Step-wise and pre-organization induced synthesis of a crossed alkene-bridged nisin Z DE-ring mimic by ring-closing metathesis†‡

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This paper describes two approaches for the synthesis of a crossed alkene-bridged mimic of the thioether ring system of the nisin Z DE-fragment. The first approach comprised the stepwise total synthesis featuring a cross metathesis and a macrolactamization on a solid support followed by a ring-closing metathesis in solution. *Via* this route the title compound was obtained in an overall yield of 7% (85% on average for 16 reaction steps). In the second approach, the linear precursor peptide was subjected to ring-closing metathesis and the bicyclic peptide with the correct side chain connectivity pattern was obtained in yields up to 95%. The preferred formation of the bicyclic crossed alkene-bridged mimic of the DE-ring suggests a favorable pre-organization of the linear precursor peptide.

Introduction

The antimicrobial peptide nisin Z belongs to the lantibiotics which form an important natural class of antibiotics.[1] A general feature of the lantibiotics is the presence of lanthionine residues as the cyclic constraint to give the peptide its specific bioactive conformation (Fig. 1).[2] The lanthionine moiety is introduced *via* an enzymatically-assisted,[3] posttranslational modification *via* a chemoselective Michael-addition of a cysteine residue toward a dehydrated serine (dehydroalanine, Dha) or a dehydrated threonine (dehydrobutyrine, Dhb) residue to give the lanthionine (Lan) or the 3-methyllanthionine (MeLan) moiety, respectively (Fig. 2).[4]

The lanthionine moiety or thioether (sulfide) bridge as a natural constraint in bioactive peptides can be replaced by an alkene- or alkyne-bridge in order to increase the metabolic stability of the newly designed peptide-derived antibiotics. Recently, it was shown by us that such thioether bridges could successfully be replaced by either alkene/alkyne-bridges or by a combination of both alternative conformational constraints.[5,6]

In the DE-ring system of nisin the amino acid side chains cross each other (connectivity pattern: [1→4], [3→6]) implying that an alkene mimic of this ring system may be difficult to synthesize.[7] The most straightforward route towards the crossed alkene-bridged DE-ring mimic is a direct synthesis from the linear peptide RCM-precursor containing the required allylglycine residues. However, it was assumed that this approach may result in a complex reaction mixture of three bicyclic products, in addition to monocyclic intermediates, starting material and alkene-isomerization products. From such a mixture the desired product would have be isolated and its structure proven, a non-trivial task.

Hence, a step-wise approach was developed; it was considered that this would more likely lead to the desired crossed alkene-bridged DE-ring mimic. The planned route featured cross metathesis[8] and macrolactamization reactions on a solid support, followed by a RCM reaction in solution. As such this represents the first example of RCM being applied for the synthesis of a crossed alkene-bridge[9,10] to obtain mimics of thioether-bridges containing lantibiotics.

Results and discussion

The envisaged route (Scheme 1) started by attachment[11] of Fmoc-Alg-OH to plain Argogel® (resin **1**), enabling the determination of the loading.[12] The Fmoc group was replaced by a Boc functionality (resin **2**) to introduce orthogonality of the protecting groups (*vide infra*). Then, the putative alkene-bridge of ring E was synthesized by a cross metathesis in the presence of 2nd generation Grubbs' catalyst[13] (Fig. 3) in 1,1,2-trichloroethane at 60 °C with Fmoc-Alg-OH and resin **2**.

Fortunately, it was found that protection of the carboxyl moiety of the Fmoc-protected allylglycine residue was not required, since this would complicate the synthesis substantially. At this stage of the synthesis (resin **3**), a third orthogonal protecting group was necessary. Therefore, after removal of the Fmoc group, azidoalanine hydroxysuccinimide ester (N_3-Ala-ONSu) was coupled, in which the azide was a masked amino group, orthogonal to the Fmoc and Boc-group.[14] This enabled us to complete the peptide sequence of ring E (resin **4**→**6**). The advantage of succinimide esters in this and the next steps is that they can be used in the presence of a free carboxylic acid moiety. Next, acidolysis of the Boc-group by TFA was followed by coupling of Fmoc-Asn(Trt)-ONSu. After removal of the Fmoc-group (resin **5**), Fmoc-Alg-ONSu was coupled to obtain resin **6**.

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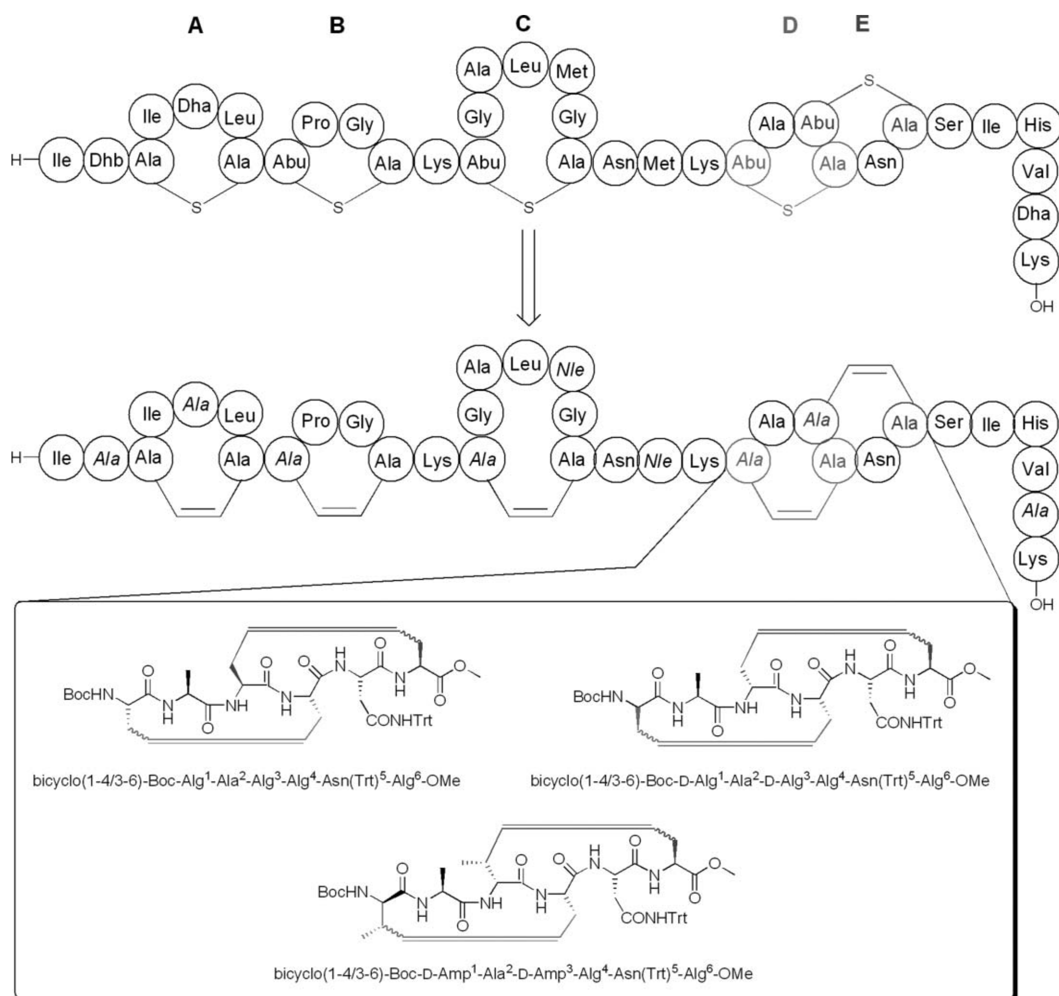


Fig. 1 Amino acid sequence of nisin Z and its alkene-bridged mimic, the crossed DE-ring system is shown in more detail.

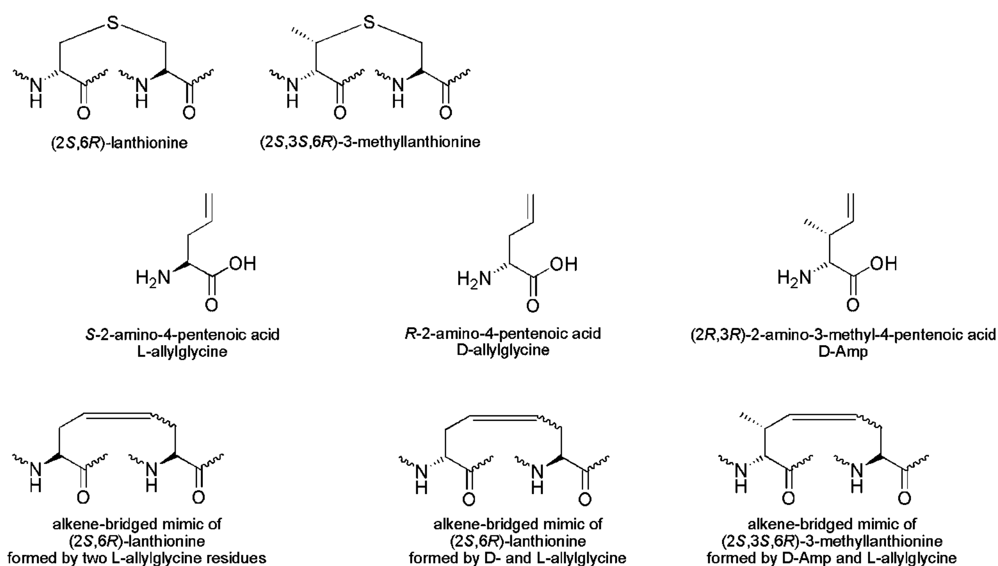
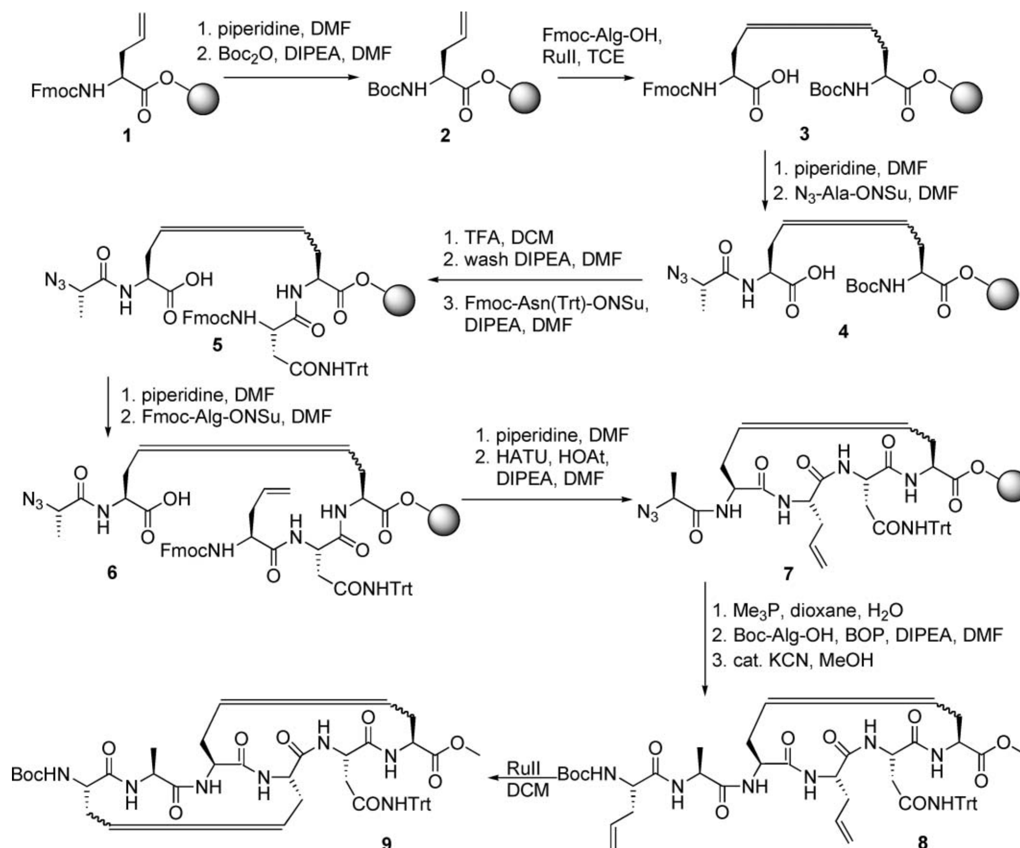


Fig. 2 Structures of the (2*S*,6*R*)-lanthionine and (2*S*,3*S*,6*R*)-3-methyllanthionine moieties and their corresponding alkene-bridged mimics formed by (*S*)-2-amino-4-pentenoic acid, (*R*)-2-amino-4-pentenoic acid and (2*R*,3*R*)-2-amino-3-methyl-4-pentenoic acid. (Note: The stereochemistry of the chiral centers is the same, their *R*/*S* configuration designations are opposite due to the CIP rules.)



Scheme 1 Step-wise solid phase synthesis of the alkene-bridged DE-ring mimic.

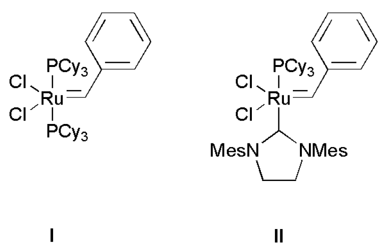


Fig. 3 Structures of the first (**I**) and second (**II**) generation Grubbs' ring-closing metathesis catalyst.

Now, the sequence of the E-ring (resin **6**) was completed and at this stage, the Fmoc group was removed followed by extensive washing with DIPEA to remove any residual piperidine in order to avoid truncation resulting from formation of a piperidinyl amide. Lactamization between residues Alg3 and Alg4 to complete ring E was performed on the resin with HATU–HOAt–DIPEA in DMF[15] which resulted in resin **7**. Reduction of the azide functionality under Staudinger conditions gave the free amine.[14] Finally, Boc-Alg-OH was coupled with BOP–DIPEA and then the resulting resin was treated with a catalytic amount of KCN in methanol to give the monocyclic fully protected peptide ester **8** in 11% overall yield after purification (86% on average per reaction step).

The correct side chain to side chain connectivity of ring E was confirmed by NMR analysis and the correct fragmentation pattern was found by mass analysis (LCES-TOF MS-MS) (Fig. 4).[16]

Peptide **8** was treated with 2nd generation Grubbs' catalyst to give the desired bicyclic peptide **9** in 50% yield. NMR analysis (^{1}H-500 MHz, COSY, TOCSY and ROESY) in combination with MS-MS experiments proved that the correct ring structure was formed and thus that the previously introduced alkene bridge of the E-ring was not converted into different metathesis products. Based on their distinct spin system, Ala2 and Asn5 could be assigned in first instance (Fig. 5A, upper and middle panel). Furthermore, three ROEs (Fig. 5A, lower panel) were visible which provided sequential assignments. ROE-1 is a cross peak between the N*H* of Ala2 and the αC*H* of Alg1, ROE-2 is a cross peak between the N*H* of Alg4 and the αC*H* of Alg3, and ROE-3 is a cross peak between the N*H* of Alg6 and the αC*H* of Asn5 (Fig. 4). The TOCSY data (the whole spectrum is given in Fig. 1 of the Supporting Information‡) proved the correct side chain connectivity pattern (Fig. 5B).

Since we had now the desired bicyclic **9** as a reference available, it was possible to evaluate the feasibility of the 'straightforward' approach using linear precursor peptide **10** directly in RCM (Scheme 2).

Protected peptide **10** was obtained after solid phase peptide synthesis using Fmoc–^{t}Bu protocols followed by purification in 69% yield. This peptide was now treated with 2nd generation Grubbs' catalyst. After 2 h a sample was taken from the reaction mixture and the catalyst was immediately removed by filtration over a small silica plug. The remaining reaction mixture was refluxed overnight after addition of more catalyst. First, the reaction intermediates in the sample were analyzed and purified by HPLC and characterized by LCES-TOF MS-MS.[16] The observed mass in combination with the obtained fragmentation pattern enabled the elucidation of the structure of the formed monocylic intermediates.

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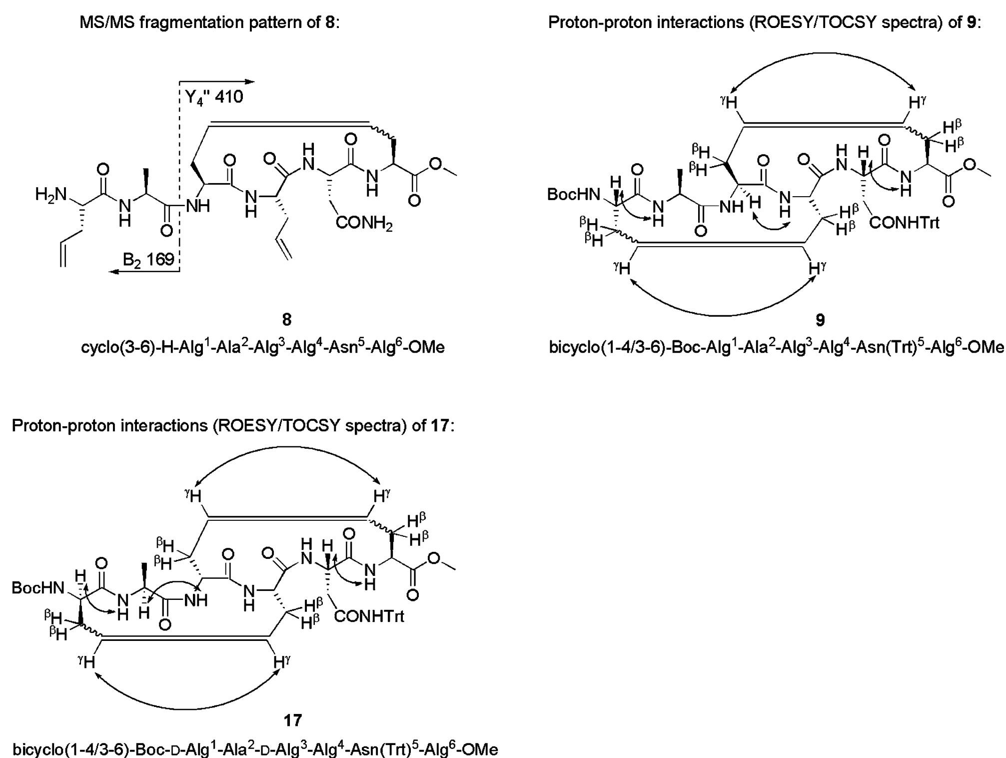
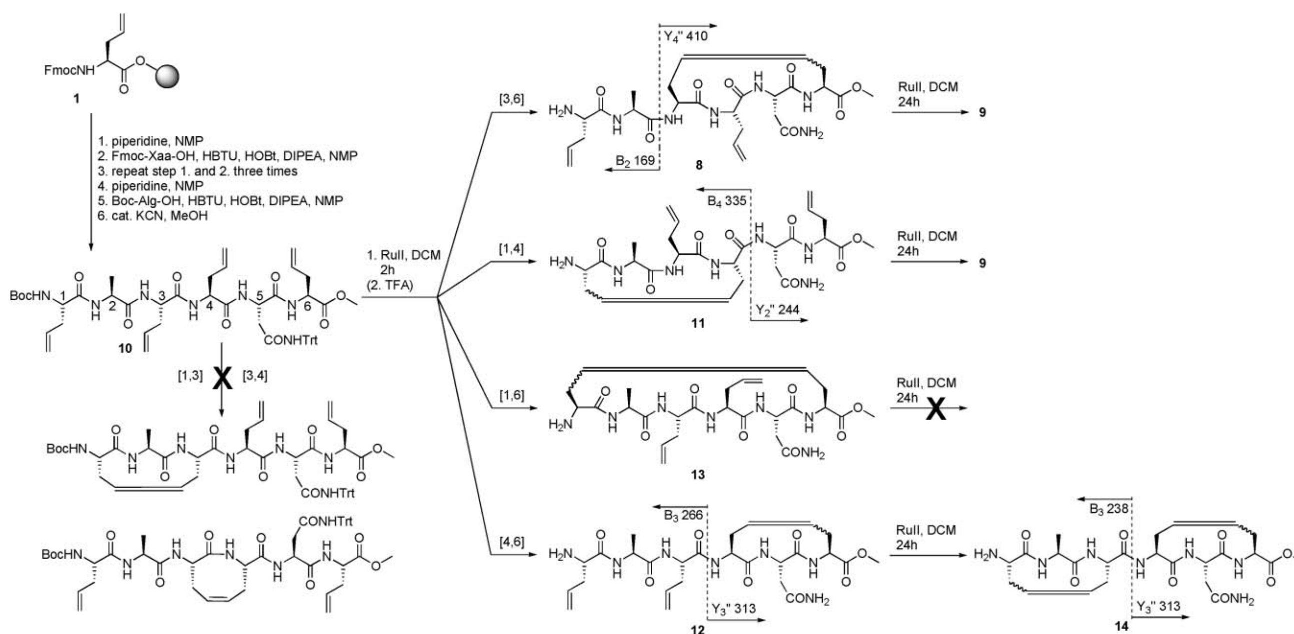


Fig. 4 MS-MS fragmentation pattern of peptide **8**[16] and the observed γ-proton and ROESY sequential connectivities evidencing the correct bicyclic structure of **9**.



Scheme 2 Intermediates in the one step double ring-closing metathesis pathway of the alkene bridged DE-ring mimic. (Note: Before the peptide could be characterized by MS-MS, both protecting groups (Boc and Trt) were removed by treatment with TFA. This treatment is represented by (2. TFA) and is carried out for analysis purposes only (also in Schemes 3 and 4).)

Theoretically, six monocyclic intermediates could have been formed, however, only four (**8**, **11–13**) corresponding to the [3,6], [1,4], [4,6] and [1,6] RCM products were found (Scheme 2). Remarkably, the [1,3] RCM-product was not observed, whereas the [4,6] RCM product was. Absence of the [3,4] RCM product might be explained by reluctance of the *trans*-amide bond to assume a cisoid geometry necessary for the eight membered ring of this product.[17] The unique fragmentation pattern of each RCM-product enabled unambiguous determination of the position of the cyclic constraint.[16] The ratio of product formation **8** : **11** : **12** : **13**

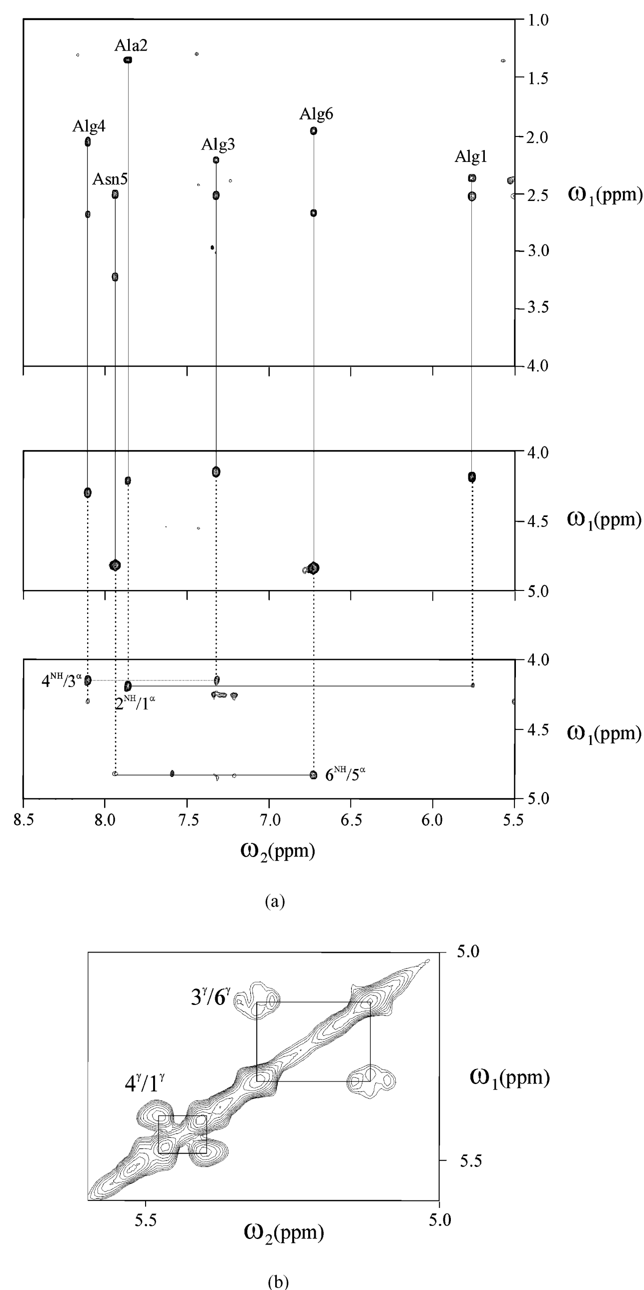


Fig. 5 (A) The upper panel (Cβ*H*–N*H* cross-peaks) and middle panel (Cα*H*–N*H* cross-peaks) are expansions of the TOCSY spectrum of bicyclic peptide **9**. The lower panel is a part of the ROESY spectrum of bicyclic peptide **9**. The observed sequential Cα*H*–N*H* connectivities are indicated. (B) Expansion of the TOCSY spectrum of bicyclic peptide **9** showing the γ-proton connectivities of Alg4–Alg1 and Alg3–Alg6 which proves the bicyclic structure of **9**.

was found to be *circa* 1 : 4 : 1 : 2 and thus the reaction mixture contained approximately 60% of the desired intermediates **8** and **11**. A pure statistical distribution—assuming the formation of six possible RCM-products—would only have resulted in formation of approximately 33% of **8** and **11**. Molecular mechanics calculations were in agreement with the preferential formation of **8** and **11**, since the energy of these intermediates appeared to be significantly lower.[18]

Next, the products obtained after refluxing overnight were isolated and purified. Only two of the three possible bicyclic compounds—based on the formed monocyclic compounds in the reaction mixture sample—were observed. Both monocyclic products **8** and **11** cyclized to the desired bicyclic product **9**. Intermediate **12** cyclized to product **14** *i.e.* the [1,3]–[4,6] product. Not unexpectedly, (*vide supra*) [1,6] product **13** was not converted to a bicycle, since this would require formation of a [3,4] cycle, which is probably difficult. Thus, the desired bicyclic product was obtained in 72% yield as compared to only 19% of one other bicyclic product (**14**). The preferred formation of monocyclic products **8** and **11** and the ensuing bicyclic product hints at a favorable pre-organization of the linear peptide for formation the DE-ring alkene mimic, which in view of their ring size (two 14-membered rings) might be close to an α-helical structure.[19,20] It is tempting to speculate that this pre-organization might also play a role in construction of the natural DE-ring system in nisin itself.

The natural DE-ring is a pair of (2*S*,3*S*,6*R*)-3-methyllanthionine with a crossed side chain to side chain connectivity pattern (Fig. 1 and 2). This implies that the stereochemistry of each amino acid forming the 3-methyllanthionine moiety corresponds to that of (2*S*,3*S*)-threonine (≡ D-Thr) and (*R*)-cysteine (≡ L-Cys). Along these lines, two additional mimics of the DE-ring have been designed, the first one, bicyclic peptide **17** (Scheme 3), has the same stereochemistry as nisin at the α-position and is therefore derived from D-allylglycine at residues 1 and 3. The second one, bicyclic peptide **21** (Scheme 4), has in addition the same stereochemistry at the β-position ((2*R*,3*R*)-2-amino-3-methyl-4-pentenoic acid ≡ D-Alg(*R*-βMe)(D-Amp) as nisin at residue 1 and 3).[21]

Precursor peptide **15** with D-allylglycine residues at position 1 and 3 respectively, was synthesized on the solid phase in 90% yield (Scheme 3). This peptide was treated with 2nd generation Grubbs' catalyst and after 2 h a sample was drawn, purified over silica gel and analyzed by MS-MS. The remaining reaction mixture was refluxed for additional 16 h after addition of more catalyst. Mass analysis of the sample showed a *single* peak apparently corresponding to a bicyclic peptide with crossed side chains, since MS-MS did not result in peptide fragment ions. On HPLC, three major peaks could be identified which converged into one single peak after hydrogenolysis of the sample. NMR analysis proved the homogeneity of the hydrogenated sample. These results suggested that ring-closure of **15** resulted in a mixture of, at least, three *cis*/*trans* isomers of a single bicyclic peptide.

Also with this bicyclic peptide **17**, ^{1}H TOCSY and ROESY experiments were performed to determine the connectivity pattern of the side chains (see Fig. 2 of the Supporting Information‡). Three ROEs (Fig. 4) (different from those found in **9**) provided sequential assignments. ROE-1 is a cross peak between the N*H* of Ala2 and the αC*H* of D-Alg1, ROE-2 is a cross peak between the N*H* of D-Alg3 and the αC*H* of Ala2, and ROE-3 is a cross peak between the N*H* of Alg6 and the αC*H* of Asn5. The TOCSY data proved the correct side chain connectivity pattern (see Fig. 3 of the Supporting Information‡). Thus, from these experiments it can be concluded that RCM of **15** resulted predominantly (>95%) in the desired bicyclic peptide **17** in less than 2 h reaction time (Fig. 4).

As an additional proof, precursor peptide **15** was also treated with the 1st generation Grubbs' catalyst[22] (Fig. 3). After 2 h, an aliquot of the reaction mixture was analyzed (while the remaining reaction mixture was refluxed overnight after the addition of

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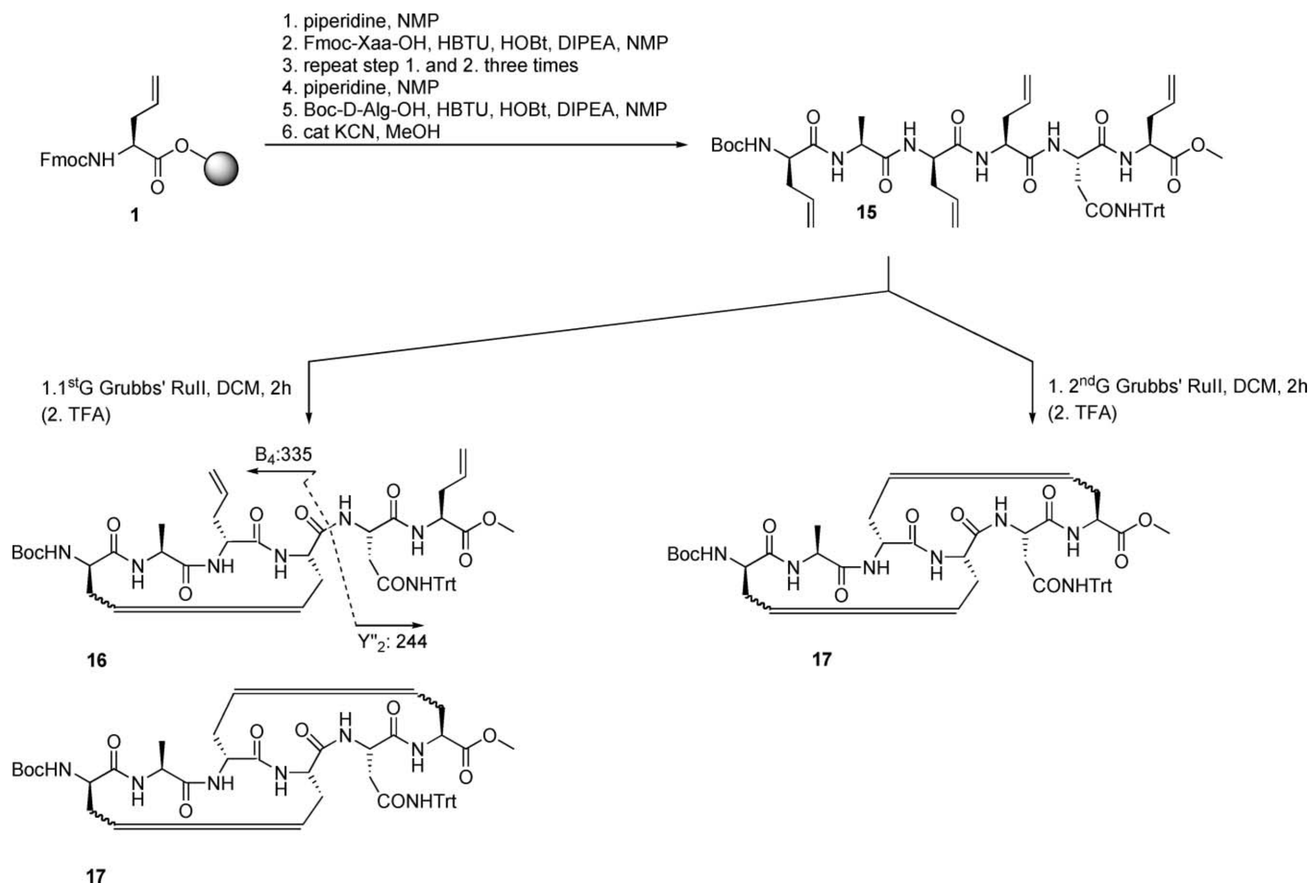


Scheme 3 RCM-intermediates starting from peptide **15**.

more catalyst) and only two products were identified: monocyclic intermediate **16** and bicyclic peptide **17** (Scheme 3). However, after refluxing overnight, both peptides were still present in a ratio **16** : **17** of 1 : 2. Apparently, ring-closing with the less reactive 1st generation Grubbs' catalyst did not result in complete conversion. Moreover, monocyclic intermediate **16**, corresponding to RCM product [1,4], is probably preferred since this intermediate product was also predominantly formed in the double ring-closing metathesis reaction of peptide **10** (Scheme 2).

Finally, precursor peptide **18** was synthesized in which (2*R*,3*R*)-2-amino-3-methyl-4-pentenoic acid[23] (D-Alg(*R*-βMe); D-Amp) was incorporated at positions 1 and 3 (Scheme 4) corresponding to the stereochemistry of the α- and β-carbons in the 3-methyllanthionine residue of native nisin Z.

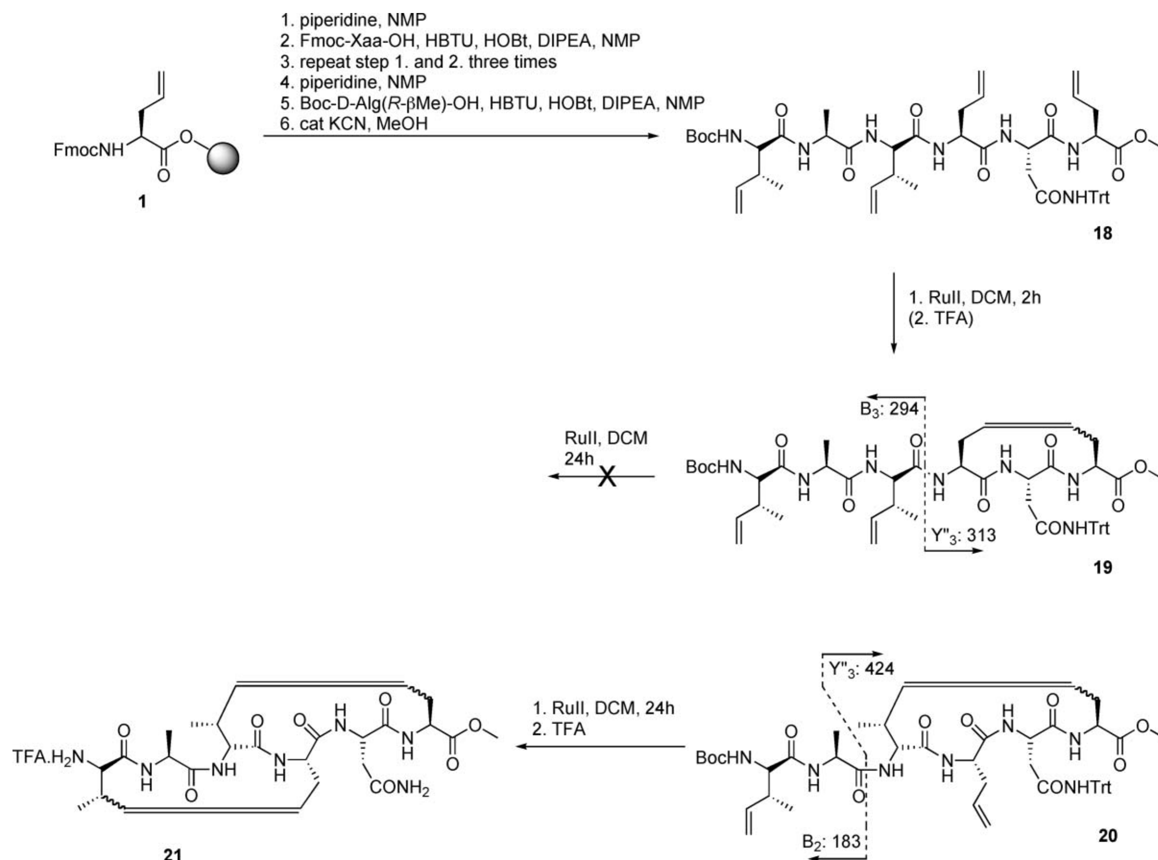
Treatment of peptide **18** with 2nd generation Grubbs' catalyst resulted predominantly in the formation of monocyclic peptide **19** (>90%) with the incorrect [4,6] connectivity pattern and the desired, correctly folded [3,6] monocyclic intermediate was isolated in only 5%.

Preference for the formation of **19** can be explained by the reduced steric hindrance in the RCM reaction, since in formation of either the [1,4] or [3,6] RCM product a β-substituted allylglycine derivative has to be incorporated. The decreased reactivity of β-substituted allylglycine compared to allylglycine in RCM has been recently described in the literature.[24] Longer reaction times did not change the outcome of this one step double ring-closing metathesis pathway. Monocyclic intermediate **19** did not react further since two β-substituted allylglycine residues were sterically too hindered for any subsequent RCM reaction. However, correctly folded intermediate product **20** was converted into bicyclic peptide **21**, unfortunately, this product was formed in very small amounts (as judged by LC-MS-MS) which hampered isolation and further analysis and characterization.

To address the issue whether an alkene-bridge is a good mimic of the thioether moiety of the lanthionine functionality, modeling studies using MacroModel[18] were carried out. The conformational search for obtaining the global minimum of compounds **9**, **17** and **21** was carried out in chloroform since, the ring-closing metathesis reactions as well as the NMR experiments were carried out in comparable apolar solvents. From each structure the energy content (kJ mol^{-1}) of the global minimum of the *E*,*E*; *E*,*Z*; *Z*,*E* and the *Z*,*Z* geometry of the double bond was calculated and are shown in Table 1.

In compound **9**, with all-L stereochemistry of the peptide backbone, the order of energy content was: $Z,Z \approx E,Z < E,E \ll Z,E$. In the case of compound **17**, with the same backbone stereochemistry as nisin, this rank order was found to be exactly reversed: $Z,E < E,E \ll E,Z < Z,Z$. Not unexpectedly, if **17** was compared with compound **21**, with the same backbone and side chain stereochemistry as nisin, the order of energy content of the four *E*/*Z* conformers was nearly identical: $Z,E \ll E,E \approx Z,Z \approx E,Z$.

In contrast to formation of **9**, ring-closing metathesis leading to **17** was very fast and efficient since no side products with undesired



Scheme 4 RCM-intermediates starting from peptide **18**.

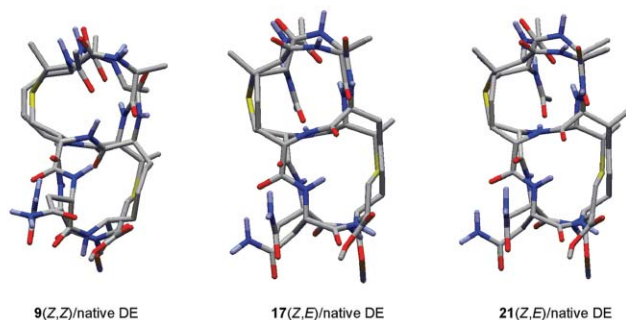
Table 1 Modeling studies using MacroModel[18] of the bicyclic alkene-bridged DE-ring mimics of nisin

Compound[a]	Double bond geometry	Energy (kJ mol^{-1})
9	*E*,*E*	291.8
9	*E*,*Z*	286.2
9	*Z*,*E*	298.5
9	*Z*,*Z*	285.9
17	*E*,*E*	263.2
17	*E*,*Z*	276.1
17	*Z*,*E*	261.3
17	*Z*,*Z*	279.8
21	*E*,*E*	312.1
21	*E*,*Z*	314.6
21	*Z*,*E*	299.9
21	*Z*,*Z*	313.5
Native nisin DE	—	298.1

[a] The conformational search to obtain the global minimum was carried out with N^{α}-acetylated hexapeptide methyl ester derivatives with a free asparagine side chain.

different side-chain to side-chain connectivities were found. In general, the energy content of the isomers of **17** were significantly lower than those of **9** (261.3 respectively 285.9 kJ mol^{-1}) which may partially explain the faster and more selective RCM reaction.

There is a good superimposition of the lowest energy conformers of each RCM product (**9**(*Z*,*Z*), **17**(*Z*,*E*) and **21**(*Z*,*E*)) with the DE-ring system of native nisin as is shown in Fig. 6. The overlay was based on the superimposition of carbon atoms αC1, αC3, αC4 and αC6 of each derivative. The following RMS-values were calculated: **9**(*Z*,*Z*)–native nisin DE: 0.79 Å, **17**(*Z*,*E*)–native nisin DE: 1.27 Å and **21**(*Z*,*E*)–native nisin DE: 1.43 Å. These results as well as the overall view of the superimposition may imply that an alkene-bridge is at least a reasonable mimic of a thioether moiety.



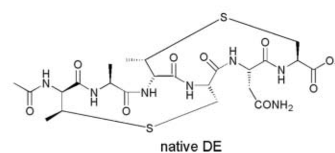


Fig. 6 Superimposition of the lowest energy conformers of bicyclic alkene-bridged mimics **9**, **17** and **21** with the native DE-fragment. The overlay was based on the superimposition of carbon atoms αC1, αC3, αC4 and αC6 of each derivative.

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In conclusion, we have prepared three alkene-bridged mimics of the structurally most challenging part of the nisin Z sequence comprising the DE-ring system. The stepwise total synthesis featuring a cross metathesis and a macrolactamization on the solid support followed by a ring-closing metathesis in solution resulted in the first DE-ring mimic (all-L stereochemistry) with an overall yield of 7% (85% on average for 16 reaction steps). In view of the complexity of the products, this approach was an absolute prerequisite for obtaining the necessary reference compound in order to evaluate whether RCM of linear peptide RCM-precursors would also lead to the desired mimics. Indeed, it was found that there was a preferred formation of the intermediate monocyclic mimic, resulting in a preferred formation of the bicyclic alkene mimic (all-L stereochemistry), which might be explained by invoking a certain degree of pre-organization of linear peptide RCM-precursor. This was further evidenced by two additional ring-closing metathesis reactions. A second DE-ring mimic was synthesized, addressing the same backbone-stereochemistry as in native nisin, and this peptide was found to undergo the double RCM reaction leading to a single compound with the correct [1→4], [3→6] side chain connectivity pattern with an increased overall yield (95%) compared to the all-L derivative. However, the precursor peptide corresponding to the stereochemistry of the α- and β-carbons in the 3-methyllanthionine residues of the native DE-ring system in nisin Z, cyclized only in trace amounts into a third DE-ring mimic since the β-substituted allylglycine residues were sterically too hindered for efficient RCM reaction.

Experimental

For general methods and procedures and details of the computational modeling, see the Supporting Information‡.

Cross metathesis reaction

ArgoGel-OH resin was loaded with Fmoc-Alg-OH using the method of Sieber.[11] The Fmoc group enabled determination of the loading and also the efficiency of the coupling reaction.[12] Fmoc-Alg-O-ArgoGel® (0.36 mmol g^{-1}, 0.7 g, 0.25 mmol) was washed with DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min). The Fmoc group was removed by treatment with 20% piperidine in DMF (3 × 10 mL, 8 min) and the resin was subsequently washed with DMF (3 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min). Reprotection of the free amine was performed with Boc_2O (764 mg, 3.5 mmol) in DMF (10 mL) in the presence of DIPEA (1.2 mL, 4.7 mmol) for 2 h at room temperature. The deprotection–reprotection steps were monitored with the Kaiser test.[25] The resin containing Boc-Alg-O-ArgoGel was washed with DMF (3 × 10 mL, 2 min), and DCM (3 × 10 mL, 2 min). The obtained resin was swelled in 1,1,2-trichloroethane (20 mL), Fmoc-Alg-OH (768 mg, 2.28 mmol) was added and the reaction mixture was purged with N_2 at 60 °C for 20 min. Then, 2nd generation Grubbs' catalyst (129 mg, 0.16 mmol) was added and the obtained reaction mixture was allowed to react overnight at 60 °C under a nitrogen atmosphere. Subsequently, the resin was washed with DCM (6 × 10 mL, 2 min) and diethyl ether (3 × 10 mL, 2 min) and dried under vacuum. The yield of the cross-metathesis reaction was calculated from an Fmoc determination[12] and was found to be 56% (0.20 mmol g^{-1}).

Synthesis of the monocyclic peptide 8 (Boc-Alg^{1}-Ala^{2}-*cyclo*(3→6)[Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}]-OMe)

The resin loaded with the cross-metathesis product (0.7 g, 0.14 mmol) was washed with DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min), subsequently, the Fmoc group was removed by treatment with 20% piperidine in DMF (3 × 10 mL, 8 min) and the resin was washed with DMF (3 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min). The resin was suspended in DMF (15 mL) and N_3-Ala-OSu[14,26] (80 mg, 0.68 mmol) was coupled to the free amine in the presence of DIPEA (215 μL, 1.2 mmol). After 2 h the resin was washed with DMF (3 × 10 mL, 2 min) and DCM (3 × 10 mL, 2 min), and the resin was suspended in TFA–DCM (10 ml, 1 : 1 v/v) for 20 min to remove the Boc group. Subsequently, the resin was washed with DCM (6 × 10 mL, 2 min), DIPEA–DCM (1 : 9 v/v; 3 × 10 mL, 2 min) and DCM (3 × 10 mL, 2 min). Then, Fmoc-Asn(Trt)-OSu (695 mg, 1.0 mmol) in DMF (10 mL) followed by DIPEA (215 μL, 1.2 mmol) were added. After 1 h the coupling was complete according to the Kaiser test. The resin was washed with DMF (3 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min) and the Fmoc group was removed with piperidine in DMF (1 : 4 v/v; 3 × 10 mL, 8 min). After washing with DMF (3 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min), Fmoc-Alg-ONSu (438 mg, 1.0 mmol) was coupled for 1 h. After Fmoc removal and washing of the resin, an extra washing step with DIPEA–DMF (1 : 9 v/v; 3 × 10 mL, 2 min) was carried out to remove any residual piperidine salt. The macrocyclization was carried out with HATU[27] (176 mg, 0.46 mmol), HOAt[27] (63 mg, 0.46 mmol) in the presence of DIPEA (241 μL, 1.4 mmol) in DMF (10 mL) in 16 h at room temperature to obtain the ring E. The resin was washed with DMF (5 × 10 mL, 2 min) and all remaining free amines were acetylated with acetic anhydride (47 μL, 0.5 mmol) with pyridine (81 μL, 1.0 mmol) as base in DMF (5 mL) for 30 min subsequently followed by extensive washing of the resin with DMF (3 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min), DMF (3 × 10 mL, 2 min) and dioxane (3 × 10 mL, 2 min). The N-terminal azide was converted into the corresponding amine by treatment with trimethylphosphine (1 M in toluene; 1.5 mL, 1.5 mmol) in dioxane–H_2O (4 : 1 v/v) for 1 h. Then, the resin was washed with dioxane (6 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min) and DMF (3 × 10 mL, 2 min) followed by the addition of Boc-Alg-OH (151 mg, 0.7 mmol), BOP (310 mg, 0.7 mmol) and DIPEA (244 μL, 1.4 mmol) in DMF (10 mL). After 1 h, the resin was washed with DMF (3 × 10 mL, 2 min), DCM (3 × 10 mL, 2 min), DMF (3 × 10 mL, 2 min) and MeOH (3 × 10 mL, 2 min). The peptide was cleaved from the resin by a catalytic amount KCN in methanol (15 mL) during 16 h. The resin was filtered and washed with methanol (3 × 10 mL). The filtrate was concentrated *in vacuo* and the residue was purified by column chromatography with DCM–MeOH as eluent (97 : 3 → 9 : 1 v/v) followed by preparative HPLC to yield 16.1 mg (overall yield 11%, average yield per step: 86%) of pure monocyclic peptide **8**. R_f: 0.51 (DCM–MeOH 9 : 1 v/v)[28]; R_t: 18.1 min; EI-MS: m/z 920.75 $[M + H]^+$, 942.90 $[M + Na]^+$, 820.65 $[(M - C_5H_8O_2) + H]^+$; ^1H NMR ($CDCl_3$–CD_3OH 14.5 : 1 v/v at 283 K, 500 MHz): δ Alg1: 5.62 (m, 1H, γCH), 5.42 (d (J 6.99 Hz), 1H, NH), 5.06 (m, 2H, δCH_2), 4.09 (m, 1H, αCH), 2.45 (m, 2H, βCH_2), 1.41 (s, 9H, $(CH_3)_3$-Boc); Ala2: 7.44 (d (J 6.99 Hz), 1H, NH), 4.34 (m, 1H, αCH), 1.28 (d (J 7.02 Hz), 3H, βCH_3); Alg3: 7.62

(d (J 6.99 Hz), 1H, NH), 5.18 (m, 1H, γCH), 4.36 (m, 1H, αCH), 2.50/2.36 (double m, 2H, βCH_2); Alg4: 7.83 (d (J 8.0 Hz), 1H, NH), 5.62 (m, 1H, γCH), 5.06 (m, 2H, δCH_2), 4.26 (m, 1H, αCH), 2.46 (m, 2H, βCH_2); Asn(Trt)5: 7.82 (d (J 6.99 Hz), 1H, CONH), 7.62 (s, 1H, CON*H*Trt), 7.30-7.15 (br m, 15H, arom H Trt), 4.70 (m, 1H, αCH), 3.11/2.46 (double m, 2H, βCH_2); Alg6: 6.87 (d (J 7.5 Hz), 1H, NH), 5.18 (m, 1H, γCH), 4.83 (m, 1H, αCH), 3.72 (s, 3H, $COOCH_3$), 2.60/2.09 (double m, 2H, βCH_2).

Synthesis of the bicyclic peptide 9 (Boc-*bicyclo*(1→4,3→6)[Alg^{1}-Ala^{2}-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}]-OMe)

Peptide **8** (7 mg, 8 μmol) was dissolved in DCM (3 mL) and refluxed in a nitrogen atmosphere during 30 min then followed by the addition of 2nd generation Grubbs' catalyst (1 mg, 1.1 μmol) and the reaction mixture was allowed to react for 4 h. The solvent was removed *in vacuo* and the residue was purified by column chromatography with DCM–MeOH as eluent (97 : 3 → 9 : 1 v/v) to obtain bicyclic peptide **9** in 50% (4 mg) yield. R_f: 0.42 (DCM–MeOH 9 : 1 v/v); R_t: 17.1 min; EI-MS: m/z 892.80 $[M + H]^+$, 915.60 $[M + Na]^+$; ^1H NMR ($CDCl_3$–CD_3OH 14.5 : 1 v/v at 283 K, 500 MHz): δ Alg1: 5.77 (d, 1H, NH), 5.40 (m, 1H, γCH), 4.18 (m, 1H, αCH), 2.53/2.37 (double m, 2H, βCH_2), 1.45 (s, 9H, $(CH_3)_3$-Boc); Ala2: 7.86 (d, 1H, NH), 4.21 (m, 1H, αCH), 1.35 (double d (J 7.02 Hz), 3H, βCH_3); Alg3: 7.32 (d, 1H, NH), 5.32 (m, 1H, γCH), 4.15 (m, 1H, αCH), 2.53/2.21 (double m, 2H, βCH_2); Alg4: 8.10 (d, 1H, NH), 5.48 (m, 1H, γCH), 4.30 (m, 1H, αCH), 2.68/2.06 (double m, 2H, βCH_2); Asn(Trt)5: 7.94 (d, 1H, CONH), 7.57 (s, 1H, CON*H*Trt), 7.31–7.18 (br m, 15H, arom H Trt), 4.82 (m, 1H, αCH), 3.23/2.51 (double m, 2H, βCH_2); Alg6: 6.73 (d, 1H, NH), 5.12 (m, 1H, γCH), 4.84 (m, 1H, αCH), 3.75 (s, 3H, $COOCH_3$), 2.67/1.96 (double m, 2H, βCH_2).

Boc-Alg^{1}-Ala^{2}-Alg^{3}-Alg^{4}-Asn^{5}(Trt)-Alg^{6}-OMe (10)

Peptide **10** was synthesized on an Applied Biosystems 433A peptide synthesizer using the FastMoc protocol[29] on Fmoc-Alg-O-ArgoGel® on a 0.25 mmol scale. Each synthetic cycle consisted of N^{α}-Fmoc removal by a 10 min treatment with 20% piperidine in NMP, a 6 min NMP wash, a 45 min coupling step with 1.0 mmol of preactivated Fmoc amino acid in the presence of 2 equivalents DIPEA, and a 6 min NMP wash. N^{α}-Fmoc amino acids were activated *in situ* with 1.0 mmol HBTU–HOBt[30] (0.36 M in NMP) in the presence of DIPEA (2.0 mmol). The peptide was detached from the resin by treatment with a catalytic amount of KCN in MeOH. After washing the resin with MeOH (3 × 10 mL) the filtrate was concentrated *in vacuo* and the residue was purified by column chromatography with DCM–MeOH as eluent (97 : 3 → 9 : 1 v/v) to yield 187 mg (69%) of the linear peptide **10**. R_f: 0.57 (DCM–MeOH 9 : 1 v/v); R_t: 18.9 min; EI-MS m/z 948.65 $[M + H]^+$, 970.70 $[M + Na]^+$; ^1H NMR ($CDCl_3$–CD_3OH 14.5 : 1 v/v at 283 K, 500 MHz): δ Alg1: 5.67 (m, 1H, γCH), 5.50 (d (J 6.99 Hz), 1H, NH), 5.07 (d (J 11.5 Hz), 2H, δCH_2), 4.06 (m, 1H, αCH), 2.48/2.31 (double m, 2H, βCH_2), 1.44 (s, 9H, $(CH_3)_3$-Boc); Ala2: 7.55 (d (J 6.99 Hz), 1H, NH), 4.22 (m, 1H, αCH), 1.28 (d (J 7.02 Hz), 3H, βCH_3); Alg3: 7.43 (d (J 6.99 Hz), 1H, NH), 5.67 (m, 1H, γCH), 5.07 (d (J 11.5 Hz), 2H, δCH_2), 4.21 (m, 1H, αCH), 2.56/2.43 (double m, 2H, βCH_2); Alg4: 7.34 (d (J 6.99 Hz), 1H, NH), 5.67 (m, 1H, γCH), 5.07 (d (J 11.5 Hz), 2H, δCH_2), 4.35 (m, 1H, αCH), 2.53/2.31 (double m, 2H, βCH_2); Asn(Trt)5: 7.90 (s, 1H, CON*H*Trt), 7.69 (d, 1H, CONH), 7.27–7.19 (br m, 15H, arom H Trt), 4.74 (m, 1H, αCH), 2.88/2.78 (double m, 2H, βCH_2); Alg6: 7.55 (d (J 6.99 Hz), 1H, NH), 5.67 (m, 1H, γCH), 5.07 (d (J 11.5 Hz), 2H, δCH_2), 4.47 (m, 1H, αCH), 3.70 (s, 3H, $COOCH_3$), 2.46/2.42 (double m, 2H, βCH_2).

Boc-D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn^{5}(Trt)-Alg^{6}-OMe (15)

Peptide **15** was synthesized on an Applied Biosystems 433A peptide synthesizer using the FastMoc protocol[29] on Fmoc-Alg-O-ArgoGel® on a 0.25 mmol scale as described for **10**. Peptide **15** was purified by column chromatography with DCM–MeOH as eluent (95 : 5 v/v) to yield 224 mg (90%). R_f: 0.57 (DCM–MeOH 9 : 1 v/v); R_t: 35.9 min[31]; EI-MS m/z 948.55 $[M + H]^+$, 970.70 $[M + Na]^+$; ^1H NMR ($CDCl_3$–CD_3OH 14.5 : 1 v/v at 283 K, 500 MHz): δ D-Alg1: 5.93/5.73 (m, 1H, γCH), 5.75 (m, 1H, NH), 5.14/5.01 (double m, 2H, δCH_2), 4.14 (m, 1H, αCH), 2.52/2.17 (double m, 2H, βCH_2), 1.44 (s, 9H, $(CH_3)_3$-Boc); Ala2: 7.54 (m, 1H, NH), 4.14 (m, 1H, αCH), 1.23 (d (J 7.02 Hz), 3H, βCH_3); D-Alg3: 7.54 (m, 1H, NH), 5.93/5.73 (double m, 1H, γCH), 5.14/5.01 (double m, 2H, δCH_2), 4.14 (m, 1H, αCH), 2.52/2.17 (double m, 2H, βCH_2); Alg4: 7.54 (m, 1H, NH), 5.93/5.73 (double m, 1H, γCH), 5.14/5.01 (double m, 2H, δCH_2), 4.14 (m, 1H, αCH), 2.52/2.17 (double m, 2H, βCH_2); Asn(Trt)5: 7.96 (d, 1H, NH), 7.88 (s, 1H, CON*H*Trt), 7.27–7.17 (br m, 15H, arom H Trt), 4.72 (m, 1H, αCH), 2.71 (m, 2H, βCH_2); Alg6: 7.62 (d, 1H, NH), 5.93/5.73 (double m, 1H, γCH), 5.14/5.01 (double m, 2H, δCH_2), 4.44 (m, 1H, αCH), 3.70 (s, 3H, $COOCH_3$), 2.52/2.17 (double m, 2H, βCH_2).

Synthesis of the bicyclic peptide 17 (Boc-*bicyclo*(1→4,3→6)[D-Alg^{1}-Ala^{2}-D-Alg^{3}-Alg^{4}-Asn(Trt)^{5}-Alg^{6}]-OMe)

Peptide **15** (67 mg, 70 μmol) was dissolved in 1,2-dichloroethane (25 mL) and heated to 60 °C in a nitrogen atmosphere during 30 min then followed by the addition of 2nd generation Grubbs' catalyst (11 mg, 13 μmol) and the reaction mixture was allowed to react for 2 h. A sample was taken from the reaction mixture and the catalyst was immediately removed by filtration over a small silica plug. The remaining reaction mixture was allowed to react overnight after addition of more catalyst (7 mg, 8 μmol). The solvent was removed *in vacuo* and the residue was purified by column chromatography with DCM–MeOH as eluent (97 : 3 → 9 : 1 v/v) and isolated in 80% yield (50 mg). R_f: 0.53 (DCM–MeOH 9 : 1 v/v); R_t: 27.3 min[31]; EI-MS m/z 892.8 $[M + H]^+$, 915.5 $[M + Na]^+$; ^1H NMR ($CDCl_3$–CD_3OH 14.5 : m1 v/v at 283 K, 500 MHz): δ D-Alg1: 6.52 (br s, 1H, NH), 5.45/5.32 (double m, 1H, γCH), 4.44 (m, 1H, αCH), 2.73/2.27 (double m, 2H, βCH_2), 1.51 (s, 9H, $(CH_3)_3$-Boc); Ala2: 7.01 (br s, 1H, NH), 4.01 (m, 1H, αCH), 1.05 (d (J 7.02 Hz), 3H, βCH_3); D-Alg3: 8.00 (m, 1H, NH), 5.22/4.69 (double m, 1H, γCH), 4.57 (m, 1H, αCH), 2.73/2.02 (double m, 2H, βCH_2); Alg4: 6.66 (d (J 6.99 Hz), 1H, NH), 5.45/5.32 (double m, 1H, γCH), 4.57 (m, 1H, αCH), 2.80/2.26 (double m, 2H, βCH_2); Asn(Trt)5: 8.00 (m, 1H, NH), 7.58 (s, 1H, CON*H*Trt), 7.25–7.18 (br m, 15H, arom H Trt), 4.78 (m, 1H, αCH), 3.24/2.87 (br m, 2H, βCH_2); Alg6: 6.72 (d (J 6.99 Hz), 1H, NH), 5.22/4.69 (double m, 1H, γCH), 4.68 (m, 1H, αCH), 3.74 (s, 3H, $COOCH_3$), 2.54/2.01 (double m, 2H, βCH_2).

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Boc-D-Alg(*R*-βMe)[1]-Ala[2]-D-Alg(*R*-βMe)[3]-Alg[4]-Asn[5](Trt)-Alg[6]-OMe (18)

Peptide **18** was obtained with a yield of 82% (210 mg). R_f: 0.64 (DCM–MeOH 9 : 1 v/v); R_t: 18.9 min; EI-MS *m*/*z* 976.8 $[M + H]^+$, 999.55 $[M + Na]^+$; ^{1}H NMR ($CDCl_3$–CD_3OH 14.5 : 1 v/v at 283 K, 500 MHz):*δ* D-Alg(*R*-βMe)1: 5.70/5.56 (double m, 1H, γCH), 5.46 (d (*J* 6.99 Hz), 1H, NH), 5.11/5.03 (double m, 2H, δCH_2), 4.04 (m, 1H, αCH), 2.81/2.36 (double m, 1H, βCH), 1.44 (s, 9H, $(CH_3)_3$-Boc), 1.06 (d (*J* 6.87 Hz), 3H, γ'CH_3); Ala2: 7.87 (d (*J* 6.99 Hz), 1H, NH), 4.32 (m, 1H, αCH), 1.26 (d (*J* 7.02 Hz), 3H, βCH_3); D-Alg(*R*-βMe)3: 7.32 (d (*J* 6.99 Hz), 1H, NH), 5.70/5.56 (double m, 1H, γCH), 5.11/5.03 (double m, 2H, δCH_2), 3.89 (m 1H, αCH), 2.81/2.36 (double m, 1H, βCH), 0.99 (d (*J* 6.87 Hz), 3H, γ'CH_3); Alg4: 7.45 (d (*J* 6.99 Hz), 1H, NH), 5.70/5.56 (double m, 1H, γCH), 5.11/5.03 (double m, 2H, δCH_2), 4.42 (m, 1H, αCH), 2.81/2.36 (double m, 2H, βCH_2); Asn(Trt)5: 7.90 (d (*J* 6.99 Hz), 1H, NH), 7.87 (s, 1H, CON*H*Trt), 7.28–7.18 (br m, 15H, arom H Trt), 4.71 (m, 1H, αCH), 2.93 (m, 2H, βCH_2); Alg6: 7.60 (d (*J* 6.99 Hz), 1H, NH), 5.70/5.56 (double m, 1H, γCH), 5.11/5.03 (double m, 2H, δCH_2), 4.42 (m, 1H, αCH), 3.70 (s, 3H, $COOCH_3$), 2.81/2.36 (double m, 2H, βCH_2).

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