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Alkene- and alkyne-bridged mimics of nisin as potential peptide-based antibiotics

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Abstract

Here we highlight our recent results of the application of ring-closing metathesis and ring-closing alkyne metathesis (RCAM) in biologically relevant biomolecules in order to introduce alkene- and/or alkyne-bridges as novel covalent constraints for stabilizing their bioactive conformation and increasing their metabolic stability.

Keywords: Ring-closing alkene metathesis; Ring-closing alkyne metathesis; Peptidomimetics; Peptide-based antibiotics

1. Introduction

Stable conformational constraints, such as thioether- or disulfide bridges are very important for the biological activity of peptides. Replacement of these natural constraints by synthetic isosteres, to increase metabolic stability or receptor-binding affinity of bioactive peptides, has recently received much attention in our group [1]. Examples of such synthetic isosteres are alkene- or alkyne-bridges which can be incorporated in peptides by ring-closing metathesis (RCM) or ring-closing alkyne metathesis (RCAM), respectively. Both metathesis reactions display an extraordinary functional group tolerance and high yield of cyclization which emphasizes their usefulness for the introduction of alkene/alkyne-based conformational constraints into peptides [2].

As a model compound for our studies nisin Z (**1**) has been chosen. Nisin Z belongs to the lantibiotics, a natural class of antimicrobial peptides [3]. Five consecutive thioether bridges, which are introduced biosynthetically by posttranslational modification [4], form the general feature of this class of antimicrobial peptides (Fig. 1). These thioethers play an important role in the dual mode of action of nisin as a bactericide. Nisin binds via its N-terminus, comprising the ABC-ring system, to lipid II, which is an essential precursor for cell wall biosynthesis. As a result, the C-terminus, comprising the knotted DE-ring, can form pores in the phospholipid membrane. This ultimately results to cell leakage and causes a collapse of the vital ion gradients across the membrane [5]. This unique binding target and mode of action makes nisin and its fragments an attractive lead compound for the development of novel peptide-based antibiotics.

Therefore, we planned a synthesis of alkene/alkyne-bridged nisin A-, B-, C- and DE-ring mimics and combinations thereof to explore their potential binding affinities towards lipid II.

2. Experimental

2.1. *Boc-Alg[1]-Ile[2]-Ala[3]-Leu[4]-Alg[5]-OMe (**11**)*

This peptide was synthesized in solution. Yield: 1.56 g (70% over 7 steps); R_t: 17.5 min (on Adsorbosphere XL (C8 90 Å 5 μm, 250 mm × 4.6 mm) in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5, v/v) in 20 min at 1 mL/min); R_f (DCM/MeOH 9:1, v/v): 0.55; ^{1}H NMR ($CDCl_3/CD_3OH$ 14.5:1, v/v, 500 MHz) Alg[1]: δ 6.05 (d, 1H, NH), 5.68 (m, 1H, γCH), 5.01 (m, 2H, δCH_2), 4.51 (m, 1H, αCH), 2.39 (m, 2H, βCH_2), 1.44 (s, 9H, $(CH_3)_3$-Boc); Ile[2]: 7.34 (d, 1H, NH), 4.66 (m, 1H, αCH), 1.77 (m, 1H, γCH), 1.54 (m, 1H, γCH), 1.06 (m, 1H, βCH), 0.84 (m, 6H, γ′CH_3/δCH_3); Ala[3]: 8.45 (d, 1H, NH), 4.93 (m, 1H, αCH), 1.32 (d, 3H, βCH_3); Leu[4]: 8.19 (d, 1H, NH), 4.68 (m, 1H, αCH), 1.68 (m, 1H, γCH), 1.60

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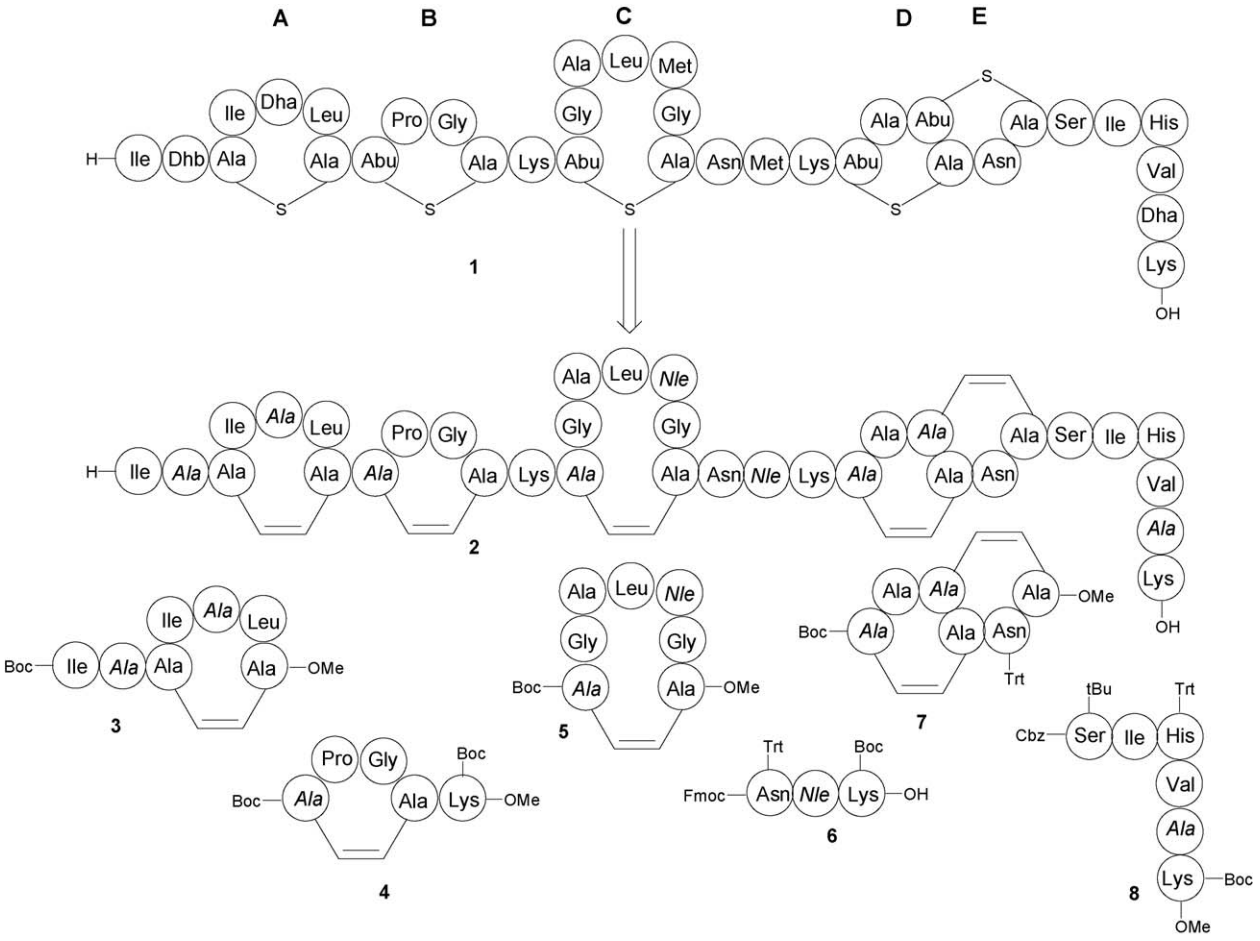


Fig. 1. Amino acid sequence of nisin Z (**1**) and a retrosynthetic analysis of the alkene-bridged nisin Z mimics **2**, **3**, **4**, **5** and **7**.

(m, 2H, βCH$_2$), 0.84 (m, 6H, δ'CH$_3$/δCH$_3$); Alg^5: 8.04 (d, 1H, NH), 5.68 (m, 1H, γCH), 5.01 (m, 2H, δCH$_2$), 4.68 (m, 1H, αCH), 3.74 (s, 3H, OCH$_3$), 2.39 (m, 2H, βCH$_2$), ES-MS: calcd for $C_{31}H_{54}N_5O_8$ 624.4, found *m/z*: $[M+H]^+$ 624.7, $[M+Na]^+$ 646.7, $[(M\text{-}C_4H_8)+H]^+$ 568.6, $[(M\text{-}C_5H_8O_2)+H]^+$ 524.7.

2.2. *Boc-cyclo[Alg^1-Ile^2-Ala^3-Leu^4-Alg^5]-OMe (**12**)*

Linear pentapeptide **11** (410 mg, 0.66 mmol) was dissolved in DCM (250 mL) and the solution was flushed with N_2 for 30 min. The solution was heated to reflux and a solution of second generation Grubbs ("RuII") catalyst (55.6 mg, 0.066 mmol) in DCM (1 mL) was added. The obtained mixture was refluxed overnight under a nitrogen atmosphere. After evaporation of the solvent, the product was purified by column chromatography (DCM/MeOH 97.5:2.5 → DCM/MeOH 95:5, v/v). The *E*/*Z* isomers could be partially separated during purification. Cyclic pentapeptide **12** was obtained as a brownish foam. Yield: 296 mg (76%); R_t: 16.4 min (*Z* isomer) and 20.8 min (*E* isomer) (on Adsorbosphere XL (C8 90 Å 5 μm, 250 mm × 4.6 mm) in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5, v/v) in 20 min at 1 mL/min); R_f(DCM/MeOH 9:1, v/v): 0.52 and 0.49; ^{1}H NMR ($CDCl_3/CD_3OH$ 14.5:1, v/v, 500 MHz) *E* isomer Alg^1: *δ* 5.72 (d, 1H, NH), 5.36 (m, 1H, γCH), 4.14 (m, 1H, αCH), 2.39 (m, 2H, βCH$_2$), 1.45 (s, 9H, (CH$_3$)$_3$-Boc); Ile^2: 7.81 (d, 1H, NH), 4.12 (m, 1H, αCH), 1.88 (m, 1H, γCH), 1.57 (m, 1H, γCH), 1.15 (m, 1H, βCH), 0.92 (m, 6H, γ'CH$_3$/δCH$_3$); Ala^3: 7.46 (d, 1H, NH), 4.24 (m, 1H, αCH), 1.49 (d, 3H, βCH$_3$); Leu^4: 8.15 (d, 1H, NH), 4.11 (m, 1H, αCH), 1.88 (m, 1H, γCH), 1.75 (m, 2H, βCH$_2$), 0.92 (m, 6H, δ'CH$_3$/δCH$_3$); Alg^5: 7.28 (d, 1H, NH), 5.36 (m, 1H, γCH), 4.50 (m, 1H, αCH), 3.75 (s, 3H, OCH$_3$), 2.39 (m, 2H, βCH$_2$); *Z* isomer Alg^1: *δ* 5.88 (d, 1H, NH), 5.25 (m, 1H, γCH), 4.69 (m, 1H, αCH), 2.37 (m, 2H, βCH$_2$), 1.48 (s, 9H, (CH$_3$)$_3$-Boc); Ile^2: 9.05 (br, 1H, NH), 4.96 (m, 1H, αCH), 1.88 (m, 1H, βCH), 1.62 (m, 2H, γCH$_2$), 0.84 (m, 6H, γ'CH$_3$/δCH$_3$); Ala^3: 8.96 (br, 1H, NH), 4.79 (m, 1H, αCH), 1.31 (d, 3H, βCH$_3$); Leu^4: 7.91 (br, 1H, NH), 4.96 (m, 1H, αCH), 1.62 (m, 3H, γCH/βCH$_2$), 0.84 (m, 6H, δ'CH$_3$/δCH$_3$); Alg^5: 8.04 (br, 1H, NH), 5.25 (m, 1H, γCH), 4.96 (m, 1H, αCH), 3.80 (s, 3H, OCH$_3$), 2.69/2.16 (m, 2H, βCH$_2$); ES-MS: calcd for $C_{29}H_{50}N_5O_8$ 596.4, found *m/z*: $[M+H]^+$ 596.7, $[M+Na]^+$ 618.7, $[(M\text{-}C_4H_8)+H]^+$ 540.5, $[(M\text{-}C_5H_8O_2)+H]^+$ 496.4.

2.3. Boc-Ile[1]-Ala[2]-cyclo[Alg[3]-Ile[4]-Ala[5]-Leu[6]-Alg[7]]-OMe (3)

Cyclic pentapeptide **12** (269 mg, 0.45 mmol) was treated with TFA to remove the Boc-group. The obtained TFA-salt was dissolved in DMF (6 mL) and dipeptide Boc-Ile-Ala-OH (200 mg, 0.6 mmol) followed by DIPEA (262 μL, 1.46 mmol) and BOP (228 mg, 0.6 mmol) were added. After stirring for 16 h the solvent is evaporated and the residue is triturated with EtOAc. On TLC two spots were visible corresponding to the two isomers (*E*/*Z*). Yield: 284 mg (80%). R_t: 16.8 min and 20.2 min (on Adsorbosphere XL (C8 90 Å 5 μm, 250 mm × 4.6 mm) in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5, v/v) in 20 min at 1 mL/min); R_f(DCM/MeOH 9:1, v/v): 0.42 and 0.39; ^{1}H NMR ($CDCl_3/CD_3OH$ 14.5:1, v/v, 500 MHz) Ile[1]: δ 5.80 (d, 1H, NH), 3.89 (m, 1H, αCH), 1.80 (m, 1H, βCH), 1.50/1.16 (m, 2H, γCH_2), 1.46 (s, 9H, $(CH_3)_3$-Boc), 0.92 (m, 6H, γ′CH_3/δCH_3); Ala[2]: 8.10 (d, 1H, NH), 4.28 (m, 1H, αCH), 1.71 (d, 3H, βCH_3); Alg[3]: 7.71 (d, 1H, NH), 5.33 (m, 1H, γCH), 4.38 (m, 1H, αCH), 2.55–2.45 (m, 2H, βCH_2); Ile[4]: 7.61 (d, 1H, NH), 4.08 (m, 1H, αCH), 1.92 (m, 1H, βCH), 1.53/1.22 (m, 2H, γCH_2), 0.92 (m, 6H, γ'CH_3/δCH_3); Ala[5]: 7.80 (d, 1H, NH), 4.22 (m, 1H, αCH), 1.41 (d, 3H, βCH_3); Leu[6]: 7.65 (d, 1H, NH), 4.34 (m, 1H, αCH), 1.68 (m, 2H, βCH_2), 1.35 (m, 1H, γCH), 0.92 (m, 6H, δ′CH_3/δCH_3); Alg[7]: 7.70 (d, 1H, NH), 5.33 (m, 1H, γCH), 4.61 (m, 1H, αCH), 3.76 (s, 3H, OCH_3), 2.55–2.45 (m, 2H, βCH_2); ES-MS: calcd for $C_{38}H_{66}N_7O_{10}$ 780.5, found *m*/*z*: $[M+H]^+$ 780.8, $[M+Na]^+$ 803.0, $[(M\text{-}C_4H_8)+H]^+$ 725.2.

2.4. Bicyclic peptide (7)

Peptide **19** (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 second 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 eluens (97:3 → 9:1, v/v) to obtain bicyclic peptide **7** with 50% yield (4 mg). R_t: 17.1 min (on Adsorbosphere XL (C8 90 Å 5 μm, 250 mm × 4.6 mm) in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5, v/v) in 20 min at 1 mL/min); R_f: 0.42 (DCM/MeOH 9:1, v/v); ^{1}H NMR ($CDCl_3/CD_3OH$ 14.5/1, v/v at 283 K, 500 MHz) δ Alg[1]: 5.74 (d, 1H, NH), 5.38 (m, 1H, γCH), 4.20 (m, 1H, αCH), 2.89/2.81 (m, 2H, βCH_2), 1.45 (s, 9H, $(CH_3)_3$-Boc); Ala[2]: 7.84 (d, 1H, NH), 4.27 (m, 1H, αCH), 1.32 (d, 3H, βCH_3); Alg[3]: 7.32 (d, 1H, NH), 5.29 (m, 1H, γCH), 4.12 (m, 1H, αCH), 2.65/2.33 (m, 2H, βCH_2); Alg[4]: 8.10 (d, 1H, NH), 5.45 (m, 1H, γCH), 4.20 (m, 1H, αCH), 2.65/2.02 (m, 2H, βCH_2); Asn(Trt)[5]: 7.90 (d, 1H, CONH), 7.57 (broad s, 1H, CONHTrt), 7.31–7.18 (m, 15H, arom H Trt), 4.80 (m, 1H, αCH), 3.18/2.64 (m, 2H, βCH_2); Alg[6]: 6.70 (d, 1H, NH), 5.09 (m, 1H, γCH), 4.80 (m, 1H, αCH), 3.75 (s, 3H, OCH_3), 2.50/1.90 (m, 2H, βCH_2); ES-MS: calcd for $C_{48}H_{57}N_7O_{10}$ 891.4, found *m*/*z*: $[M+H]^+$ 892.80 $[M+Na]^+$, 915.60.

2.5. Boc-Alg[1]-Ala[2]-Alg[3]-Alg[4]-Asn[5](Trt)-Alg[6]-OMe (21)

Linear precursor peptide **21** was synthesized on an Applied Biosystems 433A peptide synthesizer using the FastMoc protocol on Fmoc-Alg-O-ArgoGel® on a 0.25 mmol scale. 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 eluens (97:3 → 9:1, v/v) to yield 187 mg (69%) protected hexapeptide **21**. R_t: 18.9 min (on Adsorbosphere XL (C8 90 Å 5 μm, 250 mm × 4.6 mm) in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5, v/v) in 20 min at 1 mL/min); R_f: 0.57 (DCM/MeOH 9:1, v/v); ^{1}H NMR ($CDCl_3/CD_3OH$ 14.5/1, v/v at 283 K, 500 MHz): δ Alg[1]: 5.67 (m, 1H, γCH), 5.50 (d, 1H, NH), 5.07 (m, 2H, δCH_2), 4.06 (m, 1H, αCH), 2.48/2.31 (m, 2H, βCH_2), 1.44 (s, 9H, $(CH_3)_3$-Boc); Ala[2]: 7.55 (d, 1H, NH), 4.22 (m, 1H, αCH), 1.28 (d, 3H, βCH_3); Alg[3]: 7.43 (d, 1H, NH), 5.67 (m, 1H, γCH), 5.07 (m, 2H, δCH_2), 4.21 (m, 1H, αCH), 2.56/2.43 (m, 2H, βCH_2); Alg[4]: 7.34 (d, 1H, NH), 5.67 (m, 2H, γCH), 5.07 (m, 2H, δCH_2), 4.35 (m, 1H, αCH), 2.53/2.31 (m, 2H, βCH_2); Asn(Trt)[5]: 7.90 (broad s, 1H, CONHTrt), 7.69 (d, 1H, CONH), 7.27–7.19 (m, 15H, arom H Trt), 4.74(m, 1H, αCH), 2.88/2.78 (m, 2H, βCH_2); Alg[6]: 7.55 (d, 1H, NH), 5.67 (m, 1H, γCH), 5.07 (m, 2H, δCH_2), 4.47 (m, 1H, αCH), 3.70 (s, 3H, $COOCH_3$), 2.46/2.42 (m, 2H, βCH_2); ES-MS: calcd for $C_{52}H_{65}N_7O_{10}$ 947.6, found *m*/*z*: $[M+H]^+$ 948.65, $[M+Na]^+$ 970.70.

2.6. General procedure for ring-closing alkyne metathesis

All RCAM reactions were carried out under Ar in flame-dried glassware using Schlenk techniques. Precursor peptide **30** (46 mg, 70 μmol) was dissolved in dry toluene (200 mL), and the tungsten-alkylidyne catalyst (^{t}BuO)$_3$W≡C^{t}Bu (4 mg, 9 μmol) was added. The obtained reaction mixture was heated to 80 °C and stirred for 2 h. The reaction was monitored by TLC until no changes in product distribution could be observed. Then, H_2O (1 mL) was added to quench the catalyst, and the solvent was removed by evaporation. The residue was purified by column chromatography with DCM/MeOH (97.5:2.5, v/v) as the eluent. Cyclic pentapeptide **31** was obtained as an off-white powder in 42% yield (18 mg). R_f: 0.56 (DCM/MeOH 9:1, v/v); ^{1}H NMR ($CDCl_3/CD_3OH$ 14.5/1, v/v at 283 K, 500 MHz) δ Bug[1]: 5.84 (d, 1H, NH), 4.27 (m, 1H, αCH), 2.67 (m, 2H, βCH_2), 1.45 (s, 9H, $(CH_3)_3$-Boc); Ile[2]: 7.42 (m, 1H, NH), 4.18 (m, 1H, αCH), 1.91 (m, 1H, βCH), 1.58 (m, 1H, γCH), 1.06 (m, 1H, γCH), 0.92 (m, 6H, γ'CH_3/δCH_3); Ala[3]: 7.72 (broad s, 1H, NH), 4.09 (m, 1H, αCH), 1.51 (m, 3H, βCH_3); Leu[4]: 7.98 (d, 1H, NH), 4.18 (m, 1H, αCH), 1.81 (m, 1H, γCH), 1.62 (m, 2H, βCH_2), 0.92 (m, 6H, δCH_3/δ'CH_3); Bug[5]: 7.42 (m, 1H, NH), 4.60 (m, 1H, αCH), 3.79 (s, 3H, OCH_3); 2.67 (m, 2H, βCH_2); ES-MS calcd for $C_{29}H_{47}N_5O_8$ 594.4, found *m*/*z*: 594.7 $[M+H]^+$, 538.6 $[(M\text{-}C_4H_8)+H]^+$, 494.5 $[(M\text{-}C_2H_5O_2)+H]^+$; HRMS: calcd for $C_{29}H_{47}N_5O_8Na$ 616.33218, found 616.33223.

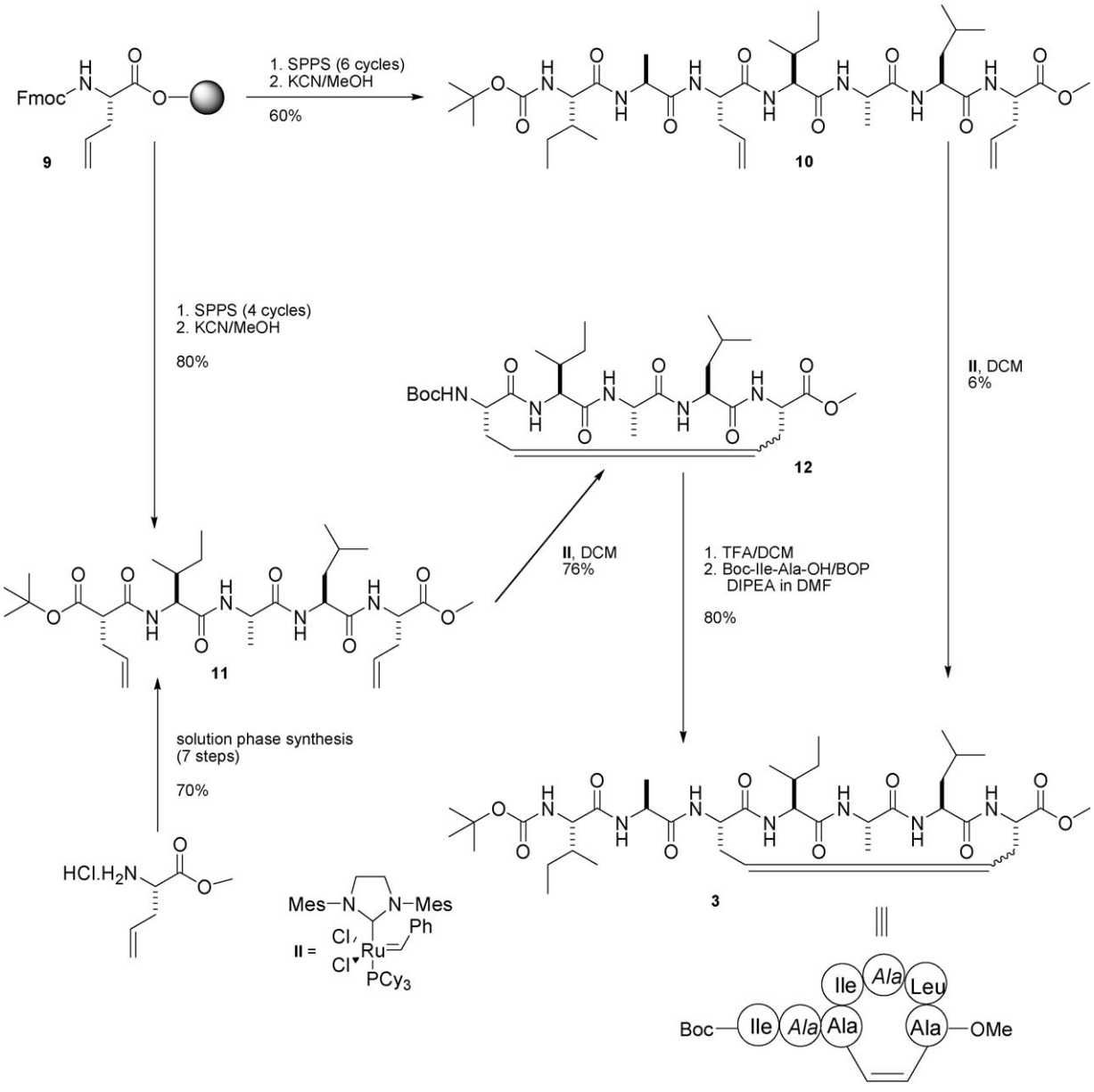
3. Results and discussion

3.1. Synthesis strategy

The retrosynthetic analysis of the alkene-bridged nisin Z mimic (**2**) is given in Fig. 1. To simplify the synthesis, the dihydrobutyrine (Dhb) and dehydroalanine (Dha) residues have been replaced by alanine residues [6]. Additionally, to avoid any impurities caused by oxidation of the methionine residues, these have been replaced by norleucine. This is a generally accepted isosteric replacement. Our strategy for the total synthesis of the AB(C)-fragment was based on the linear synthesis of the peptide sequence either on the solid-phase or in solution followed by RCM in solution using the second generation Grubbs catalyst [7]. After purification and characterization of each individual cyclic peptide, the AB(C)-fragment is assembled by stepwise synthesis in solution. As an example the synthesis of fragment A (**3**) is described in more detail.

3.2. Synthesis of fragment A

Linear peptide **10**, comprizing the open fragment A, was synthesized on plain ArgoGel® Resin using the Fmoc/tBu solid-phase synthesis methodology (Scheme 1). Compound **10** was obtained as the protected N^{α}-Boc heptapeptide methyl ester after cleavage of the peptide by treatment of the resin with a catalytic amount of KCN in methanol. The poor solubility of this fragment hampered the cyclization, since DMF is necessary as a co-solvent which deactivated the RCM-catalyst [8], resulting in a very low isolated yield (6%) of **3**. Therefore, pentapeptide **11** was synthesized and subjected to RCM to obtain cyclic inter-



Scheme 1. Synthesis of alkene-bridged fragment A (**3**).

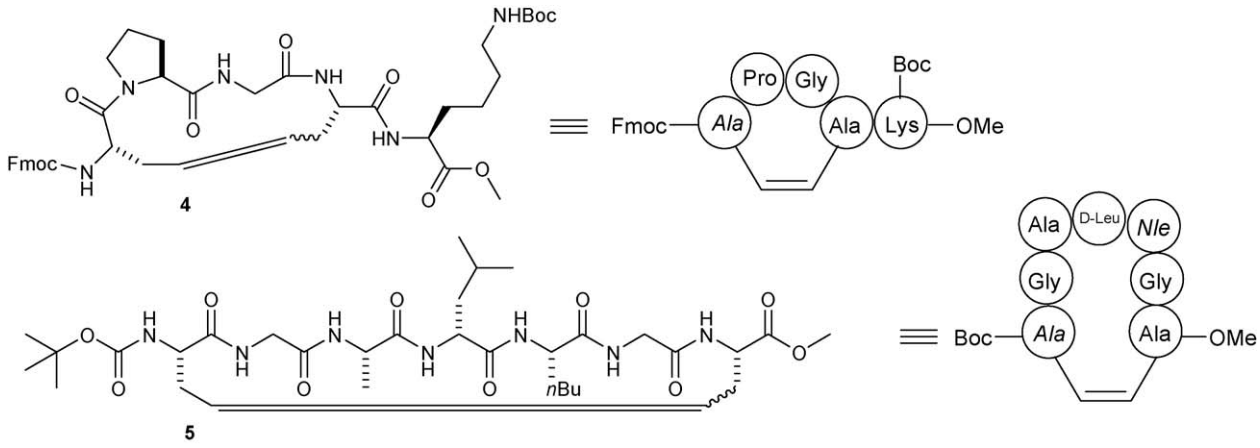
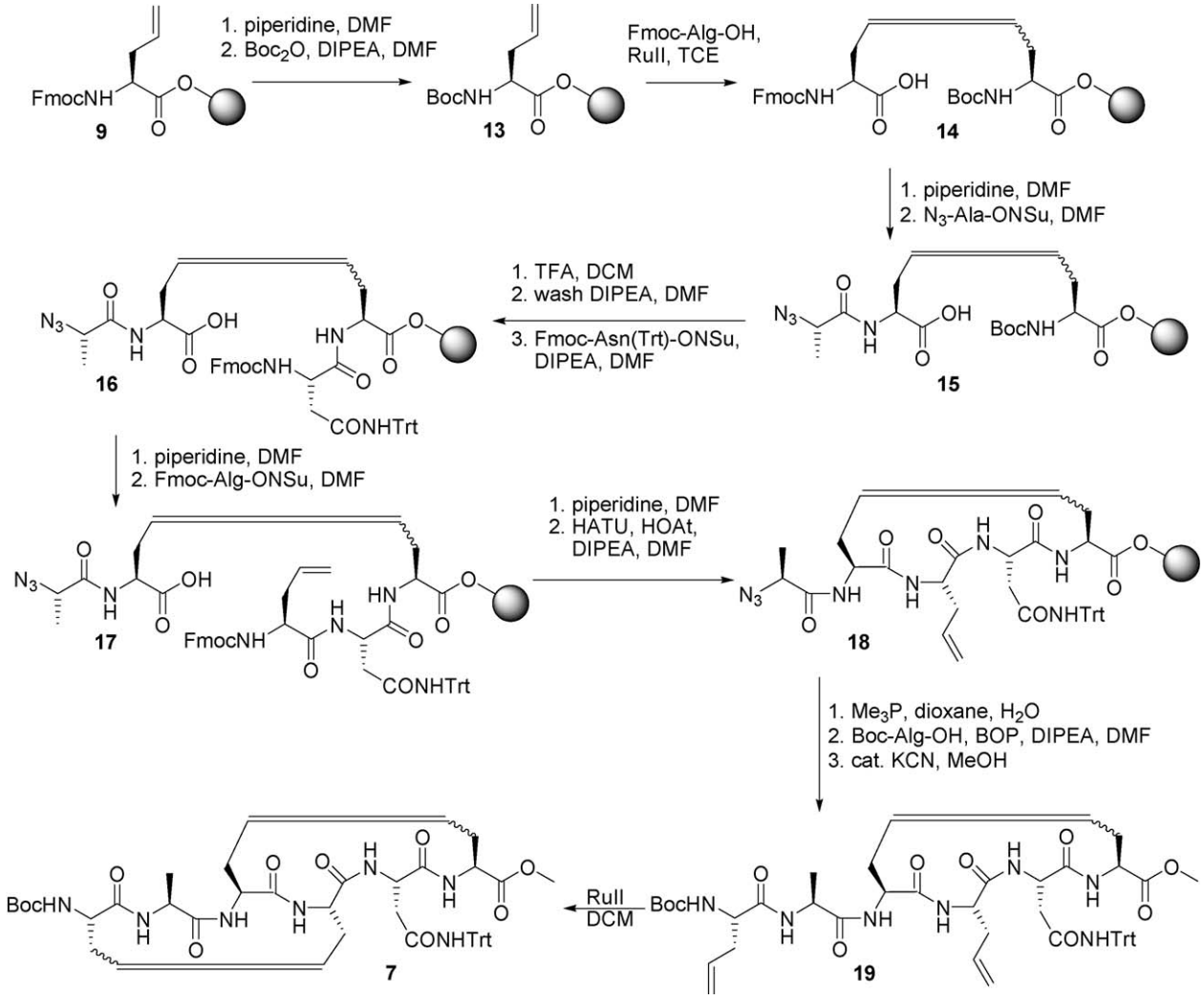


Fig. 2. Structural formulas of alkene-bridged fragment B (**4**) and C (**5**).

mediate **12** in 76% yield [9]. It should be mentioned that at this stage of the synthesis the *E*/*Z* isomers could only partially separated by column chromatography, therefore, the synthesis was continued with a mixture of isomers [10]. Cyclic peptide **12** was subsequently treated with TFA for removal of the Boc-group and then coupled to dipeptide Boc-Ile-Ala-OH to afford fragment A (**3**) in an overall yield of 80%. Fragments B (**4**) and C (**5**) were obtained in 31, and 40% overall yield, respectively (Fig. 2).



Scheme 2. Step-wise synthesis featuring subsequent cross metathesis and ring-closing metathesis to afford alkene-bridged fragment DE (**7**).

3.3. Synthesis of fragment DE

In the DE-ring system of nisin the sulfide bridges formed by the amino acid side chains cross each other (connectivity pattern: [1 → 4], [3 → 6]). As a consequence, an alkene mimic of this ring system is particularly difficult to synthesize [11] and consecutive formation of the DE-ring system as might be the case in the construction of the A-, B-, C-ring system did not seem possible at first glance [1d].

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 (Alg) residues. However, it was assumed that this approach would probably result in a complex reaction mixture of three bicyclic products, in addition to monocyclic intermediates, starting material and alkene-isomerization products. From this mixture the desired product had to be isolated and its structure proven, which is not trivial. Therefore, a step-wise synthetic approach was developed, featuring a cross metathesis [12] on the solid support, which would lead unambiguously to the desired crossed alkene-bridged DE-ring mimic. As such this represents the first example [1d] of RCM applied to the synthesis of a crossed alkene-bridge for obtaining mimics of thioether-bridges [13] containing lantibiotics.

The envisaged route (Scheme 2) started by attachment of Fmoc-Alg-OH to plain Argogel® (resin **9**), and the Fmoc group was replaced by a Boc functionality (resin **13**) to introduce orthogonality of the protecting groups (vide infra). Then, the putative alkene bridge of ring E was synthesized by a cross metathesis in 1,1,2-trichloroethane at 60 °C with Fmoc-Alg-OH and resin **13**. At this stage of the synthesis (resin **14**), 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. This enabled us to complete the peptide sequence of ring E (resin **15** → **17**).

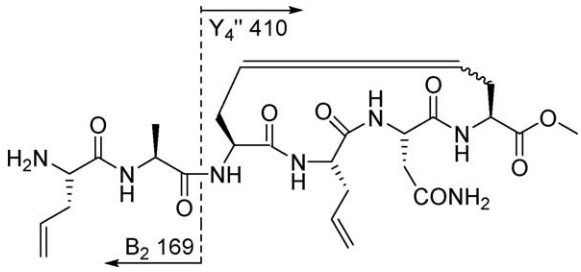
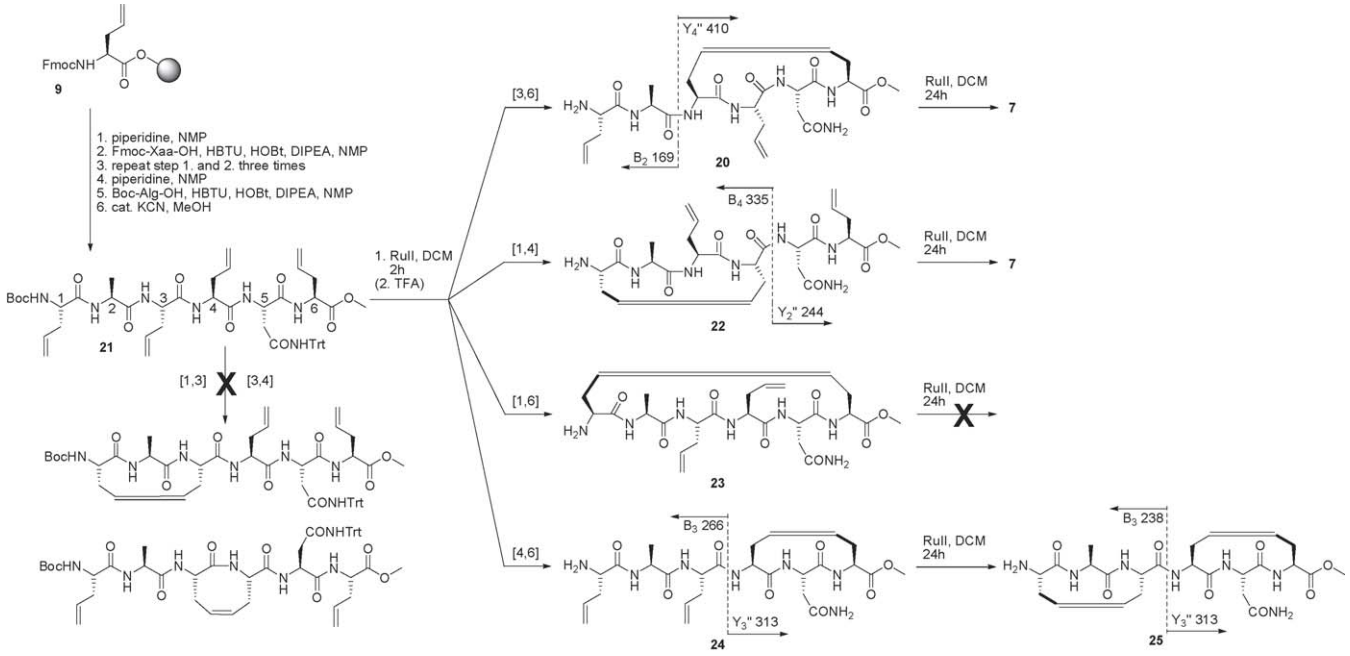


Fig. 3. MS/MS fragmentation pattern of fragment E.

Lactamization between residues Alg3 and Alg4 to afford ring E was performed on resin with HATU/HOAt/DIPEA in DMF. Finally, Boc-Alg-OH was coupled with BOP/DIPEA and then the resulting resin **18** was treated with a catalytic amount of KCN in methanol to give the monocyclic fully protected peptide ester **19** 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 (^{1}H-500 MHz, TOCSY, NOESY and ROESY) and the correct fragmentation pattern was found by mass analysis (LCES–TOF MS/MS) (Fig. 3) [14]. Peptide **19** was treated with second generation Grubbs catalyst to give the desired bicyclic peptide **7** in 50% yield. NMR analysis 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.

Since we had now the desired bicyclic **7** as a reference at hand, it was possible to evaluate the feasibility of the "straight-



Scheme 3. Possible and observed intermediates (as determined by LC–MS/MS) in the one step double ring-closing metathesis leading to alkene-bridged DE fragment **7**.

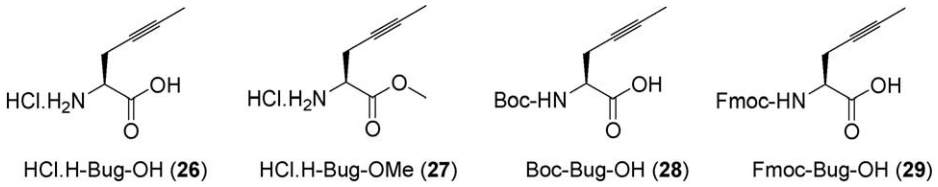


Fig. 4. (*S*)-2-amino-4-hexynoic acid (2-butyneglycine, Bug) and its derivatives.

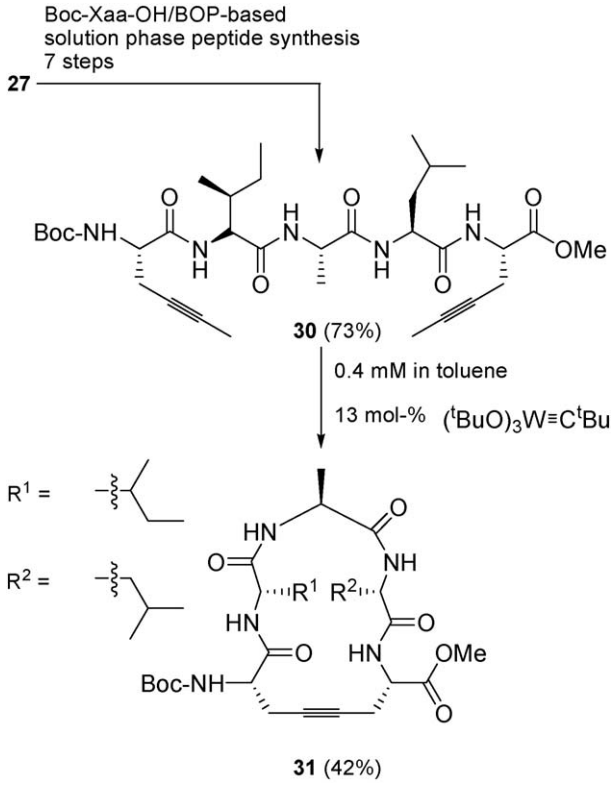
forward" approach using linear precursor peptide **21** directly in RCM (Scheme 3) [1d]. Protected peptide **21** was obtained after solid-phase peptide synthesis using Fmoc/tBu protocols followed by purification in 69% yield. This peptide was now treated with second generation Grubbs catalyst. After 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 [14]. The observed mass in combination with the obtained fragmentation pattern enabled the elucidation of the structure of the formed monocylic intermediates. Theoretically, six monocyclic intermediates could have been formed, however, only four (**20**, **22**–**24**) corresponding to the [3,6], [1,4], [1,6] and [4,6] RCM products were found (Scheme 3). The unique fragmentation pattern of each RCM-product enabled unequivocal determination of the position of the cyclic constraint. The ratio of product formation **20**:**22**:**23**:**24** was found to be ca. 1:4:2:1 and thus the reaction mixture contained approximately 60% of the desired intermediates **20** and **22**. A purely statistical distribution – assuming the formation of six possible RCM-products – would only have led to formation of ca. 33% of **20** and **22**. Next, the products obtained after refluxing overnight were isolated and purified. Only two of the three possible bicylic compounds – based on the formed monocyclic compounds in the reaction mixture sample – were observed. Both monocyclic products **20** and **22** cyclized to the desired bicyclic product **7**. Intermediate **24** cyclized to product **25** i.e. the [1,3]–[4,6] product. Thus, the desired bicyclic product was obtained in 72% yield as compared to only 19% of one other bicyclic product (**25**). The preferred formation of monocyclic products **20** and **22** 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.

So far, we successfully synthesized the individual alkene-bridged ring-fragments of nisin. However, these fragments were obtained as mixtures of *E*/*Z* isomers, which could not easily separated. Coupling of these fragments to full length nisin will result in a mixture of theoretically 32 isomers. Since our alkene-bridged mimics of nisin will be tested for their affinity towards lipid II, an undefined mixture of *E*/*Z* hampers the structure-activity relationship. Therefore, we applied ring-closing alkyne metathesis, since the formed triple bond can be unequivocally reduced to either the *E* or *Z* conformation [2e,15]. We synthesized alkyne-bridged nisin mimics to control the stereochemical purity of the double bond in the corresponding alkene-bridged nisin mimics which will be used in the synthesis of full length alkene-bridged nisin mimic [16].

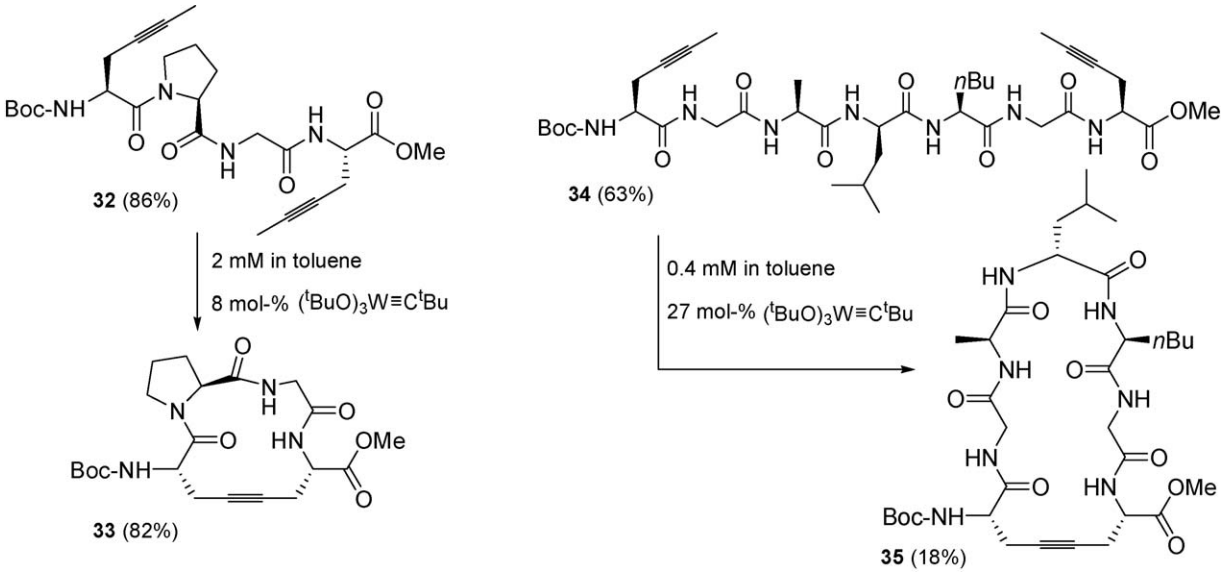
3.4. Alkyne-bridged nisin mimics

The synthesis of the required (*S*)-2-amino-4-hexynoic acid (2-butyneglycine: "Bug", **26**) for incorporation in the RCAM precursors was carried out according to the method of Belokon [17], subjecting the Ni(II) complex of the Schiff base derived from glycine and (*S*)-2-(*N*′-(*N*-benzylprolyl)-amino)benzophenone to alkylation with 1-bromo-2-butyne in the presence of base. Acid **26** was converted to the required derivatives (**27**–**29**) for solution- and solid-phase peptide synthesis (Fig. 4).

Linear RCAM-precursor peptide **30**, corresponding to the sequence of the A-ring in nisin, was synthesized in solution starting from HCl·H-Bug-OMe (**27**) in seven steps with an overall yield of 73%. RCAM of **30** (0.04 mM) was performed in the presence of the tungsten–alkylidyne complex $(tBuO)_3W{\equiv}CtBu$

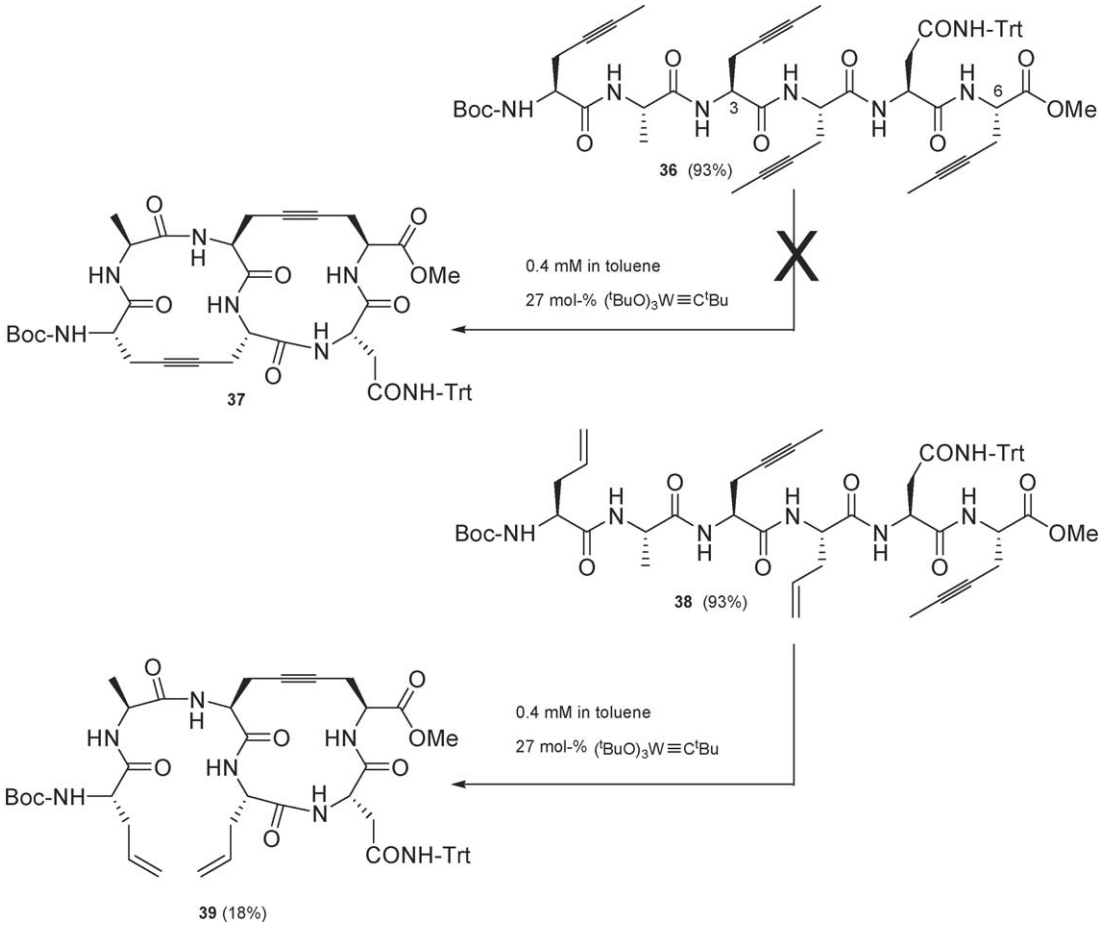


Scheme 4. Synthesis of alkyne-bridged fragment A (**31**).



Scheme 5. Synthesis of thealkyne-bridged fragments B (**33**) and C (**35**).

as catalyst [18] in toluene at 80 °C to give alkyne bridged cyclic peptide **31** in a yield of 42% (Scheme 4). When this reaction was carried out at a higher concentration, lower yields were obtained and oligomerization was a dominant side reaction. The synthesis of **31** is the first example of RCAM of a peptide without any preorganization of the backbone as was the case for proline or β-turn motifs containing sequences [19].



Scheme 6. Approaches to the synthesis of the crossed alkyne-bridged (D)E fragment.

Treatment of Boc-Bug-Pro-Gly-Bug-OMe (**32**) with the tungsten-alkylidyne catalyst resulted in cyclic tetrapeptide **33**, as the alkyne mimic of ring B (Scheme 5), in a yield of 82%. This increased yield, as compared to that of **31**, can be explained by a certain degree of preorganization induced by the proline residue, in agreement with literature data [19]. Moreover, the alkyne ring-closure leading to **33** was also faster (45 min) than that affording **31** (2 h).

The RCAM-precursor Boc-Bug-Gly-Ala-D-Leu-Nle-Gly-Bug-OMe (**34**) as the alkyne mimic of fragment C was synthesized in an overall yield of 63%. The isolated yield was somewhat lower than those of the linear counterparts of fragment A and B, respectively, mainly due to the low solubility of **34** and its precursors. Based on earlier experience, we decided to replace the central leucine by a D-leucine residue, in order to favor a turn-like conformation, since it was known that the sequence with all L-amino acid residues did not cyclize at ring-closing metathesis conditions in the presence of second generation Grubbs Ru-catalyst. Nevertheless, treatment of **34** with the tungsten-alkylidyne catalyst resulted in cyclic heptapeptide **35** (Scheme 5) in only 18% yield. The main product that was isolated consisted of insoluble oligomers. The next challenge was to synthesize the alkyne-bridged DE ring system. Two approaches have been developped to achieve this (Scheme 6).

Our first approach to synthesize the crossed DE-ring system was based on the pre-organization induced synthesis of both ring systems in a single RCM reaction step as was recently reported for the synthesis of bisalkene DE-ring mimic **7** (Scheme 3) [1d]. Therefore, the RCAM precursor **36** with the amino acid sequence comprising the DE-ring with four alkyne moieties was synthesized: Boc-Bug-Ala-Bug-Bug-Asn(Trt)-Bug-OMe (**36**, Scheme 6). Unfortunately, upon RCAM, no mono-cyclic intermediates or bicyclic **37** were formed. After 24 h the formation of polymeric material was observed, the conversion was still incomplete and starting material was recovered. Therefore, Boc-Alg-Ala-Bug-Alg-Asn(Trt)-Bug-OMe (**38**) containing two allylglycine residues and two 2-butyneglycine residues, was synthesized. Since the ruthenium alkene metathesis catalyst and the tungsten alkyne metathesis catalyst can operate orthogonally to each other, it was envisioned to first treat **38** with the tungsten-alkylidyne catalyst in order to synthesize the mono-cyclic alkyne-bridged peptide **39**. After RCAM of precursor peptide **38** under high dilution (250 μM) conditions for 90 min, the reaction mixture was quenched by the addition of H_2O. HPLC analysis showed that two products were formed in a ratio of 3:2, which could be separated by preparative HPLC. According to NMR and LC–MS/MS, **39** was the major product which was obtained in 18% isolated yield (5 mg). As a side product a dimer (connected through Bug3 and Bug6-residues) was identified by LC–MS/MS. Due to the extremely low solubility of **39** the reduction of the triple bond and the subsequent alkene ring-closing metathesis reaction were unsuccessful.

4. Conclusion

We synthesized the alkene-bridged derivatives of the nisin A,- B-, C-, AB, and ABC-ring systems in which the alkene-moiety was a mimic of the natural thioether bridge. Moreover, we developped a step-wise synthesis for the controlled synthesis of a bicylic alkene-bridged mimic of the DE-ring system in which both ring-systems cross each other. Based on the step-wise synthesis, we showed that the correctly folded DE-ring could be obtained in a single reaction step involving a double ring-closing metathesis reaction. The preferred formation of the desired bicyclic mimic may be due to a considerable degree of pre-organization of the linear peptide RCM precursor. Also we successfully applied ring-closing alkyne metathesis to the synthesis of alkyne-bridged peptides, representing the A-, B-, C- and (D)E-ring of nisin, in yields ranging from 18 to 82%. The alkene/alkyne-bridged fragments of nisin will be tested for their potency to bind to lipid II and therefore possible antibiotic activity. Preliminary results from binding assays revealed that the alkene-bridged nisin mimics (*E/Z* mixtures) were still able to bind lipid II albeit with a lower affinity than nisin. Stereoselective reduction of the triple bond leading to derivatives with a defined geometry of the double bond may lead to an additional improvement of lipid II binding activity and affinity.

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[9] To obtain fragment A on a larger scale, we also synthesized pentapeptide **11** in solution starting from HCl·H-Alg-OMe. During the solution phase synthesis the intermediates were purified by aqueous acid/base extractions. Finally, pentapeptide **11** was obtained in 70% overall yield (3.5 mmol scale).

[10] The product was obtained in a ratio *E*:*Z* of 1:2.8. The *E* isomer could be characterized with a multiple decoupling NMR experiment. (This technique was used to assign the different coupling constants, see: J.L. Stymiest, B.F. Mitchell, S. Wong, J.C. Vederas, Org. Lett. 5 (2003) 47.). The J_{AB} value for the *E* isomer is 15.3Hz, whereas the value of the *Z* isomer could not be determined.

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