Semi-synthesis of nisin-based peptide antibiotics

Semi-synthese van op nisine gebaseerde peptiden-antibiotica
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 16 december 2013 des middags te 12.45 uur

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te Den Helder

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‘What if I had never tried it’ – *Valentino Rossi (“the Doctor”)*

Cover design: Jack Slootweg

Front: Close up of a nissin brake calliper

Back: Backbone structure representation of nisin by a chain of AntaFlu candy wrappers

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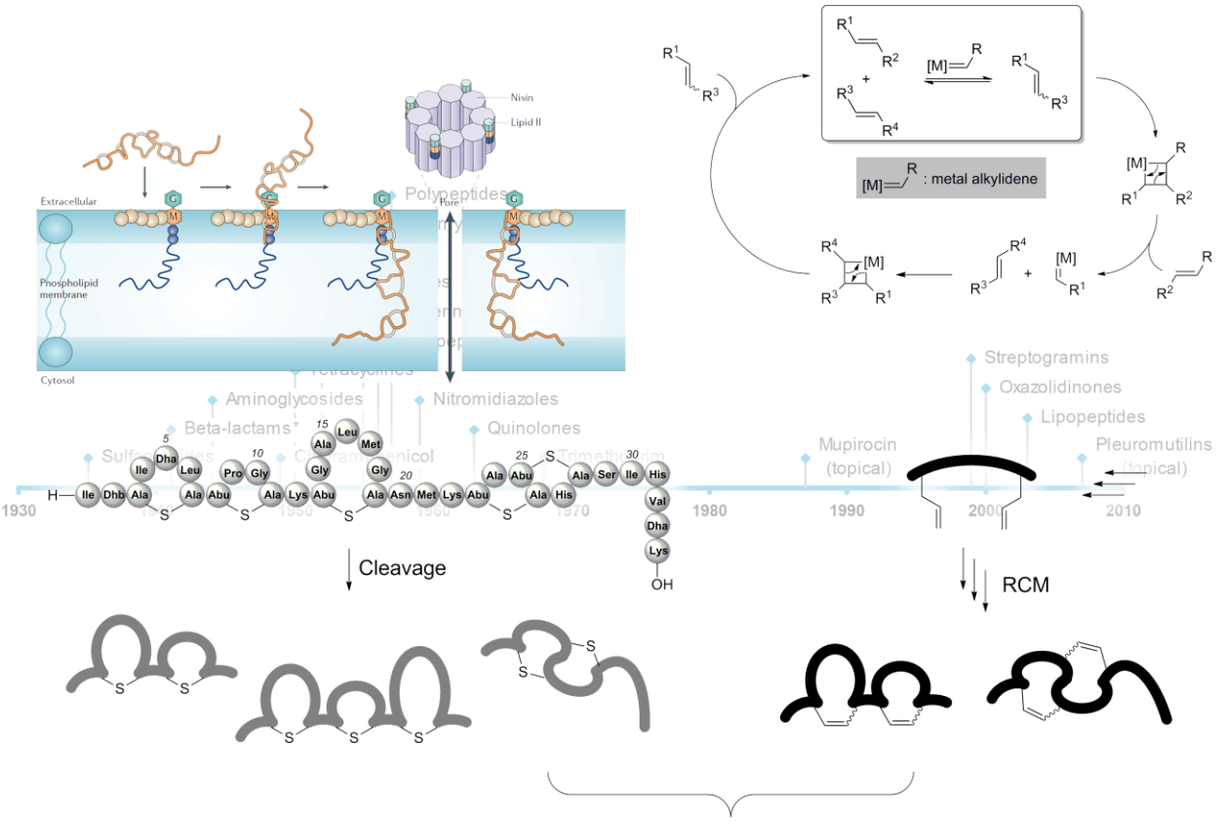
General Introduction



1.1 Search for novel antibiotics

Bacterial infections are a huge treat to public health, especially the numerous cases of infections that have been reported in recent years caused by antibiotic resistant strains, the so-called "superbugs". In this context, Methicillin resistant *Staphyloccocus aureus* (MRSA) is probably the most well-known superbug, but also infections caused by other strains that belong to the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeroginosa, and Enterobacter species)* cause significant morbidity and mortality.[1]

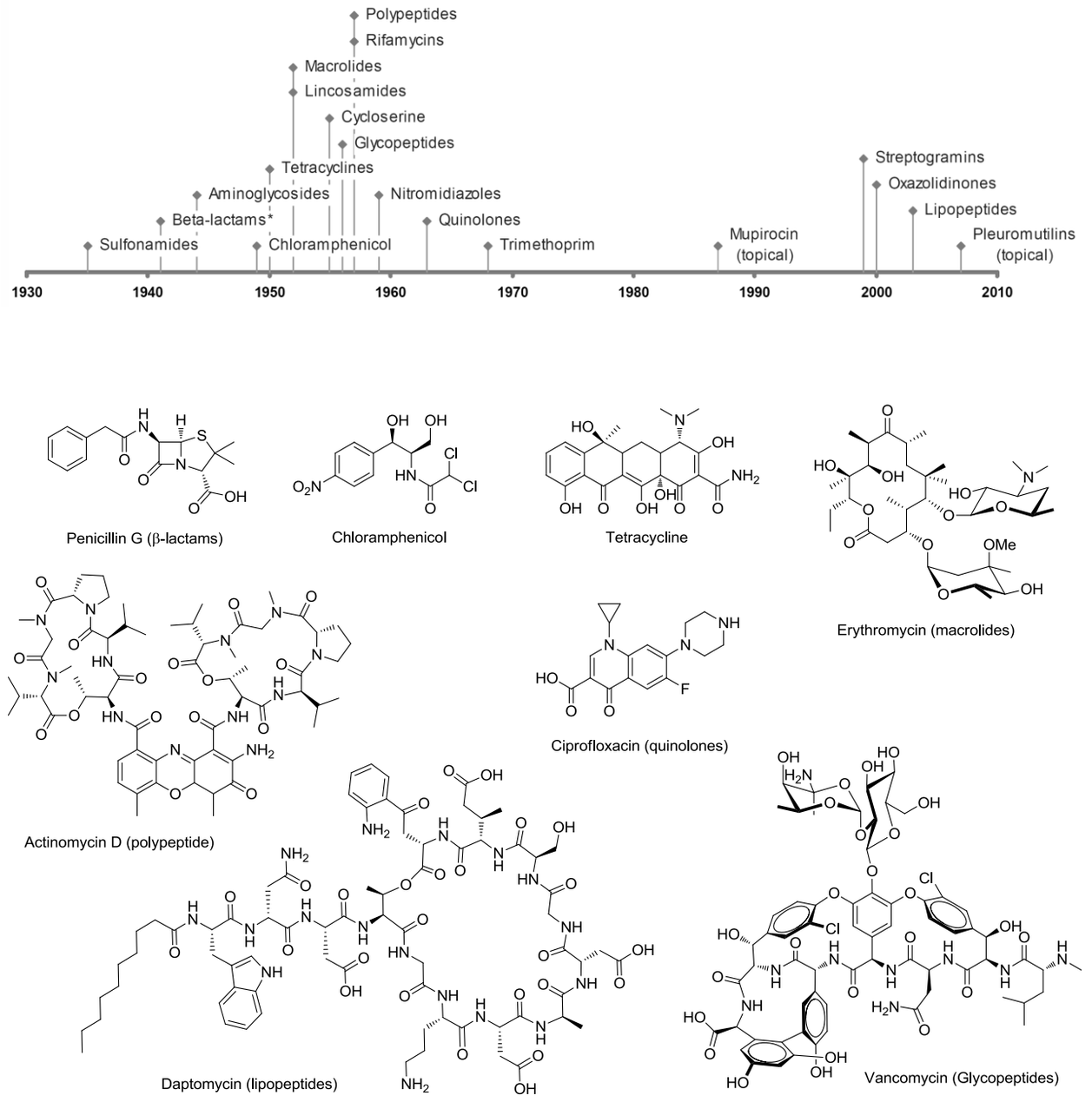


Figure 1 An overview of different classes of antibiotics discovered from 1930 until 2008. Adapted from[2]

Especially the number of strains that become resistant to vancomycin, like vancomycin-resistant *Enterococcus* (VRE), vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-resistant *Staphylococcus aureus* (VRSA), is very worrying, since vancomycin has been considered as the last resort antibiotic for many years. The situation is even more worsening, since from 1962 only two new classes of antibiotics reached the market, while between 1930 and 1962 fourteen new classes were discovered (Figure 1).[3] So there is an urgent need in exploring all possibilities for developing novel classes of potential antibiotics. One of the most recent FDA-approved antibiotics is daptomycin[4], a lipopeptide belonging to the class of antimicrobial peptides (AMPs). AMPs are evolutionarily designed peptides which protect multicellular host organisms against microbial pathogens.[5] A classic example is the AMP anoplin, a decapeptide found in the venom of the solitary wasp, which exhibits a broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria.[6]

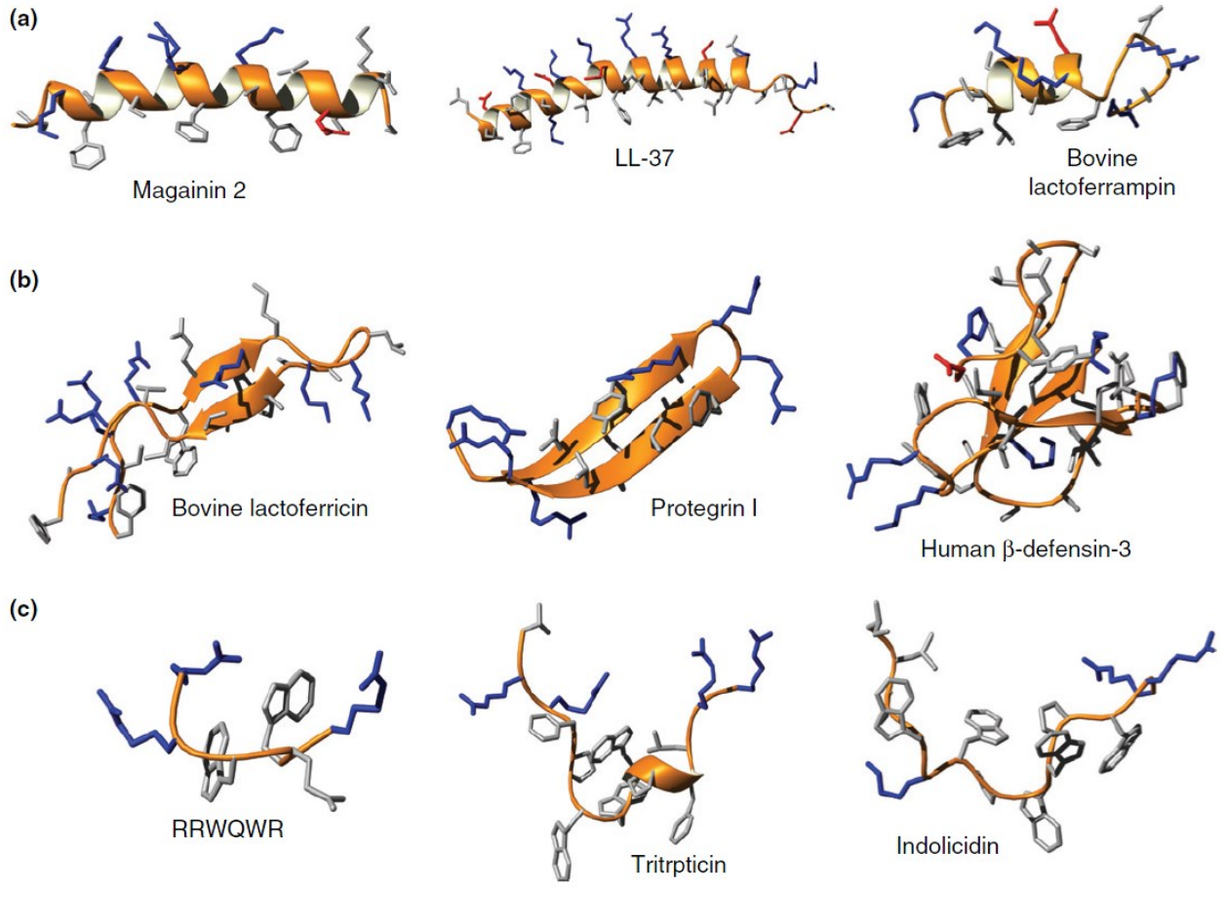


Figure 2. An overview of the major structural classes of AMPs, **(a)** α-helical, **(b)** β-sheet and **(c)** extended conformation peptides. Reprinted by permission from[7]. Copyright 2001 Elsevier, Trends in Biotechnology.

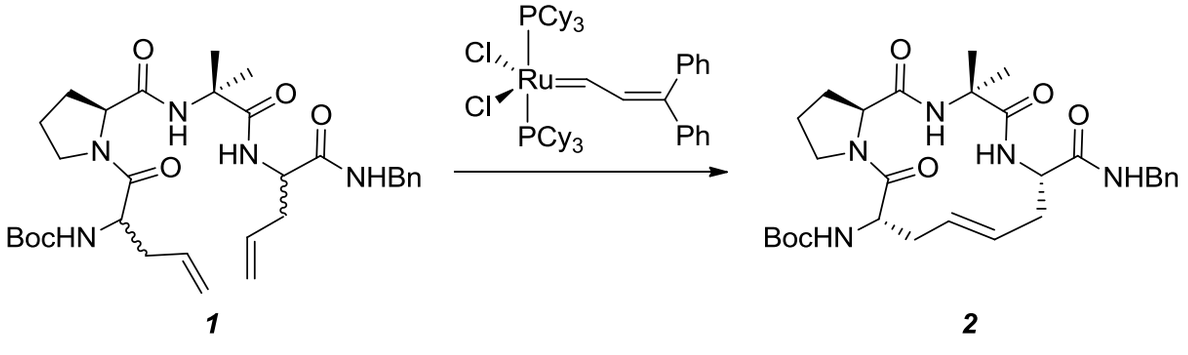
There is a large variety of AMPs, short linear peptides to large complex peptides with many structural elements. The majority of AMPs can be divided into three structural classes: α-helical, β-

sheet and extended conformation peptides (as shown in Figure 2), and AMPs generally act via membrane permeabilization of bacterial membranes.[7] Because AMPs act on bacterial membranes, they often are lipophilic, containing are large number of hydrophobic residues. Lipopeptides, like daptomycin, are peptides containing a lipid tail (see Figure 1), which is believed to act as a membrane anchor. When this lipid tail is truncated or removed, the activity of daptomycin drops dramatically,[8] showing the importance of high membrane affinity of AMPs. Development of stable resistance to AMPs has occurred to a much lesser extent than modern antibiotics, this fact is presumably explained that bacteria have been exposed to AMPs for millions of years. Hence, AMPs are a promising group of novel antibiotics and their application or even development in more drug-like molecules which should be investigated.[9]

1.2 Peptides as drug-like molecules

Although, peptides and/or proteins are not ideal drug molecules according to Lipinski's rule of five,[10] this class of bioactive molecules, generally referred to as "biologics", emerged last decade as a result of their high potency and selectivity to their molecular targets.[11] While biologics have the potential for fewer (off-target) side-effects, which is often the biggest drawback for small-molecule drugs, the metabolic stability of proteins/peptides is usually of major concern in their use as therapeutics.

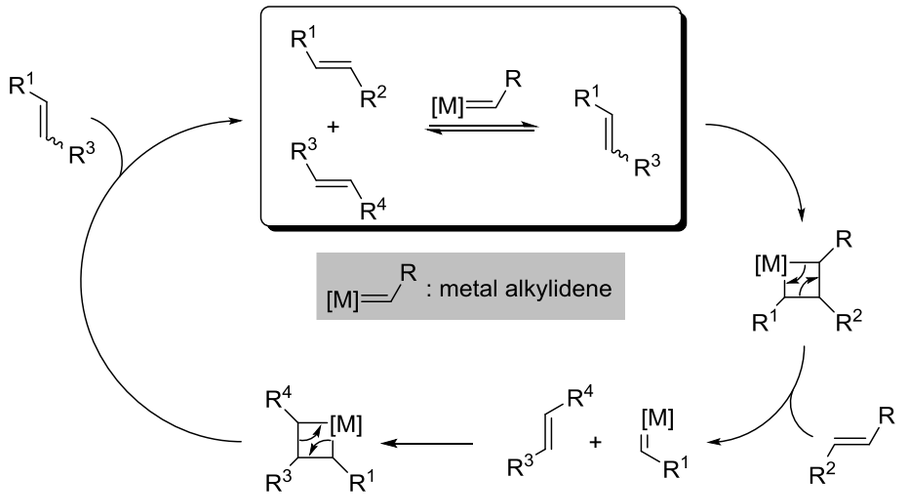
To tackle this problem of metabolic instability, chemists in the field of peptidomimetics have searched for methods to come up with approaches to stabilize peptides by for instance modifications of the peptide backbone like; β-peptides and γ-peptides, peptide bond replacements or synthesizing peptoids.[12] Another promising approach in order to increase the stability of a peptide is cyclization, which is often seen in natural products as an evolutionary design to enhance activity as well as stability. About two decades ago, the Grubbs' group reported the synthesis of cyclic peptide **2** as an effective mimic for a disulfide-stabilized β-turn peptide using ring-closing metathesis (RCM), or olefin metathesis (Scheme 1).[13] This method proved to be an excellent tool to synthesize stabilized peptides (*vide infra)*.



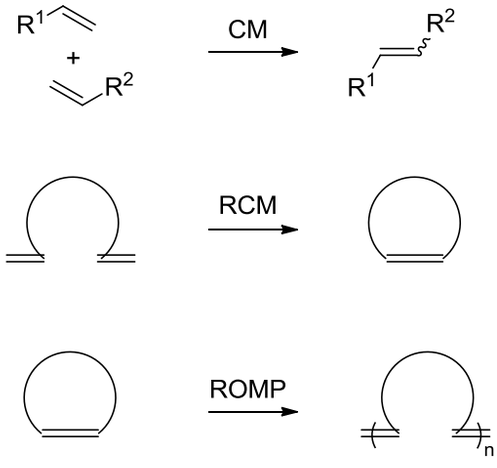
Scheme 1. Synthesis of cyclic tetrapeptide **2** via RCM as a mimic of a disulfide-stabilized β-turn.[13]

1.3 Olefin metathesis

Olefin metathesis is the metal-catalyzed redistribution of carbon-carbon double bonds, and is named from the Greek word "*μετάθεση*", which means change of position.[14] The very first olefin metathesis reaction was reported by Anderson and Merckling more than fifty years ago, since they described the carbon-carbon bond rearrangement reaction in the titanium-catalyzed polymerization of norbornene.[15] It was not until 1971 that Hérisson and Chauvin[16] proposed the mechanism of olefin metathesis, which was subsequently validated by experimental evidence by the groups of Casey[17], Katz[18] and Grubbs[19]. According to this mechanism, olefin metathesis proceeds through metallacyclobutane intermediates as shown in Scheme 2.



Scheme 2 Catalytic cycle of olefin metathesis. A [2+2]-cycloaddition of an alkene double bond to a transition metal alkylidene occurs to form a metallacyclobutane intermediate. The intermediate metallacyclobutane can cyclorevert to give the original species or a new alkene and alkylidene. Adapted from[13]



Scheme 3. Most common types of olefin metathesis reactions: cross-metathesis (CM), ring-closing metathesis (RCM) and ring-opening metathesis polymerization (ROMP).

The most common types of olefin metathesis reactions are given in Scheme 3, namely: cross-metathesis (CM), ring-closing metathesis (RCM), and ring-opening metathesis polymerization (ROMP). The gained interest in metathesis reactions boosted the development of metathesis catalysts as shown in Figure 3. One of the first well-defined, highly active molybdenum-based catalyst was introduced by Schrock and coworkers.[20] This Mo-species displayed superb metathesis activity and even nowadays it is particularly used for sterically demanding substrates. However, the Schrock catalyst is very sensitive to oxygen and moisture, and does not tolerate certain polar or protic functional groups, making it less widely applicable.

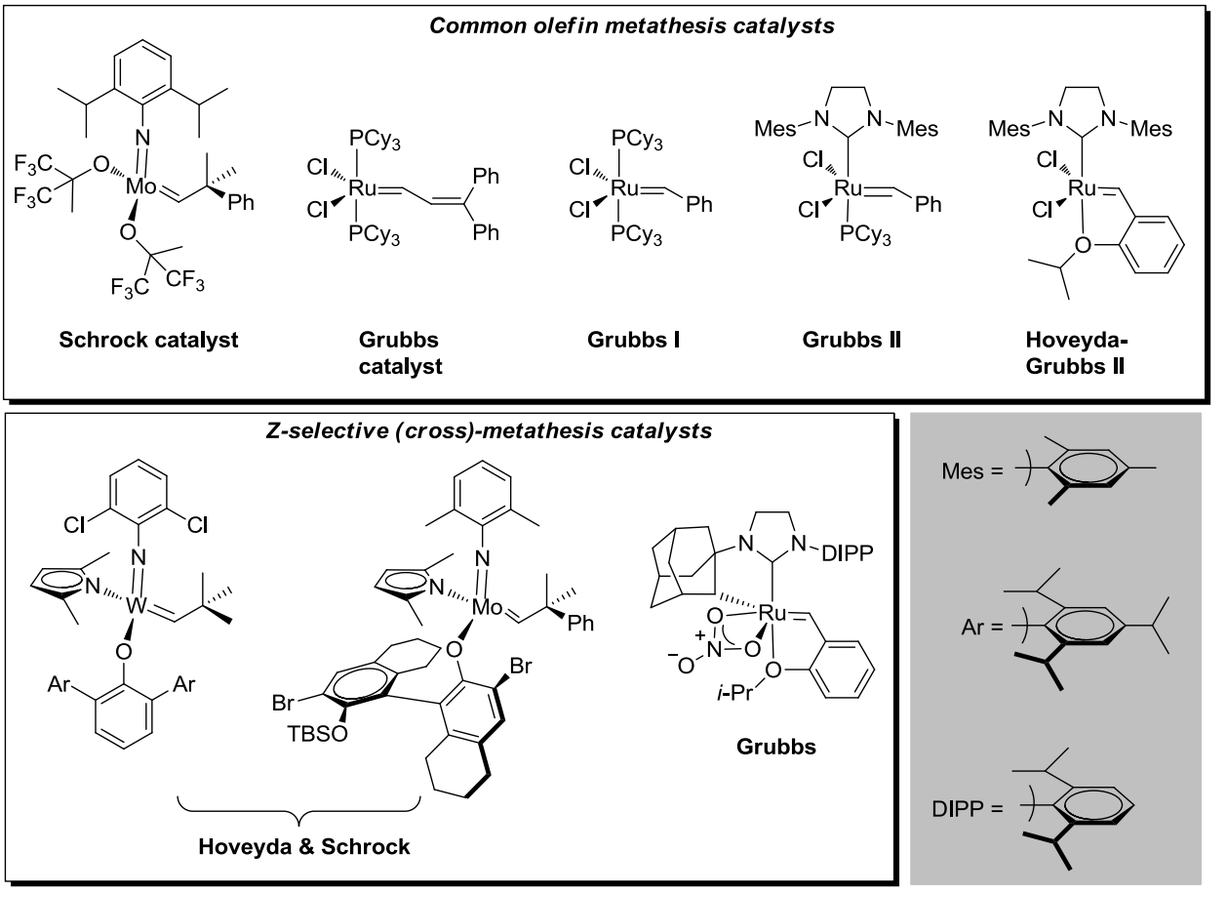


Figure 3. Structural formulas of the most common metathesis catalysts and some recently developed Z-selective metathesis catalysts.

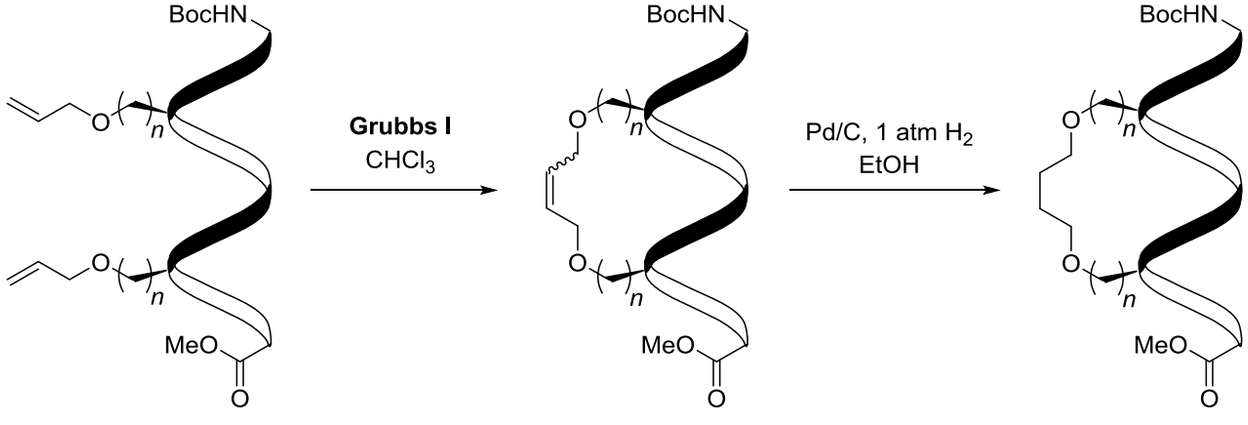
Subsequently, Grubbs and coworkers introduced the first well-defined ruthenium-based metathesis catalyst (Grubbs' catalyst),[21] which was further optimized as first generation Grubbs catalyst (Grubbs I)[22] by replacement of the alkylidene moiety. Although the ruthenium-based catalyst was less active than the molybdenum-based catalyst, the Grubbs catalyst showed remarkable high tolerance for a large variety of functional groups, which allowed its application in

a plethora of syntheses. Further improvement of the Grubbs I catalyst was achieved by substituting one of the tricyclohexylphosphine ligands by a bulky *N*-heterocyclic carbene (NHC) ligand to access the Grubbs second generation catalyst (Grubbs II)[23]. This Ru-species displayed improved catalytic activity, while its high functional group tolerance and thermal stability was maintained. Finally, replacement of the second phosphine ligand for a bidentate alkylidene, led to the second generation Hoveyda-Grubbs catalyst (Hoveyda-Grubbs II)[24] with an even higher thermal stability.

The development of metathesis catalysts is still in progress and researchers are now focusing on the development of catalysts for asymmetric metathesis reactions.[25] Over the years, olefin metathesis has developed as one of the most powerful and reliable methods for carbon-carbon bond formation among others for the total synthesis of many natural products.[14b] Chauvin, Schrock, and Grubbs were awarded for their contributions with the Chemistry Nobel Prize in 2005.

1.4 Stabilizing peptides using ring-closing metathesis

Inspired by the work of Grubbs, other groups in the field of peptidomimetics started to explore the use of RCM in the synthesis of structured peptides.[26] Katzenellebogen and co-workers[27] designed a 10-membered lactam peptide to mimic a type I beta-turn and successfully cyclized the peptide using RCM, while the group of Gmeiner[28] reported the RCM-mediated synthesis of lactam-bridged type IVa beta turn mimics. To stabilize the α-helical conformation of peptides, Grubbs group used modified (homo)serine residues having an O-allyl ether as the RCM precursor to obtain a peptide with a covalently constrained 3_{10} helix (Scheme 4).[29]



Scheme 4. Synthesis of covalently constrained peptide helices (**5**) accessed by RCM.[29a]

Verdine and co-workers went a step further by exploring the use of RCM for the stabilization of helical propensity and metabolic stability of peptides, as demonstrated by

synthesizing cross-linked mimics, varying in ring size and position, in a series of the C-peptide sequence of ribonuclease A. This was achieved by using α,α-dialkylated amino acids, in combination with an all hydrocarbon cross-link (Figure 4).[30] This approach was coined by the term "peptide stapling".

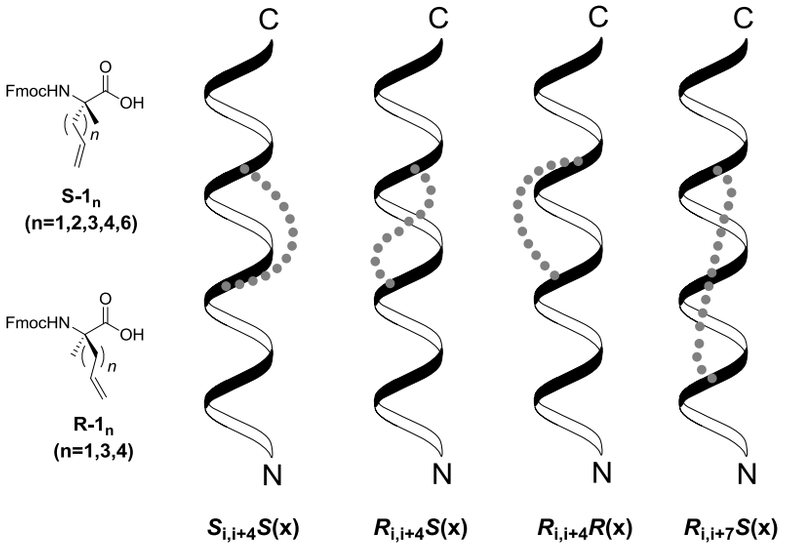


Figure 4. General strategy for the synthesis of, for instance *i*+4 or *i*+7 cross-linked helices by incorporating *R* or *S* α-methylated amino acids bearing olefinic side-chains of varying length followed by RCM.[30]

A slightly different approach was followed by Arora et al., who synthesized a cross-linked peptide via a carbon-carbon bond acting as a hydrogen bond surrogate instead cross-linking at the position of the amino acid side chain. Strikingly, the parent peptide did not exhibit any α-helicity in sharp contrast to the cross-linked peptide, which adapted a stable α-helix structure over a wide temperature range.[31] Nowadays, "stapling" of peptides is regarded as a powerful tool and has great potential as a general method to improve the drug-like properties of therapeutic peptides.[32] A recent example is the discovery of ATSP-7041, an α-helical stapled peptide which is a potent and selective dual inhibitor of MDM2 and MDMX and is planned for 2014 to go into phase I clinical trials.[33]

1.5 Mimics of natural cyclic constraints using RCM

Covalent cross-links are also found in Nature in order to reduce flexibility and to increase stability. Probably the most well-known example in this context is the presence of disulfide bonds in proteins and peptides to conserve the three-dimensional conformation. Two groups of highly structured peptides are characterized by a network of intertwined disulfide bridges. The first group resembles the family of conotoxins[34]. These peptides have been found in the venom of marine snails and are currently used as pain relieving agents. The second class is a family of plant-derived peptides called cyclotides[35]. Especially the class of cyclotides show a remarkable stability -as a result of head-to-

tail cyclization in combination with three disulfide bridges- against thermal and enzymatic treatments that would destroy non-constrained proteins.[36]

Although disulfide bonds generally stabilize the structure of the peptide, they are known to be fragile under reducing conditions. For this reason, peptide chemists investigated the possibility to use RCM to mimic disulfide containing peptides, by the replacement of the disulfide bridge with a so called "dicarba-bond" to afford redox-stable peptidic analogs. This strategy was already demonstrated by the group of Grubbs who synthesized a dicarba analog of a fragment of glutoredoxin[37]. Various research groups successfully applied this strategy to synthesize biologically active dicarba mimics of some other naturally occurring disulfide containing peptides like atosiban, an oxytocin analog[38] which is shown in Figure 5, but also leucocin A[39], Sunflower Trypsin Inhibitors[40], octreotide[41] and conotoxins[42] as the most representative examples.

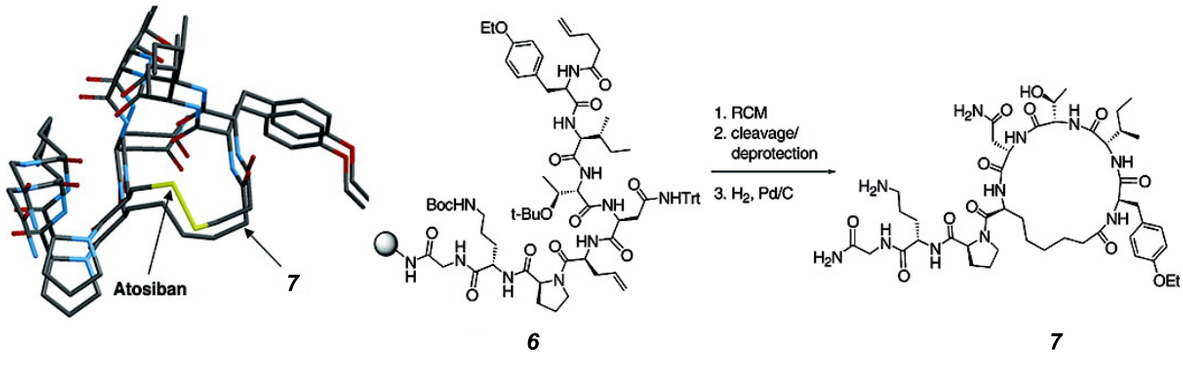


Figure 5. Synthesis of a dicarba atosiban analogue **7** via RCM on-resin. At the left side the energy minimized structures of **7** and atosiban are overlaid to show that a disulfide can be perfectly mimicked by a C-C bond isostere. Reprinted by permission from.[38] Copyright 2003 American Chemical Society

A disulfide bond is a natural cyclic constraint, and also other covalent constraints can be found in natural peptides like lactones, lactams, biaryl(ether) and thio ethers. Especially the latter group is of particular interest, since thio ether bonds are found in a class of antimicrobial peptides called lantibiotics, thereby forming a cyclic constraint highly important for activity and stability. Moreover, lantibiotics are considered to be a valuable alternative for the traditional antibiotics.[43]

1.6 The lantibiotic nisin

The name lantibiotics is derived from a class of antimicrobial peptides that contain a thio ether-bridged dipeptide moiety, a lanthionine (Scheme 5, structure **11**), while in general lanthionine-containing peptides are called lanthipeptides. Lantibiotics are divided into two different classes according to their structural features. Type-A lantibiotics are cationic, elongated peptides, up to 34 residues long, which act on the bacterial cell membrane, with the lantibiotic nisin as its most

representative example, as shown in Figure 6. Type-B lantibiotics have a more globular structure, up to 19 residues in length, and act through disruption of enzymatic functions, like cinnamycin.[44]

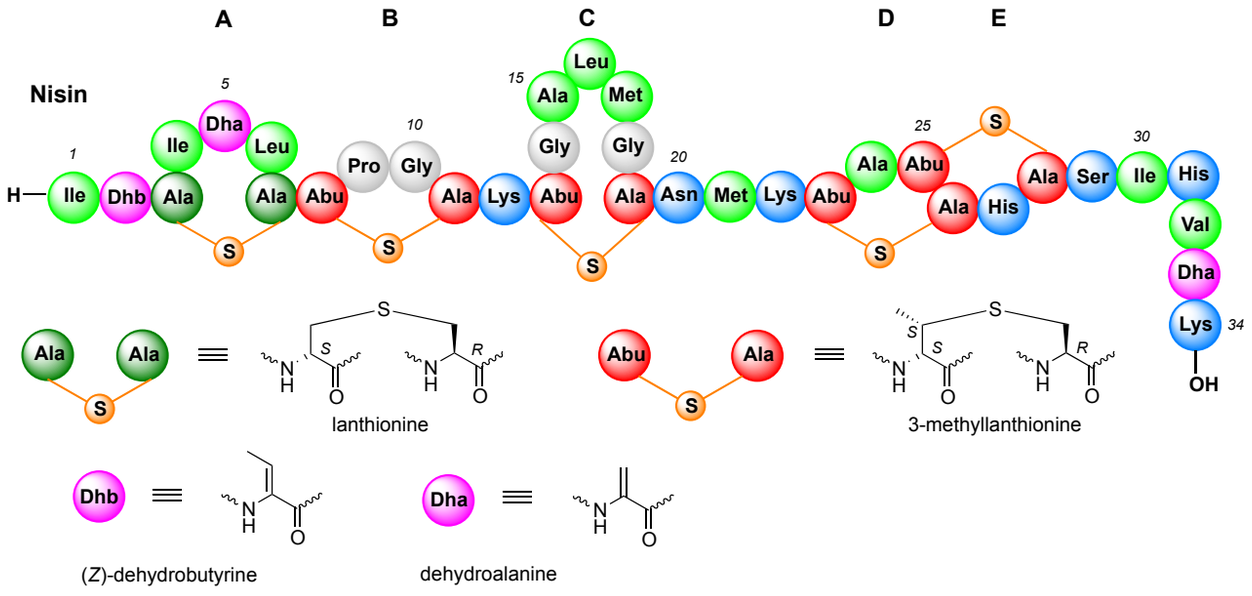
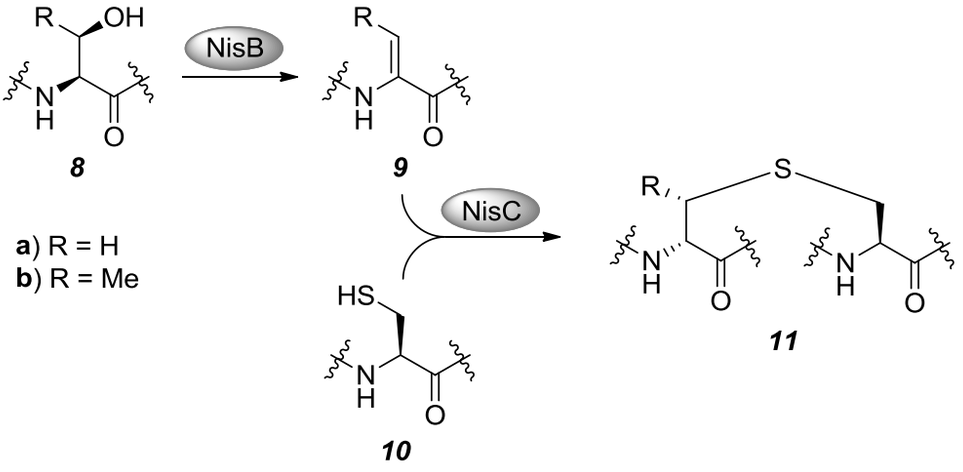


Figure 6 Schematic representation of nisin A

Lantibiotics are ribosomally synthesized peptides and undergo a series of enzyme-catalyzed post-translational modifications to yield their biologically active form. Actually, therefore, the name 'ribosomally synthesized and post-translationally-modified peptides' (RiPPs) was proposed to describe for instance the class of lantibiotics, in order to take away confusion in historical alternating nomenclature.[45]



Scheme 5. Schematic representation of the lanthionine **11** biosynthesis. After dehydration of Ser **8a** / Thr **8b** catalyzed by NisB to yield Dha **9a** / Dhb **9b**, a Michael-type addition of the side chain thiol of Cys **10** results in the stereoselective formation of Lan **11a** / MeLan **11b**.

Nisin is produced by strains of *Lactococcus lactis*. The prepeptide NisA is ribosomally synthesized, followed by sequence-specific dehydrations of serine and threonine residues to obtain dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, catalyzed by NisB (Scheme 5). Then, NisC catalyzes the region- as well as stereo-selective thio ether formation by Michael additions in which the upstream cysteine residues and the dehydrated amino acids are involved, forming the five (methyl)lanthionine rings, which give the peptide its structural and conformational rigidity.[46]

Nisin exerts a dual mode of action to kill Gram-positive bacteria. Firstly, it inhibits cell wall synthesis by binding via its N-terminal AB(C)-ring system toward lipid II, which is an essential cell wall precursor for cross-linking to give the cell membrane mechanical strength, and thereby inhibiting the bacterial cell-wall synthesis.[47] Secondly, after binding toward lipid II, the C-terminal DE-ring fragment inserts into the bacterial membrane, leading to permeabilization of the cell membrane by forming pores that disrupt vital ion gradients as explained in Figure 7.[48] The nisin-lipid II interaction was revealed using NMR spectroscopy and as illustrated at Figure 8. It has been found that nisin binds the pyrophosphate moiety of lipid II, forming a so-called pyrophosphate-cage with its AB-ring system. Also, it has been observed that the interaction is dominated by backbone amides of nisin by forming hydrogen bonds to the pyrophosphate moiety.[49]

Although wild-type nisin only exhibits activity against Gram-positive bacteria, a number of bioengineered nisin mutants showed activity against some Gram-negative bacteria strains.[50] Since the mode-of-action of nisin was uncovered, as a specific binder of lipid II,[47] to interfere with the bacterial cell-wall cross-linking, nisin caught the attention to be a promising antibiotic candidate.[51] Moreover, bacteria that were resistant against vancomycin (which also targets lipid II), were still susceptible toward nisin, an indication nisin targets a different part of lipid II, making it a very attractive template molecule for the design of a new class of antibiotics.

The total synthesis of nisin was achieved by Shiba and coworkers in 1988.[52] The desired lanthionine moieties were introduced via the corresponding disulfide-bridged peptide and subsequent desulfurization to yield the lanthionine-bridged peptide. Via a different approach, Tabor and co-workers reported the syntheses of lanthionine-containing analogs of ring C and the cross-bridged DE-ring of nisin. In their approach, a suitable orthogonally protected lanthionine was used as a building block in SPPS and peptide cyclization was achieved via macrolactimization.[53] The chemical syntheses of the other lantibiotics like lactocin S and the two components of Lacticin 3147 were reported by the group of Vederas,[54] and the synthesis of analogs op epilancin 15X by the group of van der Donk,[55] via a similar approach using suitable protected lanthionine building blocks. Also, a deeper understanding of the biosynthesis of lanthipeptides led to the development of in vivo and in vitro biotechnological methods to produce lanthionine-containing peptides.[46]

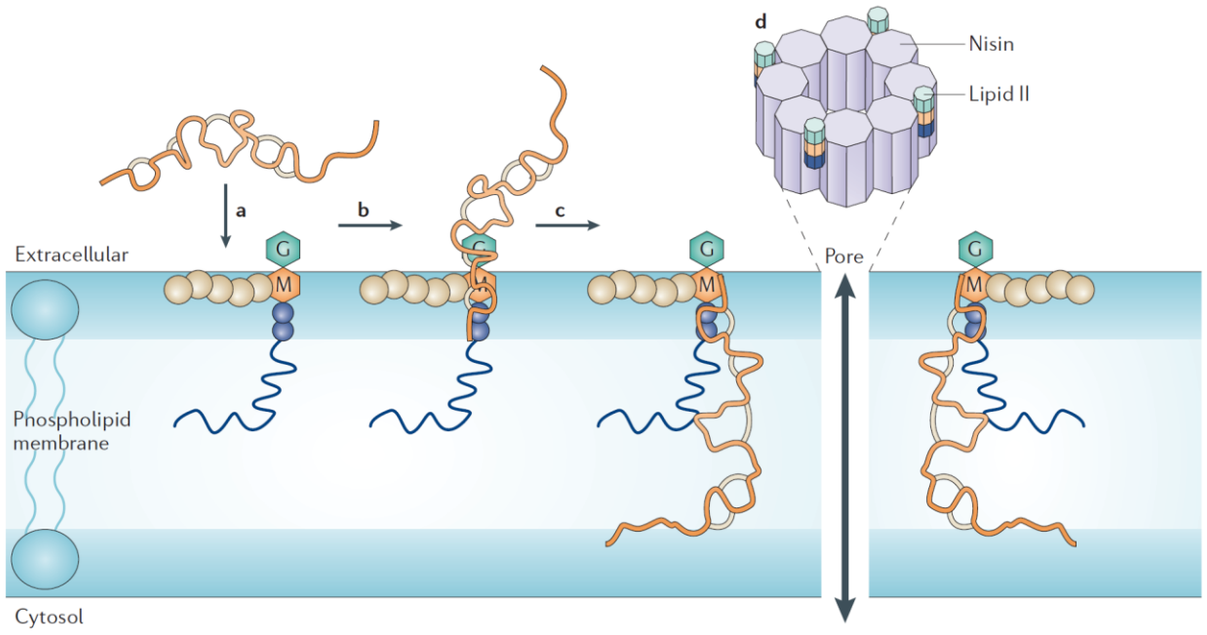


Figure 7. Model for the targeted pore-formation mechanism of nisin. After reaching the bacterial membrane (**a**), nisin binds to lipid II via its N-terminal rings (**b**). This is followed by the insertion of C-terminus of nisin into the membrane (**c**) eventually forming a stable pore-complex consisting of eight nisin molecules and four lipid II molecules (**d**). Reprinted by permission from.[48c] Copyright 2006 Nature Publishing group, Nature Reviews Drug Discovery.

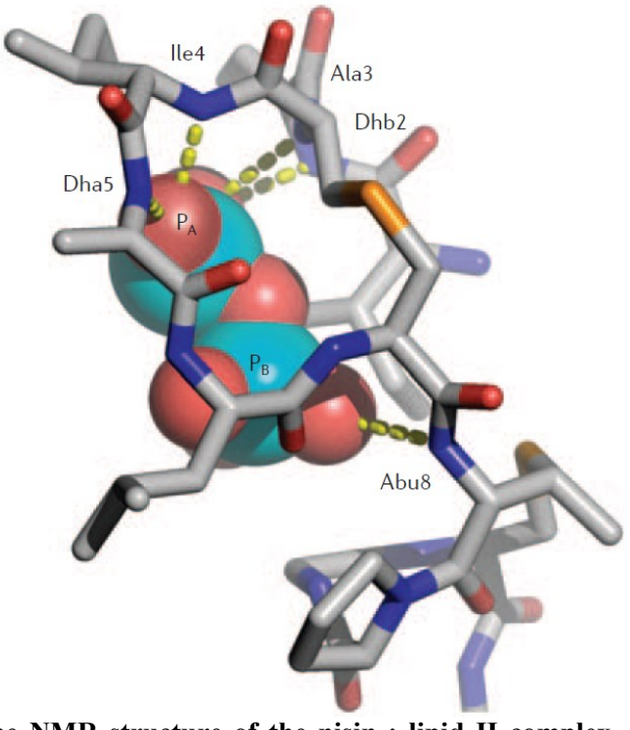
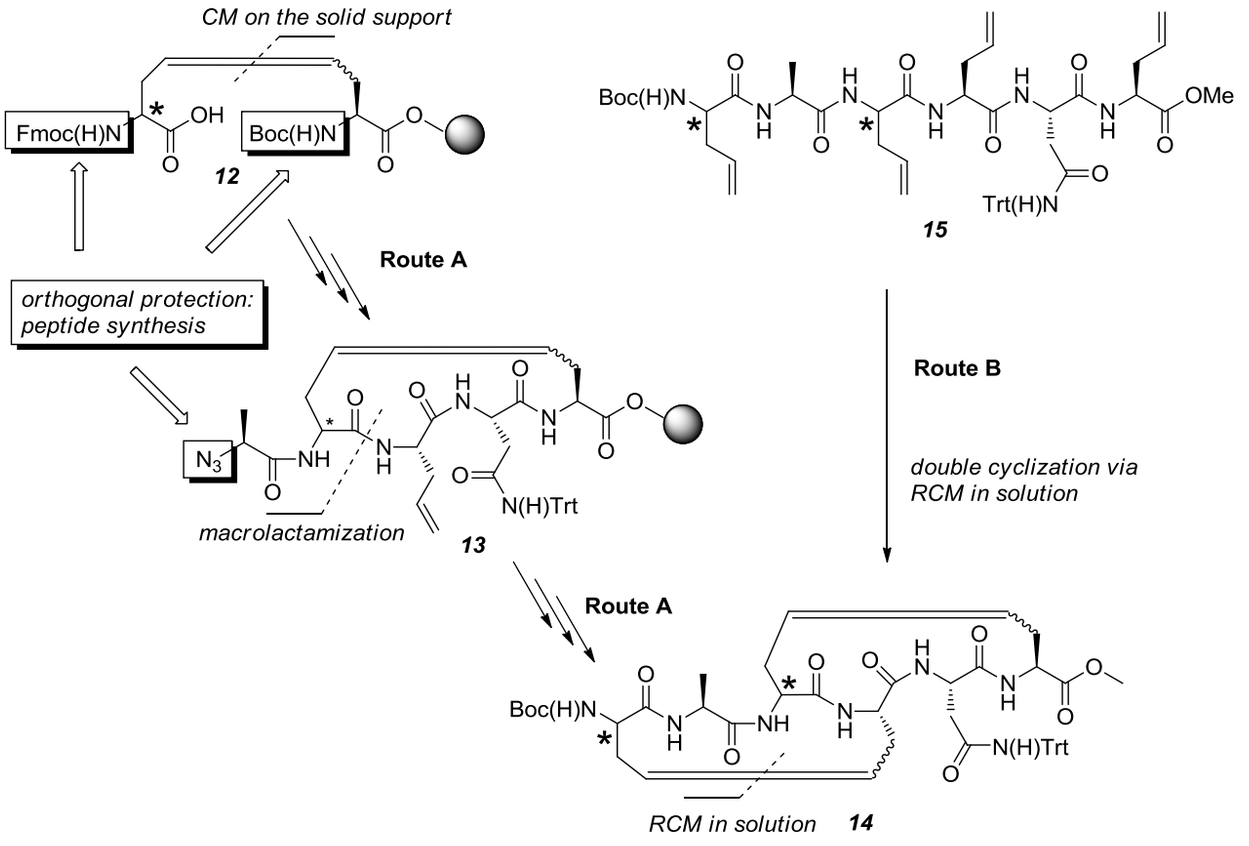


Figure 8. Zoom in of the NMR structure of the nisin : lipid II complex. The nisin AB ring system interacts via de backbone amides with the pyrophosphate moiety of lipid II via four defined hydrogen bonds (yellow). Reprinted by permission of.[48c] Copyright 2006 Nature Publishing Group, Nature Reviews Drug Discovery.

Although the lanthionine is a stable linkage, it is sensitive toward oxidation. Wilson-Stanford et al.[56] showed that oxidation of the thio ether moieties in nisin to the corresponding sulfoxides led to a complete loss of antimicrobial activity. So, converting lanthionines into stable analogs is a valid approach to improve the stability of nisin, without interfering too much with its activity as an antimicrobial peptide. Therefore, our group started a program to synthesize oxidation-resistant nisin mimics by using RCM, replacing the oxidation-sensitive lanthionine bond by a stable "dicarba-bond".[57] Replacement of lanthionines with a stable dicarba-bond was also investigated by Vederas and coworkers with the synthesis of a dicarba-analog of lacticin 3147 A2 peptide. Although synthetically successful, the dicarba-analog did not show any bioactivity. In another study, Vederas and coworkers synthesized biologically active Oxa-lacticin A2, in which the sulfur atom of the lanthionine moiety was replaced with an oxygen atom to increase the stability of the peptide.[58]

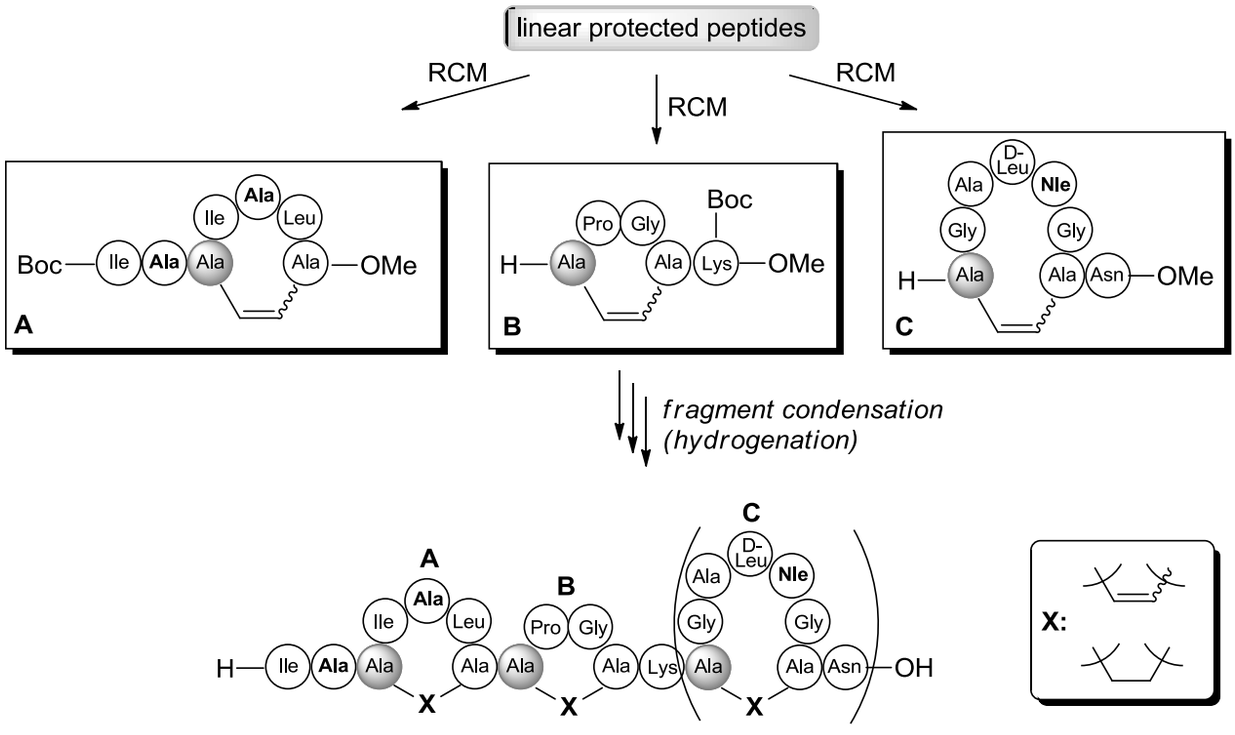
1.7 Dicarba-bond as a bioisoster of lanthionines

Previously, a dicarba mimic of the cross-bridged DE-ring of nisin Z was synthesized featuring RCM, via two different synthetic routes as shown in Scheme 6.[57a] Route A was followed for the synthesis of bicyclic product **14** via a step-wise approach featuring CM and macrolactamization on the solid support, followed by RCM in solution. The first (3→6) dicarba-bridge was synthesized via CM and an orthogonal protection group strategy was used to build up the monocyclic peptide. After cleavage of the peptide from the resin, the second dicarba-bridge was introduced using RCM in solution, yielding the all L-bicyclic peptide **14**. Route B comprised of the SPPS-mediated synthesis of a hexalinear peptide as the RCM-precursor **15**, and subsequently RCM in solution. RCM reactions using Grubbs II yielded some monocyclic intermediates, however, the correctly cyclized all L-bicyclic peptide **14** could be isolated by HPLC as the major reaction product. The same strategy was used for the synthesis of a dicarba mimic of the nisin DE-ring using the natural backbone stereochemistry, with a is D-amino acid at position 1 and 3.[57b] Strikingly, route B afforded bicyclic peptide **14** as single bicylic product after treatment with Grubbs II as was confirmed by MS-MS analysis, showing that the native peptide backbone stereochemistry was very important for the formation of the desired cross-bridged structure. HPLC analysis of the product showed mainly three product peaks, which were a result of different *E*/*Z* diastereoisomers.



Scheme 6. Two synthesis routes for the preparation of dicarba nisin DE-ring 14. Chiral centers marked with an asterisk have either the L-configuration (all L-peptide) or D-configuration (native peptide backbone stereochemistry).

The synthesis of dicarba-mimics of the AB(C)-rings of nisin was also successfully achieved in which RCM was used as the cyclization step.[57c] A series of individual A-, B-, C-linear RCM-precursor peptides was prepared using solution phase or SPPS approaches and they were cyclized in the presence of Grubbs II catalyst, yielding a mixture of *E*/*Z* diastereoisomers. By means of fragment condensation, dicarba nisin AB- and ABC mimics were synthesized and the double bonds were reduced to the corresponding alkane-bridges in order obtain a single compound instead of a mixture of diastereoisomers. Native N-terminal nisin fragments inhibit pore formation activity of full-length nisin by competitive binding of lipid II. Biological evaluation showed that the nisin dicarba AB(C)-mimics were able to inhibit pore-formation activity of nisin, an indication that these mimics displayed affinity toward lipid II. The most active mimic had the native peptide backbone stereochemistry and an alkane-bridge. Although, all dicarba-mimics were not as active as the native N-terminal fragments, they were the first example of biologically active compounds that mimicked the thio ether moiety in lanthionine-containing peptides.



Scheme 7. Synthesis of a series of dicarba analogs of nisin AB(C). Residues in grey have either the L-configuration (all L-peptide) or D-configuration (native peptide backbone stereochemistry).

1.8 Aim and outline of this thesis

The ultimate goal of the research described in this thesis is to synthesize hybrid nisin mimics consisting of a native nisin fragment, containing lanthionine bridges, coupled to a synthetic nisin fragment with dicarba-bonds, as illustrated in Figure 9. Biological evaluation of these hybrid nisin mimics will shed light on the importance of every single ring system for e.g. antimicrobial activity and membrane interactions. Moreover, incorporating dicarba-bridged DE-ring **14** into a hybrid nisin construct would allow biological evaluation as a full-length nisin mimic, since there is no bioactivity reported for only the C-terminal fragment of nisin. Although the chemical syntheses of nisin and nisin fragments have been reported in the literature, the methods are rather sophisticated, elaborative and time consuming.[60]

For the synthesis of our hybrid mimics, we decided to follow a different approach, namely to obtain native nisin fragments using semi-synthesis by preparing native nisin fragments via known enzymatic digestion and chemical degradation methods.[61] Ultimately, the native nisin fragments will be ligated to synthetic dicarba nisin mimics and the obtained nisin hybrid mimics will be evaluated in bioactivity assays.

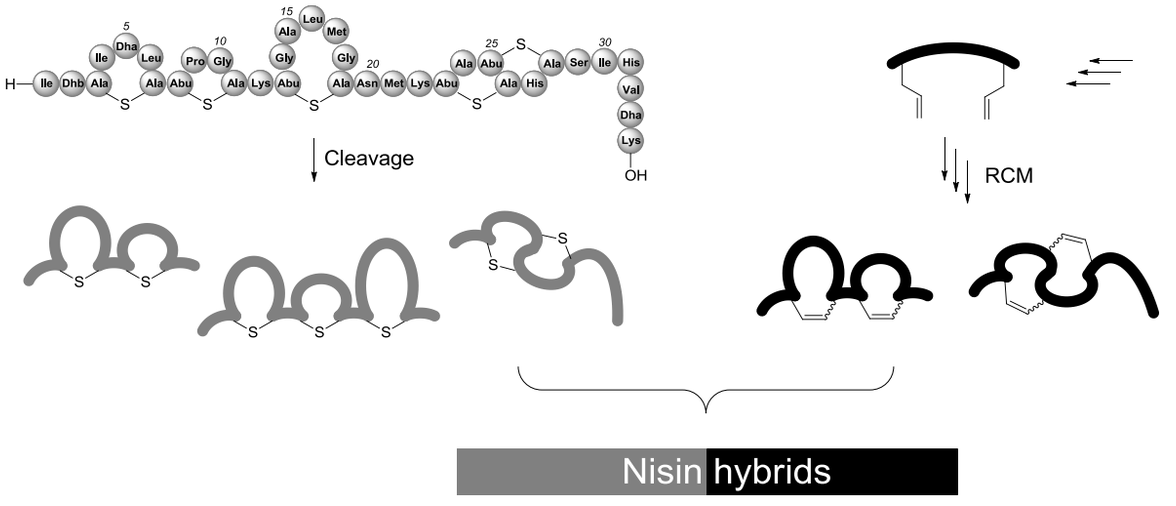


Figure 9 Simplified schematic representation of the aim of this thesis. Native nisin fragments (shown in grey) can be prepared from nisin by several cleavage methods. Synthetic dicarba fragments (shown in black) can be synthesized starting from the linear precursor peptides and subsequent cyclization via RCM. Native and synthetic nisin fragments are combined to afford nisin hybrids.

Chapter 2, describes the synthesis, via the previously reported strategy, of the dicarba-bridged DE-ring via RCM starting from the linear peptide RCM-precursor. A mixture of four diastereoisomers (which have different alkene bond configurations: *ZZ, ZE, EZ, EE*) was obtained and the diastereoisomers could be separated using preparative HPLC. Each diastereoisomer was individually characterized using 2D NMR spectroscopy analysis using TOCSY, ROESY and HSQC. N and C-terminal functionalization of the DE-ring building block was successfully performed, by introducing *N*-α-azido lysine to the N-terminus, while the C-terminus was functionalized with a properly protected lysine residue. Finally, the azide functionality was used in Cu(I)-catalyzed click chemistry to demonstrate the versatility of orthogonal ligations of azide-functionalized DE-ring alkene-bridged mimics.

Chapter 3 describes the development of a convenient method for the scalable purification of nisin from commercially available nisin preparations suitable for preparative purposes. Commercially available nisin, which only contains 2.5% nisin, was enriched via a convenient precipitation method using an organic/aqueous biphasic system. Enriched nisin was treated with two proteases, trypsin and chymotrypsin, to obtain nisin AB (1-12) and nisin ABC (1-20), respectively. Both cleavage reactions were followed in time using analytical HPLC and LC-MS, and the digestion products were identified and two previously unknown cleavage sites were found for trypsin as well as chymotrypsin. To obtain the C-terminal native nisin fragment (22-34), enriched nisin was treated with cyanogen bromide, resulting in cleavage of nisin at Met21. Finally, enriched nisin was cleaved by chymotrypsin followed by cyanogen bromide treatment in a two-step procedure affording the truncated nisin DE-tail (22-31) fragment. All the desired fragments were

obtained in good yields and are valuable building blocks to be used for the semi-synthesis of hybrid mimics.

The synthesis of a hybrid nisin peptidomimetic is described in **Chapter 4**. A nisin hybrid was designed containing a native ABC-part (nisin 1-20) and a synthetic alkene-bridged DE-ring to study the activity of the synthetic DE-ring in relation to the native lanthionine bridged DE-ring. Native nisin ABC, afforded by enzymatic cleavage of nisin, was functionalized at the C-terminus with propargylamine, as the alkyne moiety (affording a 'glycine' residue at position 21). A nisin hybrid mimic was prepared via Cu(I)-catalyzed click chemistry by ligation of the ABC-alkyne with the N-α-azidolysine-dicarba-DE ring.

This nisin mimic was able to bind lipid II, an indication that it could be active as an antimicrobial agent. Unfortunately, however, it showed no pore-formation activity. In order to improve the nisin hybrid regarding membrane permeabilization, an amino alkyne derived from leucine was prepared in two steps from Boc-Leu-OH using the Bestmann reagent. This alkyne was coupled to the C-terminus of native nisin ABC, affording a leucine side chain at position 21. *Via* an optimized synthetic alkene-bridged DE-ring construct: N-α-azidolysine-dicarba-DE-ring-lysine-NHMe, was ligated to the native nisin ABC-Leu-alkyne to afford an optimized nisin hybrid mimic. Bacterial growth inhibition tests showed that this mimic is only 10-fold less active than native nisin, an indication that the native DE-ring can be replaced by a dicarba mimic. However, membrane leakage activity was still absent.

The synthesis of an optimized dicarba-mimic of the nisin AB-ring fragment, containing the native dehydro residues, is described in **Chapter 5.** By a combination of SPPS and solution phase synthesis, dicarba ring A and dicarba ring B were synthesized via RCM, while the required dehydrobutyrine (Dhb) and dehydroalanine (Dha) residues were introduced as their corresponding precursors, threonine and serine, respectively. After fragment condensation of ring A with ring B, dehydration of threonine-2 and serine-5 within the suitably protected peptide was achieved using EDCI and CuCl. The optimized nisin AB mimic was tested for lipid II binding affinity and compared to native nisin AB and a dicarba mimic in which the Dha/Dhb residues were replaced by alanine. This study showed that optimized dicarba nisin AB mimic was as active as the native AB-ring fragment in contrast to its alanine-containing congener.

To study nisin's mode of action in more detail, functionalization of native nisin with reporter molecules is of great importance. Also multimerization of native nisin to obtain multivalent structures and conjugation of nisin to other bioactive molecules can be very attractive to attack bacteria on multiple targets. The synthesis of such bioconjugates is described in **Chapter 6**. Orthogonal ligation methods like Cu(I)-catalyzed click chemistry are ideal to conjugate large unprotected peptides and therefore it was decided to prepare a nisin-alkyne derivative, by C-

terminal functionalization of nisin with propargylamine. Two fluorescent nisin derivatives and a nisin dimer were successfully prepared by using two fluorescently labeled azides and a bis-azide, respectively. Biological evaluation made clear that all conjugates showed that membrane permeabilization as well as antimicrobial activity were in the same range as native nisin which proved that the chemical modification at the C-terminus was tolerated, prerequisite that these nisin-derived molecular constructs can be used as a tool in biological studies to uncover further details of the mode of action as a pore-forming agent.

Finally, **Chapter 7** describes the synthesis of an Fmoc protected enantiomerically pure lipophilic amino acid (Laa) which contains a nine carbon atom hydrophobic side chain. Fmoc-Laa can be introduced into any peptide sequence using standard SPPS to increase the lipophilicity of a peptide without sacrificing important polar segments of a peptide like for instance the N and C-termini. The rationale for this design was that an increase of lipophilicity could enhance membrane affinity of membrane acting peptides and increase their potential as possible novel drug-like compounds. Fmoc-Laa-OH was synthesized in four steps from L-glutamic acid and the fatty side chain was introduced via a Wittig olefination. The antimicrobial decapeptide anoplin was chosen as a model peptide to test this rationale. Three lipophilic anoplin analogs (Ano-Laa02, Ano-Laa06 and Ano-Laa10) were prepared in which either leucine or isoleucine was replaced by Laa at position 2, 6 or 10, respectively. Biological evaluation using membrane leakage experiments, bacterial growth inhibition studies and hemolytic assays revealed that the lipophilic analogs were more active up to one order of magnitude compared to anoplin, while bacterial selectivity was retained. Therefore, these results suggest that Fmoc-Laa-OH could be used as a general approach to increase membrane affinity thereby improving antimicrobial properties of the modified peptide.

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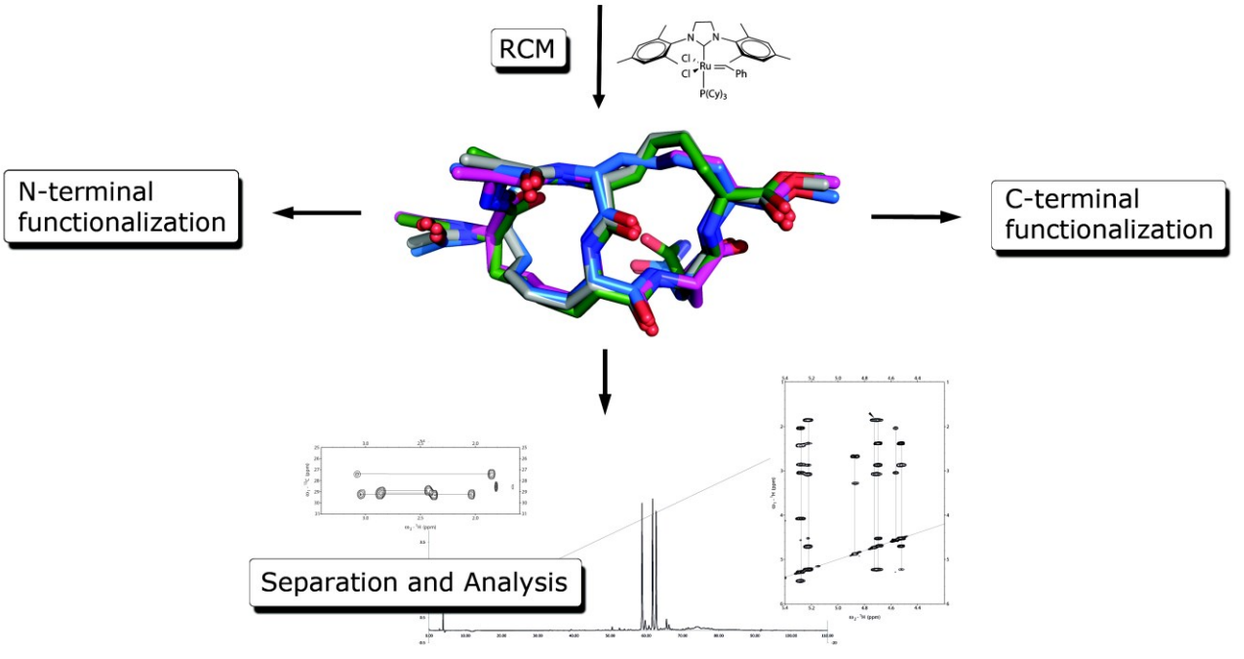
Chapter 2

Synthesis and structural characterization of the individual diastereoisomers of a cross-stapled alkene-bridged nisin DE-ring mimic



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2.1 Introduction

Nisin belongs to the class of lantibiotics, a family of antimicrobial peptides that is ribosomally synthesized by Gram-positive bacteria to act against competing microorganisms.[1] A common structural characteristic of lantibiotics is the presence of the amino acid lanthionine, which give these peptides their structural and conformational stability. The lanthionine contains a sulfide bridge that is formed after a series of enzyme-catalyzed post-translational modifications, like dehydration of serine and threonine residues followed by the stereoselective intramolecular Michael addition of the sulfhydryl of an adjacent cysteine residue to result in a D-configuration of the newly formed stereocenter.[2] Although nisin is widely used in dairy products as food preservatives, it is unstable at neutral or basic pH and readily oxidizes[3a] and reacts with water or thiol-containing nucleophiles.[3b] Therefore, we initiated a program to find more oxidation resistant lantibiotics in which the thio ether bridge is replaced by a 'carbon bridge', either based on an alkyne, alkene, or alkane moiety, which can be conveniently introduced by ring-closing metathesis (RCM)[4] approaches, and successfully synthesized biologically active nisin AB and ABC fragments.[5,6]

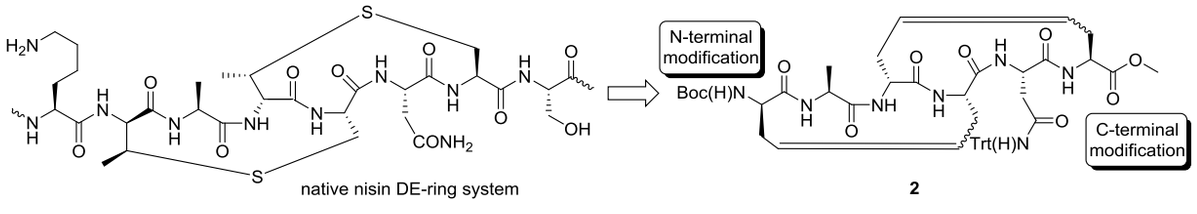


Figure 1. Design of a cross-stapled alkene-bridged protected hexapeptide as an advanced synthon to prepare mimics of the native DE-ring of the lantibiotic nisin.

The DE-ring of nisin is of special interest since it contains a crossed (or interlocking) thio ether bridge, as shown in Figure 1. This interlocking motif is a common feature in a number of lantibiotics and it is known to play an important role in the pore-forming activity of nisin.[7] The synthesis of this knotted thio ether ring structure has been achieved in the total synthesis of nisin by Fukase et al.,[8] and more recently via a solid phase approach as reported by Mothia et al.[9] Carbon-bridged analogs of the interlocking DE-ring were synthesized by our group using RCM as the penultimate carbon-carbon bond formation reaction via a pre-organization approach and starting from a linear precursor peptide in a one-step bi-cyclization approach.[10]

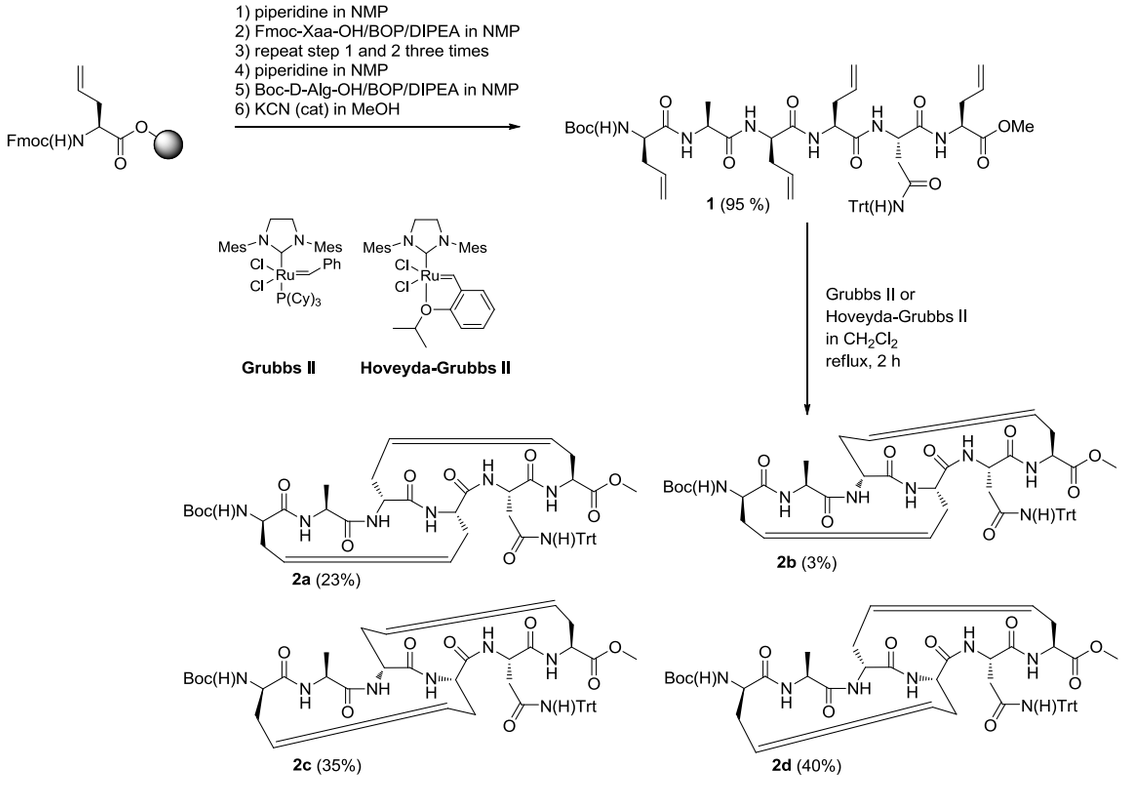
Herein, the synthesis, isolation and structural characterization of the four individual diastereoisomers of a cross-stapled[11] DE-ring mimic using HPLC, NMR and MS analysis is reported. Furthermore, the bicyclic hexapeptide was used as a versatile synthon and modified at its C- and N-terminus, among others, with an azide moiety to access a building block suitable for

Cu(I)-catalyzed alkyne-azide cycloaddition-based ligation reactions to be used in semi-synthesis approaches to arrive at improved nisin-based antibiotic peptides.

2.2 Results and Discussion

2.2.1 Synthesis

The solid phase synthesis of hexapeptide **1** (Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe) was performed as previously described (Scheme 1).[10b] Ring-closing metathesis of **1**, either with the first[12a] or second[12b] generation Ru-based Grubbs catalyst, resulted predominantly in the formation of the bicyclic hexapeptide **2** (bicyclo[1-4/3-6]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe) with a side chain to side chain connectivity pattern that corresponds to the native DE crossed-bridged thio ether ring system.[10] Since RCM leads to an *E*/*Z* mixture of the double bond,[13] hexapeptide **2** was expected to consist of four diastereoisomers. Based on analytical HPLC (Figure 2A), peptide **2** could be separated by preparative HPLC into four individual diastereoisomers **2a**-**d**, and according to analytical HPLC, MS and NMR, they could be assigned (*vide infra*) as bicyclo[Z^{1-4}/Z^{3-6}]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2a**), bicyclo[Z^{1-4}/E^{3-6}]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2b**), bicyclo[E^{1-4}/Z^{3-6}]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2c**), and bicyclo[E^{1-4}/E^{3-6}]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2d**), respectively (Scheme 1).



Scheme 1. RCM of hexapeptide **1** to bicyclo[1-4/3-6]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2a**-**d**).

The peak height in the analytical HPLC in combination with the isolated yield of each individual diastereoisomer: **2a** 23%, **2b** 3%, **2c** 35%, and **2d** 40%, might indicate a certain degree of preorganization of the linear peptide or a configurational prevalence for a double bond as cyclic constraint. This observation was further evidenced by RCM of **1** with the second generation Hoveyda-Grubbs catalyst[14] since an identical mixture of diastereoisomers **2a-d** compared to the second generation Grubbs catalyst was found by analytical HPLC (see Figure 2B).

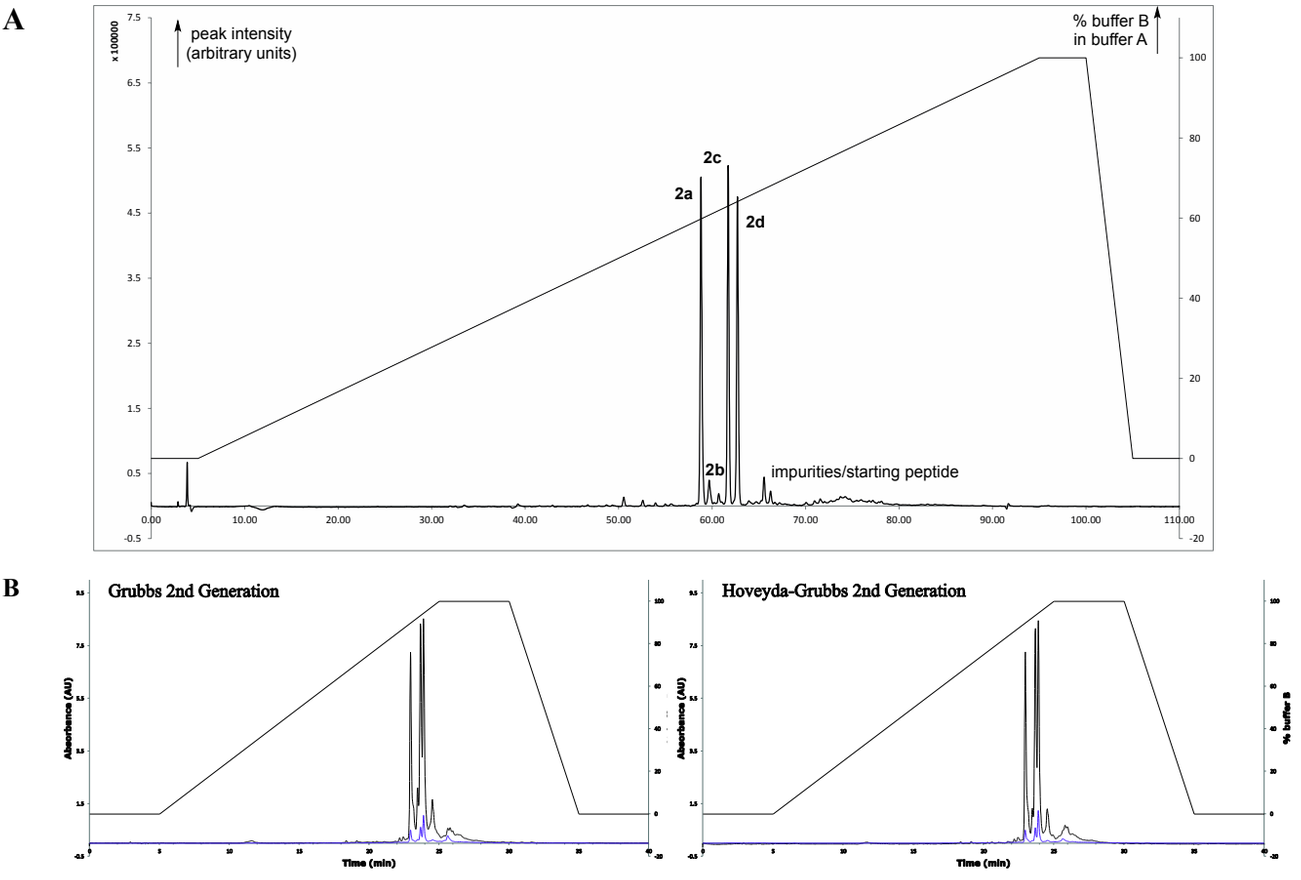


Figure 2. A) HPLC trace of bicyclo[1-4/3-6]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2**); the four individual diastereoisomers **2a-d** are indicated. **B)** HPLC chromatographs of RCM reactions mixtures of bicyclo[1-4/3-6]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2**) using Grubbs 2nd generation and Hoveyda-Grubbs 2nd generation catalyst. The peptide samples were eluted through a short silica-plug as a pre-purification step to remove any Ru-species.

It is desirable to proceed the syntheses with a single diastereoisomer, therefore bi-stapled peptide **2** was subjected to hydrogenation to obtain an alkane bridged bicyclic peptide. It turned out that hydrogenation was rather far from trivial. The first attempt was the conventional heterogeneous Pd/C catalyst (10% Pd content) in an H_2 atmosphere (55 psi (3.8 bar)) using the Parr apparatus. According to LC-MS (Figures 3), the mixture consisted mainly of starting material, a compound with only one reduced double bond. Although the desired di-alkane bridged peptide could be

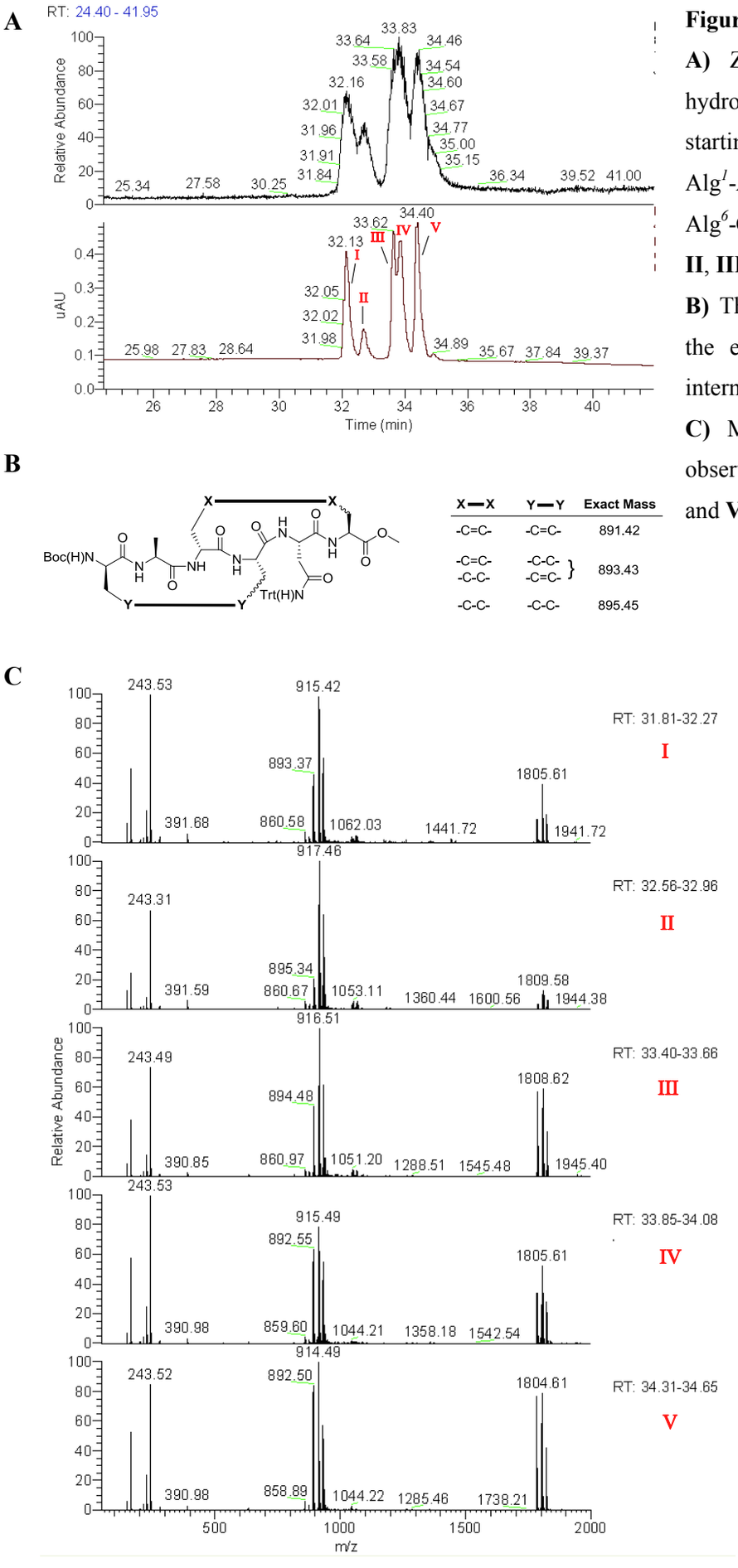


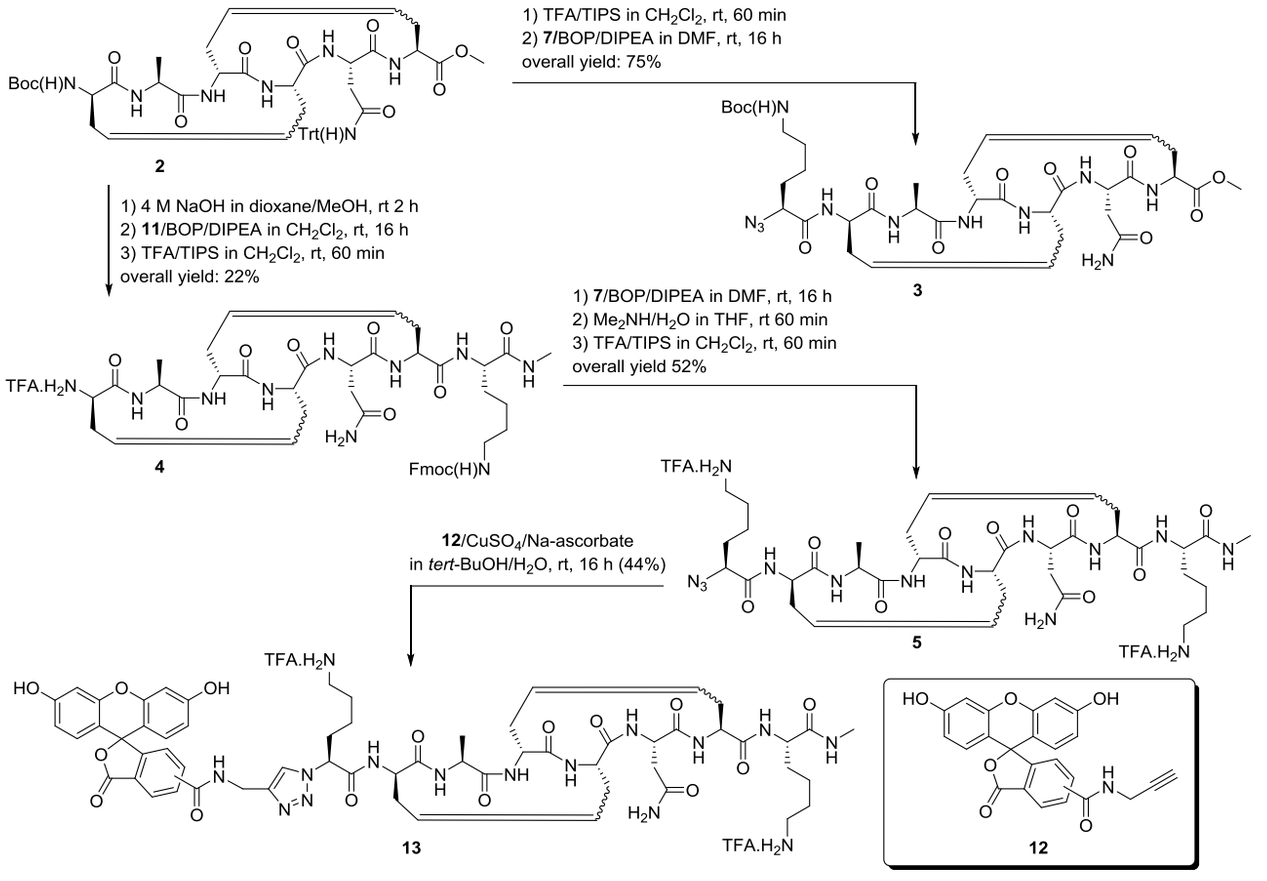
Figure 3.

A) Zoom-in of LC-MS data of hydrogenation reaction mixture starting with bicyclo[1-4/3-6]-Boc-D-Alg[1]-Ala[2]-D-Alg[3]-Alg[4]-Asn(Trt)[5]-Alg[6]-OMe (**2**) shows five peaks, **I**, **II**, **III**, **IV** and **V** respectively.

B) The chemical structure represent the expected product and reaction intermediates.

C) MS spectra derived from the observed five peaks (**I**, **II**, **III**, **IV** and **V**) from LC-MS measurements.

identified, the amount was too low for isolation. Optimization of the hydrogenation by using quantitative amounts of catalyst, different catalyst species, like Pd/C Degussa type or $Pd(OH)_2$ (Pearlman's catalyst), or longer reaction times, was not successful. Attempts to apply the Rh-based homogeneous Wilkinson's catalyst[15] and two recently developed transfer hydrogenation methods via diimide/2-nitrobenzene-sulfonohydrazide[16] and 5-ethylriboflavin/hydrazine,[17] were also not successful. Therefore, the N- and C-terminal modifications of bicyclic peptide **2** were performed as a mixture of the diastereoisomers, as shown in Scheme 2.

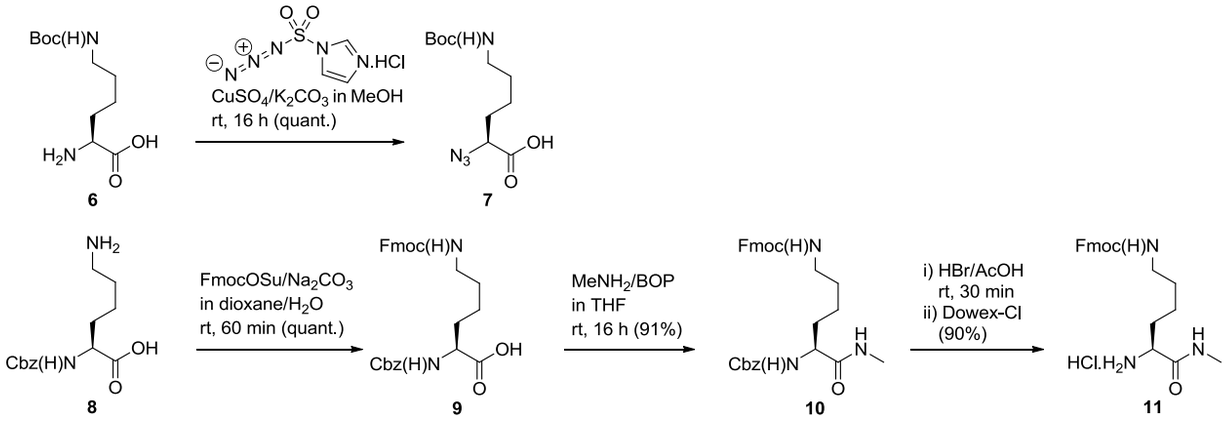


Scheme 2. Application of bicyclo[1-4/3-6]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2**) as an advanced synthon for N- and C-terminal modification.

The DE-ring in native nisin variants is N-terminally flanked by a lysine residue. Therefore, it was decided to couple N_3-Lys(Boc)-OH (**7**) since the azido moiety is not only a protecting group for amines, it can also be used for selective Cu(I)-catalyzed cycloaddition reactions with functionalized alkynes (CuAAC), a reaction that is also known as the most prominent example of "click" chemistry.[18,19] To achieve this, bicyclic peptide **2** was treated with TFA in CH_2Cl_2 with TIS as scavenger, to remove the *N*-α-Boc group and the trityl functionality from the side chain of asparagine. The free α-amine was then coupled to N_3-Lys(Boc)-OH (**7**) with BOP/DIPEA as coupling reagents and peptide **3** was obtained in 75% overall yield after column chromatography.

Azide **7** was prepared in quantitative yield from commercially available H-Lys(Boc)-OH (**6**) by using imidazole-1-sulfonyl azide hydrochloride (Scheme 3) as the diazotransfer reagent as described by Goddard-Borger.[20] Then, peptide ester **3** was saponified by treatment with Tesser's base[21], however, due to premature precipitation of the starting compound, the conversion into the corresponding carboxylate went not to completion. Besides of that, acidic aqueous work-up of the reaction mixture was severely hampered by the low solubility of the desired peptide acid in common organic solvents, like EtOAc, CH_2Cl_2 and $CHCl_3$. To remedy this solubility issue, the sequence of reaction steps was reversed. Thus, peptide ester **2** was treated with Tesser's base and after work-up the corresponding peptide acid was isolated in near quantitative yield without purification.

Although the C-terminus of nisin comprises the hexapeptide ~Ser-Ile-His-Val-Dha-Lys-OH, a ~Lys-NHMe moiety was coupled, since this truncated C-terminus was described previously to retain antimicrobial activity of a series of subtilin derivatives, a closely related lantibiotic with an identical cross-bridged DE-ring system, while slightly shorter nisin analogs had a severe loss of antimicrobial activity.[22,23] For this purpose, H-Lys(Fmoc)-NHMe (**11**) was designed and synthesized (Scheme 3) for C-terminal modification. The Fmoc functionality was used to protect the ε-amino group to introduce orthogonality with respect to the *N*-α-Boc group.



Scheme 3. Synthesis of amino acid building blocks **7** and **11**.

After a BOP/DIPEA-mediated coupling of **11** to the peptide ester of **2** in DMF as solvent, bicyclo[1-4/3-6]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-Lys(Fmoc)-NHMe was isolated and directly dissolved in CH_2Cl_2 containing TFA/TIS to remove the Boc- and Trt functionalities to give peptide **4** as the corresponding trifluoroacetate salt in an overall yield of 22% (~60% per step) after purification by preparative HPLC. Then, N_3-Lys(Boc)-OH (**7**) was coupled to amine **4** in the presence of BOP/DIPEA. The Fmoc-protected octapeptide was subsequently treated with an aqueous solution of dimethylamine with THF as co-solvent to remove the Fmoc group. Finally, Boc

group removal with TFA afforded azido peptide **5** in 52% overall yield (~80% per step) after purification by preparative HPLC and characterization by mass spectrometry.

To demonstrate the versatility of the α-azide moiety in a Cu(I)-catalyzed "click" reaction, azide **5** was treated with carboxyfluorescein-functionalized alkyne **12** in the presence of $CuSO_4$/sodium ascorbate and *tert*-BuOH/H_2O as solvent (Scheme 2). After purification by preparative HPLC and characterization by LC-MS, "click" peptide **13** was obtained in 44% yield.

2.2.2 Structural assignments

After isolation by preparative HPLC, the individual diastereoisomers were assigned by 2D NMR techniques, comprising TOCSY (mixing time 60 ms), HSQC and ROESY (mixing time 500 ms) spectra. The ^{1}H and ^{13}C assignments are given in Table 1 and 2, respectively. Unfortunately, due to the low isolated amount of diastereoisomer **2b** its ^{13}C chemical shifts could not be reliably assigned. The individual diastereoisomers were characterized as the four possible geometric isomers, namely *Z*/*Z* (**2a**), *Z*/*E* (**2b**), *E*/*Z* (**2c**), and *E*/*E* (**2d**) cross-stapled alkene-bridged DE-ring mimetics. The geometry of the double bonds was identified as the *E* (*trans*)- or *Z* (*cis*) configuration by quantification of the scalar coupling constants of the vicinal protons, $^{3}J_{H^{\gamma}H^{\gamma}}$. In case of compounds **2a**-**d**, a coupling constant of ~4 Hz was observed for a *Z* configuration, while a coupling constant of ~11 Hz was typical for an *E* configuration.[24]

All γ-protons of the diastereoisomers **2a**, **2b**, **2c** and **2d** were assigned based on the TOCSY-spectra by making use of the observed cross-peak intensity between the αNH/αCH and the respective γ-protons. Interestingly, the 2D NMR spectra clearly showed differences in the ^{1}H chemical shifts, however the overall pattern was comparable. The presence of the two dicarba-bonds results in inter-residue cross-peaks in the TOCSY spectrum as shown for the Hα/Hγ region of D-Alg[3] (X3) and Alg[6] (X6) of the *E*/*E* diastereoisomer **2d** in Figure 4. An overview of all observed TOCSY cross-peaks of dicarba bridge X3-X6 (D-Alg[3]-Alg[6]) in the structure of peptide **2d** is illustrated in Figure 5. A unique feature of these configurational isomers, was the very distinct signals of the β-protons (Hβ1 and Hβ2 as exemplified in Figure 4) of the bridged allylglycine (Alg) residues. In each diastereoisomer these β-protons had very distinct chemical shifts, with $\Delta\delta_H$ up to 1.20 ppm. At ω_2 = 4.52 ppm, a cross-peak (indicated with green labels) between X6-Hα and the X6 β-protons was observed at ω_1 = 2.86 (X6-Hβ2) and 2.38 ppm (X6-Hβ1), respectively. The HSQC spectrum confirmed that these protons were indeed bound the same carbon atom: at ω_1 = 29.2 ppm (Figure 6). Furthermore, it became clear from Figure 4 (from the part of the spectrum below the diagonal at ω_2 = 4.52 ppm and from the pattern at ω_2 = 5.23 ppm labeled in red), that a cross-peak existed between X6-Hα (ω_2 = 4.52 ppm) and the γ-protons that form the alkene-bridge:

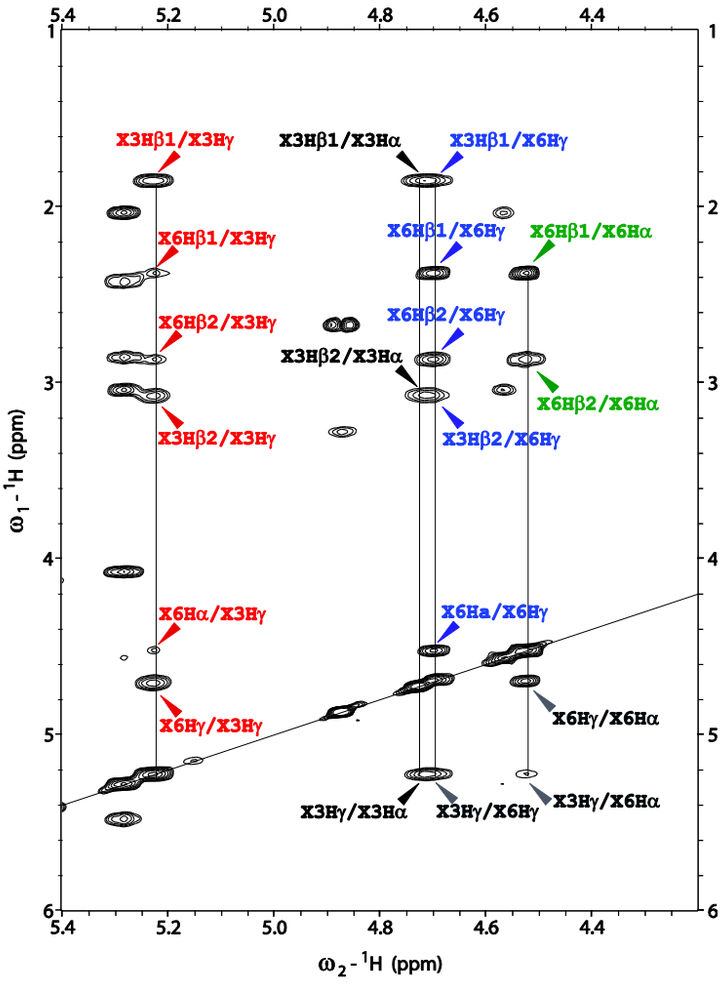
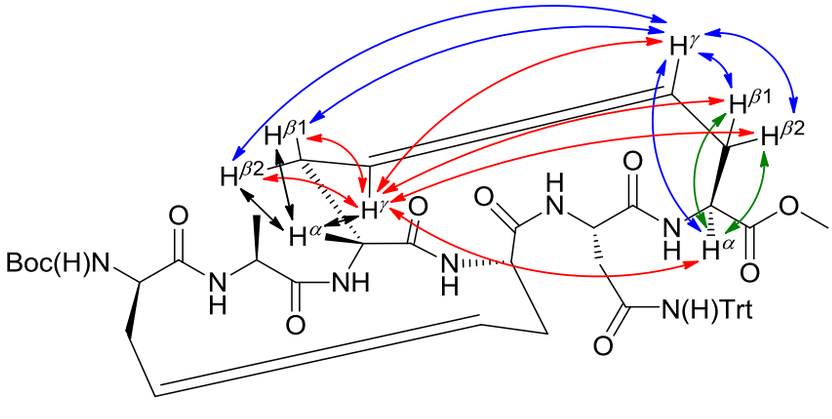


Figure 4. Detail of the TOCSY spectrum of 2d which indicates the side chain to side chain connectivity pattern. The cross-peaks are labeled with different colors; red, black, blue and green, marking peak patterns at ω2 = 5.23 ppm, 4.73 ppm, 4.71 ppm and 4.52 ppm, respectively. The grey labeled peaks are diagonal mirror peaks.



bicyclo[$E^{1\text{-}4}/E^{3\text{-}6}$]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (**2d**)

Figure 5. Overview of the cross-peaks in dicarba-bridge X3-X6 (D-Alg^{3}-Alg^{6}) of the TOCSY spectrum mapped on the chemical structure of **2d**. Each colored arrow (red, black, blue and green) indicates a cross-peak of dicarba bridge X3-X6 in the TOCSY spectrum.

at ω_1 = 5.23 (X3-Hγ) and 4.71 ppm (X6-Hγ), respectively. At ω_2 = 4.73 ppm (black labels), cross-peaks were observed between X3-Hα and the two X3 β-protons: ω_1 = 3.07 (X3-Hβ2), 1.85 ppm (X3-Hβ1), and to X3 alkene γ-proton at 5.23 ppm, respectively, to indicate the correct side-chain to side-chain connectivity pattern: X3-X6 = D-Alg[3]-Alg[6]. Also, the HSQC spectrum confirmed that the two protons at 1.85 ppm and 3.07 ppm were X3 β-protons, both connected to X3-Cβ at ω_1 = 27.41 ppm (Figure 6).

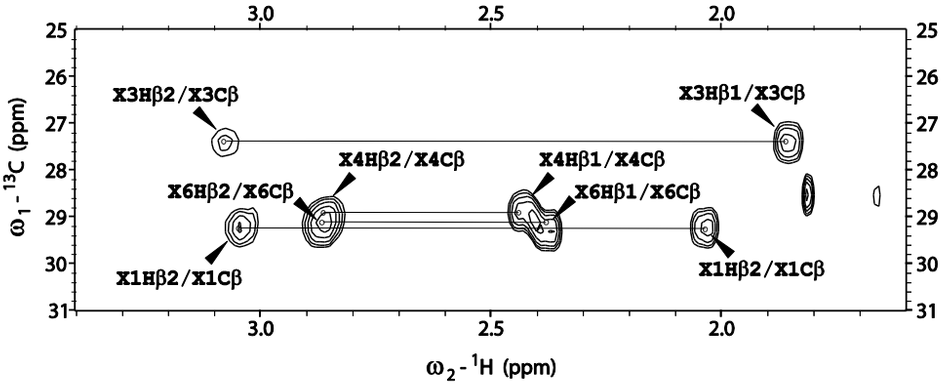


Figure 6. Detail of the HSQC spectrum of **2d**.

Table 1. ^{1}H Chemical shift assignments of hexapeptides **2a-d** in $CDCl_3/CD_3OH$ 95:5 v/v at *T* = 298 K

Xaa-residue	compound	αCH	βCH_x	γCH ($J_{1\text{-}2}$ (Hz))	αNH	other
D-Alg[1]	**2a**	4.41	2.25/2.70	5.45 (4.1)	6.55	1.51 $(CH_3)_3$ Boc
	2b	4.27	2.24/2.65	5.56 (6.8)	6.33	1.48 $(CH_3)_3$ Boc
	2c	4.61	2.05/3.04	5.46 (10.9)	6.96	1.52 $(CH_3)_3$ Boc
	2d	4.57	2.04/3.04	5.28 (10.5)	6.95	1.52 $(CH_3)_3$ Boc
Ala[2]	**2a**	4.02	1.04		7.06	
	2b	4.36	1.30		7.27	
	2c	3.64	1.06		6.80	
	2d	4.01	1.31		7.02	
D-Alg[3]	**2a**	4.55	2.03/2.70	4.75 (4.0)	8.03	
	2b	4.67	1.96/3.06	5.36 (n.d.)	8.70	
	2c	4.72	1.96/2.76	4.63 (4.0)	8.04	
	2d	4.73	1.85/3.07	5.23 (10.3)	8.66	
Alg[4]	**2a**	4.54	2.25/2.75	5.30 (4.1)	6.69	
	2b	4.08	2.48	5.15 (6.8)	6.50	
	2c	4.35	2.51/2.94	5.29 (10.9)	7.46	
	2d	4.08	2.43/2.85	5.48 (10.5)	7.56	
Asn[5]	**2a**	4.76	2.86/3.21		8.01	7.63 (CON*H*); 7.18-7.33 arom trityl
	2b	4.87	2.67/3.14		7.69	7.18 (CON*H*); 7.17-7.35 arom trityl
	2c	4.75	2.74/3.33		8.53	7.16 (CON*H*); 7.17-7.31 arom trityl
	2d	4.87	2.68/3.29		7.84	7.75 (CON*H*); 7.17-7.33 arom trityl
Alg[6]	**2a**	4.67	2.01/2.52	5.21 (4.0)	6.74	3.74 (OCH_3)
	2b	4.52	2.41/2.84	4.87 (n.d.)	6.83	3.74 (OCH_3)
	2c	4.76	1.92/2.60	5.23 (4.0)	6.74	3.73 (OCH_3)
	2d	4.52	2.38/2.87	4.71 (10.3)	6.81	3.73 (OCH_3)

Table 2. ^{13}C Chemical shift assignments of hexapeptides **2a-d** in $CDCl_3/CD_3OH$ 95:5 v/v at T = 298 K.

Xaa-residue	compound	αC	βC	γC	other
D-Alg[1]	**2a**	54.6	35.9	129.4	28.5 $(CH_3)_3$ Boc
	2c	53.4	28.5	125.6	28.3 $(CH_3)_3$ Boc
	2d	53.9	29.3	130.8	28.5 $(CH_3)_3$ Boc
Ala[2]	**2a**	49.1	16.2		
	2c	50.1	15.5		
	2d	50.7	15.9		
D-Alg[3]	**2a**	52.7	34.3	130.0	
	2c	51.3	34.2	129.4	
	2d	52.7	27.4	125.9	
Alg[4]	**2a**	53.0	33.5	129.3	
	2c	54.2	28.6	129.4	
	2d	57.3	29.0	126.6	
Asn[5]	**2a**	50.0	37.4		127.9-129.5 arom trityl
	2c	49.1	36.5		127.2-128.6 arom trityl
	2d	49.4	37.2		127.9-129.6 arom trityl
Alg[6]	**2a**	51.0	34.8	130.0	52.9 (OCH_3)
	2c	50.5	34.6	127.4	52.5 (OCH_3)
	2d	52.0	29.2	130.4	52.8 (OCH_3)

2.2.3 Biological evaluation

It is known that nisin interacts with anionic phospholipid membranes and this interaction is followed by insertion and pore formation. Previously, Breukink and co-workers reported that this nisin-membrane interaction is initiated via the C-terminus of nisin.[25] To investigate if the cross-stapled bicyclic octapeptide **5** is a functional mimic of the C-terminal fragment of nisin, a growth inhibition assay of the Gram-positive bacterium *Bacillus subtilis* as well as interaction studies with carboxyfluorescein (CF)-loaded large unilamellar vesicles (LUVs) were performed. Peptide-induced membrane leakage was measured by monitoring the release of CF by fluorescence spectroscopy, exemplified by the membrane-active antimicrobial peptide anoplin, H-Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH_2.[26] As a model for an abundant bacterial cell membrane, LUVs consisting of an equimolar amount the zwitterionic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and the anionic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were used.

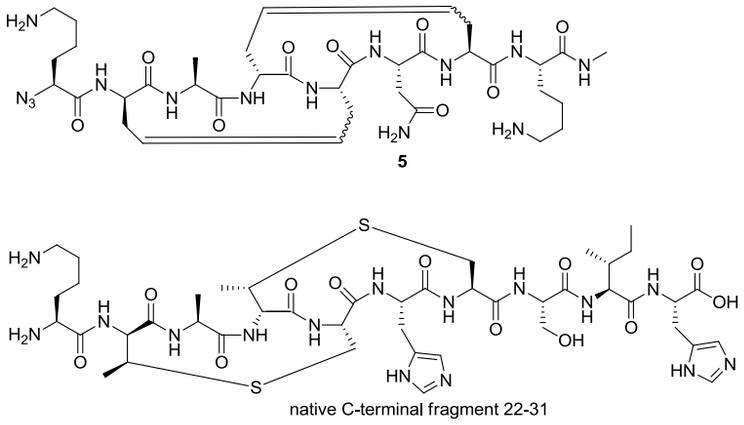


Figure 7. Chemical structures of octapeptide **5** and the control peptide, native C-terminal fragment 22-31.

The CF-loaded DOPC/DOPG LUVs were treated with **5** (at 25 μM); unfortunately, under these conditions no fluorescence could be observed. Since it is not known if the native C-terminal fragment 22-31 (Figure 7) does interact with anionic model membranes, this fragment was prepared a control peptide via digestion of full length nisin with chymotrypsin followed by cyanogen bromide cleavage.[27] Also this fragment (at 25 μM) did not result in any fluorescence.

Alternatively, membrane leakage was also studied on vesicles consisting of DOPC spiked with 0.2% lipid II. Nisin (at 1 nM) was able to induce ~46% membrane leakage, while the native nisin 22-31 fragment (at 25 μM) as well as the octapeptide **5** (at 25 μM) gave no membrane leakage. In addition to this, an antimicrobial activity test was performed as a microtitre broth dilution assay. Nisin, as a positive control peptide, was found to have a minimal inhibitory concentration (MIC) of 0.21 μM, while both the native C-terminal fragment 22-31 and octapeptide **5** were inactive (MIC >500 μM). Apparently, based on these two assays, it became clear that both C-terminal nisin peptides, the native C-terminal fragment 22-31 as well as octapeptide **5** were too short and require N-terminal amino acid residues for their antimicrobial activity and membrane permeabilizing properties.[28]

2.3 Conclusions

In conclusion, the synthesis of a cross-stapled (1→4/3→6) nisin DE-ring mimic via ring-closing metathesis starting from a linear hexapeptide containing four allylglycine residues was described. This approach resulted in the isolation of bicyclic peptides with the correct side chain to chain side connectivity pattern, irrespective of the applied catalytic Ru-species: Grubbs-II or Hoveyda-Grubbs-II. The four individual diastereoisomers were isolated by HPLC, characterized by MS, and unambiguously assigned by NMR regarding the geometry of the two alkene double bonds: [$Z^{1\text{-}4}/Z^{3\text{-}6}$] **2a**, [$Z^{1\text{-}4}/E^{3\text{-}6}$] **2b**, [$E^{1\text{-}4}/Z^{3\text{-}6}$] **2c** and [$E^{1\text{-}4}/E^{3\text{-}6}$] **2d**. These bicyclic hexapeptides were versatile advanced synthons in peptide chemistry since N- and C-terminally functionalized derivatives were obtained, especially an octapeptide N-terminally decorated with an azide functionality, to access orthogonal bioconjugation reactions via Cu(I)-catalyzed azide-alkyne cycloaddition chemistry (CuAAC). The alkene-bridged DE-ring mimic as well as the native nisin DE-ring fragment were not able to induce membrane leakage and were inactive in an antimicrobial assay. Therefore, the synthesis and biochemical evaluation of constructs comprising the native ABC-ring fragment ligated to the alkene-bridged DE-ring mimic will be highly interesting to design new nisin peptidomimetics with improved properties, like stability against oxidation, membrane permeabilization, and antimicrobial activity.

2.4 Experimental

2.4.1 Chemicals, instruments and general methods

Chemicals were used as obtained from commercial sources without further purification unless stated otherwise. The solvents were obtained as peptide synthesis grade and stored on molecular sieves (4 Å). Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40-63 μm). TLC was performed on Merck precoated silica gel 60F254 glass plates. Compounds were visualized by UV-quenching, ninhydrin, or Cl_2/TDM.[30] ^{1}H NMR spectra were acquired on a Varian Mercury 300 MHz or a Varian Inova 500 MHz spectrometer in $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm) or DMSO (2.50 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of triplet (dt), and broad. ^{13}C-NMR spectra were acquired on a Varian Mercury 75 MHz or a Varian Inova 125 MHz spectrometer in $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm), or DMSO-d_6 (39.5 ppm). Analytical HPLC was performed on an automated HPLC system (Shimadzu) equipped with a UV/vis detector operating at λ = 220/254 nm using an Alltech Alltima C8 column (pore size: 100 Å, particle size: 5 μm, 250 × 4.6 mm) at a flow rate of 1 mL/min (from 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min (method A), respectively 60 min (method B). Preparative HPLC was performed on an automated preparative HPLC system (Applied Biosystems) equipped with a UV/vis detector operating at λ = 214 nm using an Alltech Alltima C8 column (pore size: 100 Å, particle size: 10 μm, 250 × 22 mm) at a flow rate of 12.0 mL/min (from 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 90 min). ESI-MS was performed on a Shimadzu LCMS-QP8000 electrospray ionization mass spectrometer operating in a positive ionization mode.

2.4.2 Synthesis

Boc-D-Alg^1-Ala^2-D-Alg^3-Alg^4-Asn(Trt)^5-Alg^6-OMe (1)

This peptide was synthesized (on a 1.00 mmol scale) as described previously.[10b] Yield: 900 mg (95%); R_t = 25.78 min; ESI-MS calcd $C_{52}H_{65}N_7O_{10}$ 947.48, found *m/z* $[M+H]^+$ 948.30, $[M+Na]^+$ 970.35.

bicyclo[1-4/3-6]-Boc-D-Alg^1-Ala^2-D-Alg^3-Alg^4-Asn(Trt)^5-Alg^6-OMe (2)

The linear precursor peptide **1** (473 mg, 0.50 mmol) was dissolved in CH_2Cl_2 (100 mL) and the solution was gently purged for 20 min with dry N_2 while heated to reflux. Then, Hoveyda-Grubbs II

catalyst (50 mg, 80 μmol) was added and the obtained reaction mixture was refluxed for 16 h. Subsequently, the solvent was removed by evaporation and the residue was purified by column chromatography (CH_2Cl_2/MeOH 96:4 v/v → CH_2Cl_2/MeOH 9:1 v/v) to give bicyclic peptide **2** in 93% yield (416 mg of an off-white solid) as a mixture of diastereoisomers. To separate peptide **2** in its individual diastereoisomers **2a-d**, peptide **2** (416 mg, 0.47 mmol) was dissolved in DMF (6 mL) and diluted with 0.1 % TFA in CH_3CN/H_2O 1:1 v/v (34 mL) and purified by preparative HPLC. Pure fractions were analyzed by analytical HPLC and mass spectrometry, pooled and lyophilized. The total amount of recovered peptide material was 242 mg (54%).

bicyclo[*Z*$^{1\text{-}4}$/*Z*$^{3\text{-}6}$]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (2a)

The amount of isolated product was 55 mg (23%). R_t = 22.95 min; ESI-MS calcd $C_{48}H_{57}N_7O_{10}$ 891.42, found *m/z* $[M+H]^+$ 892.70, $[2M+H]^+$ 1785.05, $[2M+Na]^+$ 1805.95.

bicyclo[*Z*$^{1\text{-}4}$/*E*$^{3\text{-}6}$]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (2b)

The amount of isolated product was 7 mg (3%). R_t = 23.15 min; ESI-MS calcd $C_{48}H_{57}N_7O_{10}$ 891.42, found *m/z* $[M+H]^+$ 892.90, $[M+Na]^+$ 915.50, $[2M+H]^+$ 1785.10, $[2M+Na]^+$ 1806.10.

bicyclo[*E*$^{1\text{-}4}$/*Z*$^{3\text{-}6}$]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (2c)

The amount of isolated product was 84 mg (35%). R_t = 23.77 min; ESI-MS calcd $C_{48}H_{57}N_7O_{10}$ 891.42, found *m/z* $[M+H]^+$ 892.90, $[M+Na]^+$ 915.50, $[2M+H]^+$ 1785.05, $[2M+Na]^+$ 1806.70.

bicyclo[*E*$^{1\text{-}4}$/*E*$^{3\text{-}6}$]-Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe (2d)

The amount of isolated product was 96 mg (40%). R_t = 23.95 min; ESI-MS calcd $C_{48}H_{57}N_7O_{10}$ 891.42, found *m/z* $[M+H]^+$ 892.80, $[M+Na]^+$ 914.65, $[2M+H]^+$ 1784.50, $[2M+Na]^+$ 1806.05.

bicyclo[1-4/3-6]-N_3-Lys(Boc)-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn^{5}-Alg^{6}-OMe (3)

Bicyclic peptide **2** (23 mg, 26 μmol) was dissolved in CH_2Cl_2/TFA 1:1 v/v (2 mL) and to the bright yellow solution, TIS (20 μL) was added as a scavenger, after which the reaction mixture turned into a slightly brownish solution. After stirring for 1 h at room temperature, MeOH (2 mL) was added and the reaction mixture was evaporated to dryness and the crude residue was coevaporated with MeOH (3 × 2 mL) and $CHCl_3$ (3 × 2 mL), to remove any residual TFA. Then, the corresponding peptide amine was dissolved in DMF (1 mL) and to this solution, azide **7** (11 mg, 39 μmol), BOP (17 mg, 39 μmol), followed by DIPEA (23 μL, 0.13 mmol, 5 equiv), were added, and the reaction mixture was stirred for 16 h at room temperature. Subsequently, the volatiles were removed by evaporation, and the residue was purified by column chromatography (CH_2Cl_2/MeOH 8:2 v/v) to

give peptide **3** as an off-white solid in 75% yield (16 mg). R_t = 13.35 min; ESI-MS calcd $C_{30}H_{45}N_{11}O_9$ 703.34 (after TFA treatment), found *m/z* $[M+H]^+$ 704.05, $[2M+H]^+$ 1407.00.

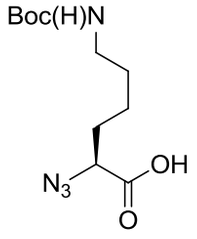
bicyclo[1-4/3-6]-H-D-Alg^1-Ala^2-D-Alg^3-Alg^4-Asn^5-Alg^6-Lys(Fmoc)-NHMe·TFA (4)

Bicyclic peptide **2** (120 mg, 135 μmol) was dissolved in Tesser's base[21] (dioxane/MeOH/4N NaOH 14:5:1 v/v/v; 13.5 mL) to saponify the methyl ester. After 2 h of stirring at room temperature, the reaction mixture was concentrated in vacuo and the residue was acidified to pH 1 by aq. 1N $KHSO_4$. The aqueous phase was extracted with CH_2Cl_2 (3 × 5 mL) and the combined organic layers were evaporated in vacuo. This residue was dissolved in DMF (4 mL), and HCl·H-Lys(Fmoc)-NHMe (**11**) (56 mg, 134 μmol), BOP (58 mg, 137 μmol), followed by DIPEA (71 μL, 408 μmol, 3 equiv) were added and the obtained reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed by evaporation and the residue was taken up in TFA/TIS/CH_2Cl_2 10:10:0.2 v/v/v (2 mL) for 1 h at room temperature to remove the Boc group. The peptide, as the corresponding trifluoroacetate, was precipitated from MTBE/hexane 1:1 v/v (20 mL) and after centrifugation (5 min at 3500 rpm) the peptide was decanted and the pellet was suspended a second time with MTBE/hexane 1:1 v/v (20 mL) and centrifuged. Finally, the crude peptide pellet was dissolved in TFA (2 mL) and diluted with CH_3CN/H_2O 1:1 v/v (8 mL) and purified by preparative HPLC. Peptide **4** was obtained as a white fluffy powder in 22% overall yield (30 mg). R_t = 18.80-19.07 min (mixture of four diastereoisomers); ESI-MS calcd $C_{45}H_{58}N_{10}O_{10}$ 898.43, found *m/z* $[M+H]^+$ 899.75, $[M+Na]^+$ 921.25, $[(M-C_{15}H_{10}O_2)+H]^+$ 677.25.

bicyclo[1-4/3-6]-N$_3$-Lys-D-Alg^1-Ala^2-D-Alg^3-Alg^4-Asn^5-Alg^6-Lys-NHMe·2TFA (5)

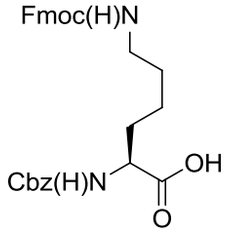
Bicyclic peptide amine **4** (30 mg, 30 μmol) was dissolved in DMF (2 mL) and to this solution, N_3-Lys(Boc)-OH (**7**) (10 mg, 37 μmol), BOP (13 mg, 31 μmol), followed by DIPEA (16 μL, 92 mmol, 3.1 equiv), were added. The obtained reaction mixture was stirred for 16 h at room temperature and subsequently concentrated in vacuo. The residue was dissolved in THF (2 mL) and directly used in the next step by treatment with dimethylamine (40% solution in H_2O; 2 mL) for 2 h at room temperature to remove the Fmoc group. Then, the reaction mixture was evaporated to dryness and the residue was coevaporated with toluene (3 × 5 mL) and $CHCl_3$ (3 × 5 mL). Finally, the obtained residue was dissolved in TFA/CH_2Cl_2 1:1 v/v (2 mL) and the solution was stirred for 1 h at room temperature followed by evaporation in vacuo. The residual oil was triturated with MTBE/hexane 1:1 v/v (10 mL) and after centrifugation (5 min at 3500 rpm) the peptide was decanted and the pellet was resuspended in MTBE/hexane 1:1 v/v (10 mL) and centrifuged. The crude peptide was dissolved in 0.1% TFA in CH_3CN/H_2O (95:5 v/v; 3 mL) and purified by semi-preparative HPLC to give peptide **5** as a white fluffy powder in 52% overall yield (16 mg). R_t = 13.15-14.57 min

(mixture of four diastereoisomers); ESI-MS calcd $C_{36}H_{58}N_{14}O_9$ 830.45, found *m/z* $[M+H]^+$ 831.40, $[M+2H]^{2+}$ 416.35.



N_3-Lys(Boc)-OH (7)

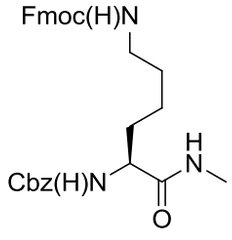
Commercially available H-Lys(Boc)-OH (**6**) (2.46 g, 10 mmol) was subjected to a diazotransfer in the presence of imidazole-1-sulfonyl azide hydrochloride (2.49 g, 12 mmol, 1.2 equiv), $CuSO_4 \cdot 5H_2O$ (25 mg, 0.1 mmol, 0.01 equiv), and K_2CO_3 (3.04 g, 27 mmol, 2.7 equiv) in MeOH (60 mL) according to the procedure of Goddard-Boger and Stick.[20] Next, the reaction mixture was concentrated and the residue was partitioned between EtOAc (50 mL) and aq. 1N $KHSO_4$ (50 mL). Subsequently, the aqueous phase was extracted with EtOAc (2 × 50 mL) and the combined organic layers were washed with aq. sat. NaCl (40 mL), dried (Na_2SO_4), and evaporated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) to give N_3-Lys(Boc)-OH as a colorless oil in quantitative yield (2.72 g). R_f(CH_2Cl_2/MeOH 97:3 v/v): 0.25; ^{1}H-NMR (300 MHz, DMSO-d_6, 298 K): δ 1.28-1.38 (m, 13H, γCH_2/δCH_2 Lys (2 × 2H)/$(CH_3)_3$ Boc (9H)), 1.52-1.76 (m, 2H, βCH_2 Lys), 2.88 (dt (J_{vic} = 6.3 Hz, J_{gem} 6.1 Hz) 2H, εCH_2 Lys), 4.01 (m, 1H, αCH Lys), 4.75 (broad t (J = 5.4 Hz) 1H, NH urethane) ^{13}C-NMR (75 MHz, DMSO-d_6, 298 K): δ 22.7, 28.3, 29.0, 30.6, 39.6, 61.4, 77.4, 155.6, 171.9.



Cbz-Lys(Fmoc)-OH (9)

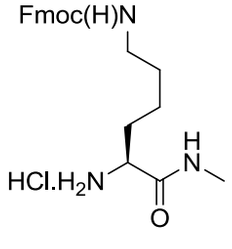
Cbz-Lys-OH (**8**) (2.80 g, 10 mmol) was dissolved in H_2O (40 mL) and a solution of Fmoc-ONSu (3.37 g, 10 mmol) in dioxane (50 mL) was added. The pH of the obtained aqueous mixture was adjusted to 8.5 by the portion-wise addition of a solution of $Na_2CO_3 \cdot 10H_2O$ (5.75 g, 20 mmol) in H_2O (15 mL) during 2 h, while the reaction mixture was stirred at room temperature. Then, the reaction mixture was neutralized by adding aq. 1N HCl and subsequently concentrated to half its original volume. The aqueous phase was acidified to pH 2 with aq. 1N HCl and extracted with EtOAc (80 mL). The EtOAc solution was subsequently washed with aq. 1N HCl (2 × 50 mL), aq. sat. NaCl (2 × 50 mL), dried (Na_2SO_4), and evaporated to dryness. The solid residue was coevaporated with $CHCl_3$ (3 × 40 mL) and dried under vacuum to give **9** as an off-white powder in quantitative yield (5.01 g). R_f(CH_2Cl_2/MeOH/AcOH 90:10:0.1 v/v/v): 0.46; ^{1}H-NMR (300 MHz, $CDCl_3$, 298 K): δ 1.26-1.41 (m, 4H, γCH_2/δCH_2 Lys), 1.68 (m, 2H, βCH_2 Lys), 2.99-3.09 (m, 2H, εCH_2 Lys), 4.15 (d (J = 6.3 Hz), 1H, CH Fmoc), 4.35-4.40 (m, 3H, αCH Lys/CH_2 Fmoc), 5.06 (m, 3H, εNH urethane/CH_2 benzyl), 5.74 (d (J = 7.6 Hz), 1H, αNH urethane), 7.22-7.72 (m, 13H, arom Fmoc (8H)/arom benzyl

(5H)); ^{13}C-NMR (75 MHz, $CDCl_3$, 298 K): δ 21.8, 22.2, 28.9, 29.1, 31.6, 40.4, 47.1, 53.6, 66.5, 66.9, 67.3, 119.9, 124.7, 124.9, 127.0, 127.6, 128.0, 128.1, 128.4, 136.1, 141.2, 143.6, 143.8, 156.3, 156.7, 175.5, 175.9.



Cbz-Lys(Boc)-NHMe (10)

Cbz-Lys(Fmoc)-OH (**9**) (1.97 g, 3.92 mmol) was dissolved in THF (60 mL) followed by the addition of BOP (1.72 g, 4.05 mmol) and methylamine (2M solution in THF; 5.91 mL). The clear reaction mixture turned into a viscous suspension and after stirring for 3 h at room temperature it was concentrated in vacuo. The residue was taken up in $CHCl_3$/MeOH 50:1 v/v (150 mL) and the organic layer was subsequently washed with aq. 1N HCl (3 × 50 mL), aq. 5% $NaHCO_3$ (3 × 50 mL) and aq. sat. NaCl (3 × 50 mL). To avoid premature precipitation of the desired compound it was required to add MeOH (3 mL) to the organic phase after each washing step. Then, the $CHCl_3$/MeOH layer was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by recrystallization from MeOH and amide **10** was obtained as a white solid in 91% yield (1.83 g). R_f(CH_2Cl_2/MeOH 9:1 v/v): 0.60; ^{1}H-NMR (300 MHz, DMSO-d_6, 298 K): δ 1.21-1.59 (m, 6H, βCH_2/γCH_2/δCH_2 Lys), 2.60 (d (*J* = 4.1 Hz), 3H; NHCH_3), 2.98 (m, 2H, εCH_2 Lys), 3.91 (m, 1H, αCH Lys), 4.19 (t (*J* = 6.6 Hz), 1H, CH Fmoc), 4.29 (d (*J* = 6.6 Hz), 2H, CH_2 Fmoc), 5.01 (s, 2H, CH_2 benzyl), 7.27-7.44 (m, 16H, arom Fmoc (8H)/arom Cbz (5H)/εNH urethane (1H)/αNH urethane (1H)/N*H*CH_3 amide (1H)); ^{13}C-NMR (75 MHz, DMSO-d_6, 298 K): δ 23.8, 26.5, 30.0, 32.6, 41.0, 47.8, 55.7, 66.2, 66.4, 121.0, 126.1, 128.5, 128.7 (2 lines), 129.3, 138.0, 141.7, 144.9, 156.9, 157.1, 173.3.



HCl·H-Lys(Fmoc)-NHMe (11)

Cbz-Lys(Boc)-NHMe (**10**) (1.00 g, 1.94 mmol) was dissolved in a solution of 30 % HBr in glacial acetic acid (10 mL) to remove the Cbz group and after 30 min of stirring at room temperature, the reaction mixture was evaporated to dryness and subsequently coevaporated with H_2O (3 × 10 mL) to remove any residual acid. The corresponding hydrobromide was dissolved in H_2O (30 mL) and passed through a short column of DOWEX-® 1X8 strongly basic anion exchange resin (chloride form). The effluent was concentrated in vacuo and the residual oil was triturated with $CHCl_3$ to give hydrochloride **11** as a white solid in 89% yield (0.72 g). R_f(CH_2Cl_2/MeOH/Et_3N 90:10:0.1 v/v/v): 0.18; ^{1}H-NMR (300 MHz, DMSO-d_6, 298K): δ 1.26-1.43 (m, 4H, γCH_2/δCH_2 Lys), 1.69 (m, 2H; βCH_2 Lys), 2.65 (d (*J* = 4.1 Hz), 3H, NHCH_3), 2.97 (q (*J* = 6.3 Hz) 2H, εCH_2 Lys), 3.69 (t, (*J* = 6.0 Hz), 1H, αCH Lys), 4.15 (d (*J* = 6.3 Hz), 1H, CH Fmoc), 4.19-4.31 (m, 3H, CH/CH_2 Fmoc), 5.06 (s, 2H, CH_2

benzyl), 7.29-7.91 (m, 9H, arom Fmoc (8H)/εNH urethane (1H)), 8.24 (broad s, 3H, αNH_2·HCl), 8.52 (broad s, 1H, N$H$$CH_3$ amide); ^{13}C-NMR (75 MHz, DMSO-d_6, 298 K): δ 22.0, 25.9, 29.4, 31.3, 39.1, 47.2, 52.7, 65.7, 120.6, 125.6, 127.5, 128.1, 141.2, 144.4, 156.6, 169.7.

Compound 13

In a microwave vial, a mixture of alkyne-functionalized 5/6-carboxyfluorescein (**12**) (1.7 mg, 4.1 μmol) and azido peptide **5** (3.2 mg, 3.9 μmol) was dissolved in *tert*-BuOH/H_2O 1:1 v/v (500 μL). Then, aq. 10 mM $CuSO_4$·$5H_2O$ (40 μL, 0.40 μmol) followed by aq. 100 mM sodium ascorbate (40 μL, 4.0 μmol), were added and the vial was capped, placed in a microwave reactor and the reaction mixture was irradiated for 20 min at 80°C. Finally, the reaction mixture was diluted with 0.1% TFA in CH_3CN/H_2O 95:5 v/v (2 mL) and purified by semi-preparative HPLC to give triazole **13** as a bright yellow solid in 44% yield (2.0 mg). R_t = 17.28 min; ESI-MS calcd $C_{60}H_{73}N_{15}O_{15}$ 1243.54, found *m/z* $[M+H]^+$ 1244.80, $[M+2H]^{2+}$ 622.90, $[M+3H]^{3+}$ 415.65.

Native nisin-fragment (22-31)[27]

R_t = 15.97 min (method B); ESI-MS calcd. $C_{44}H_{69}N_{15}O_{12}S_2$: 1063.47, found: *m/z* 1064.65 $[M+H]^+$, 533.10 $[M+2H]^{2+}$, 355.70 $[M+3H]^{3+}$.

2.5.3 Biological Evaluation

Vesicle leakage experiments

Carboxyfluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment according to a literature procedure.[25] The LUVs consisted of a equimolar amount of the zwitterionic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and the anionic lipid 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG). The peptide-induced leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA) at 20 °C. A solution (1.0 mL) of CF-loaded vesicles (20 μM final concentration) in buffer (10 mM Tris/HCl pH = 7.0, 100 mM NaCl) was added to a quartz cuvette and fluorescence was measured (A_0). After 20 s, a buffer solution (25 μL) containing the peptide of interest (stock: 1 mM; final: 25 μM) was added and peptide-induced membrane leakage was followed during 60 s (A_{60}), after which a buffer solution (10 μL) of Triton-X (stock: 20%; final: 0.2%) was added to induce total leakage of the vesicles (A_{Total}). The % of peptide-induced membrane leakage was calculated by: $((A_{60} - A_0)/(A_{Total} - A_0)) \times 100\%$. All measurements were performed in duplo. In case of anoplin (H-Gly-

Leu-Leu-Lys-Arg-Ile-Lys-The-Leu-Leu-NH_2), the final concentration was 43 μM (50 μg/mL) and in case of nisin, the final concentration was 1 nM.

Growth inhibition assay

Bacillus subtilis was used for determination of antimicrobial activity. The MIC of each peptide was determined using a broth micro-dilution assay adapted from a literature procedure as previously described by Hancock.[30] Peptide stock solutions were prepared at a concentration of 100 μM to 1000 μM peptide in 0.2% bovine serum albumin and 0.01% acetic acid. Serial three-fold solutions of the peptides were made in 0.2% bovine serum albumin and 0.01% acetic acid. To each well was added, 50 μL of the test bacteria in tryptic soya broth to a final concentration of 2×10^6 CFU/mL and 50 μL of the peptide in the different concentrations. After incubation for 24 h at 37 °C at 120 rpm in Certomat incubator, the OD at 630 nm was measured. The MIC (expressed in μM) of each peptide was read as the lowest concentration of peptide that inhibited visible growth of bacteria. All measurements were performed in duplicate.

2.5 References and Notes

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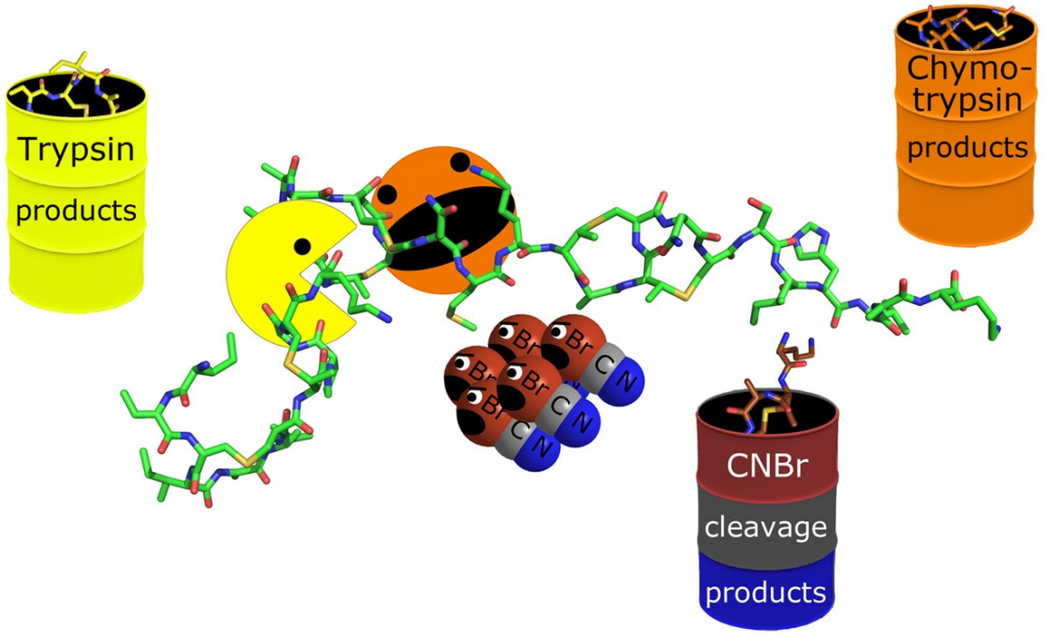
Chapter 3

Scalable purification of the lantibiotic nisin and isolation of chemical/enzymatic cleavage fragments suitable for semi-synthesis



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3.1 Introduction

Nisin is an antimicrobial peptide and belongs to the class of lantibiotics, a family of peptides that is produced by Gram-positive bacteria, like *Lactococcus lactis*, as a chemical defence system against competing microorganisms. Lantibiotics are ribosomally synthesized peptides and undergo a series of enzyme-catalyzed post-translational modifications (PTMs). Among these PTMs are the sequence-specific dehydration of serine and threonine residues to afford dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, and the stereo-selective thio ether cyclization to form the (methyl)lanthionine ring, which bestow these peptides their structural and conformational rigidity (see Figure 1).[1]

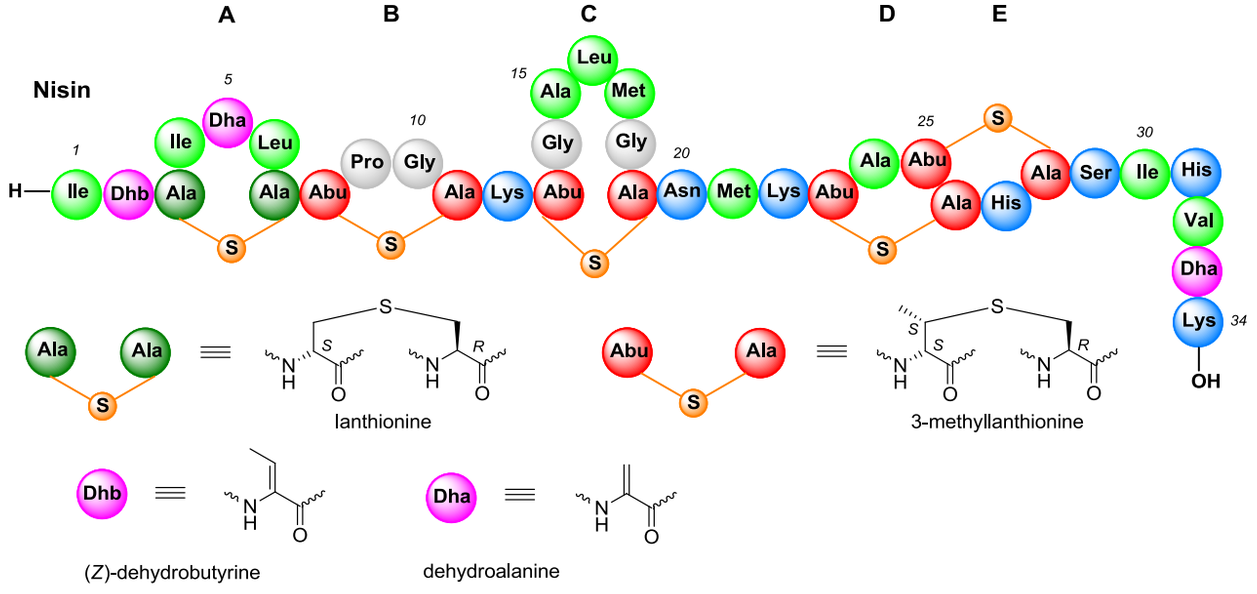


Figure 1. Schematic representation of the lantibiotic nisin, containing unnatural amino acids Dhb (*Z*-dehydrobutyrine), Dha (dehydroalanine), Lan (lanthionine) and MeLan (3-methyllanthionine).

Nisin exerts a dual mode of action to kill Gram-positive bacteria.[2] Firstly, it inhibits cell wall synthesis by binding via its N-terminal AB(C)-ring system toward lipid II,[3] which is an essential cell wall precursor for cross-linking to provide the cell membrane mechanical strength. Secondly, nisin permeabilizes the cell membrane by forming pores that disrupt vital ion gradients, since after binding toward lipid II, the C-terminal DE-ring fragment inserts into the bacterial membrane.[4] Nisin is widely used in diary products as a food preservative and has demonstrated promising chemotherapeutic potential to prevent growth of resistant *Staphylococcus aureus* or *Streptococcus pneumoniae*.[5] To improve both the drug-like properties of nisin and the chemical stability of the lanthionine moiety, we started a program to uncover more stable lantibiotics analogs by replacing the thio ether bridge by a 'carbon bridge' introduced by ring-closing metathesis as well as by combining native nisin fragments with chemically synthesized nisin fragments via semi-synthesis.[6] The latter approach requires a convenient and scalable purification method of commercially

available nisin preparations since relatively large amounts of enriched nisin (and nisin-derived fragments) are necessary in our studies.

Generally, nisin is purified by using cation exchange chromatography at acidic conditions and eluted at high salt concentrations followed by trichloroacetic acid induced precipitation and washing steps with water-miscible organic solvents to remove any residual NaCl and trichloroacetic acid.[7] To avoid these labor-intensive chromatographic techniques, we were looking for a method in which a single extraction step would be sufficient to enrich the nisin peptide from commercially available nisin preparations. Inspired by a report of Burianek and Yousef,[8] we optimized the extraction step to obtain nisin samples with a high peptide content and used these samples in enzymatic and chemical degradation studies to isolate the desired nisin fragments to be used in semi-synthesis.

3.2 Results and Discussion

3.2.1 Scalable enrichment of nisin

In this study, two commercially available nisin preparations were used and according to the manufacturer's specifications both samples contained NaCl (~75%), dairy proteins (~22.5%) and nisin (~2.5%).[9] Analytical HPLC traces of these samples (at 1 mg powder/mL buffer A), as shown in Figure 2 (black line traces), indicated in each sample the presence of two peaks (R_t = 25.97 and 26.17 min, respectively) with a relatively low intensity, and based on MALDI-TOF analysis, the major peak could be assigned to nisin (R_t = 26.17 min, $[M+H]^+$ 3352.84) while the minor peak was identified as nisin-Ser33, a non-dehydrated nisin precursor peptide (R_t = 25.97 min, $[M+3H]^{3+}$ 1124.58 ($[M+H]^+$ 3371.74).

To enrich nisin from these preparations, the method of Burianek and Yousef,[8] which was previously used to extract bacteriocins from a culture broth, was adapted and optimized for the isolation of nisin. For this purpose, the nisin preparation was suspended in water and dichloromethane was added. At the water/organic solvent interface, a brownish precipitate was formed which was harvested by centrifugation and decantation, and subsequently lyophilized. This obtained fluffy solid was further purified by dissolution in H_2O/MeCN (95:5 v/v) and filtration through a small column of Celite, which gave a clear and almost colorless solution. After lyophilization, an off-white powder was obtained which was ten-fold enriched in nisin as judged by HPLC (at 1 mg powder/mL buffer A), as shown in Figure 2 (dashed line traces), corresponding to a purity of 34%. This extraction/precipitation approach could be easily scaled-up to a 40-80 gram scale to isolate approximately 2 g of the enriched nisin peptide.

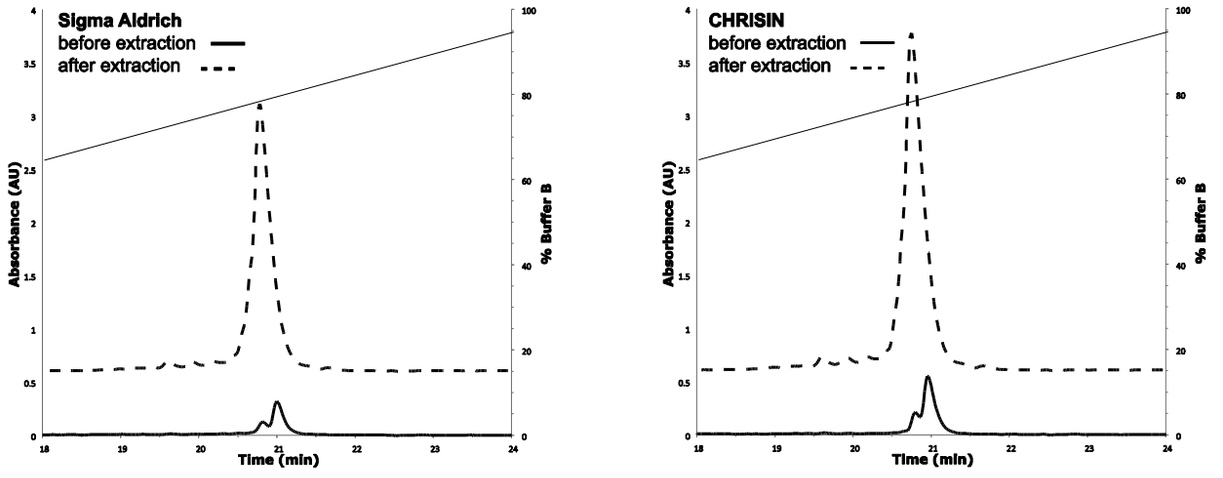


Figure 2. Analytical HPLC traces of nisin from Sigma-Aldrich (left) and CHRISIN® (right). These HPLC traces indicate the nisin content before extraction (line) and after extraction (dashed line), in which both samples have a concentration of 1 mg solid material/mL buffer. (The purification was performed with 2 g nisin preparation.)

3.2.2 Enzymatic digestion of nisin

Previous studies performed by Chan *et al.* indicated that nisin was susceptible toward the proteases trypsin and chymotrypsin.[10] Since trypsin recognizes basic residues, like arginine, lysine and histidine, nisin was found to be cleaved at positions Lys12, Lys22, His28 and His31, respectively. Therefore, nisin-fragment (1-12) could be obtained by tryptic digestion of nisin and isolated by preparative HPLC. Enriched nisin was treated with trypsin for 64 h and the progress of the hydrolysis was followed by analytical HPLC (Figure 3A) while the degradation fragments were identified by LC-MS.

As shown in Figure 4A, tryptic digestion of nisin was almost complete after 64 h, while the individual peaks could be assigned to their corresponding fragments (Table 1). The most abundant peak corresponded to nisin-fragment (1-12) an indication that cleavage site Lys12-Abu13 was preferentially recognized by trypsin, while Lys22-Abu23 bound less efficiently into the active site of trypsin since nisin-fragments (23-34) and (23-31) were only present in small quantities (Figure 4A, Table 1). Surprisingly, and not described previously, it was found that the Asn20-Met21 sequence was also susceptible toward trypsinolysis, since nisin-fragments (21-34), (21-31), and (13-20) were observed and identified based on their molecular mass (Figure 4A, Table 1). Especially the relatively convenient isolation of nisin-fragment (13-20), corresponding to the C-ring (Figure 1), is highly interesting since its chemical synthesis is far from trivial.[11] To the best of our knowledge, the enzymatic accessibility of the nisin C-ring fragment has not been reported earlier.

The digestion of nisin by chymotrypsin was described as a reliable approach to isolate nisin-fragment (1-20), corresponding to the ABC-ring fragment (Figure 1), since the Asn20-Met21

dipeptide sequence is easily cleaved by chymotrypsin.[10] However, we experienced that isolation of nisin-fragment (1-20) by preparative HPLC resulted in disappointingly low yields since many, and previously unknown digestion fragments, hampered efficient purification of the desired (1-20) fragment. Therefore, the digestion of nisin by chymotrypsin was monitored in time by LC-MS (Figure 4B).

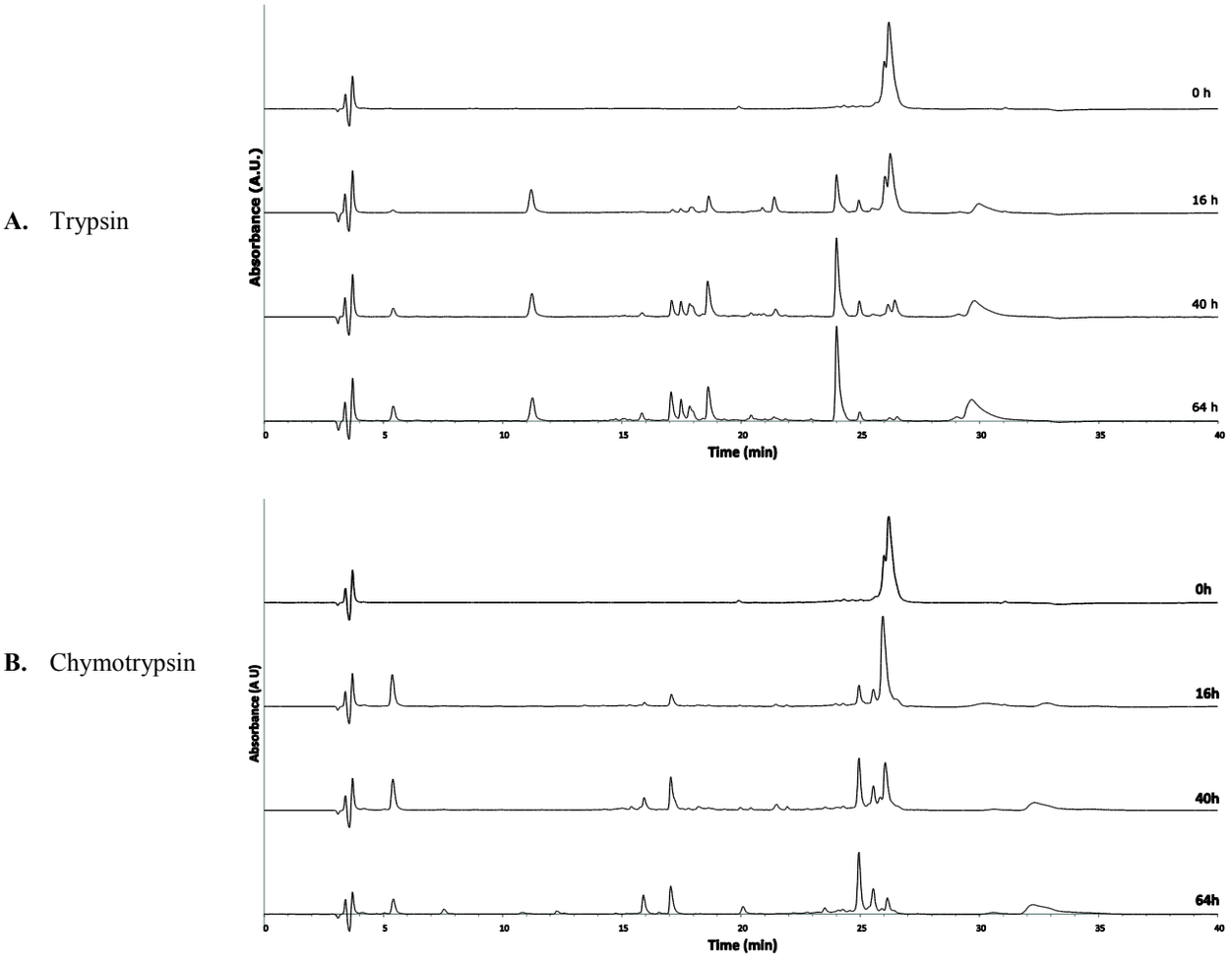


Figure 3 Overview of analytical HPLC traces of nisin cleavage using trypsin (**A**.) and chymotrypsin (**B**.) at time points 0h, 16h, 40h and 64h.

Already after 16 h of incubation, full-length nisin was quantitatively converted into nisin-fragment (1-31), while nisin-fragment (1-20) was only present in a trace amount. This implied that the His31-Val32 sequence was preferentially recognized by chymotrypsin, while Asn20-Met21 was only hydrolyzed as part of the nisin (1-31) sequence. After 64 h of digestion, nisin-fragment (1-20) was observed as the major peak by analytical HPLC (Figure 4B). However, two other peaks were also observed which could be identified as nisin-fragments (1-21) and (21-31), respectively, an indication that the sequence Met21-Lys22 was cleaved by chymotrypsin (Figure 4B, Table 1). The presence of trypsin as an impurity could be excluded, since fragment (1-12) was not found. Also, the Met21-Lys22 cleavage site has not been reported earlier. The presence of this competing

cleavage site resulted in a more complex mixture from which the desired nisin-fragment (1-20) had to be isolated.

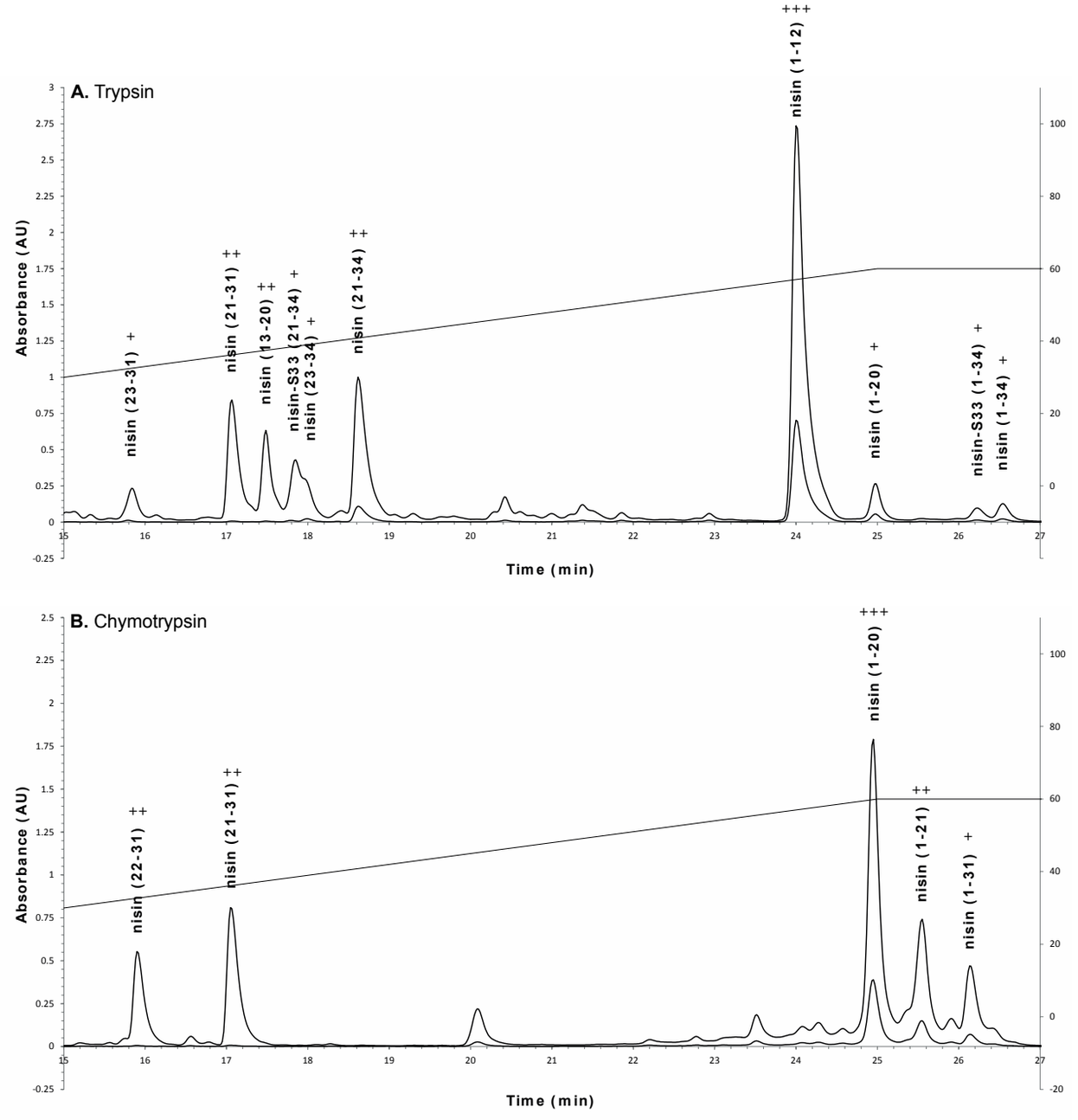


Figure 4. Analytical HPLC traces after digestion for 64 h of nisin by trypsin (**A.**) and chymotrypsin (**B.**). The peaks are marked with the corresponding nisin fragments identified by LC-MS. The peak intensity is indicated as + (low), ++ (medium) and +++ (high). Note: the peak at R_t ~20.1 min (Figure 3B) could not be characterized due to a poor ionization in the LC-MS analysis.

Table 1. HPLC retention times of nisin-fragments formed during enzymatic cleavage of full length nisin by trypsin and chymotrypsin.

Fragments	**R_t**	**$[M+H]^+$**	**Trypsin[c]**			**Chymotrypsin[c]**		
	min[a]	found/calcd[b]	digestion time			digestion time		
			16 h	40 h	64 h	16 h	40 h	64 h
nisin 23-31	15.82	936.41/935.37	-	-	+	-	-	-
nisin 22-31	15.90	1065.31/1063.47				-	+	++
nisin 21-31	17.05	1196.11/1195.44	-	+	++	+	++	++
nisin 13-20	17.47	748.88/747.30	-	+	++			
nisin-Ser33 21-34	17.82	1510.01/1508.71	-	+	+	-	-	-
nisin 23-34	17.92	1232.47/1231.56	-	+	+			
nisin 21-34	18.62	1491.96/1490.69	+	++	++	-	-	-
nisin 1-12	24.00	1150.42/1149.57	++	+++	+++			
nisin 1-20	24.95	1880.14/1878.86	+	+	+	+	++	+++
nisin 1-21	25.53	1006.88/1005.95[d]				+	++	++
nisin 1-31	26.12	1528.93/1528.68[d]	+	+	+	+++	++	+
nisin-Ser33 1-34	26.02	1124.58/1124.19[e]	++	+	+	-	-	-
nisin 1-34	26.25	1118.65/1118.18[e]	++	+	+	-	-	-

[a]Retention times were measured on an Alltech C4 Prosphere column using Method B.

[b]The calculate mass value corresponds to the [M] value.

[c]The peak intensity according to HPLC is quantified as follows: none (-), low (+), medium (++), and high (+++).

[d]Molecular mass corresponds to the $[M+2H]^{2+}$ value. [e]Molecular mass corresponds to the $[M+3H]^{3+}$ value.

3.2.3 Cyanogen bromide cleavage of nisin

The peptide backbone of nisin can be chemically cleaved by treatment with cyanogen bromide, due to the presence of methionine residues at position 17 and 21, while the thio ether in the lanthionine rings resists these reaction conditions.[12] Using cyanogen bromide, the nisin-fragment (22-34), representing the interlocked DE-ring system (Figure 1), can also be obtained which is an important building block in our semi-synthesis approach since cyanogen bromide cleavage will result in a native C-terminal fragment with a free α-amino terminus and an N-terminal fragment with a C-terminal homoserine (Hse) lactone (products **II** and **III**) via intermediate **IIa**, as shown in Scheme 1.[13]

Nisin was treated with an excess of cyanogen bromide in aqueous formic acid and the progress was monitored by HPLC (Figure 5). After 18 h, a typical HPLC trace of the cleavage mixture as shown in Figure 5 was observed. Two peak areas were observed with a retention time of approximately 17.5 and 24.5 min, representing nisin-fragments (22-34) and (1-21), respectively. The first peak area consisted of the four DE-ring fragments (22-34) **a-d**, while the second peak area

represented at least three modified ABC-ring fragments (1-21) **e**, **f-I**, and **f-II**, and full length nisin Met17Hse/Met21Hse derivatives **g-I-IV**, as judged by LC-MS (Figure 6). Further analysis by mass spectrometry of fragments **a**-**d** showed that fragment **a** and **c** corresponded to nisin-Ser33-fragment (22-34) and the desired nisin-fragment (22-34), respectively. The latter fragment could be purified and isolated by preparative HPLC in a reasonable yield of 22%.

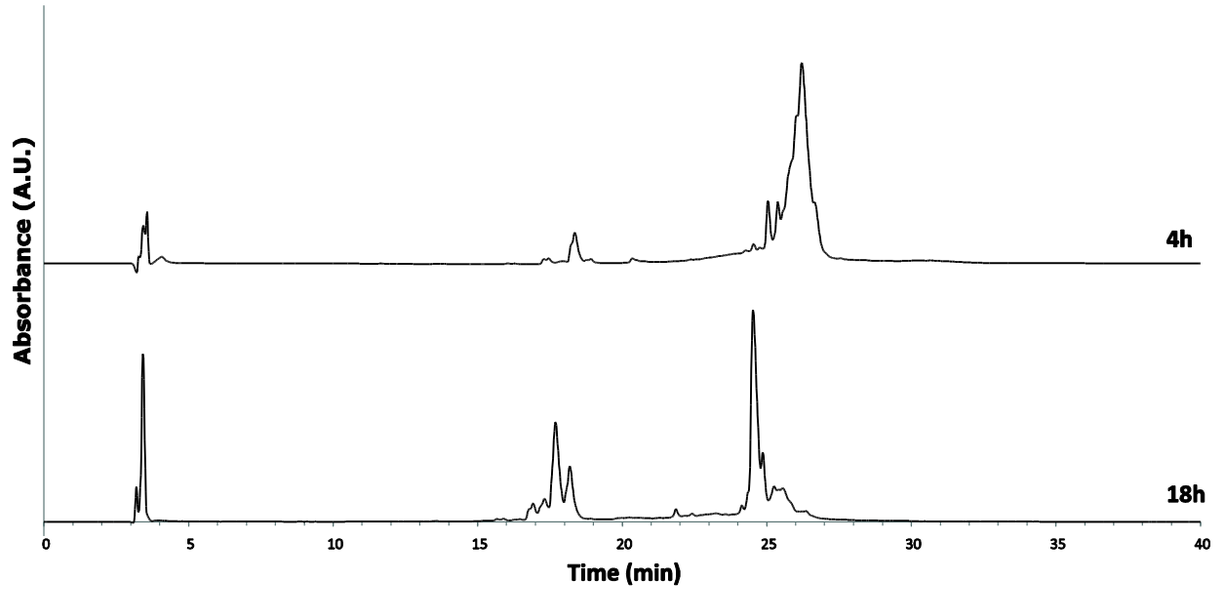


Figure 5. Analytical HPLC traces of nisin at 4h and 18h cleavage by cyanogen bromide.

Interestingly, fragments **b** and **d** had a molecular mass of 28 amu higher than what could be expected from these native nisin fragments. Based on a literature report,[14] it was assumed that this shift in molecular mass corresponded to a formylated serine residue, as a formyl ester, in which residues Ser29/Ser33 were involved. The two peaks in Figure 6 marked with an **f** (R_t = 24.52 and 24.75 min, respectively) could be assigned to either nisin-fragment (1-21) Met17Hse-lacton/Met21Hse-lacton/open C-ring (**f-I**) or nisin-fragment (1-21) Met17Hse/Met21Hse-lacton (**f-II**). While shoulder peak **e** was assigned to be nisin-fragment (1-21) Met17Hse/Met21Hse-lacton/open C-ring. The presence of full length nisin Met17Hse/Met21Hse derivatives **g-I-IV** was explained by assuming that cyclic intermediate **I** (Scheme 1), prior to rearrangement into intermediate **IIa**, underwent hydrolysis without cleavage of the peptide backbone, since the γCH_2 of methionine becomes highly electrophilic due to *S*-alkylation, as applied in the synthesis of Freidinger lactam derivatives.[15] Alternatively, such Met/Hse interconversion phenomena have been previously reported in case of Met-Ser/Thr sequences,[16] in which the hydroxyl side chain functionality attacks the cyclic intermediate **IIb** resulting in Met/Hse interconversion (product **I**) prior to amide bond cleavage.

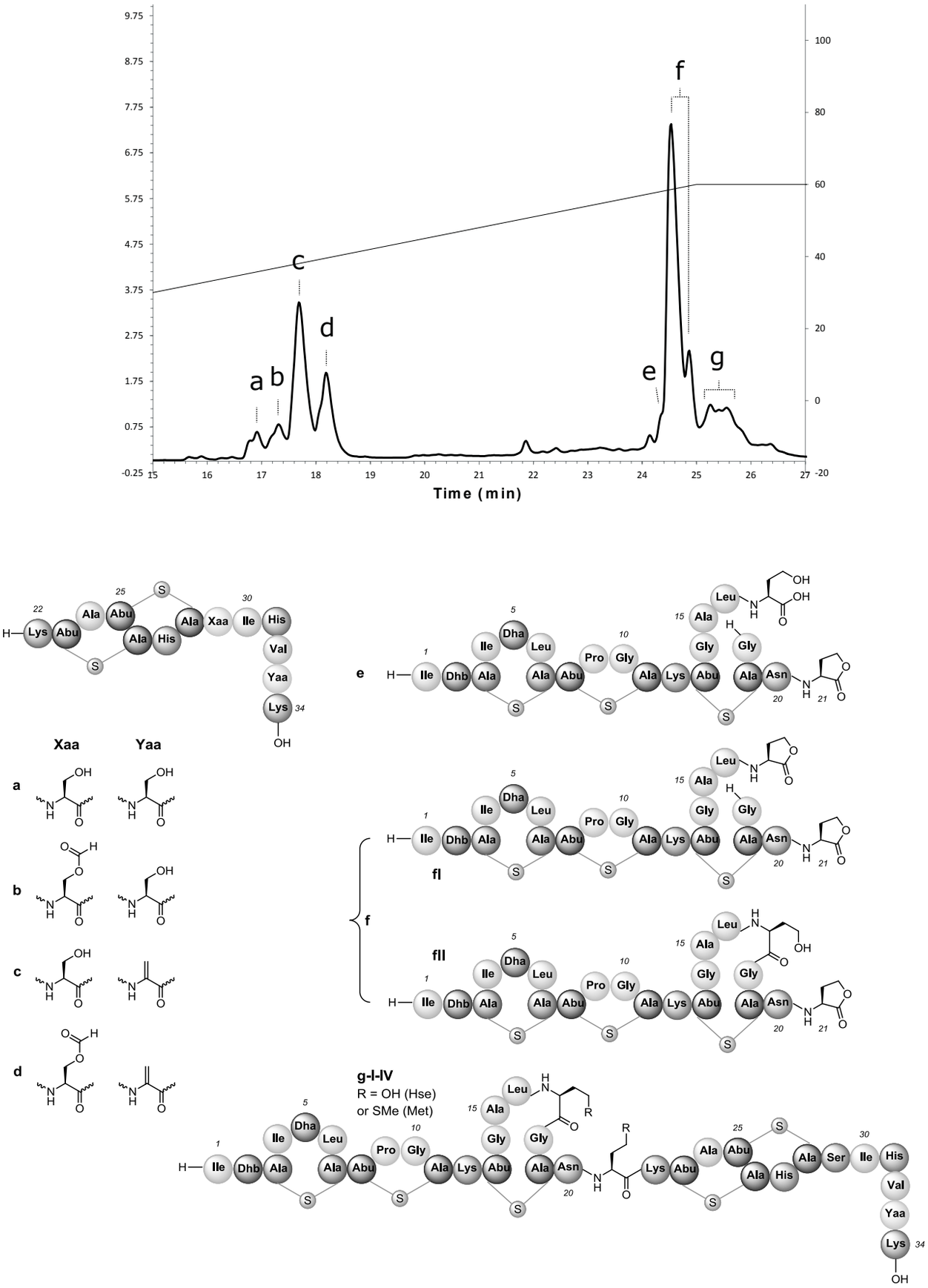
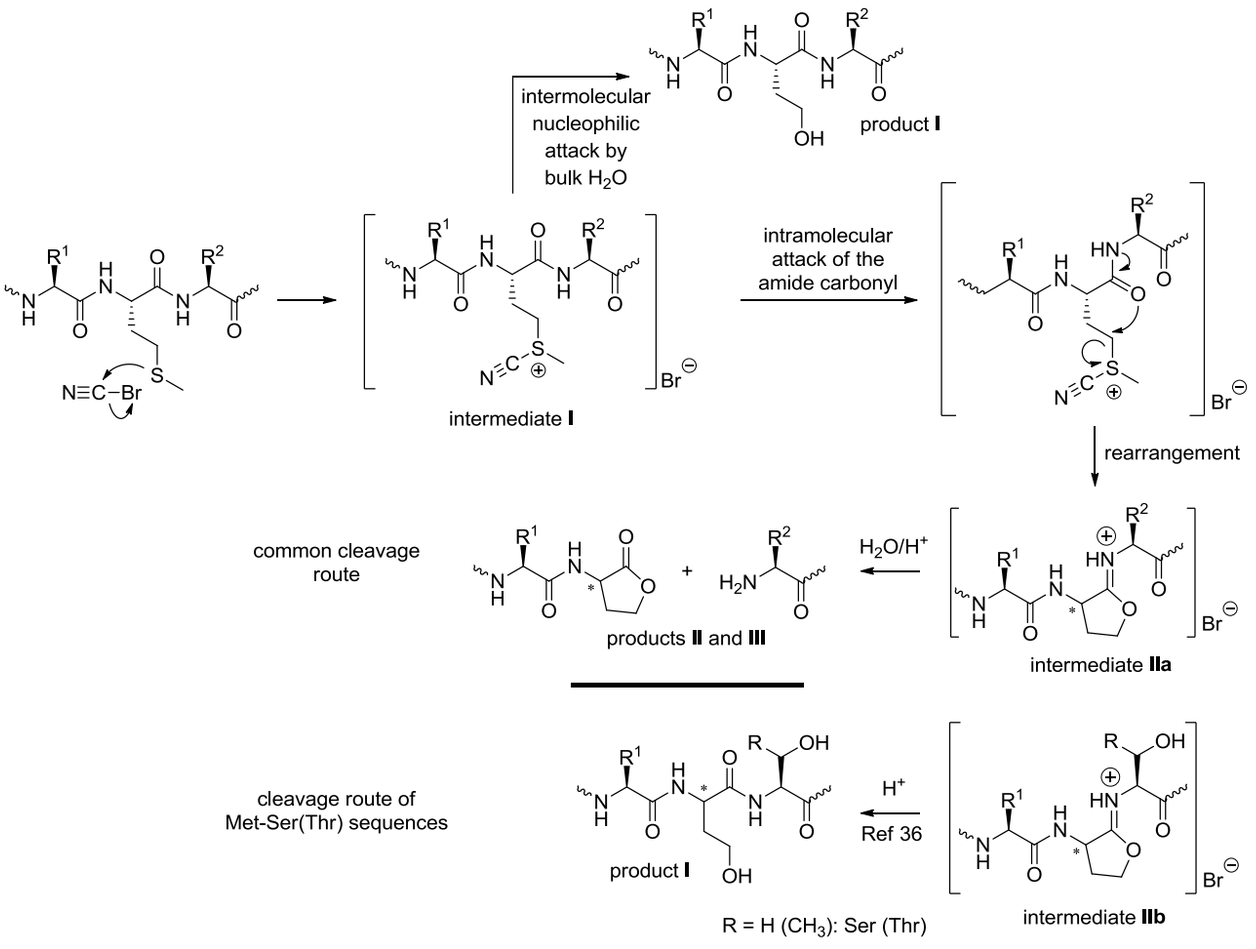


Figure 6. Analytical HPLC trace of cyanogen bromide cleavage of nisin after 18 h of incubation. The observed products (**a**, **b**, **c**, **d**, **e**, **f-I**, **f-II** and **g-I-IV**) were identified by LC-MS.



Scheme 1. CNBr cleavage mechanism and escape routes of methionine-containing peptides. Note: the asymmetric carbon atom (indicated with an asterisk) is relatively sensitive toward racemization, therefore the chirality is not defined.

3.2.4 Combining enzymatic and chemical cleavage

As discussed in the previous section, cyanogen bromide treatment of nisin resulted in a mixture of four DE-ring fragments (22-34). In an effort to isolate the truncated nisin DE-ring fragment (22-31), a combination of chymotrypsin digestion followed by cyanogen bromide treatment was tried. For this purpose, nisin was digested by chymotrypsin for 16 h to generate nisin-fragment (1-31). The enzyme was denaturated by acidification while nisin-fragment (1-31) was treated with cyanogen bromide in aqueous formic acid during 20 h. This two-step protocol resulted in the identification by analytical HPLC and LC-MS of C-terminally truncated nisin-fragment (22-31) and its subsequent isolation by preparative HPLC in a yield of 64%.

3.2.5 Cleavage sites within nisin

Figure 7 summarizes the cleavage sites within nisin. Three trypsin cleavage sites were identified, Lys12-Abu13, Asn20-Met21, and Lys22-Abu23, of which the Asn20-Met21 site has not been

described earlier. Chymotrypsin digestion was found to occur at Asn20-Met21, Met21-Lys22 and His31-Val32, respectively. The latter dipeptide sequence was preferentially recognized by chymotrypsin, while the Met21-Lys22 sequence was found to be an unprecedented cleavage site. Chemical degradation of nisin by cyanogen bromide at Met17-Gly18 and Met21-Lys22 sequences did not always result in peptide backbone cleavage since full length nisin derivatives containing Met/Hse interconversions were also found.

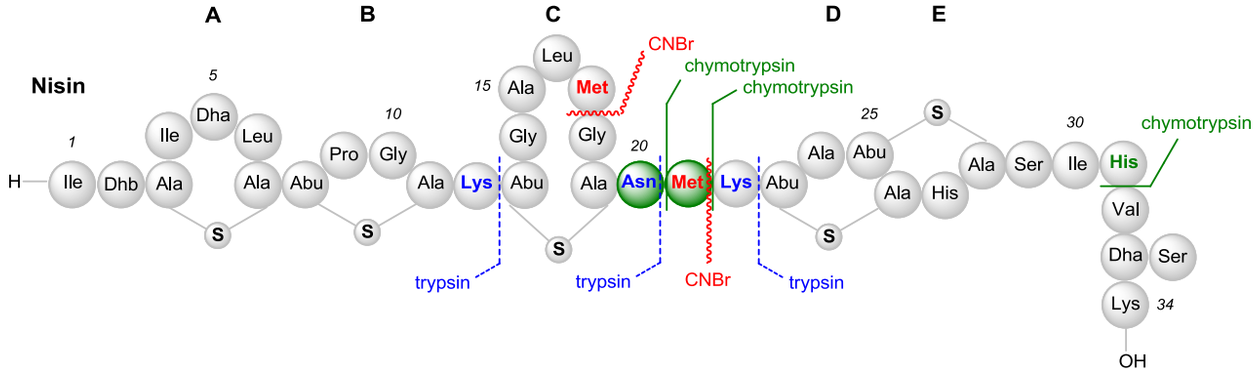


Figure 7. Cleavage sites of nisin for chymotrypsin (green), trypsin (blue) and CNBr (red).

3.3 Conclusions

Herein, an efficient and scalable purification method of nisin from commercially available nisin preparations was described. This method relied on an extraction/precipitation approach using a biphasic system resulting in an at least ten-fold enrichment of the nisin antimicrobial peptide. In the preparation of the desired nisin fragments, two unprecedented cleavage sites in nisin were found: the Asn20-Met21 sequence for trypsin, and the Met21-Lys22 sequence for chymotrypsin. The latter enzyme recognized within nisin preferentially the His31-Val32 sequence, resulting in a C-terminally truncated nisin derivative, which was only then cleaved by chymotrypsin at residues Asn20-Met21. Trypsinolysis of nisin conveniently led to the isolation of nisin-fragment (13-20), corresponding to the C-ring fragment, in contrast to its rather tedious chemical synthesis.[17] In this study the following nisin fragments were isolated and fully characterized, (1-12): AB-ring, (1-20): ABC-ring, (22-34): DE-ring, and (22-31): truncated DE-ring, as valuable building blocks for semi-synthesis to achieve improvement of the antimicrobial properties of newly engineering nisin-derived peptidomimetics.[18]

3.4 Experimental

3.4.1 Chemicals, instruments and general methods

Chymotrypsin, trypsin, cyanogen bromide (5.0 M in acetonitrile) and nisin (from *Lactococcus lactis*) were obtained from Sigma-Aldrich B.V. (Zwijndrecht, The Netherlands), CHRISIN® (a standardized nisin preparation)[9a] was obtained from Chr. Hansen A/S Hørsholm, Denmark) while all other chemicals and reagents were obtained from commercial sources and used without further purification, unless stated otherwise. HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Analytical HPLC was performed on a Shimadzu automated HPLC system equipped with a UV/vis detector operating at 220/254 nm. Preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/vis absorbance detector. The HPLC mobile phase consisted of buffer A (0.1% TFA in MeCN/H_2O 5:95 v/v) and buffer B (0.1% TFA in MeCN/H_2O 95:5 v/v). HPLC analyses were run at a flow rate of 1.0 mL/min with a linear gradient of buffer B (100% in 20 min) starting from 100% buffer A (total run time was 40 min): Method A, or with a linear gradient of buffer B (60% in 20 min) starting from 100% buffer A (total run time was 40 min): Method B, by using an Alltech C4 Prosphere column (pore size: 300 Å, particle size: 5 μm, 250 × 4.6 mm) or an Alltech C8 Alltima column (pore size: 300 Å, particle size: 5 μm, 250 × 4.6 mm). Preparative HPLC runs were performed at a flow rate of 12 mL/min with a linear gradient of buffer B (100% in 60 min) starting from 100% buffer A (total run time was 80 min) using an Alltech C4 Prosphere column (pore size: 300 Å, particle size: 10 μm, 250 × 22 mm). Peptides were characterized using electro-spray ionization mass spectrometry (ESI-MS) on a Shimadzu QP8000 single quadrupole bench top mass spectrometer in a positive ionization mode. LC-MS analyses were performed on a Thermo-Finnigan LCQ Deca XP Max ion trap mass spectrometer coupled to a Shimadzu analytical HPLC system. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS spectra were recorded on a Shimadzu Axima-CFR apparatus using α-cyano-4-hydroxycinnamic acid as the matrix, while human ACTH(18-39) was used as an external reference (monoisotopic $[M+H]^+$ 2465.198).

3.4.2 Synthesis

Scalable enrichment of nisin

CHRISIN® (40 g, containing approximately 1 g of nisin) was suspended in H_2O (1000 mL) and vigorously stirred for 15 min at room temperature before CH_2Cl_2 (800 mL) was added. Instantaneously, a copious white precipitate was formed at the interface of the yellowish colored aqueous layer and the colorless organic layer. The obtained emulsion was transferred to 50 mL tubes and centrifuged (15 min at 2500 rpm) after which the desired nisin peptide fraction formed a brownish solid pellet at the interface of both layers. The liquid was decanted and the peptide pellet

was dried at room temperature under reduced pressure to remove any residual solvent. The crude nisin peptide was subsequently redissolved in demineralized H_2O (500 mL) followed by filtration through a small column of Celite to remove traces of aggregated peptide and a colorless and clear solution was obtained, which was concentrated *in vacuo* to a final volume of approximately 150 mL followed by lyophilization. This nisin preparation was obtained as an off-white powder (850 mg) and used without further purification in the enzymatic digestion and chemical cleavage reactions. R_t = 20.77 min (C4 Prosphere, Method A), MALDI-TOF: calcd. $C_{143}H_{230}N_{42}O_{37}S_7$: 3351.55, found: *m/z* 3352.84 $[M+H]^+$, 3391.77 $[M+K]^+$.

Nisin-fragment (1-12)

Nisin (60 mg) was dissolved in an aqueous buffer (50 mL; 25 mM NaOAc, 5 mM TRIS-acetate, 5 mM $CaCl_2$, pH 7.0) and the solution was cooled on ice. Then, trypsin (5 mg) was added and the obtained reaction mixture was allowed to warm to room temperature. The enzymatic digestion was performed at 30 °C. To monitor the progress of the digestion, after 16 h, an aliquot was analyzed by HPLC and an extra amount of trypsin (5 mg) was added and incubated for another 24 h at 30 °C, the addition/incubation cycle was repeated once. After 64 h of digestion, the reaction mixture was acidified using aqueous 1 N HCl to pH 4 followed by the addition of MeCN (3 mL) to avoid excessive foaming while the solvents were removed by evaporation *in vacuo*. Finally, the concentrated digestion sample (5 mL, 10 mg 'nisin'/mL) was loaded onto the preparative HPLC column to isolate the nisin-fragment (1-12), which was obtained as a white fluffy powder (6.5 mg, 93 %). R_t = 23.93 min (C4 Prosphere, Method B), R_t = 24.02 min (C8 Alltima, Method B), ESI-MS calcd. $C_{51}H_{83}N_{13}O_{13}S_2$: 1149.57, found: *m/z* 1151.30 $[M+H]^+$, 1172.65 $[M+Na]^+$, 575.75 $[M+2H]^{2+}$.

Nisin-fragment (1-20)

Nisin (60 mg) was dissolved in a TRIS-acetate buffer (50 mL, 25 mM, pH 7.5) and the solution was cooled on ice. Then, α-chymotrypsin (5 mg) was added and the obtained reaction mixture was allowed to warm to room temperature. The enzymatic digestion, monitoring, and work-up were performed as decribed for nisin-fragment (1-12). The nisin-fragment (1-20) was isolated by preparative HPLC and obtained as a white fluffy powder (5 mg, 44 %). R_t = 24.85 min (C4 Prosphere, Method B), R_t = 24.63 min (C8 Alltima, Method B), ESI-MS calcd. $C_{80}H_{130}N_{22}O_{22}S_4$: 1878.86, found: *m/z* 1879.90 $[M+H]^+$, 940.45 $[M+2H]^{2+}$.

Nisin-fragment (22-34)

Nisin (50 mg) was dissolved in aqueous 70% formic acid (10 mL) and to this solution, CNBr (150 µL, as a 5 M solution in MeCN) was added and the obtained colorless reaction mixture was stirred

for 18 h at room temperature in the dark. The progress of the CNBr-induced cleavage was monitored by analytical HPLC. Then, the reaction mixture was diluted with MeCN (3 mL) and the solution was concentrated *in vacuo* to an end volume of approximately 1 mL. The evaporated amount of CNBr was quenched with an aqueous solution (4 M) of NaOH. The sample was diluted with H_2O/MeCN (4 mL, 3:1 v/v) and loaded onto the preparative HPLC column to isolate nisin-fragment (22-34) as a white fluffy powder (2 mg, 29%). R_t = 17.87 min (C4 Prosphere, Method B), R_t = 17.65 min (C8 Alltima, Method B), ESI-MS calcd. $C_{58}H_{93}N_{19}O_{15}S_2$: 1359.65, found: *m/z* 1360.00 $[M+H]^+$, 681.15 $[M+2H]^{2+}$, 454.05 $[M+3H]^{3+}$.

Nisin-fragment (22-31)

Nisin (130 mg) was dissolved in a TRIS-acetate buffer (70 mL, 25 mM, pH 7.5) and the solution was cooled on ice. Then, α-chymotrypsin (7.5 mg) was added was added and the obtained reaction mixture was allowed to warm to room temperature. The enzymatic digestion was performed at 30 °C. After 16 h of digestion, the reaction mixture was acidified using aqueous 1 N HCl to pH 4 followed by the addition of MeCN (3 mL) to avoid excessive foaming while the solvents were removed by evaporation *in vacuo* to obtain an end-volume of approximately 500 µL. Then, the solution was diluted with aqueous 70% formic acid (17.5 mL) and CNBr (300 µL, as a 5 M solution in MeCN) was added and the obtained reaction mixture was stirred for 18 h at room temperature in the dark. The work-up was identical as described for nisin-fragment (22-34). Nisin-fragment (22-31) was obtained as a white fluffy powder (9 mg, 64%). R_t = 15.75 min (C4 Prosphere, Method B), R_t = 15.97 min (C8 Alltima, Method B), ESI-MS calcd. $C_{44}H_{69}N_{15}O_{12}S_2$: 1063.47, found: *m/z* 1064.65 $[M+H]^+$, 533.10 $[M+2H]^{2+}$, 355.70 $[M+3H]^{3+}$.

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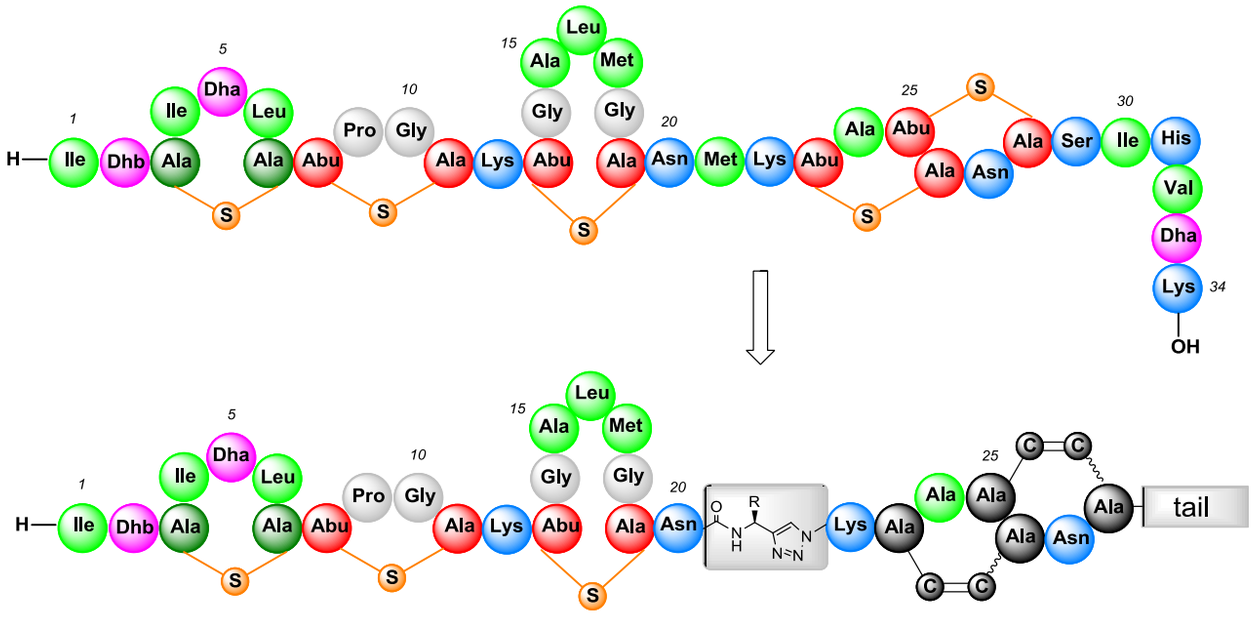
Chapter 4

Semi-synthesis of a biologically active nisin hybrid containing native lanthionines as well as a cross-stapled synthetic DE-fragment



Slootweg, J. C.; Quarles van Ufford, H.C.; Peters, N.; Breukink, E.; Liskamp, R. M. J.; Rijkers, D. T. S. *manuscript in preparation*.

4.1 Introduction

The lanthionine or thio ether moiety is a characteristic feature of the antimicrobial peptide nisin, since it is part of five cyclic constaints, namely the A-, B-, C- and the intertwined DE-ring systems, which are crucial for the bioactive conformation of nisin.[1] Lanthionine peptides, like nisin, subtilin, and lacticin 481, are synthetically accessible,[2] their synthesis however, especially of the cross-bridged DE-ring of nisin[3], is rather challenging.[4] Moreover, lanthionines are sensitive toward oxidation, and the oxidization of the sulfides to the corresponding sulfoxides is detrimental for activity since the antimicrobial activity of nisin is completely lost.[5]

It is known from the literature that thio ether bridges can be successfully mimicked by dicarba bridges. Such dicarba bridges can be conveniently incorporated in peptides via ring-closing metathesis (RCM)[6] and these carbon-carbon isosteres have been successfully applied by us[7] and others[8] to mimic lanthionines as metabolically stable analogs. Previously, it has been shown that dicarba analogs of the nisin AB(C)-ring fragments bind lipid II, although with a lower affinity than native nisin.[7d] However, the membrane interaction of dicarba DE-ring analogs could not be determined individually, since pore-formation of the C-terminus is a concerted action of the ABC-fragment and the DE-ring system. To determine the activity of the dicarba DE-ring analog a nisin hybrid was designed that contained the native nisin N-terminus comprising the ABC-fragment and a synthetic DE-ring construct in order to test its activity as a full-length hybrid. Thus, native nisin ABC fragments obtained via enzymatic digestion, were C-terminally modified with an alkyne moiety and subsequently chemo-selectively ligated to azide-functionalized dicarba DE-ring mimics.

For this approach, Cu(I)-catalyzed click chemistry[9] was chosen as the orthogonal ligation since it is a very efficient reaction at mild reaction conditions. This strategy will result in a nisin hybrid that contains a triazole moiety as an amide bond isostere in the peptide backbone of nisin at the so-called hinge region, an important position within the nisin sequence where the ABC- and DE-fragments are linked together. However, triazoles and peptide amide bonds have similar properties with respect to rigidity, size, and hydrogen bonding characteristics, and the replacement of a peptide amide bond by a triazole moiety has been studied previously, for instance, the synthesis of peptide foldamers,[10] and the fragment based synthesis of cystatin A[11], where the afforded triazole-analogs showed comparable bioactivities as their native counterparts. Herein, we report the synthesis of two nisin hybrids in combination with their biological activity using model membrane carboxyfluorescein leakage experiments and bacterial growth inhibition assays.

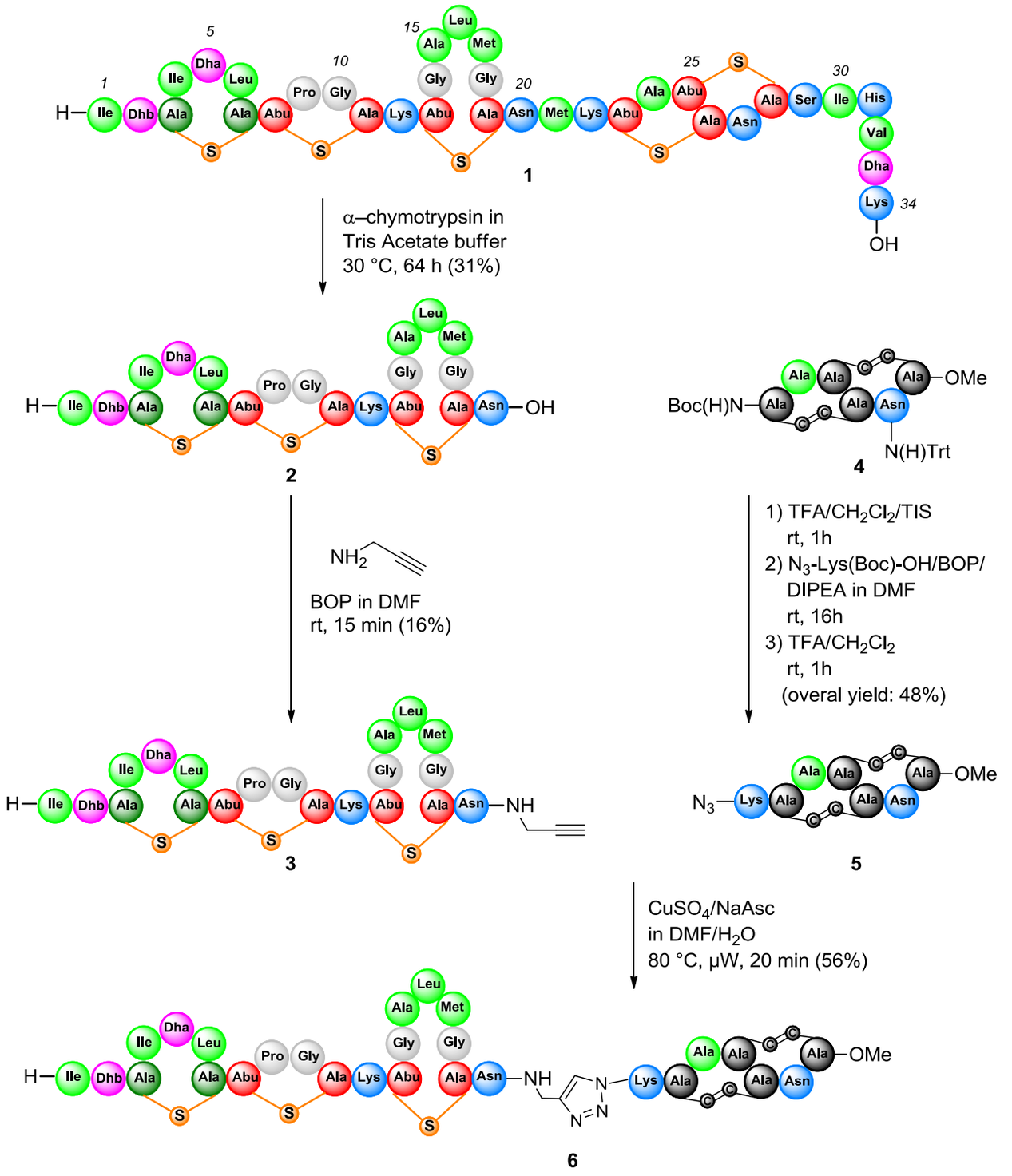
Figure 1. The triazole moiety as a peptide bond mimic. Picture adapted by permission from Valverde *et al.*[11], Copyright 2002, John Wiley and Sons, Angewandte Chemie International Edition.

4.2 Results and discussion

4.2.1 Synthesis of a hybrid nisin mimic

In first instance, nisin A was purified from commercially available Chrisin using the precipitation method as described in Chapter 3, to access the native nisin-ABC fragment (Scheme 1). Nisin (**1**) was treated with α-chymotrypsin and from this digestion mixture, nisin-fragment (1-20) **2** was isolated by preparative HPLC in 31% yield. Nisin-fragment (1-20) **2** has only one free carboxylic acid, thus it is expected that modification of the C-terminus with an amino alkyne would be rather straightforward, however, nisin rapidly degrades at conditions above pH 8. Taking this into account, it was decided to couple the unprotected nisin-fragment (1-20) **2** with relative inexpensive propargylamine, and to drive the reaction to completion in a relatively short reaction time, a large excess (100 equiv) was used. Thus, the C-terminus of nisin-fragment (1-20) **2** was treated with propargylamine in the presence of BOP as coupling reagent in DMF and the reaction mixture was quenched after 15 min by acidification. The C-terminally peptide alkyne was identified by LC-MS and isolated by preparative in 16% yield.

As the first attempt to synthesize a nisin hybrid, an azide-containing DE-ring dicarba analog was designed starting from DE diastereoisomer *E*/*E* **4** (as described in Chapter 2). TFA treatment of **4** removed the Boc as well as the trityl group, to yield the deprotected peptide which was directly coupled to α-azidolysine in the presence of BOP. After silica gel purification, followed by a final deprotection step and subsequently purification by preparative HPLC, azido-DE-ring **5** was obtained in a modest yield of 48%. As the last step in the synthesis, nisin (1-20)-alkyne **3** and azido-DE-ring **5** were chemo-selectively conjugated via Cu(I)-catalyzed click chemistry in the presence of $CuSO_4$ and sodium ascorbate in DMF/H_2O as the solvent system. Gratifyingly, the ligation proceeded very well and after preparative HPLC purification, nisin mimic **6** could be afforded in 56% yield.



Scheme 1. Synthesis route for nisin hybrid **6**.

4.2.2 Biological evaluation of nisin hybrid ***6***

The bioactivity of this nisin hybrid was tested in a model membrane experiment[12], to determine the ability of this nisin derivative to form pores like native nisin. For this purpose, large unilamellar vesicles (LUVs) composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and containing 0.2% of lipid II as the natural target of nisin were loaded with carboxyfluorescein (CF) as a fluorophore, and peptide-membrane interaction was measured by monitoring the release of CF by

fluorescence spectroscopy. It turned out that mimic **6** did not show any pore-formation activity up to 1000-fold higher concentration than native nisin (data not shown). However, mimic **6** was still able to bind lipid II, as shown in a competitive binding assay since it was able to compete with native nisin for the binding-site of lipid II.[7d] As a control, also nisin-fragment (1-20) **2** was tested, which is known to compete with native nisin in this experimental set-up. It was observed that mimic **6** was competitive to native nisin comparable to **2** (Figure 2), an indication that although mimic **6** had no pore-formation activity, the lipid II binding activity of the N-terminal part of mimic **6** was retained. Thus, although the synthesis of a nisin hybrid **6** was successfully performed, the C-terminal part (comprising the DE-rings) needed to be optimized in order to display pore-formation activity.

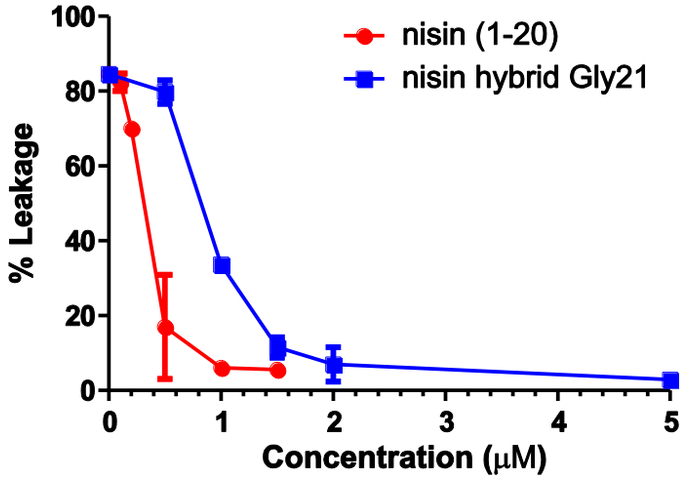


Figure 2. Overview of native nisin (1 nM) induced leakage in a competitive assay after incubation with different concentrations of nisin (1-20) fragment **2** (circles) and nisin hybrid **6 (**squares).

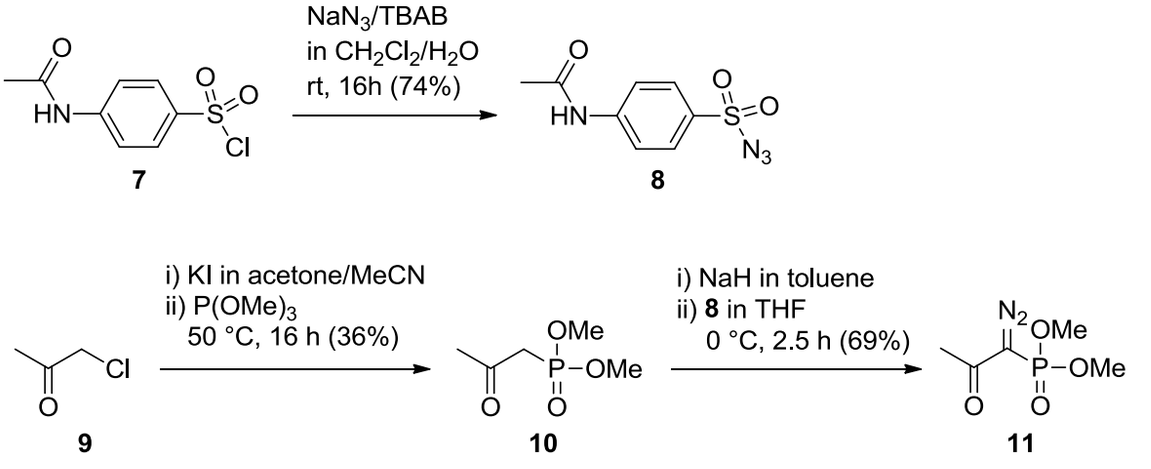
4.2.3 Optimization of the nisin hybrid

The synthetic DE fragment as used in nisin hybrid **6** represents nisin-fragment (22-28), while the sequence of native nisin is six residues longer: -Ser-Ile-His-Val-Dha-Lys-OH (nisin 29-34). Truncation studies of nisin showed that C-terminally truncated variants were slightly less active as an antimicrobial agent,[13] however some truncations led to a complete loss of pore-formation activity as was recently described for nisin-fragment (1-28).[14] This insight might explain the lack of pore-formation activity as found with nisin hybrid **6**. The sharp contrast between antimicrobial activity and absence of pore-formation is explained by the dual mode of action of nisin since nisin analogs without pore-formation activity still are bacteriostatic agents caused by lipid II binding and thus inhibiting the bacterial cell-wall synthesis.[15] Another lantibiotic, and a close family-member of nisin, subtilin, has a high structural homology as well as similar biological activity compared to nisin.[16] Paresot *et al.* showed that a truncated analog, subtilin (1-29), containing only Lys29 at its C-terminal sequence, displayed both antimicrobial and pore-formation activity.[17] Therefore, it was

decided to extend the synthetic DE-ring with a C-terminal lysine residue. The synthesis of the extended dicarba DE-ring **16** was described in Chapter 2.

Another modification that was introduced into nisin hybrid mimic **6** was the triazole moiety, which replaced the native peptide bond between Met21-Lys22. Since propargylamine was used as an amino-alkyne, an important hydrophobic side chain represented by Met21 in native nisin was also removed. This hydrophobic residue is however, conserved among the six known natural nisin variants and is represented either by methionine (nisin A, Z and F) or leucine (nisin Q, U and U2). Furthermore, structure-activity-relationship studies of nisin described the importance of this hydrophobic residue since a nisin analog with a Met21 to Gly21 mutation had no pore-formation activity.[18] These data suggested it was crucial to synthesize nisin analogs which include a hydrophobic residue on position 21 to retain pore-formation activity. For this purpose, methionine- and leucine-derived amino alkynes were synthesized via conversion of the corresponding amino aldehydes using the Bestmann-Ohira reagent.[19]

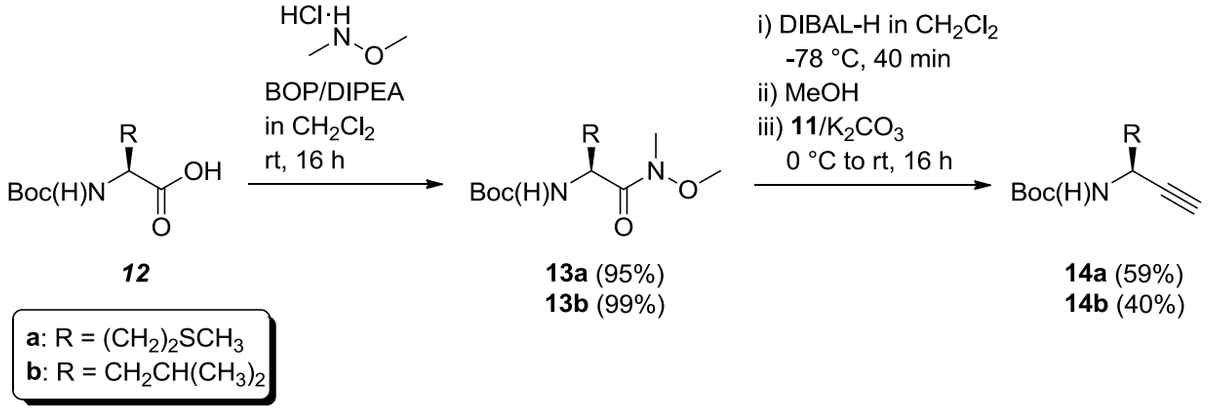
The Bestmann-Ohira reagent was synthesized according to a literature procedure[20] (Scheme 2) starting from sulfonyl chloride **7** which was transformed into sulfonyl azide **8** under phase-transfer conditions in a good yield (74%). Oxophosphonate **10** was synthesized by the in situ prepared iodoacetone from chloroacetone **9**, which reacted with trimethylphosphite via a Michaelis-Arbuzov reaction. Then, oxophosphonate **10** was deprotonated with sodium hydride and subsequently treated with sulfonyl azide **8** to give the Bestmann-Ohira reagent **11** after purification by silica gel chromatography in a reasonable yield (69%).



Scheme 2. Synthesis of Bestmann-Ohira reagent **11**.

The next steps started with Boc-protected methionine **12a** or leucine **12b**, to arrive at the corresponding Weinreb amides (**13a** and **13b)** via a BOP-mediated coupling of *N*,*O*,-dimethylhydroxylamine, in excellent yields (>95%) as shown in Scheme 3. Subsequently, the Weinreb amides were reduced to the corresponding Boc-protected amino aldehydes in the presence

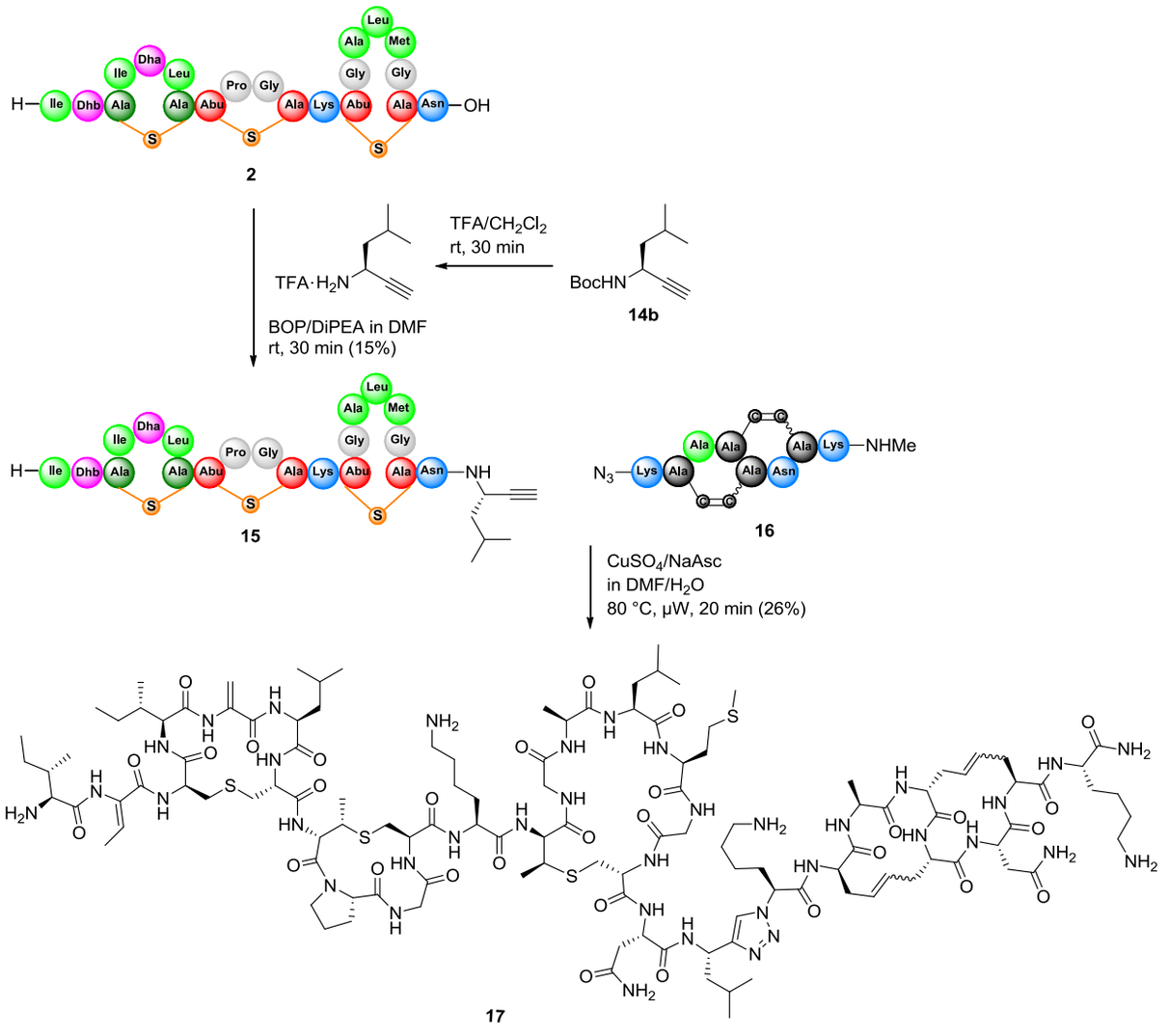
of DIBAL-H, immediately followed by a reaction with Bestmann-Ohira reagent **11**, affording methionine- and leucine-derived alkynes **14a** and **14b**, respectively, in yields between 40 to 59%. For practical reasons, it was decided to incorporate the leucine-derived alkyne **14b** into the native nisin ABC fragment.



Scheme 3. Synthesis of methionine and leucine derived amino alkynes **14a** and **14b**, respectively.

The synthesis of the optimized nisin hybrid, as shown in Scheme 4, started with a TFA treatment to remove the Boc group of amino alkyne **14b**, and after a simple workup, the amine was used in the BOP-mediated coupling step. Via a similar procedure as described to obtain alkyn **3**, the Leu-derived amino alkyne was coupled to nisin-fragment (1-20) **2** using an excess of only 25 equiv to avoid difficult purification of **15**. The coupling of Leu-derived amino alkyne proceeded rather slow compared to propargylamine and only trace amounts of alkyne **15** were observed after a reaction time of 15 min. Increasing the reaction time to 30 min did improve product formation and alkyne **15** could be isolated in an overall yield of 15%.

However, also the formation of a side product was observed. This side product corresponded to a molecule with a mass 18 amu lower than alkyne **15**. This side product could not explained by assuming lactam formation of α-amine/Lys12 and the C-terminal carboxylate. A more reasonable explanation would be a dehydration of the asparagine side chain. It is known from the literature that carbodiimide-based coupling reactions with unprotected asparagine residues result in dehydration of the amide of the side chain to the corresponding cyano moiety. It was expected that BOP-mediated couplings were devoid of the side reaction since the highly activated intermediate was transferred into the mildly active hydroxybenzotriazole ester. However, a report by Gausepohl *et al.*[21] showed that also BOP-mediated couplings result in partial dehydration of asparagine, which might explain the presence of this side product. Finally, alkyne **15** was coupled via Cu(I)-catalyzed click chemistry to azide **16** as the optimized DE-ring mimic and nisin hybrid **17** could be isolated by preparative HPLC in a yield of 26% and high purity, as shown in Scheme 4 and Figure 3.



Scheme 4. Synthesis route for nisin hybrid **17**.

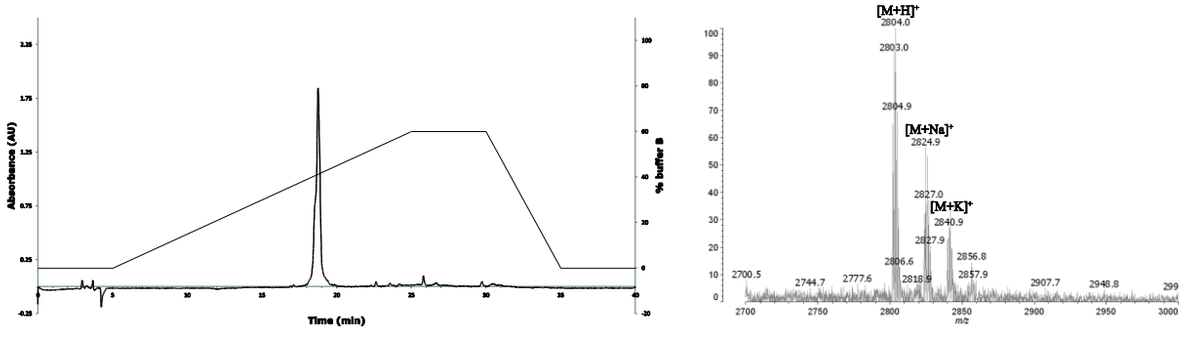


Figure 3. Analytical HPLC trace and MALDI-TOF mass spectrum of nisin hybrid **17**.

*4.2.4 Biological evaluation of nisin hybrid **17***

To investigate the bioactivity of nisin hybrid **17**, it was tested for pore-formation activity as well as in a growth inhibition assay. Unfortunately, nisin hybrid **17** did not show any pore-formation activity, an indication that the improved design did not lead to a functional C-terminal mimic.

Apparently, incorporation of the hydrophobic leucine residue at position 21 in combination with Lys29 was still not sufficient for mimickry. The triazole moiety that replaced the amide bond between Met21-Lys22 within the so called hinge region (Asn20-Met21-Lys22) of nisin, is a crucial motif for pore-formation activity, as demonstrated by mutation studies by Wiedemann et al.[30] They showed that changing Met21 into Gly (nisin M21G) or Pro (nisin M21P), resulted in a complete loss of pore-forming activity. Interestingly, the antimicrobial activity (or in vivo activity) of the nisin M21G and M21P mutants, as determined as the minimum inhibitory concentration (MIC), was approximately three-fold lower than nisin.

Therefore, native nisin and nisin hybrid **17** were tested in a growth inhibition assay in the presence of *Bacillus subtilis* and the observed MIC values were 0.62 μM and 4.07 μM, respectively. An indication that, although nisin hybrid **17** did not have pore-formation activity, the in vivo activity was reduced by a factor <10. This was a promising result since its activity was in the same range as previously shown for truncated nisin (1-29) and subtilin (1-29) peptides with a similar size as nisin hybrid **17**.[25,29] Moreover, Chan et al. showed that –compared to nisin– shorter nisin fragments, e.g. nisin (1-20), will lose their activity, approximately 50-100 fold, while nisin (1-12) was ~250 less active.[25] As a control experiment, nisin fragments (1-12) as well as (1-20) (see Chapter 3) were tested for their potency to inhibit the growth of *Bacillus subtilis*. Their MIC values were determined and found to be respectively 50 μM, an ~80-fold reduction in activity, and 6.2 μM, which is only a factor 10 less active than native nisin. This means that the activity of nisin-fragment (1-20) and nisin hybrid **17** were comparable, a strong indication that the synthetic C-terminal part in the hybrid did not contribute to the overall biological activity.

Table 1. Biological activities of nisin hybrid **17** compared to native nisin and native nisin fragments.

Peptide	**MIC (μM)**[a]	**Pore-formation**
	B. subtilis	**activity**[b]
Nisin	0.62	+
nisin hybrid Leu21 **17**	4.07	-
Nisin 1-12	50.0	-
Nisin 1-20	6.20	-

[a]Antimicrobial activity is expressed as the minimal inhibitory concentration (MIC).

[b]Activity in model membrane system measuring peptide induced CF leakage.

4.3 Conclusions

A versatile synthesis route to incorporate synthetic nisin DE-ring dicarba analogs to obtain a full-length nisin hybrid via semi-synthesis was developed. For this approach, native nisin-fragment (1-

20) was obtained by enzymatic digestion of nisin A and subsequently C-terminally functionalized with two different amino alkynes, propargylamine and a leucine-derived amino alkyne. By using Cu(I)-catalyzed click chemistry, two nisin hybrids, **6** and **17**, were synthesized and their bioactivity was evaluated in a model membrane leakage experiment and a bacterial growth inhibition assay. The first generation nisin hybrid **6** as well as the improved nisin hybrid **17** did not show activity in the model membrane experiment, an implication that the synthetic C-terminal part was not a functional mimic of the C-terminal DE-ring of nisin. However, hybrid **6** was an active competitive binder of lipid II and inhibited nisin-induced pore formation. Bacterial growth inhibition experiments showed that the hybrid **17** was approximately 8-fold less active than nisin. However, this activity was comparable with the N-terminal ABC-fragment (nisin 1-20) suggesting that the in vivo activity of the improved mimic was most likely to reside within the functional N-terminal part. Since, two modifications were introduced into the hybrid mimic, the triazole moiety as well as the dicarba bridges, it cannot be concluded which modification was responsible for the loss of pore-formation. In the outlook, at the end of this thesis (see page 146), some alternative derivatives to mimic full length nisin are proposed to answer the question to what extend each modification is responsible for the observed reduction in bioactivity.

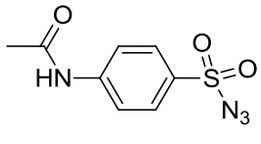
4.4 Experimental

4.4.1 Chemicals, instruments and general methods

Unless stated otherwise, all chemicals were obtained from commercial sources and used without further purification. Piperidine, *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), *tert*-butyl methyl ester (MTBE), trifluoroacetic acid (TFA), and HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and were used as obtained. DMF, NMP, and CH_2Cl_2 were dried on molecular sieves (4 Å) prior to use. Coupling reagent, benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP), and *N*-α-9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids were purchased from GL Biochem Ltd (Shanghai, China). Triisopropylsilane (TIS) was obtained from Merck (Darmstadt, Germany). Solution phase reactions were monitored by TLC on Merck precoated silica gel 60F254 glass plates. Spots were visualized by UV light, ninhydrin and Cl_2/TDM. Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40-63 μm). ^{1}H NMR data were acquired on a Varian Mercury 300 MHz apparatus with $CDCl_3$ as solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm) or DMSO-d_6 (2.50 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), and broad (br). ^{13}C-NMR data were acquired on a Varian Mercury 75 MHz apparatus with $CDCl_3$

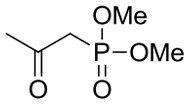
or DMSO-d_6 as solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm) and DMSO-d_6 (39.5 ppm). Analytical HPLC was performed on a Shimadzu automated HPLC system equipped with a UV/vis detector operating at 220/254 nm. Preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/vis absorbance detector. The buffer system for HPLC consisted of buffer A: 0.1% TFA in MeCN/H_2O 5:95 v/v, and buffer B: 0.1% TFA in MeCN/H_2O 95:5 v/v. For analytical HPLC a flow rate of 1.0 mL/min with a linear gradient of buffer B (100% in 20 min) from 100% buffer A (Method A) or a linear gradient of buffer B (60% in 20 min) from 100% buffer A (Method B), were used with a total run time of 40 min using an Alltech C4 Prosphere column (pore size: 300 Å, particle size: 5 µm, 250 × 4.6 mm) or an Alltech C18 Prosphere column (pore size: 300 Å, particle size: 5 µm, 250 × 4.6 mm). Preparative HPLC was run with a flow rate of 12 mL/min using a linear gradient of buffer B (100% in 60 min) from 100% buffer A with a total runtime of 80 min by using an Alltech C4 Prosphere column (pore size: 300 Å, particle size: 10 µm, 250 × 22 mm). Semi-preparative HPLC runs used a flow rate of 5 mL/min with a linear gradient of buffer B (100% in 60 min) from 100% buffer A with a total runtime of 80 min by using an Alltech C18 Prosphere column (pore size: 100 Å, particle size: 10 µm, 250 × 10 mm). Peptides were characterized using electrospray ionization mass spectrometry (ESI-MS) on a Shimadzy QP8000 single quadrupole mass spectrometer in a positive ionization mode. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra were measured on a Shimadzu Axima-CFR apparatus using α-cyano-4-hydroxycinnamic acid as the matrix, while human ACTH(18-39) was used as an external reference (monoisotopic $[M+H]^+$ 2465.198).

4.4.2 Synthesis



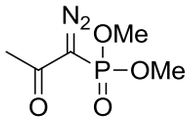
_p_-Acetamidobenzenesulfonyl azide 8

p-Acetamidobenzenesulfonyl chloride **7** (46.8 g, 200 mmol) was dissolved in CH_2Cl_2 (350 mL) and the obtained solution was stirred at room temperature. After the addition of *tert*-butyl ammonium bromide (140 mg, 0.50 mmol) as a phase-transfer catalyst, a solution of sodium azide (20 g, 307 mmol) in water (100 mL) was added. The obtained bi-phasic reaction mixture was vigorously stirred at room temperature overnight. Then, the organic layer was separated and subsequently washed with water (3 × 60 mL), dried (Na_2SO_4), filtered, and evaporated *in vacuo*. Azide **8** was obtained in 61% yield (29.5 g). R*f* 0.24 (CH_2Cl_2/acetone 95:5 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.23 (s, 3H, $COCH_3$), 7.85 (m, 4H, arom CH), 8.41 (s, 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 24.6, 119.6, 128.8, 132.2, 144.1, 169.4.



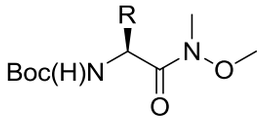
Dimethyl 2-oxopropylphosphate 10

To a stirred suspension of KI (33.2 g, 200 mmol) in acetone/MeCN (120 mL, 3:4 v/v), chloroacetone **9** (15.9 mL, 200 mmol) was added and the mixture was stirred for 50 min at room temperature. Then, trimethylphosphite (23.6 mL, 200 mmol) was added and the obtained suspension was stirred overnight at room temperature. The reaction mixture was heated to 50 °C, and stirring was continued for 1 h to ensure complete conversion. Subsequently, The suspension was filtered over Celite and the residue was rinsed with acetone (2 × 20 mL). The filtrate was concentrated under reduced pressure and the residue was purified by vacuum distillation (product fraction was collected at 101 °C at 3.5 mbar). Ketone **10** was obtained as a colorless oil in 36% yield (12.0 g). R_f 0.65 (CH_2Cl_2/MeOH 9:1 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.32 (s, 3H, $COCH_3$), 3.11 (d ($J_{H,P}$ = 22.8 Hz), 2H, CH_2), 3.80 (d ($J_{H,P}$ = 11.2 Hz), 6H, OCH_3); ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 31.0, 41.0, 42.7, 52.7/52.8, 199.4 (two lines).



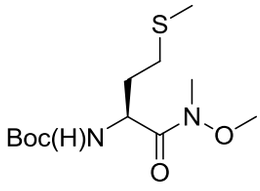
Dimethyl 1-diazo-2-oxopropylphosphate (Bestmann-Ohira reagent) 11

Ketone **10** (11.8 g, 70.9 mmol) was dissolved in toluene (70 mL) and this solution was cooled to 0 °C (ice-bath), after which NaH (2.95 g, 65.5 mmol) was added to the mixture portion-wise. To this cooled (0 °C) mixture, a solution of *p*-acetamidobenzenesulfonyl azide **8** (15.8 g, 65.5 mmol) in THF (23 mL) was added drop-wise. Initially, a highly viscous yellow suspension was obtained and the reaction mixture was stirred for 2.5 h at 0 °C. Then, the reaction mixture was allowed to warm up to room temperature and subsequently diluted with Et_2O (100 mL). The suspension was filtered over Celite and the filtrate was evaporated *in vacuo*. Finally, the residue was purified by silica column chromatography (EtOAc/hexane 1:1 v/v) to afford diazo compound **11** as a colorless oil in 69% yield (9.40 g). R_f 0.74 (CH_2Cl_2/MeOH 9:1 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 2.28 (s, 3H, $COCH_3$), 3.85 (d ($J_{H,P}$ = 11.9 Hz), 6H, OCH_3).



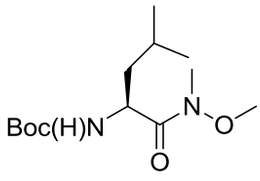
General procedure for the synthesis of Weinreb amides 13ab.

The corresponding Boc-protected amino acid **12** (18 mmol) was dissolved in CH_2Cl_2 (120 mL). To this solution BOP (7.97 g, 18 mmol) and DIPEA (6.12 mL, 36 mmol) were added, followed by the addition of *N*,*O*-dimethylhydroxylamine hydrochloride (6.12 mL, 18 mmol) and the obtained reaction mixture was stiired at room temperature overnight. Then, the solvent were removed *in vacuo*, and the residue was redissolved in EtOAc (200 mL) and this solution was subsequently washed with aq. 1N $KHSO_4$ (2 × 120 mL), aq. 1N $NaHCO_3$ (2 × 120 mL), and brine (1 × 100 mL). After drying (Na_2SO_4), filtration, and evaporation of the solvent *in vacuo*, the corresponding Weinreb amides **13ab** were obtained as colorless oils in high yield and purity.



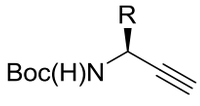
Boc-Met-Weinreb amide (13a)

Yield: 95% (5.00 g); R_f 0.38 (CH_2Cl_2/acetone 95:5 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 1.44 (s, 9H, $(CH_3)_3$ Boc), 1.68-2.05 (m, 2H, βCH_2), 2.10 (s, 3H, SCH_3), 2.40-2.75 (m, 2H, γCH_2), 3.22 (s, 3H, NCH_3), 3.79 (s, 3H, OCH_3), 4.69-4.89 (m, 1H, αCH), 5.27 (d (J = 8.7 Hz), 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 15.4, 23.1, 28.3, 30.1, 32.2, 32.4, 49.8, 61.6, 79.6, 95.4, 155.5, 172.6, 174.4.



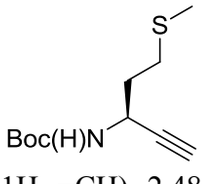
Boc-Leu-Weinreb amide (13b)

Yield: 99% (4.89 g); R_f 0.52 (CH_2Cl_2/acetone 95:5 v/v); ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 0.95 (dd (J_{gem} = 10.6 Hz, J_{vic} = 6.6 Hz), 6H, (δCH_3/δ'CH_3), 1.57-1.44 (s, 9H, $(CH_3)_3$ Boc), 1.45 (m, 2H, βCH_2), x.xx (m, 1H, γCH), 3.20 (s, 3H, NCH_3), 3.79 (s, 3H, OCH_3), 4.72 (m, 1H, αCH), 5.12 (d (J = 9.1 Hz), 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 21.5, 23.2, 24.6, 28.2, 32.0, 41.9, 48.8, 61.42, 79.3, 155.5, 173.8.



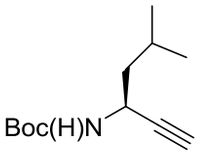
General procedure for the synthesis of the amino alkynes 14a and 14b

The corresponding Weinreb amide **13** (10.7 mmol) was dissolved in CH_2Cl_2 (80 mL) and the solution was cooled to -78 °C on a dry ice/acetone bath. A solution of DIBAL-H (1M in CH_2Cl_2, 12.8 mL, 12.8 mmol) was added drop-wise and the obtained reaction mixture was stirred for 30 min. Then, the reaction mixture was quenched by adding MeOH (21 mL) and the mixture was allowed to warm up to 0 °C by means of an ice bath. Subsequently, solid K_2CO_3 (3.00 g, 21.4 mmol) followed by a solution of Bestmann-Ohira reagent (**11**) in CH_2Cl_2 (2.41 g in 20 mL, 12.6 mmol) were added and the reaction mixture was stirred 1 h at 0 °C followed by 16 h at room temperature. Finally, the reaction mixture was filtrated over Celite and the residue was rinsed with CH_2Cl_2/MeOH (8:2 v/v, 2 × 20 mL) and the filtrate was concentrated *in vacuo* and the residue was purified by silica column chromatography (CH_2Cl_2/hexane 6:4 v/v) and the corresponding alkynes **14ab** were obtained as colorless oil in a yield of 40 to 59%.



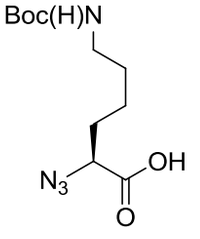
Boc-Met alkyne 14a

Yield: 59% (1.45 g); R_f 0.52 (CH_2Cl_2); ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 1.45 (s, 9H, $(CH_3)_3$ Boc), 1.95 (m, 2H, βCH_2), 2.12 (s, 3H, SCH_3), 2.31 (d (J = 1.8 Hz), 1H, ≡CH), 2.48-2.79 (m, 2H, γCH_2), 4.56 (m, 1H, αCH), 4.84 (broad s, 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 15.5, 28.3, 30.0, 35.4, 42.5, 71.7, 80.1, 82.7, 154.7.



Boc-Leu alkyne 14b

Yield: 40% (0.90 g), R_f 0.60 (CH_2Cl_2), ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 0.94 (dd (J_{gem} = 6.6, J_{vic} = 2.8 Hz), 6H, δCH_3/δ'CH_3), 1.44 (s, 9H, $(CH_3)_3$ Boc), 1.52 (m, 2H, γCH/βCH_2 (1H)), 1.78 (m, 1H, βCH_2 (1H)), 2.25 (d (J = 2.2 Hz), 1H, ≡CH), 4.43 (m, 1H, αCH), 4.63 (broad s, 1H, NH); ^{13}C-NMR (75 MHz, $CDCl_3$) δ = 21.9, 22.7, 25.0, 28.4, 41.3, 45.2, 70.7, 79.9, 83.8, 154.8.



N_3-Lys(Boc)-OH

Commercially available H-Lys(Boc)-OH (2.46 g, 10 mmol) was converted into its corresponding azide via a diazotransfer in the presence of imidazole-1-sulfonyl azide hydrochloride (2.49 g, 12 mmol, 1.2 equiv), $CuSO_4 \cdot 5H_2O$ (25 mg, 0.1 mmol, 0.01 equiv), and K_2CO_3 (3.04 g, 27 mmol, 2.7 equiv) in MeOH (60 mL) according to the procedure of Goddard-Boger and Stick.[22] After completion of the reaction, the reaction mixture was concentrated under reduced pressure and the residue was partitioned between EtOAc (50 mL) and aq. 1N $KHSO_4$ (50 mL). Subsequently, the aqueous phase was extracted with EtOAc (2 × 50 mL) and the combined organic layers were washed with aq. sat. NaCl (40 mL), dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) to give N_3-Lys(Boc)-OH as a colorless oil in quantitative yield (2.72 g). R_f 0.25 (CH_2Cl_2/MeOH 97:3 v/v); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 1.28-1.38 (m, 13H, γCH_2/δCH_2 (2 × 2H)/$(CH_3)_3$ Boc (9H)), 1.52-1.76 (m, 2H, βCH_2), 2.88 (dt (J_{vic} = 6.3 Hz, J_{gem} = 6.1 Hz), 2H, εCH_2), 4.01 (m, 1H, αCH Lys), 4.75 (broad t (J = 5.4 Hz), 1H, NH urethane); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 22.7, 28.3, 29.0, 30.6, 39.6, 61.4, 77.4, 155.6, 171.9.

Azido-DE-Ring 5

Bicyclic peptide **4**, as its *E*/*E* isomer (compound **2d** Chapter 2, 23 mg, 26 μmol) was dissolved in CH_2Cl_2/TFA (3 mL, 1:1 v/v) and to the bright yellow solution, TIPS (30 μL) was added as a scavenger, after which the reaction mixture turned into a slightly brownish reaction mixture. After stirring for 1 h at room temperature, MeOH (3 mL) was added and the reaction mixture was evaporated to dryness and the crude residue was coevaporated with MeOH (3 × 3 mL) and $CHCl_3$ (3 × 3 mL) to remove any residual TFA. Then, the corresponding peptide amine was dissolved in DMF (2 mL) and to this solution, N_3-Lys(Boc)-OH (8.7 mg, 32 μmol), BOP (14.1 mg, 32 μmol), followed by DIPEA (28 μL, 0.16 mmol, 5 equiv) were added, and the reaction mixture was stirred for 16 h at room temperature. Subsequently, the volatiles were removed by evaporation, and the residue was purified by column chromatography (CH_2Cl_2/MeOH 8:2 v/v) and the pure fractions

were combined and subsequently evaporated to dryness. The resulting compound was dissolved in TFA/CH_2Cl_2 (2 mL, 1:1 v/v) and the obtained reaction mixture was stirred for 1 h at room temperature followed by concentration *in vacuo*. The residual oil was triturated with MTBE/hexane (10 mL, 1:1 v/v) and the solid peptide was collected by centrifugation (5 min at 3500 rpm) and the resulting pellet was resuspended in MTBE/hexane (10 mL, 1:1 v/v) and centrifuged. The crude peptide was dissolved in 0.1% TFA in CH_3CN/H_2O (3 mL, 95:5 v/v) and purified by preparative HPLC to give peptide **xx** as a white fluffy powder in 48% overall yield (12 mg). R_t = 15.37 min (Alltech C18 Prosphere, Method A); ESI-MS calcd $C_{30}H_{45}N_{11}O_9$ 703.34, found *m/z* $[M+H]^+$ 704.05, $[2M+H]^+$ 1407.00, $[2M+Na]^+$ 1430.25.

Nisin-fragment (1-20) 2

Nisin A (370 mg, 37.5 μmol) was dissolved in a Tris-acetate buffer (250 mL, 25 mM, pH 7.5) and the solution was cooled on ice. Then, α-chymotrypsin (37 mg) was added and the obtained reaction mixture was allowed to warm to room temperature. The enzymatic digestion was performed at 30 °C. After 16 h of incubation, an aliquot was analysed by HPLC to monitor the progress of the digestion, and an extra amount of trypsin (18 mg) was added and the digestion was continued for another 24 h at 30 °C. The addition/incubation cycle was repeated once. After 64 h of digestion, the reaction mixture was acidified with aq. 1N HCl to pH 4 and MeCN (3 mL) was added to avoid excessive foaming while the solvents were removed by evaporation *in vacuo*. Finally, the concentrated digestion sample was diluted with a mixture of H_2O/MeCN/TFA (20 mL, 60:40:1 v/v/v), centrifuged (5 min at 12,000 rpm) and the supernatant was loaded (in 5 mL portions) onto the preparative HPLC column to isolate the nisin-fragment (1-20) **2**, as a white fluffy powder in 31% yield (22 mg). R_t = 24.87 min (Alltech C4 Prosphere, Method B); ESI-MS calcd $C_{80}H_{130}N_{22}O_{22}S_{44}$ 1879.86, found *m/z* $[M+H]^+$ 1879.00, $[M+2H]^{2+}$ 940.45.

ABC-alkyne 3

Nisin (1-20) fragment **2** (5.8 mg, 3.1 μmol) was dissolved in DMF (50 μL). To this solution, BOP (1.5 mg, 3.4 μmol) and propargylamine (23 μL, 341 μmol) were added and the obtained reaction mixture was vortexed for 15 min at room temperature. The reaction mixture was acidified by the addition of TFA (100 μL) and after removal of the volatiles by evaporation, the residue was dissolved in buffer A (2 mL) and purified by semi-prep-HPLC. The peptide alkyne was obtained as a white fluffy powder in 16% yield (1.3 mg). R_t = 19.36 min (Alltech C18 Prosphere, Method A); ESI-MS calcd $C_{80}H_{130}N_{22}O_{22}S_{44}$ 1916.89, found *m/z* $[M+H]^+$ 1816.5, $[M+Na]^+$ 1939.5, $[M+K]^+$ 1955.5.

ABC-Leu-alkyne 15

The procedure started with the deprotection of Boc-Leu-alkyne **14b**. Boc-Leu-alkyne **14b** (66.6 mg, 315 μmol) was dissolved in TFA/CH_2Cl_2 (2 mL, 1:1 v/v) and the solution was stirred for 30 min at room temperature. The mixture was subsequently concentrated *in vacuo* and the residue was taken up in aq. 1N $NaHCO_3$ (3 mL) and the free amine was extracted with Et_2O (3 × 3 mL) and the combined organic layers were evaporated to dryness. Native nisin (1-20) fragment **2** (12.0 mg, 6.3 μmol) was dissolved in DMF (100 μL). To this solution the amino-alkyne followed by BOP (3.1 mg, 7.0 μmol) and DIPEA (2.6 μL, 15 μM) were added, and the obtained reaction mixture was vortexed for 40 min at room temperature. The mixture was acidified with aq. 1N HCl to pH 4 and diluted with buffer A (2 mL). The peptide alkyne **15** was isolated by prep-HPLC (C4 Prosphere) as a white fluffy powder in 15% yield (1.9 mg). R_t = 26.62 min (Alltech C4 Prosphere, Method B); ESI-MS calcd $C_{87}H_{141}N_{23}O_{21}S_4$ 1971.96, found *m/z* $[M+H]^+$ 1972.4, $[M+Na]^+$ 1995.4, $[M+K]^+$ 2011.4.

General procedure for the synthesis of nisin hybrid 6 and 17 via click chemistry.

Peptide alkyne **3** or **15** (0.42 μmol) and azide **5** or **16** (0.50 μmol) were dissolved in DMF (20 μL). An aqueous solution of $CuSO_4 \cdot 5H_2O$ (50 μL (0.80 μmol Cu^{2+}), 9.0 mg (36.0 μmol) $CuSO_4 \cdot 5H_2O$/2.25 mL H_2O) followed by solid sodium ascorbate (0.5 mg, 2.5 μmol) and H_2O (500 μL) were added and the Cu(I)-click reaction was performed at 80 °C under microwave irradiation for 20 min. The solvents were evaporated *in vacuo*, and the residue was redissolved in H_2O/MeCN/TFA (1 mL, 75:25:0.1 v/v/v) and purified by semi-preparative HPLC (C18 Prosphere). After lyophilization, nisin hybrid **6** and **17** were obtained as white fluffy powders.

Nisin hybrid 6

Yield 54% (0.6 mg); R_t = 18.95 min (Alltech C18 Prosphere, Method A); MALDI-TOF MS calcd $C_{113}H_{178}N_{34}O_{30}S_4$ 2620.24, found *m/z* $[M+H]^+$ 2621.6.

Nisin hybrid 17

Yield 26% (0.3 mg); R_t = 18.73 min (Alltech C4 Prosphere, Method B); MALDI-TOF MS calcd $C_{123}H_{199}N_{37}O_{30}S_4$ 2803.41, found *m/z* $[M+H]^+$ 2804.0, $[M+Na]^+$ 2824.9.

4.4.3 Biological evaluation

Vesicle leakage experiments

Carboxyfluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment according to a literature procedure.[24] The peptide-induced

leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA). A solution (1.0 mL) of CF-loaded vesicles (20 μM final concentration) in buffer (50 mM Tris/HCl pH 7.0, 100 mM NaCl) was added to a quartz cuvette and fluorescence was measured (A_0). The % of peptide-induced leakage was calculated by: $((A_{60}-A_0)/(A_{Total})-A_0))$ x 100%. All measurements were performed in duplo.

Standard assay. After 20 s, a buffer solution (1 μL) containing the peptide of interest (from a freshly prepared stock solution: 1 μM, final: 1 nM) was added and peptide-induced membrane leakage was followed during 60 s (A_{60}), after which a buffer solution (10 μL) of Triton-X (stock: 20%, final 0.2%) was added to induce total leakage of the vesicles (A_{Total}).

Competitive assay. The peptide of interest in buffer solution (5 μM final concentration) was added and fluorescence was measured (A_0). After 20 s, a buffer solution (1 μL) containing nisin (from a freshly prepared stock solution: 1 μM, final: 1 nM) was added and peptide-induced membrane leakage was followed during 60 s (A_{60}), after which a buffer solution (10 μL) of Triton-X (stock: 20%, final 0.2%) was added to induce total leakage of the vesicles (A_{Total}).

Growth inhibition assay

A *Bacillus subtilis* strain was used for determination of the antimicrobial activity. The MIC value of each peptide was determined using a broth micro-dilution assay adapted from a literature procedure as previously described by Hancock. Peptide stock solutions were prepared at a concentration of 100 μM peptide in 0.2% bovine serum albumin (BSA) and 0.01% acetic acid. Serial three-fold solutions of peptide were made in 0.2% BSA and 0.01% acetic acid. To each well was added, 50 μL of the test bacteria in tryptic soya broth to a final concentration of 2×10^6 CFU/mL and 50 μL of the peptide at different concentrations. After incubation for 24 h at 37 °C and shaken at 120 rpm in a Certomat incubator, the OD at 630 nm was measured. The MIC value (expressed in μM) of each peptide was read as the lowest concentration of peptide that inhibited visible growth of bacteria. All measurements were performed in duplo.

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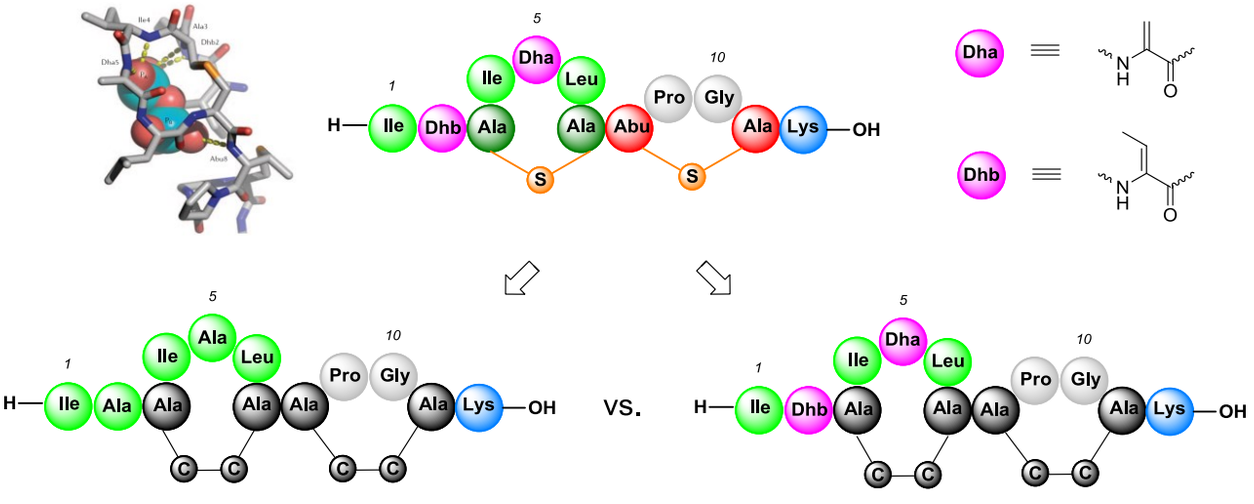
Chapter 5

Synthesis of nisin AB dicarba analogs using ring-closing metathesis: Influence of backbone C-alpha hybridization on bioactivity



Slootweg, J. C.; van Herwerden, E. F.; van Doremalen, M. D.; Breukink, E.; Liskamp, R. M. J.; Rijkers, D. T. S. *manuscript in preparation*

5.1 Introduction

Nisin belongs to the class of lantibiotics, a family of antimicrobial peptides that is ribosomally synthesized by Gram-positive bacteria to act against competing microorganisms. A common structural characteristic of lantibiotics is the presence of the amino acid lanthionine, which give these peptides their structural and conformational stability. The lanthionine moiety contains a sulfide bridge that is formed after a series of enzyme-catalyzed post-translational modifications, like dehydration of serine and threonine residues followed by the stereoselective intramolecular Michael addition of the sulfhydryl of an adjacent cysteine residue to result in a D-configuration of the newly formed stereocenter.[1]

The N-terminal part of nisin comprising the AB(C) ring system which is of particular interest with respect to its structure and binding properties of lipid II. Nisin has a specific interaction with lipid II, an important bacterial component for cell-wall synthesis as a cross-linking entity to give the peptidoglycan layer mechanical strength.[2] The structure of the complex between nisin–lipid II has been solved by NMR spectroscopy (Figure 1).[3] It was shown that the N-terminal AB-ring system of nisin interacts with the pyrophosphate moiety of lipid II, in which the AB-ring system forms a so called pyrophosphate cage. The antimicrobial activity of nisin is twofold, inhibition of the bacterial cell-wall synthesis and permeabilization of the bacterial membrane by pore-formation. In both cases the interaction between nisin and lipid II plays a crucial role. Moreover, it was shown that a truncated nisin analog (nisin (1-12)) containing only the AB-ring system, displayed bacteriostatic activity,[4] an indication that nisin-AB and other lantibiotics with a comparable N-terminus hold great promise as novel peptide antibiotics.[5]

Although nisin is widely used in dairy products as a food preservative, it is unstable at neutral or basic pH and readily reacts with water or thiol-containing nucleophiles.[6] Moreover, the lanthionine moieties are sensitive toward oxidation, resulting in a loss of activity.[7] Previously, a program started to synthesize nisin analogs by ring-closing metathesis[8], where the native lanthionines were replaced by dicarba bridges, to increase metabolic stability and potency.[9] In the literature several studies describe the successful replacement of the thio ether bridges by dicarba analogs. However, their activity is generally lower compared to the native analogs.[9d] The reduced activity could be a result of replacing the lanthionines by dicarba bonds as described for Lacticin 3147 dicarba analogues[10], since there is a slight difference in the bridge topology, with respect to the ring size and -structure. However, particularly for the nisin AB dicarba mimics, dehydro residues dehydrobutyrine and dehydroalanine were replaced by L-alanine for practical reasons, which could also be an explanation for the reduced activity. As apparent from the NMR structure of the nisin–lipid II complex the interaction is dominated by hydrogen bonding of the backbone amides of nisin with the pyrophosphate moiety (Figure 1). This suggests a strong influence of the

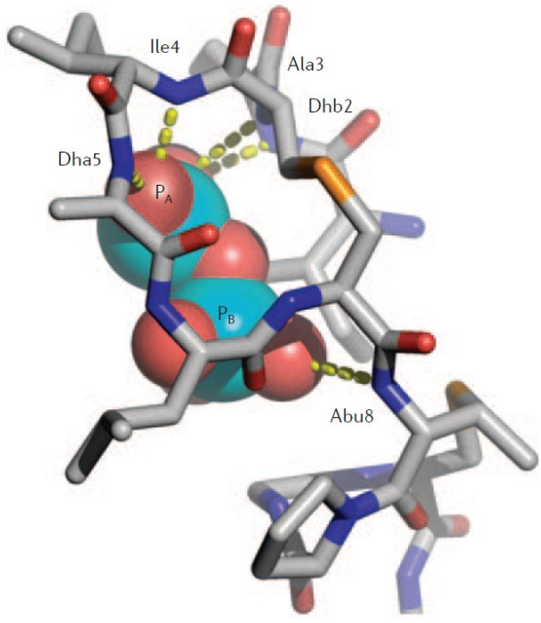


Figure 1. NMR structure of the nisin-lipid II complex. The interaction is dominated by hydrogen bonding of the backbone amide protons of Dhb2, Ala3, Ile4, Dha5 and Abu8 to pyrophosphate moiety of lipid II. Reprinted by permission from[2d]. Copyright 2006 Nature Publishing Group, Nature Reviews Drug Discovery.

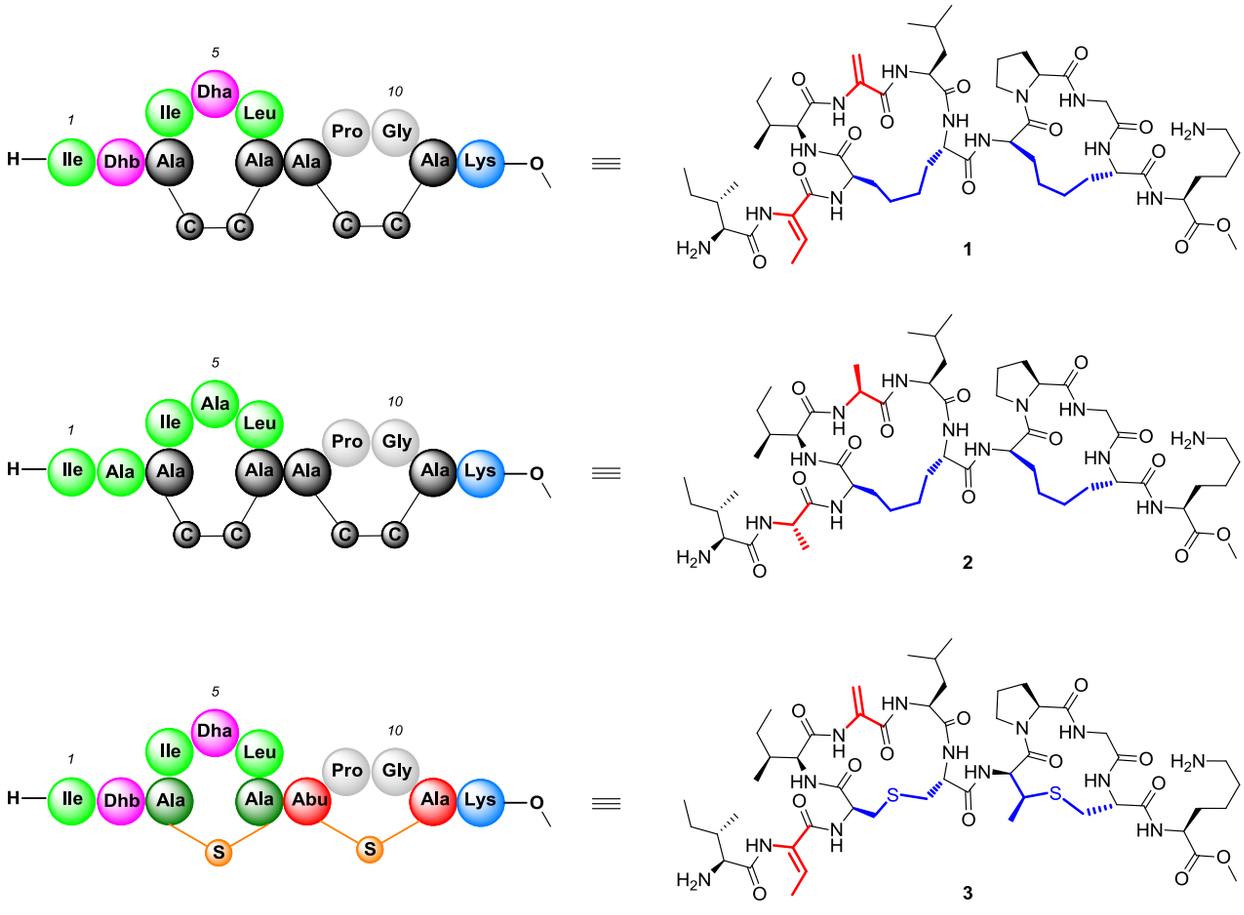


Figure 2. Chemical structures of nisin AB dicarba dehydro **1**, nisin AB dicarba Ala **2** and native nisin AB-OMe **3**.

backbone conformation of nisin for the affinity toward lipid II. The dehydro residues contain a sp^2 hybridized Cα carbon atom in contrast to the sp^3 hybridized Cα carbon atom of alanine, which might have important structural implications on the peptide backbone in terms of three-dimensional orientation and rigidity.

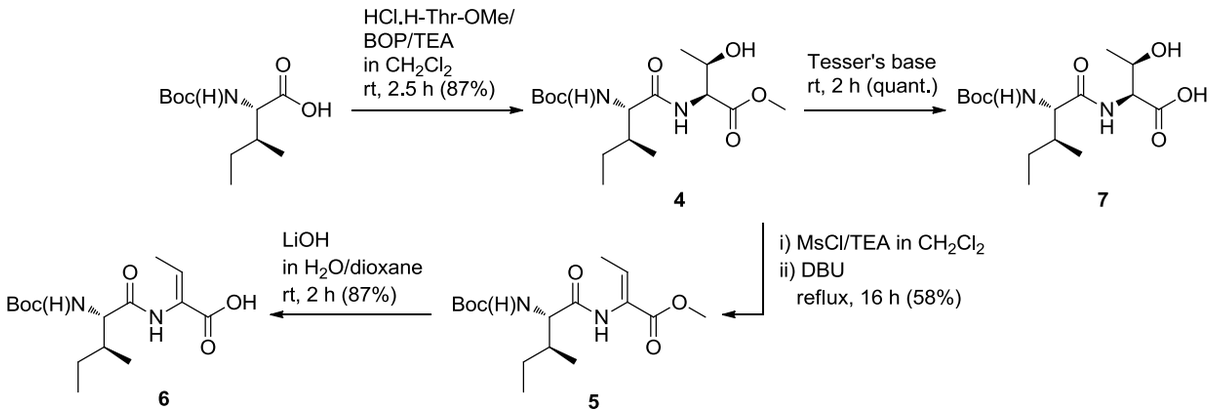
Although the presence of dehydro residues at position 2 and 5 is common in related lantibiotics with a similar AB-ring motif, this feature is, however, not conserved among the class of lantibiotics, for instance, epidermin[11] and gallidermin[12] do not have dehydro residues at position 2 and 5, they have been replaced by Ala/Phe, respectively. This suggests that the presence of an sp^2 hybridized Cα carbon atom at position 2 and 5 is not necessarily essential, however it may be a tool to improve the activity of the AB dicarba analogs. Moreover, to the best of our knowledge, a direct comparison between the binding affinities of an AB-ring motif for lipid II, with or without the presence of dehydro residues, has not been reported in the literature. Therefore, two nisin AB dicarba analogs, Dhb2/Dha5 (**1**) and Ala2/Ala5 (**2**), were synthesized to study their affinity toward lipid II in comparison with native nisin AB (**3**) (Figure 2).

5.2 Results and Discussion

5.2.1 Synthesis

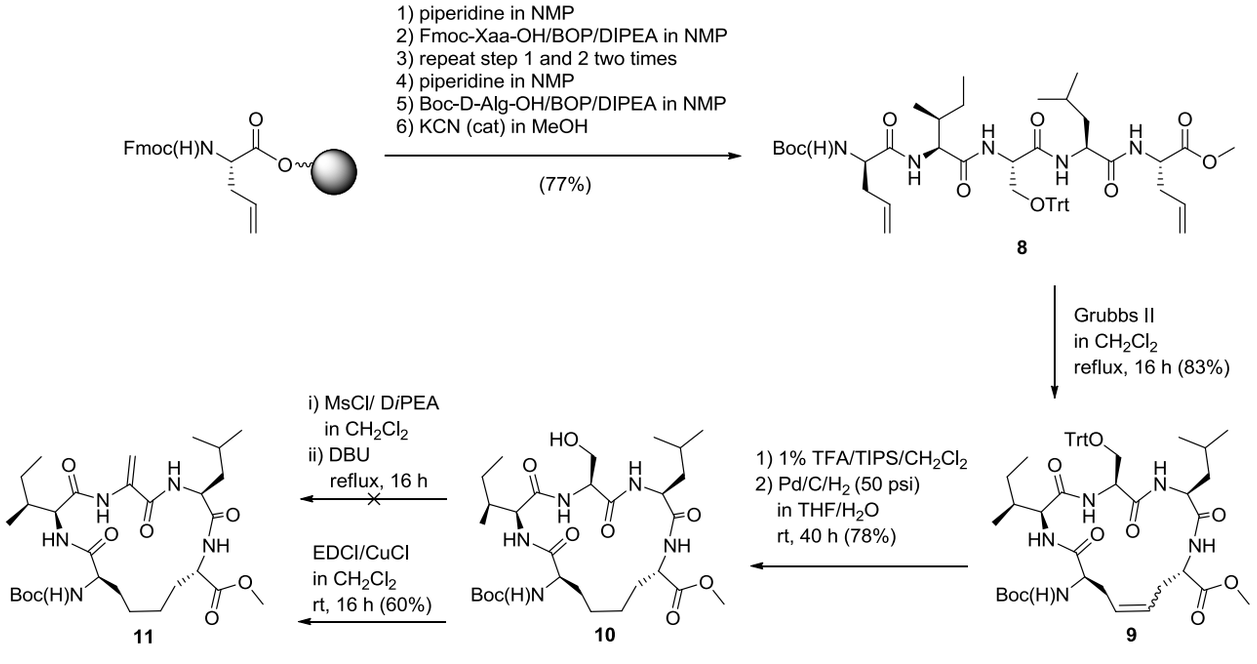
It was decided to synthesize the nisin AB dicarba analogs via three key intermediate fragments, namely an N-terminal dipeptide, ring-fragment A and ring-fragment B, and the nisin AB dicarba analogs were assembled via fragment condensation. The synthesis of the dipeptide **6** started with Boc-Ile-OH which was coupled to H-Thr-OMe to obtain dipeptide Boc-Ile-Thr-OMe **4** in a good yield (87%), as shown in Scheme 1. Then, the side chain hydroxy functionality of Boc-Ile-Thr-OMe was treated with mesyl chloride in the presence of base to generate in situ the corresponding mesylate, followed by β-elimination by adding DBU at reflux conditions in dichloromethane to yield dehydrobutyrine derivative **5** (58%). Subsequently, saponification of methyl ester **5** in the presence of lithium hydroxide provided Boc-Ile-Dhb-OH **6** (87%), which was ready for fragment condensation.

For the synthesis of ring-fragment A, in first instance linear pentapeptide **8** (Boc-D-Alg^{1}-Ile^{2}-Ser(Trt)^{3}-Leu^{4}-Alg^{5}-OMe) was synthesized via Fmoc/^{t}Bu SPPS similarly as previously described (Scheme 2).[9d] Ring-closing metathesis of **8** afforded alkene bridged cyclopeptide **9** as a mixture of two diastereoisomers (*E*/*Z*) in a good yield (83%). Then, efforts to convert diastereoisomers **9** as the trityl protected peptide, into the corresponding alkane-bridged peptide in the presence of H_2 and Pd/C as catalyst were unsuccessful, even when longer reaction times were applied, or at higher H_2 pressure (50 psi) and high catalyst loadings.



Scheme 1. Synthesis route for Boc-Ile-Dhb-OH **6**.

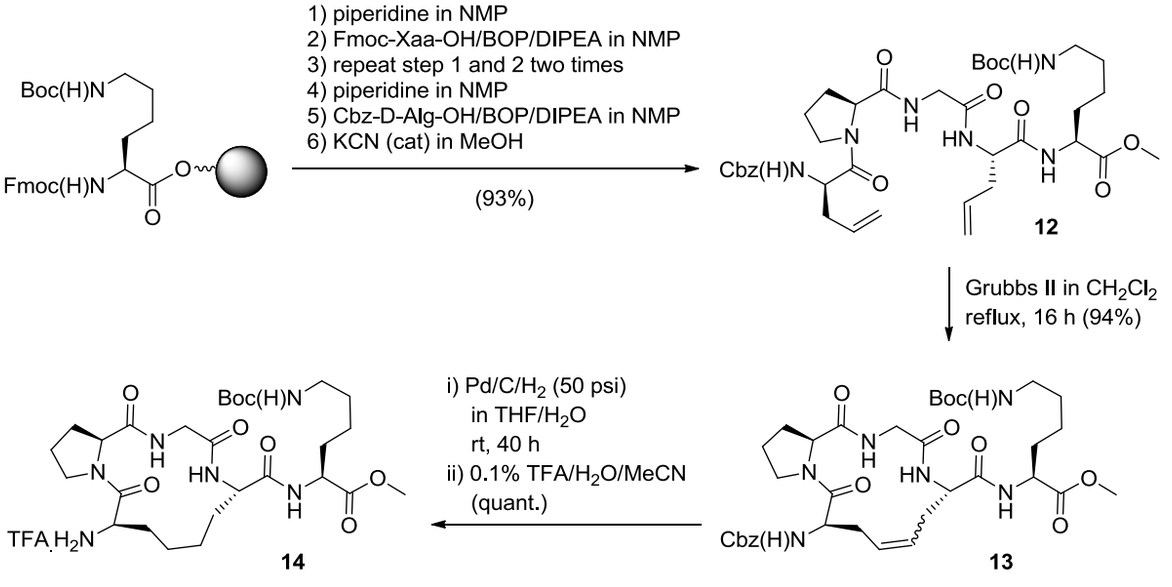
It was found that the bulkiness of the trityl group was somehow hindering hydrogenation and needed to be removed before reducing the alkene-moiety. Hence, removal of the trityl group using mild acid cleavage conditions as the first step was followed by a simple work-up, and reduction of the carbon-carbon double bond proceeded relatively easy in the presence of H_2 (50 psi) and Pd/C as a catalyst, and alkane-bridged ring-A **10** was isolated after column chromatography in a good yield (78%). Similarly as described for the synthesis of dipeptide **4**, cyclic peptide **10** was treated with mesyl chloride/DBU to synthesize dehydropeptide **11**. By this approach, only trace amounts of dehydropeptide **11** were observed, together with starting material and degradation products.



Scheme 2. Synthesis route for cyclo[1-5]-Boc-D-Alg^{1}-Ile^{2}-Dha^{3}-Leu^{4}-Alg^{5}-OMe **11**.

Optimization of the β-elimination reaction by changing reaction conditions, like temperature, reaction time, and equivalents of reagents did not result in dehydropeptide formation. Fortunately, by switching to another known method using EDCI and copper(I)-chloride,[13] dehydration was achieved and dehydropeptide **11** was isolated after preparative HPLC purification in 60% yield, as shown in Scheme 2.

The synthesis of ring-fragment B **14** started with the preparation of the linear pentapeptide Cbz-D-Alg[1]-Pro[2]-Gly[3]-Alg[4]-Lys(Boc)[5]-OMe **12** by SPPS as previously described and shown in Scheme 3. Ring-closing metathesis of linear peptide **12** in the presence of second generation Grubbs catalyst[14] in dichloromethane at reflux conditions gave the alkene-bridged peptide **13** as an *E*/*Z* diastereoisomeric mixture in an excellent yield (94%) after column chromatography. Then, alkene-bridged peptide **13** was treated with H_2 (at 50 psi) in a Parr apparatus with Pd/C as catalyst to reduce the alkene and to remove the N-terminal Cbz group in a single step, and after a work-up, ring-fragment B **14** was obtained as its corresponding trifluoro acetate in a quantitative yield. With the three required building blocks in hands, dipeptide **6**, ring-fragment A **11** and ring-fragment B **14**, the endgame of the fragment assembly via solution phase synthesis to obtain Dhb2/Dha5 nisin-AB dicarba analog **1** had begun.

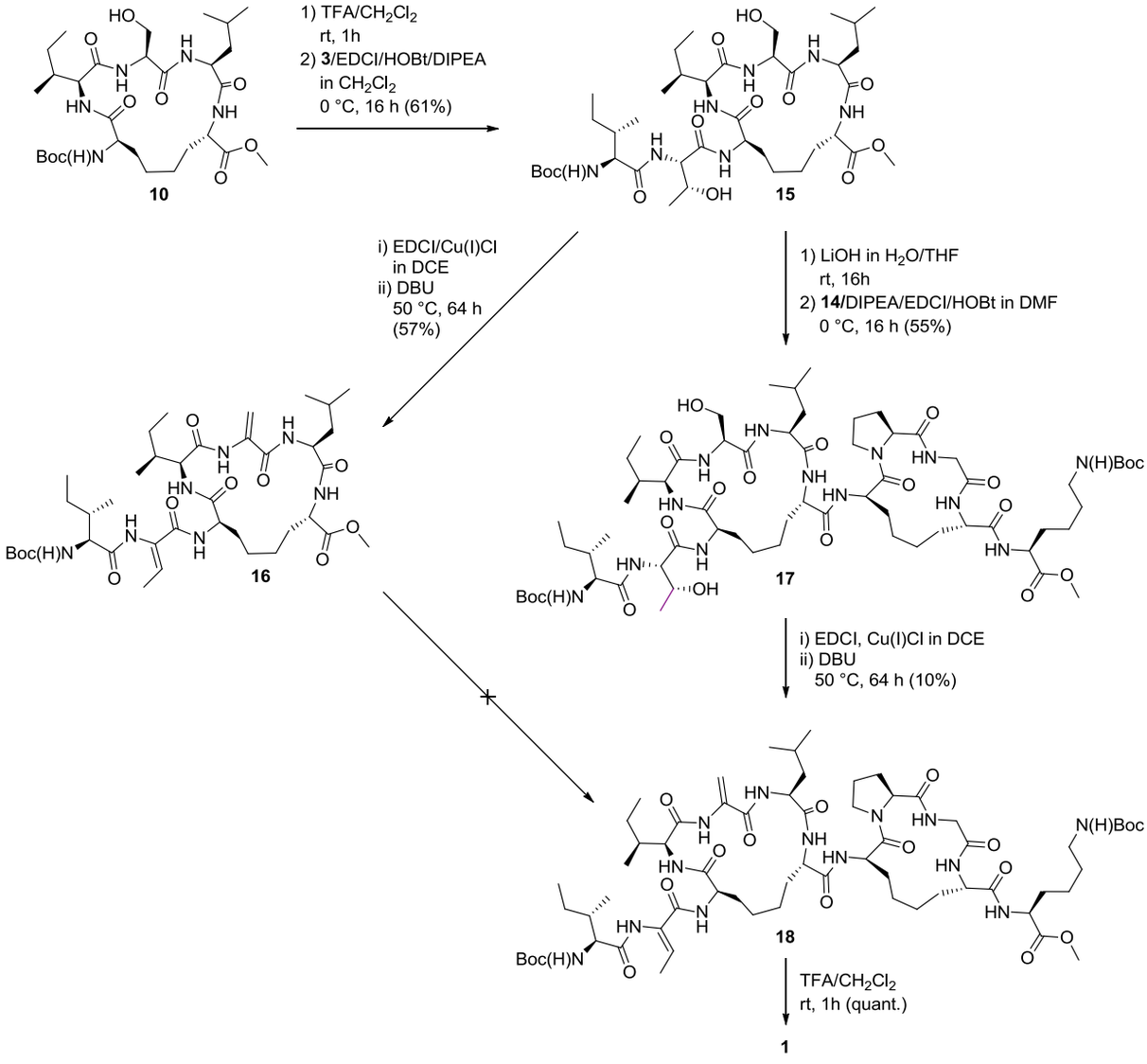


Scheme 3. Synthesis route for cyclo[1-4]-H-D-Alg[1]-Pro[2]-Gly[3]-Alg[4]-Lys(Boc)[5]-OMe.TFA **14**.

The reliable syntheses of a variety of peptides containing dehydro amino acids have been described in the literature.[15] Nevertheless, a test reaction to investigate the compatibility of the Michael acceptor in dipeptide **6** with amide bond formation reactions, was performed with H-Val-OMe and BOP/DIPEA or EDCI/HOBt as coupling reagents. Unexpectedly, under these conditions the starting materials were predominantly converted into Michael adduct rather than the desired

tripeptide Boc-Ile-Dhb-Val-OMe (data not shown). Apparently, upon activation of the carboxylic acid, the Michael acceptor became more reactive due to the electron-withdrawing effect induced during peptide bond formation. For this reason, it was decided to introduce the dehydrobutyrine moiety at a later stage in the synthesis to avoid peptide couplings at a C-terminal dehydro amino acid. Therefore, Boc-Ile-Thr-OMe **4** was saponified by Tesser's base affording Boc-Ile-Thr-OH **7**, which was used for the next steps (Scheme 1). As a result of this change in synthesis strategy, it was also decided to couple peptide **10**, with an unprotected serine residue, to dipeptide **7**. This enabled a double dehydration in one single synthesis step.

To this end, the N-terminal Boc group of cyclic peptide **10** was removed by treatment with TFA and after adding DIPEA, the free amine was coupled to dipeptide **7** in the presence of EDCI/HOBt as coupling reagents to gave peptide **15** in 61% yield after preparative HPLC (Scheme 4). Then, peptide **15** was subjected to dehydration conditions using EDCI/CuCl in dichloromethane.



Scheme 4. Synthesis route for AB dicarba dehydro **1**

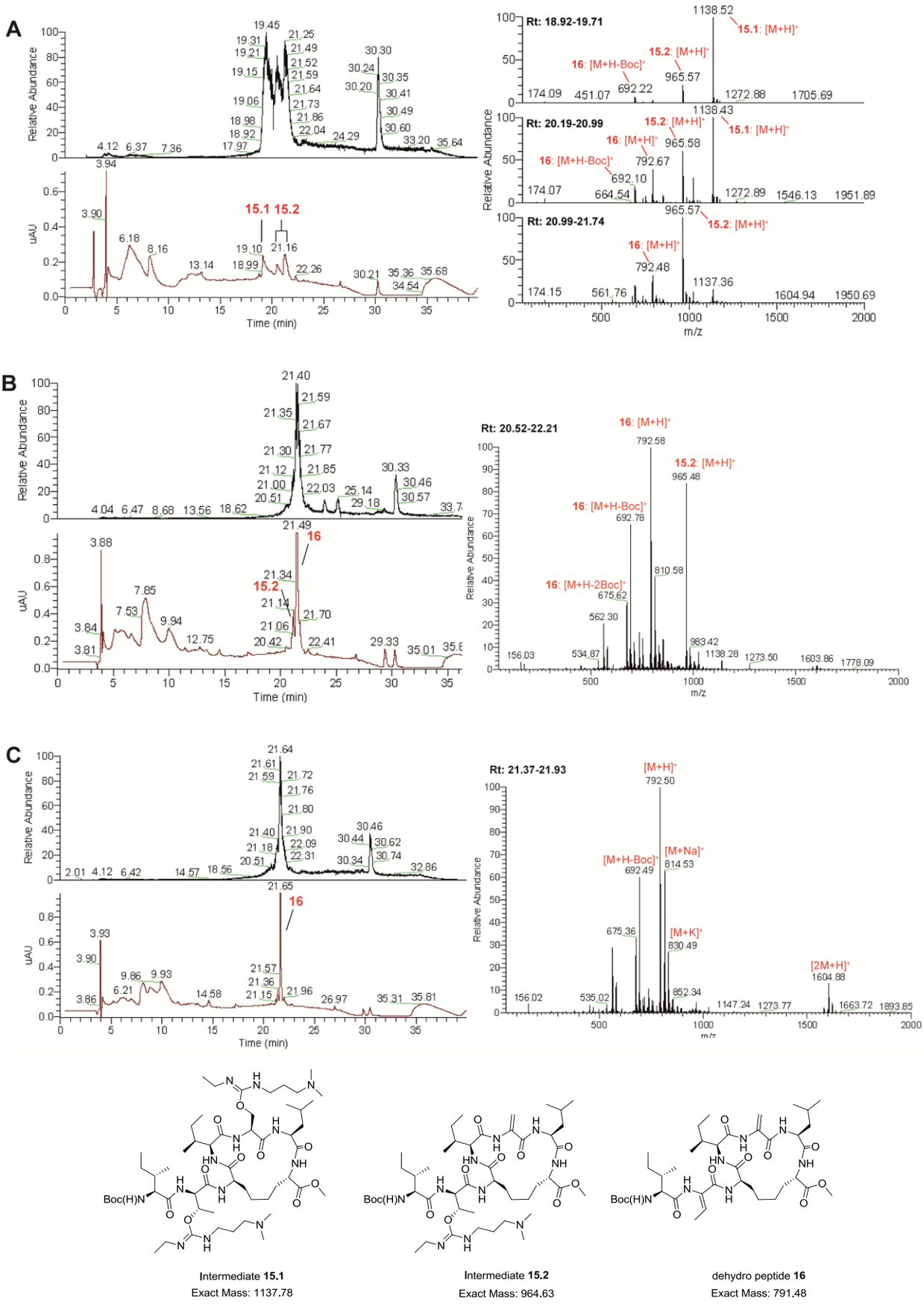
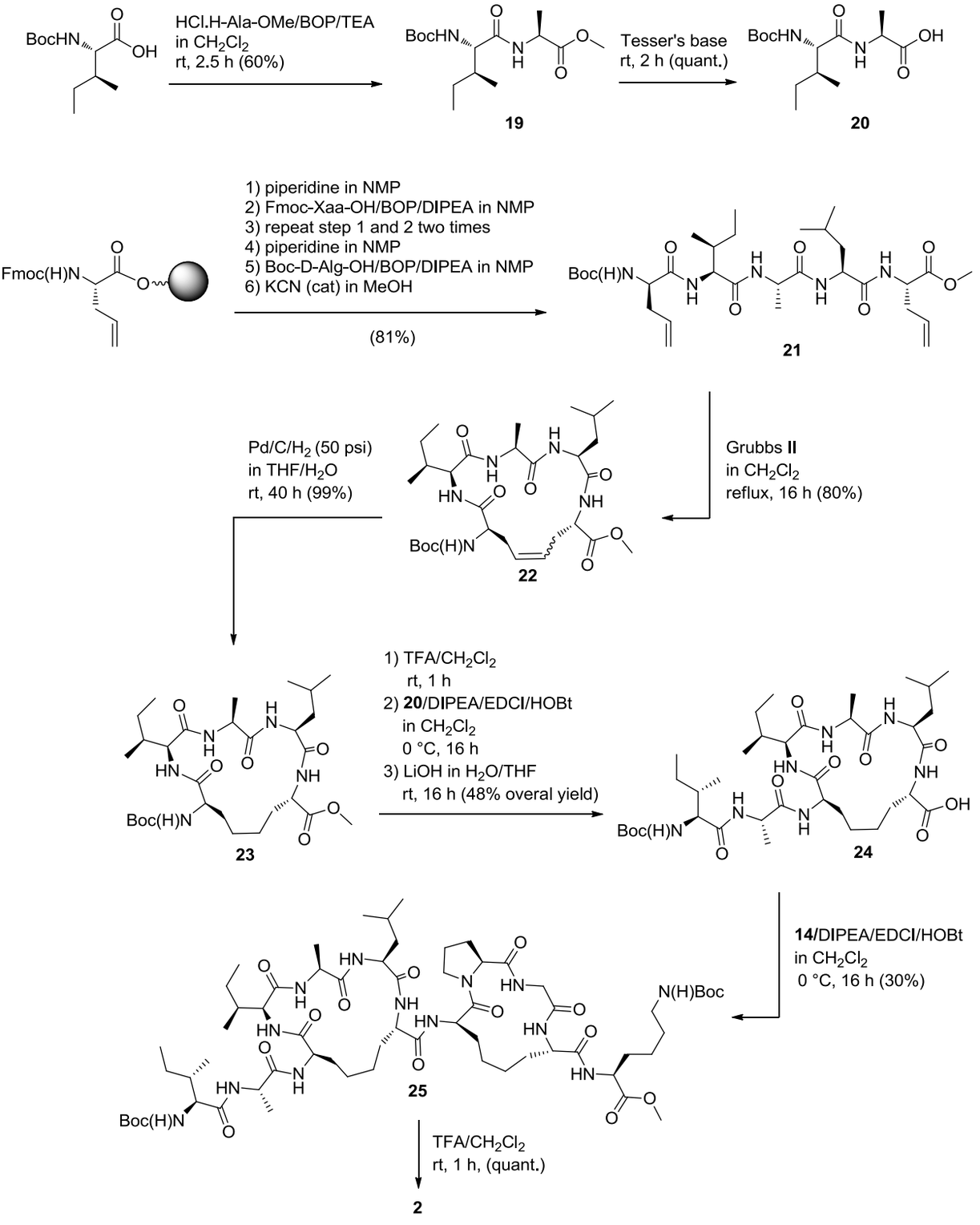


Figure 3. LC-MS data of the crude reaction mixture to afford Boc-Ile1-Dhb2-cyclo[3-7]-D-Alg3-Ile4-Dha5-Leu6-Alg7-OMe 16 after 16 h (**A**), 40 h (**B**) and 64 h (**C**) of reaction time.

Under these reaction conditions only trace amounts of the desired dehydropeptide were obtained. LC-MS analysis of the reaction mixture confirmed that the starting material was converted into intermediate products, in which one or two EDCI moieties were attached to peptide **15**, as shown in Figure 3A/3B. By increasing the equivalents of EDCI/CuCl, a complete conversion of the starting material could be realized, and finally, by switching to 1,2-dichloroethane as the solvent, and heating the reaction mixture to 50 °C, in the presence of DBU, almost complete conversion of the intermediate products into the desired dehydropeptide **16** was achieved (Figure 3C). Encouraged by this result, the final steps to synthesize the nisin AB analog were initiated and started with the LiOH-mediated saponification of peptide **16**. Although, saponification of peptide **16** was successful, unfortunately a significant part of the product was rehydrated, due to reaction with hydroxide with one of the Michael acceptors. This was somewhat surprising and not in line with the reported literature and our earlier observations. To avoid this side reaction, it was decided to assemble the AB dicarba fragment first, followed by the dehydration reaction as final step. Hence, peptide **15** was saponified and the corresponding acid was coupled cyclopeptide **14** in the presence of EDCI/HOBt and peptide **17** was obtained in a reasonable yield (55%) after preparative HPLC. Dehydration of peptide **17** was achieved by treatment with EDCI/CuCl, subsequently followed by β-elimination induced by DBU, according to the similar procedure for the synthesis of dehydropeptide **16**. The Boc-protected dehydropeptide **18** was purified by preparative HPLC, and after treatment with TFA to remove both Boc-functionalities, Dhb2/Dha5 nisin AB dicarba analog **1** was successfully synthesized.

As a control peptide, Ala2/Ala5 nisin AB dicarba analog **2** was synthesized in which the two dehydro residues, dehydrobutyrine and dehydroalanine, were replaced by an L-alanine residue. The synthesis started with the preparation of Boc-Ile-Ala-OMe **19** via a BOP coupling of Boc-Ile-OH and HCl.H-Ala-OMe in the presence of TEA as the base. Methyl ester **19** was quantitatively saponified in peptide acid Boc-Ile-Ala-OH **20** as shown in Scheme 5. Then, linear pentapeptide **21** (Boc-D-Alg^{1}-Ile^{2}-Ala^{3}-Leu^{4}-Alg^{5}-OMe) was synthesized on the solid phase as previously described and was isolated in 81% yield. Cyclization by ring-closing metathesis of linear pentapeptide **21** in the presence of Grubbs II catalyst gave both diastereoisomers of alkene-bridged ring-fragment A **22** in 80% yield after column chromatography. Reduction of the alkene was achieved by treatment with H_2 (at 50 psi) in a Parr apparatus with Pd/C as the catalyst and alkane-bridged ring-fragment A **23** was obtained in near quantitative yield. The synthesis was continued by removal of the N-terminal Boc group of cyclic peptide **23** by treatment with TFA, and after the addition of DIPEA, the free amine was coupled to dipeptide acid **20** in the presence of EDCI/HOBt as coupling reagents. The cyclic heptapeptide was extracted via an aqueous work-up, saponified using lithium hydroxide, and peptide acid **24** was isolated after preparative HPLC in 48% yield. Finally, peptide

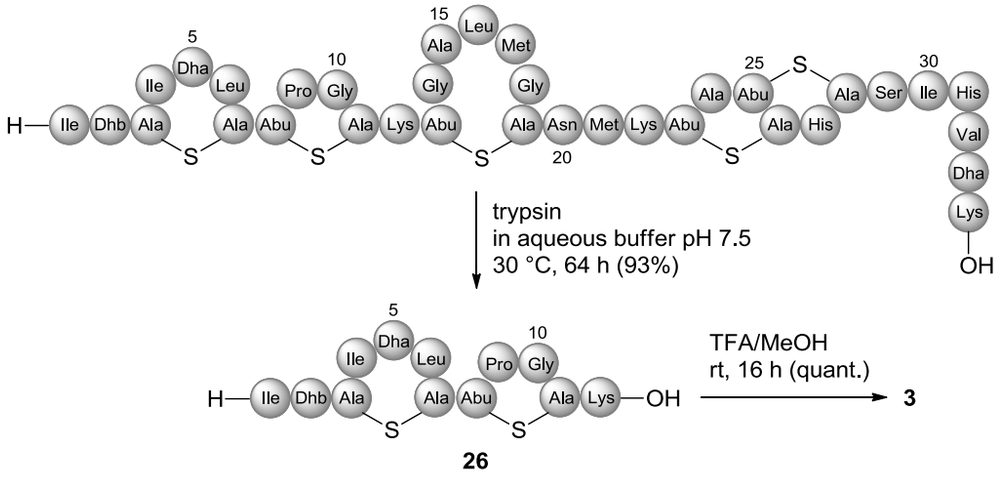
amine **14** was successfully coupled to acid **24** and protected Ala2/Ala5 nisin dicarba analog **25** was isolated (30% yield) by preparative HPLC and subsequently treated with TFA to remove both Boc-functionalities and Ala2/Ala5 nisin-AB-dicarba-Ala **2** was obtained in quantitative yield.



Scheme 5. Synthesis route for nisin AB dicarba Ala **2**.

The native nisin AB-fragment **3** was prepared as the ultimate control peptide to compare the influence of the dicarba-bridge on the bioactivity. For this purpose, nisin AB-fragment **26** was

prepared by trypic digestion of nisin as described in chapter 3 and was isolated after preparative HPLC in 35% yield as shown in Scheme 6. The carboxylic acid moiety was converted into the methyl ester by treatment with TFA in MeOH and nisin AB-fragment **3**, was obtained in quantitative yield.



Scheme 6. Semi-synthesis route for native nisin AB-OMe **3**.

5.2.2 Biological Evaluation

The synthesized nisin AB constructs **1**-**3** were tested for their lipid II binding affinity by evaluating their potency to inhibit nisin-induced pore-formation in a model membrane leakage experiment.[16] For this purpose, large unilamellar vesicles (LUVs) composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 0.2% of lipid II were loaded with carboxyfluorescein (CF) as a water-soluble fluorophore. Peptide-membrane interaction was measured by monitoring the release of CF by fluorescence spectroscopy. The vesicles were treated with nisin (200 pM) and ~70% of membrane permeabilization (CF-leakage) was observed (Figure 4). Then, the vesicles were incubated with one of the nisin AB peptides **1**-**3** (5 μM) prior to the addition of nisin (200 pM), and the resulting membrane permeabilization was measured.

Addition of native-AB **3** resulted in a reduced CF-leakage to ~25%, confirming that native-AB **3** was indeed a competitive binder of lipid II. It turned out that Ala2/Ala5 nisin AB-dicarba analog **2** also inhibited the activity of nisin, albeit with a reduced potency, since a CF-leakage of ~39% was obtained, which was in agreement with the data reported previously.[9d] Gratifyingly, Dhb2/Dha5 nisin AB-dicarba analog **1** was found to be more potent compared to Ala2/Ala5 nisin AB-dicarba analog **2**, since the nisin-induced membrane leakage dropped to ~26%, showing that incorporation of dehydrobutyrine and dehydroalanine in the nisin AB-ring fragment caused an increase in affinity toward lipid II. Moreover, the measured activity was comparable with native-AB **3**, an implication that the dicarba bridge is an excellent mimic for the lanthionine bridge for the nisin AB-ring system.

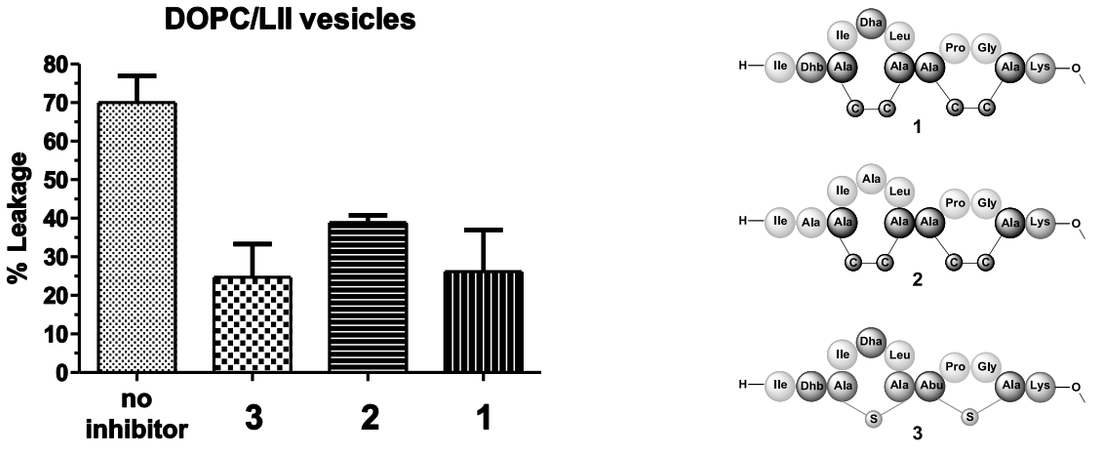


Figure 4. Pore-formation activity of nisin without competitor, and with nisin AB-OMe **3**, nisin AB dicarba Ala **2** and AB dicarba dehydro **1** measured in a model membrane leakage experiment.

5.3 Conclusions

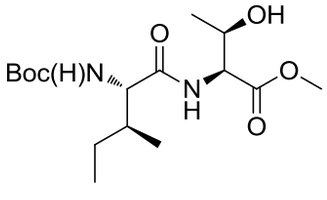
A reliable synthesis to obtain two nisin AB dicarba analogs (1 and 2) via a combination of SPPS, ring-closing metathesis, and solution phase chemistry was developed via a [2+5+5] fragment assembly strategy. Ring-closing metathesis turned out to be an efficient method for macrocyclization of these nisin-derived peptides, since all steps were performed in high yielding (>80% isolated yield). The dehydro residues, dehydrobutyrine and dehydroalanine, were introduced as their precursor residues (threonine and serine) in various peptide constructs and dehydration was performed either by mesylation/DBU-induced β-elimination or via EDCI/CuCl (EDCI/CuCl/DBU). During the syntheses it turned out that dehydration with EDCI/CuCl/DBU was the most effective approach. In contrast to the literature procedures, it was difficult to perform peptide couplings and saponifications in the presence of dehydropeptides, since side-reactions primarily based on Michael additions occurred. However, changing the synthesis strategy by performing the dehydration reactions as the penultimate step was successful. The two nisin AB dicarba analogs (**1** and **2**) were tested for lipid II binding affinity using a model membrane leakage experiment and their potency to inhibit nisin-induced membrane leakage was evaluated and compared to native nisin AB 3. Mimic **1** was equipotent as native-AB **3**, while mimic **2** was less active. From these studies two important conclusions can be drawn. Firstly, introducing dehydro analogs in nisin AB-fragments increased the activity expressed as the affinity toward lipid II, and secondly, dicarba analogs are excellent isosteres to mimic the thio ether bridge in lanthionine peptides.

5.4 Experimental

5.4.1 Chemicals, instruments and general methods

Chemicals were used as obtained from commercial sources without further purification unless stated otherwise. The solvents were obtained as peptide synthesis grade and stored on molecular sieves (4 Å) prior to use. Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40-63 μm). TLC was performed on Merck precoated silica gel 60F254 glass plates. Spots were visualized by UV quenching, ninhydrin, or Cl_2/TDM[17]. ^{1}H-NMR data were acquired on a Varian Mercury 300 MHz or a Varian Inova 500 MHz spectrometer in $CDCl_3$ or DMSO-d_6 as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm) or DMSO (2.50 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of triplet (dt), and broad (br). ^{13}C-NMR data were acquired on a Varian Mercury 75 MHz in $CDCl_3$ or DMSO-d_6 as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm), or DMSO-d_6 (39.5 ppm). Analytical HPLC was performed on an automated HPLC system (Shimadzu) equipped with a UV/vis detector operating at 220/254 nm using an Alltech Prosphere C4 column (pore size: 300 Å, particle size: 5 μm, 250 × 4.6 mm) at a flow rate of 1 mL/min (from 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min. Preparative HPLC was performed on an automated preparative HPLC system (Applied Biosystems) equipped with a UV/vis detector operating at 214 nm using an Alltech prosphere C4 column (pore size: 300 Å, particle size: 10 μm, 250 × 22 mm) at a flow rate of 12.0 mL/min (from 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 90 min). ESI-MS was performed on a Shimadzu LCMS-QP8000 electrospray ionization mass spectrometer operating in a positive ionization mode. LC-MS analyses were performed on a Thermo-Finnigan LCQ Deca XP Max ion trap mass spectrometer coupled to a Shimadzu analytical HPLC system.

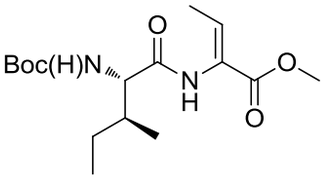
5.4.2 Synthesis



Boc-Ile-Thr-OMe 4

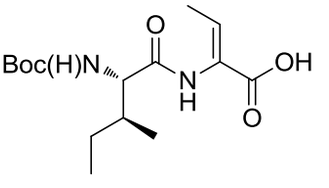
HCl.H-Thr-OMe (2.58 g, 15.2 mmol) was dissolved in CH_2Cl_2 (150 mL) and to this solution, Boc-Ile-OH (3.52 g, 15.2 mmol), BOP (6.72 g, 15.2 mmol) followed by TEA (6.36 mL, 45.6 mmol) were successively added. The reaction mixture was stirred for 2.5 h at room temperature after which the solvent was removed *in vacuo* and the residue was redissolved in EtOAc (300 mL). The obtained solution was washed with aq. 1N $KHSO_4$ (3 × 200 mL), aq. 1N $NaHCO_3$ (3 × 200 mL), and brine (2 × 150 mL), dried (Na_2SO_4), filtered and the solvent was removed *in vacuo*. The residue was

purified by column chromatography (CH_2Cl_2/MeOH 98.5:2.5 v/v), which afforded a white crystalline solid in 70% yield (4.61 g). R_f 0.15 (CH_2Cl_2/MeOH 97.5:2.5 v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.90-0.97 (m, 6H, CγH_3 Ile/ CδH_3 Ile), 1.21 (d (J = 6.4 Hz), 3H, CγH_3 Thr), 1.44 (s, 9H, Boc), 1.57 (m, 1H, CβH Ile), 1.83 (m, 2H, CγH_2 Ile), 2.80 (s, 1H, OH), 3.77 (s, 3H, OMe), 3.97 (m, 1H, CαH Ile), 4.36 (m, 1H, CβH Thr), 4.62 (dd (J_{gem} = 9.0 Hz, J_{vic} = 2.4 Hz), 1H, CαH Thr), 5.11 (d (J = 8.1 Hz), 1H, BocNH), 6.74 (d (J = 8.9 Hz), 1H, NH Thr); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 11.1, 15.3, 19.7, 24.8, 28.2, 37.0, 52.4, 57.3, 59.3, 68.0, 79.9, 156.0, 171.1, 172.5.



Boc-Ile-Dhb-OMe 5

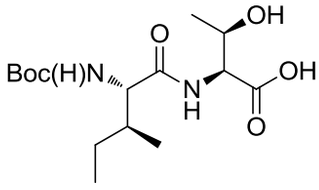
To a solution of Boc-Ile-Thr-OMe **4** (347 mg, 1.0 mmol) in CH_2Cl_2 (20 mL), MsCl (120 µL, 1.5 mmol) and TEA (210 µL, 1.5 mmol) were added, after which the reaction mixture was stirred for 1 h. Subsequently, DBU (230 µL, 1.5 mmol) was added and the reaction mixture was stirred for 16 h under reflux conditions. After cooling the reaction mixture to room temperature, the organic layer was washed with aq. 1N $KHSO_4$ (3 × 10 mL), dried (Na_2SO_4), filtrated and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/hexane 8:2 v/v), and the title compound was obtained as a white crystalline solid in 58% yield (190 mg). R_f 0.73 (CH_2Cl_2/MeOH 97.5:2.5 v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.94 (t (J = 7.4 Hz), 3H, CδH_3 Ile), 1.00 (d (J = 6.8 Hz), 3H, CγH_3 Ile), 1.22 (m, 2H), 1.45 (s, 9H, Boc), 1.49-168 (m, 2H, CγH_2 Ile), 1.77 (d (J = 7.2 Hz), 3H, CγH_3 Dhb), 1.96 (m, 1H, CβH Ile), 3.76 (s, 3H, OMe), 3.98-4.16 (m, 1H, CαH Ile), 5.03 (br s, 1H, BocNH), 6.83 (q (J = 7.2 Hz), 1H, CβH Dhb), 7.32 (s, 1H, NαH Dhb); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 11.5, 14.7, 15.6, 24.7, 28.3, 37.0, 52.3, 59.6, 80.2, 125.8, 134.4, 155.8, 164.7, 170.0.



Boc-Ile-Dhb-OH 6

Boc-Ile-Dhb-OMe **5** (180 mg, 0.55 mmol) was dissolved in THF (10 mL), after which a solution of LiOH in H_2O (10 mL; 0.11 N) was added, and the reaction mixture was stirred for 2 h. Then, dioxane was evaporated *in vacuo*, the aqueous mixture was acidified using aq. 1N $KHSO_4$, and the product was extracted with CH_2Cl_2 (4 × 10 mL). After drying (Na_2SO_4), and evaporation of the solvent *in vacuo*, the product was obtained as a white solid in 87% yield (145 mg). R_f 0.41 (CH_2Cl_2/MeOH/AcOH 95:5:0.1 v/v/v); ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD 95:5 v/v) δ = 0.91 (t (J = 6.9 Hz), 3H, CδH_3 Ile), 1.00 (d (J = 6.8 Hz), 3H, CγH_3 Ile), 1.45 (s, 9H, Boc), 1.52 (m, 2H, CγH_2 Ile), 1.79 (d (J = 6.7 Hz), 3H, CγH_3 Dhb), 2.02 (m, 1H, CβH Ile), 4.20 (dd (J_{gem} = 9.4 Hz, J_{vic} = 5.4 Hz), 1H, CαH Ile), 5.25/6.63 (d (J = 6.8 Hz), 1H, BocNH), 6.91 (q (J = 7.0 Hz), Cβ Dhb), 7.66/7.90 (s, 1H, NH Dhb);

^{13}C NMR (75.5 MHz, $CDCl_3/CD_3OD$ 95:5 v/v) δ = 11.1, 14.6, 15.3, 24.5, 28.2, 37.3, 59.0, 80.1, 125.9, 135.0, 156.2, 166.6, 170.6.



Boc-Ile-Thr-OH 7

Boc-Ile-Thr-OMe **4** (867 mg, 2.50 mmol) was dissolved in Tesser's base (15 mL, 3.0 mmol) after which the reaction mixture was stirred for 2 h. The reaction mixture was concentrated *in vacuo*, and the remaining aqueous layer was acidified using aq. 1N $KHSO_4$, and the product was extracted with CH_2Cl_2 (4 × 20 mL). After drying the CH_2Cl_2 solution (Na_2SO_4) and removing the solvents *in vacuo*, the title compound was afforded as a white powder in quantitative yield (830 mg). R_f 0.25 (CH_2Cl_2/MeOH/AcOH 90:10:0.1 v/v/v); ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$ 99:1 v/v) δ = 0.53-1.02 (m, 6H, CδH_3 Ile/CγH_3 Ile), 1.03-1.66 (m, 14H, Boc / CγH_3 Thr / CγH_2 Ile), 1.70-2.00 (m, 1H, CβH Ile), 2.80 (s, 1H, OH), 3.77 (s, 3H, OMe), 3.97 (m, 1H, CαH Ile), 4.36 (m, 1H, CβH Thr), 4.62 (dd (J_{gem} = 9.0 Hz, J_{vic} = 2.4 Hz), 1H, CαH Thr), 5.11 (d (J = 8.1 Hz), 1H, BocNH), 6.74 (d (J = 8.9 Hz), 1H, NH Thr); ^{13}C NMR (75.5 MHz, $CDCl_3/CD_3OD$ 99:1 v/v) δ = 11.2, 15.4, 19.4, 24.7, 28.3, 36.8, 58.4, 67.9, 80.0, 156.2, 172.8, 175.8.

Solid phase peptide synthesis general method

Protected linear peptides were synthesized manually at 0.90 mmol scale on Argogel resin. ArgoGel-OH resin was loaded with Fmoc-Xaa-OH using the method of Sieber.[18] The Fmoc group enabled determination of the loading and also the efficiency of the coupling reaction.[19] Each synthetic cycle consisted of the following steps. *Fmoc removal:* the resin (~3.0 g, ~0.90 mmol) was treated with a 20% solution of piperidine in NMP (25 mL, 3 × 8 min). The solution was removed by filtration and the resin was washed with NMP (25 mL, 3 × 2 min) and CH_2Cl_2 (25 mL, 3 × 2 min). The presence of free α-amino functionalities was checked by the Kaiser[20] test (blue beads). *Coupling step:* a mixture of Fmoc-Xaa-OH (3.6 mmol, 4 equiv), BOP (3.6 mmol, 4 equiv) and DIPEA (7.2 mmol, 8 equiv) in NMP (30 mL) was added to the resin and the suspension was mixed by bubbling N_2 through the reaction mixture for 45 min. Reagents and solvents were removed by filtration and the resin was subsequently washed with NMP (25 mL, 3 × 2 min) and CH_2Cl_2 (25 mL, 3 × 2 min). Completion of the coupling (absence of free α-amino functionalities) was checked by the Kaiser test[25] (colorless beads). *Peptide cleavage*: the resin was washed with MeOH (25 mL, 3 × 2 min) and subsequently swirled in MeOH (30 mL) with a catalytic amount of KCN for 16 h at room temperature. Then, the resin was removed by filtration and the residual MeOH solution was concentrated *in vacuo* yielding the crude peptide.

Boc-D-Alg^1-Ile^2-Ser(Trt)^3-Leu^4-Alg^5-OMe 8

The resin (3.01 g) was loaded with Fmoc-Alg-OH using the Sieber method with 0.30 mmol/g resin and SPPS was performed according to the procedure described in the general section solid phase peptide synthesis. As the final coupling step, Boc-D-Alg-OH (784 mg, 3.64 mmol), BOP (1.62 g, 3.64 mmol) and DIPEA (1.3 mL, 7.28 mmol) in NMP (30 mL) were used, to yield an N-terminally Boc-protected peptide. The crude peptide was purified by column chromatography (CH_2Cl_2/MeOH 95:5 v/v) and obtained as an off-white solid in 77% yield (613 mg) R_f 0.41 (CH_2Cl_2/acetone 9:1 v/v); R_t = 25.78 min; ESI-MS calcd for $C_{50}H_{67}N_5O_9$ 881.49, found *m/z*; $[M+Na]^+$ 904.55, $[2M+Na]^+$ 1786.95, $[M-Trt+H]^+$ 640.50.

cyclo[1-5]-Boc-D-Alg^1-Ile^2-Ser(Trt)^3-Leu^4-Alg^5-OMe 9

The linear precursor peptide **8** (251 mg, 0.28 mmol) was dissolved in CH_2Cl_2 (150 mL) and the solution was heated to reflux and gently purged by N_2 for 20 min. Then, Grubbs II catalyst (13 mg, 15 μmol) was added and the obtained reaction mixture was refluxed for 16 h. Subsequently, the solvent was removed by evaporation and the residue was purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) to give the title compound as a brownish solid in 83% yield (201 mg). R_f 0.29 (CH_2Cl_2/MeOH 97:3 v/v); R_t = 24.78 min; ESI-MS calcd for $C_{50}H_{67}N_5O_9$ 853.46, found *m/z* $[M+Na]^+$ 876.45,$[M-Trt]^+$ 612.40, $[2M+Na]^+$ 1731.30.

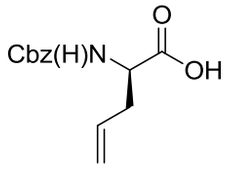
cyclo[1-5]-Boc-D-Alg^1-Ile^2-Ser(H)^3-Leu^4-Alg^5-OMe 10

First, cyclo-alkene-ringA **9** (312 mg, 0.37 mmol) was dissolved in a mixture of CH_2Cl_2/TIS/TFA (19 mL, 94:5:1 v/v/v) and the mixture was stirred for 1 h at room temperature. After coevaporation with MeOH (3 × 10 mL), the trityl-deprotected peptide was afforded and was used in the next step without further purification. The crude peptide was dissolved in THF/H_2O (44 mL, 4:1 v/v) and placed in a Parr Apparatus reaction vessel. After addition of Pd/C catalyst (10 mg, 10% Pd w/w), the reaction mixture was shaken in a H_2 atmosphere (50 PSI H_2 pressure) for 40 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, which was rinsed with THF (2 × 2 mL) and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (CH_2Cl_2/MeOH 95:5 v/v) and the title compound was afforded as a brownish solid in a good yield: 78% (177 mg). R_f 0.53 (CH_2Cl_2/MeOH 9:1 v/v); R_t = 19.27 min; ESI-MS calcd for $C_{29}H_{51}N_5O_9$ 613.37, found *m/z* $[M+H]^+$ 614.20, $[2M+Na]^+$ 1249.70, $[M+H-C_4H_8]^+$ 558.00.

cyclo[1-5]-Boc-D-Alg^1-Ile^2-Dha^3-Leu^4-Alg^5-OMe 11

A flame-dried flask was purged with N_2 gas and charged with a solution of cyclo-ringA-precursor **10** (150 mg, 244 μmol) in CH_2Cl_2 (4.0 mL) under a N_2 atmosphere. Subsequently, copper(I)

chloride (7.3 mg, 74 μmol) and EDCI.HCl (51 mg, 266 μmol) were added and the suspension was stirred for 16 h at room temperature. The organic layer was washed with H_2O (2 × 3 mL) and after drying (Na_2SO_4), the solvent was removed *in vacuo*. The residue was purified by preparative HPLC and the title compound was afforded as an off-white solid in a good yield: 60% (87 mg). R_f 0.65 (CH_2Cl_2/MeOH 9:1 v/v); R_t = 21.31 min; ESI-MS calcd for $C_{29}H_{49}N_5O_8$ 595.36, found *m/z* $[M+H]^+$ 596.46, $[M+Na]^+$ 618.45, $[M+K]^+$ 634.30.45, $[2M+Na]^+$ 1214.30, $[M+H\text{-Boc}]^+$ 496.00, $[M+H\text{-}C_4H_8]^+$ 540.40.



Cbz-D-Alg-OH

H-D-Alg-OH (1.13 g, 10 mmol) was dissolved in aq. 2M NaOH (5 mL) and the solution was vigorously stirred. Then, benzyl chloroformate (1.71 mL, 12 mmol) and aq. 2M NaOH (5.5 mL) were simultaneously added dropwise for 2 h to the reaction mixture and stirring was continued at room temperature for 16 h. Then, CH_2Cl_2 (15 mL) was added to the reaction mixture which was acidified with aq. 1N HCl until pH 1–2. The aqueous layer was extracted with CH_2Cl_2 (2 × 10 mL), dried (Na_2SO_4) and concentrated *in vacuo* affording Cbz-D-Alg-OH as a colorless oil in a good yield of 79% (1.97 g). R_f 0.75 ($CHCl_3$/MeOH/AcOH 90:9:1 v/v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.34-2.76 (m, 2H, CβH_2), 4.49 (dd (J_{gem} = 13.0 Hz, J_{vic} 6.0 Hz), 1H, CαH), 5.03-5.23 (m, 4H, CδH_2 alkene / OCH_2 benzyl), 5.32 (d (*J* = 7.9 Hz), 1H, NH), 5.55-5.94 (m, 1H, CγH alkene), 7.33 (m, 5H, Ar-H benzyl), 9.47 (s, 1H, COOH); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 36.3, 53.1, 67.2, 119.8, 128.1, 128.3, 128.5, 132.7, 136.0, 156, 176.4.

Cbz-D-Alg^1-Pro^2-Gly^3-Alg^4-Lys(Boc)^5-OMe 12

The resin (3.25 g) was loaded with Fmoc-Alg-OH using the Sieber method with 0.30 mmol/g resin and SPPS was performed according to the procedure described in the general section solid phase peptide synthesis. As the final coupling step, Cbz-D-Alg-OH (957 mg, 3.84 mmol), BOP (1.70 g, 3.84 mmol) and DIPEA (1.37 mL, 7.68 mmol) in NMP (30 mL) were used, to yield an N-terminally Cbz-protected peptide. The crude peptide was purified by column chromatography (CH_2Cl_2/MeOH 95:5 v/v) affording a yellowish oil in a very good yield (93%, 666 mg). R_f 0.58 (CH_2Cl_2/acetone 9:1 v/v); R_t = 21.35 min; ESI-MS calcd for $C_{37}H_{54}N_6O_{10}$ 742.39, found *m/z*; $[M+Na]^+$ 764.60, $[M+H\text{-Boc}]^+$ 643.95.

cyclo[1-4]-Cbz-D-Alg^1-Pro^2-Gly^3-Alg^4-Lys(Boc)^5-OMe 13

The linear precursor peptide **12** (252 mg, 0.34 mmol) was dissolved in CH_2Cl_2 (100 mL) and the solution was heated to reflux and gently purged by N_2 for 20 min. Then, Grubbs II catalyst (14 mg,

16 μmol) was added and the obtained reaction mixture was refluxed for 16 h. Subsequently, the solvent was removed by evaporation and the residue was purified by column chromatography (CH_2Cl_2/MeOH 95:5 v/v) to give the title compound as its diastereoisomeric mixture (*E*,*Z*) as a brownish solid in quantitative yield (242 mg). R_f 0.53 (CH_2Cl_2/MeOH 9:1 v/v); R_t = 19.52-20.38 min; ESI-MS calcd for $C_{35}H_{52}N_6O_{10}$ 716.37, found *m/z* $[M+Na]^+$ 737.50, $[M+H\text{-Boc}]^+$ 615.10.

cyclo[1-4]-H-D-Alg^1-Pro^2-Gly^3-Alg^4-Lys(Boc)^5-OMe.TFA 14

The Cbz protected alkene ring-AB peptide **13** (54 mg, 76 μmol) was dissolved in THF/H_2O (8 mL, 4:1 v/v) and placed in a Parr Apparatus reaction vessel. After addition of Pd/C catalyst (11 mg, 10% Pd w/w), the reaction mixture was shaken in a H_2 atmosphere (50 PSI H_2 pressure) for 40 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, which was rinsed with THF (2 × 2 mL) and the filtrate was concentrated *in vacuo*. The residue was taken up in H_2O/MeCN/TFA (15 mL, 75:25/0.1 v/v/v) and the mixture was lyophilized which afforded the title compound as an off-white fluffy powder in quantitative yield (51 mg). R_f 0.22 (CH_2Cl_2/MeOH/TEA 85:15:0.1 v/v/v); R_t = 16.68 min; ESI-MS calcd for $C_{27}H_{46}N_6O_8$ 582.34, found *m/z* $[M+H]^+$ 583.45, $[M+Na]^+$ 605.35, $[M+K]^+$ 621.35, $[2M+H]^+$ 1165.60, $[M\text{-Boc}]^+$ 483.05.

Boc-Ile^1-Thr^2-cyclo[3-7]-D-Alg^3-Ile^4-Ser^5-Leu^6-Alg^7-OMe 15

Boc-protected peptide **10** (51 mg, 82 μmol) was dissolved in CH_2Cl_2/TFA (2 mL, 1:1 v/v) and the reaction mixture was stirred at room temperature for 1 h in order to remove the Boc group. Then, the solvents were removed *in vacuo*, followed by coevaporation of the residue with $CHCl_3$ (2 × 5 mL), and further drying at high vacuum. The residue was dissolved in CH_2Cl_2 (5 mL) and DIPEA (14 μL, 82 μmol) followed by HOBt (12 mg, 89 μmol) were added and the reaction mixture was cooled to 0 °C. Subsequently, Boc-Ile-Thr-OH **7** (33 mg, 99 μmol) and EDCI.HCl (16 mg, 83 μmol) were added and the reaction mixture was stirred for 16 h and was allowed to warm up to room temperature. The reaction mixture was concentrated *in vacuo* and the residue was redissolved in EtOAc (5 mL), and the solution was washed with aq. 1N $KHSO_4$ (3 × 4 mL), aq. 1N $NaHCO_3$ (3 × 4 mL) and brine (1 × 4 mL). After drying (Na_2SO_4) and subsequently removing the solvent *in vacuo*, peptide **15** was afforded as a white solid in a reasonable yield of 61% (41 mg). R_t = 20.68 min; ESI-MS calcd for $C_{39}H_{69}N_7O_{12}$ 827.50, found *m/z* $[M+H]^+$ 828.85, $[M+Na]^+$ 851.05, $[2M+Na]^+$ 1678.35, $[2M+Na\text{-Boc}]^+$ 1579.15, $[M\text{-Boc}]^+$ 729.05.

Boc-Ile^1-Dhb^2-cyclo[3-7]-D-Alg^3-Ile^4-Dha^5-Leu^6-Alg^7-OMe 16

A flame-dried flask was purged with N_2 gas and charged with **15** (8.9 mg, 10.7 mmol) which was dissolved in 1,2-dichloroethane (2.0 mL) under a N_2 atmosphere. Subsequently, copper(I) chloride

(1.0 mg, 10.1 μmol) and EDCI.HCl (5.8 mg, 30.4 μmol) were added and the suspension was stirred for 16 h at room temperature and the reaction progress was followed by LC-MS. After 16 h starting material as well as reaction intermediates were observed, and after the addition of an extra amount of EDCI.HCl (2.5 mg, 13.1 μmol) stirring of the reaction mixture was continued at room temperature for 40 h. LC-MS analysis showed complete conversion of the starting material, however, reaction intermediates were observed rather than the desired product **16**. Therefore, DBU (1.5 μL, 10.0 μmol) was added to the reaction mixture and stirring was continued at 50 °C for 24 h, which resulted in a complete conversion of all reaction intermediates. Subsequently, the organic solution was washed with aq. 1N $KHSO_4$ (2 × 1 mL) and after drying (Na_2SO_4), the solvent was removed *in vacuo*. The residue was redissolved in *tert*-BuOH/H_2O (5 mL, 1:1 v/v) and subsequently lyophilized to afford the title compound as a white fluffy powder in a yield of 57% (4.8 mg). R_t = 21.65 min; ESI-MS calcd for $C_{39}H_{65}N_7O_{10}$ 791.97, found *m/z* $[M+H]^+$ 792.51, $[M+Na]^+$ 814.53, $[2M+Na]^+$ 1604.88, $[M+H\text{-Boc}]^+$ 692.50.

Boc-Ile^1-Thr^2-bicyclo[3-7/8-11]-D-Alg^3-Ile^4-Ser^5-Leu^6-Alg^7-D-Alg^8-Pro^9-Gly^{10}-Alg^{11}-Lys(Boc)^{12}-OMe 17

Peptide **15** (15 mg, 18 μmol) was dissolved in THF (500 μL), and a solution of LiOH in H_2O (400 μL, 0.1M) was added, and the reaction mixture was stirred for 16 h at room temperature. Then, THF was evaporated *in vacuo*, and the aqueous layer was acidified by aq. 1N $KHSO_4$, followed by extraction with CH_2Cl_2 (3 × 2 mL). The solvents were removed *in vacuo*, and the peptide acid was obtained. In a separate flask, trifluoro acetate **14** (12 mg, 17.6 μmol) was dissolved in DMF (500 μL) and DIPEA (3.5 μL, 18 μmol) was added to obtain **17** as the free amine, and this solution was added to the peptide acid followed by the addition of HOBt (3 mg, 22 μmol). After cooling the reaction mixture to 0 °C, EDCI.HCl (4 mg, 21 μmol) was added and the reaction mixture was stirred for 16 h, and was allowed to warm up to room temperature. Then, the reaction mixture was diluted with a mixture of H_2O/MeCN/TFA (3 mL, 60:40:0.1 v/v/v) and peptide **17** was purified by preparative HPLC as a white fluffy powder in 55% yield (13 mg) over two steps. R_t = 20.65 min; ESI-MS calcd for $C_{65}H_{111}N_{13}O_{19}$ 1377.81, found *m/z* $[M+H]^+$ 1379.10, $[M+Na]^+$ 1400.95, $[M+K]^+$ 1417.35, $[M+H\text{-Boc}]^+$ 1279.50, $[M+2H\text{-Boc}]^{2+}$ 639.75.

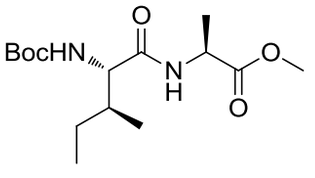
Boc-Ile^1-Dhb^2-bicyclo[3-7/8-11]-D-Alg^3-Ile^4-Dha^5-Leu^6-Alg^7-D-Alg^8-Pro^9-Gly^{10}-Alg^{11}-Lys(Boc)^{12}-OMe 18

A flame-dried flask was purged with N_2 gas and charged with **17** (6.0 mg, 4.4 mmol) which was dissolved in 1,2-dichloroethane (500 μL) under a N_2 atmosphere. Subsequently, copper(I) chloride (1.0 mg, 10 μmol) and EDCI.HCl (4.2 mg, 22 μmol) were added and the suspension was stirred for

16 h at room temperature and the reaction progress was followed by LC-MS. After 16 h an additional amount of copper(I) chloride (0.5 mg, 5 μmol) and EDCI.HCl (2.0 mg, 10.4 μmol) were added. After 40 h of stirring at room temperature, a mixture of DBU/1,2-dichloroethane (7 μL, 1:9 v/v) was added to the reaction mixture and stirring was continued at 50 °C for 24 h. Then, the solvents were evaporated *in vacuo* and the residue was redissolved in a mixture of H_2O/MeCN/TFA (2 mL, 60:40:0.1 v/v/v) to isolate peptide **18** by preparative HPLC as a white fluffy powder in 10% yield (0.6 mg). R_t = 22.23 min; ESI-MS calcd for $C_{65}H_{107}N_{13}O_{17}$ 1341.79, found *m/z* $[M+H]^+$ 1342.95, $[M+Na]^+$ 1365.10, $[M+K]^+$ 1381.15, $[M+H\text{-Boc}]^+$ 1242.35, $[M+2H\text{-Boc}]^{2+}$ 622.45.

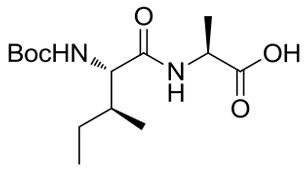
H-Ile[1]-Dhb[2]-bicyclo[3-7/8-11]-D-Alg[3]-Ile[4]-Dha[5]-Leu[6]-Alg[7]-D-Alg[8]-Pro[9]-Gly[10]-Alg[11]-Lys[12]-OMe.2TFA 1

Protected peptide **18** (0.5 mg, 0.37 μmol) was suspended in CH_2Cl_2 (250 μL) and after the addition of TFA (250 μmol), the solution was stirred at room temperature for 1 h. The solvents were evaporated *in vacuo* and the residue was coevaporated with MeOH (2 × 1 mL), dissolved in *tert*-BuOH/H_2O (5 mL, 1:1 v/v) and subsequently lyophilized to afford peptide **1** as a white fluffy powder in quantitative yield (0.5 mg). R_t = 18.17 min; ESI-MS calcd for $C_{55}H_{91}N_{13}O_{13}$ 1141.69, found *m/z* $[M+Na]^+$ 1164.60, $[M+2H]^+$ 571.65.



Boc-Ile-Ala-OMe 19

HCl.H-Ala-OMe (1.40 g, 10.0 mmol) was dissolved in CH_2Cl_2 (100 mL), and to this solution Boc-Ile-OH (1.70 g, 10.0 mmol), BOP (4.42 g, 10.0 mmol) and TEA (4.2 mL, 30.0 mmol) were added, and the obtained reaction mixture was stirred for 2.5 h. then, the solvent was removed *in vacuo* and the residue redissolved in EtOAc (200 mL). The organic solution was washed with aq. 1N $KHSO_4$ (3 × 150 mL), aq. 1N $NaHCO_3$ (3 × 150 mL), and brine (2 × 150 mL), dried (Na_2SO_4), filtered followed by evaporation *in vacuo*. The residue was purified by column chromatography (EtOAc/hexane 3:7 v/v) to give dipeptide **19** as a white crystalline solid in 60% yield (1.90 g). R_f 0.29 (EtOAc/hexane 4:6 v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.87 (t (*J* = 7.4 Hz), 3H, CδH$_3$ Ile), 0.91 (d (*J* = 6.8 Hz), 3H, CγH$_3$ Ile), 1.03-1.21 (m, 1H, CβH Ile), 1.36 (d (*J* = 7.2 Hz), 3H, CβH$_3$ Ala), 1.40 (s, 9H, Boc), 1.52/1.82 (m, 2H, CγH$_2$ Ile), 3.70 (s, 3H, OMe), 3.96 (m, 1H, CαH Ile), 4.54 (quintet (*J* = 7.2 Hz), 1H, CαH Ala), 5.17 (d (*J* = 8.8 Hz), 1H, BocNH), 6.68 (d (*J* = 6.8 Hz), 1H, NH Ala); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 11.3, 15.3, 18.1, 24.7, 28.2, 37.3, 47.9, 52.3, 59.0, 79.7, 156.0, 171.2, 173.0.



Boc-Ile-Ala-OH 20

Boc-Ile-Ala-OMe **19** (791 mg, 2.5 mmol) was dissolved in Tesser's base (15 mL, 3.0 mmol) and the reaction mixture was stirred for 2 h at room temperature. The reaction mixture was concentrated *in vacuo*, and the aqueous layer was acidified by aq. 1N $KHSO_4$ and extracted with CH_2Cl_2 (4 × 20 mL). The combined organic layers were dried (Na_2SO_4) and evaporated *in vacuo* to give peptide acid **20** as a white powder in quantitative yield (760 mg). R_f 0.33 (CH_2Cl_2/MeOH/AcOH 90:10:0.1 v/v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.87-0.95 (m, 6H, CδH_3 Ile/ CγH_3 Ile), 1.82 (m, 1H, CβH Ile), 1.40-1.48 (m, 12H, CβH_3 Ala/Boc), 1.16/15.1 (m, 2H, CγH_2 Ile), 4.03 (m, 1H, CαH Ile), 4.58 (m, 1H, CαH Ala), 6.13 (br s, 1H, BocNH), 6.95 (br s, 1H, NH Ala); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 11.0, 15.2, 17.8, 24.7, 28.2, 37.1, 48.0, 59.0, 80.1, 156.0, 172.2, 175.4.

Boc-D-Alg^1-Ile^2-Ala^3-Leu^4-Alg^5-OMe 21

The resin (1.03 g) was loaded with Fmoc-Alg-OH using the Sieber method with 0.30 mmol/g resin and SPPS was performed according to the procedure described in the general section solid phase peptide synthesis. As the final coupling step, Boc-D-Alg-OH (253 mg, 1.18 mmol), BOP (522 mg, 1.18 mmol) and DIPEA (0.41 mL, 2.36 mmol) in NMP (10 mL) were used to yield an N-terminal Boc protected peptide. The crude peptide was purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) and obtained as an off-white solid in 81% yield (187 mg). R_f 0.55 (CH_2Cl_2/MeOH 9:1 v/v); ESI-MS calcd for $C_{50}H_{67}N_5O_9$ 881.49, found *m/z*; $[M+Na]^+$ 904.35, $[2M+Na]^+$ 1787.45, $[M-Trt+H]^+$ 640.24.

cyclo[1-5]-Boc-D-Alg^1-Ile^2-Ala^3-Leu^4-Alg^5-OMe 22

The linear precursor peptide **21** (100 mg, 0.19 mmol) was dissolved in CH_2Cl_2 (50 mL) and the solution was heated to reflux and gently purged by N_2 for 20 min. Then, Grubbs II catalyst (12 mg, 14 μmol) was added and the obtained reaction mixture was refluxed for 16 h. Subsequently, the solvent was removed by evaporation and the residue was purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) to give the title compound as a brownish solid in 80% yield (76 mg). R_f 0.53 (CH_2Cl_2/MeOH 9:1 v/v).

cyclo[1-5]-Boc-D-Alg^1-Ile^2-Ala^3-Leu^4-Alg^5-OMe 23

Cyclo-alkene-ringA **22** (48 mg, 81 μmol) was dissolved in THF/H_2O (6 mL, 4:1 v/v) and placed in a Parr Apparatus reaction vessel. After addition of Pd/C catalyst (6 mg, 10% Pd w/w), the reaction mixture was shaken in a H_2 atmosphere (50 PSI H_2 pressure) for 40 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, which was rinsed with THF (2 × 2 mL)

and the filtrate was concentrated *in vacuo*. The residue redissolved in *tert*-BuOH/H_2O (10 mL, 1:1 v/v) and subsequently lyophilized, to give peptide **23** as an off-white fluffy powder in quantitative yield (48 mg). R_f 0.53 (CH_2Cl_2/MeOH 9:1 v/v); ESI-MS calcd for $C_{29}H_{51}N_5O_8$ 597.37, found *m/z* $[M+H]^+$ 598.35, $[M+Na]^+$ 620.50, $[2M+Na]^+$ 1217.80.

Boc-Ile^1-Ala^2-cyclo[3-7]-D-Alg^3-Ile^4-Ala^5-Leu^5-Alg^7-OH 24

Boc-protected peptide **23** (48 mg, 80 µmol) was dissolved in CH_2Cl_2/TFA (2 mL, 1:1 v/v) and the obtained reaction mixture was stirred at room temperature for 1 h. The solvents were removed *in vacuo*, and the residue was coevaporated with $CHCl_3$ (2 x 5 mL) followed by drying in high vacuum. Subsequently, the residue was dissolved in CH_2Cl_2 (5 mL) and DIPEA (14 µL, 82 µmol) followed by HOBt (11 mg, 81 µmol) were added and the reaction mixture was cooled to 0 °C. Then, Boc-Ile-Ala-OH **20** (29 mg, 96 µmol) and EDCI.HCl (16 mg, 83 µmol) were added and the reaction mixture was stirred for 16 h and was allowed warm up to room temperature. The reaction mixture was concentrated *in vacuo* and the residue was redissolved in EtOAc (5 mL). This solution was washed with aq. 1N $KHSO_4$ (3 × 4 mL), aq. 1N $NaHCO_3$ (3 × 4 mL) and brine (1 × 4 mL), dried (Na_2SO_4), and evaporated *in vacuo*, to give the heptapeptide ester as a white solid was obtained (35 mg). The crude ester was dissolved in THF (1.5 mL) and a solution of LiOH in H_2O (1.0 mL; 0.1M) was added, and the reaction mixture was stirred for 16 h at room temperature. Then, THF was evaporated *in vacuo* and the aqueous mixture was acidified by aq. 1N $KHSO_4$, and extracted with CH_2Cl_2 (3 × 5 mL). The solvent was removed *in vacuo*, and after preparative HPLC, peptide acid **24** was obtained as a fluffy white powder in 48% yield (30 mg). R_t = 20.72 min; ESI-MS calcd for $C_{37}H_{65}N_7O_{10}$ 767.48, found *m/z* $[M+H]^+$ 768.30, $[M+Na]^+$ 790.55, $[2M+Na]^+$ 1558.05.

Boc-Ile^1-Ala^2-bicyclo[3-7/8-11]-D-Alg^3-Ile^4-Ala^5-Leu^6-Alg^7-D-Alg^8-Pro^9-Gly^{10}-Alg^{11}-Lys(Boc)^{12}-OMe 25

Trifluoro acetate **14** (5.0 mg, 7.4 µmol) was dissolved in DMF (300 µL) and DIPEA (1.5 µL, 8.6 µmol) was added to obtain **14** as the free amine. To this solution, peptide acid **23** (6.0 mg, 7.7 µmol) was added followed by the addition of HOBt (2.0 mg, 14.8 µmol). The reaction mixture was cooled to 0 °C and EDCI.HCl (2.0 mg, 10.4 µmol) was added and the reaction mixture was stirred for 16 h, and allowed to warm up to room temperature. Then, the reaction mixture was diluted with a mixture of H_2O/MeCN/TFA (2 mL, 60:40:0.1 v/v/v) and peptide **25** was purified by preparative HPLC as a white fluffy powder in a yield of 30% (3.0 mg). R_t = 21.63 min; ESI-MS calcd for $C_{64}H_{109}N_{13}O_{17}$ 1331.81, found *m/z* $[M+H]^+$ 1332.70, $[M+Na]^+$ 1354.75, $[M+K]^+$ 1370.60, $[M+H\text{-Boc}]^+$ 1232.95, $[M+2H\text{-Boc}]^+$ 617.50, $[M+2Na\text{-}2Boc]^+$ 589.55.

H-Ile[1]-Ala[2]-bicyclo[3-7/8-11]-D-Alg[3]-Ile[4]-Ala[5]-Leu[6]-Alg[7]-D-Alg[8]-Pro[9]-Gly[10]-Alg[11]-Lys[12]-OMe.2TFA 2

Protected peptide **25** (2.0 mg, 1.5 μmol) was suspended in CH_2Cl_2 (250 μL) and TFA (250 μL) was added, the resulting reaction mixture was stirred at room temperature for 1 h. Then, the solvents were evaporated *in vacuo* followed by coevaporation with MeOH (2 × 1 mL), the residue was dissolved in *tert*-BuOH/H_2O (5 mL, 1:1 v/v) and subsequently lyophilized, to give peptide **2** as a white fluffy powder in quantitative yield (2.0 mg). R_t = 16.85 min; ESI-MS calcd for $C_{54}H_{93}N_{13}O_{13}$ 1131.70, found *m/z* $[M+H]^+$ 1132.85, $[M+Na]^+$ 1153.90, $[M+K]^+$ 1170.65, $[M+2H]^+$ 567.00.

native nisin 1-12 26

Yield: 6.5 mg. R_t = 24.00 min; ESI-MS calcd. $C_{51}H_{83}N_{13}O_{13}S_2$: 1149.57, found: *m/z* $[M+H]^+$ 1151.5, $[M+Na]^+$ 1172.65, $[M+2H]^{2+}$ 575.75.

native nisin 1-12-OMe 3

Native nisin 1-12 (1.5 mg, 1.7 μmol) was dissolved in MeOH (400 μL) and TFA (100 μL) was added. The reaction mixture was stirred for 16 h at room temperature and the reaction progress was followed by LC-MS. The solvents were evaporated *in vacuo* followed by coevaporation with MeOH (2 × 1 mL), and the residue was dissolved in *tert*-BuOH/H_2O (5 mL, 1:1 v/v) and subsequently lyophilized, to give peptide ester **3** as a white fluffy powder in quantitative yield (1.5 mg). R_t = 19.53 min; ESI-MS calcd for $C_{52}H_{85}N_{13}O_{13}S_2$ 1163.58, found *m/z* $[M+H]^+$ 1164.90, $[M+Na]^+$ 1186.55, $[M+2H]^+$ 582.80.

5.4.3 Vesicle leakage experiments

Carboxyfluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment as previously described.[16] The peptide-induced leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA). A solution (1.0 mL) of CF-loaded vesicles (1 μM final concentration) in buffer (50 mM tris/HCl pH = 7.0, 100 mM NaCl) was added to a quartz cuvette. Then the peptide of interest in buffer solution (5 μM final concentration) was added and fluorescence was measured (A_0). After 20 s, a buffer solution (1 μL) containing the nisin (freshly prepared stock: 200 nM; final: 0.2 nM) was added and peptide-induced membrane leakage was followed during 60 s (A_{60}), after which a buffer solution (10 μL) of Triton-X (stock: 20%; final 0.2%) was added to induce total leakage of the vesicles (A_{Total}). The % of peptide-induced leakage was calculated by: $((A_{60}-A_0)/(A_{Total})-A_0))$ x 100%. All measurements were performed in duplo.

5.5 References and Notes

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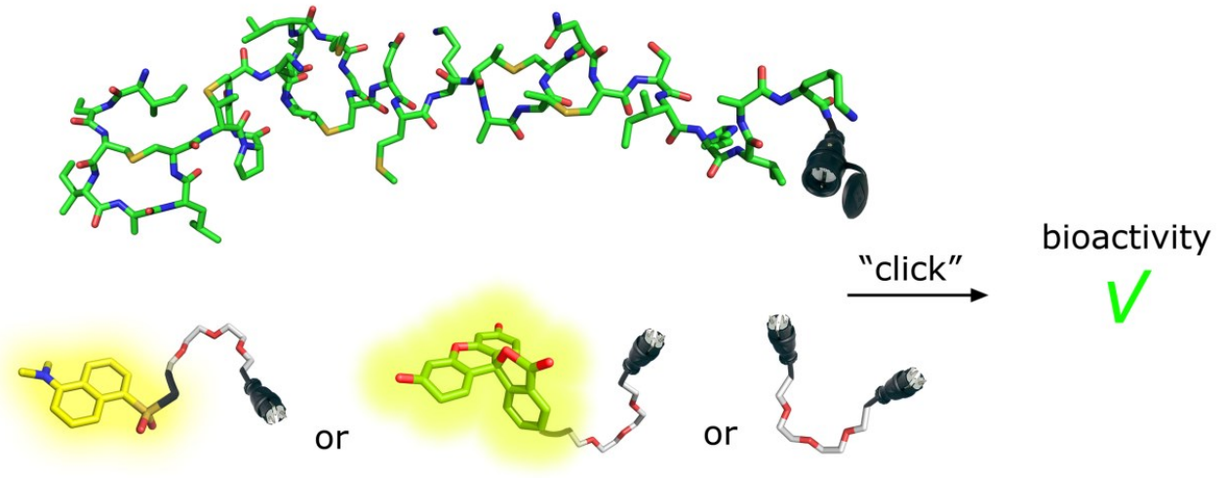
Chapter 6

Synthesis, antimicrobial activity, and membrane permeabilizing properties of C-terminally modified nisin conjugates accessed by CuAAC



Slootweg, J.C.;[‡] van der Wal, S.;[‡] Quarles van Ufford, H. C.; Breukink, E.; Liskamp, R.M.J.; Rijkers, D.T.S. *Bioconjugate Chem.* **2013**, *provisionally accepted*

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6.1 Introduction

The antimicrobial peptide nisin belongs to the class of lantibiotics,[1] a potent natural subset of antibiotic molecules characterized by specifically modified amino acids. Lantibiotics are ribosomally synthesized peptides and undergo several post-translational modifications induced by a variety of enzymes, comprising regiospecific dehydration of serine and threonine residues and subsequent stereoselective intramolecular cyclization of these dehydroamino acids with cysteine residues.[2] These post-translational modifications lead to the unique structural characteristics of lantibiotics, since the presence of dehydrated amino acids in combination with lanthionines (thio ether bridges), give these peptides a rigid conformation which is of utmost importance for their antimicrobial activity.

Nisin consists of 34 amino acid residues and has an elongated structure with an overall positive charge and contains five cyclic constrained A-, B-, C- as well as the knotted DE-ring systems (Figure 1). Nisin is a very potent antimicrobial peptide, active at nanomolar concentrations against a broad spectrum of Gram-positive bacteria, for example as a food additive (E234) and, among others, in antimicrobial materials and coatings.[3] Nisin has a two-fold mode of action. Firstly, it inhibits bacterial cell-wall synthesis, by binding of the N-terminal AB-ring fragment to lipid II, an essential precursor cross-linking molecule during the bacterial cell-wall synthesis. Secondly, after binding to lipid II, the C-terminus inserts into the phospholipid membrane forming a pore-complex, which leads to a collapse of vital ion gradients and ultimately results in bacterial cell death.[4] Although the molecular interaction of nisin and lipid II is well described, details of nisin-induced pore formation still remain elusive and to study this process, fluorescently labeled nisin derivatives are important tools for the chemical biologist.

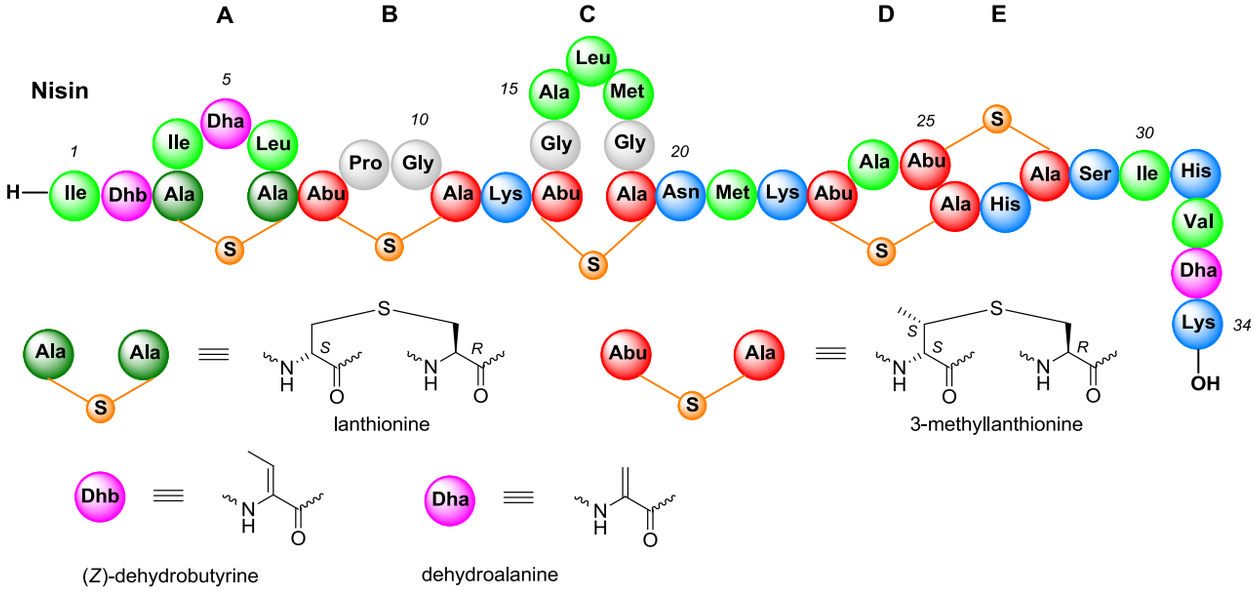


Figure 1. Schematic representation of the structure of nisin

Selective functionalization of nisin is rather difficult due to the presence of several reactive side chain functionalities. Although the total synthesis of nisin[5] and syntheses of nisin analogs[6] have been published, it is not a realistic approach to design a unique synthesis route for each single modified analog. Since nisin contains four primary amines, the N-terminal α-amino group and three ε-amines of Lys12, 22, and 34, respectively (Figure 1), the regio-selective modification of these amine functionalities is, however, far from trivial. Nevertheless, the selective N-terminal α-amine modification has been successfully performed making use of differences in pK_a-values of these amines by careful p*H* control.[7] However, a crucial feature of the antimicrobial activity of nisin resides in the binding of the N-terminal AB-ring system to lipid II and any modification of the N-terminus will interfere with this critical interaction, resulting in a significantly reduced bioactivity. Indeed, Veronese and coworkers showed that PEG-ylation of the N-terminal α-amine functionality of nisin resulted in a complete loss of antimicrobial activity.[8]

Another functional moiety within nisin as a conjugation target is bestowed by the dehydrated residues (Dhb2, Dha5 and Dha33, see Figure 1), in which the Cα-Cβ double bond in combination with the carbonyl of the amide bond forms a typical Michael acceptor and can be used in chemo-selective conjugation reactions, like thiol-ene 'click' approaches.[9] Within this context, Liu and Hansen showed that although nisin rapidly reacted with thiols, presumably via these Michael acceptors, the resulting conjugate did not show any antimicrobial activity.[10]

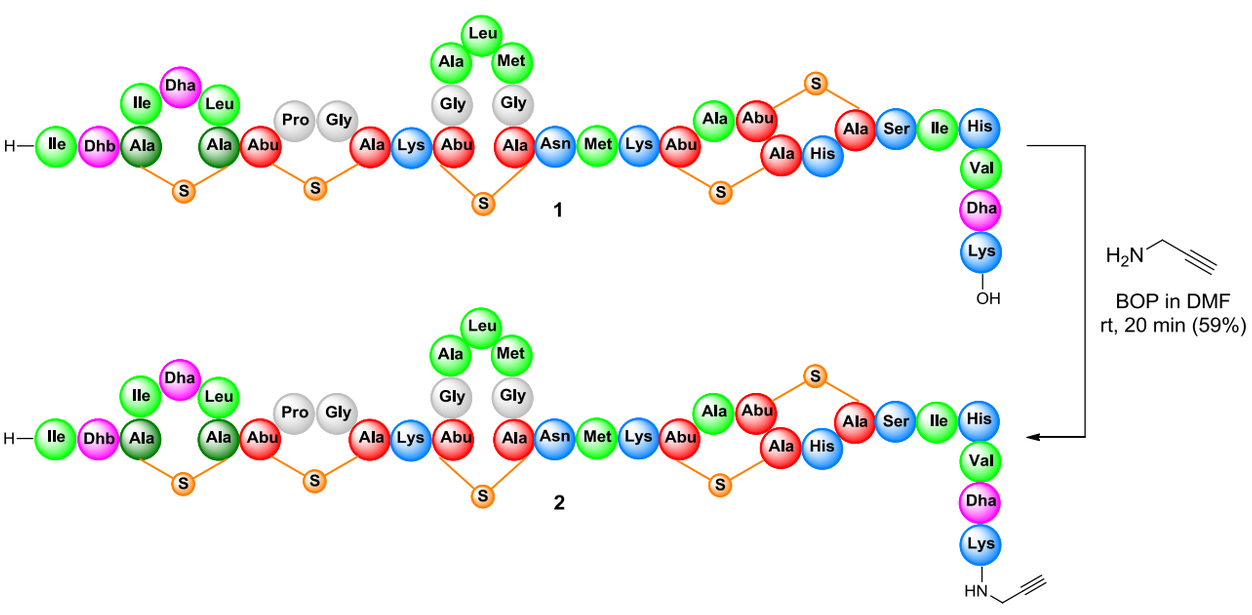
Since nisin contains only a single carboxylic acid moiety as part of the C-terminus, this functionality can be conveniently used for the regio-selective modification of the antimicrobial peptide, and several examples have been reported in literature in which fluorescent molecules[9d,11], biotin labels[12] and amino-PEG chains[8] have been used. Modification of this carboxylic acid functionality by amide bond formation is not always ideal, since often excess of relatively expensive amine derivatives have to be used. Furthermore, the need for using a large excess of amine makes the synthesis of multivalent constructs or complex conjugates such as surface tethered nisin far from trivial, as the excess of the amine may be difficult to remove from these complex molecular constructs. However, a stoichiometric amount of the amine may not be sufficient for an efficient coupling and side reactions may occur, such as self-condensation. Here we present a more convenient approach, namely the C-terminal modification of nisin with a cheap amine of which excess can be easily removed and that after coupling can be used as a bio-orthogonal conjugation handle for chemoselective coupling of the reporter molecule of choice. With respect to this, the Cu(I)-catalyzed alkyne-azide cycloaddition reaction (CuAAC)[13] is ideal, since nisin can be conveniently derivatized with propargylamine and the resulting peptide-alkyne can be conjugated in stoichiometric amounts to a variety of functionalized azides.

Thus, we describe the convenient synthesis of a C-terminally functionalized nisin-alkyne derivative and its subsequent application in Cu(I)-catalyzed cycloaddition chemistry with two fluorescent azides and a bis-azide which afforded, two fluorescently labeled nisin derivatives and a dimeric nisin construct, respectively, all in good yields (31-52%). The dimer construct was synthesized to show the potential of this strategy for multimerization of nisin. All nisin conjugates were biologically active as potent inhibitors of bacterial growth and retained their membrane permeabilizing properties.

6.2 Results and discussion

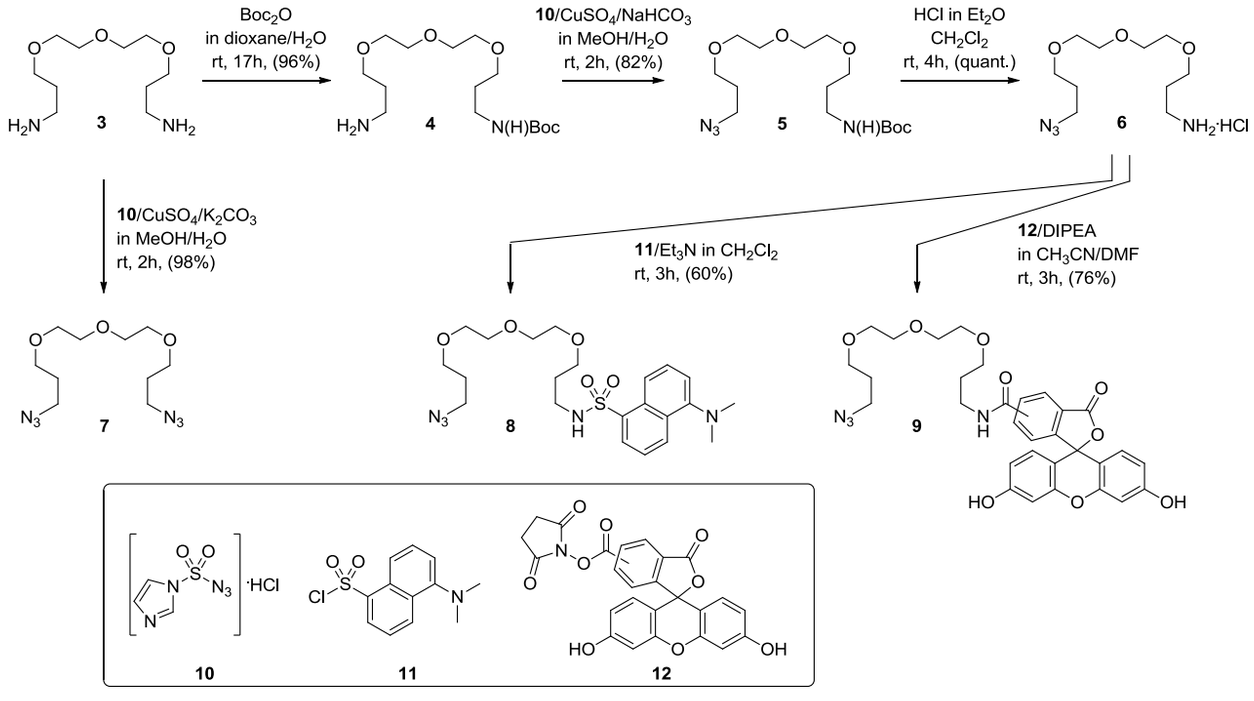
6.2.1 Synthesis

Nisin was purified from a commercial available nisin preparation, which contains only 2.5% nisin, via an extraction procedure as was recently reported.[14] Nisin **1** was used as such, this is without any protection groups and its C-terminal carboxylate was reacted with a 25-fold excess of inexpensive propargylamine in the presence of BOP as the coupling reagent (Scheme 1). This reaction can be easily scaled-up and nisin alkyne **2** was conveniently isolated by preparative HPLC to give the corresponding peptide alkyne in good yield (59%) and high purity (>90%). For labeling nisin-alkyne **2** with fluorescent reporter molecules, it was decided to synthesize the fluorescent azides **8** and **9**, (Scheme 2) representing a dansyl- and a carboxyfluorescein-derivative, respectively. A short PEG-spacer was introduced to avoid any steric hindrance and might improve the solubility of the azide derivatives.



Scheme 1. Synthesis of nisin-alkyne **2**

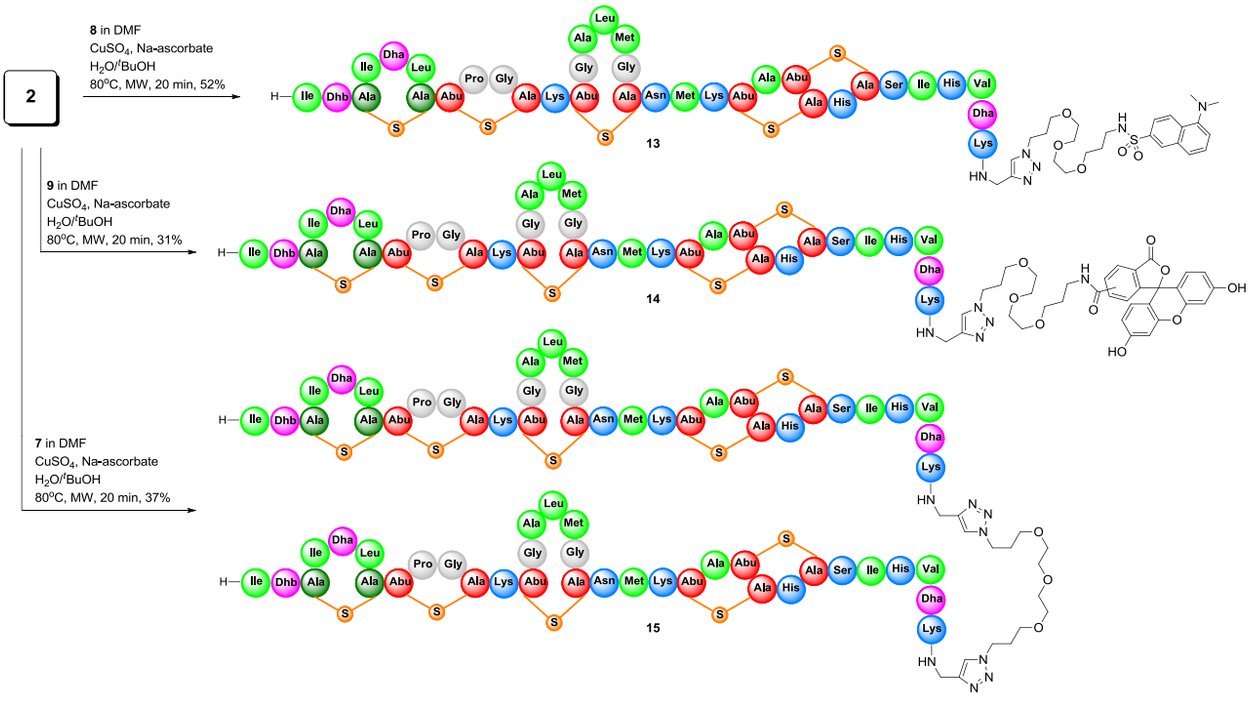
Their synthesis started with the mono-protection of *O*,*O'*-bis(3-aminopropyl)diethylene glycol **3** in the presence of Boc_2O to give carbamate **4** in excellent yield (96%). For the conversion into the corresponding azide, **4** was treated with imidazole-sulfonyl azide hydrochloride (**10**) as the diazotransfer reagent in the presence of Cu^{2+} according to Goddard-Borger and Stick,[15] and azide **5** was obtained in a high yield of 82%. After the quantitative removal of the Boc-group by treatment of **5** with a solution of dry HCl in diethyl ether, hydrochloride **6** was reacted with dansyl chloride **11** or the active *N*-hydroxysuccinimidyl ester **12** to give dansyl-azide **8** and carboxyfluorescein-azide **9**, in a yield of 60 and 76%, respectively, as shown in Scheme 2. In parallel with these syntheses, bis-azide **7** was prepared from bis-amine **3** in the presence of **10**/Cu^{2+} in a nearly quantitative yield (98%).



Scheme 2. Synthesis of azides **7 – 9**

With the azides **7**-**9** in hand, the stage was set to couple them to nisin-alkyne **2** via CuAAC-based bioconjugation (Scheme 3). In a first attempt, dansyl-azide **8** was reacted with alkyne **2** by using 0.1 equiv $CuSO_4$ and 0.5 equiv sodium ascorbate in *tert*-BuOH/H_2O at 80 °C by microwave irradiation for 20 min.[16] Under these reaction conditions, incomplete conversion of the starting materials was observed by analytical HPLC (data not shown). Prolonging the reaction time to 60 min, did not increase the conversion, instead, nisin degradation products were observed. Gratifyingly, increasing the number of equivalents of $CuSO_4$ and sodium ascorbate, improved the conversion, and best results were obtained when 2 equiv $CuSO_4$ and 5 equiv sodium ascorbate were used, and heating the reaction mixture to 80 °C by microwave irradiation for 20 min. These

optimized reaction conditions were applied, and nisin-alkyne **2** was conjugated to dansyl-azide **8** and carboxyfluorescein-azide **9** to give, after preparative HPLC, the fluorescent nisin conjugates **13** and **14**, respectively, in good to acceptable yields (52 and 31%) and in excellent purity. The same protocol was used for the synthesis of nisin dimer **15** in which bis-azide **7** was reacted with alkyne **2** and conjugate **15** was isolated in 37% yield as a single peak according to analytical HPLC.



Scheme 3. Synthesis of nisin-dansyl **13**, nisin-CF **14**, and nisin dimer **15**, *via* Cu(I)-catalyzed click chemistry.

6.2.2 Growth Inhibition Assays

As a first test to evaluate the biological activity of the newly synthesized nisin-analogs, a growth inhibition assay was performed. Two different Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, were used and the minimal inhibitory concentration (MIC) was determined for each construct using a broth micro-dilution assay,[17] and the obtained MIC-values are given in Table 1. As the positive control, nisin **1** (purified by preparative HPLC) was used. Generally, the obtained MIC-values of the nisin conjugates **2**, and **13**-**15** were in the same range as nisin. Interestingly, the activity of nisin-dansyl **13** was slightly improved (one dilution-step (1:3) lower) against *B. subtilis*, while the activity of nisin-CF **14** was approximately 10-fold less against *S. aureus* (Table 1). These results showed that C-terminal modifications were well-tolerated since the found MIC-values were in the same range as unmodified nisin. The importance of C-terminal

fluorescently labeled nisin analogs[9] as molecular tools to shed more light on its mechanism of action has been recently shown in studies by Scherer *et al.*[11a] and Desobry *et al.*[11b]

Table 1. Biological activities of nisin and the synthesized nisin conjugates

Peptide	MIC (μM)[a]	
	B. subtilis	*S. aureus*
Nisin (**1**)	0.62	5.56
Nisin-alkyne (**2**)	0.62	5.56
Nisin-dansyl (**13**)	0.21	5.56
Nisin-CF (**14**)	1.85	50
Nisin-dimer (**15**)	1.85	5.56

[a]Antimicrobial activity is expressed as the Minimal Inhibitory Concentration (MIC).[17]

6.2.3 Membrane Leakage Assays

In a second biochemical assay, nisin conjugates **2**, and **13**-**15** were tested for their interaction with model membrane systems.[19] For this purpose, large unilamellar vesicles (LUVs) were loaded with carboxyfluorescein (CF) as the fluorophore, and the ability of the nisin conjugates to induce membrane permeability was measured by monitoring the release of CF by fluorescence spectroscopy. LUVs composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a zwitterionic lipid mimicking mammalian membranes, spiked with 0.2% lipid II as the natural target of nisin, were treated with nisin **1** (at 1 nM) and ~46% of CF leakage was observed (as shown in Figure 2). Membrane lysis induced (at 1 nM) by nisin-alkyne **2**, nisin-dansyl **13**, nisin-CF **14**, and nisin-dimer **15** was found to be ~49, ~31, ~16, and ~51% respectively. Interestingly, a slightly increased pore-formation activity was observed for nisin-dimer **15**, while the fluorescently labeled nisin conjugates **13** and **14** induced less CF-leakage, compared to nisin **1**.

Nisin's mode of action (*vide supra*) is based on the interaction with lipid II followed by pore formation. This pore-complex is believed to be a specific complex formed by eight nisin and four lipid II molecules in a 2:1 nisin:lipid II stoichiometry.[4c] Based on this model, nisin dimer **15** was designed and it was expected that this dimer should be more active than the nisin monomer due to multivalency.[18] Apparently, formation of the nisin pore-complex did not benefit from the presence of nisin dimer **15** since both monomer as dimer were equally active expressed as their MIC values. Nevertheless, retention of leakage activity was observed despite the increased steric hindrance caused by dimerization.

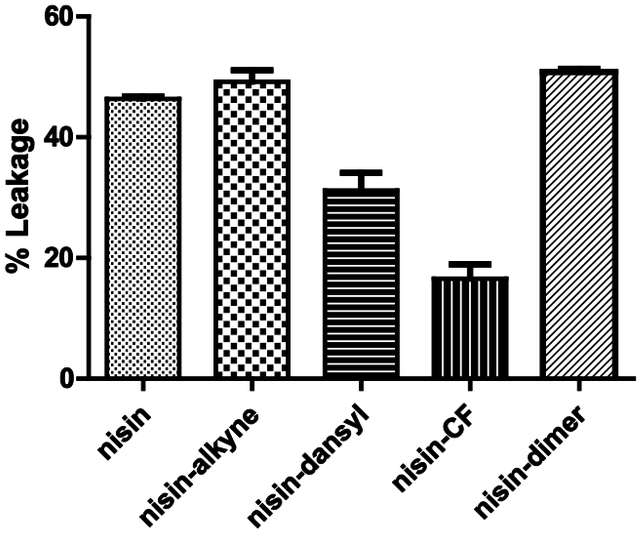


Figure 2. Lysis of large unilamellar vesicles expressed in percentage of leakage induced by nisin and nisin-conjugates.

As mentioned above, nisin binds lipid II embedded in the bacterial cell membrane via the N-terminal AB-ring fragment, and directs its C-terminal part into the phospholipid membrane to form pores and thereby lyse the bacterial cell. However, there are nisin mutants with an altered or even truncated C-terminus,[20] and other related lantibiotics like mutacin 1140, mutacin B-Ny266 and mersacidin,[4e] with a similar AB-ring fragment as nisin, that do not have pore-forming abilities, but still are potent antibiotics. This alternative mechanism of antimicrobial activity has been unraveled by Breukink *et al.*[4d] since lantibiotics with a functional AB-ring fragment bind lipid II and thereby interfere with cell division and act as a bactericidal agent to kill bacteria. Generally, the newly synthesized nisin conjugates **2**, and **13-15** retained their biological activity compared to nisin **1**. However, subtle differences in activity may be explained by a combined interpretation of the measured MIC-values (Table 1) and the membrane permeabilization assay (Figure 2). Most pronounced in this case was the reduced activity of nisin-CF **14** since a ten-fold lower activity against *S. aureus* as well as the lowest pore-forming capacity might suggest that the C-terminal modification with a carboxyfluorescein moiety interfered with lipid II binding and membrane insertion.

6.3 Conclusions

In conclusion, a versatile synthesis route toward C-terminally functionalized nisin derivatives was developed. The introduction of an alkyne moiety enabled access to a wide variety of nisin conjugates, since a diverse set of functionalized azides can by efficiently coupled via the Cu(I)-catalyzed alkyne-azide cycloaddition reaction. The newly synthesized nisin conjugates were active in a bacterial growth inhibition assay as well as in a membrane permeabilizing assay. The retained

biological activities suggest that these nisin conjugates can be used as –conveniently accessible– fluorescent probes as molecular tools to increase the insight of the mechanistic details to understand the mode of action of nisin and other related lantibiotic peptides. Moreover, the synthesis of well-defined covalent nisin dimers has been demonstrated for the first time and it might prove interesting to investigate different orientation and multivalency patterns of the nisin constructs to improve antimicrobial activity. Furthermore, to address the interest in surface modification with antimicrobial peptides, the C-terminal conjugation of nisin-azide to alkyne-functionalized surfaces or coatings is highly recommended.

6.4 Experimental

2.4.1 Chemicals, instruments and general methods

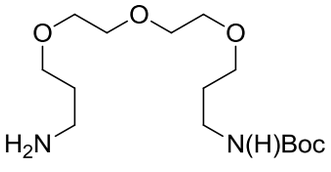
Unless stated otherwise, all chemicals were obtained from commercial sources and used without further purification. The active ester, 5(6)-carboxyfluorescein-ONSu (**12**), was purchased from Thermo Scientific (Breda, The Netherlands). All reactions were performed at ambient temperature and under an air atmosphere, unless stated specifically at each entry. *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF) and HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and used as supplied, with the exception of DMF and CH_2Cl_2, which were dried over molecular sieves (4 Å) prior to use. Progress of reactions was monitored by TLC on Merck precoated silica gel 60F254 glass plates. Spots were visualized by UV quenching and staining with ninhydrin. Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40-63 μm). ^{1}H NMR spectra were acquired on a Varian Mercury 300 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m), and broad (br). ^{13}C-NMR data were acquired on a Varian Mercury 75.5 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal $CDCl_3$ (77.0 ppm). Microwave reactions were performed in a Biotage Initiator microwave reactor, equipped with a temperature and pressure control, in sealed vessels of 0.5-2 mL. Analytical HPLC was performed on a Shimadzu automated HPLC system equipped with a UV/Vis detector operating at 220/254 nm. Preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/VIS absorbance detector. The buffer system for HPLC consisted of buffer A: 0.1% TFA in MeCN/H_2O 5:95 v/v and buffer B: 0.1% TFA in MeCN/H_2O 95:5 v/v. For analytical HPLC a flow rate of 1.0 mL/min with a linear gradient of buffer A to buffer B (60% in 20 min) was used with a total run time of 40 min using an Alltech C8 Alltima column (pore size: 100 Å, particle size: 5 μm, 250 × 4.6 mm). Preparative runs used a flow rate of 12 mL/min with a linear gradient of buffer B

(60% in 60 min) from 100% buffer A with a total runtime of 80 min using an Alltech C18 Prosphere column (pore size: 300 Å, particle size: 10 µm, 250 × 22 mm). Semi-preparative HPLC runs were performed at a flow rate of 5 mL/min with a linear gradient of buffer B (60% in 60 min) from 100% buffer A with a total runtime of 80 min using a Grace HP C18 Prosphere column (pore size: 300 Å, particle size: 10 µm, 250 × 10 mm). Peptides were characterized using ElectroSpray Ionization Mass Spectrometry (ESI-MS) on a Shimadzu QP8000 single quadrupole mass spectrometer in a positive ionization mode. Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) MS spectra were recorded on a Shimadzu Axima-CFR with α-cyano-4-hydroxycinnamic acid as the matrix, while human ACTH(18-39) was used as an external reference (monoisotopic $[M+H]^+$ 2465.198).

2.4.2. Synthesis

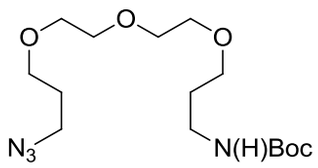
Nisin-alkyne 2

Nisin **1**[14] (250 mg, ~25 µmol) was suspended in DMF (1000 µL) and to this brown suspension, propargylamine (120 µL, 1.88 mmol, 25 equiv) and BOP (33.2 mg, 75.0 µmol) were added and the obtained reaction mixture was shaken for 20 min. Then, the reaction mixture was neutralized by the drop-wise addition of aq. 1M HCl and after evaporation of the solvents *in vacuo*, the residue was dissolved in HPLC buffer (2 mL, A/B 3:1 v/v) and subsequently purified by preparative HPLC. After lyophilization of the pure fractions, nisin-alkyne **2** was obtained as a white fluffy powder with 59% yield (50 mg). R_t: 23.93 min; MALDI-TOF-MS calcd. for $C_{146}H_{233}N_{43}O_{36}S_7$: 3388.58, found *m/z* 3390.11 $[M+H]^+$, 3412.06 $[M+Na]^+$.



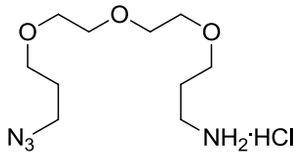
***O*-(3-aminopropyl)-*O*'-(*N*-Boc-aminopropyl)diethylene glycol 4**

A solution of Boc_2O (3.4 g, 15.6 mmol) in dioxane (100 mL) was added drop-wise to a solution of *O*,*O'*-bis(3-aminopropyl)diethylene glycol **3** (24.0 g, 109.2 mmol) in dioxane (100 mL) and the reaction mixture was stirred for 17 h. After removing the solvent *in vacuo*, the residual oil was dissolved in water (150 mL) and filtered over HyFlo to remove the bis-carbamate side-product. The water layer was extracted with EtOAc (3 × 150 mL) and the combined organic layers were dried (Na_2SO_4), filtrated, and concentrated *in vacuo* to give carbamate **4** as a colorless syrup with an excellent yield of 96% (4.8 g, 14.92 mmol). ^{1}H NMR (300 MHz, D_2O) δ = 3.54 (s, 8H, O-CH_2 (4 × 2H)), 3.47 (t (*J* = 6.6 Hz), 4H, ~O-C*H*$_2$-CH_2-CH_2~), 3.01 (t (*J* = 6.7 Hz), 2H, C*H*$_2$-N(H)Boc), 2.55 (t (*J* = 7.0 Hz), 2H, C*H*$_2$-NH_2), 1.61 (m, 4H, ~O-CH_2-C*H*$_2$-CH_2~), 1.30 (s, 9H, Boc). This ^{1}H-NMR spectrum was in agreement with the reported literature data.[21]



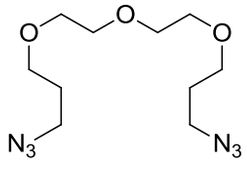
***O*-(3-azidopropyl)-*O*'-(*N*-Boc-aminopropyl)diethylene glycol 5**

Amine **4** (1.75 g, 5.5 mmol) was dissolved in MeOH (50 mL). Then, a solution of $CuSO_4 \cdot 5H_2O$ (27 mg, 0.11 mmol) and $NaHCO_3$ (1.40 g, 16.4 mmol) in H_2O (25 mL) was subsequently added to the solution of **4** in MeOH and a clear blue reaction mixture was obtained. The diazotransfer reagent imidazole-sulfonyl azide hydrochloride **10**[33] (1.40 g, 6.55 mmol) was added portion-wise and the conversion from amine **4** into azide **5** was complete after 2 h of stirring according to TLC analysis. Subsequently, the pale green reaction mixture was concentrated *in vacuo* to approximately 25 mL and the aqueous layer was extracted with EtOAc (3 × 20 mL). The combined organic layers were subsequently washed with aq. 1M $KHSO_4$ (3 × 40 mL), aq. 5% $NaHCO_3$ (3 × 40 mL) and brine (3 × 40 mL), dried (Na_2SO_4), filtrated, and concentrated under reduced pressure to give azide **5** in high yield (82%) as a colorless oil (1.55 g, 4.5 mmol). R_f (MeOH/$CHCl_3$/AcOH 89:10:1 v/v/v): 0.67; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.02 (s, 1H, N(*H*)Boc), 3.72-3.49 (m, 12H, O-CH_2 (6 × 2H)), 3.40 (t (*J* = 6.7 Hz), 2H, C*H*$_2$-N_3), 3.22 (m, 2H, C*H*$_2$-NHBoc), 1.96-1.81 (m, 2H, ~CH_2-C*H*$_2$-CH_2-N_3), 1.75 (m, 2H, ~CH_2-C*H*$_2$-CH_2-N(H)Boc), 1.44 (s, 9H, Boc); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 156.1, 78.9, 70.5, 70.5, 70.3, 70.2, 69.5, 67.9, 48.4, 38.4, 29.6, 29.0, 28.4.



***O*-(3-azidopropyl)-*O*'-(aminopropyl)diethylene glycol hydrochloride 6**

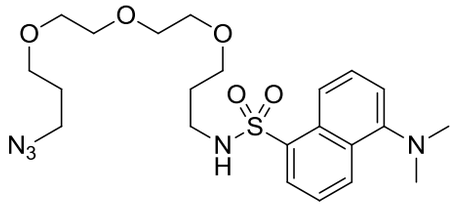
Boc-protected amine **5** (1.55 g, 4.48 mmol) was dissolved in CH_2Cl_2 (40 mL) and a saturated solution of dry HCl in Et_2O (20 mL) was added. Boc-removal was complete after 4 h of stirring and the reaction mixture was subsequently evaporated to dryness. The residue was coevaporated with MeOH (3 × 10 mL) and CH_2Cl_2 (3 × 10 mL) to remove any residual acid and hydrochloride **6** was obtained in quantitative yield (1.26 g). R_f (CH_2Cl_2/MeOH 9:1 v/v): 0.16; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.11 (s, 3H, ~N*H*$_2$·*H*Cl), 3.63-3.52 (m, 8H, O-CH_2 (4 × 2H)), 3.47 (m, 4H, ~O-C*H*$_2$-CH_2-CH_2~ (2 × 2H)), 3.35 (m, 2H, C*H*$_2$-N_3), 3.09 (m, 2H, C*H*$_2$-NH_2·HCl), 1.98 (m, 2H, ~CH_2-C*H*$_2$-CH_2-N_3), 1.77 (m, 2H, ~CH_2-C*H*$_2$-CH_2-NH_2·HCl); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 70.7, 70.5, 70.4, 70.2, 69.3, 68.1, 48.6, 39.0, 29.2, 27.1.



***O,O'*-bis(3-azidopropyl)diethylene glycol 7**

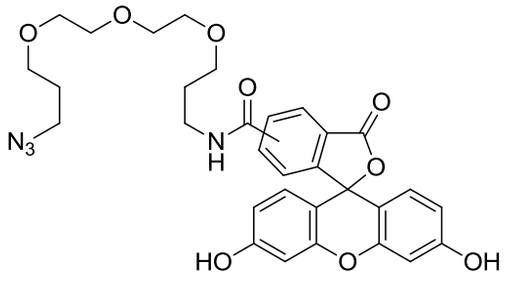
O,*O*'-Bis(3-aminopropyl)diethylene glycol (**3**) (1.10 g, 5.0 mmol) was dissolved in MeOH (40 mL) and a solution containing $CuSO_4$ (15 mg, 0.05 mmol) and K_2CO_3 (2.07 g, 15.0 mmol) in H_2O (20 mL) was added resulting in a clear blue reaction mixture. Then, imidazole-sulfonyl azide hydrochloride **10**[33] (2.51 g, 12.0

mmol) was added portion-wise and the diazotransfer reaction was complete after 2 h. The greenish reaction mixture was concentrated to approximately 20 mL by evaporation and the residue was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with aq. 1M $KHSO_4$ (3 × 50 mL) followed by brine (2 × 50 mL), dried (Na_2SO_4), filtrated and evaporated under reduced pressure to give the bis-azide **7** as a yellowish oil in 98% yield (1.33 g, 4.9 mmol). R_f (CH_3Cl_3/MeOH/AcOH 89:10:1 v/v/v): 0.88; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.69-3.59 (m, 8H, O-CH_2 (4 × 2H)), 3.55 (t (*J* = 6.0 Hz), 4H, ~O-C*H*$_2$-CH_2-CH_2~ (2 × 2H)), 3.40 (t (*J* = 6.5 Hz), 4H, C*H*$_2$-N_3 (2 × 2H)), 1.86 (quintet (*J* = 6.3 Hz), 4H, ~*O*-CH_2-C*H*$_2$-CH_2~ (2 × 2H)); ^{13}C NMR (75 MHz, $CDCl_3$) δ 70.3, 70.1, 67.7, 48.2, 29.0.



***O*-(3-azidopropyl)-*O*'-(*N*-dansyl-aminopropyl)diethylene glycol 8**

Hydrochloride **6** (1.0 g, 3.5 mmol) was dissolved in CH_2Cl_2 (30 mL) and to this solution Et_3N (1.1 mL, 8.0 mmol) followed by dansyl chloride (**11**) (1.0 g, 3.8 mmol) were added, and the obtained reaction mixture was allowed to stir for 3 h. Subsequently, the reaction mixture was concentrated *in vacuo,* and the residue was redissolved in EtOAc (50 mL) and this solution was washed with aq. sat. NH_4OAc (2 × 30 mL). Then, the EtOAc solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo* and the resulting residual oil was purified by column chromatography using a gradient of acetone (2.5% → 10% v/v) in CH_2Cl_2/Et_3N (100:0.1 v/v) as the eluens to afford azide **8** as a yellow oil in 60% yield (1.0 g, 2.1 mmo). R_f ($CHCl_3$/MeOH/AcOH 89:10:1 v/v/v): 0.75; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.53 (d (*J* = 8.5 Hz), 1H, dansyl-CH), 8.32 (d (*J* = 8.6 Hz), 1H, dansyl-CH), 8.24 (d (*J* = 7.3), 1H, dansyl-CH), 7.53 (m, 2H, dansyl-CH), 7.18 (d (*J* = 7.5 Hz), 1H, dansyl-CH), 5.66 (s, 1H, SO_2N*H*), 3.63 (m, 6H), 3.57-3.30 (m, 8H), 3.03 (m, 2H, C*H*$_2$-$NHSO_2$), 2.89 (s, 6H, $N(CH_3)_2$), 1.83 (quintet (*J* = 6.3 Hz), 2H, ~CH_2-C*H*$_2$-CH_2-N_3), 1.66 (m, 2H, ~CH_2-C*H*$_2$-CH_2-$NHSO_2$~); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 151.9, 135.0, 130.1, 129.9, 129.7, 129.4, 128.2, 123.2, 119.1, 115.1, 70.6, 70.5, 70.3, 70.1, 70.0, 67.8, 48.5, 45.4, 42.2, 29.1, 28.6.



***O*-(3-azidopropyl)-*O*'-(*N*-(5(6)-carboxyfluoresceinyl)-aminopropyl)diethylene glycol 9**

Hydrochloride 6 (90 mg, 0.32 mmol) was dissolved in CH_3CN/DMF (10 mL, 9:1 v/v) and to this solution, DIPEA (220 µL, 1.26 mmol) followed by active ester 5(6)-carboxyfluorescein-ONSu **12** (100 mg, 0.21 mmol) were added. The reaction mixture was stirred for 3 h and subsequently concentrated *in vacuo*. The

residue was redissolved in EtOAc (10 mL) and the solution was washed with aq. 1M $KHSO_4$ (2 × 8 mL). The EtOAc solution was dried (Na_2SO_4), filtrated and evaporated under reduced pressure and the obtained residue was further purified by column chromatography (CH_2Cl_2/MeOH 92.5:7.5 v/v) to give compound **9** as a greenish oil in 76% yield (94 mg, 0.16 mmol). R_f ($CHCl_3$/MeOH/AcOH 89:10:1 v/v/v): 0.40; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD 95:5 v/v) δ = 8.35 (s), 8.10 (d (*J* = 8.0 Hz)), 7.98 (s, 1H, NH), 7.87 (m), 7.75 (m), 7.58 (s), 7.16 (d (*J* = 7.9 Hz)), 6.74-6.55 (m, 2H), 6.55-6.37 (m, 4H), 4.46 (broad s, 2H, ~OH), 3.68-3.30 (m, 14H, O-CH_2 (6 × 2H)/NH-CH_2), 3.25 (m, 2H, N_3-CH_2), 1.86-1.70 (m, 4H, ~CH_2-CH_2-CH_2~); ^{13}C NMR (75.5 MHz, $CDCl_3$/CD_3OD 95:5 v/v) δ = 169.4, 169.3, 166.4, 160.4, 152.9, 152.8, 140.6, 136.3, 134.1, 129.2, 127.7, 125.6, 125.0, 123.9, 123.3, 113.2, 109.9, 103.0, 70.3, 70.0, 69.9, 69.7, 67.9, 48.3, 38.8, 28.9, 28.8, 28.7, 28.4.

Nisin-dansyl 13

A stock solution of dansyl-azide **8** (3.8 mg, 7.9 μmol) in DMF (144 μL) and a stock solution of $CuSO_4$·$5H_2O$ (13.2 mg, 52.9 μmol) in H_2O (2.64 mL) were prepared. Then, nisin-alkyne **2** (1.7 mg, 0.5 μmol) was dissolved in *tert*-BuOH/H_2O (450 μL, 1:1 v/v) and to this solution, an aliquot of the dansyl-azide stock solution (10 μL, 0.55 μmol, 1.10 equiv) and $CuSO_4$ stock solution (50 μL, 1.00 μmol, 2.0 equiv), respectively were added, followed by the addition of solid sodium ascorbate (0.49 mg, 2.5 μmol, 5.0 equiv). This reaction mixture was heated to 80 °C by means of microwave irradiation during 20 min. Subsequently, the reaction mixture was diluted with buffer A (1.5 mL) and the product was purified using semi-preparative HPLC. The pure fractions were identified by analytical HPLC and lyophilized to obtain nisin conjugate **13** as a white fluffy powder in 52% overall yield (1.0 mg). R_t 24.47 min; MALDI-TOF-MS calcd. for $C_{168}H_{266}N_{48}O_{41}S_8$: 3867.80, found *m/z* 3869.53 $[M+H]^+$, 3891.42 $[M+Na]^+$, 3906.25 $[M+K]^+$.

Nisin-CF 14

A stock solution of compound **9** (2.6 mg, 4.2 μmol) in DMF (76 μL) and a stock solution of $CuSO_4$·$5H_2O$ (18.3 mg, 73.3 μmol) in H_2O (3.66 mL) were prepared. Then, nisin-alkyne **2** (1.7 mg, 0.50 μmol) was dissolved in *tert*-BuOH/H_2O (450 μL, 1:1 v/v) and to this solution, an aliquot of the stock solution containing compound **9** (10 μL, 0.55 μmol, 1.10 equiv), $CuSO_4$ stock solution (50 μL, 1.00 μmol, 2.0 equiv), respectively were added, followed by the addition of solid sodium ascorbate (0.49 mg, 2.50 μmol, 5.0 equiv). The obtained reaction mixture was heated to 80 °C by microwave irradiation during 20 min. Then, the reaction mixture was diluted with buffer A (1.5 mL) and the product was purified using semi-preparative HPLC. The pure fractions were identified by analytical HPLC and lyophilized to obtain nisin conjugate **14** as a yellowish fluffy powder in

31% overall yield (0.6 mg). R_t 24.93 min; MALDI-TOF-MS calcd. for $C_{177}H_{265}N_{47}O_{45}S_7$: 3992.79, found *m/z* 3993.5 $[M+H]^+$, 4014.82 $[M+Na]^+$, 4029.92 $[M+K]^+$.

Nisin-dimer 15

A stock solution of bis-azide **7** (3.2 mg, 11.8 μmol) in DMF (235 μL) and a stock solution of $CuSO_4{\cdot}5H_2O$ (16.4 mg, 65.7 μmol) in H_2O (1.64 mL) were prepared. Then, nisin-alkyne **2** (3.4 mg, 1.0 μmol, 1.0 equiv) was dissolved in *tert*-BuOH/H_2O (450 μL, 1:1 v/v) and to this solution an aliquot of the bis-azide stock solution (10 μL, 0.50 μmol, 1.0 equiv of azide) and $CuSO_4$ stock solution (50 μL, 2.0 μmol, 2.0 equiv), respectively were added, followed by the addition of solid sodium ascorbate (0.99 mg, 5.0 μmol, 5.0 equiv). The obtained reaction mixture was heated to 80 °C by microwave irradiation during 20 min. Then, the reaction mixture was diluted with buffer A (1.5 mL) and the product was purified using semi-preparative HPLC. The pure fractions were identified by analytical HPLC and lyophilized to obtain nisin dimer **15** as a white fluffy powder in 37% overall yield (1.3 mg). R_t 24.60 min; MALDI-TOF-MS calcd. for $C_{302}H_{486}N_{92}O_{75}S_{14}$: 7054.60 (average mass), found *m/z* 7055.5 $[M+H]^+$, 7077.3 $[M+Na]^+$, 7093.0 $[M+K]^+$.

6.4.3 Biological Evaluation

Growth inhibition assay

Strains used for determination of antimicrobial activity included the American Type Culture Collection (ATCC) strain *Staphylococcus aureus* ATCC 259923 and *Bacillus subtilis*. The minimal inhibitory concentration (MIC) of each peptide was determined using a broth micro-dilution assay adapted from a literature procedure as previously described by Hancock.[17] Peptide stock solutions were prepared at a concentration of 100 μM peptide in 0.2% bovine serum albumin (BSA) and 0.01% acetic acid. Serial three-fold dilutions of the peptides were made in 0.2% BSA and 0.01% acetic acid. Two wells were filled with 50 μL of the test bacterium in Mueller-Hinton broth (for *S. aureus*) or in tryptic soya broth (for *B. subtilis*) to a final concentration of 2×10^6 CFU/mL and 50 μL of the peptide solution with a different concentration was added, while the third well was filled with medium (without bacteria) as the negative control. The measured samples had a final concentration of 50 μM, 16.7 μM, 5.56 μM, 1.85 μM, 0.62 μM, 0.21 μM, and 0.07 μM. After incubation for 24 h at 37 °C at 120 rpm in a Certomat incubator, the OD at 630 nm was measured. The MIC (expressed in μM) of each peptide was read as the lowest concentration of peptide that was able to inhibit visible bacterial growth. All measurements were performed in duplicate.

Vesicle Leakage Experiments

Carboxyfluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment according to a literature procedure.[19] The peptide-induced leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA). A solution (1.0 mL) of CF-loaded vesicles (20 μM final concentration) in buffer (50 mM TRIS-HCl, pH 7.0 containing 100 mM NaCl) was added to a quartz cuvette and fluorescence was measured (A_0). After 20 s, a sample solution containing the peptide of interest (1 μL from a freshly prepared stock solution of 1 μM resulted in a final concentration of 1 nM) was added and peptide-induced membrane leakage was followed during 60 s (A_{60}), after which a stock solution of Triton-X (10 μL of a stock solution (20%) resulted in a final concentration of 0.2%) was added to induce total leakage of the vesicles (A_{Total}). The % of peptide-induced leakage was calculated by: $((A_{60} - A_0)/(A_{Total} - A_0)) \times 100\%$. Each nisin-conjugate was measured in two independent experiments and the given leakage value is an average of the two determinants.

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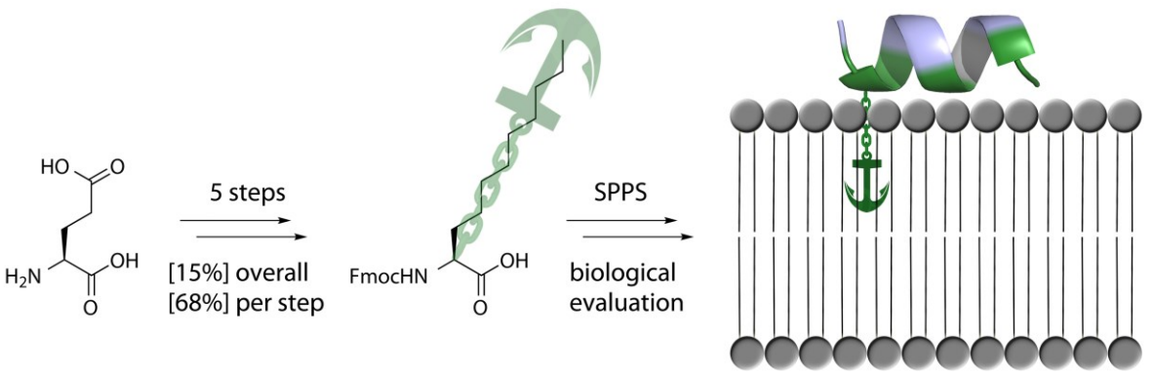
Chapter 7

Improving the biological activity of the antimicrobial peptide anoplin by membrane anchoring through a lipophilic amino acid derivative



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7.1 Introduction

There is a growing need for the development of novel antibiotics, or for improving upon existing ones, as established antibiotic compounds continue to lose ground in the struggle against resistant bacteria. Of particular concern are the Methicillin resistant *Staphyloccous aureus* (MRSA) and vancomycin resistant *Enterococcus faecium* (VRE) infections that continue to rise. Clearly compounds active against these resistant classes are urgently required.[1]

The recently discovered host-defensive antimicrobial peptides (AMPs), have considerable promise as novel antibacterial agents.[2] Their mode of action is (target-unspecific) permeabilization of bacterial membranes which induces little stable resistance, since it is very difficult for a bacterium to change its membrane composition in order to counteract the activity of AMPs. This might explain the great potential of AMPs as lead compounds for the development of the next generation peptide-based antibiotic drug molecules, like the FDA-approved lipopeptides Caspofungin **1**[3] and Daptomycin **2**,[4] which are active against fungal and bacterial infections, respectively (Figure 1).

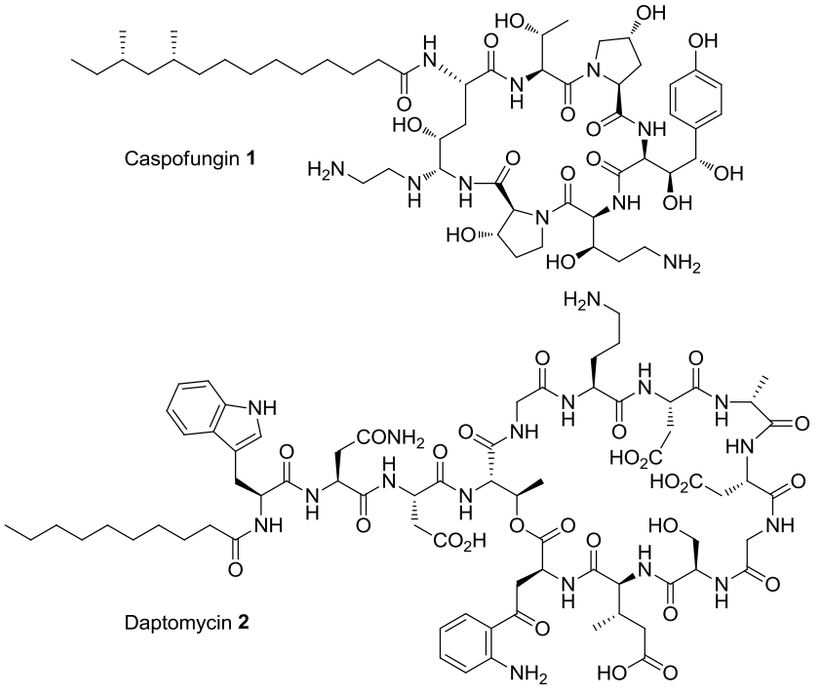


Figure 1. Structural formula of Caspofungin **1** and Daptomycin **2**.

Lipopeptides form a subclass of antimicrobial peptides in which a lipophilic alkyl chain acts as a membrane anchor. The length of the alkyl chain is highly important for the bioactivity, since analogs with a truncated alkyl chain show a dramatic decrease in antimicrobial activity.[5,6] Acylation of the N-terminal α-amino functionality is a well-known approach to increase membrane affinity.[7-12] However, such *N*-acylation results in a non-charged amino terminus, while a positively charged

N-terminus, in combination with a peptide sequence that is rich in arginine and lysine residues, is often important for activity and selectivity.[10] This is especially the case when the peptide is meant to interact with negatively charged bacterial membranes, and not with overall neutral mammalian membranes.

To increase the membrane affinity for membrane-acting antimicrobial peptides, without sacrificing these important positively charged backbone/side chain functionalities, a lipophilic amino acid derivative ((*S*)-2-aminoundecanoic acid) has been designed and synthesized that can be incorporated at any position of the peptide sequence. Herein, we report that the antimicrobial decapeptide anoplin, H-Gly^{1}-Leu^{2}-Leu^{3}-Lys^{4}-Arg^{5}-Ile^{6}-Lys^{7}-Thr^{8}-Leu^{9}-Leu^{10}-NH_2, was modified at residues Leu^{2}, Ile^{6}, and Leu^{10}, with the lipophilic amino acid residue, and that these anoplin derivatives were found to be ten times more active compared to native anoplin, while their selectivity towards microbial membranes remained unaffected.

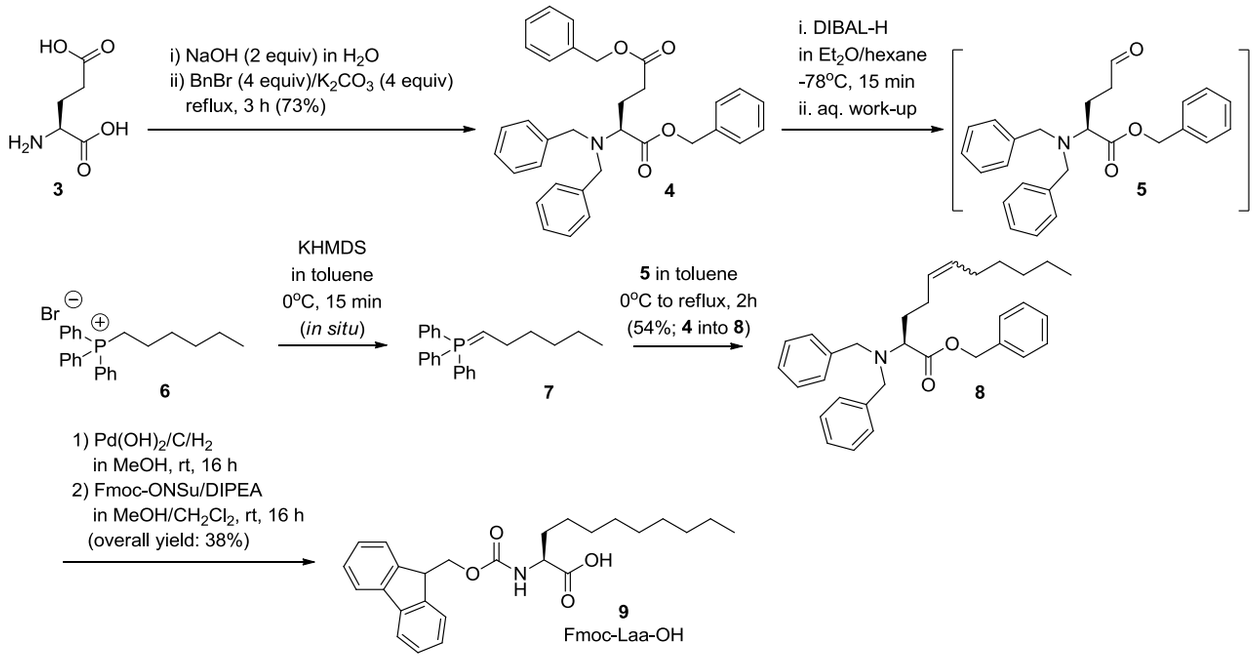
7.2 Results and discussion

7.2.1 Synthesis

The lipophilic amino acid derivative (*S*)-2-aminoundecanoic acid was synthetically accessible from L-glutamic acid **3** in only four steps, as shown in Scheme 1.[13,14] To this end, **3** was treated with benzyl bromide under basic conditions[15] to obtain the *N*,*N*,*O*,*O*-perbenzylated Bzl-*N*(Bzl)Glu(OBzl)-OBzl derivative **4** in a good yield of 73%. Then, diester **4** was subjected to a controlled DIBAL-H mediated reduction, according to the method of Martín *et al.*,[16] to afford aldehyde **5**. To check the presence of the aldehyde functionality, the crude reaction product was analyzed by ^{1}H NMR ($CDCl_3$, 300 MHz) and the aldehyde peak could be identified at δ 9.58 ppm, and aldehyde **5** was used without further purification, in the subsequent Wittig reaction.

In a separate flask, hexyltriphenylphosphonium bromide **6** was treated with hexamethyldisilazane potassium salt (KHMDS) in toluene to generate *in situ* ylide **7** and after 15 min at 0 °C, aldehyde **5** was added, and the reaction mixture was heated under reflux conditions for 2 h to give alkene **8** in an overall yield of 54% after purification by column chromatography. In the original literature procedure,[16] the Wittig reaction was performed at -78 °C (and gradually raising the temperature to 0 °C), however, it turned out that for a complete conversion of the starting materials, reaction a reflux temperature was required. In the next step, hydrogenation of the double bond and the simultaneous removal of the benzylic protecting groups of compound **8** was initially attempted with 10%-Pd on activated charcoal in a hydrogen atmosphere in *tert*-BuOH/H_2O as the solvent. Under these conditions the reaction did not go to completion, even when at 55 psi (3.8 bar) hydrogen pressure. Gratifyingly, hydrogenation in the presence of Pearlman's catalyst (10%-

$Pd(OH)_2$ on activated carbon) in MeOH, a complete conversion was obtained. The desired (*S*)-2-aminoundecanoic acid was difficult to isolate due to the amphiphatic character in its zwitterionic form. Therefore, protection of the α-amino group was performed in $MeOH/CH_2Cl_2$ (after removal of the catalyst by filtration) in the presence of Fmoc-ONSu and DIPEA as base. Finally, *N*-α-(9-fluorenylmethyloxycarbonyl)-(*S*)-2-aminoundecanoic acid (Fmoc-Laa-OH) **9** was obtained in a modest yield of 38% (62% per step) after aqueous work-up and purification by column chromatography.



Scheme 1. Synthesis of *N*-α-(9-fluorenylmethyloxycarbonyl)-(*S*)-2-aminoundecanoic acid (Fmoc-Laa-OH) **9** from L-glutamic acid **3**.

A previously reported structure-activity relationship study of anoplin performed by Hansen *et al.*[17] indicated that any substitution of the lysine or arginine residues by alanine or modification of the α-amino terminus resulted in anoplin derivatives with an increased hemolytic activity. Furthermore, antimicrobial activity was completely lost when the leucine residues at position 2 and 10, or the isoleucine at position 6, were replaced by alanine (see Figure 2A for amino acid numbering). These data indicate that the overall charge of +4 is essential for selectivity, while hydrophobicity is required for activity. Therefore, we hypothesized that incorporation of (*S*)-2-aminoundecanoic acid on position 2, 6, or 10 might increase activity while selectivity remained unaffected. The rationale for this design strategy is illustrated in Figure 2B, since the sites of modification form a hydrophobic patch as indicated by the helical wheel representation of the anoplin sequence. To test this hypothesis, anoplin derivatives **11** – **13** were synthesized, and their activity/selectivity profile was analyzed and compared to anoplin **10** and *N*-decanoylated anoplin **14**

(Figure 2). The peptides were synthesized on a Tentagel S-RAM resin using the Fmoc/^{t}Bu SPPS protocol with BOP/DIPEA as coupling reagents. Crude peptides **10** – **14** were purified by preparative HPLC and characterized by analytical HPLC and mass spectrometry.

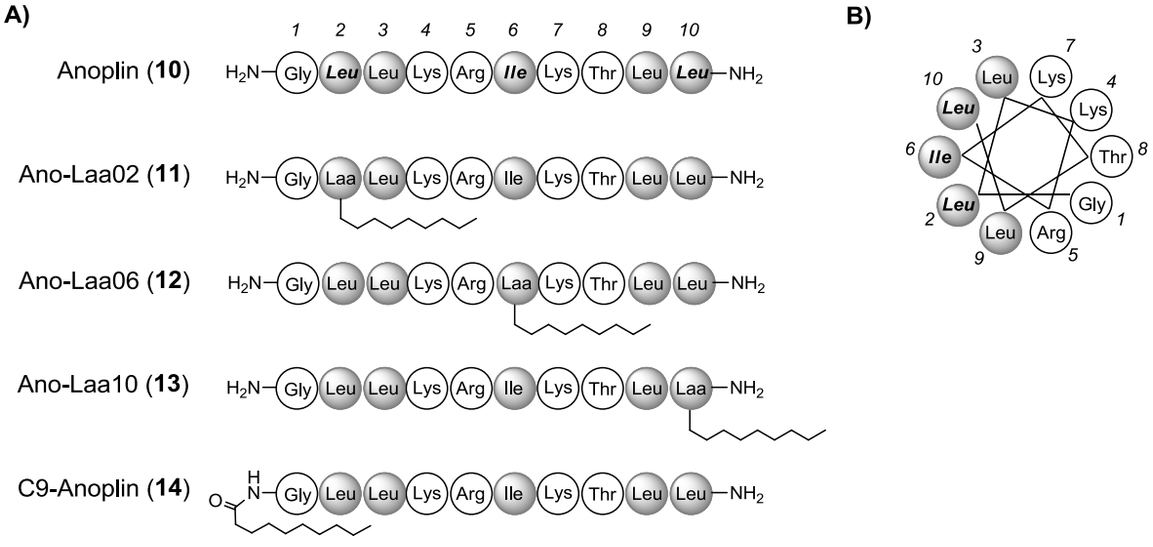


Figure 2. **A**) Schematic representation of anoplin and the four newly synthesized lipophilic analogs. Herein, Laa ((*S*)-2-aminoundecanoic acid) has been incorporated at positions 2 (**11**), 6 (**12**), and 10 (**13**), respectively. As a control, an *N*-α-decanoyl-anoplin derivative (**14**) has been prepared. **B**) Helical wheel representation of anoplin that indicates the amphiphilic character of this antimicrobial peptide, since the membrane-acting amino acid residues form a hydrophobic patch.

7.2.2 Biological evaluation

The peptides **10** – **14** were tested for their potency to inhibit bacterial growth, expressed as their minimal inhibition concentration (MIC), of a Gram-negative (*E. coli*) as well as a Gram-positive (*S. aureus*) bacterium. Selectivity against bacterial membranes was measured by exposing red blood cells to the peptides in a hemoglobin leakage assay (EC50). Anoplin **10** was active against both strains with a MIC value of 41 and 20.6 μg/mL, respectively, and highly selective against bacterial membranes since no hemolysis was observed up to 500 μg/mL (Table 1 and Figure 3). The lipophilic anoplin analogs **11** – **13** were 4 to 8 times more active against *E. coli* and even 10 times more active against *S. aureus* compared to anoplin (Table 1). As can be seen from Figure 3, the hemolytic activity of analogs **11** – **13** was higher than anoplin (39 to 108 μg/mL versus >500 μg/mL), however, the concentration at which 50% lysis will occur (EC50) was even one order of magnitude higher than the respective MIC values, an indication that these lipophilic anoplin derivatives were still selective toward bacterial membranes (Table 1). The specificity of antimicrobial agents toward bacterial membranes is expressed as the therapeutic index (TI), and is calculated by the ratio of hemolytic activity (EC50 in μg/mL) and antimicrobial activity (MIC in

μg/mL).[18] Thus a larger value in therapeutic index implies an increased antimicrobial specificity. The TI values are shown in Table 1, and especially anoplin derivative **11** was found to be very promising regarding its antimicrobial specificity compared to anoplin. The *N*-decanoylated anoplin derivative **14** is an example of a non-selective antimicrobial peptide since its EC50 value was found to be in the same concentration range as its MIC value: 5 versus 2.5 μg/mL, while its TI value was rather low (2.0) as shown in Table 1 and Figure 3.

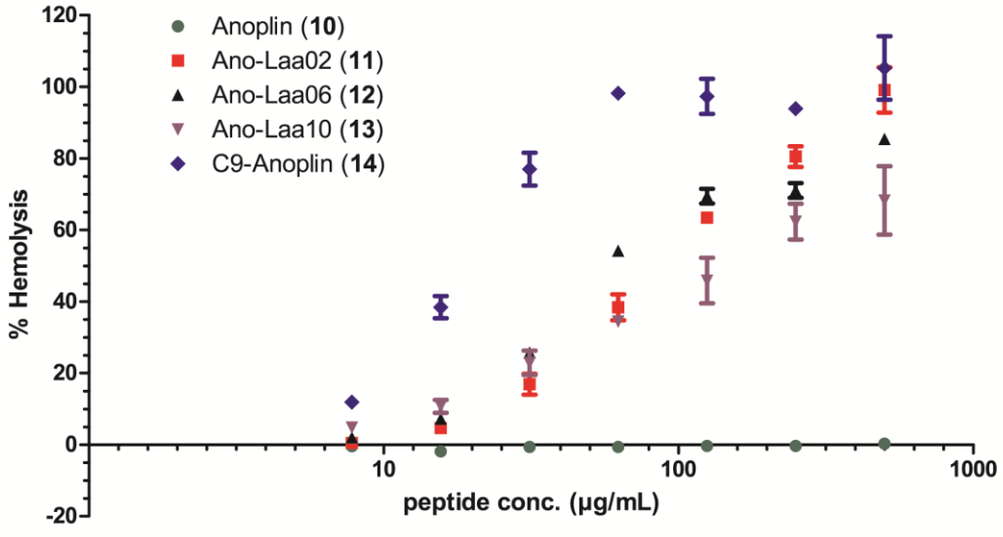


Figure 3. Hemolytic activity of anoplin compared with its hydrophobic analogs.

Table 1. Physico-chemical properties and biological activities of anoplin and its derivatives.

Peptide	**M_w**	**R_t**	**MIC**		**MIC**		**EC50**
	(Da)	**(min)**[a]	**(μg/mL)**[b]		**(μg/mL)**[b]		**(μg/mL)**[c]
			E. coli	TI[d]	*S. aureus*	TI[d]	
Anoplin (**10**)	1052.8	18.3	41	>12.2	20.6	>24.3	>500
Ano-Laa02 (**11**)	1222.9	20.3	5.2-10.3	10.5-20.8	2.5	43.2	~108
Ano-Laa06 (**12**)	1222.9	20.3	10.3-20.6	1.9-3.8	2.5	15.6	~39
Ano-Laa10 (**13**)	1222.9	20.2	10.3-20.6	3.7-7.5	2.5	30.8	~77
C9-Anoplin (**14**)	1306.9	22.2	2.5	2.0	2.5	2.0	~5

[a]Retention times were determined on a C8 column (for details see experimental section). [b]Antimicrobial activity is expressed as the minimal inhibitory concentration (MIC). [c]Hemolytic activity is expressed as the concentration of the peptide that induces 50% hemolysis (EC50). [d]Therapeutic Index = EC50 (in μg/mL)/MIC (in μg/mL); larger values indicate an improved antimicrobial specificity.[21]

In a second biochemical assay, peptides **10** – **14** were tested for their interaction with model membrane systems.[19] For this purpose, large unilamellar vesicles (LUVs) were loaded with carboxyfluorescein (CF) as the fluorophore, and the ability of the peptides to induce membrane permeability was measured by monitoring the release of CF by fluorescence spectroscopy. LUVs composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a zwitterionic lipid membrane mimicking mammalian cell membranes, was treated with anoplin **10** (at 50 μg/mL) and ~41% of CF leakage was observed (as shown in Figure 4A). Membrane lysis induced by anoplin derivatives **11** – **13** was found to be in the same order (47, 45, and 48%, respectively), while CF leakage induced by the *N*-decanoylated anoplin derivative **14** was increased to approximately 62% (Figure 4A).

As a model for an abundant bacterial cell membrane, LUVs consisting of an equimolar amount DOPC and the anionic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were used. The interaction of anoplin with DOPC/DOPG vesicles resulted in approximately 35% CF leakage, while membrane lysis induced by anoplin derivatives **11** – **13** was increased (48 to 52%), as shown in Figure 4B. Interaction of the *N*-decanoylated anoplin derivative **14** with DOPC/DOPG vesicles resulted in almost 100% CF leakage, an indication that this peptide was highly unspecific, since it had also the highest leakage-% of DOPC vesicles (Figure 4). Based on these leakage experiments it became clear that the lipophilic anoplin-derived peptides **11** – **13** displayed an increased permeabilization activity towards anionic lipid containing membranes most likely due to an increased affinity for these membranes, while *N*-acylation increased membrane affinity but lead to a loss of selectivity.

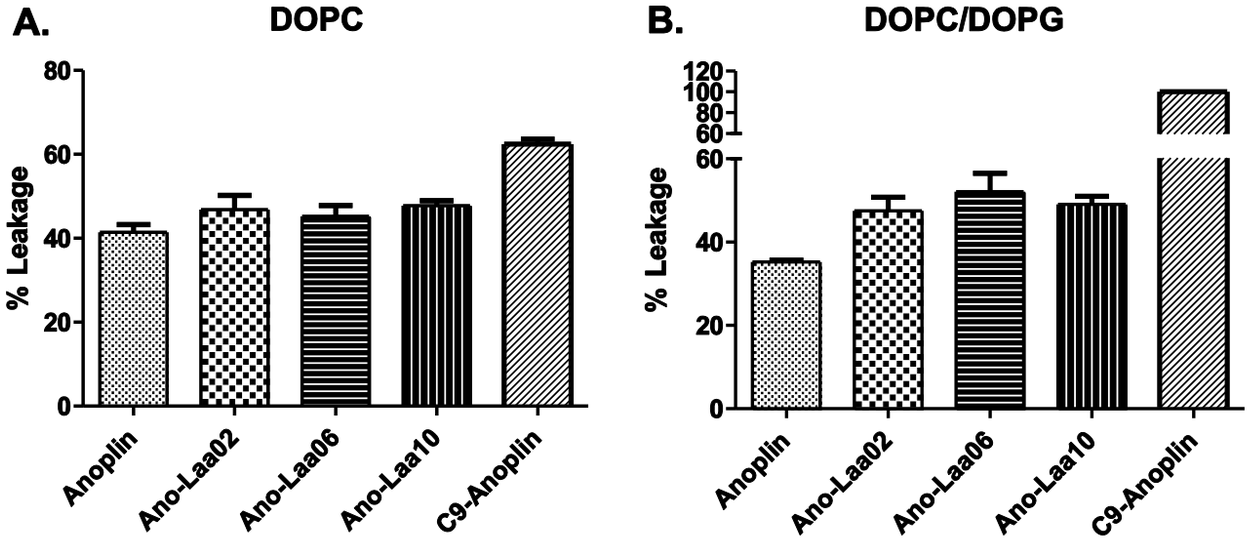


Figure 4. Lysis of large unilamellar vesicles expressed in a percentage of leakage induced by anoplin and its hydrophobic analogs as measured on neutral DOPC vesicles (**A.**) and negatively charged DOPC/DOPG vesicles (**B.**), at a peptide concentration of 50 μg/mL.

7.3 Conclusions

In conclusion, an efficient five-step synthesis of *N*-α-(9-fluorenylmethyloxycarbonyl)-(*S*)-2-aminoundecanoic acid starting from L-glutamic acid is reported, including its application as building block in solid phase peptide synthesis. The incorporation of this lipophilic amino acid in an antimicrobial peptide increase the affinity of this peptide toward bacterial cell membranes, likely by acting as a membrane anchor. Using this approach, antimicrobial specificity was preserved and in one particular case (anoplin derivative **11**) highly promising compared to native anoplin. These data may imply that amino acid substitution by a lipophilic residue could be a general approach to improve drug-like properties of membrane-interacting antimicrobial peptides.

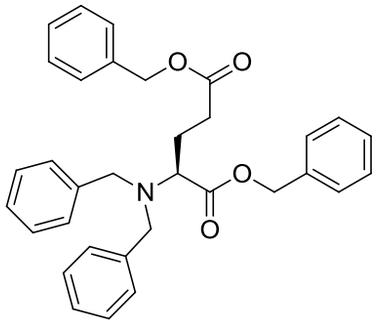
7.4 Experimental

7.4.1 Chemicals, instruments and general methods

Unless stated otherwise, all chemicals were obtained from commercial sources and used without further purification. Piperidine, *N*,*N*-diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), *tert*-butyl methylester (MTBE), trifluoroacetic acid (TFA) and HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and dried on 4Å molecular sieves (DMF, NMP and CH_2Cl_2) prior to use. The coupling reagent benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and *N*-α-9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids were purchased from GL Biochem Ltd (Shanghai, China). Triisopropylsilane (TIS) was obtained from Merck (Darmstadt, Germany). Rink resin, Tentagel S RAM (theoretical loading: 0.25 mmol/g), was purchased from RAPP Polymere (Tübingen, Germany). Solid phase peptide synthesis was performed in plastic syringes with a polyethylene frit. Solution phase reactions were monitored by TLC on Merck precoated silica gel 60F254 glass plates. Spots were visualized either by UV quenching, ninhydrin, or staining with Cl_2/TDM.[20] Solid phase reactions were monitored with the Kaiser test[21] and the bromophenol blue test (BPB).[22] Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40-63 μm). ^{1}H NMR spectra were acquired on a Varian Mercury 300 MHz spectrometer with $CDCl_3$ as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), and broad (br). ^{13}C NMR spectra were acquired on a Varian Mercury 75.5 MHz with $CDCl_3$ as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm). Analytical HPLC was performed on a Shimadzu automated HPLC system equipped with a UV/vis detector operating at 220/254 nm. Preparative HPLC runs were

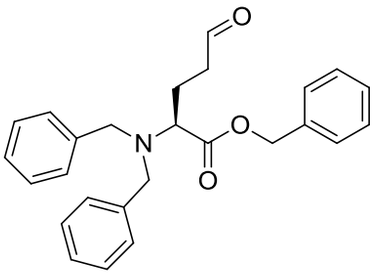
performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/vis absorbance detector. The mobile phase for HPLC consisted of buffer A: 0.1% TFA in MeCN/H_2O 5:95 v/v and buffer B: 0.1% TFA in MeCN/H_2O 95:5 v/v. For analytical HPLC a flow rate of 1.0 mL/min with a linear gradient of buffer B (100% in 20 min) from 100% buffer A was used with a total run time of 40 min using an Alltech C8 Alltima column (pore size: 300Å, particle size: 5 μm, 250 × 4.6 mm). Preparative HPLC runs were performed at a flow rate of 12 mL/min with a linear gradient of buffer B (100% in 60 min) from 100% buffer A with a total run time of 80 min using an Alltech C8 Alltima column (pore size: 300Å, particle size: 10 μm, 250 × 22 mm). Peptides were characterized using electro-spray ionization mass spectrometry (ESI-MS) on a Shimadzu QP8000 single quadrupole bench top mass spectrometer in a positive ionization mode.

7.4.2 Building block synthesis



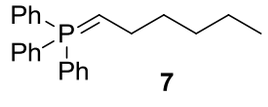
***N,N,O,O*-perbenzylated Bzl-N(Bzl)Glu(OBzl)-OBzl (4)[15]**

L-Glutamic acid **3** (5.00 g, 34 mmol), K_2CO_3 (18.8 g, 136 mmol) and NaOH (2.75 g, 68 mmol) were dissolved in water (30 mL). This solution was stirred and heated till reflux, while benzyl bromide (25.0 mL, 136 mmol) was added dropwise via a dropping funnel and after complete addition of benzyl bromide, the obtained reaction mixture was refluxed for 3 h, while the progress of the reaction was monitored by TLC (hexane/EtOAc 1:1 v/v). Subsequently, the reaction mixture was cooled to room temperature and the aqueous layer was extracted with Et_2O (3 × 80 mL), the combined organic layers were washed with water (50 mL) and brine (50 mL), and dried (Na_2SO_4). After filtration, the Et_2O solution was concentrated *in vacuo* to afford a yellowish oil. The residual oil was purified by column chromatography on silica gel (hexane/EtOAc 7:1 v/v) and diester **4** was obtained as a colorless oil. Yield: 12.6 g (73%); R_f 0.46 (hexane/EtOAc 8:2 v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.07 (q (*J* = 7.5 Hz), 2H, γCH_2), 2.34 (m, 1H, βCH_2), 2.50 (m, 1H, βCH_2), 3.41 (t (*J* = 7.7 Hz), 1H, αCH), 3.50 (d (*J* = 13.7 Hz), 2H, *N*-CH_2 benzyl), 3.88 (d (*J* = 13.7 Hz), 2H, *N*-CH_2 benzyl), 4.97 (dd (J_{vic} = 12.4 Hz, J_{gem} = 15.4 Hz), 2H, *O*-CH_2 benzyl), 5.20 (dd (J_{vic} = 12.2 Hz, J_{gem} = 33.3 Hz), 2H, *O*-CH_2 benzyl), 7.17-7.42 (m, 20H, arom CH benzyl); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ = 24.4, 30.6, 54.5, 59.7, 66.1, 66.2, 127.0, 128.1, 128.3 (two lines), 128.5 (two lines), 128.6, 128.9, 135.9 (two lines), 172.1, 172.7.



Aldehyde 5[16]

Diester **4** (6.16 g, 12.3 mmol) was dissolved in freshly distilled anhydrous Et_2O (50 mL) and the solution was cooled to -78 °C thereby gently flushing the reaction vial with dry nitrogen. Under vigorous stirring, DIBAL-H, as a solution in hexane (1 M, 14.5 mL, 14.5 mmol) was slowly added by a syringe and after the addition was complete, the reaction was stirring for an additional 15 min at -78 °C. Then, the reaction mixture was quenched with H_2O (0.65 mL), and the mixture was allowed to warm to room temperature and stirring was continued for an additional 30 min. Subsequently, the reaction mixture was dried (Na_2SO_4), filtered and the solvent was evaporated *in vacuo* and the crude aldehyde **5** was obtained as a yellowish oil and was used without further purification in the next synthesis step. The formation of the aldehyde was confirmed by proton NMR (in $CDCl_3$) since the ~C*H*O signal at δ 9.58 ppm was visible which was in accordance to the literature.[5] Yield: 4.35 g (10.8 mmol corresponding to 89%); R_f 0.39 (hexane/EtOAc 8:2 v/v).



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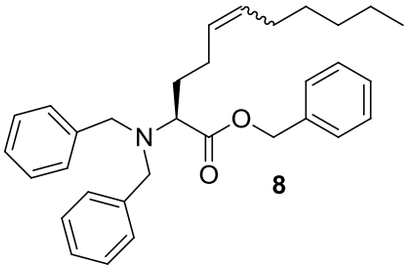
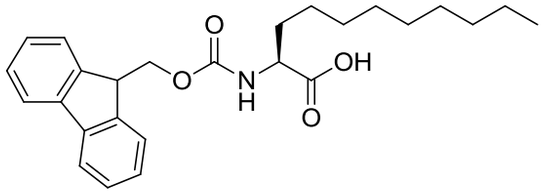


8

Ylide 7 and **alkene 8**

Hexyltriphenylphosphonium bromide **6** (6.92 g, 16.2 mmol) was suspended in dry toluene (80 mL) and the suspension was cooled to 0 °C thereby gently flushing the reaction vial with nitrogen. A solution of hexamethyldisilazane potassium salt (KHMDS) in toluene (0.5 M, 27.6 mL, 13.8 mmol) was added and the reaction mixture turned into a bright orange color to yield in situ ylide **7**. After 15 min of stirring at 0 °C, a solution of aldehyde **5** (4.35 g, 10.8 mmol) in ice-cold toluene (10 mL) was added dropwise. Then, the reaction mixture was heated to reflux conditions. On TLC, the conversion was complete and the reaction mixture was quenched with saturated aq. NH_4Cl (100 mL) and alkene **8** was isolated from the aqueous phase by extraction with Et_2O (3 × 20 mL). The combined organic layers were washed with brine (100 mL), dried (Na_2SO_4), filtered and the solvents were evaporated *in vacuo*. The residue was purified by column chromatography (CH_2Cl_2/hexane 1:1 v/v) and alkene **8** was obtained as a colorless oil. Yield: 3.08 g (54% in two steps, 73% per step); R_f 0.61 (hexane/EtOAc 8:2 v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.86 (t (*J* = 6.9 Hz), 3H, CH_3), 1.12-1.35 (m, 6H, ~(CH_2)$_3$-CH_3), 1.65-2.35 (m, 6H, βCH_2 (2H)/~CH_2-CH=CH-CH_2~ (4H)), 3.38 (t (*J* = 7.3 Hz), 1H, αCH), 3.53 (d (*J* = 13.9 Hz), 2H, *N*-CH_2 benzyl), 3.90 (d (*J* = 13.9 Hz), 2H, *N*-CH_2 benzyl), 5.11-5.38 (m, 4H *O*-CH_2 benzyl (2H)/~C*H*=C*H*~ (2H)), 7.21-7.40 (m, 15H, CH arom); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.1, 22.5,

24.1, 27.1, 29.3, 29.7, 31.4, 54.5, 60.6, 65.9, 126.9, 128.2 (two lines), 128.4, 128.5, 128.8 (two lines), 130.7, 136.1, 139.5, 172.7.



_N_-α-(9-fluorenylmethyloxycarbonyl)-(_S_)-2-aminoundecanoic acid 9

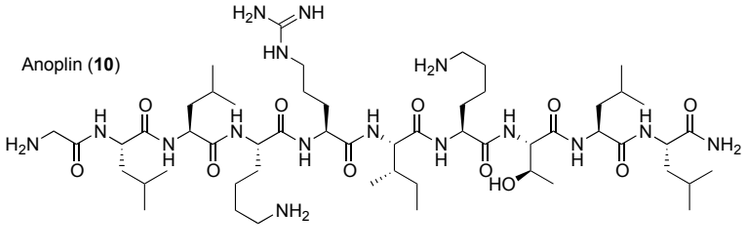
Fully protected alkene **8** (2.50 g, 5.3 mmol) was dissolved in MeOH (50 mL) and placed in a Parr Apparatus reaction vessel. After addition of $Pd(OH)_2/C$ (125 mg, 5% Pd w/w), the reaction mixture was shaken in an H_2 atmosphere (55 psi H_2 pressure) for 16 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, which was rinsed with MeOH (2 × 10 mL). The filtrate was concentrated *in vacuo* to a volume of approximately 40 mL. Then, CH_2Cl_2 (40 mL) was added subsequently followed by Fmoc-ONSu (1.79 g, 5.3 mmol) and DIPEA (1.85 mL, 10.6 mmol), and the obtained suspension was stirred for 16 h at room temperature. Then, the solvents were removed by evaporation *in vacuo* and the residue was redissolved in EtOAc (80 mL) and the solution was washed with aq. 1 N HCl (3 × 60 mL) and brine (2 × 40 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The residue was purified by column chromatography using a gradient of hexane/EtOAc 1:1 v/v to 100% EtOAc as the eluent system and *N*-α-(9-fluorenylmethyloxycarbonyl)-(*S*)-2-aminoundecanoic acid **9** (Fmoc-Laa-OH) was afforded as a white solid in 38% yield (62% per step, 0.85 g). R_f 0.16 (hexane/EtOAc 1:1 v/v), $[\alpha]_D$ +4.8 (*c* = 1 in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$) δ = 0.87 (t (*J* = 6.7 Hz), 3H, CH_3), 1.34 (broad s, 14H, CH_2), 1.69 (m, 1H, βCH_2), 1.89 (m, 1H, βCH_2), 4.22 (t (*J* = 6.9 Hz), 1H, CH Fmoc), 4.44 (m, 3H, αCH (1H)/CH_2 Fmoc (2H)), 5.23 (d (*J* = 8.2 Hz), 1H, NH), 7.28-7.77 (m, 8H, arom CH Fmoc); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 14.1, 22.7, 25.2, 29.1, 29.3, 29.4, 29.5, 31.9, 32.3, 47.2, 53.8, 67.1, 120.0, 125.1, 127.1, 127.7, 141.3, 143.7, 143.8, 156.1, 177.4.

7.4.3. Solid phase peptide synthesis

The anoplin-derived peptides **10** – **14** were synthesized manually via the Fmoc/^{t}Bu protocol on an Fmoc-Rink Amide TentaGel resin (0.25 mmol/g). Each synthetic cycle consisted of the following steps. *Fmoc removal:* the resin (1 g, 0.25 mmol) was treated with a 20% solution of piperidine in NMP (10 mL; 3 × 8 min). The solution was removed by filtration and the resin was washed with NMP (10 mL; 3 × 2 min) and CH_2Cl_2 (10 mL; 3 × 2 min). The presence of free α-amino functionalities was checked either by the Kaiser test (blue beads) or the BPB test (blue-green beads). *Coupling step:* a mixture of Fmoc-Xxx-OH (1 mmol, 4 equiv), BOP (1 mmol, 4 equiv) and DIPEA (2 mmol, 8 equiv) in NMP (20 mL) was added to the resin and the suspension was mixed

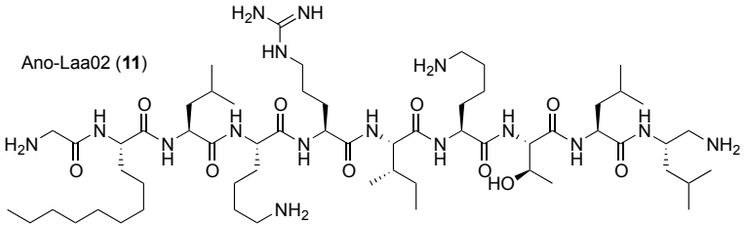
by bubbling N_2 through the reaction mixture for 45 min. Fmoc-Laa-OH (**9**) was coupled by using the following conditions: Fmoc-Laa-OH (0.5 mmol, 2 equiv), BOP (0.5 mmol, 2 equiv) and DIPEA (1 mmol, 4 equiv) in NMP (10 mL) for 90 min. Reagents and solvents were removed by filtration and the resin was subsequently washed with NMP (10 mL; 3 × 2 min) and CH_2Cl_2 (10 mL; 3 × 2 min). Completion of the coupling (absence of free α-amino functionalities) was checked either by the Kaiser test or the BPB test (colorless beads in both cases). *TFA cleavage:* the resin was swirled in a mixture of TFA/TIS/H_2O (10 mL; 95:2.5:2.5 v/v/v) for 3 h at room temperature. Then, the resin was removed by filtration and the residual TFA solution was diluted with ice-cold MTBE/hexane (1:1 v/v) to precipitate the peptide. The supernatant was removed after centrifugation and the peptide pellet was washed twice with MTBE/hexane. The crude peptide was dissolved in *tert*-BuOH/H_2O (1:1 v/v) and lyophilized. The crude peptides were purified by preparative HPLC and the pure peptide fractions were pooled and lyophilized. Finally, the peptides were analyzed by analytical HPLC and characterized by ESI-MS.

Anoplin, H-Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH_2 (10)



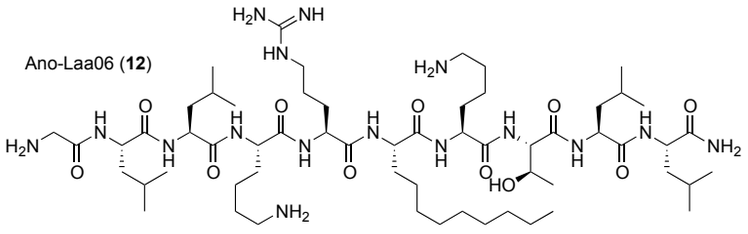
Yield after HPLC purification: 45 mg; R_t = 18.34 min (C8 Alltima); ESI-MS calcd. for $C_{54}H_{104}N_{16}O_{11}$: 1052.81, found: *m/z* 1153.95 $[M+H]^+$, 1176.55 $[M+Na]^+$, 577.45 $[M+2H]^{2+}$, 485.20 $[M+3H]^{3+}$.

Ano-Laa02, H-Gly-*Laa*-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH_2 (11)



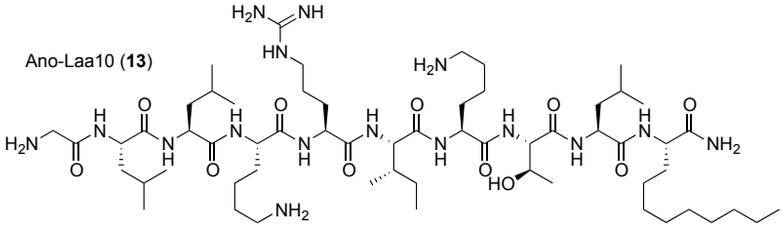
Yield after HPLC purification: 40 mg; R_t = 20.32 min (C8 Alltima); ESI-MS calcd. for $C_{59}H_{114}N_{16}O_{11}$: 1222.89, found: *m/z* 1223.80 $[M+H]^+$, 612.50 $[M+2H]^{2+}$, 406.70 $[M+3H]^{3+}$.

Ano-Laa06, H-Gly-Leu-Leu-Lys-Arg-*Laa*-Lys-Thr-Leu-Leu-NH_2 (12)



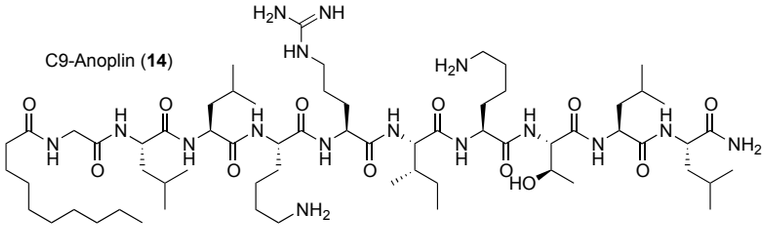
Yield after HPLC purification: 25 mg; R_t = 20.27 min (C8 Alltima); ESI-MS calcd. for $C_{59}H_{114}N_{16}O_{11}$: 1222.89, found: *m/z* 1224.20 $[M+H]^+$, 612.50 $[M+2H]^{2+}$, 406.85 $[M+3H]^{3+}$.

Ano-Laa10, H-Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-*Laa*-NH_2 (13)



Yield after HPLC purification: 18 mg; R_t = 20.22 min (C8 Alltima); ESI-MS calcd. for $C_{59}H_{114}N_{16}O_{11}$: 1222.89, found: *m/z* 1224.20 $[M+H]^+$, 612.50 $[M+2H]^{2+}$, 406.70 $[M+3H]^{3+}$.

C_9-Anoplin, Dec-Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH_2 (14)



This anoplin-derivative was synthesized on Fmoc-Rink-Tentagel resin (400 mg, 0.1 mmol) according to the general procedure for solid phase peptide synthesis. Prior to resin cleavage, the free α-amino functionality was acylated with decanoic acid (69 mg, 0.4 mmol, 4 equiv) in the presence of BOP (177 mg, 0.4 mmol, 4 equiv) and DIPEA (139 µL, 0.8 mmol, 8 equiv) as the coupling reagents in NMP (2 mL) for 45 min at room temperature. Then the peptide was deprotected and cleaved from the resin according to the general procedure for solid phase peptide synthesis. Yield after HPLC purification: 20 mg; R_t = 22.15 min (C8 Alltima); ESI-MS calcd. for $C_{64}H_{122}N_{16}O_{12}$: 1306.94, found: *m/z* 1308.40 $[M+H]^+$, 1329.90 $[M+Na]^+$, 654.05 $[M+2H]^{2+}$, 437.55 $[M+3H]^{3+}$.

7.4.4 Biological Evaluation

Growth inhibition assay

Strains used for determination of antimicrobial activity included the two American Type Culture Collection (ATCC) strains *E. coli* ATCC 8739 and *S. aureus* ATCC 259923. The MIC of each peptide was determined using a broth micro-dilution assay adapted from a literature procedure as previously described by Hancock.[23] Peptide stock solutions were prepared at a concentration of 330 μg/mL peptide in 0.2% bovine serum albumin and 0.01% acetic acid. Serial two-fold solutions of the peptides were made in 0.2% bovine serum albumin and 0.01% acetic acid in sterile 96-well polypropylene microtiter plates. To each well was added, 50 μL of the test bacteria in Mueller-Hinton broth to a final concentration of 2×10^6 CFU/mL and 50 μL of the peptide in the different concentrations. After incubation for 24 h at 37 °C and shaken at 120 rpm in Certomat incubator, the OD at 630 nm was measured. The MIC (expressed in μg/mL) of each peptide was read as the lowest concentration of peptide that inhibited visible growth of bacteria. All measurements were performed in duplicate and validated using two independent experiments.

Hemolytic activity assay

Sheep blood erythrocytes were washed three times with PBS buffer by centrifugation for 5 min (2000 rpm) and subsequent aspiration. A suspension of erythrocytes in PBS was prepared, where the OD_{414} of a 1/50 dilution was 0.3 (~100 μL in 10 mL PBS). Serial two-fold dilutions of the peptide in PBS (50 μL) were added to each well of a round-bottom polypropylene microtiter plate followed by the subsequent addition of 50 μL of an erythrocytes suspension in PBS to a final peptide concentration ranging from 500 μg/mL to 7.8 μg/mL. After the microtiter plate was incubated at 37 °C for 1 h, the plate was centrifuged for 10 min (2000 rpm). A flat-bottom plate was filled with 100 μL demi-water and after the supernatant (25 μL) of the round-bottom plate was transferred to the flat-bottom plate the absorption at 414 nm was measured. The blank was evaluated from PBS (A_{blank}) and 100% hemolysis was evaluated with addition of demi-water to the erythrocytes ($A_{100\%}$). The hemolysis percentage was calculated as follows: $[(A_{peptide} - A_{blank})/(A_{100\%} - A_{blank})] \times 100\%$. All measurements were performed in duplicate and the peptide concentrations causing 50% hemolysis (EC50, expressed in μg/mL) were determined from the dose-response curves.

Vesicle leakage experiments

Carboxyfluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment according to a literature procedure.[19] The peptide-induced

leakage of CF from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM instruments Inc., USA) at 20 °C. A solution (1.0 mL) of CF-loaded vesicles (25 μM final concentration) in buffer (10 mM Tris/HCl pH = 7.0, 100 mM NaCl) was added to a quartz cuvette and fluorescence was measured (A_0). After 20 s, a buffer solution (50 μL) containing the peptide of interest (stock: 1 mM; final: 50 μM) was added and peptide-induced membrane leakage was followed during 220 s (A_{220}), after which a buffer solution (10 μL) of Triton-X (stock: 20%; final: 0.2%) was added to induce total leakage of the vesicles (A_{Total}). The % of peptide-induced membrane leakage was calculated by: $((A_{220} - A_0)/(A_{Total} - A_0)) \times 100\%$.

7.5 References and notes

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Chapter 8

Summary and outlook

8.1 Summary

There is a growing need for novel antibiotics since there are more and more cases of infections caused by resistant bacteria, for example, Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Enteroccocus* (VRE). Possible novel antibiotics are antimicrobial peptides, which cause bacterial membrane permeabilization and therefore have great promise since it is hard for bacteria to become resistant against this mechanism of action. With respect to this a very promising antimicrobial peptide is nisin, which belongs to the class of lantibiotics. Lantibiotics are ribosomally synthesized cationic peptides that contain several unnatural amino acids like dehydroalanine (Dha), dehydrobutyrine (Dhb) and have multiple cyclic structures derived by thioether bonds or lanthionines.

Nisin is a 34 residue long peptide containing five lanthionine ring structures: A, B, C and the interlocking DE ring, which are crucial for antimicrobial activity. Nisin exhibits low nanomolar activity against Gram-positive bacteria and its mode of action is twofold. Firstly, nisin binds with its N-terminus (AB ring fragment) to the bacterial membrane by interacting with lipid II, a very important bacterial cell wall component, thereby inhibiting the bacterial cell wall synthesis. Secondly, while the N-terminus is bound to lipid II, the C-terminus (C and DE ring fragments) of nisin inserts into the membrane forming a pore complex leading to the loss of vital ion gradients and thereby causing bacterial cell death (Figure 1).

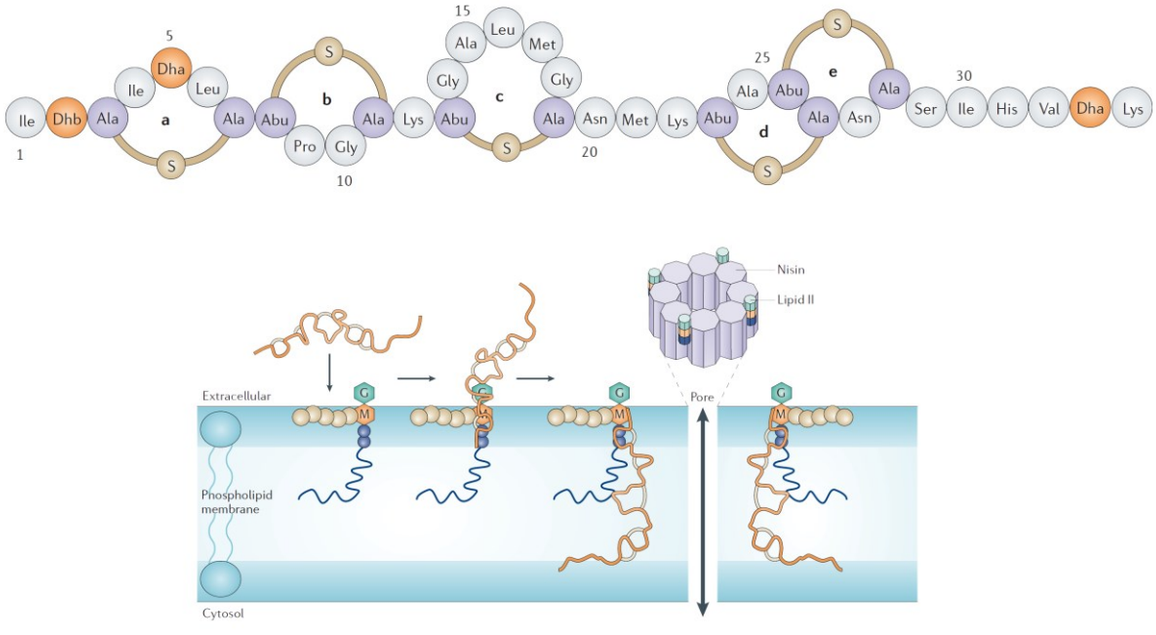

Figure 1. Nisin interacts with the bacterial membrane by binding to lipid II. After insertion of the C-terminal DE-ring fragment into the membrane a lipid II-nisin pore complex is formed. Picture reprinted by permission from Breukink *et al.,* Copyright 2006 Nature Publishing group, Nature Reviews Drug Discovery.

Although nisin holds a promising template for novel peptide-based antibiotics, synthesis of nisin-like structures is not trivial and especially the introduction of the lanthionine rings is challenging. Moreover, the lanthionine bridges are oxidation sensitive and the approach as described in this research to replace the lanthionines by dicarba bonds via ring-closing metathesis (RCM) could be used to access stabilized nisin-like structures via (chemical) synthesis. Also, the investigation of the importance of each individual ring structure, especially at the C-terminus, and synthesis of nisin mimics will give more insight into the potential of nisin as an improved antibiotic.

Chapter 2 describes the synthesis of an alkene-bridged nisin DE ring mimic via RCM using allylglycine (Alg) residues. A linear protected hexapeptide, Boc-D-Alg-Ala-D-Alg-Alg-Asn(Trt)-Alg-OMe, was synthesized via solid phase peptide synthesis (SPPS) and via RCM a bicylic, 1→4, 3→6 interlocking peptide, was synthesized mimicking the DE-ring of nisin. The mimic contains two rotational restricted alkene bonds and a mixture of four diastereoisomers (which have different alkene bond configurations: *ZZ, ZE, EZ, EE*) were obtained and the diastereoisomers could be separated using preparative HPLC. Each diastereoisomer was individually characterized using 2D NMR analysis using TOCSY, ROESY and HSQC spectroscopy. Furthermore, N and C-terminal functionalization was demonstrated by introducing *N*-α-azido lysine to the N-terminus and the C-terminus was functionalized with a properly protected lysine residue. Finally, the azide functionality was used in Cu(I)-catalyzed click chemistry to demonstrate the possibility of orthogonal ligations of DE-Ring alkene-bridged mimics.

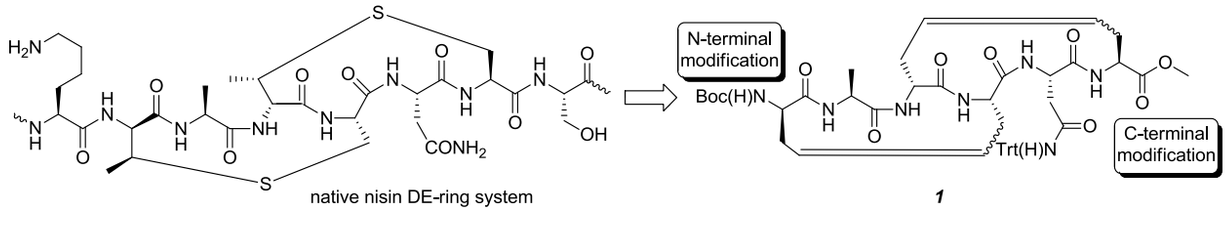

Figure 2. Design of an alkene-bridged DE-Ring mimic where the oxidation sensitive lanthionines have been replaced by alkene bonds.

Chapter 3 describes the development of a convenient method for the purification of nisin from commercial available nisin sources suitable for preparative purposes. A nisin preparation is commercially available as a mixture of NaCl (75%), milk proteins (22.5%) and contains only ~2.5% nisin. Nisin was enriched via a convenient precipitation method using an organic/aqueous biphasic system. This method affords an enriched nisin batch with a high purity without using any sort of column chromatography making it a very fast and convenient method. To demonstrate the use of this batch, enriched nisin was treated with two proteases, trypsin and chymotrypsin, to obtain

nisin AB (1-12) and nisin ABC (1-20), respectively. The cleavage reaction was followed in time using analytical HPLC and LC-MS to investigate the hydrolysis process, and two previously unknown cleavage sites were indentified. Also, enriched nisin was treated with cyanogen bromide, cleaving nisin at Met21, affording the nisin DE-tail (22-34). Finally, enriched nisin was cleaved by chymotrypsin followed by cyanogen bromide in a two-step procedure affording the truncated nisin DE-tail (22-31). Accessing these native nisin fragments is of great value to investigate the importance of the individual ring systems for bioactivity by combining native fragments with synthetic nisin mimics, obtaining nisin hybrid structures as shown in the following chapters.

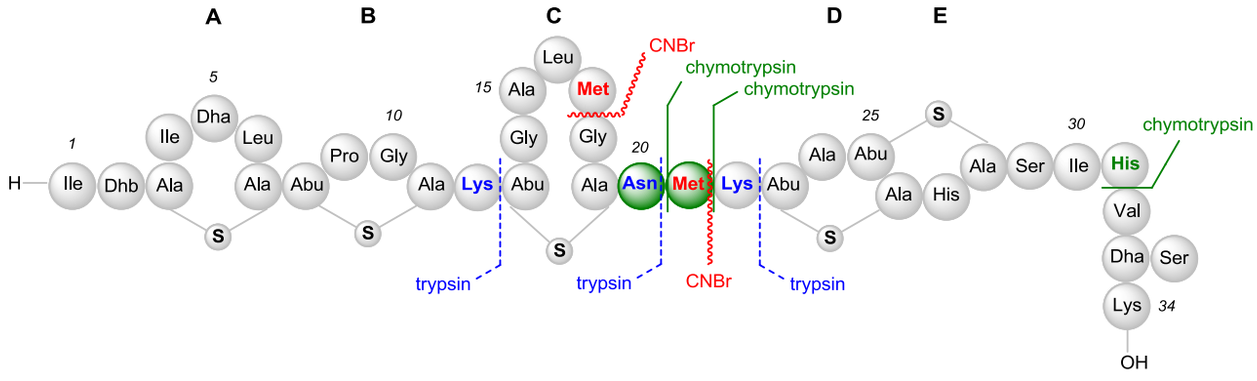

Figure 3. Overview of cleavage sites of nisin A observed for chymotrypsin (line), trypsin (dashed line) and CNBr (wavy line).

The synthesis of a hybrid nisin peptidomimetic is described in **chapter 4**. The pore-formation activity of nisin is derived from its C-terminus, since C-terminal truncations result in the loss of pore-formation activity. As a first attempt, a hybrid nisin mimic was designed containing a native ABC-part (nisin 1-20) and a synthetic alkene-bridged DE-ring to study the activity of the synthetic DE-ring in relation to the native lanthionine bridged DE-ring. Native nisin ABC was prepared by enzymatic digestion of nisin with chymotrypsin. Native nisin ABC was functionalized at the C-terminus with propargylamine, as the alkyne moiety. A nisin hybrid mimic was prepared via Cu(I)-catalyzed click chemistry via ligation of the ABC-alkyne with the N-α-azidolysine-dicarba-DE ring and the bioactivity was tested.

This nisin mimic was able to bind lipid II, an indication that it was an active antimicrobial agent, however, it showed no pore-formation activity. This “simplified” nisin mimic contained an achiral linker at position 21 derived from propargylamine, mimicking the amino acid glycine and a previous mutation study showed a dramatic decrease in pore-formation activity when replacing the hydrophobic methionine-21 into glycine-21. Furthermore, a recent paper indicated the importance of the C-terminal tail next to nisin’s DE-ring, where truncated versions show a decrease in pore-

formation activity. To improve the nisin peptidomimetic, an amino alkyne derived from leucine was prepared in two steps from Boc-Leu-OH using the Bestmann reagent. This more sterically hindered amino alkyne was coupled to the C-terminus of native nisin ABC via an optimized procedure, affording a leucine side chain at position 21. An optimized synthetic alkene-bridged DE-ring construct: N-α-azidolysine-dicarba-DE-ring-lysine-NHMe, was ligated to the native nisin ABC-Leu-alkyne to afford an optimized nisin hybrid mimic. Unfortunately, also the optimized mimic did not show any pore-formation activity. Bacterial growth inhibition tests showed that this mimic is only 10-fold less active than native nisin, however, also native nisin ABC fragment displayed a similar activity, an indication that the C-terminus of the mimic did not contributed significantly to the antimicrobial activity and needs further optimization.

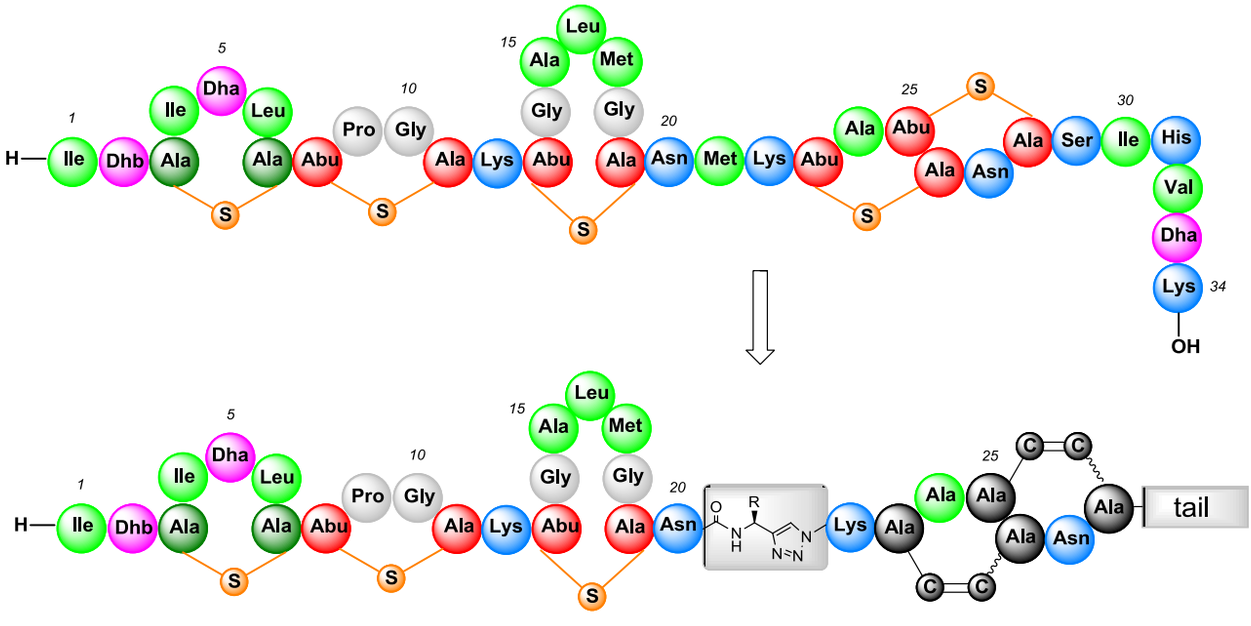

Figure 4. Schematic representation of a hybrid nisin mimic. Native nisin ABC was ligated to a synthetic alkene-bridged DE-ring using Cu(I)-catalyzed click chemistry.

Chapter 5 describes the synthesis of an improved dicarba bridged nisin AB (nisin 1-12) mimic via RCM. Previously, in our group, the synthesis of a "simplified" dicarba AB mimic was achieved in which two lanthionine rings were replaced by dicarba bonds and the two dehydro amino acids (Dhb2 and Dha5) were replaced by L-alanine. The AB mimics were active in binding lipid II, however, they were less potent than native nisin AB. Since the interaction of nisin AB to lipid II is dominated by the backbone amides of nisin AB, the backbone conformation is very important and the sp^2 hybridized Cα atoms of the dehydro amino acids, rather than the sp^3 hybridized Cα of Ala, may play a crucial role in this recognition process.

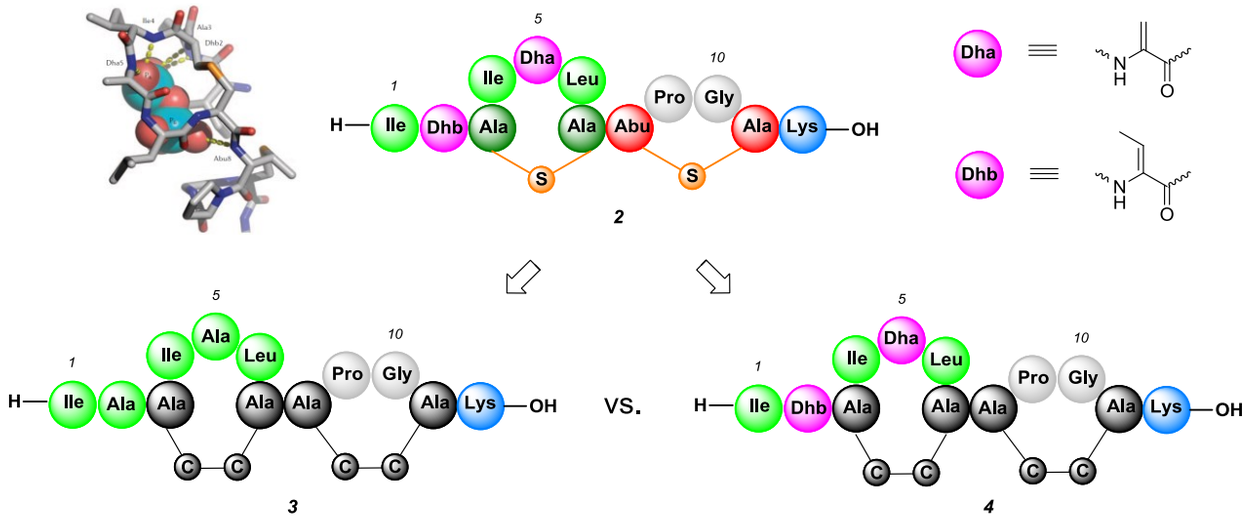

Figure 5. Nisin AB dicarba mimics with incorporation of native dehydro amino acids.

By a combination of SPPS and solution phase synthesis, dicarba ring A and dicarba ring B were synthesized via RCM, and Dhb and Dha were introduced as their precursor form threonine and serine, respectively. After fragment condensation of ring A with ring B, dehydration of Thr2 and Ser5 on the protected peptide was achieved using EDCI and $CuCl_2$. The optimized nisin AB mimic **4** was tested for lipid II binding affinity and compared to native nisin AB **2** and a dicarba mimic **3** in which the Dhb/Dha residues were replaced by alanine. This study showed that optimized dicarba nisin AB mimic **4** was as active as the native AB-ring fragment **2** in contrast to its alanine-containing congener.

Functionalization of native nisin with reporter molecules can be of great importance to understand nisin's mode of action in more detail. Also conjugation of native nisin to obtain multimeric structures and to other bioactive molecules can be very attractive to attack bacteria on multiple targets. The synthesis of such conjugates is described in **chapter 6**. Orthogonal ligation methods like Cu(I)-catalyzed click chemistry are ideal to conjugate large unprotected peptides and therefore it was chosen to prepare a nisin-alkyne derivative. Two fluorescent nisin derivatives were prepared by reacting a dansyl-azide or a carboxyfluorescein-azide derivative with the nisin-alkyne construct using $CuSO_4$, sodium ascorbate in H_2O/*tert*-BuOH mixtures. Furthermore, nisin-alkyne was successfully conjugated to a two-armed "dendritic" structure to synthesize a nisin dimer. All conjugates showed antimicrobial activity in the same range as native nisin which proved that the chemical modifications at the C-terminus were tolerated, an implication that the molecular constructs can be used in biological studies.

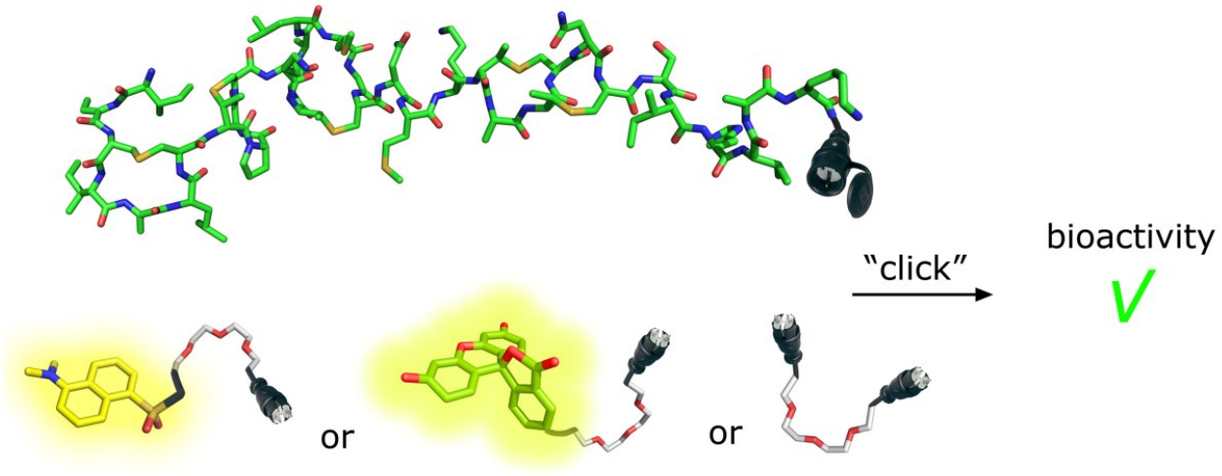

Figure 6. Synthesis of nisin conjugates via Cu(I)-catalyzed click chemistry.

Chapter 7 describes the synthesis of an Fmoc protected enantiomerically pure lipophilic amino acid (Laa) which contains a nine carbon atom hydrophobic side chain. Fmoc-Laa can be introduced into any peptide sequence using standard SPPS to increase the lipophilicity of a peptide without sacrificing important polar segments of a peptide like for instance the N and C-termini. The rationale for this design was that an increase of lipophilicity could enhance membrane affinity of membrane acting peptides and increase their potential as possible novel drug-like compounds. Fmoc-Laa-OH was synthesized in four steps from L-glutamic acid and the fatty side chain was introduced via a Wittig olefination.

The antimicrobial decapeptide anoplin was chosen as a model peptide to test this rationale. Three lipophilic anoplin analogs (Ano-Laa02, Ano-Laa06 and Ano-Laa10) were prepared in which either leucine or isoleucine was replaced by Laa at position 2, 6 or 10, respectively. All three lipophilic analogs show a dramatic increase in antimicrobial activity up to 4-8 times better for *E. coli* (Gram-negative) and over one order of magnitude for *S. aureus* (Gram-positive) compared to anoplin. Although the hemolytic activity was increased for the lipophilic analogs, the concentration at which 50% lysis will occur (EC_{50}) was still one order of magnitude higher than the determined MICs. As a second proof, anoplin and the lipophilic analogs were tested in a model membrane system using carboxyfluorescein loaded large unilaminar vesicles (LUVs) and their lytic activity was determined. The lipophilic analogs showed a higher lytic activity with respect to anoplin, in agreement with the observed MIC values. Introduction of Laa into anoplin clearly showed a positive effect and the results suggest that Fmoc-Laa-OH could be used as a general approach to increase membrane affinity.

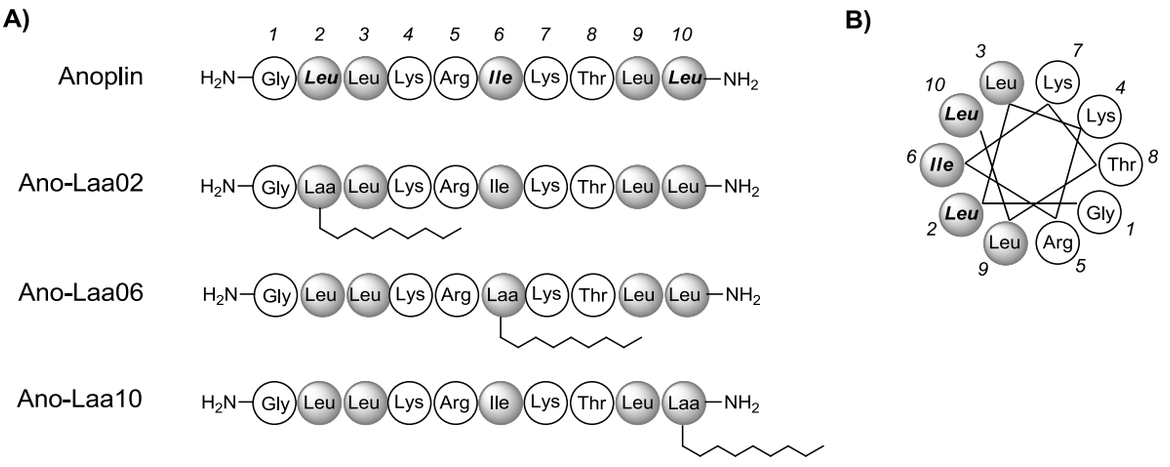

Figure 7. A schematic representation of anoplin and the three lipophilic analogs (left). A helical wheel presentation (right) of the proposed active conformation interacting met membranes. Positions 2, 6 and 10 are located at the proposed hydrophobic membrane acting side.

8.2 Outlook

8.2.1 Structural insight into the DE-ring system

The results described in **chapter 2** are focused on the separation, characterization and functionalization of dicarba nisin DE-ring mimics. NMR spectroscopy techniques were used to determine the sequential connectivity of the diastereoisomers, and the alkene-bond configurations were assigned. Elucidation of the three-dimensional structure of the dicarba DE-ring mimics is a very interesting topic, which was not proceeded into much detail in this thesis.

X-ray crystallography would be an excellent method to determine the three-dimensional structure of this bicyclic cross-bridged structure. For this, high quality crystals of the diastereoisomers have to be obtained in order to measure X-ray diffraction. The Boc and Trityl-protected DE-ring structures showed to be crystalline, since crystals were accidently obtained after slow evaporation of organic solvents in a NMR-tube as is illustrated in Figure 8 (left side). The obtained crystals were not of sufficient quality, therefore, some preliminary experiments were performed to obtain better crystals, however, crystallization attempts using organic solvents did not afford crystals of higher quality. The protected diastereoisomers were treated in acidic deprotection conditions to obtain the unprotected peptides and preliminary crystallization experiments using the hanging drop vapor diffusion method via an aqueous buffer system were performed. Although, a few conditions afforded crystals (Figure 8 right side), unfortunately, they proved to be not suitable for X-ray diffraction measurements. It would be highly interesting to determine the three-dimensional structure of these DE-ring mimics and a focused approach using a library screen of crystallization conditions could lead to suitable crystals for X-ray diffraction.

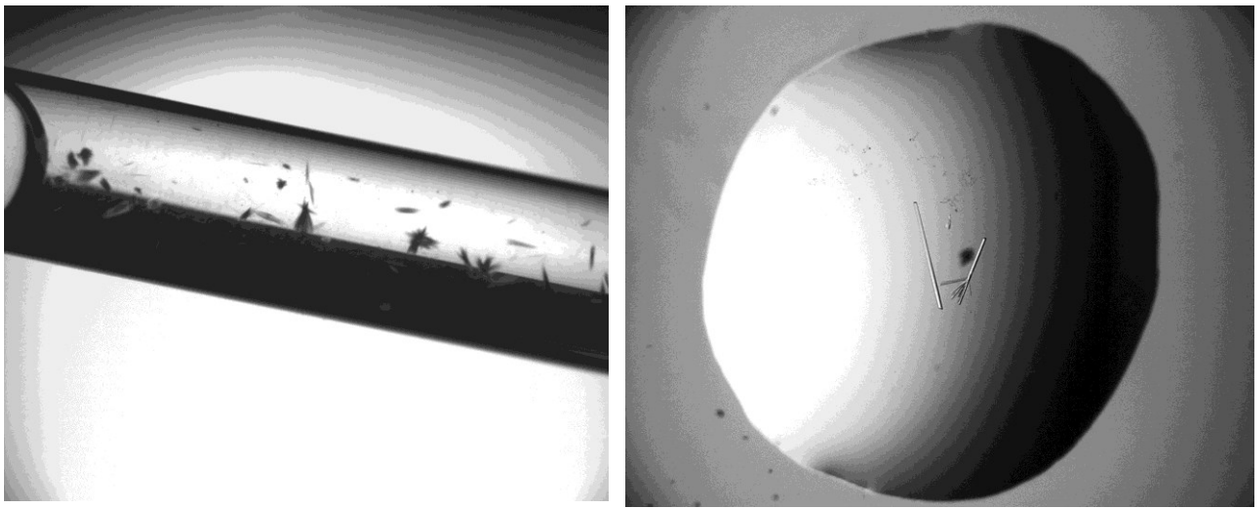

Figure 8. Crystals of protected dicarba (*Z,Z*-)DE-ring mimic in a NMR-tube (left) and crystals of deprotected dicarba (*Z,Z*-)DE-ring mimic obtained in an aqueous droplet.

Another approach to get three-dimensional structural insights into the dicarba DE-ring mimics is using NMR spectroscopy. NMR techniques as NOESY or ROESY show 'through-space' contacts between nuclei, usually protons, and this data can be used to model a structure using distance restraints derived from the NOESY/ROESY data (Figure 9). The obtained NMR spectra of the dicarba DE-ring mimics could therefore be used to determine the structure using NMR. Although there are known protocols for structure calculations of proteins using NMR distance restraints, these protocols have to be adapted in order to use it for peptidomimetics, because of the presence of many unnatural bonds compared to native peptides and proteins. Especially, because the NMR spectra of the four diastereoisomers are fully characterized, it is worthwhile investigating the possibility to determine the structure of dicarba DE-ring mimic using NMR data.

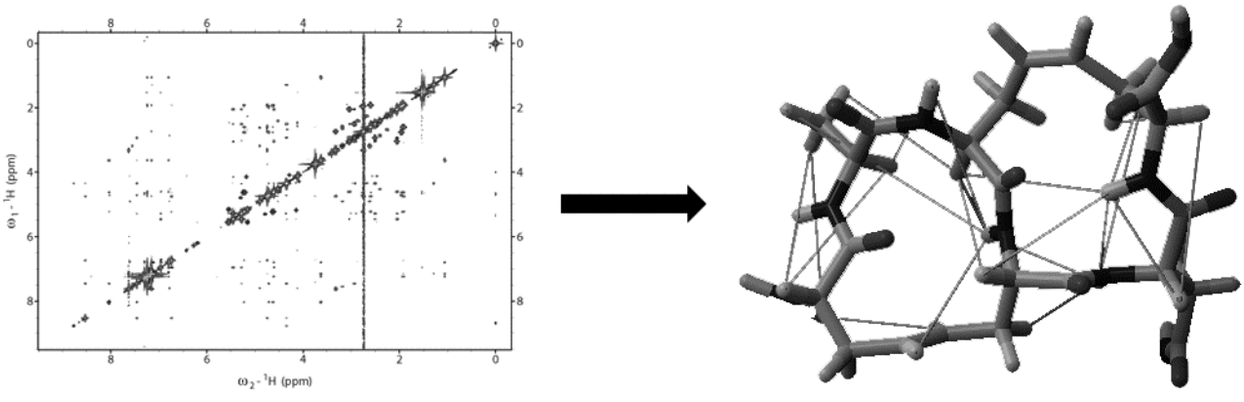

Figure 9. Overview of the procedure of structure calculations using NMR data. Proton-proton contacts observed in NOESY/ROESY spectra can be implemented as distance restrains (displayed as lines between two protons) in structure calculations.

8.2.2 Influence of a triazole moiety as a peptide bond surrogate in the hinge-region of nisin

The hybrid mimics described in **chapter 4** showed antimicrobial activity determined in competitive leakage experiments and bacterial growth inhibition studies. Unfortunately, no membrane pore-formation was observed, which suggests that the hybrid mimics have an active N-terminus, which binds lipid II, however they have an inactive C-terminus, which is responsible for membrane pore-formation, as a result by the chemical modifications. The introduced synthetic C-terminal part, comprising the DE-ring, is different from the native C-terminus in three areas. Firstly, the C-terminus was truncated from a native hexapeptide (-Ser-Ile-His-Val-Dha-Lys-OH) to one amino acid lysine. Secondly, the lanthionine bridges were replaced by dicarba bridges. And lastly, The peptide bond between Met21-Lys22 was replaced by a triazole moiety as a result of using Cu(I)-catalyzed click chemistry for the ligation of native nisin ABC and synthetic DE-ring.

Truncation studies of nisin show the importance of the C-terminal tail for pore-formation activity, however, a truncated derivative of subtilin, which is a close nisin family member, consisting of a single lysine residue as C-terminus did show pore-formation activity. Hence, it is likely that the short C-terminal tail of the hybrid nisin mimic is not responsible for the loss in pore-formation activity. The introduction of stable dicarba bridges as a replacement of the native lanthionines could influence the structure of the DE-ring, since it increases the ring-size by one atom, and thereby possibly disabling pore-formation activity. Although the dicarba-bridge increases the size of the corresponding ring by one atom, there are differences in the carbon-sulfur bonds versus carbon-carbon double bonds, with respect to size and rigidity, which might lead to only a slight increase in overall ring-size. Moreover, replacement of the lanthionines in the AB(C)-rings of nisin did afford biologically active mimics, showing that dicarba bonds can replace lanthionines in a biological context.

The last modification, the replacement of peptide bond Met21-Lys22 into a triazole, could possibly explain the loss in pore-formation activity. The triazole moiety has been used as a peptide-bond surrogate in a number of studies yielding biologically active peptidomimetics. However, the Met21-Lys22 peptide bond is located in the so-called hinge region of nisin, which is important for pore-formation activity as has been demonstrated by mutation studies. These studies showed that for instance mutation of Met21 into Gly21 or Pro21, both mutations which influence the peptide backbone flexibility, yielded a biologically active nisin mutant, however, lacking pore-formation activity.

To test if the triazole moiety is responsible for the loss of pore-formation activity in our nisin hybrid, a nisin derivative could be synthesized in which only the Met21-Lys22 peptide bond is replaced by a triazole moiety, and the retrosynthetic route is shown in Figure 10. For the synthesis of nisin triazole mimic **5**, a native C-terminal fragment (22-34) bearing a N-terminal azide has to be

prepared, which can be subsequently ligated using Cu(I)-catalyzed click chemistry to nisin ABC-Leu-alkyne **6** (prepared in chapter 4). Azido-DE-ring **7** can be prepared by performing a diazotransfer reaction on (side chain) protected DE-ring **8** and subsequent cleavages of protection groups. Cyanogen bromide cleavage, similar as described in chapter 3, of native nisin in which all free amines are protected with a suitable non-acid labile protecting group, should afford protected DE-ring **8**. Bioactivity studies of nisin triazole mimic **5**, especially testing its pore-formation activity, should elucidate if peptide-bond Met21-Lys22 can be replaced by a triazole.

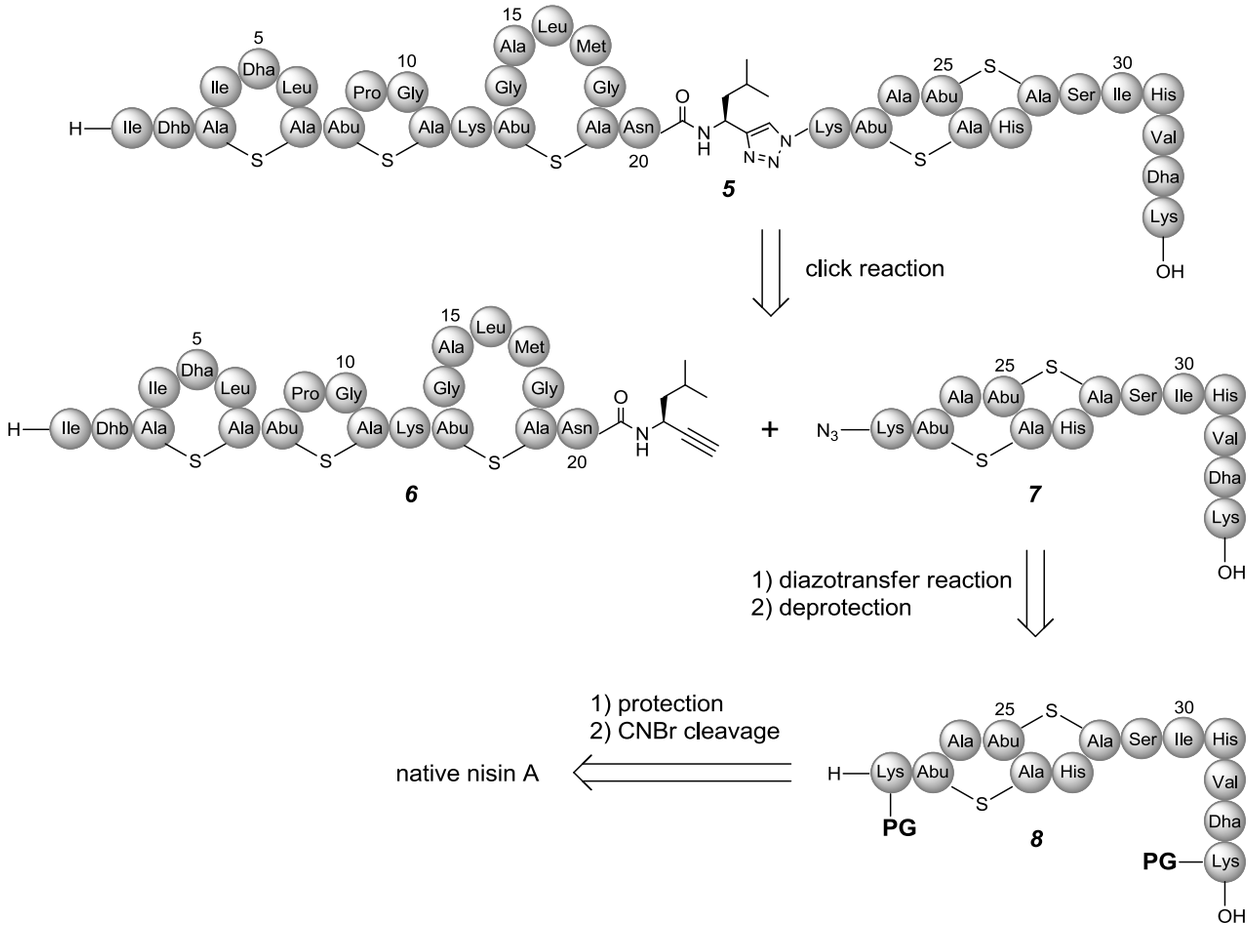

Figure 10. Retrosynthetic route for the preparation of native nisin containing a triazole moiety as a peptide bond surrogate.

8.2.3 Increase nisin AB activity via "membrane anchoring"

Chapter 5 describes the synthesis of an optimized dicarba mimic of nisin AB. It was demonstrated that dicarba nisin AB mimic in which the dehydro residues were replaced by alanines had a reduced activity in lipid II binding compared to native nisin AB. Strikingly, dicarba nisin AB containing dehydro residues, showed a comparable activity for lipid II when compared to native nisin, suggesting that incorporation of the dehydro residues in nisin AB mimics is important to maximize activity. However, the dehydro residues in nisin are responsible for the low stability of nisin at

higher pH or in the presence of nucleophiles, since the dehydro residues can act as a Michael acceptor. Therefore, nisin analogs which are lacking dehydro residues are expected to have an increased stability which is an important aspect in the design of nisin based peptide antibiotics.

A different approach to maximize or enhance the activity of antimicrobial peptides is to increase lipophilicity and thereby membrane affinity as is described in **chapter 7**. It was observed that incorporation of a lipophilic amino acid (Laa) into the antimicrobial peptide anoplin enhanced the antimicrobial activity up to tenfold. Therefore, incorporation of LAA into dicarba nisin AB mimic to afford Laa-nisin AB mimic (Figure 11), could increase the activity, while the stability is also increased compared to native nisin AB. The position of the lipophilic moiety in the peptide can have influence on the gain in activity, as can be seen in chapter 7, so a library of Laa-nisin AB mimics should be synthesized to investigate the optimal position of the membrane anchor.

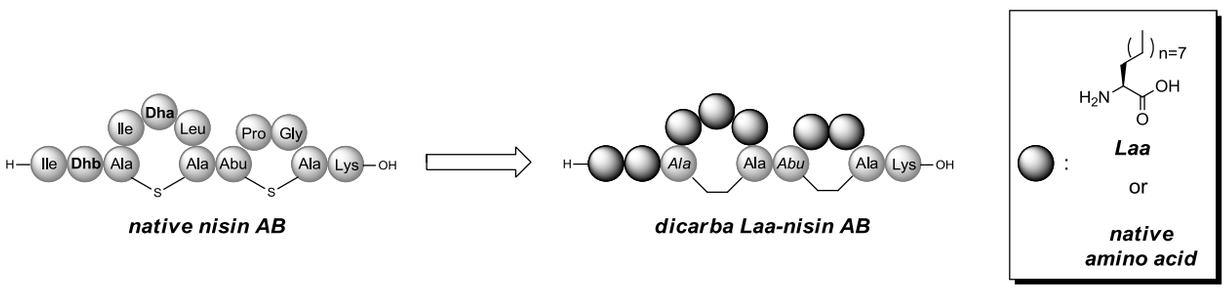

Figure 11. Design of lipophilic dicarba analogs of nisin AB by incorporating of lipophilic amino acid (Laa) as a membrane anchor.

Appendices

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Nederlandse samenvatting

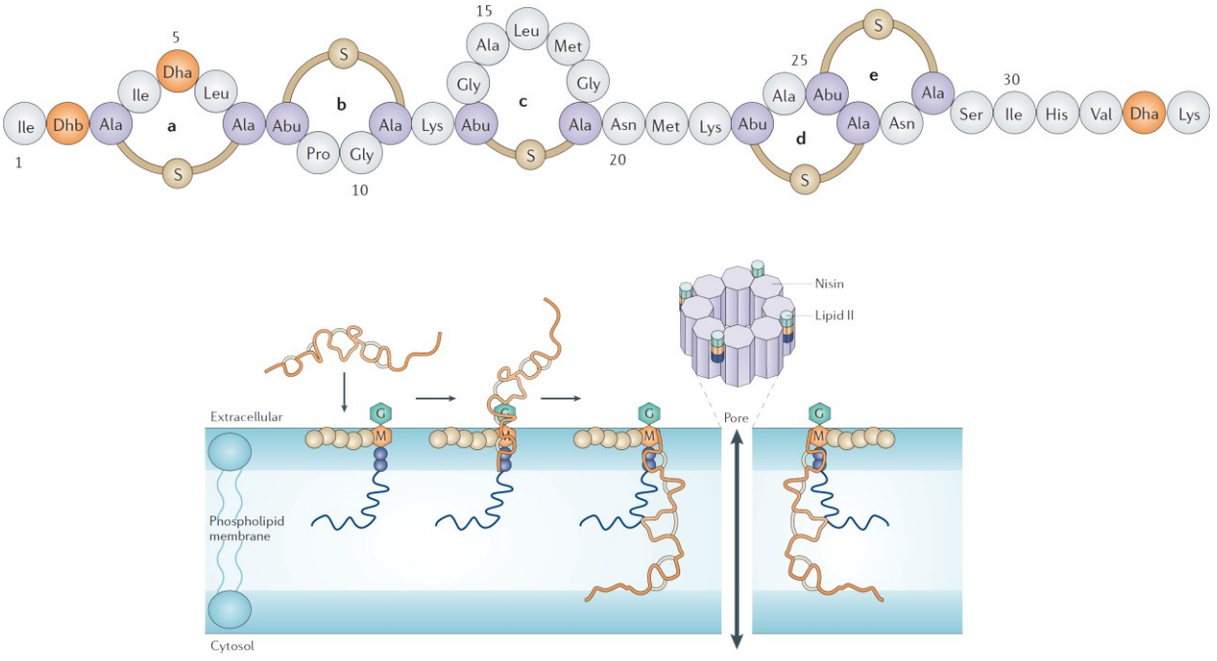
Semi-synthese van op nisine gebaseerde peptiden-antibiotica

Er is een groeiende vraag naar nieuwe antibiotica, omdat er steeds meer gevallen bekend zijn van infecties die worden veroorzaakt door resistente bacteriën, bijvoorbeeld, Methicilline Resistente *Staphylococcus aureus* (MRSA) en Vancomycine Resistente *Enteroccus* (VRE). Veelbelovende nieuwe antibiotica zijn antimicrobiële peptiden (AMPs), welke het membraan van bacteriën verstoren en daardoor mogelijk heel geschikt zijn als nieuwe antibiotica, omdat bacteriën tegen dit werkingsmechanisme moeilijk resistentie kunnen ontwikkelen. Een veelbelovend AMP is nisine, welke behoort tot de klasse van de lantibiotica. Lantibiotica zijn post-ribosomale, kationische peptiden en bevatten verscheidene onnatuurlijke aminozuren, zoals, dehydroalanine (Dha) en dehydrobutyrine (Dhb). Daarnaast kenmerken lantibiotica zich door het feit dat er meerdere cyclische structuren, gevormd door een thioether brug, ook wel lanthioninen genoemd, aanwezig zijn.

Nisine is peptide opgebouwd uit 34 aminozuurresiduen met daarin vijf lanthionine ringstructuren, de A-, B-, C- en de verknoopte DE-ring fragmenten. Deze vijf ringstructuren zijn cruciaal voor de antimicrobiële activiteit. Nisine vertoont activiteit in het lage nM-bereik gericht tegen Gram-positieve bacteriën en het werkingsmechanisme is hierbij tweeledig. Ten eerste bindt nisine met de N-terminus (het AB-fragment) aan het bacteriële membraan door een interactie aan te gaan met lipide II, een belangrijke component van de bacteriële celwand, en daarmee remt nisine de bacteriële celwandsynthese. In het tweede mechanisme steekt nisine, eenmaal gebonden via de N-terminus aan lipide II, de C-terminus in het bacteriële membraan en vormt een poriecomplex met lipide II wat leidt tot een efflux van allerlei ionen en dus tot het verlies van belangrijke cellulaire ion-gradiënten en vervolgens het afsterven van de bacterie (Figuur 1).

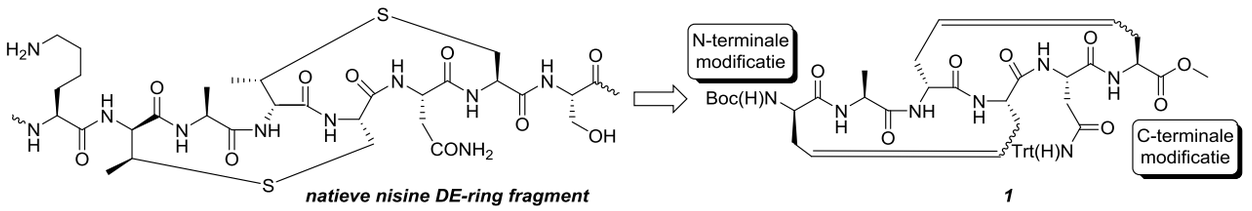
Hoewel nisine een veelbelovend startpunt is voor nieuwe, op peptiden-gebaseerde antibiotica, is de chemische totaalsynthese van nisine bepaald niet triviaal en vooral het introduceren van de lanthioninen is erg uitdagend. Bovendien zijn de lanthioninen gevoelig voor oxidatie wat leidt tot inactivatie van nisine. Door deze lanthioninen te vervangen met stabiele koolstof-koolstof (zogenaamde ‘dicarba’) bruggen, die via ringsluitingsmetathese (RSM) geïntroduceerd kunnen worden, kunnen stabiele op nisine-gelijkende structuren gesynthetiseerd worden. Vervolgens kan hiermee onderzocht worden wat de bijdrage is van elke individuele ring met betrekking tot de bioactiviteit van nisine, en in het bijzonder wat betreft het C-terminale DE-

ring fragment. Bovendien leidt de synthese van dit type nisine-mimetica tot meer inzicht in de toepasbaarheid van deze gemodificeerde peptiden als mogelijke nieuwe antibiotica.



Figuur 1. Nisine heeft een interactie met het bacteriële membraan door binding met lipide II. Nadat het C-terminale gedeelte van nisine in het membraan steekt, vormen nisine en lipide II een poriecomplex. Dit figuur is met toestemming overgenomen uit Breukink *et al.*, Copyright 2006 Nature Publishing group, Nature Reviews Drug Discovery.

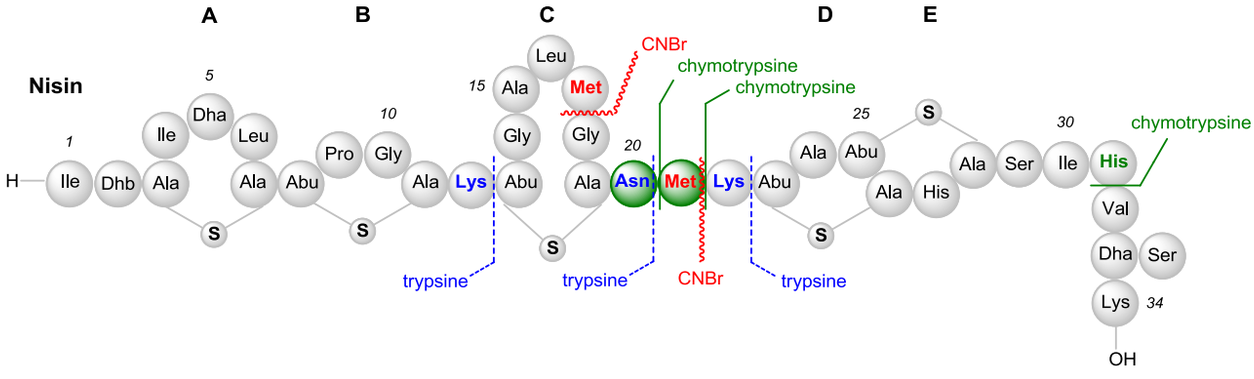
Hoofdstuk 2 beschrijft de synthese van een alkeen-gebrugd nisine DE-ring mimeticum **1** (zie Figuur 2) verkregen via RSM vanuit een lineair peptide met vier allylglycine (Alg) residuen. Het lineaire hexapeptide, Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}-OMe, werd gesynthetiseerd via vaste drager peptidesynthese en via RSM werd de verknoopte bicyclische, 1→4, 3→6 structuur, verkregen als een nisine DE-ring mimeticum. Omdat het mimeticum twee starre alkeen-bindingen heeft werd een mengsel van vier diastereoisomeren verkregen (elk met andere configuratie van de alkeen-binding : *Z*/*Z*, *Z*/*E*, *E*/*Z*, *E*/*E*). Dit mengsel van diastereoisomeren werd met behulp van HPLC gezuiverd en elk individueel diastereoisomeer werd gekarakteriseerd met behulp van twee-dimensionale NMR-spectroscopie analysetechnieken, waaronder TOCSY, ROESY en HSQC. Tevens werd de mogelijkheid aangetoond om de N- en C-termini te functionaliseren, zoals de koppeling van *N*-α-azido lysine aan de N-terminus en een beschermd lysine-derivaat aan de C-terminus. Tenslotte werd de azide-functionaliteit van het mimeticum gebruikt in een Cu(I)-gekatalyseerde 'klik' reactie om de toepasbaarheid in orthogonale ligaties van deze alkeen-gebrugde nisine DE-ring mimetica te demonstreren.



Figuur 2. Ontwerp van een alkeen-gebrugd DE-ring mimeticum (**1**) waarin de oxidatie-gevoelige lanthioninen zijn vervangen door alkeen-bruggen.

In **hoofdstuk 3** staat de ontwikkeling van een zuiveringsmethode voor nisine uit commercieel verkrijgbare nisine-preparaten beschreven. Nisine is commercieel verkrijgbaar als een mengsel bestaande uit NaCl (75%), melkeiwitten (22.5%) en slechts 2.5% nisine. Nisine werd hieruit verrijkt via een precipitatiemethode met een organisch oplosmiddel/water systeem. Met deze methode werd snel en efficiënt grote hoeveelheden nisine met een hoge zuiverheid verkregen zonder hierbij gebruik te maken van kolomchromatografie.

Om het gebruik van dit verrijkte nisine-preparaat te demonstreren werd het behandeld met twee verschillende proteasen, trypsine en chymotrypsine, waarmee de fragmenten nisine AB (1-12) en nisine ABC (1-20) verkregen werden. De enzymatische digesties werden bestudeerd met analytische HPLC en LC-MS om hiermee inzicht te verwerven in dit hydrolyseproces en hierbij werden twee niet eerder beschreven 'knip-posities' gevonden (Figuur 3). De verkregen peptiden-mengsels werden geanalyseerd en uiteindelijk gezuiverd met HPLC om de gewenste fragmenten (1-12), respectievelijk (1-20) te isoleren.



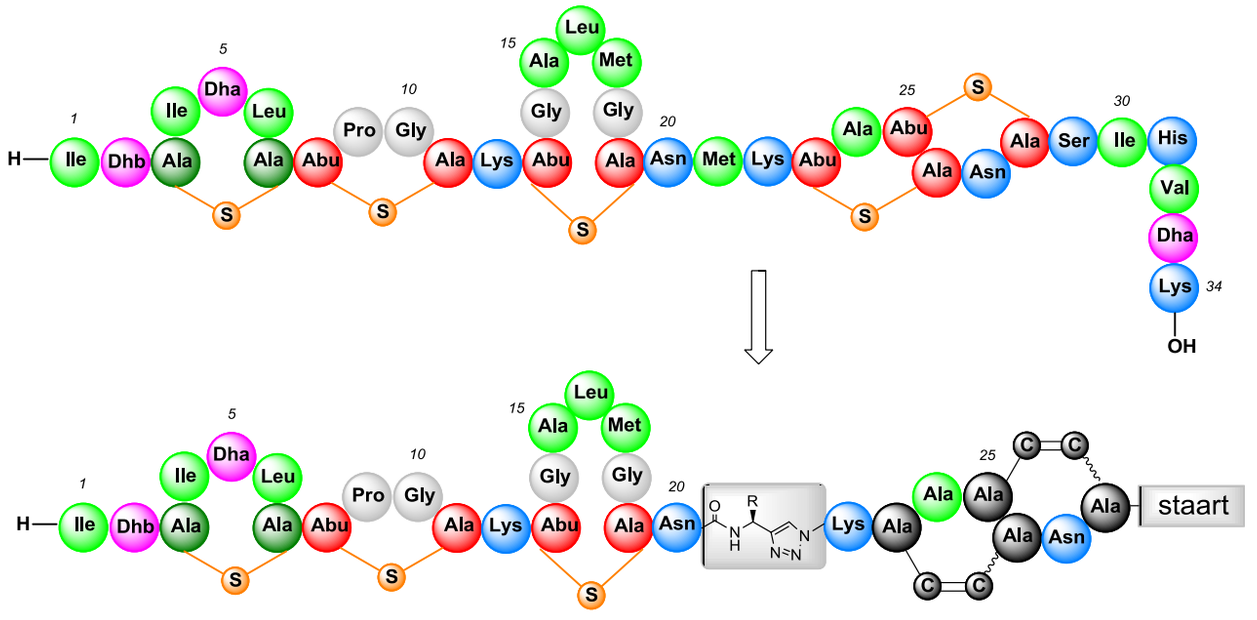
Figuur 3. Overzicht van de splitsingsposities van nisine A zoals waargenomen voor chymotrypsine (doorgetrokken lijn), trypsine (gestippelde lijn) en cyanogeen bromide (golflijn).

Het nisine-preparaat werd tevens met cyanogen bromide behandeld, waarbij nisine op positie methionine-21 gesplitst wordt, en werd het C-terminale DE-fragment van nisine (22-34) geïsoleerd. Als laatste werd nisine gesplitst via een tweestaps procedure, door eerst een behandeling met

chymotrypsine te ondergaan gevolgd door een reactie met cyanogeen bromide. Hiermee kon een ingekort DE-fragment (22-31) verkregen worden. De mogelijkheid tot het verkrijgen van natieve nisine fragmenten is waardevol voor dit onderzoek om de bijdrage van de individuele ring-structuren aan de bioactiviteit te bestuderen. Dit laatste gebeurt aan de hand van het synthetiseren van hybride nisine-structuren zoals deze in de volgende hoofdstukken beschreven zijn.

De synthese van hybride nisine peptidomimetica is beschreven in **hoofdstuk 4**. De porie-vormende activiteit van nisine is gelegen in de C-terminus, omdat bij nisine varianten met een verkorte C-terminus de porie-vormende activiteit verloren is gegaan. In een eerste poging werd een hybride nisine mimeticum ontworpen door een natief ABC-gedeelte (nisine 1-20) te combineren met een alkeen-gebrugde DE-ring om zo het aandeel in de bioactiviteit van het synthetische DE-mimeticum te bestuderen en deze activiteit te vergelijken met die van nisine (zie Figuur 4). Natief nisine ABC werd verkregen door enzymatische digestie van nisine A met chymotrypsine. Natief nisine ABC werd C-terminaal voorzien van een propargylamine eenheid, om zo het alkyn-gefunctionaliseerde nisine ABC te verkrijgen. Vervolgens werd via Cu(I)-gekatalyseerde "klik" chemie het *N*-α-azidolysine-dicarba-DE-fragment gekoppeld om zodoende het nisine hybride te verkrijgen.

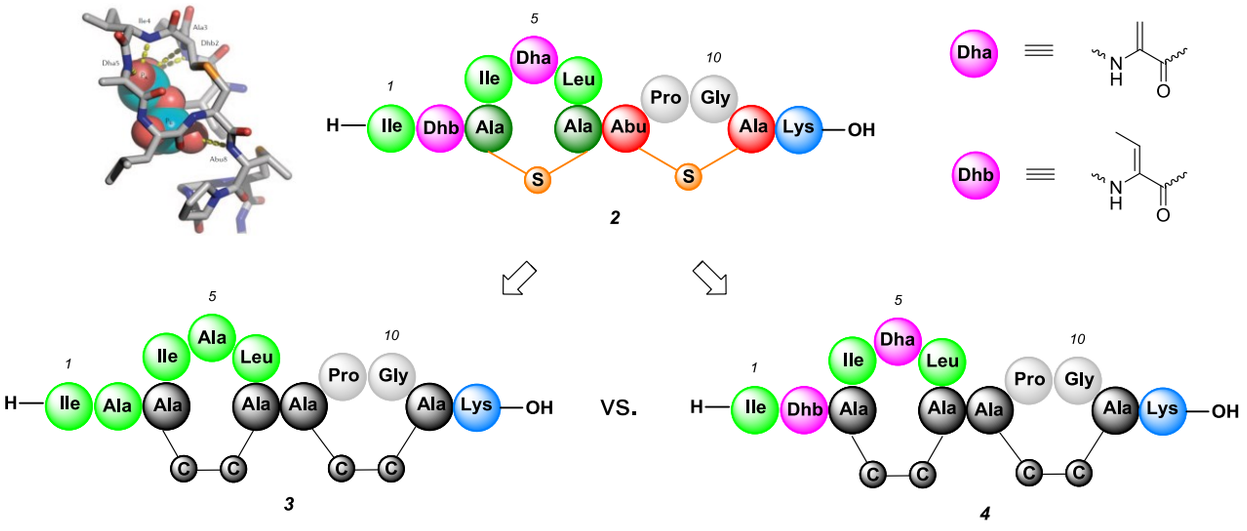
Het nisine mimeticum was in staat aan lipide II te binden, een indicatie dat het antibacteriële werking bezit, echter het vertoonde geen porie-vormende activiteit. Dit mimeticum bevatte een achirale linker, afkomstig van propargylamine, op de positie van residu 21. Deze linker bootst glycine na en volgens een mutatiestudie met nisine zorgde een mutatie van het hydrofobe residu methionine-21 in glycine-21 voor een sterk verminderde porie-vormende activiteit. Ook werd er in een recente studie aangetoond dat een (ingekorte) C-terminale sequentie cruciaal is voor de porie-vormende activiteit. Om tot een verbeterd nisine mimeticum te komen, werd een amino-alkyn derivaat, gebaseerd op methionine of leucine, gesynthetiseerd via een tweestaps methode vanuit Boc-Met-OH of Boc-Leu-OH, gebruikmakend van het Bestmann-Ohira reagens. Het leucine amino-alkyn werd aan de C-terminus gekoppeld van nisine ABC om uiteindelijk nisine ABC-alkyn te verkrijgen met een leucine zijketen op positie 21. Vervolgens werd hieraan via een Cu(I)-gekatalyseerde 'klik' reactie *N*-α-azidolysine-dicarba-DE-ring-lysine-NHMe gekoppeld. Helaas bleek ook dit mimeticum inactief te zijn als porie-vormer. Echter, de antimicrobiële activiteit bleek een factor 10 lager te zijn dan natief nisine. Ter controle werd het ABC-fragment getest en hieruit bleek dat de waargenomen activiteit van het nisine mimeticum vergelijkbaar was met die van het ABC-fragment, een indicatie dat de bijdrage van het alkeen-gebrugde DE-ring mimeticum aan de biologische activiteit zeer gering was.



Figuur 4. Schematische representatie van een hybride nisine mimeticum. Natief nisine ABC werd geligeerd aan het alkeen-gebrugde DE-ring mimeticum via Cu(I)-gekatalyseerde "klik" chemie.

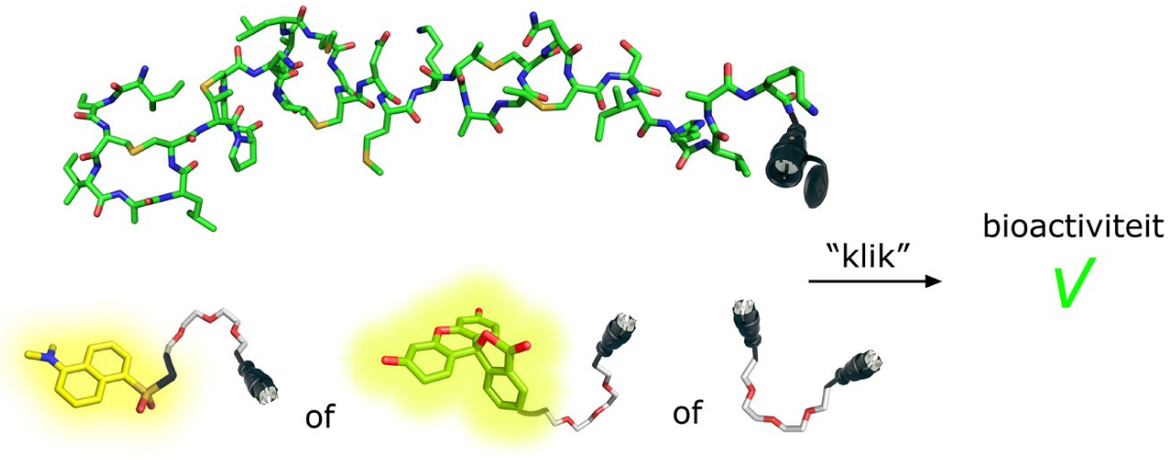
Hoofdstuk 5 beschrijft de synthese van een dicarba-gebrugd nisine AB (1-12) mimeticum via RSM. In een eerdere studie was een serie dicarba AB mimetica gesynthetiseerd waarin de twee dehydro-aminozuren (Dhb2 en Dha5) waren vervangen door een alanine residu. Deze nisine AB mimetica waren actief met betrekking tot het binden van lipide II, echter, de activiteit was lager ten opzichte van natief nisine AB. Omdat de interactie van nisine AB met lipide II voor een groot deel bepaald wordt door de amidebindingen in de peptideketen, is de conformatie van de peptideketen erg belangrijk waarbij de sp^2-gehybridiseerde Cα atomen van de dehydro-aminozuren een belangrijke rol spelen. Een peptideketen met alanine op posities 2 en 5 heeft alle Cα atomen in de sp3-hydridisatietoestand en is hierdoor minder star en mogelijk minder goed in staat lipide II met hoge affiniteit te binden.

Door een combinatie van vaste drager- en vloeistoffase-synthese werden dicarba ring A en dicarba ring B gesynthetiseerd via RSM, en werden threonine respectievelijk serine gebruikt als precursor bouwstenen voor dehydrobutyrine en dehydroalanine. Na het koppelen van ring A aan ring B via fragmentcondensatie, werden Thr2 en Ser5 gedehydrateerd via de chemicaliën-combinatie, EDCI en $CuCl_2$. AB-fragmenten **2-4,** afgebeeld in Figuur 5, werden getest in een lipide II bindingsexperiment en het bleek dat mimeticum **4** even actief was vergeleken met het natieve fragment **2**, terwijl mimeticum **3** een beduidend lager affiniteit voor lipide II vertoonde.



Figuur 5. Nisine AB dicarba mimetica waarin de natieve lanthionine bruggen zijn vervangen door dicarba-bruggen en ook de dehydro aminozuren Dhb2 en Dha5 zijn opgenomen.

De functionalisatie van nisine met een indicator-molecuul kan zeer wenselijk zijn voor het in detail bestuderen van het werkingsmechanisme van nisine. Ook de multimerisatie van nisine om multivalente structuren te verkrijgen, of het conjugeren van nisine aan andere bioactieve moleculen, kan heel interessant zijn in het bestrijden van bacteriën gericht op meerdere methoden van aanpak. De synthese van zulke conjugaten is beschreven in **hoofdstuk 6**. Het gebruik van orthogonale ligatie methoden, zoals de Cu(I)-gekatalyseerde azide-alkyn koppeling, is ideaal voor het conjugeren van grote onbeschermde peptiden vanwege de milde reactie condities.

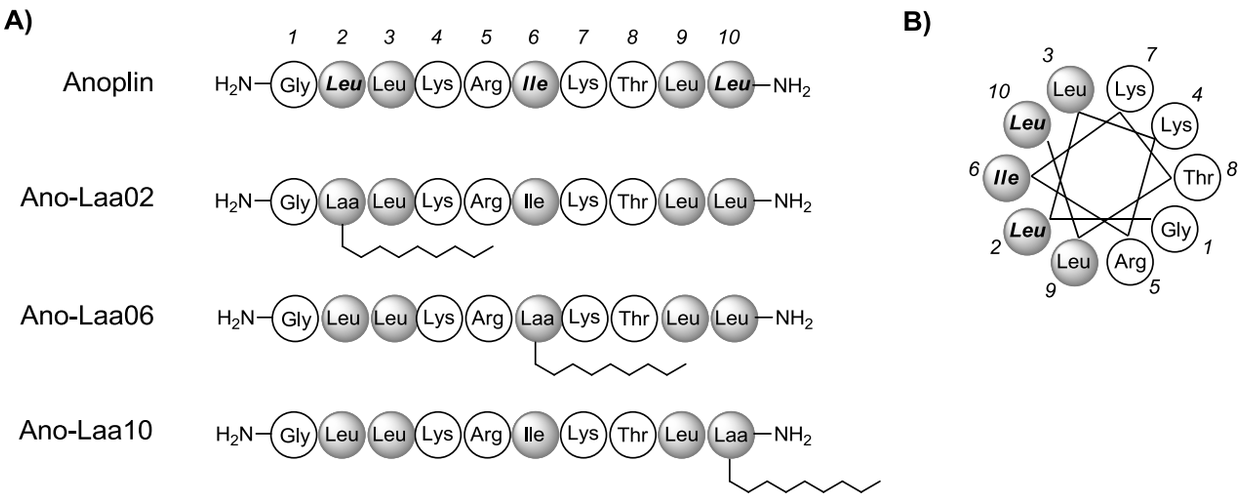


Figuur 6. Synthese van nisine-conjugaten via 'klik' chemie.

Twee fluorescente nisin derivaten werden gesynthetiseerd door dansyl-azide respectievelijk carboxyfluoresceine-azide te koppelen aan het nisine-alkyn. Daarnaast werd het nisine-alkyn geconjugeerd aan een bis-azide om zodoende een nisine-dimeer te verkrijgen. Alle conjugaten vertoonden zowel antimicrobiële- alsook membraan-permeabilisatie activiteit, vergelijkbaar met natief nisine. Met deze aanpak werd het bewijs geleverd dat de C-terminale modificaties getolereerd werden met betrekking tot de biologische activiteit en dat de moleculaire constructen gebruikt kunnen worden in biochemische studies.

Hoofdstuk 7 beschrijft de synthese van een Fmoc-beschermd, chiraal, en lipofiel aminozuur (Laa), welke een zijketen van negen koolstofatomen bevat. Fmoc-Laa-OH kan in elk gewenste peptideseqentie geïntroduceerd worden met behulp van vaste drager synthese om de lipofiliciteit van het peptide te verhogen zonder dat hierbij eventuele belangrijke polaire segmenten van het peptide, bijvoorbeeld de N- en C-termini, opgeofferd hoeven te worden. De basale gedachte achter deze aanpak was dat het verhogen van de lipofiliciteit van een AMP een verbeterde membraan-affiniteit kan opleveren, en zo mogelijk ook verbeterde eigenschappen als potentieel nieuw antibioticum. Fmoc-Laa-OH werd gesynthetiseerd in vier reactiestappen vanuit L-glutaminezuur en de lipofiele zijketen werd via een Wittig reactie geïntroduceerd.

Anoplin, een uit tien aminozuren bestaand AMP, werd hiervoor gekozen om als een model peptide te dienen waarmee het ontwerp experimenteel werd getest. Drie lipofiele anoplin analoga (Ano-Laa02, Ano-Laa06 en Ano-Laa10) werden gesynthetiseerd op de vaste drager waarbij een leucine- danwel een isoleucine-residu werd vervangen door Laa bouwsteen en wel op posities 2, 6 en 10. Alle drie de lipofiele analoga vertoonden een sterk verhoogde antimicrobiële activiteit tot wel 4-8 keer beter voor *E. coli* (Gram-negatief) en meer dan tien keer hoger tegen *S. aureus* (Gram-positief) in vergelijking tot anoplin. Ondanks het feit dat de hemolytische activiteit van de lipofiele analoga ook verhoogd was, was de concentratie waarbij 50% hemolyse plaatsvindt meer dan tienmaal hoger dan de concentratie waarbij bacteriegroei geremd wordt, de zogenaamde minimale inhibitie concentratie (MIC). Als een tweede bewijs werden de lipofiele analoga samen met anoplin getest op een model membraansysteem, met carboxyfluoresceïne geladen vesicles, waarbij de membraan-verstorende activiteit van het peptide werd bepaald. Ook hier vertoonden de lipofiele analoga een verhoogde activiteit ten opzichte van anoplin, zonder daarbij aan selectiviteit te verliezen. Deze resultaten suggereren dat de verhoging van de lipofiliciteit van een antimicrobieel peptide door het introduceren van het lipofiele aminozuur Laa mogelijk als een algemene methode toegepast kan worden voor het verhogen van de membraanaffiniteit van peptiden.



Figuur 7. Een schematische weergave van anoplin en de drie lipofiele analoga (links). Een ’helix wiel’ representatie (rechts) van de verwachte helix structuur van anoplin wanneer het een interactie heeft met een membraan. Posities 2, 6 en 10 zijn gelokaliseerd op de hydrofobe, de veronderstelde membraan-bindende zijde van anoplin.

List of abbreviations

Å	Ångström
AcOH	acetic acid
AMP	antimicrobial peptide
aq.	aqueous
Ar	aromatic
Boc	tert-butyloxycarbonyl
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
BPB	bromophenol blue
^{t}Bu	*tert*-butyl
Bzl	benzyl
Cbz	carboxybenzyl
CF	carboxyfluorescein
CFU	colony-forming unit
CuAAC	copper (I)-catalyzed azide-alkyne cycloaddition
Cy	cyclohexyl
d	doublet
δ	chemical shift
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC `	dicyclohexyl carbodiimide
dd	double doublet
Dha	dehydroalanine
Dhb	(*Z*)-dehydrobutyrine
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPG	1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol
dt	doublet of triplets
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
equiv	equivalents

ESI-MS	electrospray ionization mass spectroscopy
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
Fmoc	9-fluorenylmethyloxycarbonyl
Fmoc-ONSu	9-fluorenylmethyloxycarbonyl *N*-hydroxysuccinimide ester
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Hz	hertz
IC_{50}	half maximal inhibitory concentration
KHMDS	hexamethyldisilazane potassium salt
Laa	lipophilic amino acid
LC-MS	liquid chromatography mass spectroscopy
LUVs	large unilamellar vesicles
m	multiplet
MALDI-TOF	matrix-assisted laser desorption/ionization - time of flight
MeCN	acetonitrile
MeOH	methanol
min	minutes
MS	mass spectrometry
MTBE	methyl-tert-butyl ether
m/z	mass over charge ratio
Mes	2,4,6-trimethylphenyl
NMP	*N*-methylpyrrolylidone
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
OD_{630}	optical density at 630 nm
p	para
PEG	polyethylene glycol
ppm	parts per million
q	quartet
RCM	ring-closing metathesis
R_f	retention factor
R_t	retention time

ROESY	rotating frame nuclear overhauser enhancement spectroscopy
s	singlet
SPPS	solid phase peptide synthesis
t	triplet
TBAB	tert *n*-butylammonium bromide
TEA	triethylamine
TOCSY	total correlation spectroscopy
TDM	*N,N,N',N'*-tetramethyl-4,4'-diaminodiphenylmethane
tert-BuOH	*tert*-butanol
TFA	trifluoroacetic acid
THF	tetrahedrofuran
TIS	triisopropylsilane
TLC	thin layer chromatography
TMS	trimethylsilane
Trt	triphenylmethyl (or trityl)
Tris	tris(hydroxymethane)aminomethane
UV	ultraviolet
v	volume
vis	visible
Xaa	amino acid

Amino acids

Ala	A	alanine	Leu	L	leucine
Alg		allylglycine	Lys	K	lysine
Arg	R	arginine	Met	M	methionine
Asn	N	asparagine	Phe	F	phenylalanine
Asp	D	aspartic acid	Pro	P	proline
Cys	C	cysteine	Ser	S	serine
Gln	Q	glutamine	Thr	T	threonine
Glu	E	glutamic acid	Trp	W	tryptophan
Gly	G	glycine	Tyr	Y	tyrosine
His	H	histidine	Val	V	valine
Ile	I	isoleucine			

Curriculum Vitae

Jack Slootweg werd geboren op 2 oktober 1984 te Den Helder. In 2003 behaalde hij zijn VWO diploma met het profiel Natuur en Gezondheid aan het Molenplein te Den Helder. Datzelfde jaar begon hij de bachelor opleiding Scheikunde aan de Universiteit Utrecht. Na het behalen van het bachelor-diploma in 2007 werd begonnen met de prestige master Biomolecular Sciences eveneens aan de Universiteit Utrecht. Een major onderzoeksstage van 9 maanden werd uitgevoerd in de disciplinegroep Medicinal Chemistry and Chemical Biology van Prof. Dr. Rob Liskamp aan de Universiteit Utrecht onder begeleiding van Dr. Ir. Dirk Rijkers. Vervolgens werd de tweede onderzoeksstage uitgevoerd binnen de vakgroep NMR Spectroscopy van Prof. Dr. Rolf Boelens aan de Universiteit Utrecht onder begeleiding van Dr. Hans Wienk en Dr. Gert Folkers. Na het behalen van het master-diploma in 2009 startte hij met zijn promotieonderzoek onder begeleiding van Prof. Dr. Rob Liskamp en Dr. Ir. Dirk Rijkers in de disciplinegroep Medicinal Chemistry and Chemical Biology aan de Universiteit Utrecht. Het hierbij verrichte onderzoek is beschreven in dit proefschrift. Daarnaast zijn de behaalde resultaten ook gepresenteerd op nationale en internationale symposia waaronder het 31^{e} European Peptide Symposium te Kopenhagen (september 2010) en het 32^{e} European Peptide Symposium te Athene (september 2012). Vanaf juli 2013 is de auteur werkzaam als post-doc in de disciplinegroep Bioinorganic Chemistry van Prof. Dr. Nils Metzler-Nolte aan de Ruhruniversität te Bochum (Duitsland).

List of Publications

Slootweg, **J**. **C**.; van Schaik, T. B.; Quarles Van Ufford, H. C.; Breukink, E.; Liskamp, R. M. J.; Rijkers, D. T. S., Improving the biological activity of the antimicrobial peptide anoplin by membrane anchoring through a lipophilic amino acid derivative, *Bioorg. Med. Chem Lett.* **2013**, *23*, 3749-3752.

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‡These authors contributed equally to this work

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Abstracts

Slootweg, **J**. **C**.; Rijkers, D. T. S.; Liskamp, R. M. J., Synthesis and structural characterization of all four diastereoisomers of the crossed alkene-bridged nisin DE-ring mimics. *J. Pept. Sci.* **2010**, *16*, 45-45.

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UK-Netherlands Joint AMP Symposium, **2011**, Durham, United Kingdom:

Slootweg, **J**. **C**.; Liskamp, R. M. J.; Rijkers, D. T. S., Synthesis and structural characterization of all four diastereoisomers of the crossed alkene-bridged nisin DE-ring mimics.

32nd European Peptide Symposium, **2012**, Athens, Greece:

Slootweg, **J**. **C**.; Liskamp, R. M. J.; Rijkers, D. T. S., Synthesis, structural characterization and application of crossed alkene-bridged nisin DE-ring mimics for semi-synthesis of nisin hybrid mimics.

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Slootweg, **J**. **C**.; Liskamp, R. M. J.; Rijkers, D. T. S., Combining nature and synthesis for the preparation of novel peptide antibiotics.

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