
<td>MBI-594</td>
<td>Micrologix</td>
<td>Acne</td>
<td>Phase II</td>
</tr>
<tr>
<td>Oral</td>
<td>Protegrin analogue (IB-367)</td>
<td>Intrabiotics</td>
<td>Mucositis</td>
<td>Phase III</td>
</tr>
<tr>
<td>Oral</td>
<td>Histatin analogue (P-113)</td>
<td>Demegen</td>
<td>Gingivitis</td>
<td>Phase II</td>
</tr>
<tr>
<td>Systemic</td>
<td>Heliomycin</td>
<td>Entomed</td>
<td>Antifungal</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Systemic</td>
<td>Human lactoferricin</td>
<td>AM Pharma</td>
<td>Antibacterial</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Systemic</td>
<td>BPI</td>
<td>Xoma</td>
<td>Meningococcal meningitis</td>
<td>Phase III</td>
</tr>
</tbody>
</table>

Bactericidal Permeability Increasing protein.
3.1 Tryptophan and Arginine - rich AMPs

In this work special attention will be turned on a specific subset of the AMPs - the tryptophan (Trp) and arginine (Arg) rich peptides [36-38]. These residues possess some specific chemical properties that make them suitable for antimicrobial peptides. Tryptophan for example [39, 40] has preference for the interfacial region of lipid bilayers, while Arginine residues endow the peptides with cationic charges and hydrogen bonding properties crucial for interaction with the abundant anionic components of the bacterial membrane.

Arg and Trp residues are found in unusually high proportion in many antimicrobial peptides. Their roles have been investigated but it is not entirely clear which specific properties they bring to the antimicrobial peptides, apart from their positive charge and hydrophobic bulk, respectively.

Trp residues have been placed at many different and contrasting positions on hydrophobicity scales of amino acids. In certain cases, Trp is considered hydrophobic due to its uncharged sidechain, while on the other hand, it is observed that Trp residues do not reside in the hydrocarbon region of lipid bilayers and consequently it is placed towards the more hydrophilic side of the scale. Another important factor is the extensive π–electron system of the aromatic indole side chain that gives rise to a significant quadrupole moment (Fig. 3).

In combination, these two residues participate in cation –π interactions, thereby facilitating the peptide–membrane interactions. This has been observed for the antimicrobial peptide from human Lactoferrin [41, 42], possibly restraining the peptide structure in a suitable conformation to interact with the bacterial membrane. These unique properties make the Arg- and Trp- rich AMPs highly active even at very short peptide lengths [43].
The π-electron system of Trp results in negatively charged clouds that can participate in cation–π interactions that occur in proteins between the negatively charged electron cloud of any aromatic residue and various cationic species, such as ions or side chains of positively charged amino acids as Arg (Fig. 3).

Interestingly, in the stacked conformation, the Arg side chain is able to form almost as many hydrogen bonds with the surrounding water molecules as when it is not involved in any cation–π interactions. Those interactions make the entry of Arg into the hydrophobic environment inside a lipid bilayer energetically more favourable.

Other simulations highlight the importance of Trp residues in membrane–peptide interactions as well. The studied pentapeptides show that the Trp residues can associate with the positively charged choline head groups of the lipid bilayer. The hydrogen-bonding partners are no longer available, to the Trp residues, when the peptide inserts further into the hydrocarbon core.

Furthermore, the large and bulky shape of the indole side chain disrupts the hydrophobic interactions of the lipid acyl chains, when it buries deeper into the hydrocarbon core of a lipid bilayer.
3.2 Short tryptophan and arginine AMPs

Surprisingly, highly active arginine and tryptophan rich AMPs were synthesised ranging in size from 11 to 5 amino acid residues\(^{[37, 43]}\) in contrast to the natural cationic peptides, which generally contain 12 to 50 amino acids.

In order to elucidate the main structural requirement for such short AMPs, Strøm et al. have synthesised large number of those peptides. The amino acid sequences of the peptides were based on previous studies of longer bovine and murine lactoferricin derivatives. Most of the peptides showed strong inhibitory action against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, and the Gram-positive bacterium *Staphylococcus aureus*. For the most active derivatives, the minimal inhibitory concentration values observed for the Gram-negative bacteria were 5 µg/ml (3.5 µM), whereas it was 2.5 µg/ml (1.5 µM) for the Gram-positive bacterium.

They observed that, it was essential for the antimicrobial activity that the peptides contained a minimum of three tryptophan and three arginine residues, and carried a free *N*-terminal amino group and an amidated *C*-terminal end. Furthermore, a minimum sequence size of seven amino acid residues was required for a high antimicrobial activity against *Pseudomonas aeruginosa*. The insertion of additional arginine and tryptophan residues into the peptides resulted only in small variations in the antimicrobial activity, whereas replacement of a tryptophan residue with tyrosine in the hepta- and hexapeptides resulted in reduced antimicrobial activity, especially against the Gram-negative bacteria. The peptides were non-haemolytic, making them highly potent as prospective antibiotic agents (Tab. 3).
Tab. 3 Antimicrobial and haemolytic activities of short arginine and tryptophan rich peptides

<table>
<thead>
<tr>
<th>Name of peptide</th>
<th>Amino acid sequence (single letter code)</th>
<th>MIC E. coli</th>
<th>MIC P. aeruginosa</th>
<th>MIC S. aureus</th>
<th>Haemolysis EC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undeca 9</td>
<td>RRWYRWARMR-NH₂</td>
<td>10 (5.8)</td>
<td>7.5 (4.4)</td>
<td>5 (2.9)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Deca 1</td>
<td>YRWWRWARW-NH₂</td>
<td>10 (6.2)</td>
<td>7.5 (4.6)</td>
<td>2.5 (1.5)</td>
<td>720 (444)</td>
</tr>
<tr>
<td>Nona 1</td>
<td>YRWARWWR-NH₂</td>
<td>10 (7.0)</td>
<td>5 (3.5)</td>
<td>5 (3.5)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Octa 1</td>
<td>RRWYRWWR-NH₂</td>
<td>5 (3.7)</td>
<td>5 (3.7)</td>
<td>2.5 (1.8)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Octa 2</td>
<td>RRWWRWWR-NH₂</td>
<td>7.5 (5.4)</td>
<td>5 (3.6)</td>
<td>2.5 (1.8)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Hepta 1</td>
<td>RWWRRWWR-NH₂</td>
<td>10 (8.1)</td>
<td>7.5 (6.1)</td>
<td>2.5 (2.0)</td>
<td>920 (748)</td>
</tr>
<tr>
<td>Hepta 2</td>
<td>RWWRYWR-NH₂</td>
<td>10 (8.3)</td>
<td>10 (17)</td>
<td>2.5 (2.1)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Hexa 1</td>
<td>RWRWRW-NH₂</td>
<td>5 (4.8)</td>
<td>5 (19)</td>
<td>5 (4.8)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Hexa 2</td>
<td>WWRWRW-NH₂</td>
<td>10 (9.3)</td>
<td>10 (19)</td>
<td>5 (4.7)</td>
<td>590 (549)</td>
</tr>
<tr>
<td>Hexa 3</td>
<td>YWRWRW-NH₂</td>
<td>20 (19)</td>
<td>20 (48)</td>
<td>10 (9.5)</td>
<td>600 (571)</td>
</tr>
<tr>
<td>Hexa 4</td>
<td>RWRYRW-NH₂</td>
<td>50 (49)</td>
<td>50 (78)</td>
<td>10 (9.8)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Penta 1</td>
<td>WRWRW-NH₂</td>
<td>15 (17)</td>
<td>15 (56)</td>
<td>10 (11)</td>
<td>N.h.</td>
</tr>
<tr>
<td>Penta 2</td>
<td>RWRWR-NH₂</td>
<td>200 (233)</td>
<td>50 (58)</td>
<td>50 (58)</td>
<td>N.h.</td>
</tr>
</tbody>
</table>

**MIC** - Minimal Inhibitory Concentration in µg/ml and (µM).

**EC<sub>50</sub>** - concentration in µg/ml and (µM) required for 50% haemolysis.

N.h. - no haemolytic activity within the concentration range tested, i.e. up to 1000 µg/ml.

The susceptibility of AMPs to proteolysis and the high production cost are some of the limitations to their commercial use as antibiotics. Therefore, such shorter, modified peptides with similar or enhanced activities are desirable.

In a recent paper, the Metzler-Nolte group identified the first active metalloocene-modified short tryptophan-arginine AMPs<sup>[49, 50]</sup> (Tab. 4).

The ferrocenyl moiety can be regarded as unit of bulk<sup>[43]</sup>, as for example the tryptophan, and was attached at the N-terminus of AMPs on a solid phase peptide synthesis (SPPS, see Peptide synthesis). Studies of the antimicrobial activities of AMPs showed that the activity was enhanced compared to the results reported by Strøm et al. (Tab. 3 and 4, in grey) and even better than the naturally occurring peptide pilosulin (20 amino acid).

Therefore, we need to go further with the synthesis of new analogues and the rationalisation of the antimicrobial activity. To succeed it, we applied new techniques, which will be presented in this work (see Proteomic approach and Quantitative proteomics).
**Tab. 4 Metallicene-modified short tryptophan-arginine AMPs**

<table>
<thead>
<tr>
<th>AMPs</th>
<th>MIC (µM)</th>
<th>E. col</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-WRWRWR-NH$_2$</td>
<td>3.9</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Cc$^+$/CO-RWRWR-NH$_2$</td>
<td>15</td>
<td>121</td>
<td>&gt;121</td>
<td></td>
</tr>
<tr>
<td>FeCO-RWRWR-NH$_2$</td>
<td>16</td>
<td>33/16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>H-RWRWRW-NH$_2$</td>
<td>4.0</td>
<td>16</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>Cc$^+$/CO-RWRWRW-NH$_2$</td>
<td>6.8</td>
<td>27</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>FeCO-RWRWRW-NH$_2$</td>
<td>57</td>
<td>7.1</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>pilosulin 2</td>
<td>2.0</td>
<td>16</td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>

$Fe=[Fe(Cp)(C5H4)]$ and $Cc^+=[Co(Cp)(C5H4)]^+$ with counter ion $CF_3CO_2^-$.
3.3 Way of action of AMPs

In general, the mechanism of action\textsuperscript{[51]} of any of these AMPs is not very well established. For many of them, there is evidence that one of the targets for the peptide is the bacterial membrane. Most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a rational explanation for their specificity for bacterial membranes (Fig. 4).\textsuperscript{[52]}

![Diagram of AMP interaction with membrane](image)

*Fig. 4 The membrane target of the AMPs*

Bacteria are the major target for antibiotics. Thus before going further, the two major classes of bacteria will be briefly presented. The two distinct classes are Gram negatives and Gram positives (Fig. 5). The cytoplasm of all bacterial cells, contain the DNA, mRNAs, ribosomes, proteins, and metabolites. Most bacteria have a single circular chromosome. The cytoplasmic membrane has a lipid bilayer structure, similar to the structure of the eukaryotic membranes. The membrane contains transport proteins, enzymes and ion pumps to maintain a membrane potential.
Gram Positive Bacteria

A Gram-positive bacterium has a thick, multilayered cell envelopes consisting mainly of peptidoglycans surrounding the cytoplasmic membrane. The peptidoglycans can be degraded by treatment with lysozyme which degrades the glycan backbone of the peptidoglycans. Without the peptidoglycans, the bacteria succumb to the large osmotic pressure differences across the cytoplasmic membrane and lyse.

Gram Negative Bacteria

Gram-negative cell envelopes are more complex than Gram-positive cell envelopes, both structurally and chemically. Structurally, a Gram-negative cell envelope contains an external layer to the peptidoglycan layer, the outer membrane, which is presented only in this class of bacteria.

The area between the external surface of the cytoplasmic membrane and the internal surface of the outer membrane is called periplasmic space. This space also contains components of the sugar transport systems and other binding proteins to facilitate the uptake of different metabolites and other compounds.
In Gram-negative bacteria, both the outer leaflet of the plasma membrane as well as the outer membrane contains anionic molecules oriented towards the exterior of the cell. This is not the case for mammalian membranes.

Hence, the cationic antimicrobial peptides will preferentially bind to the exposed negative charges of bacterial membranes, but not to the zwitterionic amphiphiles present in the extracellular monolayer of mammalian plasma membranes.

However, there is no absolute proof about how these peptides perturb the membrane and whether this membrane perturbation is related to the antimicrobial activity of the AMPs. Recent publications show that there is not always a correlation between the ability of peptides to permeabilise membranes and their antimicrobial activity [53]. It is possible that the membrane effects of these peptides are not directly related to their mechanism of action but rather simplify the manner by which they enter the cell to reach the target.

With regard to the break down of the membrane permeability, it is possible that the peptide induces complete lysis [54] of the organism by rupture of the membrane or that they perturb the membrane lipid bilayer in a way which allows leakage of certain cellular components as well as dissipating the electrical potential of the membrane and the damaging of critical intracellular targets e.g. the peptide pyrrhocoricin [55]. Another example is the amphipathic helical peptide, cecropin (Gram-negative active), which will dissipate a transmembrane electrochemical gradient at a low peptide concentration [56].

The most commonly referred model, that can explain the activity of most antimicrobial peptides, is the **Shai-Matsuzaki-Huang** (SMH) model [52, 57-59] (Fig. 6). This model proposes an interaction of the peptide with the membrane, followed by displacement of lipids or alteration of membrane structure, and in certain cases entry of the peptide into the interior of the target cell.

In general, peptides operating by the SMH mechanism kill microbes at micromolar concentrations. In contrast, the peptide **Nisin**, a 14 amino acids amphipathic peptide produced by Lactococci, is an exception and has activity at nanomolar concentrations. **Nisin** binds with high affinity to Lipid II, the fatty acyl proteoglycan anchor in the bacterial membrane, from which it subsequently diffuses into the surrounding membrane [60].
Fig. 6 The Shai-Matsuzaki-Huang model [44] Various models of antimicrobial peptide activity A, B, and C all start from the same conformation, with the peptides associating with the bacterial membrane (top left). The red part of the peptide represents a hydrophilic surface, while blue is hydrophobic; In the barrel-stave model, the peptides span the membrane and form a pore lined with peptides (A); the carpet model is characterized by the peptides lining up parallel to the membrane surface and forming a peptide carpet, as the name implies. This is followed by a detergent-like action induced by the peptides that causes a pore formation (B); the toroidal pore model creates pores that contain peptides as well as lipid molecules that are curved inwards towards the pore in a continuous fashion from the surface of the membrane. The lifetime of these pores is believed to vary. After transient pore formation, the peptides end up in both leaflets of the bilayer, which presents a mechanism of shuttling the peptides inside. Longer lived toroidal pores may have a lethal effect similar to barrel-stave pores, i.e., they dissipate proton gradients, etc. (A, B, and C reproduced from Nature Reviews Microbiology [61]) (C); In the molecular electroporation model the cationic peptides associate with the bacterial membrane and generate an electrical potential difference across the membrane. When the potential difference reaches 0.2 V, it is thought that pores will be generated through electroporation (D); the sinking raft model proposes that binding of the amphipathic peptides causes a mass imbalance and consequently, an increase in local membrane curvature. As the peptides self-associate, they sink into the membrane, creating transient pores, which result in the peptides residing in both leaflets after their resolution (E).
The direct detection of the pores in membranes with structural information has been difficult. Without labeling, a peptide pore in a lipid bilayer does not provide sufficient contrast, in either electron density or neutron scattering length density, to be detectable by X-ray, electron or neutron diffraction.

However, Huang et al. have successfully crystallised the multiplepore states of magainin and protegrin-1 and IB367 \cite{59} in membranes and have provided neutron diffraction patterns of those compounds. Interestingly, the same crystallization procedure failed to crystallise the multiple-pore state of alamethicin. Perhaps this is another indication that there is a fundamental difference between the alamethicin pore and the pores made by self-defense antimicrobial peptides.

**Conclusion**

The objective of this part was to show the interesting character of the AMPs as well as the investigation of the mechanism of those specific compounds, because the knowledge of «what or where» is the target, can give useful information for further optimisations.

In the present work we will present other novel ways to study the responses of bacteria to antibacterial compounds. We will show that, proteome analysis and protein quantification techniques are useful for both target identification and target validation. More attention on those new techniques and on the results will be paid in the parts made in cooperation with the groups of Jun. Prof. Julia Bandow and Dr. Dirk Wolters.

To synthesise the tryptophan and arginine rich AMPs we used the peptided chemistry, common technique for both natural or unnatural peptides. The next chapter will introduce the basic notions, on which this technique is based.
4. Peptide Synthesis

The formation of a peptide bond, resulting in a dipeptide, is a very simple chemical process. The two amino acids are connected by a peptide (amide) bond \[^{62}\] with the elimination of water (Scheme 1).

The synthesis of a peptide bond under mild reaction conditions can only be achieved after activation of the carboxy function of one amino acid. The second amino acid attacks the activated carboxylic group in a nucleophilic attack, with formation of the dipeptide. If the amino function of the carboxy component is unprotected, then formation of the peptide bond occurs in an uncontrolled way. Consequently, during the course of peptide synthesis all functional groups not involved in peptide bond formation must be blocked, both temporarily and reversibly.

![Scheme 1 Formation of a peptide bond](image)

Peptide synthesis \[^{63}\] is therefore a three step procedure:

1. The preparation of a partially protected amino acid. After this protection, the zwitterionic structure of the amino acids is no longer present.
2. The formation of the peptide bond occurs in two partial steps. The N-protected amino acid must be activated at the carboxy function in order to be converted into a reactive intermediate. Subsequently, the formation of the peptide bond occurs.
3. Selective or total cleavage of the protecting groups is carried out. Although total deprotection is required only when the peptide chain has been fully assembled, selective cleavage of the protecting groups is usually necessary in order to continue the peptide synthesis.
4.1 Step one - Preparation of a partially protected amino acid

Peptide synthesis becomes further complicated by the fact that ten of the proteinogenic amino acids (Ser, Thr, Tyr, Asp, Glu, Lys, Arg, His, Sec, and Cys) have functional groups in the side chain which need to be selectively protected. Because of the different requirements with respect to selectivity, a distinction must be made between the intermediary (temporary) and semipermanent protecting groups. The intermediary groups are used for temporary protection of the amino or carboxy function involved in subsequent bond formations. These groups must be cleaved selectively under conditions that do not interfere with the stability of peptide bonds already present, or that of semipermanent protecting groups at amino acid side chains. The semipermanent protecting groups are usually cleaved only at the end of the peptide synthesis or, occasionally, also at an intermediate stage.

Temporary and semipermanent (usually cleaved at the end of the peptide synthesis) protecting groups are distinguished with respect to cleavage selectivity. A temporary protecting group must fulfill the following requirements:

♦ Introduction of the protecting group leads to an amino acid derivative that is no longer present in a zwitterionic structure
♦ Cleavage must occur without hampering the stability of semipermanent protecting groups or peptide bonds
♦ Racemization must not occur during all necessary operations
♦ Stability

4.1.1 Nα-amino Protection

Protecting groups of this type are used for the Nα-amino group of all proteinogenic amino acids. In principle, an amino group can be blocked reversibly by acylation, alkylation, and alkyl-acylation. Several hundred different amino-protecting groups have been developed during the past decades which confirm the non-existence of a universal, ideal amino-protecting group. A classification can be made based on the structure of the protecting group, or on the cleavage conditions: acidolysis, base cleavage, reduction/oxidation, nucleophilic substitution, and photolysis. Because most peptides are sufficiently stable under moderately acidic conditions, amino-protecting groups with different liabilities towards acidic deblocking agents are preferred. Alternately, the base-labile functional group Fmoc (9-
fluorenylmethoxycarbonyl group) has found widespread application. Some protecting groups may be cleaved by hydrogenolysis. Protection of the amino group can also be achieved easily by acylation and by alkylation. The disadvantage of structurally and chemically simple acyl residues (e.g., acetyl-, benzoyl-, -monochloroacetyl) is that the carboxamide group of the protecting function is chemically very similar to the peptide bond, which make the selective cleavage very difficult or even impossible, and this eventually led to the development of urethane-type amino-protecting groups.

### 4.1.2 Alkoxycarbonyl-Type (Urethane-Type) Protecting Groups

A variety of urethane type amino protecting groups, which are cleavable under different reaction conditions, is available. Only the most used, during this work (Table 5) will be discussed in the following section.

**Tab. 5 Urethane type amino protecting groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Symbol</th>
<th>Structure</th>
<th>Cleavage conditions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylloxycarbonyl</td>
<td>Z</td>
<td><img src="image" alt="Structure" /></td>
<td>(H_2/Pd, HBr/AcOH, Na/liq.NH_3)</td>
<td>[65]</td>
</tr>
<tr>
<td>tert-Butoxycarbonyl</td>
<td>Boc</td>
<td><img src="image" alt="Structure" /></td>
<td>TFA, TFA/DCM, HCl/organic solvent</td>
<td>[66, 67]</td>
</tr>
<tr>
<td>Fluorenyl-9-methoxycarbonyl</td>
<td>Fmoc</td>
<td><img src="image" alt="Structure" /></td>
<td>Piperidine, DBU, 2-aminoethanol, Morpholine, liq. NH_3</td>
<td>[68, 69]</td>
</tr>
</tbody>
</table>
**tert-Butoxycarbonyl group** (Boc) is, like the Z and the Fmoc group, one of the most important amino-protecting groups. It should also be mentioned that those protecting groups safeguard racemization-free peptide bond formations during the stepwise assembly of a peptide chain starting from the C-terminus.

Reagents such as tert-Butyl-S-4,6-dimethylpyrimidyl-2-thiocarbonate, 2-tert-butoxycarbonyloximino-2-phenylacetonitrile and especially di-tert-butyldicarbonate (Boc)_2O 1 are used (Scheme 2).

Scheme 2 Boc protection of a free amino group

The Boc protecting group is compatible with most coupling methods for peptide synthesis. It can be cleaved under mild acidolytic conditions, and is resistant towards catalytic hydrogenation, alkaline hydrolysis and reduction with Na/liquid ammonia. A cleavage method frequently used is treatment with non-aqueous TFA at temperatures around 0°C, which smoothly cleaves Boc groups (Scheme 3).

Scheme 3 Mechanism of acidolytic cleavage of the Boc group

Undesired tert-butylation may occur during acidolysis at nucleophilic sites of the peptide chain. For instance, the indole system of tryptophan or the thioether group of methionine can be tert-butylated. The intermediate carbenium ions responsible for these side reactions are usually trapped using scavengers such as anisole, thioanisole or triisopropylsilan (TIS). Further deblocking reagents are BF₃·OEt₂, 2-mercaptanesulfonylic acid, aqueous TFA, or 98% formic acid. This variety of reagents can be used when other protected functional groups are present.
**9-Fluorenylmethoxycarbonyl group (Fmoc)**[^1] is the only amino-protecting group of the urethane type of widespread practical importance that can be cleaved under mildly basic conditions. Usually, 20% piperidine in DMF is enough to cleave the Fmoc group within seconds at room temperature. Other reagents, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or fluoride ion may be used as an alternative. The reaction proceeds according to a mechanism, with initial proton abstraction to give the stabilized dibenzocyclopentadienyl anion. The dibenzofulvene formed reacts with piperidine to give a stable adduct as a by-product of the cleavage reaction (Scheme 4).

![Scheme 4 Mechanism of Fmoc cleavage](image)

Interestingly, the Fmoc group is not totally inert towards standard hydrogenolytic cleavage conditions. The reagents Fmoc-Cl and, alternately, Fmoc-OSu (9-fluorenylmethyl-\(N\)-succinimidyl carbonate) have been recommended for the formation of Fmoc-protected amino acids. Although, the Fmoc group was introduced into peptide chemistry in 1970, its application in the Merrifield solid-phase peptide synthesis was reported much later. Nowadays, the Fmoc tactics is one of the most preferred synthetic concepts in solid-phase peptide synthesis. The acid stability, combined with the mildly basic cleavage conditions, allows for the application of acid-labile semipermanent protecting groups of the tert-butyl type for side-chain functionalities in solid-phase peptide synthesis.
4.1.3 Carboxamide-Type Protecting Groups

Representatives of the carboxamide-type protecting groups are the formyl group 2 (For) which can be cleaved by solvolysis, oxidation, or hydrazinolysis, and the trifluoroacetyl group 3 (Tfa), which is labile towards alkaline conditions (Fig.7).

\[
\begin{align*}
R^1 & \quad \text{N} \quad R^2 \\
\text{O} & \quad \text{H} \\
\text{2 For, } R^1 &= \text{H} \\
\text{3 Tfa, } R^1 &= \text{CF}_3
\end{align*}
\]

Fig. 7 Carboxamide Protecting Groups

4.1.4 C\text{\textalpha} -Carboxy Protection

C-terminal protection has not been investigated as extensively as N-terminal protection. C\text{\textalpha} -carboxy-protecting groups in solution phase synthesis, and also as linker moieties in solid-phase synthesis, must be orthogonal to the temporary N\text{\textalpha} -protecting group. Carboxy-protecting groups may be divided into two categories on the basis of strategic-tactic criteria:

- The first group contains protecting groups which can be removed after the end of the synthesis by regeneration of the free carboxy group.
- The second group contains derivatives that are activated towards aminolysis at the end of a synthesis, either immediately or after special chemical transformation.
4.1.5 Side chain Protection

As the side chain functionality of an amino acid may give rise to undesired side reactions, it is especially important that those side-chain functionalities are protected in any situation. In particular, $\alpha$-amino or $\alpha$-carboxy groups of respectively diamino carboxylic acids or amino dicarboxylic acids must be mentioned in this context. The $\alpha$-protecting groups of trifunctional amino acids are termed «semipermanent protecting groups», because they are usually only cleaved at the end of the peptide synthesis. The thiol group of cysteine, and usually also the guanidino group of Arginine, also require semipermanent masking. Despite this possibility of minimal protection, maximum protection is preferred in a practical sense, and this applies especially to SPPS.

4.1.6 Protection for the guanidine group of the Arg

Although the guanidino group of Arg is usually protected by protonation under normal reaction conditions due to its strongly basic character, the low solubility of the corresponding derivatives in organic solvents makes the peptide synthesis difficult. Ideally, all three side-chain nitrogen atoms should be protected, but most strategies rely on $N^{\omega}$- or $N^{\omega}$-, $N^{\delta}$-protection, respectively. Despite the existence of a variety of blocking possibilities described for the strongly basic guanidino group, no ideal protecting group exists until now.

The $\omega$-nitro group is stable toward TFA and HBr/AcOH. Treatment with liquid HF and several reductive methods (Zn/AcOH, electrolysis) or catalytic hydrogenation with Pd or Raney-nickel are suitable methods for cleavage. $\omega$-nitro arginine is prone to other side reactions during acylation and cleavage. Lactam formation (Scheme 5) and consecutive aminolysis have been observed upon activation of protected $\omega$-nitro arginine derivatives ($R^2=NO_2$).
A single arene sulfonyl moiety offers complete protection of the guanidino group. Consequently, members of this class belong to the most commonly used guanidino protecting groups (Fig.8). The 4-toluenesulfonyl group (Ts) can only be removed by acidolysis with liquid HF or by reduction with Na/liquid NH₃. Arene sulfonyl type protecting groups for the guanidino function have been modified with respect to acid lability by the introduction of electron-donating substituents on the aryl residue. Groups such as 4-methoxybenzenesulfonyl (Mbs) [71], 2,4,6-trimethylbenzenesulfonyl (Mts) [72], 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) [73], 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) [74], and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) [75] have become very popular in SPPS.

Acid lability increases in the series Tos<Mbs<Mts <Mtr <Pmc <Pbf, with Pbf displaying the best deprotection kinetics.
4.1.7 Protection of the indole of the Trp

The indole ring of tryptophan usually does not require any protecting measures. A broad variety of synthetic conditions permit the incorporation of N<sup>in</sup>-unprotected tryptophan both as the amino or as the carboxy component. The indole system is prone to oxidation by either oxygen or peroxides, and therefore an inert gas atmosphere and absolutely peroxide-free solvents are required. Side reactions at the indole ring (e.g., electrophilic aromatic substitutions) have been observed during some deprotection reactions: tert-butylolation may occur both at N<sup>in</sup> and at different positions of the indole ring upon acidolysis of tert-butyl-type protecting groups. This also applies to other reactions involving carbenium ions. In most cases, these side reactions are suppressed by the addition of scavengers. Although tryptophan may be used without side-chain protection in Fmoc protocols, problems may arise during final deprotection. While indole tert-butylolation usually is suppressed by the addition of scavengers, sulfonation by the cleavage products of arene sulfonyl-type guanidino-protecting groups may be deleterious. In these cases, Fmoc-Trp(Boc)-OH provides a viable alternative of preventing Trp sulfonation, even in the case of peptides containing multiple Arginine residues. Indole Boc protection also diminishes the propensity of trialkylsilane scavengers to reduce the indole ring to an indoline ring.

4.2 Step two-Activation of the carboxy group

As we already have said the peptide bond is formed via nucleophilic substitution between an amino group (nucleophile) and a carboxy group. The N-protected amino acid must be activated at the carboxy function in order to be converted into a reactive intermediate (Scheme 6). Therefore, the carboxy component 4 (X-R<sup>2</sup> = OH) must be activated prior to peptide bond formation. Furthermore, the peptide coupling reaction must be performed under mild conditions, and at room temperature.

Activation of the carboxy component (an increase of the electrophilicity) is achieved by the introduction of electron-accepting moieties. Groups which exert either an inductive (-I) effect or mesomeric (-M) effect (or both) decrease the electron density at the C=O group, thereby favouring the nucleophilic attack of the amino component 5. The latter attacks with its nitrogen lone pair the electrophilic position of the carboxy group to give the tetrahedral
zwitterionic intermediate 6. Peptide bond formation is then completed by dissociation of the leaving group (nucleofuge $R^2X^-$) from 6. The leaving group capacity (nucleofugicity) is another factor which influences the reaction rate (Scheme 6). [63]

![Scheme 6 General mechanism of carboxy group activation](image)

The variation of the leaving group $XR^2$ provides a broad spectrum of methods for peptide bond formation for example: acyl azides, anhydrides, carbodiimides, active esters, and acyl halides, together with phosphonium and uranium reagents (Fig. 9).
4.2.1 Active Esters

Without doubt the most common method used in the peptide chemistry is this one of the activated esters. The mechanism arising here is similar with the described by Jakubke and Voigt \cite{77} for 8-quinolyl esters \(7\) where an additional proton-accepting group stabilises a hydrogen-bonded transition state during aminolysis. This leads to high aminolytic activity and extensively decreases the racemization-prone oxazolone formation. This general principle is outlined in Scheme 7 for the aminolysis of 8-quinolyl esters as general example.

8-quinolyl esters display significantly higher aminolysis reactivity because nucleophilic attack of the amino group to the carboxy group of the ester is favoured by the assistance of the quinoline nitrogen. This nitrogen atom acts as a basic catalyst and “delivers” the attacking amino group to the reactive center. As the reaction proceeds, the tetrahedral intermediate is
further stabilized by an intramolecular hydrogen bond. Racemization of 8-quinolyl esters 8 is disfavoured, because the amide oxygen in 9 cannot intramolecularly attack the weakly electrophilic ester group to give oxazolone 10 (Scheme 8).

![Scheme 8 Disfavoured racemization of 8-quinolyl esters](image)

The same situation applies to other active esters of high practical importance, such as derivatives of HOSu (N-hydroxysuccinimide) 10 \(^{[78]}\), HOBr (1-hydroxybenzotriazole) esters 11 \(^{[79]}\) and HONde (N-hydroxy-5-norbornene-2, 3-dicarboximide) 12 \(^{[80]}\). The 7-aza analogue of HOBr, commonly referred to as N-hydroxy-7-azabenzotriazole 13 (HOAt), has been recommended as a highly efficient additive for DCC coupling reactions \(^{[75]}\) (Tab.6). Active esters of protected amino acids can be synthesized by the mixed anhydride method, the carbodiimide method, the carbonate method, and other more specialized variants. Likewise, the crystalline N-hydroxysuccinimidyl esters are characterized by high reactivity with amines and low sensitivity towards hydrolysis in aqueous/organic solvent mixtures (water/ethanol, water/dioxan, water/tetrahydrofuran, etc.) that makes peptide syntheses feasible in such media. The water-solubility of the N-hydroxysuccinimide formed upon aminolysis is a further advantage of this type of active ester.
<table>
<thead>
<tr>
<th>Group</th>
<th>Symbol</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Quinolyl</td>
<td>OQ</td>
<td><img src="image1.png" alt="Structure Image" /></td>
</tr>
<tr>
<td>N-Hydroxysuccinimidyl</td>
<td>OSu</td>
<td><img src="image2.png" alt="Structure Image" /></td>
</tr>
<tr>
<td>N-Hydroxybenzotriazoly</td>
<td>OBt</td>
<td><img src="image3.png" alt="Structure Image" /></td>
</tr>
<tr>
<td>N-Norbornene-2,3-dicarboximidoxy</td>
<td>ONdc</td>
<td><img src="image4.png" alt="Structure Image" /></td>
</tr>
<tr>
<td>N-hydroxy-9-azabenzotriazoly</td>
<td>OAt</td>
<td><img src="image5.png" alt="Structure Image" /></td>
</tr>
</tbody>
</table>
4.2.2 Phosphonium Reagents

The coupling reagents, for in-situ generation of active esters, are of considerable importance in peptide chemistry. The HOBt-derived BOP (benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate) 14 (Fig. 10) developed by Castro et al. \[81\] is a very efficient reagent, which has also been applied successfully in SPPS. The main disadvantages of this reagent are, the highly toxic and carcinogenic HMPA formed during the reaction, and the high risk of racemisation \[82\]. The PyBOP coupling reagent 15 \[83\], where the dimethylamino groups are replaced by pyrrolidine substituents, represent viable alternatives to BOP that do not produce the hazardous by-product.

Sterically hindered amino acids often can be coupled successfully using bromo-tris(pyrrolidino)phosphonium hexafluorophosphate PyBroP 16 \[84\]. These reagents do not react with α-amino groups, and so may be added directly to a mixture of the amino and carboxy component to be coupled. A tertiary amine is usually added in order to form the anion of the carboxy component. Nucleophilic attack of the carboxylate leads to a highly reactive acylphosphonium species that immediately is transformed into the HOBt ester in the presence of HOBt.

![Fig. 10 Castro reagents](image-url)
4.2.3 Uronium Reagents

Several uronium reagents (Fig.11) have been used as coupling reagents in SPPS, for segment condensations, and other purposes of peptide and protein chemistry. Such reagents are 18, 17\textsuperscript{[85-87]} and 19, which are based on HOBt 11\textsuperscript{[79]} and HOAt 13\textsuperscript{[88, 89]}, respectively (Tab. 3).

\begin{center}
\begin{align*}
\text{HBTU } X^- &= \text{PF}_6^- \text{ 17} \\
\text{TBTU } X^- &= \text{BF}_4^- \text{ 18}
\end{align*}
\end{center}

\textit{Fig. 11 Uronium Reagents}

Nucleophilic attack of the carboxylate results in an O-acyluronium species that further reacts to give the HOBt (or HOAt) active ester (Scheme 9). If a reaction of the guanidinium form of HOBt (or HOAt) is taken into consideration, it may be attacked by the carboxylate ion and then undergo a further acyl migration to give the HOBt/HOAt active ester.

\begin{center}
\textit{Scheme 9 Activation process using uronium/aminium type reagents}
\end{center}

All phosphonium and uronium salt reagents based on HOBt (or even better, on HOAt) serve as direct coupling reagents and HATU is especially suited for peptide cyclizations\textsuperscript{[90]}. 

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4.2.4 Racemization\textsuperscript{[63]}

An inherent risk of racemization is imposed on all reactions involving functional groups directly connected to the stereogenic center of $\alpha$-amino acids. This may lead to a partial or total loss of the stereochemical information. While the expression “racemization” in organic chemistry is used for the complete conversion of a single enantiomer into the racemate, it is often used in peptide chemistry also for partial or total epimerization at one chiral center, irrespective of whether a mixture of diastereomers or enantiomers is formed in the course of the process.

4.3 Step three-Peptide Cleavage from the Resin

Final cleavage of the peptide from the polymeric support usually represents the last step of SPPS. As the anchoring group (linker) usually is chosen to be compatible with the peptide synthetic operations, selective cleavage between the C-terminus of the peptide and the solid support occurs upon treatment with reagents that may concomitantly effect partial or complete deprotection of the peptide side chains.
5. Solid-Phase Peptide Synthesis (SPPS)

Ever since the early days of peptide chemistry, reactions involving peptide synthesis have been traditionally performed in solution, and many impressive results – including the total synthesis of small proteins – have been achieved using this conventional technique. Unfortunately, peptide synthesis in solution is highly labor-intensive and requires extensive knowledge with regard to the strategy and tactics in choosing protecting groups and coupling methods, as well as solving problems of solubility. One major advantage of solution-based synthesis is the high purity of the final product, though this is clearly dependent upon the purification of intermediate compounds.

The ingenious concept of peptide synthesis on a solid support, known as SPPS, was developed by Robert Bruce Merrifield in 1963 [91], and provided a major breakthrough in peptide chemistry. Merrifield was awarded the Nobel prize in 1984 for this unique invention that has revolutionized organic chemistry during the past 20 years. Today, the concept has been extended and generalized to organic synthesis on polymeric supports, which includes not only heterogeneous reactions involving an insoluble polymer, but also the application of soluble polymeric materials which allow homogeneous reactions (liquid-phase peptide synthesis) to be conducted.

In SPPS the peptide chain is assembled in the usual manner, starting from the C-terminus. The amazingly simple concept is that the first amino acid of the peptide is connected via its carboxy group to an insoluble polymer that may be easily separated from either reagents or dissolved products by the use of filtration.

The general principle is shown in Scheme 10. In step 1 an amino acid which is protected [92] at N\textsuperscript{α} is reacted with the functional group of the linker. Subsequently, the temporary protecting group is removed (step 2) and the next amino acid is coupled (step 3). Steps 2 and 3 are then repeated (step 4) until the required peptide sequence has been assembled. Finally, the covalent bond between the linker moiety and the peptide chain is cleaved. In many cases the semipermanent side chain-protecting groups may be simultaneously removed (step 5). The insoluble polymeric support is then separated from the dissolved product by filtration. The product of all the reactions (the growing peptide chain) remains bound to the support during steps 2 to 4, and excess reagents and by-products are removed by filtration. Clearly,
the Merrifield synthesis had a major influence on chemical peptide and protein synthesis as well as on solid-phase organic synthesis.

Scheme 10 \(R^1, R^2, R^n, R^{n+1}\) = amino acid side chains, if necessary, protected with semipermanent protecting group.

Unfortunately, despite the high expectations that were initially imposed on the Merrifield method, it does suffer from some limitations:

♦ The final product of a synthesis carried out on a polymeric support is only a homogeneous compound if all deprotection and coupling steps proceed quantitatively.
♦ A large excess of each amino acid component is required in the corresponding coupling reaction in order to achieve complete conversion.
♦ There is a permanent risk of undesirable side reactions during activation, coupling, and deprotection.
♦ Monitoring the reaction progress and analysis of complete conversion are difficult to perform in heterogeneous reaction systems, and are hampered by experimental error.
♦ Swelling properties of the polymeric resin and diffusion of the reagents are important parameters for the success of a solid-phase synthesis.
♦ Aggregation phenomena of the growing peptide chain may complicate the synthesis.
♦ Drastic conditions required to cleave the peptide from the polymer may also damage the final product.

Although all methods described for synthesis of peptides and organic molecules on polymeric support rely on the basic principle introduced by Merrifield, the expression “Merrifield
“synthesis” has mainly been coined for SPPS. More specialized information is available in a series of review articles and monographs [93, 94].

5.1 Solid Supports

A solid support (organic polymer), chemically inert, mechanically stable and completely insoluble in the reaction solvent together with linker gave what is called a resin. It must contain a sufficient number of reactive sites where the first amino acid of the peptide chain to be synthesized can be attached. Interactions between the peptide chains bound to the resin should be minimal.

5.1.1 Merrifield resin [95, 96]

The chloromethyl group (linker) is the classical anchoring moiety present in the Merrifield resin, and it is introduced into polystyrene/divinylbenzene (organic polymer) resins by Friedel-Crafts-type chloromethylation with an alkoxy-substituted chloromethane in the presence of tin(IV)chloride. Attachment of the first amino acid to the resin is performed as a nucleophilic substitution reaction of chloride by the amino acid carboxylate.

The resulting benzyl-type peptidyl ester can be cleaved on completion of the peptide chain assembly only with very strong acids such as liquid HF or HBr/TFA. Consequently, this type of resin is used in combination with Boc as a temporary protecting group and semipermanent TFA-stable side chain protection of the benzyl or cyclohexyl type to obtain peptides with a free carboxy group at the C-terminus (peptide acids). One disadvantage of the Merrifield resin is that ~ 1–2% of the growing peptide is cleaved from the resin during each of the repetitive acidolytic deprotection steps.
5.1.2 Wang resin [97]

The Merrifield resin can easily be converted into the Wang resin by etherification with methyl 4-hydroxybenzoate, followed by reduction of the methyl ester with LiAlH₄. This anchoring group comprises a 4-alkoxy-substituted benzyl alcohol moiety, which confers increased acid-lability onto the linker. Hence, the Wang resin is used routinely in batch Fmoc chemistry. Active esters (e.g., pentafluorophenyl esters) or carbodiimides (e.g., DCC or carbonyldiimidazol) are used for the direct attachment of the first amino acid to the resin. Cleavage of the final product proceeds smoothly with 95% TFA, and yields peptide acids with concomitant removal of tert-butyl-type side chain-protecting groups.

5.1.3 Rink amide resin [98]

The combination of a benzhydrylamine group with two additional methoxy substituents on the second aromatic ring renders the (2,4-dimethoxy)benzhydrylamine resin even more labile towards acidolysis, and allows cleavage of peptide amides with 95% TFA.
5.1.4 2-Chlorotritylchloride resin (Barlos resin) \[^{[99]}\]

Attachment of the first amino acid to the 2-chlorotritylchloride resin is accomplished upon reaction with an amino acid carboxylate salt (e.g., diisopropylethylammonium or triethylammonium salts). This procedure does not suffer from racemization, as the amino acid acts as the nucleophile and not as an electrophilic species.

The steric constraint of the 2-chlorotrityl group impedes diketopiperazine formation on the dipeptide stage \[^{[100]}\]. Cleavage occurs upon treatment with 0.5% TFA in dichloromethane or with 1, 1, 1, 3, 3, 3- hexafluoropropanol in dichloromethane. The 2-chlorotrityl handle is also suitable for the attachment of C-terminal alcohols, thiols, and amines.
5.2 Fmoc/tBu-protecting Groups Scheme (Sheppard Tactics)

The Fmoc-protecting group tactics makes use of the base lability of the fluorenyl-9-methyloxy carbonyl group (Fmoc). It is a widely applied alternative to the Boc/Bzl scheme (Merrifield Tactics) with two-dimensional orthogonality (Fig. 12).

Fmoc is cleaved by base-catalyzed elimination where the secondary amine (piperidine) also traps the dibenzofulvene initially formed in the reaction. The semipermanent side chain-protecting groups are mostly of the tert-butyl type, and can be cleaved under relatively mild reaction conditions with TFA (Tab.7). Linker moieties displaying comparable acid lability are mainly used. The following side chain-protecting groups have been used preferentially in Fmoc tactics.

Fig. 12 Fmoc/tBu tactics in SPPS according to Sheppard.
### Tab. 7 Side chain-protecting groups

<table>
<thead>
<tr>
<th></th>
<th>Boc</th>
<th>tBu</th>
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### 5.3 On-Resin Monitoring

The ninhydrin test is one of the simplest and most frequently used methods for the on-resin monitoring \[101\]. A positive color reaction, performed with a small aliquot of the resin material, indicates unconverted amino groups. This so-called Kaiser test is simple, reliable (in most cases), and requires only minutes to perform.

Titrmetric methods are also quite easy to perform. Further monitoring methods that rely on color reactions include the TNBS test \[102\], the acetaldehyde/chloroanil test \[103\], and the bromophenolblue test \[104\]. Dorman developed a protocol involving the determination of chloride ions after protonation of unconverted amino groups of the peptide with pyridinium chloride and subsequent elution of bound chloride with triethylamine \[105\]. Cleavage and total dedprotection of an aliquot of resin-bound peptide after a certain number of synthetic steps is also an important and sensible tool to check the integrity of the product. Analysis may also be performed on each synthetic step by mass spectrometry (MALDI-ToF MS or ESI) \[106\] or HPLC after each cleavage.
5.4 Automation of the Process

Robert Merrifield developed the first automated peptide synthesizer in 1966 for batch-wise peptide synthesis (Fig. 13). The original machine, which is now in the Smithsonian Museum, consists of a reactor unit and a controlling unit. The former comprises the reaction vessel and valve systems for solvents, reagents, and amino acid derivatives as well as reservoirs for all these components.

Fig. 13 Robert Bruce Merrifield and the first-generation SPPS synthesizer and new generation SPPS synthesizer

Nowadays, different types of synthesizers are commercially available. SPPS may also be performed in a continuous-flow mode using resin-filled columns.
Chapter II. Aims of the project

In this project the effect of metallocenes on the antimicrobial activity of short tryptophan (Trp) and arginine (Arg) containing antimicrobial peptides (AMPs) is studied. Since the presence of Trp and Arg residues is crucial for the AMPs activity, this part will remain unmodified.

By using convenient and straightforward solid phase peptide synthesis (SPPS) these organometallic AMPs should be obtained with good yields and purity. Introduction of either ferrocene or ruthenocene allows assessment of the effect of the metal centre on the antimicrobial activity. The measured minimum inhibitory concentration (MIC) values will be used for monitoring of the antimicrobial activity. Moreover the most active compounds will be investigated through a proteomic approach, giving information about the proteins, which are involved in the regulation of the bacterial membrane. In order to enhance the activity of the promising candidates the synthesis of multivalent species will also be explored.
Chapter III. Results and discussions

6. Peptidomimetics and peptoids

It is well known that peptides (neurotransmitters, neuromodulators and hormones) and proteins play a crucial role in all biological processes. In fact it is difficult, if not impossible, to name any biological process which does not involve those biopolymers. However, the use of peptides as drugs is limited by the following factors (Fig. 14):

- low metabolic stability towards proteolysis in the gastrointestinal tract and in serum;
- poor absorption after oral ingestion, in particular due to their relatively high molecular mass or the lack of specific transport systems or both;
- fast excretion through liver and kidneys;
- undesired effects caused by interaction of the conformationally flexible peptides with various receptors;

Since the application of peptides as drugs has been hampered by poor bioavailability or biodegradation by proteases the development of new compounds, aiming at the improvement of those disadvantages, increased.

To solve this problem peptidomimetics, compounds that act as substitutes for peptides, have been synthesised. In comparison with native peptides they have more rigid backbone and show higher metabolic stability, better bioavailability, and longer action. The peptoid is the base unit of the peptidomimetics.

Perhaps the most well known class of peptoids were the N-substituted glycine derivatives. Those compounds do not have chiral centres with the exception for proline.

\[
\begin{align*}
\text{N-substituted glycine}
\end{align*}
\]
Fig. 14 In solution peptides exist in a variety of conformations that are in dynamic equilibrium with each other. If a conformational restriction is introduced to the bioactive conformation of the peptide A and B cannot arise. Thus, the interaction with alternative receptors and peptidases is suppressed or does not occur. In this fashion a desired biological effect can be obtained. [113]
It is also realistic to assume that peptoids, due to the absence of a recurrent peptide bond, will be less sensitive to proteolytic degradation normally occurring at the N-terminus of the peptide. Indeed, that has been proven to be the case in the examples studied so far \cite{113, 114}.

Going back to the project aim which was to obtain modified active AMPs then the peptoids become excellent candidates for backbone modification due to the following advantages:

- straightforward synthesis
- easily assembled from primary amines
- possible use of SPPS

Sequence-specific peptoids are assembled by the solid-phase «submonomer method» where N-terminal secondary amine was reacted with bromoacetic acid and then via \(S_N2\) the bromide was displaced by a desired primary amine (submonomer).

The template peptide MP\textsubscript{66} (Fig. 15) contains a short sequence of tryptophan and arginine, already introduced (see Tab. 4), where a submonomer unit was introduced to give modified peptide 24 (Fig. 15, peptoid part in red).

![Chemical structure](image)

**Fig. 15 MP\textsubscript{66} and 24 with the peptide - peptoid backbone (in red)**

The submonomer unit that was introduced was the tryptamine. It is a monoamine alkaloid found in plants, fungi, and in trace amounts in the brains of mammals and is believed to play a
role as a neuromodulator or neurotransmitter. The most well-known tryptamines are Serotonin (important neurotransmitter) and Melatonin (hormone involved in regulating the sleep-wake cycle). Tryptamine alkaloids found in fungi, plants and animals are commonly used for their psychotropic effects. Many synthetic tryptamines have also been synthesised, including the migraine drug Sumatriptan.

![Chemical structures of Tryptophan, Tryptamine, Serotonin, Melatonin, and Sumatriptan](image)

6.1 AMPs with peptoid - peptide backbone on SPPS

For the SPPS «submonomer method» we used an Fmoc protected Rink amide resin. For the next step Tryptamine was modified to fit to the SPPS using an orthogonal strategy of protection. In first place the free primary amine was reacted with fluorinated acid anhydrides and then the pyrol of the indol ring was protected with Boc anhydride. Removal of the Tfa group gives the desired primary amine 22 (Scheme 11). The order of protection is very important in order to provide suitable species for SPPS.
Scheme 11 Synthesis of N-Boc-indol-Tryptamine 22

For the successful incorporation of 22, into peptides by SPPS, successive $S_N^2$ reactions were used, as described in the following procedure (Scheme 12). The resin was swollen in DMF for 30 minutes and then the solvent was drained. A mixture of piperidine/DMF (1:4) was then added (Step 1) to liberate the N-terminus. After drainage the resin was washed with DMF and methylene chloride. Kaiser test $^{[101]}$ was used to follow the deprotection of the Fmoc group resulting in a deep blue-violet colour of the solution due to the free amino group.

Scheme 12 Peptoid backbone on SPPS

Then 2 M bromoacetic acid in DMF and 2 M DIC in DMF (Step 2), were added to the unprotected resin and placed in an oven at 50°C ($2 \times 30$ min). Afterwards the bead were washed with DMF and again checked with the Kaiser test. After acylation the resin was supposed to keep original yellowish colour; nevertheless sometimes blue-violet shape was observed.
For the nucleophilic displacement a 1 M solution of 22 in DMSO was prepared and added to the resin then allowed to react for 3 hours. The resin was then filtered and washed following classical washing procedures, with DMF and methylene chloride (Step 3).

For this step the Chloranil test \[^{[115]}\] was more accurate regarding its sensitivity for detection of secondary amines. A few beads of resin were placed in a small test tube and 2-5 drops of the Chloranil test were added. The mixture was left at room temperature and the beads were inspected after 5 min and showed blue-green colour. Dark blue to green beads were proof for a positive test and colourless to yellowish bead for a negative.

In the next step, the resin was suspended in a solution of 1% AcOH in DMF and Fmoc-aminoethanal 69 (2.5 eq. relative to the resin loading) and NaBH\(_3\)CN (7.5 eq. relative to the resin loading) were added for the reductive amination (Step 4). The mixture was shaken overnight and then quenched with MeOH followed by the standard washing procedure. The condensation was controlled again with the Chloranil test. The final peptoid backbone was obtained after deprotection of the Fmoc protecting group providing a suitable fragment for standard SPPS.

The following protected amino acids (Fmoc-AA(PG)OH, Scheme 13) were reacted with TBTU/HOBt in order to form activated OBt-esters. DIPEA was added to help the deprotonation of the acid and thus the formation of the carboxy activated form. The active ester was then reacted with the deprotected resin. In the present case experience shows that double coupling is needed for better yield.

A step of coupling is followed by an Fmoc deprotection and after the final Fmoc deprotection the N-terminus of the peptide was acetylated. The resin was washed with MeOH and dried \textit{in vacuo}. The peptide was cleaved from the resin with a mixture of 95% TFA, 2.5% H\(_2\)O and 2.5% TIS (1 × 3 h).
After removal of the cleavage cocktail under reduced pressure, the crude product was precipitated from cold ether. Preparative RP-HPLC purification gave more than 90% purity and after lyophilisation 23 was obtained as a white powder. LC-MS showed a base peak at 494.2 m/z corresponding to \([M+H]^{2+}\). A more detailed description of the SPPS can be found in the experimental section.

The \(^1H\) NMR (Fig. 16) did not clearly show all the amide proton signals, due to proton/deuteron exchange with the solvent. Assignment of the signals was supported by \(^{13}C\) NMR and HH-COSY 2D NMR.
The NMR analysis was further complicated by the fact that the solvent peak overlapped with the H$^\beta$ of the Trp and the H$^{b,c}$ of the modified fragment. Another overlap was observed between the H$^\alpha$ and the H$^\alpha$ of the Arg (Fig. 17).

It is important to mention that the synthesis of this peptide is providing a relatively good yield and purity only in case of double coupling. A standard single coupling is not satisfying the criteria of yield and purity and this observation was confirmed for all tryptophan – arginine sequences. The purified compound was stored at -4 °C, and did not show degradation.
7. Ferrocene. Ferrocenyl chemistry

In 1951 a new compound containing iron and two cyclopentadienide ligands was reported and shortly afterward the structure was published independently by Wilkinson and Fischer. Its behaviour and reactivity were similar to that of benzene and Woodward proposed the name «ferrocene» for this new molecule. The term «sandwich compound» came later and is now worldwide used for a wider class of similar compounds.

It is well known that the ferrocenyl group is stable in aqueous, aerobic media and has favourable electrochemical properties. Those properties have made ferrocene and its derivatives very interesting for biological applications and for conjugation with biomolecules, as amino acids or peptides, proteins, DNA, RNA, PNA and others.

Recently, organometallic derivatives of biomolecules have attracted much attention. The metal could confer an increased stability to the conjugate in biological media and, in certain cases metal-specific modes of action were suggested, for example by the lipophilicity or redox properties of the metal complex. Replacement of a phenyl ring in the well known anticancer drug, tamoxifen, by the ferrocenyl group results in a spectacular change of the pharmacodynamic profile which can be traced to the redox activity of the organometallic group.

In early work, the antibacterial activities of the ß-lactam antibiotics, penicillin and cephalosporin, were claimed to be enhanced by replacing aromatic groups with a
ferrocenyl group; although no comprehensive data was provided. The structures of the ferrocene derivatives are shown in Fig. 18.

Furthermore, structural variations of established drugs were also reported, such as ferrocenyl aspirin \cite{127} and the anti-malarial drug ferroquine \cite{128-132} and the anti-cancer drug ferrocifen \cite{133}.

![Fig. 18 Ferrocene derivatives](image)

That’s why we had the idea to use AMPs (see Antibiotics) and ferrocene as a new class of antibacterial compounds. Moreover, metallocenes in peptide chemistry were rarely been described before due to the instability of the ferroceny moiety \cite{134-138} especially on SPPS. Fortunately, this kind of problem was avoided with the introduction of phenol in to the cleavage mixture as antioxidant, which strategy was already described by the Metzler-Nolte group \cite{49, 50}. Thus the SPPS strategy was successfully used to provide the desired product in good yield and in high purity.
7.1 Peptoid-ferroenyl AMPs on SPPS

The synthesis of ferrocenyl AMPs 24 (Fig. 19) follows the strategy used for the synthesis of 23. After the final Fmoc deprotection the N-terminus was left free. The ferrocene carboxylic acid was reacted with HATU/HOBt in order to form the activated OBt-esters. DIPEA was added to help the formation of the carboxy activated form. The active ester was then reacted with the deprotected peptide.

The resin was washed and dried and treated with a cleavage mixture of 85% TFA, 1% Phenol, to avoid oxidation of the ferrocene during the cleavage, and 0.5% of TIS. The mixture was then left for 3 hours and after removal of the cleavage cocktail under reduced pressure, the crude product was precipitated with cold ether.

![Peptoid-peptide ferrocenyl compound 24](image)

After purification by preparative RP-HPLC and lyophilization the desired product was obtained as a yellow powder. ESI shows a base peak at 579.3 m/z, which corresponds to [M+H]^{2+} (Fig. 20).
Fig. 20 HPLC trace of 24 at 220 nm and ESI (positive) with the pattern of the iron (in orange)

The pure compound was stable in air and aqueous solutions, but decomposed slowly in organic solvents like DMF, MeOH and DMSO. This decomposition can be related to ferrocene oxidation in the case of MeOH-d₄ supported by the observation of broadened NMR signals caused probably by the paramagnetic Fe³⁺.

In case of DMSO-d₆, the Cp signals of the ferrocene are clearly visible and show as expected a ratio of 1-1-2-5 (N-terminal bound ferrocene) between 4.80 and 4.65 ppm and 4.30 and 3.80 ppm, respectively (Fig. 21. case A).

Fig. 21 The ¹H NMR (400 MHz, DMSO) spectrum shows four signals for the ferrocene group (A) and the ¹H NMR (400 MHz, CD₃OD) shows signals which overlapped with other proton signals (B).
7.2 Ferrocenyl cyclic AMPs on SPPS

A cyclic Arg-Trp ferrocenyl AMP was obtained according to the synthesis of octreotide \cite{139, 140} a cyclic octapeptide. To achieve the cyclisation on SPPS another two amino acid residues were introduced to the template peptide. Two Cys(Acm) residue were used for the direct disulfide bond formation, by Ti(CF$_3$COO)$_3$ treatment, and one Gly residue to attach the ferrocenyl moiety (Scheme 14).

Scheme 14 Ferrocenyl cyclic AMPs 25 on SPPS. Tryptophan (W) and arginine (R) were protected with Boc and Pbf, respectively.
As usual, Rink amide was used for the SPPS of this peptide. HOBr/ TBTU/ DIPEA were used for the coupling of each amino acid residue and piperidine/ DMF for the Fmoc deprotection. HATU/ HOBr/ DIPEA were used only for the ferrocenyl attachment, due to the better coupling results.

The on-resin disulfide bond formation was successfully obtained by Tl(CF₃COO)₃ treatment in DMF for 24 h. After the disulfide bond formation, the peptide-resin was washed with DMF, CH₂Cl₂, and methanol and cleaved with TFA (85 %), Phenol (1%) and TIS (0.5%) for 3 h.

**Fig. 22** The IR spectrum did not show a typical thiol absorption bond (A); ESI positive moddus of 25 (B); Elman test for detection of free thiols: 1st solution - Tris dilution buffer, 2nd - DTNB but no peptide inside, 3th - DTNB and peptide and no yellow colour was observed, thus no free thiol. The probes 4-6 contain DTNB and different concentrations of DTT as controls and clearly show the detection of a free SH groups. (C)

After purification by preparative RP-HPLC and lyophilisation the desired product was obtained as a yellow powder. ESI shows a base peak at 1361.2 m/z (Fig. 22, B) corresponds to the exact molecular mass. The IR measurement did not show any thiol bond at 2600-2550 cm⁻¹ (Fig. 22, A).
Furthermore, an Ellman test for detection of free thiols was also negative (Fig. 21, C). This test uses the fact that DTNB (5,5’-dithiobis(2-nitrobenzoic acid) reacts quantitatively with aliphatic sulfhydryl groups to generate a yellow anion. Free thiol was not detected.

Additional experimental data were collected from the measured $^1$H and $^{13}$C NMR as well as from the HMBC and HMQC 2D NMR experiments. No upfield shift of the signals was observed (around 1.5 ppm or 27.1 ppm for $^1$H and $^{13}$C, respectively) which may be provoked by the presence of a free SH group (Fig. 23).

![Fig. 23 $^1$H (400 MHz, DMSO) and $^{13}$C (100 MHz, DMSO) of 25: the ferrocenyl signals are marked in blue; characteristic carbon signals of the ferrocenyl moiety (A).](image)

Proton and carbon peaks from the substituted Cp ring show an interesting pattern (Fig. 23, A). Due to the fact that the chiral center was not next to the c and c’ the two signals were with low difference in the chemical shift in the $^{13}$C NMR and in the $^1$H NMR only one signal was observed. It is important to know that the compound decomposes easily in DMSO-d$_6$ losing its non-substituted cyclopentadienyl ring and the iron atom.

The purified compound was stored at -4 °C and does not show degradation when there is no organic solvent.
8 SPPS of Ruthenocenyl AMPs 28

The use of iron as a cofactor in metabolic pathways is essential to both pathogenic microorganisms and their hosts. As compounds 24, 25 and the template peptide MP contains ferrocene, a metallocene with another metal than iron was needed to evitate possible confusion with the iron background from the bacteria. Thus we could use 28 to quantify the distribution of the peptide in different subcellular fractions (cytosol, membrane, cell wall). This analysis is currently under investigation.

27 was synthesised according to the procedure of Schotter et al. starting from commercially available ruthenocene, which was Friedel-Crafts monoacylated to 26 and then hydrolysed to give acid 27 (Scheme 15).

![Scheme 15 Synthesis of 27 according to Schotter et al.](image)

Rink amide was used for the SPPS of the peptide with HOBt/ TBTU/ DIPEA as coupling agents and piperidine/ DMF for the Fmoc deprotection. HATU/ HOBt/ DIPEA were used only for the ruthenocenyl attachment.
After the end of the synthesis the resin was washed with DMF, CH$_2$Cl$_2$, MeOH and dried. The peptide was then cleaved with TFA (85%), Phenol (1%), TIS (0.5%) for 3 hours.

After purification by preparative RP-HPLC and lyophilisation the desired product was obtained as a white powder with a very poor yield (2 mg). LC-MS shows a base peak at m/z = 573, corresponding to [M+H]$^{2+}$ (Fig. 24).

To better understand the problem occurring during the synthesis, a test reaction was setup. Compound 27 and Glycine methyl ester hydrochloride were reacted. The hydrochloride of the amino acid was first cleaved, with Et$_3$N, and then coupled with the same agents (HATU/ HOBt/ DIPEA) as used for the synthesis of 28. The product was obtained in quantitative yield and NMR and ESI were as expected. Thus this problem was due to the cleavage and it is not related to the starting material 27 or to the coupling conditions. This synthesis is still under investigation and the cleavage conditions probably need to be revised.
9. Dendritic-Branching Concepts

The origins of the dendritic branching concepts can be traced back to the initial introduction of infinite network theory by Flory\textsuperscript{[144]} and Stockmayer\textsuperscript{[145]}. In 1943, Flory introduced the term \textit{network cell}, which he defined as the most fundamental unit in a molecular network structure.

Graessley\textsuperscript{[146]} took the notion one step further by describing ensembles of these network cells as micronetworks. Extending the concept of Flory’s statistical treatment of Gaussian-coil networks, analogous species that are part of an open, branched/dendritic organization are known as \textit{branch cells} and \textit{dendritic assemblies} (designed to mimic the morphological branching of trees).

In the late 1970s, dendrimers had emerged as a new class of compounds between small-organic molecules and polymers. The synthesis of multi-branched compounds was pioneered by Vögtle\textsuperscript{[147]} and co-workers in 1978 with the so-called «cascade molecules». The field was further developed in the mid-1980s when Newkome\textsuperscript{[148]} synthesized tree-like molecules termed “arborols” and Tomalia\textsuperscript{[149]} reported the synthesis of poly(amidoamine) and coined the term “dendrimer” (greek \textit{dendri}-tree and \textit{meros}-part of) for this class of compounds. The most widely studied dendrimer families are the Fréchet-type polyether compositions\textsuperscript{[150-152]} and the two now commercially available dendrimers PAMAM, poly(amidoamine), and PPI, poly(ethyleneimine) which trade name is Astramol\textsuperscript{[150, 153]}.

Since the PAMAM (Scheme 16) polymers were commercialised they find widespread application as dendrimer-antibody conjugates in commercial immunoassays for the rapid and sensitive detection of cardiac markers indicative of heart attacks (in vitro diagnostics). They form dendrimer-DNA complexes with low cytotoxicity (in vitro gene transfection). Moreover a variety of molecules, such as drugs and other therapeutic agents, can be loaded on the PAMAM dendrimers to control the rate of release of these agents into the body (controlled drug delivery).
Scheme 16 Commercially available dendrimers PAMAM
At the moment the number of research groups focussing on the development of multivalent dendrimeric molecules increases. The multivalent conjugates enhanced the overall binding affinity compared to monovalent ligands. [147, 151]

Thus looking back to the Shai-Matsuzaki-Huang concept (see Way of action of AMPs) and comparing it to the branched morphology raised the idea to synthesise a molecule where several AMPs are attached to a core. We were interested to explore which will be the effect of those poly AMPs compounds on the bacterial membrane (Fig. 25).

Fig. 25 General structure of a branched (multivalent) molecule in comparison with the AMPs model of action

Liskamp et al. [154] have prepared for example multimers of cyclic Arg-Gly-Asp (RGD) peptides to evaluate their binding characteristics toward a specific receptor. Studies have shown that tumour uptake of a tetrameric RGD dendrimer was significantly higher, compared to that of the monomeric analogues [155].

Especially challenging is the efficient conjugation of these biologically active peptides to a dendrimer. A fast coupling reaction with high efficiency and chemoselectivity is desirable, to allow the conjugation of protected or unprotected peptides and thus a well-defined multivalent peptide dendrimer.
9.1 Branched (multivalent) molecules synthetic approaches

In the present work, the Cu\(^1\) catalysed variant of the Huisgen 1,3-dipolar cycloaddition\(^{[156]}\) of azides and alkynes to afford 1,2,3-triazoles (Scheme 17) was used (Cu-AAC).

The Huisgen cycloaddition is the reaction of a dipolarophile with a 1,3-dipolar compound that leads to 5-membered heterocycles. Dipolarophiles are alkenes and alkynes and molecules that possess related heteroatom functional groups (such as carbonyls and nitriles). 1,3-Dipolar compounds contain one or more heteroatoms and can be described as having at least one mesomeric structure that represents a charged dipole.

![Scheme 17 Mechanism of the Huisgen 1,3-Dipolar Cycloaddition](image)

In fact, the kinetic stability of alkynes and azides is directly responsible for their slow cycloaddition, which generally requires elevated temperatures and long reaction times. Good regioselectivity in the uncatalyzed Huisgen-type cycloaddition is observed for coupling reactions involving highly electron-deficient terminal alkynes, but reactions with other alkynes usually afford mixtures of the 1,4- and 1,5-regioisomers.

![1,4 and 1,5 regioisomers](image)

Since the initial discovery of Cu\(^1\) catalyzed alkyne–azide coupling, numerous systematic study have been reported in the literature, but no of optimal conditions has been reported, yet. Moreover, the conditions have varied widely, particularly with respect to generating of the active Cu\(^1\) species. Sources of Cu\(^1\) include Cu\(^1\) salts, most commonly CuI,\(^{[157]}\) in-situ
reduction of Cu$^{II}$ salts, particularly Cu$^{II}$ sulfate, and comproportionation of Cu$^{0}$ and Cu$^{II}$. Steric factors and electronic effects may also play role in the success of this «click» concept. Recent reports suggest that nitrogen-based ligands can stabilize the Cu$^{I}$ oxidation state under aerobic, aqueous conditions and promote the desired transformation. 

**Scheme 18** Mechanism of 1,4-disubstituted 1,2,3-triazoles via Cu$^{I}$ catalysed «click» concept.

Since Sharpless et al. have introduced the Cu$^{I}$-catalyzed «click» concept in 2002, interest and applications of triazoles has increased dramatically (Scheme 18). The applications ranging from therapeutics to macromolecules, combinatorial chemistry, organic synthesis, bioconjugation, and other fields are just beginning to be explored. Furthermore unprotected peptides efficiently attached to an alkyne dendrimers, using the 1,3-dipolar cycloaddition reaction, were also reported.

Although the scope of this reaction is remarkable, not every azide or alkyne gives good results. Highly electron-deficient fluorine-substituted azides react sluggishly with low yields in general. This in fact indicates that alkyne–azide coupling requires a more electron rich azide. In contrast, electron-poor alkynes actually accelerate the reaction by facilitating the formation of the metallocycle (Scheme 18). Steric constraints may also limit the success of this reaction, but only in extreme cases, otherwise alkynes and azides for the most part “click” together.
9.2 Synthesis of core precursors

Compound 29 was chosen as core precursor. This starting material has the advantages to be commercially available and to provide a relatively large number of straightforward synthesised molecules (Scheme 19). The complete synthesis of those compounds is available in the experimental part.

Scheme 19 Overview of the synthesised core precursors

Compound 34 was successfully obtained from 29 and used for the synthesised of 35 as described by Newman et al. \cite{171, 172}. Unfortunately, after numerous extractions from the aqueous phase, 35 was not isolated.
For the synthesis of compounds 36 and 37, different starting molecules were used (Scheme 20). Various strategies were applied to 29, 31 and 38, which unfortunately were unsuccessful and this data will be presented in Tab. 8. Reactions such as nucleophilic substitution (row 2 to 5 and 7); amide bond formation (row 6); Kochi reagent for copper catalysed coupling of Grignard reagents and alkyl halides (roll 1); etherification (row 8) of OH groups of phloroglucinol, which is known only in few cases and the resulting tris-ethers are accessible only in poor yield, were used.

Scheme 20
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Starting Molecule</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Li2Cl4Cu</td>
<td></td>
<td><img src="image1" alt="Image" /></td>
</tr>
<tr>
<td>2 K2CO3</td>
<td></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>3 Et3N</td>
<td></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>4 K2CO3, 80°C</td>
<td></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>5 TMS</td>
<td></td>
<td><img src="image5" alt="Image" /></td>
</tr>
</tbody>
</table>
6
$$\text{C}_{2}H_{4}COOH$$

TBTU/HOBt/DIPEA

7
$$\text{C}_{2}H_{4}Br$$

Et$_3$N

8
$$\text{C}_{2}H_{4}Br$$

K$_2$CO$_3$
Synthesis of 30

Compound 30 was easily synthesised in good yield of more than 80% via SN reaction\cite{175, 176} of bromide displacement with the azide ion. The reaction was performed with a large excess of NaN$_3$ (10 eq.) and refluxed overnight in acetone. It must be kept in mind that this step is dangerous, due to the explosive nature of the azides. The compound should not be heated when it is dry and a blast shield should be used.

After purification the compound was characterised with $^1$H, $^{13}$C NMR and GC-MS. To confirm the presence of an azide function, an IR spectrum was measured from both the starting material and the product, and the presence of an absorption band between 2160 and 2120 cm$^{-1}$ confirmed the successful azide synthesis (Fig. 26).

![Fig. 26 IR of starting material 29 and triazide 30](image)

This compound was a suitable core precursor for the «click» reaction giving three equal reaction sides.
Synthesis of 31

Three different strategies were involved in the synthesis of compound 31 - the Gabriel reaction\(^{[177]}\), reduction with LiAlH\(_4\)\(^{[175]}\) and Staudinger reaction\(^{[178]}\). Only the Staudinger reaction (Scheme 21) gives an optimal result. The mechanism is involving triphenylphosphine reacting with the azide to generate a phosphazide, which loses N\(_2\), to form an iminophosphorane.

Scheme 21 Mechanism of the Staudinger reaction

The separation of the free amine and phosphine oxide is possible due to the difference in the solubility. Phosphine oxide has a very good solubility in organic solvents and on other hand is not soluble in aqueous solution. After concentration of the crude reaction mixture H\(_2\)O was added. The aqueous phase was washed with CH\(_2\)Cl\(_2\) and toluene in order to eliminate all traces of phosphine oxide. Thus after lyophilising the aqueous solution, the product was isolated and directly used in synthesis without further purification.

The \(^1\)H and \(^{13}\)C NMR spectra were in accordance with the expectation, and ESI shows the m/z = 166 corresponding to [M+H]^+ and m/z = 331 corresponding to the dimer (Fig. 27).

Fig. 27 ESI (positive) of 31 with the base peaks corresponding to [M+H]^+ and 2[M+H]^+
Synthesis of 34[179]

Yamaguchi and al. reported this synthesis in 1981 starting from 1,3,5-tris(chloromethyl)benzene by reaction of KCN in the presence of 18-crown-6 ether to obtain 80% yield, although the reaction time was rather long (6 days)[180].

Later on the synthesis has been improved by using 29 as intermediate, with NaCN in DMSO at 40 °C affording the product in 87% yield. The reaction was shortened considerably (15 h) and the use of crown ethers was not required. The analytical data was in agreement with the literature.

Synthesis of 32

The synthesis of compound 32 was achieved via an anhydride acylation. Similar examples were described, in aqueous medium, with chemoselective acylation in the presence of phenols and thiols[181].

The trisamine 31 was efficiently acylated with the cyclic glutaric anhydrides (1-1.5 eq.) without use of acidic or basic reagents in DMF. After isolation of the acylated products, no chromatographic separation is required. The analytical data were as expected (Fig. 28).

![Image](image_url)

**Fig. 28** $^1$H and $^{13}$C NMR of 32 (CD$_3$OD, 250 MHz) the two different signals for the carbonyl are shown in the inset in red. IR spectrum shows the corresponding COOH and CONH bands.

Additional ESI (negative) data show a base peak with m/z = 506 corresponding to [M-H].
9.3 Synthesis of alkyne derivatives

It was already pointed out that not all alkynes or azides are a good combination for click reaction. Partners like highly electron deficient azides or steric constraints may drastically limit the success of this reaction.

In this chapter the synthesis of the alkyne partner will be discussed and more attention will be paid to the steric effect, the choice of catalyst and the conditions used for the performance of the click reaction. A large number of terminal alkynes (Fig. 29), commercial or synthesised, was used for the cycloaddition.

**Fig. 29 Alkyne derivatives. 44-47 and 50 and 51 are commercially available**
9.4 Synthesis of alkynyl amino acids

For the synthesis of 40, 41 and 42 commercially available esters of the corresponding amino acid were used. The methyl ester of tryptophan was not commercially available and the ester was synthesised according to Abato et al. \[182\] and purified by flash chromatography to yield 80% of 39 (Fig. 30).

Esters 39, 40, 41 and 42 were then reacted with pentynoic or hexynoic acid, which were activated with HOBt, EDAC with a catalytic amount of DMAP. All of them were purified and then characterised by NMR spectroscopy and mass spectrometry.

\[\text{Fig. 30} \] \( ^1H \text{NMR (250 MHz, CDCl}_3\) of 39 with the triplet of the alkyne proton (in red) with \(^4J = 2.6 \text{ Hz.}\)
9.5 Synthesis of ferrocenoyl derivatives

Compounds 48 and 49 were obtained by modification of ferrocene carboxylic acid. The carboxyl function of 52 readily undergoes peptide bond formation as presented in literature and confirmed from the previous peptide synthesis. As a consequence, the reaction with an amino alkyne has to result in the formation of the desired ferrocene alkyne derivative. This reaction has already been reported \[183\] and was reproducible, according to the following Scheme 22.

![Scheme 22 Synthesis of 48 and 49](image)

After the treatment of 52 with oxalyl chloride in dry DCM for 2 hours and the following removal of the reagents in vacuo, diethylpropargylamine (or propargylamine) and triethylamine were added in DCM and the mixture was stirred for 2 hours. After filtration and extraction with CHCl$_3$/H$_2$O an orange solution was obtained. Flash chromatography gave the products as orange powders. They were characterised by FAB and $^1$H and $^{13}$C NMR.
The $^1$H and $^{13}$C NMR (in CDCl$_3$) shows as expected the alkyne hydrogen atom at 2.26 ppm (triplet) and the alkyne carbon atoms at 80.3 and 71.5 ppm, respectively (Fig. 31, B). Since no chiral centre was present the Cp rings give rise to signals with intensity 2-2-5 pattern in the proton NMR spectrum (Fig. 31, A and C).
10. Catalyst and conditions for «click» chemistry

Since the introduction of the «click» concept the conditions have varied widely [157, 161, 162, 164-168, 170], particularly with respect to generation of the active Cu$^\text{I}$ species. Sources of Cu$^\text{I}$ (CuI or CuBr) and in-situ reduction of Cu$^\text{II}$ salts (CuSO$_4$ and CuOAc) to Cu$^\text{I}$, as well as addition of Cu$^\text{0}$ (copper-in-charcoal (Cu/C)) were used.

In this work the azide functionalised core and the alkynes (Scheme 23) were reacted via «click» chemistry. To prove the concept, the first experiments were carried out with alkynes according to the literature procedure of Sharpless [158] with catalytic amounts of CuSO$_4$ reduced by sodium ascorbate. Unfortunately, the Sharpless strategy as well as the one described by Astruc et al. [184] provide mixture of products which $R_f$ values were comparable on TLC. Furthermore, ESI did not show a mass corresponding to the desired product. The use of CuI as catalyst did not show any formation of product, probably due to the poor solubility of CuI. It seemed that the use of Cu$^\text{II}$ and in situ reduction to Cu$^\text{I}$ has a more beneficial effect on the reaction than the direct use of Cu$^\text{I}$.

However, the use of stoichiometric CuOAc showed improved results and low amounts of impurities. It was observed that CuOAc did not necessarily need to be solubilised before being introduced to the reaction mixture. Therefore, CuOAc was chosen as the catalyst for the subsequent click conjugation reaction with the alkynes (Scheme 23). Moreover it was found that a microwave assisted procedure [169] is providing an easy and efficient way for this cycloaddition synthesis. In this way the time for the complete triple click was reduced from days to a couple of minutes.

Different results were observed also with the change of reaction solvent. Therefore solvents like DMF, THF, dioxane, DMSO, t-BuOH and mixtures like DMF/H$_2$O (1/1), THF/H$_2$O (1/1) and tBuOH/H$_2$O (1/1) were used with objective to find the best conditions. Examples of these different conditions are presented in Tab. 9.
Scheme 23 Triple click reaction

It is interesting that the change of the catalyst and a ratio of polar solvent /H₂O (1:1) can have such an impact on the reactions as shown in row 3, 4 and 5. Finally the best results were obtained after 20 min microwave irradiation at 100°C, with a large excess of CuOAc and Na-ascorbate (see experimental part) in a mixture of DMF/H₂O (1/1). The following workup with NH₃ (25% aq. solution) helps to eliminate the excess of Cu. The product was then extracted with CH₂Cl₂. The organic phase was washed numerous times with H₂O and NH₃ (25% aq. solution) and then dried over Na₂SO₄. The R_f values of the starting material and product were often comparable on TLC and ESI analysis was used to monitor the progress of the click reaction. Flash chromatography was used (CH₂Cl₂/ MeOH; 10:1) to purify the products.
<table>
<thead>
<tr>
<th>Starting alkyne</th>
<th>Solvent</th>
<th>Catalyst</th>
<th>Base</th>
<th>T °C</th>
<th>t (min)</th>
<th>ESI Detection</th>
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</thead>
<tbody>
<tr>
<td><img src="image1" alt="42" /></td>
<td>Dioxane</td>
<td>CuOAc</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>2 branch</td>
</tr>
<tr>
<td><img src="image2" alt="42" /></td>
<td>THF/H$_2$O (1:1)</td>
<td>CuOAc</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>Product 54</td>
</tr>
<tr>
<td><img src="image3" alt="39" /></td>
<td>DMF</td>
<td>CuSO$_4$</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>No product</td>
</tr>
<tr>
<td><img src="image4" alt="39" /></td>
<td>DMF</td>
<td>CuOAc</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>2 branch</td>
</tr>
<tr>
<td><img src="image5" alt="39" /></td>
<td>DMF/H$_2$O (1:1)</td>
<td>CuOAc</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>mixture 2 and 3 branches</td>
</tr>
<tr>
<td><img src="image6" alt="39" /></td>
<td>THF/H$_2$O</td>
<td>CuOAc</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>Product 73</td>
</tr>
<tr>
<td><img src="image7" alt="47" /></td>
<td>THF/H$_2$O</td>
<td>CuOAc</td>
<td>Na ascorbate</td>
<td>100</td>
<td>20</td>
<td>Product 57</td>
</tr>
</tbody>
</table>
11. Synthesis of triple branched molecules

The first examples were performed with alkynes from Fig. 29. In the case of alkynes 44, 45, and 51, the first results did not show formation of the desired product.

In these first attempts with the alkyne alcohol 45 and alkyne amine 51, there were no protection groups on the alcohol and the amine groups. Therefore TMS and Boc protection groups were introduced to the alcohol 46 and the amine functional groups 43, respectively. The reaction monitoring shows no formation of the desired product in both cases 46 and 45.

In contrast, the simple protection of the free amino group provides 53 in more than 80 % yield. The resulting molecule was successfully isolated and purified, then analysed by ESI, and $^1$H and $^{13}$C NMR and additional 2D NMR as shown on Fig. 32.

\[ \text{Fig. 32 HMQC 2D NMR (DMSO-$d_6$, 400 MHz) of 53} \]
The proton NMR data show the expected signals and the triazole H was shifted upfield to 7.89 ppm. Mass spectrometry shows a base peak with m/z = 731, which correspond to the expected product ([M+Na]+).

For the unsuccessful results with 44, an intramolecular cyclisation may be assumed, as described by Schibli et al. [185], which gives enol lactones under reaction conditions typically applied for the CuI cycloaddition (Scheme 24). To escape this disturbing mechanism the free carboxylic acids were esterified as a precaution.

![Scheme 24 Intramolecular cyclisation in case of propargyl glycines](image)

Alkyne esters 42 and 41 were used for click reactions with microwave radiation. This strategy led to formation of the desired triply branched products 54 and 59 (see experimental part) in very good yield. The 2D NMR spectra of 54 were as expected (Fig. 33). Mass spectrometry shows base peaks at m/z=983 [M+Na]+ and m/z=1043.19 [M+Na]+, for molecules 54 and 59, respectively.

Despite the multiple trials successful results with alkyne esters 39 and 40 were not obtained (see Tab.6). In the case of 39, as reported in Tab. 6, formation of mixtures of mono-, di- or triple- substituted products was observed. The Rf values of those compounds were comparable on TLC and separation by flash chromatography was not possible.
Fig. 33 HMQC and HMBC 2D NMR (CD$_2$Cl$_2$, 400 MHz) of 54
Formation of multivalent compound was not observed at all in the case of alkyne ester 40. Product formation was not observed after microwave assistance or changes of the catalyst. This gives rise to the idea that the presence of the Pbf group may bring steric constraints which leads to this decrease in reactivity.

Then, another strategy was applied where Fmoc-Trp(Boc)-COOH was reacted with 31 via peptide coupling conditions and after deprotection of the Fmoc group the trivalent molecule 55 was finally successfully synthesised (Fig. 34).

For compound 56 peptide synthesis was used where the acid of the Fmoc-Arg(Pbf)-OH was activated with HOBt/EDAC/DMAP (1/1/10%), then added to 55 in DMF. 56 was detected using MALDI – TOF with a peak at m/z = 2917 [M+H^+] (Fig. 34). A peak at m/z = 631 arises from the excess of Fmoc-Arg(Pbf)-COOH. Probably due to the big size of the growing molecule, 56 could not be isolated.
11.1 Multivalent ferrocenyl molecules

Compounds 57 and 58 were synthesised from 50 (commercial) and 49, which was synthesised according the work of O. Brosch [186], respectively.

Compound 57 was successfully synthesised and the same was reported by the group of Astruc et al. [187]. The analytical data for this product match the reported ones.

The same strategy was used for 49 and 50. The alkynes were dissolved in DMF and then CuOAc was added in the mixture followed by the dropwise addition of a freshly prepared aq. solution of sodium ascorbate. The solution was stirred for 20 min with microwave radiation at 100 C°, then a solution of 25% aq. NH₃ was added and the mixture was diluted with CH₂Cl₂ and decanted. Compounds 57 and 58 were isolated in very good yield 90% and 40%, respectively. The ¹H NMR of 57 and 58 were as expected and shows the Cp-signals with the already described intensity pattern of 2-2-5 (Fig. 35).

Alkyne 48 did not give branched product using the same synthetic approach, as for 57 and 58.

Fig. 35 ¹H NMR (250 MHz, CD₂Cl₂) of 57 (A) and 58 (B) a, b and c in both spectra are Cp signals and triazole signal is matched with an asterics.
11.2 Multivalent peptide molecules

Suitable peptides were synthesised for the derivatives of type I and II, as shown in fig. 36. Three different resins were used to synthesise these peptides. The Rink amide is a common resin, which, after cleavage provides unprotected peptides with amidated C-terminus. Glycine preloaded Wang resin gives after cleavage, unprotected peptide with a free C-terminus. Finally Arg(Pbf) preloaded chlorotrityl chloride resin was used for the SPPS of fully protected peptides with free C-terminus.

For the click reaction (type I) microwave radiation and an excess of alkynyl peptides, were used. For the peptide coupling to the core fragment of type II the peptide synthesis conditions were used in ratio 1:3.5 (core:peptide). Despite all the changes, in the reagents and conditions, triply branched molecules were not isolated. This part of the work is still under investigation and other possible solutions are currently studied.

Fig. 36 Reaction pathways for triply peptide - branched molecules.
12. Proteomic approach to antibiotic drug discovery

In this part the biological results from the experiments effectuated by the group of Jun. Prof. Julia Bandow are summarised. Minimum inhibitory concentration (MICs) values, the bacterial response and proteomics were used to investigate the response to the tested novel compounds. Experiments were performed with the template AMPs (MP_{66} and MP_{196})^{50}, the modified AMPs (23, 24, 25 and 28) as well as with the triple branched molecules (54, 59, 57 and 73) (see General Synthesis). The bacterial response to antibiotics can give valuable insights into the antibiotic mode of action (MOA). Therefore a proteomic approach was chosen to study the cellular response towards the template peptides and the modified compounds.

Many different criteria can be considered when choosing a species. For the actual study two bacteria were chosen: *E. coli* and *B. subtilis*.

*Escherichia coli* (*E. coli*) is a Gram negative rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some can cause serious food poisoning. The harmless strains are part of the normal flora of the human organism, and can benefit their hosts by preventing the establishment of pathogenic bacteria within the intestine. The bacteria can also be grown easily and its genetics are relatively simple and easily-manipulated, making it one of the best-studied prokaryotic model organisms.

*B. subtilis* (soil bacteria) was chosen as a Gram-positive model organism because of a well studied bacterial physiology, non-pathogenic character as well as its easy cultivation and handling under laboratory conditions. Another leading criteria were the available genome sequence, also available for *E. coli*, which then makes possible mass spectrometry-based protein identification, as well as the amount of knowledge about the proteome and the general physiology of the organism.

For these organisms so called proteome maps are published on which the identity of many protein spots has already been determined as *B. subtilis*^{188} or *E. coli*^{189}. Proteomic maps

The known protein patterns and the fact that *B. subtilis* is the only organism for which a 2D-gel-based drug response reference compendium \(^{[190]}\) has been published made it an excellent choice of model organism.

12.1 An introduction to proteomics

12.1.1 Technique

Proteomics refers to the global analysis of the proteins of cells, tissues and organisms. The term «proteome» was introduced by Wasinger et al. (1995) \(^{[191]}\) to describe the expressed proteins under a defined condition at a specific point of time. Global protein analyses have been performed since the invention of two-dimensional-gel-electrophoresis (2-DE) in 1975 by O’Farrell and Klose \(^{[192, 193]}\), a method that was used also in the present work.

12.1.2 Proteomic profiling \(^{[194]}\)

At the beginning physiologically active antimicrobial concentration needs to be established. To this end, MIC values of the different analysed compounds were determined. MIC (Minimum Inhibitory Concentration) is the lowest concentration of an antimicrobial compound that inhibits the visible growth of a microorganism after overnight incubation.

For the following work a concentration was needed, where the bacterial population will be stressed but still able to respond to the stress by synthesising proteins needed for survival. To find this concentration, growth curves (Fig. 37, A) were used where the studied drugs were applied in different concentrations to find suitable concentrations. When the cells are adapting to changes in their environment they produce those proteins that are vital to the adaptive response to the imposed stress. At the same time cells often stop producing proteins that are no longer required for the non-growing cells to avoid wasting nutrients. After determination of this concentration samples for proteomic profiling were prepared.
Fig. 37 Growth curve of B. subtilis. The arrow indicates the addition of MP 66 to the medium (A). 2D SDS PAGE of Control/MP 66 (B) (This data is reproduced with permission from the Bachelor thesis of Michaela Wenzel and from the work data of Nadja Raatschen, both working under the supervision of Jun. Prof. J. Bandow)

Aliquots of the control culture and treated culture were labelled with L-[\textsuperscript{35}S]-methionine, to capture snapshots of protein synthesis 10 min after addition of the antibacterial compound. Two-dimensional gel electrophoresis (2-DE) was then used to allow the separation of the complex protein mixture into individual proteins, by exploiting two physicochemical properties of proteins - the isoelectric point and the size (Fig. 37, B).

Gels were stained with fluorescent dye then dried and exposed to phosphor screens, which generate autoradiographic images of the protein synthesis. The false-coloured red gel image of the autoradiograph represents protein synthesis in the antibiotic treated cells. It is warped to fit onto the green coloured image of the untreated control. Thus, antibiotic-induced proteins appear red, repressed proteins appear green, and proteins synthesized under both conditions appear yellow (Fig. 37, B).

After trypic digest of the proteins and elution of the trypic peptides from the gel, proteins are identified by mass spectrometry.
12.2 Pattern matching

The proteomic profile was then generated to evaluate if similar profiles are known for treatment with other antimicrobial drugs. A reference compendium of protein profiles contains annotated protein profiles that visualize differences and provide quantitation of the changes in protein expression compared to control conditions (Fig. 38).

![Reference compendium](image)

**Fig. 38** Reference compendium. Comparing protein profiles of novel compounds C to the reference compendium bacitracin (A) and valinomycin (B) can generate hypotheses about the mechanism of action (in blue- Marker proteins shared with bacitracin and valinomycin).

The largest and most diverse compendium of reference proteomic profiles has been published for *B. subtilis* [190]. It contains the profiles for 30 different agents, with antimicrobial activity, some of which were antibiotics with established as well as unknown mechanisms of action. Others were general cytotoxic agents such as detergents and DNA intercalators, which in themselves have no value as drug due to universal toxicity, but which serve to define the proteomic response to toxic mechanisms.
Results and discussions

The AMPs studied in this work, have preference to interact with the negatively charged bacterial cytoplasmic membranes, via the net positive charge that they carry (see Antibiotics). The effect of AMPs was faster in the case of Gram-positive compared to the Gram-negative bacteria. That was probably due to the missing outer membrane, in the case of the Gram-positive bacteria, which decreased the number of barriers to cross.

That idea was supported by the fact that the major part of the tested compounds was active on the Gram-positive *B. subtilis* and only MP\textsubscript{196} showed activity on the Gram-negative *E. coli*.

All tested AMPs showed antimicrobial activity. As mentioned, the MIC of peptide MP\textsubscript{196}\textsuperscript{[50]} against the Gram-positive *B. subtilis* and Gram-negative *E. coli* were 2 µg/mL and 12 µg/mL, respectively. Ferrocenyl AMP MP\textsubscript{66} was already fully characterised\textsuperscript{[50]} and here we present additional analytical data. MP\textsubscript{66} was synthesised in two different sequences, one composed only of L-amino acids and the second only with D-amino acids. L-MP\textsubscript{66} shows MIC of 7 µg/mL and D-MP\textsubscript{66} shows MIC of 5 µg/mL. The modified analogues 23, 24 and 25 of MP\textsubscript{66} have shown MIC values of 12 µg/mL, 7 µg/mL and 5 µg/mL, respectively. Compound 24 has an MIC in the same range as the ferrocenyl peptide MP\textsubscript{66} and we assume it has the same MOA as MP\textsubscript{66}. For that reason MP\textsubscript{66} was used for the proteomic pattern determination. Compounds MP\textsubscript{66}, 23 and 24 were aditionaly tested for cytotoxicity by Antonio Pinto (see Annex). These test results were negative and further IC\textsubscript{50}, testing was not efectuated.

The branched molecules 54, 59, 57 and 73 were tested on *B. subtilis* and *E. coli*. Probably due to the missing positive charges antimicrobial activity was not observed.

The newly synthesised ruthenium analogue 28 surprisingly has an enhanced MIC of 0.9 µg/mL. The difference of one order of magnitude was unexpected and difficult to explain, with the organometallic moiety. Ferrocene and ruthenocene have comparable chemical characteristics and it seemed that other unknown factors were involved in this case. Compound 28 will be used to quantify the distribution of the peptide conjugate in different subcellular fractions (cytosol, membrane, cell wall). If the mechanism of action is related to membrane damage, a significant fraction of the compound should be detected in the membrane.

Growth curves were made for compounds MP\textsubscript{66}, 25 for *B. subtilis* (Fig. 39, C and D) and MP\textsubscript{196} for *B. subtilis* and *E. coli* (Fig. 39, A and B). After determination of the physiologically
active concentrations the proteomic profiling was analysed for compounds MP$_{66}$, MP$_{196}$ and 25. The 2-DE gels show the same pattern for all tested compounds.

Fig. 39 Growth curves of B. subtilis (A) and E.coli (B) under MP$_{196}$ stress and Growth curves of B. subtilis under MP$_{66}$ and 25 stress (C and D; data reproduced with permission from the work data of Michaela Wenzel and Sarah Yanik)

12.3 Pattern matching for MP$_{66}$

Interestingly, the protein profile of B. subtilis, after treatment with MP$_{66}$ was highly similar to that of the detergent triton-X 100 (Fig. 40). At the moment protein identification by mass spectrometry is still under way. Triton-X 100 is a detergent often used in biochemical laboratories. Even a pattern partially matching with a detergent, the profile can still be very helpful to define the metabolic pathways affected. Moreover, triton X 100 has also six shared proteins markers with the Valinomycin (dodecadepsipeptide)\(^{[190]}\), which may also contribute to the elucidation of the mechanism. MP$_{66}$ also shares marker proteins with bacitracin, an
antibiotic, which inhibits cell wall biosynthesis at a membrane-bound step. Together these results suggest that MP\textsubscript{66} as triton X 100 and bacitracin, target the cytoplasmic membrane.

![2D SDS PAGE gels of protein expression under MP\textsubscript{66} and triton X 100 stress](image)

**Fig. 40** 2D SDS PAGE gels of protein expression under MP\textsubscript{66} and triton X 100 stress: Control/MP\textsubscript{66} (A) Control/Triton X-100 (B) Triton X-100/MP\textsubscript{66} (C). In circles are highlighted the proteins induced by both MP66 and Triton X-100 (This data is reproduced with permission from the Bachelor thesis of Michaela Wenzel and from the work of Nadja Raatschen, both working under the supervision of Jun. Prof. J. Bandow)
13. Quantitative proteomics in response to MP₆₆

This approach monitors the response of *C. glutamicum* to treatment with MP₆₆ (MIC<sub>MP₆₆</sub> against *C. glutamicum* = 40 µg/mL). *C. glutamicum* is a small, non-pathogenic and non-moving Gram-positive soil bacterium. It does not produce spores, grows quickly and has a fully sequenced genome, which properties make it a good model organism. Thus, the relatively well characterized physiology makes *C. glutamicum* an ideal model organism for the closely related human pathogens *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*.

For proteomic analyses, published quantification techniques were used (MudPIT - Multidimensional Protein Identification Technology \[195, 196\] in combination with a stable isotope labelling strategy \[197, 198\]) to monitor the protein expression profile of the adapting bacterium to the applied compound. To elaborate the cellular response to MP66, a proteomic outline has been performed where a compound-treated sample was compared with an untreated control (Fig. 41).

![Diagram](image)

**Fig. 41** Isolated proteins of the control and the MP66 treated cells were equally mixed with stable isotope labelled standard. Membranes of the different samples were then further processed and identified by MudPIT. Quantification was performed by calculating the peak areas of all identified peptides in the comparable samples using the internal standard peptides for normalization (factor 1 / factor 2). (This data is reproduced with permission from the work of Dr. D. Wolters)
Due to this study more than 900 protein identifications, including numerous integral membrane proteins were identified. 751 have been quantified which corresponds to a quantification efficiency of 83%. Among these, 32 proteins had a relative regulation factor after *C. glutamicum* has been stressed with MP$_{66}$. Moreover, 76 proteins have been found induced after stimulation with the compound and 80 were detected as repressed. Thus, a total number of 185 proteins of *C. glutamicum* exhibited a regulated response towards MP$_{66}$ stress. According to a COG (cluster of orthologous groups) analysis (Fig. 42), proteins of the class which are involved in amino acid transport and metabolism (COG E) turned out to be the most strongly affected ones of all.

![Quantified proteins according to COGs (Clusters of orthologous groups)](image)

*Fig. 42* Quantified proteins according to COGs (Clusters of orthologous groups)

The relatively high iron concentration caused by the ferrocene conjugated peptide might lead to the assumption that *C. glutamicum* suffers from iron excess. Beside an essential role, Fe$^{2+}$ reacts with H$_2$O$_2$ to yield the ferric state (Fe$^{3+}$), OH$^-$ and the highly reactive and damaging OH$^-$ radicals as described by the Fenton reaction.

The unregulated catalase and no elevated H$_2$O$_2$ levels demonstrate that no oxidative stress occurs after treatment with the iron containing compound MP$_{66}$.

Moreover it seems that MP$_{66}$ has a membrane related effect, as assumed before (*see Proteomics*). Not only various integral membrane proteins were regulated such as peptide permeases and transporters, which were rather down-regulated, the bacterial cell also responded by adjusting the lipid composition of its cell envelope.
Conclusions

Generally, it can be ascertained that the treatment of *C. glutamicum* with MP66 causes a cellular stress situation (*see Proteomic approach*). The down-regulated state of peptide permeases points out the inhibition of the cellular uptake of the iron conjugated peptide, which corresponds to an iron excess response. Nevertheless, no proves for oxidative stress became manifest during the study. In conclusion, membrane related cellular response to a small ferrocene-coordinated arginine and tryptophan penta-peptide has been demonstrated in this work.
This work has presented the synthesis of short tryptophan and arginine residue containing antimicrobial peptides (AMPs) as well as their metallocene derivatives. All of the AMPs were synthesised using solid phase peptide synthesis (SPPS) (see Introduction). The metallocene markers were generally introduced at the N-termini of the AMPs. These AMP derivatives were conveniently prepared using commercially available resins and amino acid building blocks (Fig.43).

Once the synthesised compounds were analysed and purified, their antimicrobial properties were studied in additional biological experiments, which were described in two last chapters of the present work (see Proteinomic approach and Quantitative proteomics). All modified compounds showed minimum inhibitory concentration (MIC) in range of 7 to 0.9 µg/mL. On
the way to this ultimate goal we obtained valuable insight in the mode of action of these small AMPs derivatives. These biological studies were made with \(B.\ subtilis\) (soil bacteria) as a Gram-positive model organism. It was chosen because of its well studied bacterial physiology, non-pathogenic character as well as the available genome sequence. This organism has published proteome map on which the identity of many protein spots has already been determined. The protein profile (see Pattern matching for \(MP_{66}\)) of \(B.\ subtilis\), after treatment with AMPs was highly similar to that of the detergent triton X-100 (Fig. 40). Moreover, triton X-100 has also shared proteins markers with valinomycin (dodecadepsipeptide) and bacitracin (antibiotic), which inhibits cell wall biosynthesis at a membrane-bound step.

Together these results suggest that \(MP_{66}\) had a similar mode of action as triton X-100 and bacitracin, which target the cytoplasmic membrane.

![Fig. 40 2D SDS PAGE gels of protein expression under \(MP_{66}\) and triton X 100 stress: Control/\(MP_{66}\) (A) Control/Triton X-100 (B) Triton X-100/\(MP_{66}\) (C). In circles are highlighted the proteins induced by both \(MP66\) and Triton X-100 (This data is reproduced with permission from the Bachelor thesis of Michaela Wenzel and from the work of Nadja Raatschen, both working under the supervision of Jun. Prof. J. Bandow)](image-url)
Surprisingly the newly synthesised ruthenium analogue 28 had an MIC value of 0.9 µg/mL whereas that of ferrocene analogue MP66 had a MIC value of 7 µg/mL. Therefore, compound 28 will be used to quantify the distribution of the peptide in different subcellular fractions (cytosol, membrane, cell wall). If the mechanism of action is related to membrane damage, a significant fraction of the compound should be detected in the membrane. At this stage final results still have to be obtained.

Another synthetic perspective in this work was the synthesis of multivalent AMPs motivated by results obtained using dendrimers. Several derivatives containing alkyne and azide functionalities were successfully synthesised and applied in reactions based on copper-catalysed 1,3 - dipolar alkyne azide cycloaddition (Cu-AAC) or «click» chemistry. This strategy provided numerous model compounds, but unfortunately was not successful in the case of peptides (Fig. 44).

**Fig. 44** Trivalent molecules synthesised via «click» chemistry or peptide coupling
Therefore a second strategy was explored to obtain multivalent AMPs. For this a peptide coupling, instead of the «click» reaction mentioned before, was used. Following this method compound 55 and 56 were synthesised. It should be emphasized that such derivatives could not be prepared by click chemistry.

Branched molecules 54, 57, 59 and 73 were tested on B. subtilis and E. coli, for their antimicrobial activity. E. coli is a Gram-negative model organism, which as B. subtilis was chosen because of its well studied physiology, non-pathogenic character and published proteome map. As is well known for AMPs, a combination of positive charges and tryptophan residues are often found in active compounds. It is therefore likely that due to the absence of this combination antimicrobial activity was not observed.

The present work describes the successful synthesis of metalloocene-AMPs conjugates. We showed that small metalloocene peptides containing tryptophan and arginine residues have activities better than the activity of naturally occurring antibiotic such as pilosulin (twenty amino acid residues), which had a MIC value of 16 µM (see Tab.4). More surprising a hexapeptide in which the ferrocene of MP_{66} was replaced by an arginine residue had an activity on an E. coli strain. This was not observed in the case of pentapeptides with or without ferrocenyl moiety. Moreover the metalloocene part also had an interesting role as observed in compound 28, which showed activity of one order of magnitude higher than all studied peptides.

In conclusion it is very important to obtain more knowledge about the mode of action of AMPs. Once the exact target is known modifications can be made to the AMP, the specificity can be further optimised and the activity enhanced. The first steps towards this have now been described for organometallic-AMP conjugates.
Chapter VI. Materials and methods

Elemental analysis

Elemental Analysis was performed on a Jena multi EA 3100 Analysator in C, H and N mode.

Infrared spectroscopy

Infrared spectra were recorded on a Tensor 27 Brucker spectrometer with Miracle-ATR (spectral resolution of 4.0 cm\(^{-1}\)). Wavenumbers were given in cm\(^{-1}\).

Mass spectrometry

The FAB mass spectra were measured on a Finnigan VG Autospec (glycerol or 3-nitrobenzyl alcohol (NBA) as matrix).

All ESI spectra (positive/negative) were measured on a Bruker Esquire 6000 (Nebulizer: 10 psi, Dry gas: 5 L/min, Dry gas: 300°C, Flow rate: 240 μL/h).

GC-MS spectra were measured on a Shimadzu GSMS-QP2010 quadrupol spectrometer with ionisation energy of 70 eV (Column: FS-OV-1-CB-0.25; Length: 25 m; ID: 0.25 mm; AD: 0.36 mm; film thickness: 0.25 μm; max.temp: 300°C; Inlet pres: 47.8 kPa; column flow: 1.02 ml/min; split ratio: 49; purge flow: 3.0mL/min; Inj. temp: 290°C).

LC-MS spectra were measured on an Agilent 1100 Series with column Zorbax SB-C18 from Agilent (Rapid resolution HT 2.1 \(\times\) 50 mm, particle resolution-1.8 μm). The used method was MeCN/H\(_2\)O with increasing MeCN, from 0 – 100%, with flow of 0.3 μL. All chromatograms were measured at 220 nm. The mass spectres were measured on Bruker Esquire 6000, on positive modus.
MALDI-TOF spectra were measured on Bruker Ultraflux III TOF/TOF (Matrix = sinapinic acid in TA-1:2solution of MeCN: H$_2$O with 0.1% TFA and probes were also dissolved in TA-solution; measurement mode: RP= reflector mode, positiv scan).

**Microwave**

The microwave experiments were effectuated on a CEM discover (Power-50 W; Ramp Time-4 min; Hold Time- 20 min; Temperature- 100 °C; Pressure- 16 bar; Stirring-on; PowerMax-on).

**NMR Spectroscopy**

NMR spectra were determined on a Brucker spectrometer, $^1$H operating at 200, 250, 400 and 600 MHz and $^{13}$C operating at 50.3, 62.8, 100.6 and 150.9 MHz. Peak positions in both $^1$H and $^{13}$C are reported in ppm relative to TMS, the internal standard. Coupling constants, $J$, are given in Hz. Individual peaks are marked as: singlet (s), broad singlet (br. s.), doublet (d), triplet (t) or multiplet (m).

**HPLC analysis and purification**

High Performance Liquid Chromatography (HPLC) was performed on a customised Varian ProStar 210 with PDA detector. The peptide was purified using a mixture of Millipore water and MeCN (Baker, HPLC grade) with 0.1% TFA (v/v), as eluents.

The analytical measurements were done on a Varian DynaMax C-18 RP column (250 × 4.6 mm), with a flow rate of 1.0 mL/min, starting at 5% MeCN/95% H$_2$O. The solvent gradient was increased linearly to 95% MeCN over 30 min.

The semi preparative runs were done on a Varian VariTide-RP column (250 × 10.0 mm), with a flow rate of 4 mL/min, starting at 10% MeCN/90% H$_2$O. The solvent gradient was increased linearly to 90% MeCN over 45 min. All chromatograms were plotted at 220 and 254 nm at room temperature.
Chemicals/Material

All chemicals were used as obtained from commercial sources. All standard peptide synthesis chemicals and solvents were analytical reagent grade or better and purchased from Novabiochem (Bad Soden, Germany) or from Iris Biotech (Marktredwitz, Germany). Resins were exclusively used in 200 mesh size, with loadings 0.68 mmol/g for Rink amide resins and 0.52 mmol/g for H-L-Arg(Pbf)-2-Chlorotrityl resins. Peptide grade DMF and CH$_2$Cl$_2$ were used for all peptide syntheses.

IBX 68

![IBX 68](image)

68 was prepared according to ref. [199,200] and the analytical data is available in [201].

NB: Work with precaution, compound 68 is potentially explosive!
Solid Phase Peptide Synthesis - general protocol for manual synthesis

**Swelling:** At the beginning of each synthesis the resin was swollen in DMF for 1 hour. When the peptide synthesis had to be interrupted, the resin was shrunk using MeOH and stored in the refrigerator overnight. Before the synthesis was continued the resin was again swollen in DMF.

**Deprotection:** The N-terminal Fmoc protection group was removed by a solution of piperidine in DMF (1:4), which was freshly prepared. Deprotection was carried out 2 × 10 minutes.

**Washing:** After each deprotection and coupling step, the resin was excessively washed (3 times for 5 minutes) with DMF and CH$_2$Cl$_2$ to remove any residual activation or deprotection reagents.

**Coupling:** Amino acids (4 fold excess) were mixed with equimolar amounts of TBTU and HOBT. Then, 9 equivalents of DIPEA were added and the whole mixture was dissolved in 1.5 mL of DMF and then activated for 5 min. Ferrocene and ruthenocene (5 fold excess) activation was effectuated with equimolar amounts of HATU and HOBT and DIPEA (9 fold excess). The coupling was performed for 45 minutes for Fmoc-Trp(Boc)-OH and 1 hour for Fmoc-Arg(Pbf)-OH and all the metallocene compounds.

**Acetylation:** N-terminal acylation was carried out by treatment with a 1:1 (v/v) mixture of Ac$_2$O (5% in DMF) and DIPEA (6% in DMF) for 2 × 5 minutes. Before acetylation the N-terminally deprotected resin was washed with DMF and DCM.

**Cleavage for Rink amide and Wang resins:** After the synthesis was finished, the resin was treated with a mixture of 95% TFA, 2.5% H2O and 2.5% TIS (6 ml/g resin) for 3 hours. If ferrocene (or ruthenocene) was bound, the cleavage mixture consisted of 85% TFA, 10% phenol and 0.5% TIS. After removal of all volatile compounds *in vacuo*, the crude product was precipitated by addition of cold Et$_2$O. After centrifugation, the peptide precipitate was
washed again with cold Et₂O and dried in air. The peptide was then dissolved in H₂O/MeCN, filtered and lyophilized.

**Cleavage for 2-Chlorotrityl resins:** After the synthesis was finished, the resin was treated with a mixture of 1% TFA in CH₂Cl₂ (6 ml/g resin) for 5 × 10 minutes. After cleavage the solvents were eliminated *in vacuo* to give the crude product.
**Cell culture and cytotoxicity**

**HeLa** - Cervical cancer cell line  
**HepG2** - Hepatocellular carcinoma cell line  
**HT-29** – Human colon adenocarcinoma cell line

HeLa, HepG2 and HT-29 cells were kept in culture with RPMI 1640 medium supplemented with 10% FCS (fetal calf serum), 2 mM $\text{L}_\text{-}$glutamine and the antibiotics penicillin (100 U mL$^{-1}$) and streptomycin (100 mg mL$^{-1}$) in 5% CO$_2$ atmosphere.

*In vitro* cytotoxicity of the AMPs compounds was studied on HeLa, HT-29 and HepG2 cells. Cell viability, which correlates with the metabolic activity of cells, was determined by the resazurin assay. In addition to the cell viability, absolute cell numbers were determined by the crystal violet assay, which can be applied after elution of resazurin. The cells were seeded in 96-well microtiterplates (MTP) coated with 0.2% of gelatine. After seeding, the cells were grown for 24 h under standard conditions. The compounds were dissolved in culture medium with 0.5% DMSO and applied to the cells in 10, 100 and 500 $\mu$M concentrations for 48 h. Every concentration was tested three fold.

Before resazurin was added to the cells they were washed three times with phenol red-free RPMI-1640 medium. Then, phenol red-free RPMI-medium with 10% resazurin was added. Absorbance at 600 nm was directly measured with a Tecan Sapphire microplate reader (Tecan, Germany) at 37°C. After 2 h of incubation at 37°C and 5% CO$_2$, the measurement was repeated. The decrease in absorbance gave the viability.

Resazurin was removed and the cells were fixated with 4% *para*-formaldehyde (PFA) in PBS (phosphate buffered saline) for 15 min at room temperature. PFA was eluted twice with PBS and membranes were permeabilised by 0.1% Triton-X100 in PBS for 10 min. Afterward an aqueous 0.04% crystal violet solution was added to the cells and the MTP was mechanically shaken for 1 h. The cells were washed seven times with H$_2$O, and crystal violet was eluted with 96% ethanol for 4 h. The absorbance was determined at 570 nm, after subtraction of 24 h pre-substance incubation absorbance values, cell biomass could be calculated. The tested compounds did not show any cytotoxic activity up to concentration of 500 $\mu$M.
Experimental part

To a solution of tryptamine (1g, 6.24 mmol, 1 eq.) in a (1:1) mixture of CH₂Cl₂/pyridine (20 mL) was added (CF₃CO)₂O (0.925 mL, 6.55 mmol, 1 eq.). After 1h at room temperature, the solvents were removed under reduced pressure. The residue was solubilised in 300 mL EtOAc and washed with aq. sat. NaHCO₃ (2 × 50 mL), aq. 1 M HCl (2 × 50 mL), brine (50 mL), dried over Na₂SO₄, filtered and concentrated to give **20** as a yellow solid (1.22 g, 76%)\[110\].

\[\text{MP} = 91.4^\circ \text{C};\]

\[^1\text{H NMR (200 MHz, CDCl}_3\text{): }\delta = 8.11 \text{ (s, 1H, H}_{\text{NH}}\text{), 7.62 - 7.04 \text{ (m, 4H, H}_{4,5,6,7}\text{), 6.37 \text{ (s, 1H, H}_{2}\text{), 3.74 - 3.65 \text{ (m, 2H, H}_{9}\text{), 3.06 \text{ (t, }^3\text{J} = 6.8 \text{ Hz, 2H, H}_{8}}\text{);}\]

\[^{13}\text{C NMR (50.3 MHz, CDCl}_3\text{): }\delta = 136.5 \text{ (C}_{7}\text{), 126.9 \text{ (C}_{3a}\text{), 122.5 \text{ (C}_{2}\text{), 122.2 \text{ (C}_{3}\text{), 119.8 \text{ (C}_{6}\text{), 118.4 \text{ (C}_{4}\text{), 111.4 \text{ (C}_{7}\text{), 109.5 \text{ (C}_{3}\text{), 40.0 \text{ (C}_{9}\text{), 24.7 \text{ (C}_{8}}\text{);}\]

\text{FAB: } m/z = 256.1 \text{ [M]}^+.\]
To a solution of 20 (0.109 g, 0.426 mmol, 1 eq.) in dry THF (10 mL) was added DMAP (48 mg, 0.022 mmol, 0.05 eq.) and (Boc)_2O (93 mg, 0.426 mmol, 1 eq.). After 1h at 38°C, the solvent was removed under reduced pressure. The residue was solubilised in 250 mL EtOAc and washed with aq. 1M HCl (2 × 20 mL), brine (2 × 50 mL), dried over Na_2SO_4, filtered and concentrated to give the desired product as yellow solid (0.152 g, 99%)^{[110]}.

**^1H NMR (200MHz, CDCl_3):** \(\delta = 7.98\) (d, \(^3J = 7.9\) Hz, 1H, H\textsubscript{NH}), 7.46 – 7.10 (m, 4H, H\textsubscript{4,5,6,7}), 6.39 (s, 1H, H\textsubscript{2}), 3.57 – 3.47 (m, 2H, H\textsubscript{9}), 2.82 (t, \(^3J = 6.7\) Hz, 2H, H\textsubscript{8}), 1.49 (s, 9H, H\textsubscript{14});

**^13C NMR (50.3MHz, CDCl_3):** \(\delta = 149.9\) (C\textsubscript{12}), 136.5 (C\textsubscript{7a}), 130.4 (C\textsubscript{3a}), 125.2 (C\textsubscript{2}), 123.8 (C\textsubscript{5}), 123.1 (C\textsubscript{6}), 119.0 (C\textsubscript{4}), 116.9 (C\textsubscript{11}), 115.9 (C\textsubscript{7}), 110.0 (C\textsubscript{3}), 84.3 (C\textsubscript{13}), 40.0 (C\textsubscript{9}), 28.6 (C\textsubscript{14}), 24.9 (C\textsubscript{8});

**FAB:** \(m/z = 356\) [M]^+.
21 (613 mg, 1.721 mmol, 1 eq.) was dissolved in a mixture of MeOH/H₂O (7:3) then K₂CO₃ (2.37 g, 17.21 mmol, 10 eq.) was added. The reaction was stirred overnight at r.t. and MeOH removed under vacuum. The organic phase was extracted with 500 mL EtOAc and washed with H₂O (2 × 20 mL), dried over Na₂SO₄, filtered and concentrated to give the desired product as a yellow oil (1.37 g, 91%).

Compounds 20, 21 and 22 were synthesised according Uno et al. ¹H NMR and ¹³C NMR spectra of 22 available in [110].

ESI: m/z = 261 [M+H]⁺.
23 was synthesised according to the SPPS - general protocol (see Materials and methods). Amino acids (4 fold excess) were mixed with equimolar amounts of TBTU and HOBt. Then, 9 equivalents of DIPEA were added and the whole mixture was dissolved in 1.5 mL of DMF and leads to activate for 5 min.

After the end of the peptide synthesis N-terminal acylation was carried out by treatment with a 1:1 (v/v) mixture of Ac₂O (5% in DMF) and DIPEA (6% in DMF) for $2 \times 5$ minutes. Before acetylation the N-terminally deprotected resin was washed with DMF and DCM.

After the synthesis was finished, the resin was cleaved with a mixture of 95% TFA, 2.5% H₂O and 2.5% TIS (6 ml/g resin) for 3 hours. After removal of all volatile compounds in vacuo, the crude product was precipitated by addition of cold Et₂O. After centrifugation, the peptide precipitate was washed again with cold Et₂O and dried in air. The peptide was then dissolved in H₂O/MeCN, filtered and lyophilized.

The pure compound was characterised after purification by HPLC.

**ESI:** $m/z = 494 \ [M+H]^2+.$
White solid $^1$H NMR (400 MHz, CD$_3$OD): $\delta = 7.59$ (d, $^3J = 7.9$ Hz, 1H, H$_{\varepsilon}$Tryptamine), 7.52 (dd, $^3J = 12.9$ Hz, 7.9, 2H, H$_{\varepsilon}$Trp), 7.37 (d, $^3J = 8.2$ Hz, 1H, H$_{\varepsilon}$Tryptamine), 7.33 (d, $^3J = 8.1$ Hz, 2H, H$_{\varepsilon}$Trp), 4.62 (dd, $^3J = 8.9$, 6.0 Hz, 1H, H$_{\varepsilon}$Arg), 4.44 (t, $^3J = 7.0$ Hz, 1H, H$_{\varepsilon}$Arg), 4.23 (dd, $^3J = 9.2$, 5.0 Hz, 1H, H$_{\varepsilon}$Arg), 4.02–3.94 (m, 3H, H$_{\alpha}$Arg), 3.47 (m, 3H, H$_{\alpha}$Tryptamine), 3.34–3.29 (m, 5H, 2H $\beta$Trp, 3H $\alpha$Trp signals overlapping with solvent peak), 3.18 (m, 4H, 2H$^\beta$Trp, 2H$^\beta$Tryptamine), 3.11 (m, 2H, H$_{\varepsilon}$Arg), 2.89 (t, $^3J = 7.1$ Hz, 2H, H$_{\varepsilon}$Arg), 2.02 (s, 3H, H$_{CH3CO}$), 1.86 (td, $^3J = 13.9$ Hz, 6.2, 1H, H$_{\beta}$Arg), 1.73–1.62 (m, 1H, H$_{\beta}$Arg), 1.60–1.47 (m, 2H, H$_{\gamma}$Arg), 1.54–1.42 (dd, $^3J = 13.1$, 5.8 Hz, 2H, H$_{\beta}$Arg), 1.19–1.14 (dt, $^3J = 13.8$, 6.8 Hz, 2H, H$_{\gamma}$Arg).

$^{13}$C NMR (100.6 MHz, CD$_3$OD) $\delta =$ 175.8 (C=O), 175.5 (C=O), 174.8 (C=O), 174.6 (C=O), 163.4 (C=O), 163.1 (C=O), 158.7 (C$_{\varepsilon}$Arg), 158.7 (C$_{\varepsilon}$Arg), 138.3 (C$_{\alpha}$Trp), 138.2 (C$_{\alpha}$Trp), 138.0 (C$_{\varepsilon}$Tryptamine), 130.5 (C$_{Tryptamine,Trp}$), 128.7 (C$_{\varepsilon}$Tryptamine), 128.7 (C$_{\varepsilon}$Trp), 128.2 (C$_{\varepsilon}$Trp), 124.5, 124.0, 122.6, 122.4, 119.9, 119.8 and 119.8 (C$_{\eta}$Arg), 119.0, 118.9 and 118.8 (C$_{\varepsilon}$Trp, Tryptamine), 112.4, 112.4 and 112.3 (C$_{\varepsilon}$Trp, Tryptamine), 57.1 (C$_{\varepsilon}$Trp), 56.3 (C$_{\varepsilon}$Tryptamine), 55.9 (C$_{\varepsilon}$Trp), 55.9 (C$_{\varepsilon}$), 55.4 (C$_{\varepsilon}$Arg), 55.6 (C$_{\beta}$), 54.5 (C$_{\varepsilon}$Arg), 41.6 and 41.5 (C$_{\alpha}$Arg), 35.8 and 35.8 (C$_{\gamma}$), 29.3,29.2 and 28.8 (C$_{\beta}$Arg), 27.9 (C$_{\beta}$Trp), 28.1 (C$_{\beta}$Trp), 25.9 (C$_{\gamma}$Arg), 25.5 (C$_{\gamma}$Arg), 22.3 (C$_{CH3est}$), 21.5 (C$_{Tryptamine}$).
was synthesised according to the SPPS - general protocol (see Materials and methods). Amino acids (4 fold excess) were mixed with equimolar amounts of TBTU and HOBr. Then, 9 equivalents of DIPEA were added and the whole mixture was dissolved in 1.5 mL of DMF and leads to activate for 5 min. Ferrocene (5 fold excess) activation was effectuated with equimolar amounts of HATU and HOBr and DIPEA (9 fold excess).

After the synthesis was finished, the resin was treated with a mixture of 85% TFA, 10% phenol and 0.5% TIS (6 ml/g resin) for 3 hours. After removal of all volatile compounds in vacuo, the crude product was precipitated by addition of cold Et₂O. After centrifugation, the peptide precipitate was washed again with cold Et₂O and dried in air. The peptide was then dissolved in H₂O/MeCN, filtered and lyophilized. The pure compound was characterised after purification by HPLC.

**ESI:** m/z = 579 [M+H]²⁺.
Yellow solid - \(^1\)H NMR (400 MHz, CD\(_3\)OD): \(\delta = 7.70–7.68 \) (d, \(^3J = 7.06 \) Hz, 1H, H\(^{\text{c2}}\)_Tryptamine), 7.61–7.59 (d, \(^3J = 7.9 \) Hz, 1H, H\(^{\text{c3}}\)_Trp), 7.54–7.52 (d, \(^3J = 7.9 \) Hz, 1H, H\(^{\text{c3}}\)_Trp), 7.44 (t, \(^3J = 7.8 \) Hz, 2H, H\(^{\text{c3}}\)_Trp), 7.35 (d, \(^3J = 8.1 \) Hz, 2H, H\(^{\text{b}}\)_Arg), 7.24–7.16 (m, 7H, H\(^{\text{b}}\)_Trp, H\(^{\text{c2}}\)_Tryptamine), 7.00 (t, \(^3J = 7.7 \) Hz, 1H, H\(^{\text{c2}}\)_Tryptamine), 6.84 (m, 2H, H\(^{\text{b}}\)_Trp), 4.66–4.63 (m, 3H, H\(^{\alpha}\)_Trp overlapping with water peak), 4.39 (br s, H\(^{\text{N}}\)_N, 1H, CONH\(_2\)), 4.32–4.28 (m, 3H, H\(^{\text{Cp}}\), overlapping with H\(^{\alpha}\)_Trp), 4.14–4.11 (t, \(^3J = 6.5 \) Hz, 1H, H\(^{\text{b}}\)_Arg), 4.03 (s, 7H, H\(^{\text{a}}\) signals overlapping with H\(^{\text{CP}}\)), 3.80 (br s, H\(^{\text{N}}\), 1H, CONH\(_2\)), 3.55–3.48 (m, 4H, H\(^{\text{c}}\)_Tryptamine), 3.40–3.14 (m, 10H, H\(^{\beta}\)_Trp, H\(^{\text{b}}\)_Trp, H\(^{\text{c2}}\)_Tryptamine, H\(^{\text{b}}\)_Arg), 2.92 (t, \(^3J = 6.0 \) Hz, 2H, H\(^{\text{a}}\)_Arg), 1.99–1.90 (m, 1H, H\(^{\text{b}}\)_Arg), 1.80–1.71 (m, 1H, H\(^{\text{b}}\)_Arg), 1.68–1.60 (m, 2H, H\(^{\text{Arg}}\)), 1.58–1.53 (m, 2H, H\(^{\text{b}}\)_Arg), 1.32–1.29 (m, 2H, H\(^{\text{Arg}}\)).

\(^{13}\)C NMR (100.62 MHz, CD\(_3\)OD): \(\delta = 175.5 \) (C=O), 174.8 (C=O), 174.8 (C=O), 174.5 (C=O), 168.0 (C=O), 162.9 (C=O), 160.9 (C\(^{\text{c}}\)_Arg), 158.8 (C\(^{\text{c}}\)_Arg), 138.6, 138.4 and 138.2 (C\(^{\text{c2}}\)_Tryptamine-Trp), 130.2 (C\(^{\text{c}}\)_Tryptamine-Trp), 128.9, 128.7 and 128.2 (C\(^{\text{c2}}\)_Tryptamine-Trp), 124.6, 125.1, 124.4, 123.7, 122.6, 120.1 (C\(^{\text{b}}\)_Trp, Tryptamine), 119.5, 119.2 and 118.5 (C\(^{\text{c3}}\)_Trp), 112.5, 112.4 and 112.3 (C\(^{\text{c2}}\)_Trp, Tryptamine), 101.6 (C\(^{\text{b}}\)_Trp, Tryptamine), 56.4 (C\(_{\text{CP ipso}}\)), 56.2 (C\(_{\text{CP ipso}}\)), 55.9 (C\(_{\text{CP}}\)), 55.2 (C\(_{\text{Arg}}\)), 56.4 (C\(_{\text{Tryptamine}}\)), 54.4 (C\(_{\text{Trp}}\)), 54.4 (C\(_{\text{CP ipso}}\)), 41.7 and 41.6 (C\(_{\text{Arg}}\)), 35.8 (C\(_{\text{c}}\)), 29.2, 29.2 and 28.9 (C\(_{\text{b}}\)_Arg), 28.1 (C\(_{\text{Trp}}\)), 25.9 and 25.6 (C\(_{\text{Arg}}\)), 21.6 (C\(_{\text{b}}\)_Tryptamine).
was synthesised according to the SPPS - general protocol (see Materials and methods). Amino acids (4 fold excess) were mixed with equimolar amounts of TBTU and HOBt. Then, 9 equivalents of DIPEA were added and the whole mixture was dissolved in 1.5 mL of DMF and leads to activate for 5 min. Ferrocene (5 fold excess) activation was effectuated with equimolar amounts of HATU and HOBt and DIPEA (9 fold excess). After the end of the ferrocene coupling, the on-resin disulfide bond was formed via treatment with Tl(CF$_3$COO)$_3$ (1.5 equiv, 1 × 24 h) in DMF. The peptide-resin was then washed with DMF, CH$_2$C1$_2$ and methanol and was treated with a mixture of 85% TFA, 10% phenol and 0.5% TIS (6 ml/g resin) for 3 hours.
After removal of all volatile compounds \textit{in vacuo}, the crude product was precipitated by addition of cold Et$_2$O. After centrifugation, the peptide precipitate was washed again with cold Et$_2$O and dried in air. The peptide was then dissolved in H$_2$O/MeCN, filtered and lyophilized. The pure compound was characterised after purification by HPLC.

\textbf{ESI:} m/z = 681 [M+2H]$^2^+$, 1361 [M]$^+$

\textbf{Yellow solid.} $^1$H NMR (400 MHz, DMSO): $\delta$ = 10.82 (s, 1H, H$^{e1}_{\text{Trp}}$), 10.74 (d, 2H, H$^{e1}_{\text{Trp}}$), 8.43 (d, $^3$J = 7.6 Hz, 1H, H$^{N}$), 8.33 (d, $^3$J = 7.1 Hz, 1H, H$^{N}$), 8.26–8.21 (m, 1H, H$^{N}$), 8.17 (d, $^3$J = 5.0 Hz, 1H, H$^{N}$), 8.11 (d, $^3$J = 7.7 Hz, 1H, H$^{N}$), 7.91–7.83 (m, 2H, H$^{N}$), 7.80 (d, $^3$J = 4.5 Hz, 1H, H$^{N}$), 7.62–7.43 (m, 4H, H$^{e3}_{\text{Trp}},$ H$^{e}_{\text{Arg}}$), 7.36–6.94 (m, 10H, H$^{e3,\eta_2,\zeta_3,\delta_{\text{Trp}}}$), 4.82 (s, 2H, H$_{\text{Cp \text{ipso}}}$), 4.66 (m, 1H, H$^{a}_{\text{Trp}}$), 4.54 - 4.40 (m, 5H, H$^{a}_{\text{Arg},\text{Trp},\text{Cys}}$), 4.37 (s, 2H, H$_{\text{Cp \text{ipso}}}$), 4.22 (s, 5H, H$_{\text{Cp}}$), 4.03–3.90 (m, 2H, H$^{\beta}_{\text{Gly}},$ H$^{a}_{\text{Arg}}$), 3.81–3.75 (dd, 1H, H$^{\beta}_{\text{Gly}}$), 3.35 (s, H$_2$O), 3.29–2.86 (m, 14H, H$^{\beta}_{\text{Trp},\text{Cys},\text{H^5}_{\text{Arg}}}$), 1.63 - 1.52 (m, 2H, H$^{\beta}_{\text{Arg}}$), 1.45–1.31 (s, 4H, H$^{\gamma}_{\text{Arg}}$), 1.20–1.09 (m, 2H, H$^{\gamma}_{\text{Arg}}$).

\textbf{$^{13}$C NMR (100.6 MHz, DMSO)}: $\delta=$171.7 (C=O), 171.2 (C=O), 171.0 (C=O), 170.8 (C=O), 170.8 (C=O), 170.2 (C=O), 170.0 (C=O), 169.9 (C=O), 156.5 (C$^\zeta_{\text{Arg}}$), 156.5 (C$^\zeta_{\text{Arg}}$), 135.9 (C$^{e2}_{\text{Trp}}$), 135.9 (C$^{e2}_{\text{Trp}}$), 135.8 (C$^{e2}_{\text{Trp}}$), 129.8 (C$^{e}_{\text{Trp}}$), 129.6 (C$^{e}_{\text{Trp}}$), 129.1 (C$^{e}_{\text{Trp}}$), 127.0 (C$^{e2}_{\text{Trp}}$), 126.9 (C$^{a2}_{\text{Trp}}$), 126.9 (C$^{a2}_{\text{Trp}}$), 123.4, 123.2 and 123.1 (C$^{\zeta3}_{\text{Trp}}$), 120.7, 120.6 and 120.5 (C$^{n2}_{\text{Trp}}$), 118.2, 118.0 and 117.9 (C$^{e2}_{\text{Trp}}$), 111.2, 111.1 and 110.9 (C$^{e2}_{\text{Trp}}$), 110.0, 109.9 and 109.4 (C$^{b1}_{\text{Trp}}$), 69.5 (C$_{\text{Cp \text{ipso}}}$), 68.7 (C$_{\text{Cp}}$), 67.6 (C$_{\text{Cp \text{ipso}}}$), 54.7 and 54.2 (C$^\zeta_{\text{Cys}}$), 52.4, 52.4 and 52.2 (C$^{e}_{\text{Trp}}$), 53.1 and 53.0 (C$^{a}_{\text{Arg}}$), 41.9 (C$^{\beta}_{\text{Gly}}$), 40.1, 40.0, 39.9 and 39.8 (C$^{b1}_{\text{Arg},\text{Cys}}$ overlapping with solvent peak), 27.3 (C$^{\beta}_{\text{Arg}}$), 26.5 and 26.5 (C$^{b}_{\text{Trp}}$), 24.7 and 24.6 (C$^{e}_{\text{Arg}}$).
To a suspension of ruthenocene (300 mg, 1.297 mmol, 1 eq.) and anhydrous AlCl$_3$ (173 mg, 1.297 mmol, 1 eq.) in CH$_2$Cl$_2$ (15 mL) was added dropwise 2-chlorobenzoylchloride (0.164 mL, 1 mmol, 0.8 eq.) at -20 °C (deep red colour). The mixture was then stirred at 0°C for 30 min and then at room temperature for 2 h. The reaction was cooled again to 0°C and H$_2$O (2 mL) was added (exothermic reaction). The two layers were decanted and aq. NaOH (10%) was added to the organic phase (colour changes to yellow). The combined organic layers were dried over Na$_2$SO$_4$, filtered and concentrated. Purification by flash chromatography (hexane/EtOAc, 4:1) afforded 26 as yellow solid (180 mg, 38%).

Mp = 116-118 °C;

$^1$H NMR (250 MHz, CDCl$_3$): $\delta$ = 7.33–7.15 (m, 3H, H$_{4,5,6,7}$), 4.86 (m, 2H, H$_{e,c'}$), 4.68 (m, 2H, H$_{b,b'}$), 4.53 (s, 5H, H$_a$).

ESI: $m/z = 370$ [M+H]$^+$. 

C$_{17}$H$_{13}$ClORu
369.97 g/mol

26
To a suspension of 26 (300 mg, 0.80 mmol, 1 eq.) and t-BuOK (358 mg, 3.20 mmol, 4 eq.) in 1,2-dimethoxyethane (8 mL) was added H₂O (16.0 µL, 0.48 mmol, 0.6 eq.). The mixture was stirred for 18h at 105°C under nitrogen and diluted with H₂O (10 mL) and Et₂O. The organic layer was extracted with 10% aq. NaOH (20 mL). The aqueous layers were combined and acidified with concentrated aq. HCl and extracted with Et₂O. The combined organic layers were dried with MgSO₄, filtered and evaporated. Purification by flash chromatography (CH₂Cl₂/MeOH, 98:2) gave 27 as a yellow solid (140 mg, 63%) [142, 143].

**Mp** = 254-256°C;

**¹H NMR (250 MHz, DMSO):** δ = 12.17 (br. s, 1H, H₉OH), 5.02 (m, 2H, Hₙₙₙₙ), 4.74 (m, 2H, Hₜₜ), 4.61 (s, 5H, Hₐ);

**¹³C NMR (63 MHz, DMSO):** δ = 171.1 (C₉₉), 76.7 (Cₙₙₙₙ), 73.2 (Cₜₜ), 72.0 (Cₐ);

**GC-MS:** m/z = 276 [M+H]⁺.
A schlenk was charged with 1,3,5-tribromomethylbenzene 29 (0.5 g, 1.401 mmol), NaN₃ (0.9 g, 15.131 mmol), and dry acetone (20 mL). The resulting solution was heated at reflux for 10 h. and allowed to cool to r.t. The reaction mixture was diluted with Et₂O (50 mL) and H₂O (50 mL) and decanted. The organic layer was washed with brine (100 mL), dried over Na₂SO₄, and concentrated (without heating) under vacuum to give crude slightly yellow oil. Purification by flash chromatography (hexane/CH₂Cl₂, 1:1) afforded 30 as slightly yellow oil (276 mg, 81%)[^15].

**Caution:** This step is dangerous; the pure compound should not be heated. A blast shield should be used.

Rₛ(DCM) = 0.61;

^1H NMR (250 MHz, CDCl₃): δ = 7.24 (s, 3H, Hₐr), 4.39 (s, 6H, HCH₂)

^13C NMR (50.32 MHz, (CD₃)₂CO): δ = 138.67 (CₗH₂), 129.12 (C₁H), 55.15 (CₗH₂N₃);

GC-MS: m/z = 243 [M]^*.
(440 mg, 1.809 mmol, 1 eq.) was dissolved in a (1:1) mixture of THF/H₂O (10 mL) and Ph₃P (712 mg, 2.713 mmol, 1.5 eq.) was then added. The solution was stirred overnight. The organic solvent was evaporated and a small amount of water (15 mL) was added. The water phase was washed with toluene (3 × 50 mL) and CH₂Cl₂ (3 × 50 mL) and the aqueous solution was lyophilised to give product as a white solid (300 mg, 93%).

**H NMR (200 MHz, D₂O):** \( \delta = 3.78 \) (s, 6H, H₅NH₂), 4.80 (s, 6H, HCH₂), 7.20 (s, 3H, HCH);

**C NMR (50.32 MHz, D₂O):** 142.9 (CCH₂), 125.5 (CCH), 45.2 (CCH₂NH₂);

**ESI:** \( m/z = 166.10 \) [M+H]+, 330 [2M]+.
The following strategies were applied to obtain the 31, but they did not provide the desired product and only the Staudinger reaction gave constant results and good yield.

*Gabriel reaction*

![Chemical structure](image)

Compound 29, 3.6 equiv. of potassium phthalimide, and 0.3 equiv. of 18[crown]-6 were dissolved in toluene under argon. The mixture was heated to 100 °C for 24 h and quenched with H₂O. The aqueous layer was extracted with CH₂Cl₂ (4 × 20 mL). The combined organic layers were dried over Na₂SO₄ and concentrated. The residue was purified by flash chromatography (CH₂Cl₂/acetone, 20:1).

The phthalimide compound was dissolved in a hot mixture of dry EtOH/toluene (2:1) and refluxed with 6 equiv. of hydrazine hydrate for 72 h. The reaction mixture was evaporated, and the residue was first suspended in ether and then shaken with a cold aq. solution of KOH (40%). The extraction was repeated four times, and the combined organic extracts were dried over Na₂SO₄ and concentrated. Unfortunately, the phthalimide could not be removed after several repetitions of the hydrazine treatment."
In a 500-mL flask, under an argon atmosphere, LiAlH₄ (3 g, 80.2 mmol) was suspended in THF (150 mL). The suspension was cooled with an ice bath, and a solution of 30 (6.5 g, 26.7 mmol) in THF (60 mL) was added dropwise over a period of 1.5h. After the addition was completed (yellow-pink suspension), the mixture was heated at reflux for 12 h. The mixture was cooled with an ice bath, and H₂O (8 mL) was slowly added. The gray mixture was stirred at r.t. for 20 min and filtered over a pad of Celite. The solvent was removed, but formation of the desired product was not observed. [175]
(50 mg, 0.303 mmol, 1 eq.) and glutaric anhydride (0.104 g, 0.909 mmol, 3 eq.) were mixed together in DMF and stirred in a microwave reactor for 1h at 50°C. MeOH was repeatedly added and then evaporated several times to dryness. The product was isolated in quantitative yield.

\[ \text{H NMR (250 MHz, MeOD): } \delta = 7.11 \text{ (s, 3H, H}_\text{Ar}), 4.34 \text{ (s, 6H, H}_\text{CH}_2), 2.44-2.24 \text{ (m, 12H, H}_\text{COCH}_2), 2.01-1.83 \text{ (m, 6H, H}_\text{CH}_2); \]

\[ \text{C NMR (63 MHz, MeOD): } \delta = 175.4 \text{ (C}_\text{CO}), 175.4 \text{ (C}_\text{CO}), 140.8 \text{ (C}_\text{Ar}), 126.7 \text{ (C}_\text{Ar}), 44.1 \text{ (C}_\text{CH}_2\text{NH}), 36.0 \text{ (C}_\text{COCH}_2), 34.1 \text{ (C}_\text{CH}_2\text{CO}), 22.4 \text{ (C}_\text{CH}_2\text{CH}_2\text{CH}_2); \]

\[ \text{ESI: m/z = 506 [M-H]}. \]
KCN (0.282 g, 4.333 mmol) was dissolved in DMSO (10 mL) at 40 °C and a solution of 29 (500 mg, 1.401 mmol) in DMSO (5 mL) was then slowly added. The reaction mixture was stirred for 15 h at 40 °C and then poured into ice-cold H₂O (50 mL). The aqueous phase was extracted with Et₂O (2 × 100 mL) and CH₂Cl₂ (2 × 100 mL). The combined organic layers were washed with brine (2 × 50 mL), dried over Na₂SO₄, filtered and concentrated to give product as a slight yellow solid (246 mg, 90%) [202].

¹H NMR (200 MHz, CDCl₃): δ = 7.30 (s, 3 H, H₆Ar), 3.80 (s, 6 H, CH₂);

¹³C NMR (50.32 MHz, DMSO): δ = 132.2 (C₆Ar,q), 127.3 (C₆Ar), 117.0 (C-CN), 23.2 (C-CH₂);

GS-MS: m/z = 195 [M]+.
Product is commercially available

\[
\begin{array}{c}
\text{HOOC} \\
\text{HOOC} \\
\text{COOH}
\end{array}
\]

\[\text{C}_{12}\text{H}_{12}\text{O}_6\]

252.22 g/mol

35 was dissolved in a mixture of con. H\textsubscript{2}SO\textsubscript{4} and H\textsubscript{2}O and refluxed for 5 h. The cooled mixture was diluted with water and extracted with ether. Unfortunately the organic phase did not contain the desired product and it was not possible to extract the product from the aqueous phase\textsuperscript{[171, 172]}.
I step

Fmoc-Trp(Boc)-OH (3.53 g, 6.7 mmol, 1 eq.) was dissolved in MeOH (20 mL), DMAP (10%, 82 mg, 0.67 mmol), and EDAC (2.57 g, 13.41 mmol, 2 eq.) were then successively added. The reaction was stirred at r.t. for 1h. The reaction was then diluted with EtOAc (500 mL) and washed with aq. HCl (1M, 500 mL), sat. aq. Na₂CO₃ (500 mL) and brine (500 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated under reduced pressure.

II step

To compound 39a was added a (1:1) solution of piperidine/DMF (15 mL). The reaction was stirred at r.t. for 30 min, after which time it was diluted with of EtOAc (500 mL). The reaction was washed with sat. aq. Na₂CO₃ (2 × 500 mL) and brine (500 mL). The organic layer was dried over Na₂SO₄, and concentrated under reduced pressure. The crude material was purified by flash chromatography (hexanes/EtOAc, 1:1,) to yield the product as colourless oil (1.64 g, 78%)[182].

R_f (hexanes/EtOAc, 1:1) = 0.3;

^1^H NMR (250 MHz, CD₂Cl₂): δ = 7.99 (d, 3J = 7.8 Hz, 1H, H₂), 7.42–7.28 (m, 2H, H₆₅), 7.09 (dt, 3J = 22.9, 7.3 Hz, 2H, H₇₄), 3.67–3.57 (m, 1H, H₉), 3.50 (s, 3H, H₁₁), 2.99 (dd, 3J = 14.3, 4.8 Hz, 1H, H₈), 2.77 (dd, 3J = 14.4, 7.6 Hz, 1H, H₈), 1.48 (s, 9H, H₁₄);

^1^3^C NMR (63 MHz, CDCl₃): δ = 175.4 (C₁₀), 149.5 (C₁₂), 135.5 (C₇₄), 130.5 (C₃), 124.5 (C₃₃), 124.1 (C₅), 122.5 (C₆), 118.9 (C₄), 116.2 (C₇), 115.3 (C₃), 83.4 (C₁₃), 54.4 (C₉), 51.9 (C₁₁), 30.5 (C₈), 28.1 (C₁₄);

ESI: m/z = 341 [M+Na]^+. 
To 39a (90 mg, 0.283 mmol, 1 eq.) were added HOBt (43 mg, 0.283 mmol, 1 eq.), EDAC (81 mg, 0.424 mmol, 1.5 eq.), DMAP (10%, 3 mg, 0.0283 mmol) and hexynoic acid (0.034 mL, 0.283 mmol, 1 eq.). The reaction was stirred at room temperature o.n., after which time the reaction was washed with sat. aq. Na₂CO₃ (2 × 100 mL) and brine (1 × 500 mL). The organic layer was dried over Na₂SO₄, and the solvent was removed under reduced pressure and directly purified by flash chromatography (EtOAc/Hexane, 1:1) to yield the product as colourless oil (70 mg, 60%).

¹H NMR (250 MHz, CD₂Cl₂): δ = 8.08 (d, ³J = 8.2 Hz, 1H, H₂), 7.50–7.15 (m, 4H, H₄,₅,₆,₇), 6.04 (d, ³J = 7.6 Hz, 1H, HNH), 4.99–4.86 (m, 1H, H₉), 3.69 (s, 3H, H₁₁), 3.33–3.13 (m, 2H, H₈), 2.30 (t, ³J = 7.4 Hz, 2H, H₁₆), 2.21 (td, ³J = 6.7, 2.1 Hz, 2H₁₈), 1.92 (t, ⁴J = 2.6 Hz, 1H, H₂₀), 1.81 (p, ³J = 6.9 Hz, 2H, H₁₇), 1.64 (s, 9H, H₁₄);

¹³C NMR (63 MHz, CDCl₃): δ = 172.8 (C₁₅), 172.7 (C₁₀), 150.2 (C₁₂), 135.9 (C₇₉), 131.1 (C₂), 125.2 (C₉), 124.7 (C₅), 123.3 (C₆), 119.4 (C₄), 115.9 (C₇), 115.5 (C₂), 84.4 (C₁₉), 83.9 (C₁₃), 69.9 (C₂₀), 53.1 (C₉), 53.1 (C₁₁), 35.5 (C₁₆), 28.8 (C₁₄), 28.0 (C₁₇), 24.6 (C₈), 18.4 (C₁₈);

ESI: m/z = 435 [M+Na]⁺.
To a solution of L-Arginine(Pbf) methyl ester hydrochloride (650 mg, 1.363 mmol, 1 eq.) in CH$_2$Cl$_2$ was added triethylamine (0.284 mL, 2.044 mmol, 1.5 eq.) for 30 min. Hexynoic acid (162 mL, 1.363 mmol, 1 eq.), HOBt (209 mg, 1.363 mmol, 1 eq.), EDAC (392 mg, 2.045 mmol, 1.5 eq.), DMAP (10%, 17 mg, 0.136 mmol) were added into the solution. The reaction was stirred at r.t. overnight, after which time the reaction mixture was washed with sat. aq. Na$_2$CO$_3$ (2 × 100 mL) and brine (1 × 500 mL). The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (EtOAc/Hexane, 1:1) gave product as colorless oil (647 mg, 89%).

$^1$H NMR (250 MHz, CD$_2$Cl$_2$): $\delta = 6.61$ (d, $^3J = 7.8$ Hz, 1H, H$_{NH}$), 6.27 (s, 1H, H$_{NH}$), 4.47 (m, 1H, H$_5$), 3.69 (s, 1H, H$_7$), 3.30–3.13 (m, 2H, H$_{11}$), 2.97 (s, 2H, H$_b$), 2.54 (s, 3H, H$_c$), 2.48 (s, 3H, H$_d$), 2.33 (t, $^3J = 7.4$ Hz, 2H, H$_2$), 2.20 (td, $^3J = 7.0$, 2.6, 2H, H$_{10}$), 2.08 (s, 3H, H$_d$), 2.01 (t, $^4J = 2.6$ Hz, 1H, H$_{14}$), 1.84–1.70 (m, 4H, H$_{4,12}$), 1.67–1.54 (m, 2H, H$_3$), 1.45 (s, 6H, H$_a$);

$^{13}$C NMR (63 MHz, CD$_2$Cl$_2$): $\delta = 173.5$ (C$_6$), 173.1 (C$_6$), 159.2, 156.8, 138.8, 132.8, 125.38, 117.9 (C$_q$ from Pbf), 87.1 (C$_{q,a}$), 83.9 (C$_{13}$), 69.6 (C$_{14}$), 52.9 (C$_7$), 43.6 (C$_b$), 35.2 (C$_{10}$), 28.8 (C$_a$), 24.8 (C$_3$), 19.6 (C$_d$), 18.2 (C$_c$), 12.7 (C$_c$);

ESI: $m/z = 535$ [M+H]$^+$. 
To a solution of hexynoic acid (0.292 mL, 2.456 mmol, 1 eq.) in CH$_2$Cl$_2$ (5 mL) was added (COCl)$_2$ (0.466 mL, 5.528 mmol, 2 eq.) and the mixture was refluxed for 1h. The clear solution was allowed to cool at r.t. and the solvent was removed under reduced pressure. The colourless oil was dissolved in CH$_2$Cl$_2$ (5 mL), L-Leucine methyl ester hydrochloride (0.446 g, 2.456 mmol, 1 eq.) and Et$_3$N (0.684 mL, 4.912 mmol, 2 eq.) were added to the solution. After 2 h of stirring at r.t. and removal of triethylamine hydrochloride by filtration, the solvent was removed under reduced pressure. The colourless oil was purified by flash chromatography (CH$_2$Cl$_2$/MeOH, 10:1) to give 42 as colourless oil (306 mg, 52 %).

$R_f$(CH$_2$Cl$_2$/MeOH, 10:1) = 0.3;

$^1$H NMR (250 MHz, CD$_2$Cl$_2$): $\delta$ = 6.45 (d, $^3$J = 8.0 Hz, 1H, H$_{NH}$), 4.53 (td, $^3$J = 8.4, 5.5 Hz, 1H, H$_7$), 3.67 (s, 3H, H$_9$), 2.31 (t, $^3$J = 7.4 Hz, 2H, H$_8$), 2.26–2.17 (m, 2H, H$_3$), 2.02 (t, $^4$J = 2.7 Hz, 1H, H$_{1, \text{alkyne}}$), 1.87–1.72 (m, 2H, H$_4$), 1.72–1.44 (m, 2H, H$_{10}$), 0.90 (dd, $^3$J = 6.2, 2.3 Hz, 7H, H$_{11,12}$);

$^{13}$C NMR (63 MHz, CD$_2$Cl$_2$): 174.0 (C$_6$), 172.6 (C$_8$), 84.1 (C$_2$), 69.4 (C$_1$), 52.6 (C$_9$), 51.2 (C$_7$), 41.7 (C$_{10}$), 35.2 (C$_3$), 25.3 (C$_4$), 24.8 and 23.1 (C$_{12}$), 22.1 (C$_{11}$), 18.2 (C$_5$);

ESI: $m/z$ = 238 [M].
(Boc)₂O (6 g, 27.49 mmol, 1.5 eq.) was solubilised in dioxane (20 mL) and was added dropwise to a mixture of 51 (1.25 mL, 19.52 mmol, 1 eq.) in dioxane cooled at 0°C. The ice bath was removed and the mixture was stirred at r.t. overnight. The solvent was removed in vacuo and the crude material was purified by flash chromatography (hexane/EtOAc, 9:1) to give the product as a white solid (4.14 g, 98%)\(^{[203]}\).

\[ R_f \text{ (hexane/EtOAc, 9:1) = 0.28}; \]

\[ \text{M} \text{p} = 39-40^\circ \text{C}; \]

\[ ^1\text{H NMR (200 MHz, CDCl}_3): \delta = 4.68 \text{ (br.s, 1H, } H_{\text{NH}}), 3.89 - 3.85 \text{ (m, 2H, } H_{\text{CH2}}), 2.19 \text{ (t, } J = 2.5 \text{ Hz, 1H,H}_{\text{alkyne}}), 1.43 \text{ (s, 9H, } H_{\text{Boc}}); \]

\[ ^13\text{C NMR (50.32 MHz, CDCl}_3): 155.4 \text{ (C}_2\text{O)}, 80.3 \text{ (C}_{\text{alkyne},q}), 71.4 \text{ (C}_{\text{alkyne}}), 30.6 \text{ (C}_{\text{CH2}}), 29.4 \text{ (C}_{\text{Boc}}); \]

\[ \text{ESI: } m/z = 178 \text{ [M+Na]}^+. \]
To a solution of 52 (300 mg, 1.304 mmol, 1 eq.) in dried and degassed CH$_2$Cl$_2$ (10 mL) was added (COCl)$_2$ (0.168 mL, 1.956 mmol, 1.5 eq.). The mixture was refluxed for 1h and the resulting clear orange solution was allowed to cool down. The solvent was removed under reduced pressure. The resulting orange solid was dissolved in CH$_2$Cl$_2$ (10 mL) and propargylamine (0.084 mL, 1.304 mmol, 1 eq.) followed by Et$_3$N (0.182 mL, 1.304 mmol, 1 eq.) were added to the solution. After 6 h of stirring at r.t. and removal of triethylamine hydrochloride by filtration, the solvent was removed under reduced pressure: The orange solid was dissolved in CHCl$_3$ (20 mL) and washed with H$_2$O (3 × 20 mL), dried over Na$_2$SO$_4$ filtered and evaporated to give product as a brown powder (113 mg, 29%).

$^1$H NMR and $^{13}$C NMR were in accordance with published data in reference $^{183}$. 

FAB: $m/z = 267.0$ [M]$^+$. 

C$_{12}$H$_{13}$FeNO
267.1 g/mol

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To a solution of 52 (0.310 g, 1.348 mmol, 1 eq.) in dried and degassed CH₂Cl₂ (10 mL) was added (COCl)₂ (0.174 mL, 2.02 mmol, 1.5 eq.). The mixture was refluxed for 1h and the resulting clear orange solution was allowed to cool down. The solvent was removed under reduced pressure. The resulting orange solid was dissolved in CH₂Cl₂ (10 mL) and diethylpropargylamine (0.181 mL, 1.348 mmol, 1 eq.) followed by Et₃N (0.188 mL, 1.348 mmol, 1 eq.) were added to the solution. After 6 h of stirring at r.t. and removal of triethylamine hydrochloride by filtration, the solvent was removed under reduced pressure: The orange solid was dissolved in CHCl₃ (20 mL) and washed with H₂O (3 × 20 mL), dried over Na₂SO₄ filtered and evaporated to give product as a brown powder (200 mg, 46%).¹⁸⁶

¹H NMR (200 MHz, DMSO): δ = 7.08 (s, 1H, H₉NH), 4.92–4.86 (m, 2H, H₉C₆₁,₂), 4.36–4.29 (m, 2H, H₉C₆₃,₄), 4.18 (s, 4H, H₉C₆₅), 3.19 (s, 1H, H₉alkyne), 2.02 (dq, ³J = 7.4 Hz, 14.7, 2H, H₉CH₂, DEPA), 1.83 (m, 2H, H₉CH₂, DEPA), 0.95 (t, ³J = 7.3 Hz, 6H, CH₃, DEPA);

¹³C NMR (50.3 MHz, DMSO): 168.2 (C₁₁CO), 86.1 (C₉alkyne, q), 76.8 (C₉C₆), 73.1 (C₉alkyne), 69.8 (C₉C₆₂,₅), 69.2 (C₉C₆), 68.4 (C₉C₆₃,₄), 55.3 (C₉DEPA, q), 29.9 (C₉CH₂, DEPA), 8.4 (C₉CH₃, DEPA);

FAB: m/z = 323.1 [M]⁺.
30 (52 mg, 0.215 mmol) and 43 (100 mg, 0.644 mmol) were dissolved in THF. Cu(OAc)$_2$(H$_2$O)$_2$ (117 mg, 0.644 mmol) was added followed by the dropwise addition of a freshly prepared solution of sodium ascorbate (256 mg, 1.29 mmol) in H$_2$O. The solution was then stirred overnight at r.t. THF was removed in vacuo and a solution of 25% aq. NH$_3$ (20 mL) was added and allowed to stir for 10 min, in order to remove all the Cu by forming Cu(NH$_3$)$_6$. The mixture was diluted CH$_2$Cl$_2$ and decanted. The organic phase was then washed with a solution of 25% aq. NH$_3$ (3 × 20 mL), H$_2$O (4 × 20 mL), dried with Na$_2$SO$_4$, filtered and evaporated to give a crude white solid (119 mg, 79%).

$^1$H NMR (400 MHz, DMSO): $\delta = 7.89$ (s, 3H, H$_8$), 7.23 (s, 6H, H$_{2,4,6,11}$), 5.53 (s, 6H, H$_7$), 4.16 (d, $^3J = 5.7$ Hz, 6H, H$_{10}$), 1.37 (s, 27H, H$_{14}$);

$^{13}$C NMR (100 MHz, DMSO): $\delta = 155.5$ (C$_{12}$), 145.8 (C$_9$), 137.2 (C$_{1,3,5}$), 127.4 (C$_{2,4,6}$), 122.7 (C$_8$), 77.9 (C$_{13}$), 52.2 (C$_7$), 35.6 (C$_{10}$), 28.2 (C$_{14}$);

Elemental analysis: calculated for C 55.92, H 6.83, N 23.71 found C 54.95, H 6.97, N 21.08;

ESI: m/z = 731 [M+Na]$^+$. 

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«Click» reactions with microwave assistance [169, 204]

\[
\begin{align*}
\text{30} & \text{ (61 mg, 0.251 mmol, 1 equiv.) and 42 (210 mg, 0.878 mmol, 3.5 equiv.) were dissolved in DMF. Then Cu(Ac)₂(H₂O)₂ (175 mg, 0.878 mmol, 3.5 equiv.) was added in the mixture followed by the dropwise addition of a freshly prepared solution of sodium ascorbate (552 mg, 2.634 mmol, 10 equiv.). The solution was stirred for 20 min in a microwave reactor at 100°C and diluted with a solution of 25% aq. NH₃ (20 mL) and let reacted for 10 min. The mixture was diluted CH₂Cl₂ and decanted. The organic phase was then washed with a solution of 25% aq. NH₃ (3 × 20 mL), H₂O (4 × 20 mL), dried with Na₂SO₄, filtered and evaporated. Purification by flash chromatography (CH₂Cl₂/MeOH, 9:1) gave product as a white solid (192 mg, 80%).}

\text{1H NMR (400 MHz, CD₂Cl₂):} \delta = 7.39 (s, 3H, H₈), 7.17 (s, 3H, H₁₄), 6.96 (s, 3H, H₂,₄,₆), 5.46 (s, 6H, H₁), 4.51 (td, \(^3J = 8.4, 5.9\) Hz, 3H, H₁₅), 3.66 (s, 9H, H₂₀), 2.71 (td, \(^3J = 7.2, 4.6\) Hz, 6H₁₀), 2.23 (t, \(^3J = 7.5\) Hz, 6H, H₁₂), 2.00–1.89 (m, 6H, H₁₆), 1.65 (m, 3H, H₁₇), 1.59–1.49 (m, 6H, H₁₁), 0.88 (dd, \(^3J = 6.5\) Hz, 3.7, 19H, H₁₈);

\text{13C NMR (100 MHz, CD₂Cl₂):} \delta = 174.2 (C₁₃), 173.2 (C₁₉), 148.3 (C₉), 137.9 (C₁₃,₅), 126.8 (C₂,₄,₆), 122.3 (C₈), 52.5 (C₇₂₀), 51.4 (C₁₅), 46.5 (C₁₆), 41.5 (C₁₂), 35.5 (C₁₀), 25.7 (C₁₁), 25.3 (C₁₇), 23.1 (C₁₈), 22.1 (C₁₈);

\text{ESI: } m/z = 983 [M+Na]^+.
\end{align*}
\]
A mixture of Fmoc-Trp(Boc)-OH (765 mg, 1.452 mmol, 3 equiv.), HOBt (222 mg, 1.452 mmol, 3 equiv.), EDAC (417 mg, 2.178 mmol, 4.5 equiv.) and DMAP (6 µg, 0.1 equiv.) in DMF, was reacted for 5 min, and then added to a solution of [31] (80 mg, 0.484 mmol, 1 equiv.) in DMF. The reaction was stirred overnight and quenched with H₂O causing the formation of a white precipitate. The solid was filtered through a medium porosity sintered-glass funnel, and the solid was repeatedly washed with H₂O and Et₂O and further dried under reduced pressure.

The resulting white solid (648 mg, 79 %) was treated with a (1:4) solution of piperidine/DMF (15 mL). After 30 min the reaction was diluted with EtOAc (500 mL) and washed with H₂O (3 x 100 mL), dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product was purified by flash chromatography (CH₂Cl₂/MeOH, 10:1, with 1% Et₃N) to give a white solid (314 mg, 80%).

^1H NMR (250 MHz, CD₂Cl₂): δ = 8.11 (d, 3J = 8.1 Hz, 3H, H₁₅), 7.84 (br s, 3H, H₈), 7.62 (d, 3J = 7.6 Hz, 3H, H₁₅), 7.49 (s, 3H, H₁₃), 7.30 (m, 3H, H₁₆), 7.20 (m, 3H, H₁₆), 7.01 (s, 3H, H₂,4,6), 4.32 (d, 3J = 4.2, 6H, H₂), 3.75 (m, 3H, H₁₀), 3.36 (d, 3J = 3.6 Hz, 1H, H₂₁), 3.30 (d, 3J = 3.8 Hz, 2H, H₂₁), 2.88 (dd, 3J = 14.4, 8.9 Hz, 6H, H₁₁), 1.64 (s, 27H, H₂₀);

^13C NMR (63 MHz, CD₂Cl₂): δ = 174.6 (C₉), 150.1 (C₁₈), 140.2 (C₁₅,3), 136.2 (C₁₇), 130.9 (C₁₄), 126.0 (C₂₄,₆), 124.9 (C₁₃), 124.9 (C₁₆), 123.0 (C₁₆), 119.8 (C₁₅), 117.2 (C₁₅), 115.7 (C₁₂), 84.2 (C₁₉), 46.4 (C Et₃N), 43.3 (C₇), 31.0 (C₁₁), 28.5 (C₂₀), 9.1 (C Et₃N);

Elemental analysis: calculated for C 66.84, H 6.79, N 12.31 found C 64.24, H 7.03, N 11.37;

ESI: m/z = 1024.49 [M+H⁺].
30 (117 mg, 0.481 mmol, 1 equiv.) and 50 (450 mg, 2.165 mmol, 4.5 equiv.) were dissolved in DMF. Then Cu(OAc)$_2$(H$_2$O)$_2$ (290 mg, 1.443 mmol, 3 equiv.) was added in the mixture followed by the dropwise addition of a freshly prepared aq. solution of sodium ascorbate (572 mg, 2.9 mmol, 6 equiv.). The solution was stirred for 20 min with microwave radiation at 100 C°, then a solution of 25% aq. NH$_3$ (20 mL) was added and let reacted for 10 min at r.t. The mixture was diluted CH$_2$Cl$_2$ and decanted. The organic phase was then washed with a solution of 25% aq. NH$_3$ (3 × 20 mL), H$_2$O (4 × 20 mL), dried with Na$_2$SO$_4$, filtered and evaporated under reduced pressure to give product as a yellow solid (168 mg, 90 %).

NMR data available in $^{[187]}$.

FAB: $m/z = 873.1$ [M$^+$].
30 (32 mg, 0.133 mmol, 1 equiv.) and 49 (150 mg, 0.464 mmol, 3.5 equiv.) were dissolved in DMF. Then Cu(OAc)$_2$(H$_2$O)$_2$ (277 mg, 1.392 mmol, 10 equiv.) was added followed by the dropwise addition of a freshly prepared solution of sodium ascorbate (552 mg, 2.78 mmol, 21 equiv.) in H$_2$O. The solution was stirred for 20 min in a microwave reactor at 100°C and diluted with a solution of 25% aq. NH$_3$ (20 mL) and let reacted for 10 min. The mixture was diluted CH$_2$Cl$_2$ and decanted. The organic phase was then washed with a solution of 25% aq. NH$_3$ (3 × 20 mL), H$_2$O (4 × 20 mL), dried with Na$_2$SO$_4$, filtered and evaporated. Purification by flash chromatography (CH$_2$Cl$_2$/MeOH, 9:1) gave product as a yellow solid (64 mg, 40%).

$^1$H NMR (250 MHz, CD$_2$Cl$_2$): $\delta$ = 7.50 (s, 3H, H$_{2,6,4}$), 7.07 (s, 3H, H$_8$), 6.92 (s, 3H, H$_{13}$), 5.52 (s, 6H, H$_7$), 4.66 (m, 6H, H$_{Cp\, ipso}$), 4.34 (m, 6H, H$_{Cp\, ipso}$), 4.22 (s, 15H, H$_{Cp\, unsubstituted}$), 2.50 (dq, $J$ = 14.5, 7.2 Hz, 6H, H$_{11'}$), 1.90 (td, $^3J$ = 14.6, 7.3 Hz, 6H, H$_{11}$), 0.75 (t, $^3J$ = 7.3 Hz, 18H, H$_{12}$);

$^{13}$C NMR (50 MHz, CD$_2$Cl$_2$): $\delta$ = 169.34 (C$_{14}$), 152.3 (C$_9$), 137.9 (C$_{1,3,5}$), 127.4 (C$_{2,4,6}$), 121.4 (C$_8$), 78.4 (C$_{Cp,q}$), 70.7 (C$_{Cp\, ipso}$), 70.2 (C$_{Cp\, unsubstituted}$), 68.7 (C$_{Cp\, ipso}$), 59.6 (C$_{7,10}$), 31.1 (C$_{11,11'}$), 8.5 (C$_{12}$);


ESI: $m/z = 1213.17$ [M+H$^+$.]
30 (19 mg, 0.077 mmol) and 41 (70 mg, 0.270 mmol, 3.5 equiv.) were dissolved in DMF. Then Cu(OAc)$_2$(H$_2$O)$_2$ (215 mg, 1.08 mmol, 14 equiv.) was added followed by the dropwise addition of a freshly prepared solution of sodium ascorbate (641 mg, 3.24 mmol, 42 equiv.) in H$_2$O. The solution was stirred for 20 min in a microwave reactor at 100°C and diluted with a solution of 25% aq. NH$_3$ (20 mL) and let reacted for 10 min. The mixture was diluted CH$_2$Cl$_2$ and decanted. The organic phase was then washed with a solution of 25% aq. NH$_3$ (3 × 20 mL), H$_2$O (4 × 20 mL), dried with Na$_2$SO$_4$, filtered and evaporated. Purification by flash chromatography (CH$_2$Cl$_2$/MeOH, 9:1) gave 59 as a white solid (50 mg, 63%).

$^1$H NMR (250 MHz, CD$_2$Cl$_2$): $\delta$ = 7.25 (m, 15H, H$_{17,17a,18,18a,19}$), 7.07 (m, 3H, H$_{2,4,6}$), 6.98 (s, 3H, H$_8$), 6.66 (m, 3H, H$_{NH}$), 5.36(s, 6H, H$_2$), 4.77(m, 3H, H$_{14}$), 3.64 (s, 9H, H$_{21}$), 2.97 (m, 12H, H$_{10,15}$), 2.53 (t, $^3$J = 7.2 Hz, 6H, H$_{11}$);

$^{13}$C NMR (63 MHz, CD$_2$Cl$_2$): $\delta$ = 172.6 (C$_{12}$), 172.0 (C$_{20}$), 147.7 (C$_9$), 137.8 (C$_{16}$), 136.9 (C$_{1,3,5}$), 129.8 (C$_{18,18a}$), 128.9 (C$_{2,4,6,17a}$), 127.4 (C$_{19}$), 122.2 (C$_8$), 53.9 (C$_{14}$), 52.7 (C$_{7,21}$), 38.2 (C$_{15}$), 35.8 (C$_{11}$), 21.8 (C$_{10}$);

Elemental analysis: calculated for C 63.52, H 5.92, N 16.46 found C 63.32, H 6.05, N 15.14;

ESI: $m/z$ = 1043.19 [M+Na$^+$].
This peptide was synthesised on 0.147 g (0.1 mmol) resin. After Fmoc removal the N-terminus of the resin was acylated with a mixture of bromoacetic acid (2M solution in DMF) and DIC (2M solution in DMF). In the next step the alkyne group was introduced via nucleophilic displacement of the brome with propargyl amine (1M solution in DMSO).

The rest of the peptide was synthesised using successive steps of coupling and deprotection (see protocol for SPPS synthesis).

After the end of the cleavage, the residue was concentrated and precipitated with hexane. The centrifuge was used to separate the solid from the resulting solution. The yellow solid was then solubilised in a (1:1) mixture of MeCN/H$_2$O, and then lyophilised.

The peptide was purified on a RP-HPLC and the mass spectrometry shows base peak at m/z = 862.3 [M+H$^+$].
This peptide was synthesised on 0.147 g of resin (0.1 mmol). After deprotection of the Fmoc the peptide synthesis used successive steps of coupling and deprotection (see protocol for SPPS synthesis).

After the end of the cleavage, the residue was concentrated and precipitated with hexane. The centrifuge was used to separate the solid from the resulting solution. The white solid was then solubilised in a (1:1) mixture of MeCN/H$_2$O, and then lyophilised.

The peptide was purified on a RP-HPLC and the mass spectrometry shows base peak at m/z = 657.2 [M+Na$^+$].
This peptide was synthesised on 0.147 g (0.1 mmol) resin. After deprotection of the Fmoc the peptide synthesis used successive steps of coupling and deprotection (see protocol for SPPS synthesis).

After the end of the cleavage, the residue was concentrated and precipitated with hexane. The centrifuge was used to separate the solid from the resulting solution. The white solid was then solubilised in a (1:1) mixture of MeCN/H₂O, and then lyophilised.

The peptide was purified on a RP-HPLC and the mass spectrometry shows base peak at m/z = 641.2 [M+Na⁺].
For the synthesis of this peptide was used 0.192 g (0.1 mmol) resin. After cleavage the free C–terminus of the peptide was esterified, in solution, using EDAC (2 eq.) and DMAP (0.1 eq.) in methanol for 3 h.

The addition of the cleavage solution is followed by change of the colour of the resin from yellow to purple.

The mass spectrometry shows base peaks at m/z = 1515.5 [M⁺] and m/z = 1537.4 [M+Na⁺].
For the synthesis of this peptide was used 0.192 g (0.1 mmol) resin. The peptide was synthesised using successive steps of coupling and deprotection. To activate the ferrocenyl carboxylic acid were used HATU (4 eq.), HOBt (4 eq.) and DIPEA (4 eq.).

The addition of the cleavage solution is followed by change of the colour of the resin from yellow to purple.

The mass spectrometry shows base peak at m/z = 1619.2 [M⁺].

Additional notes: Drops of 10% pyridine in methanol can be added into the cleavage filtrate to neutralise the TFA.
Additional notes: Drops of 10% pyridine in methanol can be added into the cleavage filtrate to neutralise the TFA.

For the synthesis of this peptide was used 0.192 g (0.1 mmol) resin. The peptide was synthesised using successive steps of coupling and deprotection (see protocol for SPPS synthesis).

The addition of the cleavage solution is followed by change of the colour of the resin from yellow to purple.

The mass spectrometry shows base peak at m/z = 1629.4 [M^+].
This peptide was synthesised on 0.147 g (0.1 mmol) resin. After deprotection of the Fmoc the peptide synthesis used successive steps of coupling and deprotection (see protocol for SPPS synthesis).

After the end of the cleavage, the residue was concentrated and precipitated with diethyl ether. The centrifuge was used to separate the solid from the resulting solution. The yellow solid was then solubilised in a (1:1) mixture of MeCN/H₂O, and then lyophilised.

The peptide was purified on a RP-HPLC and the mass spectrometry shows base peak at m/z = 1195.3 [M⁺].
Aminoethanol (508 mg, 8.317 mmol, 1 eq.) was dissolved in anhydrous THF (30 mL) and cooled down to 0°C, and then Fmoc-Cl (2.152 g, 8.317 mmol, 1 eq.) and DIPEA (1.5 mL, 8.317 mmol, 1 eq.) were added to the solution. The reaction mixture was stirred for 24 h at r.t. and then concentrated. The organic layer was washed with EtOAc and washed with H2O (2 × 100 mL), brine, dried over Na2SO4, filtered and concentrated to give 67 in quantitative yield.

\[\text{C}_{17}\text{H}_{17}\text{NO}_3\]
\[283.32 \text{ g/mol}\]

2-Aminoethanol (508 mg, 8.317 mmol, 1 eq.) was dissolved in anhydrous THF (30 mL) and cooled down to 0°C, and then Fmoc-Cl (2.152 g, 8.317 mmol, 1 eq.) and DIPEA (1.5 mL, 8.317 mmol, 1 eq.) were added to the solution. The reaction mixture was stirred for 24 h at r.t. and then concentrated. The organic layer was washed with EtOAc and washed with H2O (2 × 100 mL), brine, dried over Na2SO4, filtered and concentrated to give 67 in quantitative yield.

\[\text{H NMR (200MHz, CDCl}_3\):} \ \delta = 7.78–7.31 (m, 8H, H\text{1,2,3,4,5,6,7,8}), 5.17 (s, 1H, H\text{NH}), 4.36 (d, \ J = 6.7 \text{ Hz, 2H, H}_{10}), 4.17 – 4.11 (m, 1H, H\text{9}), 3.64 (t, \ J = 4.2 \text{ Hz, 2H, H}_{13}), 3.32 – 3.23 (m 2H, H\text{12}), 1.97 (br.s, 1H, H\text{OH});

\[\text{C NMR (50.3MHz, CDCl}_3\):} \ \delta = 144.5 (C\text{9a,8b}), 141.9 (C\text{4a,4b}), 128.3 (C\text{1,8}), 127.7 (C\text{4,5}), 125.6 (C\text{2,7}), 120.6 (C\text{3,6}), 67.4 (C\text{10}), 62.9 (C\text{13}), 47.9 (C\text{9}), 44.1 (C\text{12});

**Elemental analysis:** calculated for C 72.00, H 6.00, N 4.94, O 16.94 found C 72.31, H 5.80, N 4.76, O 17.13;

**ESI:** \(m/z = 284 \text{ [M+H}^+]\), 306 [M+Na].
1. IBX 68 (2.97 g, 10.59 mmol) was added to a solution of 67 (1 g, 3.53 mmol) in EtOAc (40 mL). After 4 h at 80 °C the reaction mixture was filtered and evaporated to give the crude aldehyde as a white solid which was immediately used in the next step.

2. 67 was solubilised in DMSO (3 mL) followed by addition of 68 (0.198 g, 0.706 mmol). The reaction was stirred for 24 h at r.t. Then H₂O was added and the suspension filtered, the organic phase was extracted with AcOEt (2 × 50 mL) and washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated to give 69 (70 mg, 70%).[205,206]
3. To a solution of **67** (1 g, 3.53 mmol, 1 eq.) in CH$_2$Cl$_2$ (15 mL) was added TEMPO (10%, 6 mg, 0.0353 mmol) and a 1 M aq. solution of KBr (0.35 mL, 0.35 mmol, 0.1 eq.). A mixture of aqueous NaOCl (12 %, 2.18 mL, 4.24 mmol, 1.2 eq.) diluted with a sodium phosphate buffer (18 mL, 0.68 M, pH = 7.4) was then slowly added (buffer prevent deprotection of the Fmoc). After 15 min, the reaction was quenched with aq. sat. Na$_2$S$_2$O$_3$ (10 mL), decanted and the aqueous phase was extracted with CH$_2$Cl$_2$ (2 × 5 mL). The combined organic layers were washed with H$_2$O (10 mL), brine (10 mL), dried over Na$_2$SO$_4$, filtered and evaporated to give crude aldehyde as a white solid which was immediately used in the next step. [207-209]

$^1$H NMR (200 MHz, CDCl$_3$): $\delta = 9.63$ (s, 1H, H$_{\text{CHO}}$), 7.79–7.31 (m, 8H, H$_{7,8,9,10,7a,8a,9a,10a}$), 5.51 (s, 1H, H$_{\text{NH}}$), 4.43 (d, $^3J = 8$ Hz, 2H, H$_4$), 4.23 (t, $^3J = 6$ Hz, 1H, H$_5$), 4.13 (d, $^3J = 4$ Hz, 2H, H$_1$);

$^{13}$C NMR (50.3 MHz, CDCl$_3$): $\delta =$ 197.1 (C$_{14}$), 158.8 (C$_{11}$), 144.3 (C$_{9a,8b}$), 141.9 (C$_{4a,4b}$), 128.4 (C$_{1,8}$), 127.7 (C$_{4,5}$), 125.6 (C$_{2,7}$), 125.6 (C$_{3,6}$), 67.8 (C$_{10}$), 52.2 (C$_{12}$), 47.7 (C$_{9}$);

ESI: m/z = 304 [M+Na$^+$].
The mixture of bromoacetic acid (3.47 g, 24.97 mmol) and sodium azide (3.41 g, 52.44 mmol) in DMSO was stirred for 24 h, then H₂O was added and the solution adjusted to pH 5 with HCl (1M). The solution was extracted with EtOAc (3 × 50 mL). The organic layer was washed with brine, dried over anhydrous Na₂SO₄ and concentrated to give a colourless liquid, which was pumped under reduced pressure to yield the acid in quantitative yield.[210-212]

^{1}H NMR (CDCl₃, 300 MHz) [212]: δ = 11.17 (1H, s); 3.99 (2H, s).

ESI: m/z = 99.97 [M-H], 140 [M+K]⁻

*Fig.X IR spectra of 70 with a characteristic bond of the azide group at 2104 cm⁻¹.*
To a solution of 39a (90 mg, 0.283 mmol) and 70 (30 mg, 0.283 mmol) were added HOBT (43 mg, 0.283 mmol), EDAC (80 mg, 0.425 mmol) and DMAP (10%, 3 mg, 0.0283 mmol). The reaction was stirred at r.t. overnight, after which time H₂O was added, then the product was extracted with EtOAc (2 × 100 mL) and washed with H₂O (2 × 100 mL). The organic layer was dried over Na₂SO₄, filtered and evaporated to give product as yellow oil (quantitative).

**1H NMR (250 MHz, CDCl₃)** δ = 8.09 (d, 3J=7.9, 1H, H₈NH), 7.43-7.19 (m, 4H, H₄,₅,₆,₇), 6.82 (d, 3J=7.7, 1H, H₂), 4.90 (m, 1H, H₉), 3.92 (s, 2H, H₁₆), 3.69 (s, 3H, H₁₁), 3.26-3.23 (m, 2H, H₈), 1.64 (s, 9H, H₁₄).

**13C NMR (63 MHz, CDCl₃)** δ = 171.6 (C₁₁,₁₀), 166.6 (C₁₂), 130.5 (C₇a), 124.9 (C₃), 124.3 (C₃a), 122.8 (C₅), 118.8 (C₆), 115.6 (C₄), 114.7 (C₇), 99.1 (C₃), 84.0 (C₁₃), 52.8 (C₁₆), 52.7 (C₉), 52.6 (C₁₁), 28.4 (C₁₄), 27.5 (C₈).

**ESI:** m/z = 424 [M+Na⁺]
(50 mg, 0.125 mmol) and 50 (39 mg, 0.187 mmol) were dissolved in DMF. Cu(OAc)$_2$(H$_2$O)$_2$ (37 mg, 0.187 mmol) was added in the mixture followed by the dropwise addition of a freshly prepared solution of sodium ascorbate (50 mg, 0.250 mmol) in H$_2$O. The solution was then stirred overnight at r.t. A solution of 25% aq. NH$_3$ (20 mL) was added and allowed to stir for 10 min, in order to remove all the Cu by forming Cu(NH$_3$)$_6$. The mixture was diluted CH$_2$Cl$_2$, and H$_2$O was added then decanted. The organic phase was then washed with a solution of 25% aq. NH$_3$ (3 × 20 mL), H$_2$O (4 × 20 mL), dried with Na$_2$SO$_4$, filtered and evaporated to give a yellow oil.

Purification by flash chromatography (Hexane/EtOAc, 1:1) gave the product as yellow oil (52 mg, 66%).

$^1$H NMR (250 MHz, CD$_2$Cl$_2$) $\delta = 8.13$ (d, $^3J=8$, 1H, H$_{NH}$), 7.40 - 7.23 (m, 5H, H$_{4,5,6,7,18}$), 6.52 (ps-d, 1H, H$_2$), 5.01 (s, 2H, H$_{16}$), 4.93 - 4.86 (m, 1H, H$_b$), 4.69 (ps-d, 2H, H$_c$), 4.31 (s, 2H, H$_b$), 4.02 (s, 5H, H$_a$), 3.69 (s, 3H, H$_{11}$), 3.38 - 3.15 (m, 2H, H$_{1H}$), 1.66 (s, 9H, H$_{14}$).

$^{13}$C NMR (63 MHz, CD$_2$Cl$_2$) $\delta = 171.8$ (C$_{C-o}$), 165.6 (C$_{C-o}$), 147.8 (C$_{C-o}$), 130.9 (C$_{18}$), 125.1 (C$_{7a}$), 124.9 (C$_3$), 123.1 (C$_{3a}$), 120.8 (C$_{17}$), 119.2 (C$_5$), 115.9 (C$_6$), 115.1 (C$_4$), 84.5 (C$_{13}$), 70.2 (C$_a$), 69.3 (C$_c$), 67.3 (C$_b$), 67.2 (C$_{b,16}$), 28.5 (C$_{14}$), 27.6 (C$_8$).

ESI: $m/z = 611$ [M$^+$].
30 (120 mg, 0.493 mmol, 1 equiv.) and 47 (244 µL, 2.220 mmol, 4.5 equiv.) were dissolved in DMF. Then Cu(OAc)$_2$(H$_2$O)$_2$ (300 mg, 1.48 mmol, 3 equiv.) was added in the mixture followed by the dropwise addition of a freshly prepared aq. solution of sodium ascorbate (600 mg 3.00 mmol, 6 equiv.). The solution was stirred for 20 min, with microwave radiation at 100 °C, then a solution of 25% aq. NH$_3$ (20 mL) was added and let reacted for 10 min at r.t. The mixture was diluted CH$_2$Cl$_2$ and decanted. The organic phase was then washed with a solution of 25% aq. NH$_3$ (3 × 20 mL), H$_2$O (4 × 20 mL), dried with Na$_2$SO$_4$, filtered and evaporated under reduced pressure to give product as a fluorescent yellow solid (93 mg, 80%).

$^1$H NMR (400 MHz, DMSO): $\delta = 8.58$ (s, 3H, H$_8$), 7.81 (dd, $^3J = 5.2, 3.3$ Hz, 6H, H$_{11,11a}$), 7.41 (m, 6H, H$_{12,12a}$), 7.32 (m, 6H, H$_{2,4,6,13}$), 5.66 (s, 6H, H$_7$);

$^{13}$C NMR (100 MHz, DMSO): $\delta = 146.6$ (C$_9$), 137.2 (C$_{1,3,5}$), 130.6 (C$_{10}$), 128.8 (C$_{12,12a}$), 127.8 (C$_{13}$), 127.2 (C$_{11,11a}$), 125.1 (C$_{2,4,6}$), 121.5 (C$_8$), 52.5 (C$_7$);

Elemental analysis: calculated for C 72.11, H 4.95, N 22.94 found C 69.39, H 4.74, N 20.7;

FAB: $m/z = 550$ [M+H$^+$.]
Literature


162


[186] O. Brosch, Ruhe Universität 1999.


Annex

HPLC and ESI spectra

This part contains additional analytical data from peptides 60 to 66.

**60**

RP-HPLC trace of 60 after purification and ESI (positive) with the pattern of iron at m/z = 862 [M+H]+.

**61**

RP-HPLC trace of 61 after cleavage and ESI (positive) with mass peak at m/z = 657 [M+Na]+.
RP-HPLC trace of 62 after cleavage and ESI (positive) with mass peak at m/z = 641 [M+Na]^+.

ESI (positive) of 63 after cleavage with m/z = 1515.5 [M]^+ with the pattern of iron.

ESI (positive) of 64 after cleavage with base peaks at m/z = 1619.2 [M]^+ with the pattern of iron.
ESI (positive) of 65 after cleavage with base peak at m/z = 1629.4 [M]^+

RP-HPLC trace after purification of 66 and ESI (positive) with base peak at m/z = 620 [M+Na]^2+ and m/z = 1195.3 [M]^+ with the pattern of iron
Cell culture and cytotoxicity (peptides 23, MP₆₆ and 24)

**HT-29** – Human colon adenocarcinoma cell line  
**HeLa** - Cervical cancer cell line  
**HepG2** - Hepatocellular carcinoma cell line
Compounds list

1

2 For, $R^1 = H$

3 Tfa, $R^1 = CF_3$

4

5

6

7

8

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12

13

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22

HBTU $X^- = PF_6^-$

TBTU $X^- = BF_4^-$