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## Synthesis of nisin AB dicarba analogs using ring-closing metathesis: influence of $sp^3$ versus $sp^2$ hybridization of the $\alpha$ -carbon atom of residues dehydrobutyrine-2 and dehydroalanine-5 on the lipid II binding affinity†

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Herein the synthesis of two nisin AB dicarba analogs is described, focusing on amino acid modifications at positions 2 and 5. The nisin mimics were synthesized by a combination of solid phase synthesis of the linear peptides, followed by macrocyclization *via* ring-closing metathesis and fragment assembly by means of solution phase chemistry. The two N-terminal nisin AB-fragment mimics contain either the native dehydrobutyrine (Dhb)/dehydroalanine (Dha) amino acid residues or alanine at position 2 and 5, respectively. The native dehydrobutyrine at position 2 and dehydroalanine at position 5 were introduced as their precursors, namely threonine and serine, respectively, and subsequent dehydration was carried out by EDCI/CuCl as the condensing agent. Both AB-fragment mimics were analyzed in a lipid II binding assay and it was found that the Ala2/Ala5 AB-mimic (**2**) showed a reduced activity, while the Dhb2/Dha5 AB-mimic (**3**) was as active as the native AB-fragment (**1**).

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## 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 sulfhy-

dryl of an adjacent cysteine residue to result in a D-configuration of the newly formed stereocenter.<sup>1</sup>

The N-terminal part of nisin comprising the AB(C) ring system 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. The structure of the complex between nisin–lipid II has been solved by NMR spectroscopy.<sup>2,3</sup> 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, an indication that nisin-AB and other lantibiotics with a comparable N-terminus hold great promise as novel peptide antibiotics.<sup>4</sup> Recent studies on the activity of nisin against clinical isolates of *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) underpins the importance for further development of nisin as an alternative antibiotic therapy.<sup>5</sup> To reach

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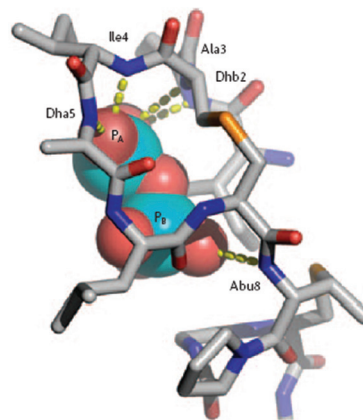
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†Electronic supplementary information (ESI) available: Copies of <sup>1</sup>H and <sup>13</sup>C NMR spectra, ESI-MS spectra, HPLC chromatograms and LC-MS data. See DOI: 10.1039/c5ob00336a

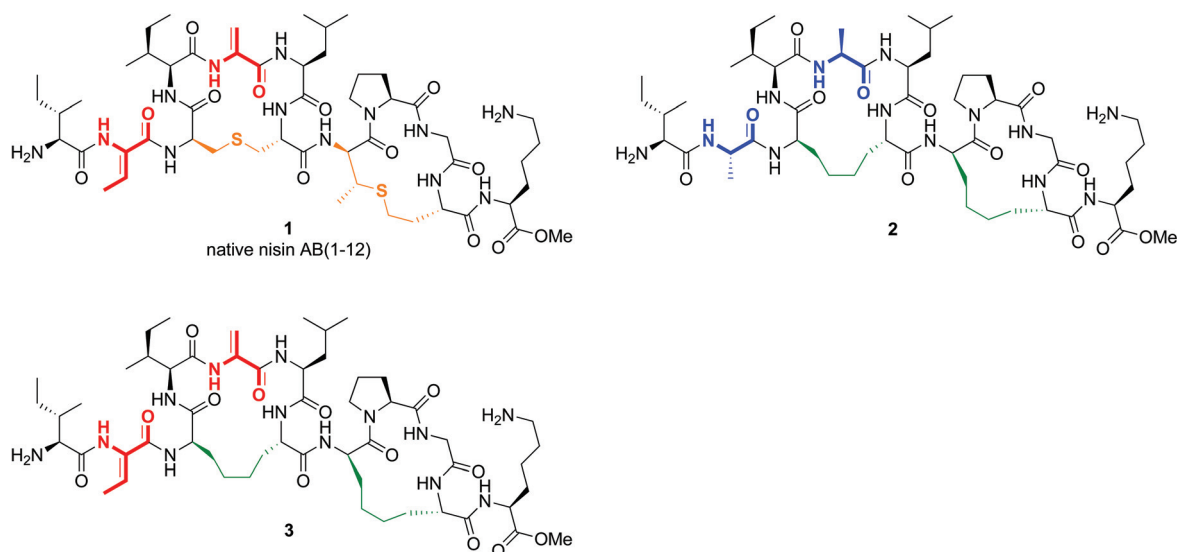
this goal, special attention should be paid toward a better pharmacokinetic profile, better metabolic stability and increased solubility and the described nisin derivatives herein could be the first step.

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.<sup>6</sup> Moreover, the lanthionine moieties are sensitive toward oxidation, resulting in a loss of activity.<sup>7</sup> Previously, we initiated a program to synthesize nisin analogs by ring-closing metathesis,<sup>8</sup> where the native lanthionines were replaced by dicarba bridges, to increase metabolic stability and potency.<sup>9</sup> 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.<sup>9,10</sup> The reduced activity could be a result of replacing the lanthionines by dicarba bonds as described for Lacticin 3147 dicarba analogues,<sup>10</sup> 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 (Fig. 1). This suggests a strong influence of the backbone conformation of nisin for the affinity toward lipid II. The dehydro residues contain a  $sp^2$  hybridized  $C\alpha$  carbon atom in contrast to the  $sp^3$  hybridized  $C\alpha$  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<sup>11</sup> and gallidermin<sup>12,13</sup> do not have dehydro residues at position 2 and 5, they have been replaced by Ala/Phe. This suggests that the presence of an  $sp^2$  hybridized  $C\alpha$  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, Ala2/Ala5 (2) and Dhb2/Dha5 (3), were synthesized to study their affinity toward lipid II and compared with its native counterpart 1, as shown in Fig. 2.



**Fig. 1** NMR structure of the nisin:lipid II complex. The interaction is dominated by hydrogen bonding (shown in yellow) of the backbone amide protons of Dhb2, Ala3, Ile4, Dha5 and Abu8 to pyrophosphate moiety of lipid II. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Struct. Mol. Biol.*, 2004, **11**, 963, copyright 2004 (see ref. 3).



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

## Results and discussion

### Synthesis

It was decided to synthesize the nisin AB dicarba analogs 2 and 3 according to the retrosynthesis as shown in Scheme 1, *via* three key intermediate fragments, namely the N-terminal dipeptides 5, 6 and 8, ring-fragments A 13–15 and 17–19, ring-fragment B 10–12, while the nisin AB dicarba analogs were assembled *via* fragment condensation.

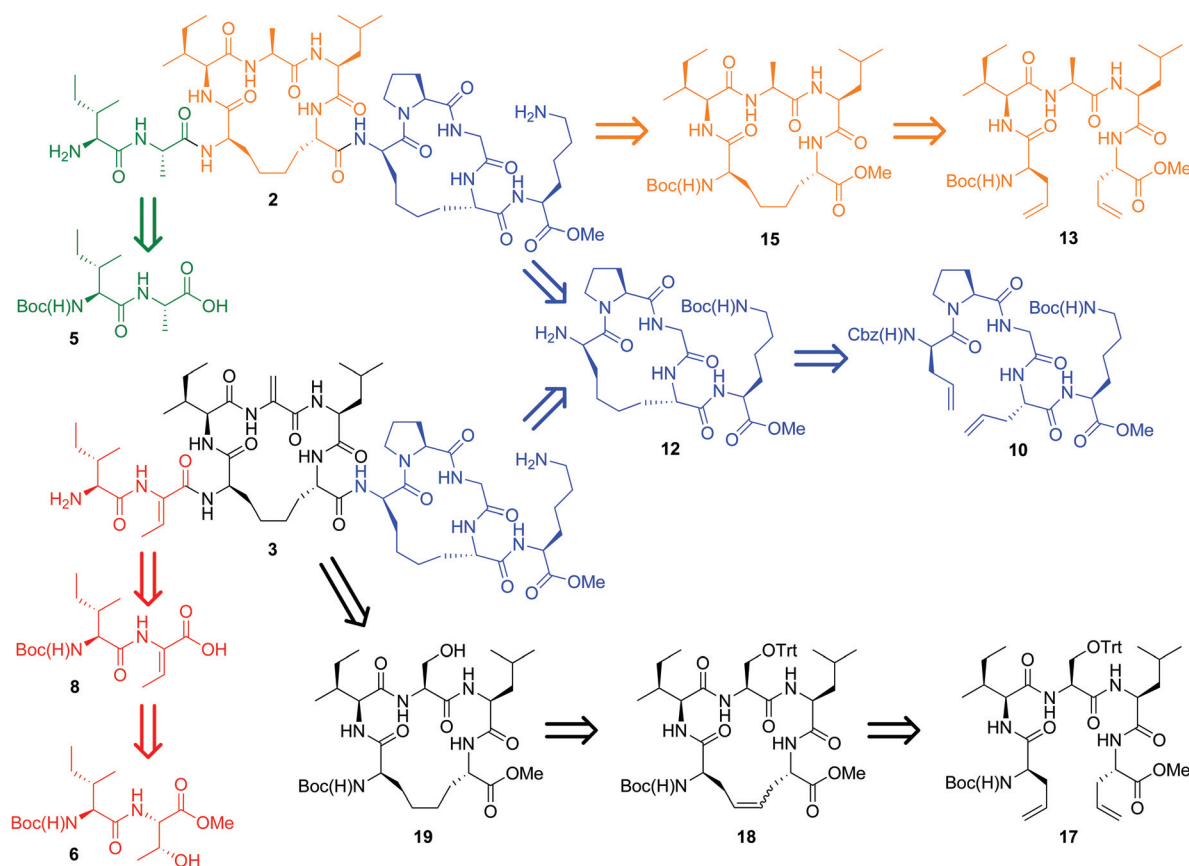
The Ala2/Ala5 nisin AB dicarba analog 2 was synthesized in which the two dehydro residues, *Z*-dehydrobutyrine (Dhb) and dehydroalanine (Dha), were replaced by an *L*-alanine residue. The synthesis started with the preparation of Boc-Ile-Ala-OMe 4 *via* a BOP coupling of Boc-Ile-OH and HCl-H-Ala-OMe in the presence of TEA as the base. Methyl ester 4 was quantitatively saponified in peptide acid Boc-Ile-Ala-OH 5 as shown in Scheme 2A.

Then, the synthesis of the dehydro dipeptide 8 started with Boc-Ile-OH which was coupled to HCl-H-Thr-OMe to obtain dipeptide Boc-Ile-Thr-OMe 6 in a good yield (70%), as shown in Scheme 2B. In the next step, 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  $\beta$ -elimination by adding DBU at reflux conditions in dichloromethane<sup>14</sup> to yield dehydrobutyrine

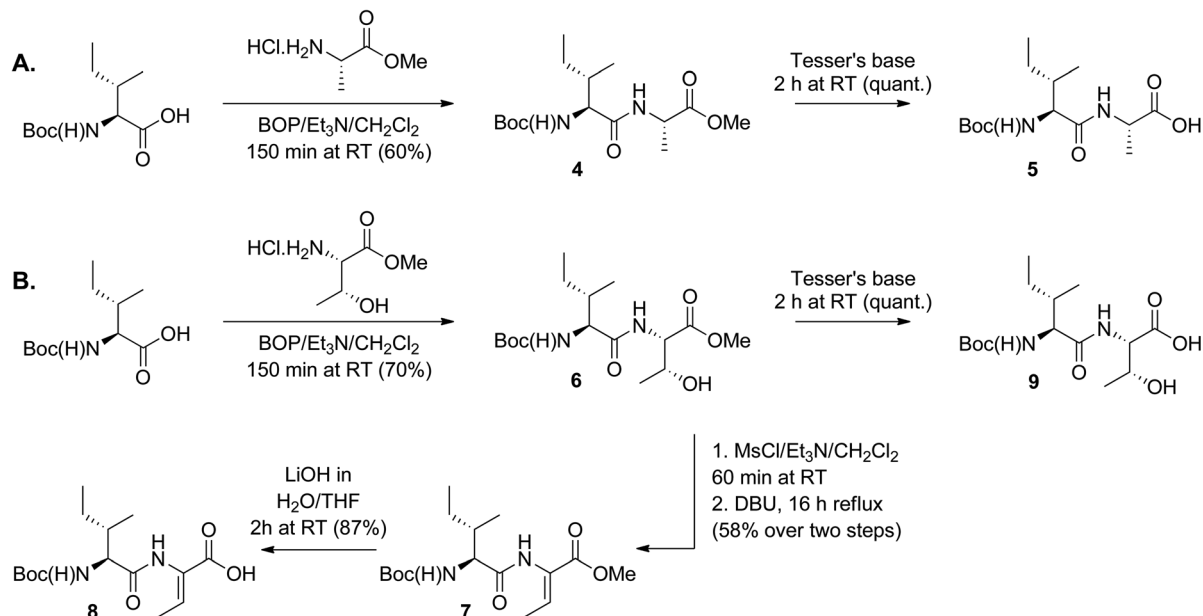
derivative 7 (58%). Subsequently, saponification of methyl ester 7 in the presence of lithium hydroxide provided Boc-Ile-Dhb-OH 8 (87%), which was ready for fragment condensation.

The synthesis of ring-fragment B 12 started with the preparation of the linear pentapeptide Cbz-D-Alg<sup>1</sup>-Pro<sup>2</sup>-Gly<sup>3</sup>-Alg<sup>4</sup>-Lys-(Boc)<sup>5</sup>-OMe 10 by SPPS shown in Scheme 3. Ring-closing metathesis of linear peptide 10 in the presence of second generation Grubbs catalyst<sup>15</sup> (5 mol%) in dichloromethane at reflux conditions gave the alkene-bridged peptide 11 as an *E/Z* diastereomeric mixture in an excellent yield (94%) after column chromatography. Then, alkene-bridged peptide 11 was treated with H<sub>2</sub> (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 (12) was quantitatively obtained as its corresponding trifluoroacetate.

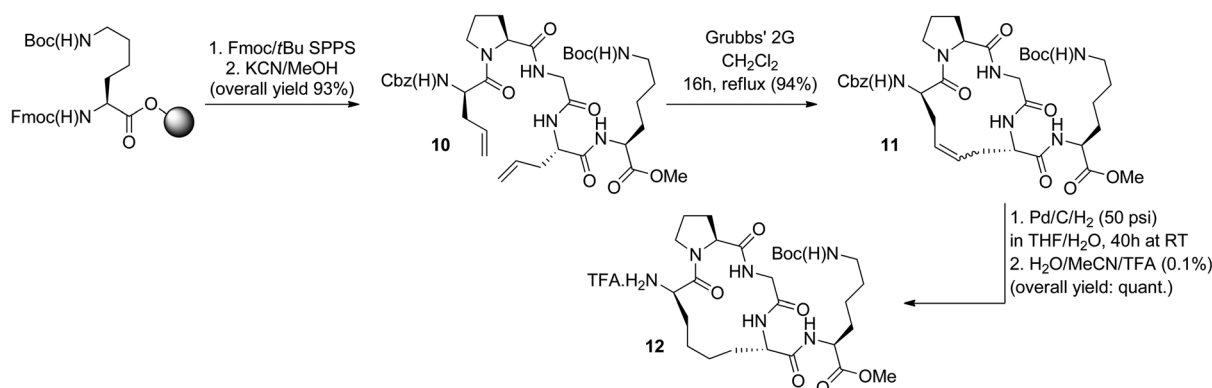
Subsequently, linear pentapeptide 13 (Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OMe) was synthesized on the solid phase and was isolated in 81% yield, as shown in Scheme 4. Cyclization by ring-closing metathesis of bis-alkene 13 in the presence of Grubbs II catalyst gave both diastereoisomers of alkene-bridged ring-fragment A 14 in 80% yield after column chromatography. Reduction of the alkene was achieved by treatment with H<sub>2</sub> (at 50 psi) in a Parr apparatus with Pd/C as the catalyst and alkane-bridged ring-fragment A 15 was obtained in quantitative yield. The synthesis was continued by removal of the



Scheme 1 Retrosynthesis of compounds 2 and 3.



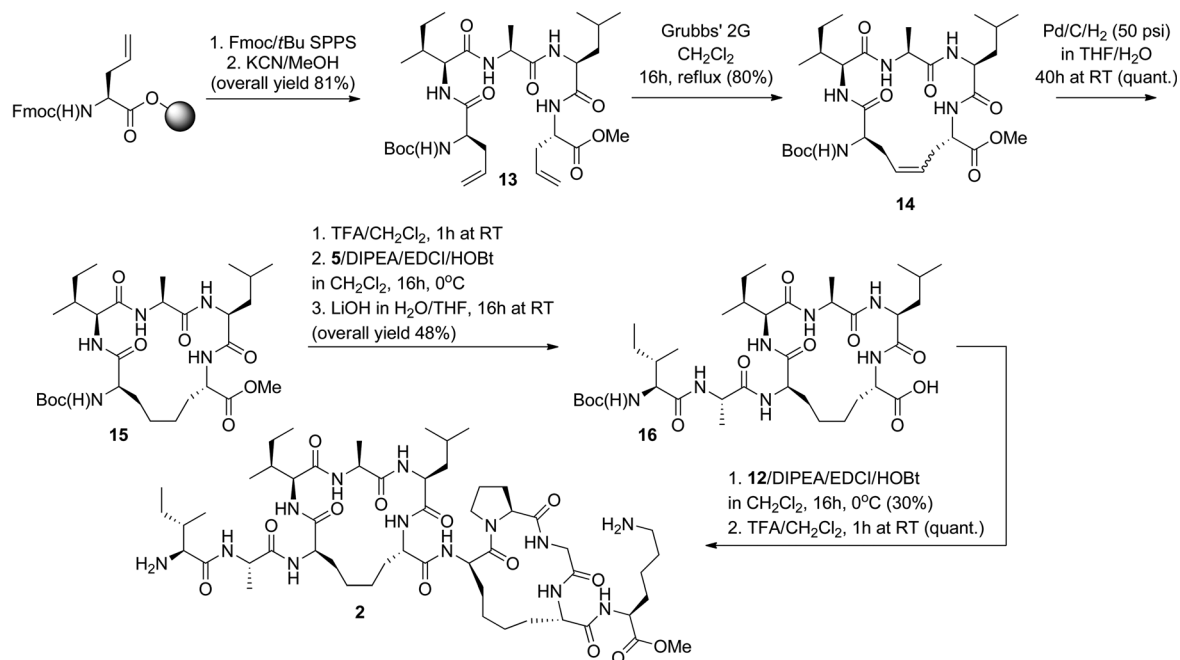
Scheme 2 Synthesis of dipeptides acids 5, 8, and 9.

Scheme 3 Synthesis route for ring-fragment B, cyclo[1-4]-H-D-Alg<sup>1</sup>-Pro<sup>2</sup>-Gly<sup>3</sup>-Alg<sup>4</sup>-Lys(Boc)<sup>5</sup>-OMe-TFA 12.

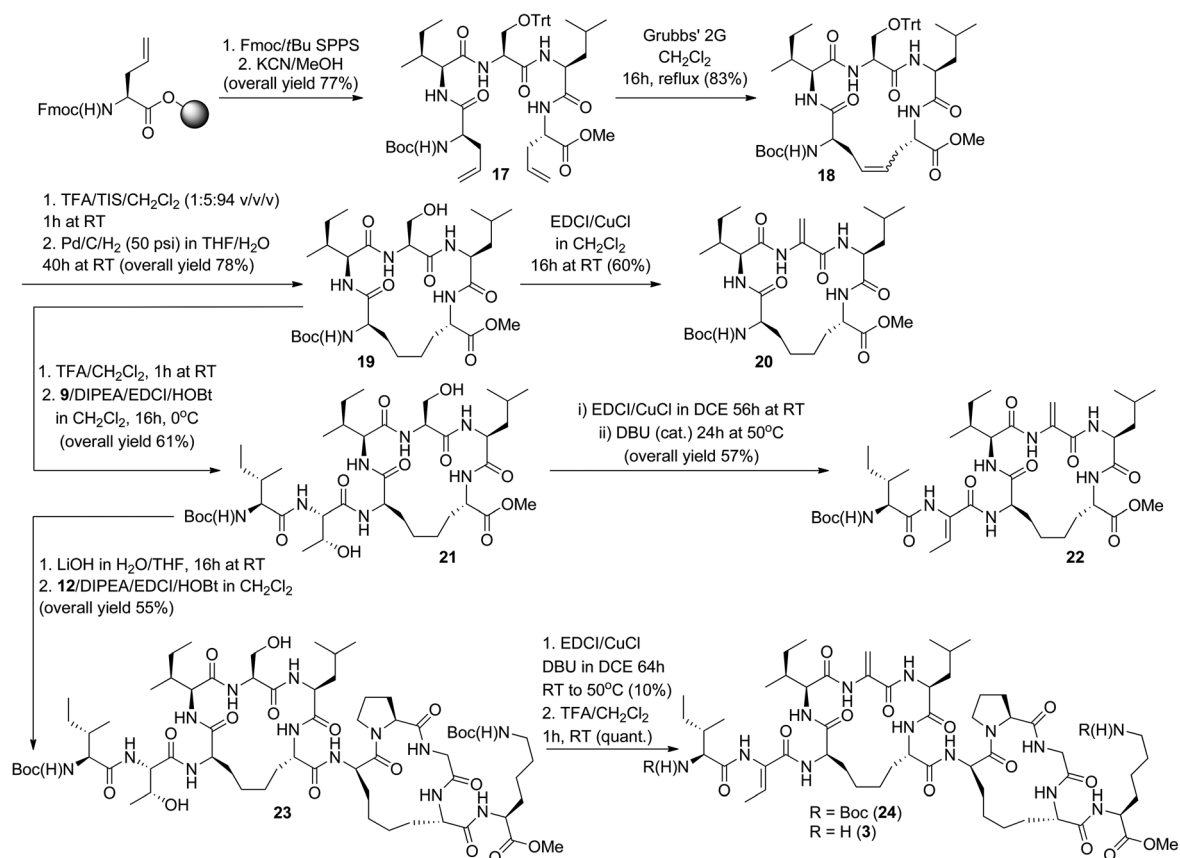
N-terminal Boc group of cyclic peptide 15 by treatment with TFA, and after the addition of DIPEA, the free amine was coupled to dipeptide acid 5 in the presence of EDCI/HOBt as coupling reagents. The cyclic heptapeptide was extracted *via* an aqueous work-up, saponified using lithium hydroxide, and peptide acid 16 was isolated after preparative HPLC in 48% overall yield. Finally, peptide amine 12 was successfully coupled to acid 16 and the protected Ala<sup>2</sup>/Ala<sup>5</sup> nisin dicarba intermediate was isolated (30% yield) by preparative HPLC and subsequently treated with TFA to remove both Boc-functionalities affording Ala<sup>2</sup>/Ala<sup>5</sup> nisin-AB-dicarba-Ala 2 in quantitative yield, as shown in Scheme 4.

For the synthesis of ring-fragment A, in first instance linear pentapeptide 17 (Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ser(Trt)<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OMe) was synthesized *via* Fmoc/<sup>t</sup>Bu SPPS as shown in Scheme 5. Ring-closing metathesis of diene 17 afforded alkene bridged cyclo-

peptide 18 as a mixture of two diastereoisomers (*E/Z*) in a good yield (83%). Then, efforts to convert alkene-bridge macrocycle 18 as the trityl protected peptide, into the corresponding alkane-bridged cyclopeptide in the presence of H<sub>2</sub> (50 psi) and Pd/C as catalyst were unsuccessful, even when longer reaction times were applied, or at higher H<sub>2</sub> pressure and high catalyst loadings. It was found that the bulkiness of the trityl group was somehow hindering hydrogenation and this protecting group needed to be removed before reducing the alkene-moiety. Hence, removal of the trityl group using mild acidic 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<sub>2</sub> (50 psi) and Pd/C as a catalyst, and alkane-bridged ring-A 19 was isolated after column chromatography in a good yield (78%). Similarly as described for the synthesis of dipeptide 6, cyclic peptide 19



Scheme 4 Synthesis route for nisin AB dicarba Ala 2.



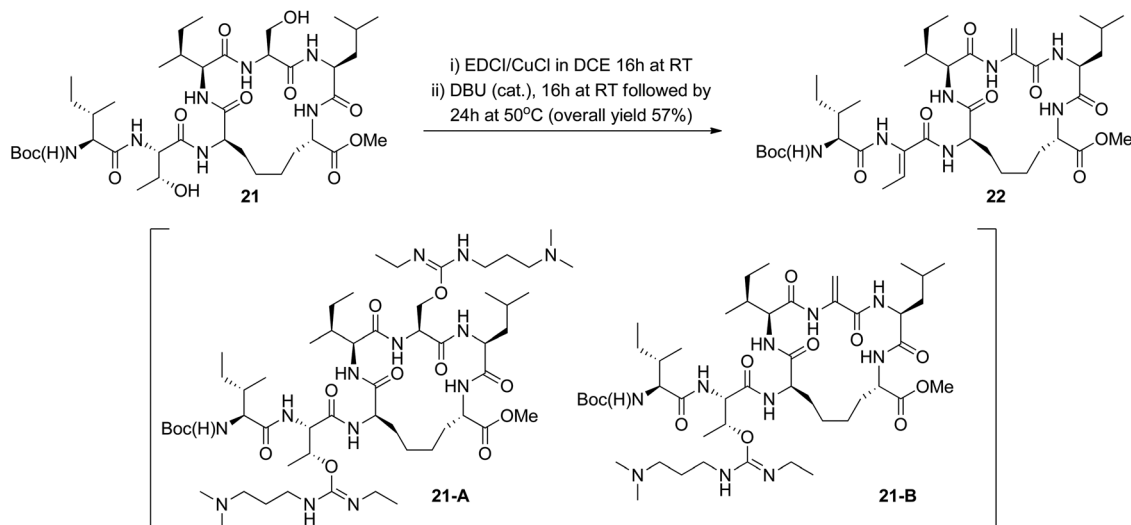
Scheme 5 Synthesis route for AB dicarba dehydro 3.



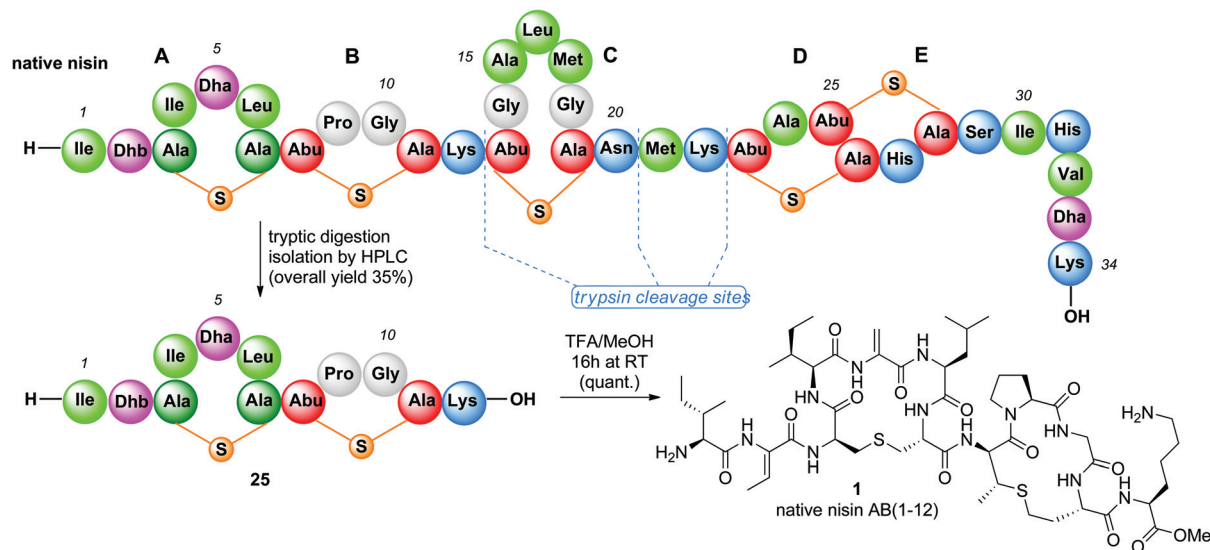
was treated with mesyl chloride/DBU to synthesize dehydropeptide **20**. However, *via* this approach, only trace amounts of dehydropeptide **20** were observed, together with starting material and degradation products. Optimization of the  $\beta$ -elimination reaction by changing reaction conditions, 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,<sup>16</sup> dehydration was achieved and dehydropeptide **20** was isolated after preparative HPLC purification in 60% yield (Scheme 5).

With the three required building blocks in hands, dipeptide **6**, ring-fragment A **20** and ring-fragment B **12**, the endgame of the fragment assembly *via* solution phase synthesis to obtain Dhb2/Dha5 nisin-AB dicarba analog **3** had begun, as shown in Scheme 5. Reliable syntheses of a variety of peptides containing dehydro amino acids have been described in the literature.<sup>14,17</sup> 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, as shown in Scheme 1, Boc-Ile-Thr-OMe **6** was saponified by Tesser's base<sup>18</sup> affording Boc-Ile-Thr-OH **9**, which was used for the next steps, as shown in Scheme 5. As a result of this change in synthesis strategy, it was also decided to couple peptide **19**, with an unprotected serine residue, to dipeptide **9**. This enabled a

double dehydration in one single synthesis step. To this end, the N-terminal Boc group of cyclic peptide **19** was removed by treatment with TFA and after adding DIPEA, the free amine was coupled to dipeptide **9** in the presence of EDCI/HOBt as coupling reagents to give peptide **21** in 61% yield after preparative HPLC (Scheme 5). Subsequently, peptide **15** was subjected to dehydration conditions using EDCI/CuCl in dichloromethane. Under these reaction conditions only trace amounts of the desired dehydropeptide **22** were obtained. LC-MS analysis of the reaction mixture confirmed that the starting material **21** was converted into intermediate products (**21-A**, **21-B**), in which one or two EDCI moieties were attached to peptide **21**, as shown in Scheme 6. 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 (**21AB**) into the desired dehydropeptide **22** was achieved (See ESI†).<sup>19</sup> Encouraged by this result, the final steps to synthesize the nisin AB analog **3** were initiated and started with the LiOH-mediated saponification of peptide **22**. Although, saponification of peptide **22** was successful, unfortunately a significant part of the product was rehydrated, due to reaction with hydroxide with one of the Michael acceptors (structure not shown). This was somewhat surprising and not in line with the reported literature<sup>14,17c</sup> and our earlier observations (conversion **7**  $\rightarrow$  **8**, Scheme 2). 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 **21** was saponified and the corresponding acid was coupled to cyclopeptide **12** in the presence of EDCI/HOBt and peptide **23** was obtained in a reasonable yield (55%) after preparative HPLC. Dehydration of peptide **23** was achieved by treatment with EDCI/CuCl, subsequently followed by  $\beta$ -elimination induced by DBU,



Scheme 6 Intermediates as found by LCMS during the dehydration of peptide **21**.



Scheme 7 Semi-synthesis route for native nisin AB-OMe 1.

according to the similar procedure for the synthesis of dehydropolypeptide 21. The Boc-protected dehydropolypeptide 24 was purified by preparative HPLC, and after treatment with TFA (to remove both Boc-functionalities), Dhb2/Dha5 nisin AB dicarba analog 3 was successfully synthesized.

The native nisin AB-fragment 1 was prepared as the ultimate control peptide to compare the influence of the dicarba-bridge on the bioactivity. For this purpose, nisin AB-fragment 25 was prepared by tryptic digestion of nisin as described<sup>4,20</sup> and could be isolated after preparative HPLC in 35% yield as shown in Scheme 7. The carboxylic acid moiety was converted into the corresponding methyl ester by treatment with TFA in MeOH and nisin AB-fragment 1, was obtained in quantitative yield.

### 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.<sup>21</sup> 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 (Fig. 3). 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 1 resulted in a reduced CF-leakage to ~25%, confirming that native-AB 1 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

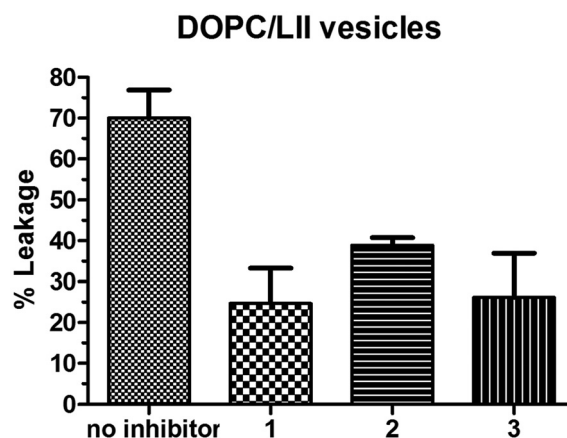


Fig. 3 Pore-formation activity of nisin without competitor, and with nisin AB-OMe 1, nisin AB dicarba Ala 2, and nisin AB dicarba dehydro 3 as measured in a model membrane leakage experiment. The following concentrations have been used, nisin: 0.2 nM; nisin AB fragments: 5 μM.

~39% was obtained, which was in agreement with the data reported previously.<sup>9</sup> Gratifyingly, Dhb2/Dha5 nisin AB-dicarba analog 3 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 dehydrobutyryne 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 1, an implication that the dicarba bridge is an excellent mimic for the lanthionine bridge for the nisin AB-ring system.<sup>22</sup>

In conclusion, a reliable synthesis to obtain two nisin AB dicarba dodecapeptides 2 and 3 *via* a combination of SPPS, ring-closing metathesis, and solution phase chemistry, was developed applying a [2 + 5 + 5] fragment assembly strategy.

It should be noted that this fragment assembly approach may introduce racemization since acyl peptides have been used as activated species in peptide bond formation. To keep racemization to the lowest possible level, amide bond formation was performed in a slightly acidic reaction environment since HOBT was added while only a single equivalent of DIPEA was added to neutralize the protonated  $\alpha$ -amine functionality. However, partial epimerization of the C-terminus cannot be completely ruled out, and we have not checked it, which may be a possible explanation for peak broadening as seen in the HPLC analysis for compounds **2** and **3** (see also ESI† on pages 16 and 17). Ring-closing metathesis turned out to be an efficient method for macrocyclization of these nisin-derived peptides, since all steps were performed in high isolated yield (>80%). The dehydro residues, dehydrobutyrine and dehydroalanine, were introduced as their precursor residues (threonine and serine) in various peptide constructs and  $\beta$ -elimination-induced dehydration was performed either by mesylation/DBU (on dipeptides) or *via* EDCI/CuCl/DBU (on more challenging dodecapeptides). 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 challenging to perform peptide couplings and saponifications in the presence of dehydro peptides, 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 **2** and **3** 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 **1**. Mimic **3** was equipotent as native nisin AB **1**, 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.

## Experimental

### 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  $\mu\text{m}$ ). TLC was performed on Merck precoated silica gel 60F254 glass plates. Spots were visualized by UV quenching, ninhydrin, or  $\text{Cl}_2/\text{TDM}$ .<sup>23</sup>  $^1\text{H-NMR}$  data were acquired on a Varian Mercury 300 MHz or a Varian Inova 500 MHz spectrometer in  $\text{CDCl}_3$  or  $\text{DMSO-d}_6$  as the solvent. Chemical shifts ( $\delta$ ) 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).  $^{13}\text{C-NMR}$  data were acquired on a Varian Mercury 75 MHz in  $\text{CDCl}_3$  or  $\text{DMSO-d}_6$  as the solvent. Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to the solvent residual signal,  $\text{CDCl}_3$  (77.0 ppm), or  $\text{DMSO-d}_6$  (39.5 ppm). Analytical HPLC was performed on an automated Shimadzu HPLC system equipped with a UV/vis detector operating at 220/254 nm using an Alltech Prosphere C4 column (pore size: 300 Å, particle size: 5  $\mu\text{m}$ , 250  $\times$  4.6 mm) at a flow rate of 1  $\text{mL min}^{-1}$  (from 100% buffer A (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  5 : 95 v/v) to 100% buffer B (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  95 : 5 v/v) in 40 min. Preparative HPLC was performed on an automated preparative Applied Biosystems HPLC system equipped with a UV/vis detector operating at 214 nm using an Alltech prosphere C4 column (pore size: 300 Å, particle size: 10  $\mu\text{m}$ , 250  $\times$  22 mm) at a flow rate of 12.0  $\text{mL min}^{-1}$  (from 100% buffer A (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  5 : 95 v/v) to 100% buffer B (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  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. LCMS analyses were performed on a Thermo-Finnigan LCQ Deca XP Max ion trap mass spectrometer coupled to a Shimadzu analytical HPLC system equipped with a UV/vis detector operating at 214 nm using an Alltech Prosphere C4 column (pore size: 300 Å, particle size: 5  $\mu\text{m}$ , 250  $\times$  4.6 mm) at a flow rate of 1  $\text{mL min}^{-1}$  (from 100% buffer A (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  5 : 95 v/v) to 100% buffer B (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  95 : 5 v/v) in 40 min. The dipeptide building blocks **4–6**, and **8, 9** were analyzed as follows: a Maisch Reprosil 120 C18-AQ column (pore size: 120 Å, particle size: 5  $\mu\text{m}$ , 250  $\times$  4.6 mm) at a flow rate of 1  $\text{mL min}^{-1}$  (from 100% buffer A (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  5 : 95 v/v) to 100% buffer B (0.1% TFA in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  95 : 5 v/v) in 48 min.

### Synthesis

**Boc-Ile-Ala-OMe (4).** To a solution of HCl-H-Ala-OMe (1.40 g, 10.0 mmol) in  $\text{CH}_2\text{Cl}_2$  (100 mL) and  $\text{Et}_3\text{N}$  (4.2 mL, 30.0 mmol), Boc-Ile-OH (1.70 g, 10.0 mmol) and BOP (4.42 g, 10.0 mmol) were added, and the obtained reaction mixture was stirred for 2.5 h at room temperature. Then, the solvent was removed *in vacuo* and the residue redissolved in EtOAc (200 mL). The organic solution was washed with aq. 1 N  $\text{KHSO}_4$  (3  $\times$  150 mL), aq. 1 N  $\text{NaHCO}_3$  (3  $\times$  150 mL), and brine (2  $\times$  150 mL), dried ( $\text{Na}_2\text{SO}_4$ ), filtered and subsequently followed by evaporation *in vacuo*. The residue was purified by column chromatography (EtOAc/hexane 3 : 7 v/v) to give dipeptide methyl ester **4** as a white crystalline solid in 60% yield (1.90 g).  $R_f$  0.29 (EtOAc/hexane 4 : 6 v/v);  $R_t$  = 30.74 min (LCMS);  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ )  $\delta$  = 0.87 (t ( $J$  = 7.4 Hz), 3H,  $\text{C}\delta\text{H}_3$  Ile), 0.91 (d ( $J$  = 6.8 Hz), 3H,  $\text{C}\gamma\text{H}_3$  Ile), 1.03–1.21 (m, 1H,  $\text{C}\beta\text{H}$  Ile), 1.36 (d ( $J$  = 7.2 Hz), 3H,  $\text{C}\beta\text{H}_3$  Ala), 1.40 (s, 9H, Boc), 1.52/1.82 (m, 2H,  $\text{C}\gamma\text{H}_2$  Ile), 3.70 (s, 3H, OMe), 3.96 (m, 1H,  $\text{C}\alpha\text{H}$  Ile), 4.54 (quintet ( $J$  = 7.2 Hz), 1H,  $\text{C}\alpha\text{H}$  Ala), 5.17 (d ( $J$  = 8.8 Hz), 1H, BocNH), 6.68 (d ( $J$  = 6.8 Hz), 1H, NH Ala);  $^{13}\text{C NMR}$  (75.5 MHz,  $\text{CDCl}_3$ )  $\delta$  = 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; ESI-MS calcd



for  $C_{15}H_{28}N_2O_5$  316.20, found  $m/z$   $[M + H]^+$  317.30,  $[M + Na]^+$  339.45,  $[(M - tBu) + H]^+$  260.90,  $[(M - Boc) + H]^+$  217.00.

**Boc-Ile-Ala-OH (5).** Dipeptide ester Boc-Ile-Ala-OMe 4 (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. 1 N  $KHSO_4$  and extracted with  $CH_2Cl_2$  ( $4 \times 20$  mL). The combined organic layers were dried ( $Na_2SO_4$ ) and evaporated *in vacuo* to give peptide acid 5 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);  $R_t$  = 26.10 min (LCMS);  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  = 0.87–0.95 (m, 6H,  $C\delta H_3$  Ile/ $C\gamma H_3$  Ile), 1.82 (m, 1H,  $C\beta H$  Ile), 1.40–1.48 (m, 12H,  $C\beta H_3$  Ala/Boc), 1.16/15.1 (m, 2H,  $C\gamma H_2$  Ile), 4.03 (m, 1H,  $C\alpha H$  Ile), 4.58 (m, 1H,  $C\alpha H$  Ala), 6.13 (br s, 1H, BocNH), 6.95 (br s, 1H, NH Ala);  $^{13}C$  NMR (75.5 MHz,  $CDCl_3$ )  $\delta$  = 11.0, 15.2, 17.8, 24.7, 28.2, 37.1, 48.0, 59.0, 80.1, 156.0, 172.2, 175.4; ESI-MS calcd for  $C_{14}H_{26}N_2O_5$  302.18, found  $m/z$   $[M + Na]^+$  325.50,  $[2M + Na]^+$  626.85,  $[(M - tBu) + H]^+$  260.90,  $[(M - Boc) + H]^+$  203.00.

**Boc-Ile-Thr-OMe (6).** 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  $Et_3N$  (6.36 mL, 45.6 mmol) were added successively. 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. 1 N  $KHSO_4$  ( $3 \times 200$  mL), aq. 1 N  $NaHCO_3$  ( $3 \times 200$  mL), and brine ( $2 \times 150$  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);  $R_t$  = 28.03 min (LCMS);  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  = 0.90–0.97 (m, 6H,  $C\gamma H_3$  Ile/ $C\delta H_3$  Ile), 1.21 (d ( $J$  = 6.4 Hz), 3H,  $C\gamma H_3$  Thr), 1.44 (s, 9H, Boc), 1.57 (m, 1H,  $C\beta H$  Ile), 1.83 (m, 2H,  $C\gamma H_2$  Ile), 2.80 (s, 1H, OH), 3.77 (s, 3H, OMe), 3.97 (m, 1H,  $C\alpha H$  Ile), 4.36 (m, 1H,  $C\beta H$  Thr), 4.62 (dd ( $J_{gem}$  = 9.0 Hz,  $J_{vic}$  = 2.4 Hz), 1H,  $C\alpha H$  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,  $CDCl_3$ )  $\delta$  = 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; ESI-MS calcd for  $C_{16}H_{30}N_2O_6$  346.21, found  $m/z$   $[M + H]^+$  346.80,  $[M + Na]^+$  368.70,  $[(M - tBu) + H]^+$  291.10,  $[(M - Boc) + H]^+$  247.25.

**Boc-Ile-Dhb-OMe (7).** To a solution of Boc-Ile-Thr-OMe 6 (347 mg, 1.0 mmol) in  $CH_2Cl_2$  (20 mL), mesyl chloride (120  $\mu$ L, 1.5 mmol) and  $Et_3N$  (210  $\mu$ L, 1.5 mmol) were added, after which the reaction mixture was stirred for 1 h at room temperature. Subsequently, DBU (230  $\mu$ L, 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. 1 N  $KHSO_4$  ( $3 \times 10$  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 over two steps

(190 mg).  $R_f$  0.73 ( $CH_2Cl_2/MeOH$  97.5 : 2.5 v/v);  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  = 0.94 (t ( $J$  = 7.4 Hz), 3H,  $C\delta H_3$  Ile), 1.00 (d ( $J$  = 6.8 Hz), 3H,  $C\gamma H_3$  Ile), 1.22 (m, 2H), 1.45 (s, 9H, Boc), 1.49–1.68 (m, 2H,  $C\gamma H_2$  Ile), 1.77 (d ( $J$  = 7.2 Hz), 3H,  $C\gamma H_3$  Dhb), 1.96 (m, 1H,  $C\beta H$  Ile), 3.76 (s, 3H, OMe), 3.98–4.16 (m, 1H,  $C\alpha H$  Ile), 5.03 (br s, 1H, BocNH), 6.83 (q ( $J$  = 7.2 Hz), 1H,  $C\beta H$  Dhb), 7.32 (s, 1H, NH amide Dhb);  $^{13}C$  NMR (75.5 MHz,  $CDCl_3$ )  $\delta$  = 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 (8).** Boc-Ile-Dhb-OMe 7 (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 at room temperature. Then, THF was evaporated *in vacuo*, and the remaining aqueous phase was acidified using aq. 1 N  $KHSO_4$ , and the peptide acid was extracted with  $CH_2Cl_2$  ( $4 \times 10$  mL). After drying ( $Na_2SO_4$ ), and evaporation of the solvent *in vacuo*, the dipeptide acid 8 was obtained as a white solid in 87% yield (145 mg).  $R_f$  0.41 (DCM/MeOH/AcOH 95 : 5 : 0.1 v/v/v);  $R_t$  = 27.28 min (LCMS);  $^1H$  NMR (300 MHz,  $CDCl_3/CD_3OD$  95 : 5 v/v)  $\delta$  = 0.91 (t ( $J$  = 6.9 Hz), 3H,  $C\delta H_3$  Ile), 1.00 (d ( $J$  = 6.8 Hz), 3H,  $C\gamma H_3$  Ile), 1.45 (s, 9H, Boc), 1.52 (m, 2H,  $C\gamma H_2$  Ile), 1.79 (d ( $J$  = 6.7 Hz), 3H,  $C\gamma H_3$  Dhb), 2.02 (m, 1H,  $C\beta H$  Ile), 4.20 (dd ( $J_{gem}$  = 9.4 Hz,  $J_{vic}$  = 5.4 Hz), 1H,  $C\alpha H$  Ile), 5.25/6.63 (d ( $J$  = 6.8 Hz), 1H, BocNH), 6.91 (q ( $J$  = 7.0 Hz),  $C\beta$  Dhb), 7.66/7.90 (s, 1H, NH amide Dhb);  $^{13}C$  NMR (75.5 MHz,  $CDCl_3/CD_3OD$  95 : 5 v/v)  $\delta$  = 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; ESI-MS calcd for  $C_{15}H_{26}N_2O_5$  314.18, found  $m/z$   $[M + Na]^+$  337.55,  $[M + K]^+$  352.85,  $[2M + Na]^+$  651.00,  $[(M - tBu) + H]^+$  258.70,  $[(M - Boc) + H]^+$  215.15.

**Boc-Ile-Thr-OH (9).** Boc-Ile-Thr-OMe 6 (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 at room temperature. The reaction mixture was concentrated *in vacuo*, and the remaining aqueous layer was acidified using aq. 1 N  $KHSO_4$ , and the acid was extracted with  $CH_2Cl_2$  ( $4 \times 20$  mL). After drying the  $CH_2Cl_2$  solution ( $Na_2SO_4$ ) and removing the solvents *in vacuo*, Boc-Ile-Thr-OH (9) 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);  $R_t$  = 24.55 min (LCMS);  $^1H$  NMR (300 MHz,  $CDCl_3/CD_3OD$  99 : 1 v/v)  $\delta$  = 0.53–1.02 (m, 6H,  $C\delta H_3$  Ile/ $C\gamma H_3$  Ile), 1.03–1.66 (m, 14H, Boc/ $C\gamma H_3$  Thr/ $C\gamma H_2$  Ile), 1.70–2.00 (m, 1H,  $C\beta H$  Ile), 2.80 (s, 1H, OH), 3.77 (s, 3H, OMe), 3.97 (m, 1H,  $C\alpha H$  Ile), 4.36 (m, 1H,  $C\beta H$  Thr), 4.62 (dd ( $J_{gem}$  = 9.0 Hz,  $J_{vic}$  = 2.4 Hz), 1H,  $C\alpha H$  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)  $\delta$  = 11.2, 15.4, 19.4, 24.7, 28.3, 36.8, 58.4, 67.9, 80.0, 156.2, 172.8, 175.8; ESI-MS calcd for  $C_{15}H_{28}N_2O_6$  332.19, found  $m/z$   $[M + H]^+$  333.10,  $[M + Na]^+$  355.15,  $[2M + Na]^+$  687.50,  $[(M - tBu) + H]^+$  276.80,  $[(M - Boc) + H]^+$  232.95.

**Solid phase peptide synthesis (SPPS).** 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.<sup>24</sup> The Fmoc group enabled determination of the loading and also the efficiency of the

coupling reaction.<sup>25</sup> 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<sub>2</sub>Cl<sub>2</sub> (25 mL, 3 × 2 min). The presence of free α-amino functionalities was checked by the Kaiser<sup>26</sup> 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<sub>2</sub> 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<sub>2</sub>Cl<sub>2</sub> (25 mL, 3 × 2 min). Completion of the coupling (absence of free α-amino functionalities) was checked by the Kaiser test (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.

**Cbz-D-Alg<sup>1</sup>-Pro<sup>2</sup>-Gly<sup>3</sup>-Alg<sup>4</sup>-Lys(Boc)<sup>5</sup>-OMe (10)**. The AgroGel resin (3.25 g) was loaded with Fmoc-Alg-OH using the Sieber method 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<sub>2</sub>Cl<sub>2</sub>/MeOH 95 : 5 v/v) affording protected pentapeptide **10** as a yellowish oil in a very good yield of 93% (666 mg). *R<sub>f</sub>* 0.58 (CH<sub>2</sub>Cl<sub>2</sub>/acetone 9 : 1 v/v); *R<sub>t</sub>* = 21.35 min; ESI-MS calcd for C<sub>37</sub>H<sub>54</sub>N<sub>6</sub>O<sub>10</sub> 742.39, found *m/z*; [M + Na]<sup>+</sup> 764.60, [(M - Boc) + H]<sup>+</sup> 643.95.

**Cyclo[1-4]-Cbz-D-Alg<sup>1</sup>-Pro<sup>2</sup>-Gly<sup>3</sup>-Alg<sup>4</sup>-Lys(Boc)<sup>5</sup>-OMe (11)**. The linear precursor peptide **10** (252 mg, 0.34 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (100 mL) and the solution was heated to reflux and gently purged by N<sub>2</sub> 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<sub>2</sub>Cl<sub>2</sub>/MeOH 95 : 5 v/v) to give the protected cyclic peptide **11** as a brownish solid and as a mixture of both diastereoisomers (*E/Z*) in quantitative yield (242 mg). *R<sub>f</sub>* 0.53 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1 v/v); *R<sub>t</sub>* = 19.52–20.38 min; ESI-MS calcd for C<sub>35</sub>H<sub>52</sub>N<sub>6</sub>O<sub>10</sub> 716.37, found *m/z* [M + Na]<sup>+</sup> 737.50, [(M - Boc) + H]<sup>+</sup> 615.10.

**Cyclo[1-4]-H-D-Alg<sup>1</sup>-Pro<sup>2</sup>-Gly<sup>3</sup>-Alg<sup>4</sup>-Lys(Boc)<sup>5</sup>-OMe-TFA (12)**. The Cbz protected alkene bridged peptide macrocycle **11** (54 mg, 76 μmol) was dissolved in THF/H<sub>2</sub>O (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<sub>2</sub> atmosphere (50 psi H<sub>2</sub> pressure) for 40 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, and the residue was rinsed with THF (2 × 2 mL) and the filtrate was concentrated *in vacuo*. The peptide

amine **12** was taken up in H<sub>2</sub>O/MeCN/TFA (15 mL, 75 : 25/0.1 v/v/v) and the solution was lyophilized which afforded alkane-bridged peptide amine **12** as an off-white fluffy powder in quantitative yield (51 mg). *R<sub>f</sub>* 0.22 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/TEA 85 : 15 : 0.1 v/v/v); *R<sub>t</sub>* = 16.68 min; ESI-MS calcd for C<sub>27</sub>H<sub>46</sub>N<sub>6</sub>O<sub>8</sub> 582.34, found *m/z* [M + H]<sup>+</sup> 583.45, [M + Na]<sup>+</sup> 605.35, [M + K]<sup>+</sup> 621.35, [2M + H]<sup>+</sup> 1165.60, [(M - Boc) + H]<sup>+</sup> 483.05.

**Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OMe (13)**. ArgoGel resin (1.03 g) was loaded with Fmoc-Alg-OH using the Sieber method 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<sub>2</sub>Cl<sub>2</sub>/MeOH 97 : 3 v/v) and protected linear peptide **13** was obtained as an off-white solid in 81% yield (187 mg). *R<sub>f</sub>* 0.55 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1 v/v); ESI-MS calcd for C<sub>50</sub>H<sub>67</sub>N<sub>5</sub>O<sub>9</sub> 881.49, found *m/z*; [M + Na]<sup>+</sup> 904.35, [2M + Na]<sup>+</sup> 1787.45, [(M - Trt) + H]<sup>+</sup> 640.24.

**Cyclo[1-5]-Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OMe (14)**. The linear precursor peptide **13** (100 mg, 0.19 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and the solution was heated to reflux and gently purged by N<sub>2</sub> 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<sub>2</sub>Cl<sub>2</sub>/MeOH 97 : 3 v/v) to give cyclic peptide **14** as a brownish solid in 80% yield (76 mg). *R<sub>f</sub>* 0.53 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1 v/v).

**Cyclo[1-5]-Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ala<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OMe (15)**. Alkene-bridged cyclic peptide **14** (48 mg, 81 μmol) was dissolved in THF/H<sub>2</sub>O (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<sub>2</sub> atmosphere (50 psi H<sub>2</sub> pressure) for 40 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, and subsequently rinsed with THF (2 × 2 mL) and the filtrate was concentrated *in vacuo*. The residue was redissolved in *tert*-BuOH/H<sub>2</sub>O (10 mL, 1 : 1 v/v) and subsequently lyophilized, to give alkane-bridged cyclic peptide **15** as an off-white fluffy powder in quantitative yield (48 mg). *R<sub>f</sub>* 0.53 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1 v/v); ESI-MS calcd for C<sub>29</sub>H<sub>51</sub>N<sub>5</sub>O<sub>8</sub> 597.37, found *m/z* [M + H]<sup>+</sup> 598.35, [M + Na]<sup>+</sup> 620.50, [2M + Na]<sup>+</sup> 1217.80.

**Boc-Ile<sup>1</sup>-Ala<sup>2</sup>-cyclo[3-7]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Ala<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-OH (16)**. Boc-protected peptide **15** (48 mg, 80 μmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub>/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<sub>3</sub> (2 × 5 mL) followed by drying in high vacuum. Subsequently, the peptide amine was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (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, dipeptide acid Boc-Ile-Ala-OH **5** (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. 1 N KHSO<sub>4</sub> (3 × 4 mL), aq. 1 N NaHCO<sub>3</sub> (3 × 4 mL) and brine (1 × 4 mL), dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated *in vacuo*, to give the heptapeptide ester as a white solid (35 mg). Then, the crude peptide ester was dissolved in THF (1.5 mL) and a solution of LiOH in H<sub>2</sub>O (1.0 mL; 0.1 M) was added, and the reaction mixture was stirred for 16 h at room temperature. After the evaporation of THF *in vacuo*, the aqueous mixture was acidified by aq. 1 N KHSO<sub>4</sub>, and the peptide acid was extracted into CH<sub>2</sub>Cl<sub>2</sub> (3 × 5 mL). The solvent was removed *in vacuo*, and after preparative HPLC, peptide acid **16** was obtained as a fluffy white powder in 48% yield (30 mg). *R*<sub>t</sub> = 20.72 min; ESI-MS calcd for C<sub>37</sub>H<sub>65</sub>N<sub>7</sub>O<sub>10</sub> 767.48, found *m/z* [M + H]<sup>+</sup> 768.30, [M + Na]<sup>+</sup> 790.55, [2M + Na]<sup>+</sup> 1558.05.

H-Ile<sup>1</sup>-Ala<sup>2</sup>-bicyclo[3-7/8-11]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Ala<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-D-Alg<sup>8</sup>-Pro<sup>9</sup>-Gly<sup>10</sup>-Alg<sup>11</sup>-Lys<sup>12</sup>-OME-2TFA (**2**). Trifluoroacetate **12** (5.0 mg, 7.4 μmol) was dissolved in DMF (300 μL) and DIPEA (1.5 μL, 8.6 μmol) was added to obtain the corresponding free amine. To this solution, peptide acid **16** (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<sub>2</sub>O/MeCN/TFA (2 mL, 60 : 40 : 0.1 v/v/v) and the bis-Boc-protected dodecapeptide was purified by preparative HPLC as a white fluffy powder in a yield of 30% (3.0 mg). *R*<sub>t</sub> = 21.63 min; ESI-MS calcd for C<sub>64</sub>H<sub>109</sub>N<sub>13</sub>O<sub>17</sub> 1331.81, found *m/z* [M + H]<sup>+</sup> 1332.70, [M + Na]<sup>+</sup> 1354.75, [M + K]<sup>+</sup> 1370.60, [(M - Boc) + H]<sup>+</sup> 1232.95, [(M - Boc) + 2H]<sup>2+</sup> 617.50, [(M - 2Boc) + 2Na]<sup>2+</sup> 589.55. The obtained protected peptide (2.0 mg, 1.5 μmol) was suspended in CH<sub>2</sub>Cl<sub>2</sub> (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<sub>2</sub>O (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*<sub>t</sub> = 16.85 min; ESI-MS calcd for C<sub>54</sub>H<sub>93</sub>N<sub>13</sub>O<sub>13</sub> 1131.70, found *m/z* [M + H]<sup>+</sup> 1132.85, [M + Na]<sup>+</sup> 1153.90, [M + K]<sup>+</sup> 1170.65, [M + 2H]<sup>2+</sup> 567.00.

**Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ser(Trt)<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OME** (**17**). The ArgoGel resin (3.01 g) was loaded with Fmoc-Alg-OH using the Sieber method with 0.30 mmol g<sup>-1</sup> 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<sub>2</sub>Cl<sub>2</sub>/MeOH 95 : 5 v/v) and linear peptide **17** was obtained as an off-white solid in 77% yield (613 mg) *R*<sub>f</sub> 0.41 (CH<sub>2</sub>Cl<sub>2</sub>/acetone 9 : 1 v/v); *R*<sub>t</sub> = 25.78 min; ESI-MS calcd for C<sub>50</sub>H<sub>67</sub>N<sub>5</sub>O<sub>9</sub> 881.49, found *m/z*; [M + Na]<sup>+</sup> 904.55, [2M + Na]<sup>+</sup> 1786.95, [(M - Trt) + H]<sup>+</sup> 640.50.

**Cyclo[1-5]-Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ser(Trt)<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OME** (**18**). The linear precursor peptide **17** (251 mg, 0.28 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (150 mL) and the solution was heated to reflux and gently purged by N<sub>2</sub> 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<sub>2</sub>Cl<sub>2</sub>/MeOH 97 : 3 v/v) to give macrocyclic peptide **18** as a brownish solid in 83% yield (201 mg). *R*<sub>f</sub> 0.29 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 97 : 3 v/v); *R*<sub>t</sub> = 24.78 min; ESI-MS calcd for C<sub>50</sub>H<sub>67</sub>N<sub>5</sub>O<sub>9</sub> 853.46, found *m/z* [M + Na]<sup>+</sup> 876.45, [(M - Trt) + H]<sup>+</sup> 612.40, [2M + Na]<sup>+</sup> 1731.30.

**Cyclo[1-5]-Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Ser<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OME** (**19**). First, trityl-protected peptide **18** (312 mg, 0.37 mmol) was dissolved in a mixture of CH<sub>2</sub>Cl<sub>2</sub>/TIS/TFA (19 mL, 94 : 5 : 1 v/v/v) and the solution 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<sub>2</sub>O (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<sub>2</sub> atmosphere (50 psi H<sub>2</sub> pressure) for 40 h at room temperature. Subsequently, the reaction mixture was filtered over Celite, subsequently rinsed with THF (2 × 2 mL) and the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95 : 5 v/v) and the alkane-bridged cyclic peptide **19** was afforded as a brownish solid in a good yield: 78% (177 mg). *R*<sub>f</sub> 0.53 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1 v/v); *R*<sub>t</sub> = 19.27 min; ESI-MS calcd for C<sub>29</sub>H<sub>51</sub>N<sub>5</sub>O<sub>9</sub> 613.37, found *m/z* [M + H]<sup>+</sup> 614.20, [2M + Na]<sup>+</sup> 1249.70, [(M - <sup>t</sup>Bu) + H]<sup>+</sup> 558.00.

**Cyclo[1-5]-Boc-D-Alg<sup>1</sup>-Ile<sup>2</sup>-Dha<sup>3</sup>-Leu<sup>4</sup>-Alg<sup>5</sup>-OME** (**20**). A flame-dried flask was purged with N<sub>2</sub> gas and charged with a solution of cyclopeptide **19** (150 mg, 244 μmol) in CH<sub>2</sub>Cl<sub>2</sub> (4.0 mL) under a N<sub>2</sub> atmosphere. Subsequently, CuCl (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<sub>2</sub>O (2 × 3 mL) and after drying (Na<sub>2</sub>SO<sub>4</sub>), the solvent was removed *in vacuo*. The crude peptide was purified by preparative HPLC and dehydropeptide **20** was afforded as an off-white solid in a good yield: 60% (87 mg). *R*<sub>f</sub> 0.65 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9 : 1 v/v); *R*<sub>t</sub> = 21.31 min; ESI-MS calcd for C<sub>29</sub>H<sub>49</sub>N<sub>5</sub>O<sub>8</sub> 595.36, found *m/z* [M + H]<sup>+</sup> 596.46, [M + Na]<sup>+</sup> 618.45, [M + K]<sup>+</sup> 634.30.45, [2M + Na]<sup>+</sup> 1214.30, [(M - <sup>t</sup>Bu) + H]<sup>+</sup> 540.40, [(M - Boc) + H]<sup>+</sup> 496.00.

**Boc-Ile<sup>1</sup>-Thr<sup>2</sup>-cyclo[3-7]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Ser<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-OME** (**21**). Boc-protected peptide **19** (51 mg, 82 μmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub>/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<sub>3</sub> (2 × 5 mL), and further drying at high vacuum. The corresponding peptide amine was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and DIPEA (14 μL, 82 μmol) followed by HOBt (12 mg, 89 μmol) were added and the obtained mixture was cooled to 0 °C. Subsequently, Boc-Ile-Thr-OH **9** (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 to room temperature. Subsequently, the reaction mixture was concentrated *in vacuo* and the residue was redissolved in EtOAc (5 mL), and this solution was washed with aq. 1 N KHSO<sub>4</sub> (3 × 4 mL), aq. 1 N NaHCO<sub>3</sub> (3 × 4 mL) and brine (1 × 4 mL). After drying (Na<sub>2</sub>SO<sub>4</sub>) followed by removing the solvent *in vacuo*, peptide **21** was obtained as a white solid in a reasonable yield of 61% (41 mg). *R*<sub>t</sub> = 20.68 min; ESI-MS calcd for C<sub>39</sub>H<sub>69</sub>N<sub>7</sub>O<sub>12</sub> 827.50, found *m/z* [M + H]<sup>+</sup> 828.85, [M + Na]<sup>+</sup> 851.05, [2M + Na]<sup>+</sup> 1678.35, [(2M - Boc) + Na]<sup>+</sup> 1579.15, [(M - Boc) + H]<sup>+</sup> 729.05.

**Boc-Ile<sup>1</sup>-Dhb<sup>2</sup>-cyclo[3-7]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Dha<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-OMe (22).** A flame-dried flask was purged with N<sub>2</sub> gas and charged with as solution of peptide **21** (8.9 mg, 10.7 μmol) in 1,2-dichloroethane (2.0 mL) under a N<sub>2</sub> atmosphere. Subsequently, copper(i) chloride (1.0 mg, 10.1 μmol) and EDCI-HCl (5.8 mg, 30.4 μmol) were added and this suspension was stirred for 16 h at room temperature and the reaction progress was followed by LCMS (See ESI<sup>†</sup>). After 16 h starting material as well as reaction intermediates (EDCI-adducts) were observed, and after the addition of an extra amount of EDCI-HCl (2.5 mg, 13.1 μmol), stirring 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 **22**. 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. 1 N KHSO<sub>4</sub> (2 × 1 mL) and after drying (Na<sub>2</sub>SO<sub>4</sub>), the solvent was removed *in vacuo*. The residue was redissolved in *tert*-BuOH/H<sub>2</sub>O (5 mL, 1 : 1 v/v) and lyophilized to afford the dehydro peptide **22** as a white fluffy powder in a yield of 57% (4.8 mg). *R*<sub>t</sub> = 21.65 min; ESI-MS calcd for C<sub>39</sub>H<sub>65</sub>N<sub>7</sub>O<sub>10</sub> 791.97, found *m/z* [M + H]<sup>+</sup> 792.51, [M + Na]<sup>+</sup> 814.53, [2M + Na]<sup>+</sup> 1604.88, [(M - Boc) + H]<sup>+</sup> 692.50.

**Boc-Ile<sup>1</sup>-Thr<sup>2</sup>-bicyclo[3-7/8-11]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Ser<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-D-Alg<sup>8</sup>-Pro<sup>9</sup>-Gly<sup>10</sup>-Alg<sup>11</sup>-Lys(Boc)<sup>12</sup>-OMe (23).** Peptide **21** (15 mg, 18 μmol) was dissolved in THF (500 μL), and a solution of LiOH in H<sub>2</sub>O (400 μL, 0.1 M) was added, and the obtained reaction mixture was stirred for 16 h at room temperature. Then, THF was evaporated *in vacuo*, and the remaining aqueous phase was acidified by aq. 1 N KHSO<sub>4</sub>, followed by extraction with CH<sub>2</sub>Cl<sub>2</sub> (3 × 2 mL). The solvents were removed *in vacuo*, and the corresponding peptide acid was obtained. In a separate flask, trifluoroacetate **12** (12 mg, 17.6 μmol) was dissolved in DMF (500 μL) and DIPEA (3.5 μL, 18 μmol) was added to 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 to room temperature. Then, the reaction mixture was diluted with a mixture of H<sub>2</sub>O/MeCN/TFA (3 mL, 60 : 40 : 0.1 v/v/v) and peptide **23** was purified by preparative HPLC as a white fluffy powder in 55% yield (13 mg) over two steps. *R*<sub>t</sub> = 20.65 min; ESI-MS calcd for

C<sub>65</sub>H<sub>111</sub>N<sub>13</sub>O<sub>19</sub> 1377.81, found *m/z* [M + H]<sup>+</sup> 1379.10, [M + Na]<sup>+</sup> 1400.95, [M + K]<sup>+</sup> 1417.35, [(M - Boc) + H]<sup>+</sup> 1279.50, [(M - Boc) + 2H]<sup>2+</sup> 639.75.

**Boc-Ile<sup>1</sup>-Dhb<sup>2</sup>-bicyclo[3-7/8-11]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Dha<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-D-Alg<sup>8</sup>-Pro<sup>9</sup>-Gly<sup>10</sup>-Alg<sup>11</sup>-Lys(Boc)<sup>12</sup>-OMe (24).** A flame-dried flask was purged with N<sub>2</sub> gas and charged with **23** (6.0 mg, 4.4 μmol) which was dissolved in 1,2-dichloroethane (500 μL) under a N<sub>2</sub> 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 LCMS. 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<sub>2</sub>O/MeCN/TFA (2 mL, 60 : 40 : 0.1 v/v/v) to isolate peptide **24** by preparative HPLC as a white fluffy powder in 10% yield (0.6 mg). *R*<sub>t</sub> = 22.23 min; ESI-MS calcd for C<sub>65</sub>H<sub>107</sub>N<sub>13</sub>O<sub>17</sub> 1341.79, found *m/z* [M + H]<sup>+</sup> 1342.95, [M + Na]<sup>+</sup> 1365.10, [M + K]<sup>+</sup> 1381.15, [(M - Boc) + H]<sup>+</sup> 1242.35, [(M - Boc) + 2H]<sup>2+</sup> 622.45.

**H-Ile<sup>1</sup>-Dhb<sup>2</sup>-bicyclo[3-7/8-11]-D-Alg<sup>3</sup>-Ile<sup>4</sup>-Dha<sup>5</sup>-Leu<sup>6</sup>-Alg<sup>7</sup>-D-Alg<sup>8</sup>-Pro<sup>9</sup>-Gly<sup>10</sup>-Alg<sup>11</sup>-Lys<sup>12</sup>-OMe·2TFA (3).** Protected peptide **24** (0.5 mg, 0.37 μmol) was suspended in CH<sub>2</sub>Cl<sub>2</sub> (250 μL) and after the addition of TFA (250 μL), 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<sub>2</sub>O (5 mL, 1 : 1 v/v) and lyophilized to afford peptide **3** as a white fluffy powder in quantitative yield (0.5 mg). *R*<sub>t</sub> = 18.17 min; ESI-MS calcd for C<sub>55</sub>H<sub>91</sub>N<sub>13</sub>O<sub>13</sub> 1141.69, found *m/z* [M + Na]<sup>+</sup> 1164.60, [M + 2H]<sup>2+</sup> 571.65.

**Native nisin(1-12)-OMe (1).** Native nisin 1-12 (1.5 mg, 1.7 μmol; *R*<sub>t</sub> = 24.00 min; ESI-MS calcd. C<sub>51</sub>H<sub>83</sub>N<sub>13</sub>O<sub>13</sub>S<sub>2</sub>: 1149.57, found: *m/z* [M + H]<sup>+</sup> 1151.5, [M + Na]<sup>+</sup> 1172.65, [M + 2H]<sup>2+</sup> 575.75) 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 co-evaporation with MeOH (2 × 1 mL), and the residue was dissolved in *tert*-BuOH/H<sub>2</sub>O (5 mL, 1 : 1 v/v) and subsequently lyophilized, to give peptide ester **1** as a white fluffy powder in quantitative yield (1.5 mg). *R*<sub>t</sub> = 19.53 min; ESI-MS calcd for C<sub>52</sub>H<sub>85</sub>N<sub>13</sub>O<sub>13</sub>S<sub>2</sub> 1163.58, found *m/z* [M + H]<sup>+</sup> 1164.90, [M + Na]<sup>+</sup> 1186.55, [M + 2H]<sup>2+</sup> 582.80.

**Biological evaluation: vesicle leakage experiments.** Carboxy-fluorescein (CF) loaded large unilamellar vesicles (LUVs) were prepared and used in a model membrane leakage experiment as previously described.<sup>9</sup> 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  $\mu\text{M}$  final concentration) was added and fluorescence was measured ( $A_0$ ). After 20 s, a buffer solution (1  $\mu\text{L}$ ) 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  $\mu\text{L}$ ) of Triton-X (stock: 20%; final 0.2%) was added to induce total leakage of the vesicles ( $A_{\text{Total}}$ ). The % of peptide-induced leakage was calculated by:  $((A_{60} - A_0)/(A_{\text{Total}} - A_0)) \times 100\%$ . All measurements were performed in duplo.

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