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Synthesis of Bicyclic Alkene-/Alkane-Bridged Nisin Mimics by Ring-Closing Metathesis and their Biochemical Evaluation as Lipid II Binders: toward the Design of Potential Novel Antibiotics

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This report describes the design, synthesis, and biochemical evaluation of alkene- and alkane-bridged AB(C)-ring mimics of the lantibiotic nisin. Nisin belongs to a class of natural antimicrobial peptides, and has a unique mode of action: its AB(C)-ring system binds to the pyrophosphate moiety of lipid II. This mode of action was the rationale for the design of smaller nisin-derived peptides to obtain novel potential antibiotics. As a conformational constraint the thioether bridge was mimicked by an alkene- or alkane isostere. The peptides of the linear individual ring precursors were synthesized on solid support or in solution, and cyclized by ring-closing metathesis in solution with overall yields of between 36 and 89%. The individual alkene-bridged macrocycles were assembled in solution by using carbodiimide-based synthesis protocols for the corresponding AB(C)-ring mimics. These compounds were tested for their binding affinity toward lipid II by evaluation of their potency to inhibit nisin-induced carboxyfluorescein release from large unilamellar vesicles. It was found that these AB(C)-ring mimics were not able to induce membrane leakage; however, they acted by inhibiting nisin-induced carboxyfluorescein release; this indicates their affinity toward lipid II. These results imply that an alkene or alkane moiety is a suitable thioether bridge mimic.

Introduction

Nisin (Scheme 1) belongs to a class of natural antimicrobial peptides called lantibiotics, which are produced by a broad range of bacteria, for example, *Bacillus*, *Lactococcus*, *Streptomyces*, and *Staphylococcus* species.[1] A general feature of these peptides is the presence of a lanthionine moiety (a thioether or sulfide bridge; Scheme 2) that gives them their specific bioactive conformation.[2]

Nisin binds with its N terminus, which consists of the AB(C)-ring system, to lipid II (Scheme 3). This enables the C terminus, which consists of the knotted DE-ring system, to form pores in the phospholipid membrane, which ultimately lead to cell leakage and collapse of the vital ion gradients across the membrane.[3]

Lipid II[4] is an essential precursor for cell-wall synthesis.[5] It consists of two amino sugars, *N*-acetyl glucosamine (Glc*N*Ac) and *N*-acetyl muramic acid (Mur*N*Ac), and a pentapeptide (usually H-Ala-D-Glu(Lys-D-Ala-D-Ala-OH)-OH-) that is attached to the carboxyl moiety of Mur*N*Ac. This glycopeptide is coupled via a pyrophosphate group to undecaprenol—a membrane-anchored carrier—to yield lipid II. The Lys-D-Ala-D-Ala-OH peptide fragment is recognized by a transpeptidase that forms interstrand peptide cross-links.[6] This cross-linking gives the bacterial cell wall its mechanical strength. The lysine residue, present in Gram-positive bacteria, is replaced by a *meso*-diaminopimelate (*m*-DAP) moiety in Gram-negative bacteria.[6c] Lipid II is an important target for many antimicrobial peptides, such

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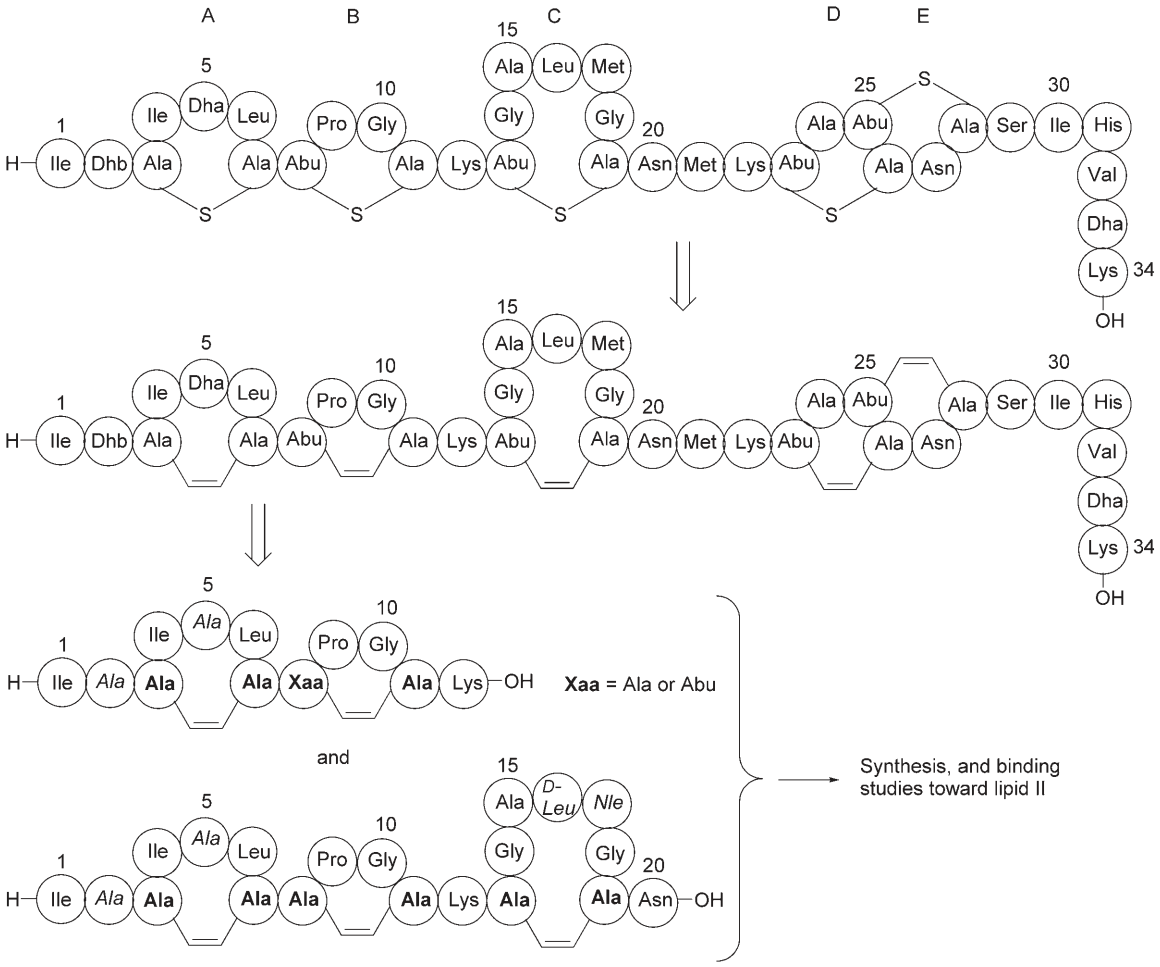


Scheme 1. Schematic representation of nisin (top) and its alkene-bridged mimics. Dhb: dihydrobutyrine; Dha: dehydroalanine; Nle: norleucine; Abu: α-aminobutyric acid. The italicized amino acids represent those changed in the alkene/alkane mimics as compared to the nisin AB(C) fragments. The bold amino acids represent the bridge heads, which are, of course, no longer actual Ala or Abu residues.

as vancomycin, ramoplanin, and nisin, and is a target for the development of new antibiotics.[7,8]

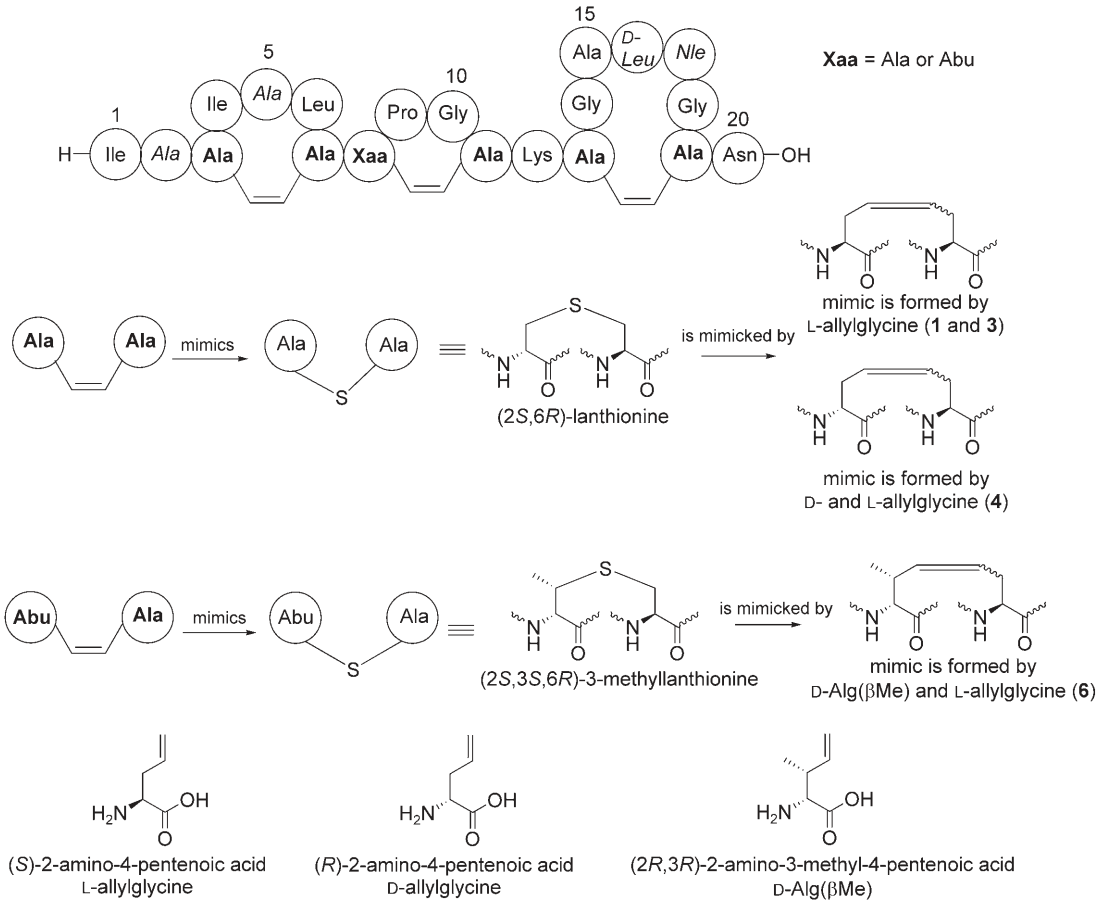
Recently, the molecular mechanism of the nisin–lipid II interaction has been unraveled by NMR spectroscopy studies.[9] It has been found that nisin binds the pyrophosphate moiety of the so-called "pyrophosphate cage" that is formed by the peptide backbone of the AB(C)-ring system. Binding of the AB(C) fragment to lipid II also results in antimicrobial activity, although to a lesser extent than full-length nisin.[10] It is thought that this binding interferes with the cross-linking in the peptidoglycan network in the bacterial cell wall.[10b,11] The formation of this complex implies that any molecule that has a binding affinity toward lipid II is a potential antibiotic.[8]

We were interested to investigate whether the repertoire of Nature, with respect to conformational constraints, such as disulfide or thioether bridges, could be extended to other promising constraints, in particular alkane-, alkene-, or alkyne constraints.[12] We describe here the design, synthesis and biochemical evaluation of nisin-derived AB(C)-ring mimics **1**–**7** (Scheme 4) for the development of novel peptide-based antibiotics.

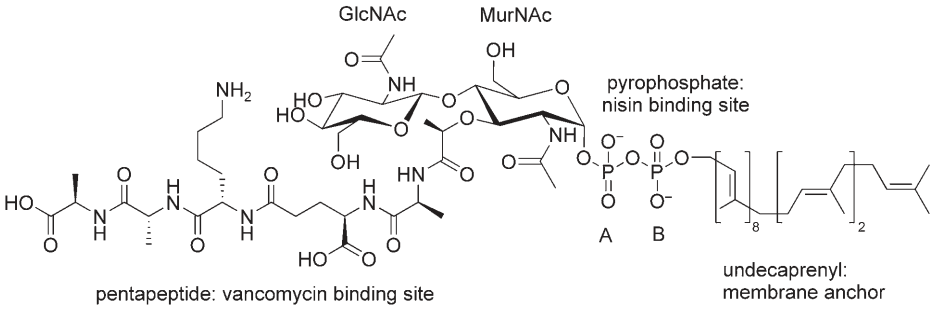
Results and Discussion

Rationale for the design

The lipid II–nisin interaction is dictated by the peptide backbone of the AB(C) fragment, as recently shown by Hsu et al.[9] Replacement of the thioether moiety by alkane-, alkene-, or alkyne cyclic constraints might result in peptide mimics of nisin with improved binding properties and/or increased metabolic stability.[12,13] A potential isostere of the thioether bridge is the alkene bridge; this can be introduced with ring-closing metathesis (RCM)[14] by using amino-acid residues that have a terminal alkene moiety in their side chain. n this study, lanthionine and 3-methyllanthionine (Scheme 2) were replaced by an alkene (**1**, **3**) or alkane (**2**) bridge, respectively, which were formed from two L-allylglycine residues (Scheme 4). As (3-methyl)lanthionine is formed from a combination of a D- and L-amino-acid residue,[15] two additional alkene-bridged mimics (**4** and **6**) were designed and synthesized. Compound **4**, an alkene mimic with a backbone stereochemistry identical to that of nisin, was obtained from a RCM-precursor that had a combination of D- and L-allylglycine residues. Compound **6** not only had the same backbone stereochemistry as nisin, but also



Scheme 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 configuration of the chiral centers is the same; their *R*/*S* signs are opposite due to the CIP rules. The italicized amino acids represent those changed in the alkene/alkane mimics as compared to the nisin AB(C) fragments. The bold amino acids represent the bridge heads, which are, of course, no longer actual Ala or Abu residues.



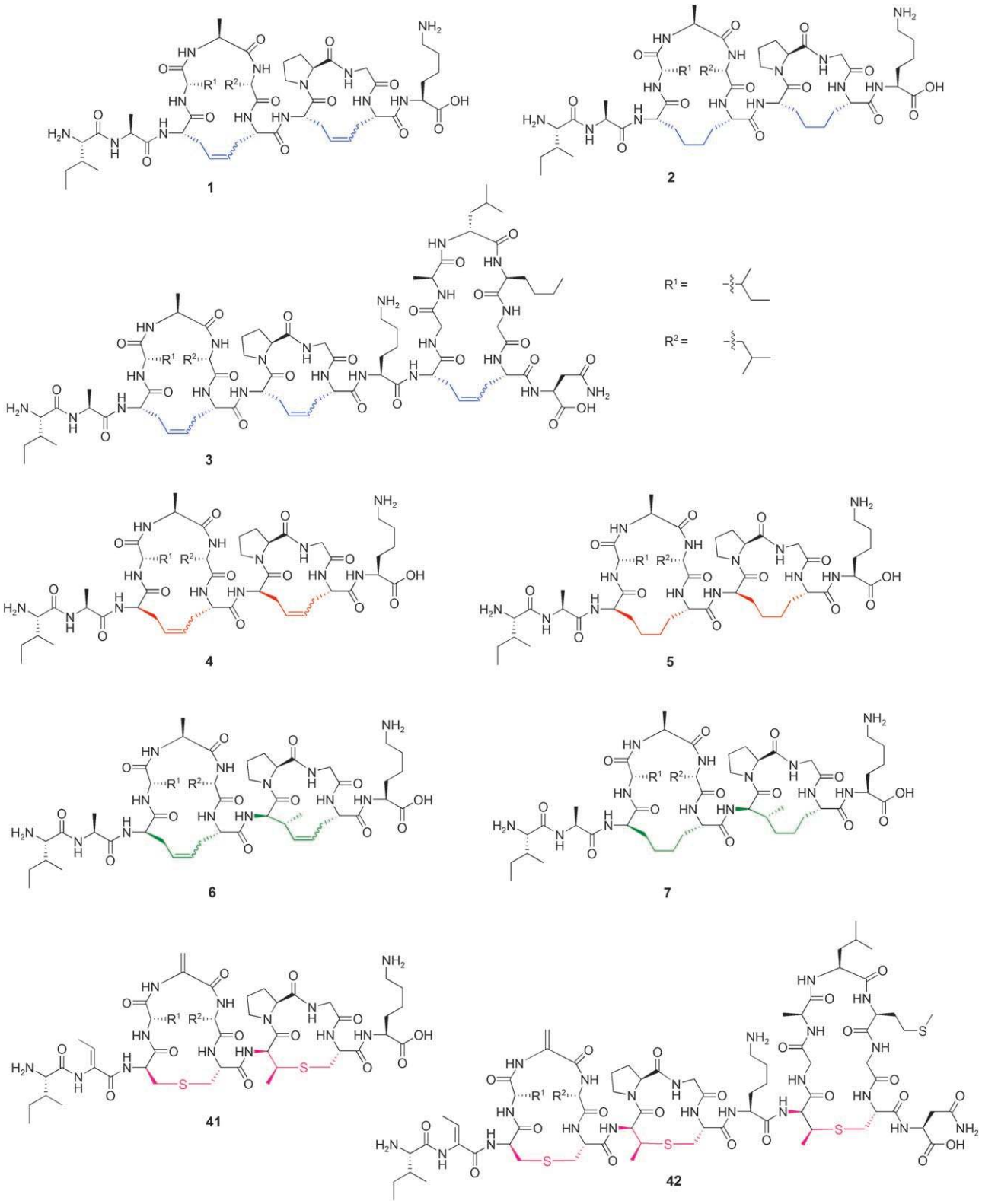
Scheme 3. Structural formula of lipid II.

the same side-chain stereochemistry. For the synthesis of this alkene-bridged 3-methyllanthionine mimic, a β-methyl-substituted D-allylglycine residue, was prepared.[16] This was incorporated with an L-allylglycine residue, followed by RCM. To avoid any *E*/*Z* conformational effects, **4** and **6** were reduced to their alkane-bridged congeners **5** and **7**, respectively.

Synthesis

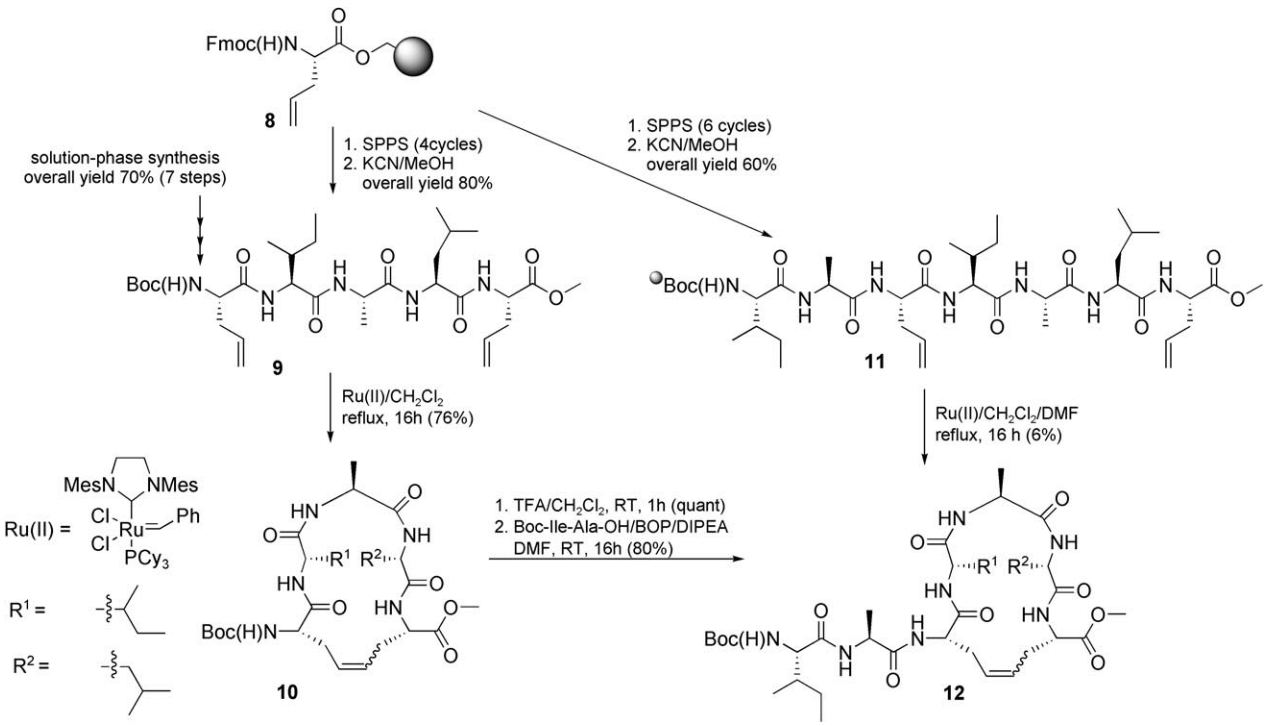
Synthesis strategy: Retro-synthesis analysis shows that a mimic of the AB(C) fragment should be attainable by the coupling of three cyclic alkene-bridge-containing peptides. To simplify the synthesis, dihydrobutyrine (Dhb)[10a] and dehydroalanine (Dha)[17] residues (fragment A) were replaced by alanine residues, and the methionine residue (fragment C) was replaced by norleucine (Nle).[18] It has been shown that these chemically more resistant residues do not influence the biological activity of full-length nisin.[10a,17] Thus, our strategy for the total synthesis of the mimics of the AB(C) fragment consisted of linear synthesis of the peptide sequences of the three individual ring fragments, either on solid phase or in solution, followed by ring-closing metathesis (RCM) in solution by using second generation Grubbs' catalyst.[19] After that, the AB(C) mimics were assembled by coupling the cyclized fragments in solution.[20]

*Synthesis of AB(C) fragment mimics **1**–**3***: The linear peptide **11**, that is, the RCM-precursor of fragment A, was synthesized on plain ArgoGel resin loaded with Fmoc-Alg-OH (**8**) by using



Scheme 4. Structural formulae of the alkene- and alkane-bridged mimics **1**–**7** of the nisin AB(C) fragments. Compound **41** is the natural AB fragment. The colored fragments highlight the alkene/alkane bridges (blue, red, and green), which mimic the two ether bridges (magenta) of the nisin AB(C) fragment.

Fmoc/*t*Bu solid-phase synthesis (Scheme 5). Peptide **11** was obtained as the protected N^{α}-Boc heptapeptide methyl ester after cleavage by treatment of the resin with KCN in methanol. The poor solubility of this fragment hampered cyclization, since DMF, which was necessary as a cosolvent, deactivated the RCM catalyst;[21] consequently, **12** was isolated in low yield (6%). Therefore, the strategy was adjusted and the shorter, more soluble, pentapeptide **9** was synthesized; this led to



Scheme 5. Synthesis of the alkene-bridged A-ring mimic **12**.

cyclic peptide **10** after RCM, in good yield (76%). At this stage the *E*/*Z* isomers could be only partially separated by column chromatography; thus, it was decided to continue the synthesis with the mixture of isomers. The product was obtained with an *E*:*Z* ratio of 1:2.8. The *E* isomer was assigned by using multiple-decoupling NMR spectroscopy[22] (J_{AB}: 15.3 Hz). Unfortunately, the *J* value of the *Z* isomer could not be determined due to the absence of a well-defined multiplet. Cyclic peptide **10** (as *E*/*Z* mixture) was treated with TFA to remove the Boc group and coupled to dipeptide Boc-Ile-Ala-OH. The desired fragment A (**12**) was obtained in an overall yield of 80%.[23]

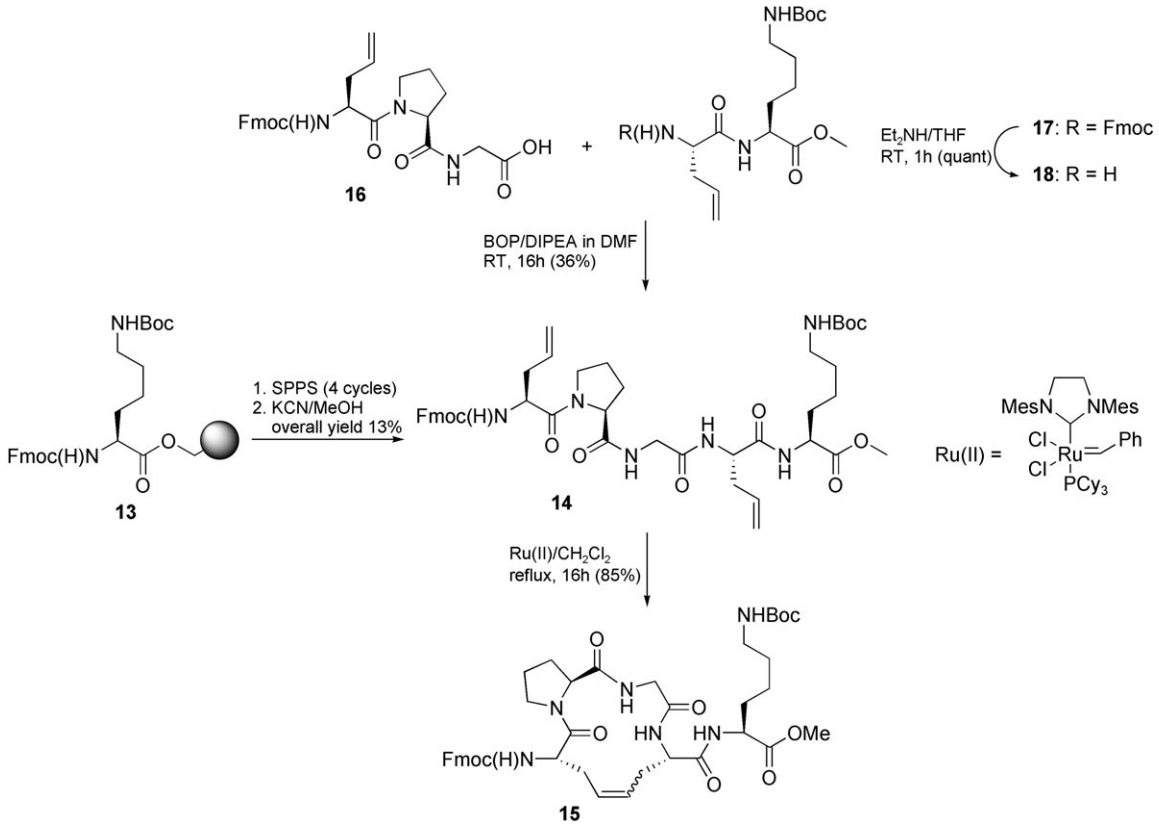
For the synthesis of fragment B (**15**), protected pentapeptide **14** was prepared from resin **13** by treatment of the peptide resin with a catalytic amount of KCN in methanol (Scheme 6). Not entirely unexpectedly, the isolated yield was rather low (13%), mainly due to cleavage of the Fmoc group under these relatively basic conditions.

Therefore, it was decided to synthesize the RCM precursor **14** in solution by coupling Fmoc-Alg-Pro-Gly-OH (**16**) to Fmoc-Alg-Lys(Boc)-OMe (**17**). Although amine **18** was prone to diketopiperazine formation[24] during the coupling with peptide acid **16**, the overall yield was better (36%) than for solid-phase synthesis (13%). Cyclization of **14** was performed by refluxing DCM in the presence of Ru catalyst to obtain **15** in 85% yield. The product appeared pure by TLC and HPLC; however, NMR analysis clearly showed the presence of several conformational isomers.

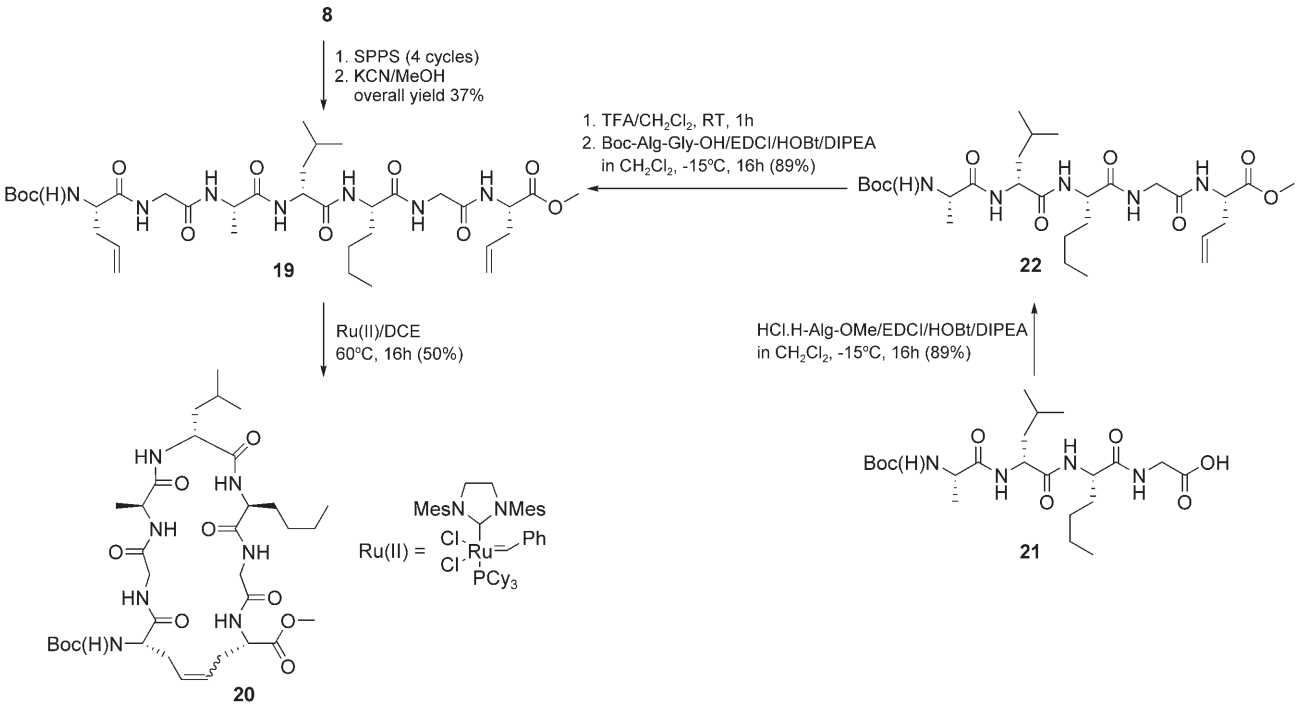
For the assembly of fragment C, protected heptapeptide **19** was prepared by solid-phase synthesis (Scheme 7). Unfortunately, attempts to cyclize Boc-Alg-Gly-Ala-Leu-Nle-Gly-Ala-OMe were unsuccessful. Incorporation of a D-*Leu* residue was necessary to achieve cyclization, and cyclic heptapeptide **20** was obtained by using 50 mol% of second generation Grubbs' catalyst. Disappointingly, the cleavage yield of **19** from the solid support was unsatisfactory due to its low solubility in MeOH. Thus, it was decided to synthesize heptapeptide **19** in solution from fragments Boc-Alg-Gly-OH and Boc-Ala-D-Leu-Nle-Gly-Alg-OMe (**22**). The latter was prepared from tetrapeptide Boc-Ala-D-Leu-Nle-Gly-OH (**21**) and HCl·H-Alg-OMe.

The AB(C) fragment was assembled in solution as shown in Scheme 8. Peptides **12**, **15**, and **20** were obtained as protected methyl esters since the cleavage from plain ArgoGel resin was performed with KCN in MeOH. In the first fragment-coupling approach, attempts were made to convert the methyl esters into hydrazides by treatment with hydrazine hydrate. These hydrazides were converted in situ into azides for a racemization-free coupling of the C-terminal amino-acid residue, as described by Curtius[25] and modified by Honzl and Rudinger.[26] However, the protected peptide hydrazides were found to be nearly insoluble. Moreover, treatment with hydrazine also reduced the double bonds of the alkene-bridged fragments **12**, **15**, and **20**. Therefore, it was decided to convert the protected peptide methyl esters into their corresponding acids, which were then coupled to the amines by carbodiimide-based coupling reagents in the presence of HOBt to suppress racemization.[27] Conversion of the peptide acids into their corresponding peptide azides with diphenylphosphorazidate[28] did not achieve satisfactory yields, therefore, this was abandoned.

Cyclic peptide **12** was saponified with LiOH in THF/H_2O,[29] and gave peptide acid **23** in 81% yield after workup. The Fmoc group of cyclic peptide **15** was removed by treatment

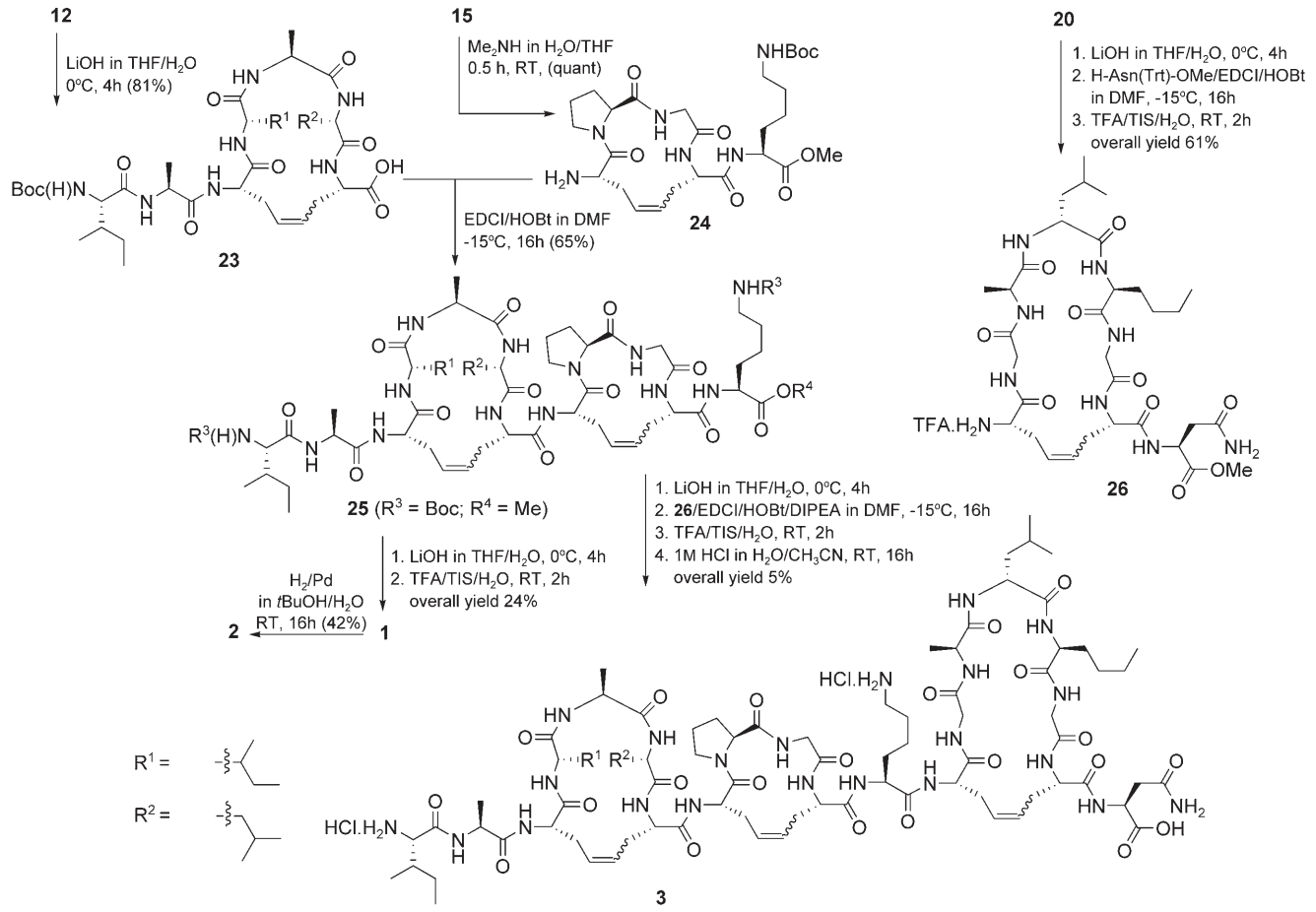


Scheme 6. Synthesis of the alkene-bridged B-ring mimic **15**.



Scheme 7. Synthesis of the alkene-bridged C-ring mimic **20**.

with aqueous dimethylamine, and **24** was obtained in a quantitative yield. The obtained alkene mimics of fragment A and B were coupled, overnight, by using EDCI/HOBt[30] as coupling reagents in DMF at −15 °C, and the bicyclic alkene mimic **25** of the AB fragment was obtained in 65% yield. An aliquot of **25** was fully deprotected and characterized with LC-MS/MS[31] and



Scheme 8. Assembly of the tricyclic alkene-bridged ABC-ring mimic **3** from the individual cyclic-ring fragments **12**, **15**, and **20**.

MALDI-TOF. The main portion of the AB fragment mimic **25** was saponified with LiOH in THF/H_2O to give the corresponding peptide acid after acidic workup. Due to its low solubility, this compound was used without further purification.

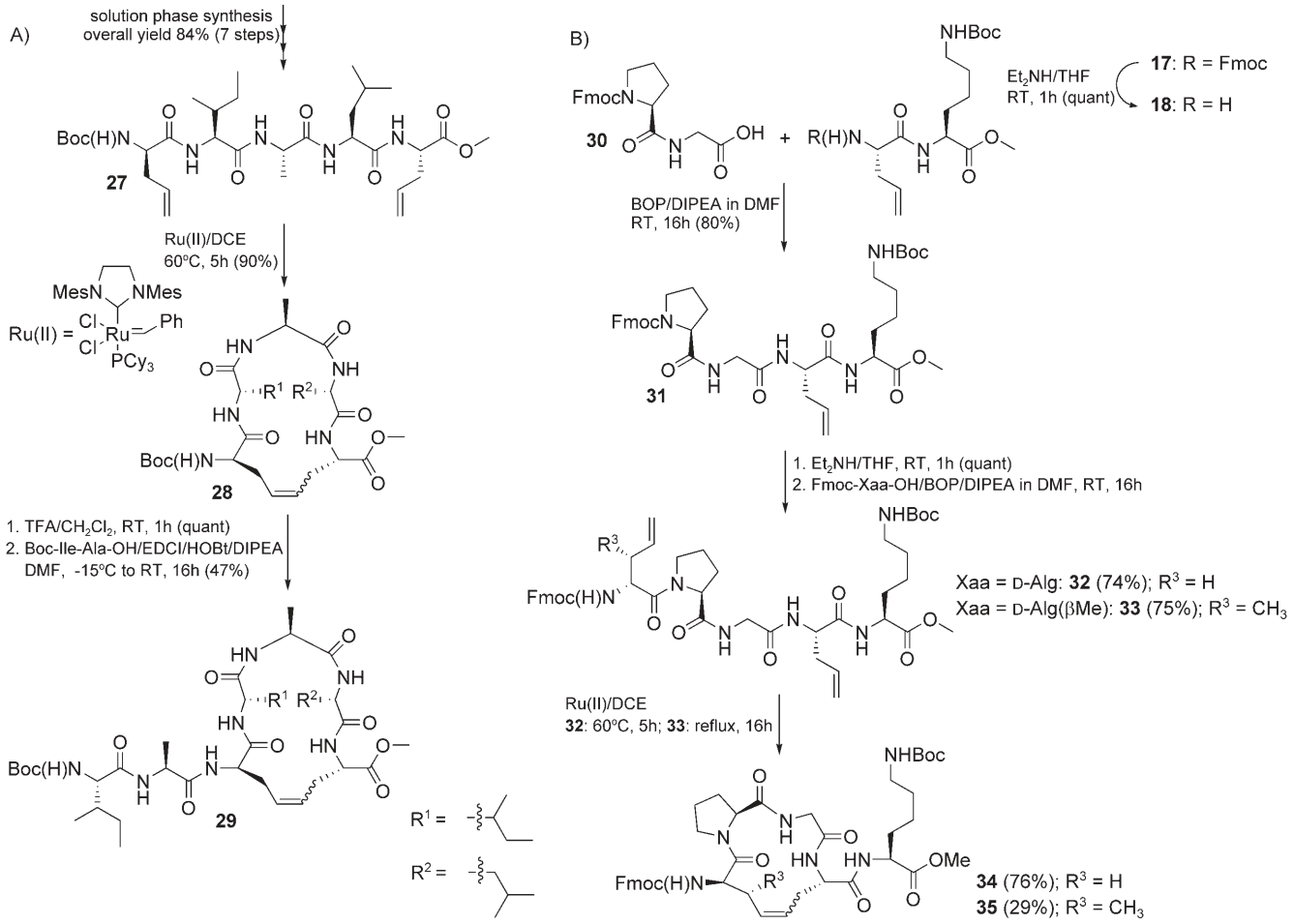
The protected AB fragment peptide acid was subjected to a second deprotection step in the presence of TFA and scavengers (TIS, H_2O) to remove both Boc groups, and the AB-ring mimic **1** was obtained. After purification by HPLC, **1** was obtained in 24% yield and characterized by mass spectrometry. A small portion was subsequently hydrogenated to give the alkane-bridged mimic **2** in 42% yield.

For final assembly of the AB(C) mimic, cyclic peptide **20** was saponified with LiOH in THF/H_2O. After workup and coupling to H-Asn(Trt)-OMe, as described above, the corresponding cyclic octapeptide was treated with TFA/TIS/H_2O to remove the Boc and Trt groups. Peptide ester **26** was obtained in 61% overall yield after trituration with MTBE/hexane.

Finally, the saponified AB-ring mimic **25** and C-ring mimic **26** were coupled by using EDCI/HOBt in DMF at −15 °C, overnight, and the product was subjected to saponification of the methyl ester. However, this also resulted in dehydration of the carboxamide functionality of the asparagine residue to the corresponding cyano derivative, as evidenced by MALDI-TOF analysis, which showed a loss of 18 amu.[32] To avoid dehydration, the crude reaction product was first treated with TFA/TIS/H_2O to remove the Boc functionalities, and then treated with 1 M HCl/CH_3CN to hydrolyze the methyl ester. This resulted in isolation of tricyclic peptide mimic **3** as its hydrochloride salt. After purification by HPLC and analysis by mass spectrometry (ES-MS, MALDI-TOF, and LC-MS/MS) the alkene-bridged mimic of the ABC-ring system **3** was obtained in 5% overall yield starting from **25** and **26**.

*Synthesis of AB fragment mimics **4**–**7***: The synthesis of **4**–**7** is shown in Schemes 9 and Scheme 10. Precursor peptide **27**, which has an N-terminal D-allylglycine residue, was synthesized in solution starting from HCl·H-Alg-OMe in seven steps in 84% overall yield (Scheme 9A). RCM of precursor **27** proceeded with increased efficiency compared to its all-L diastereomer **9** (90 vs. 76%). Cyclic peptide **28** was treated with TFA to remove the Boc functionality, and the amine was subsequently coupled to Boc-Ile-Ala-OH in the presence of EDCI/HOBt at −15 °C to minimize racemization of the alanine residue;[30] peptide **29** (fragment A) was obtained in 47% yield.

In the next step, the B fragments, which were represented by **34** (lanthionine mimic) and **35** (3-methyllanthionine mimic), were prepared by starting from the general precursor peptide **31** (Scheme 9B). Peptide **31** was synthesized by BOP-mediated coupling in 80% yield from dipeptides Fmoc-Pro-Gly-OH (**30**) and H-Alg-Lys(Boc)-OMe (**18**). The latter was obtained from **17** after treatment with diethylamine to remove the Fmoc group.



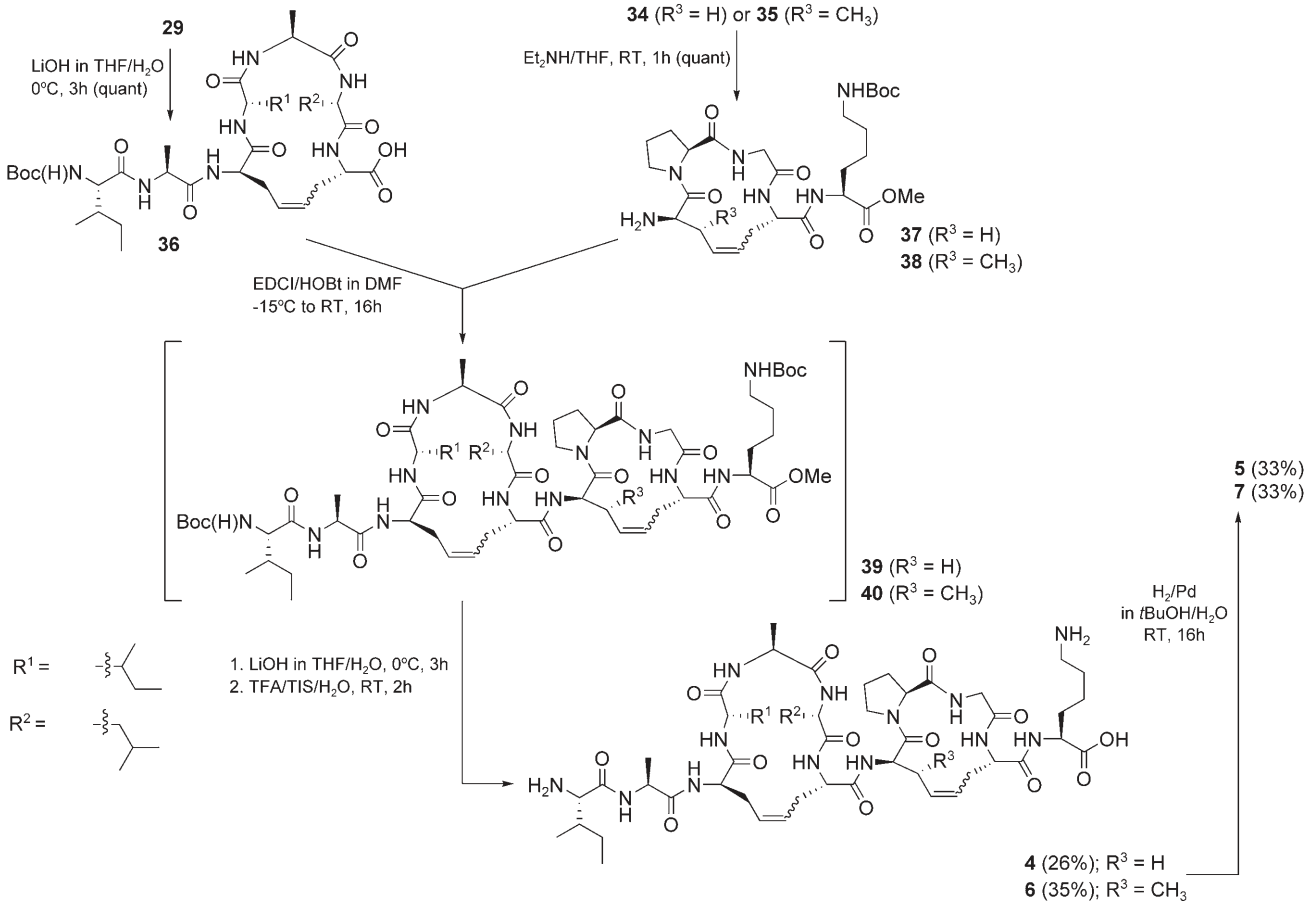
Scheme 9. Synthesis of the alkene-bridged AB-ring mimics with the stereochemistry of native nisin in the peptide backbone/side chain. A) Synthesis of fragment A; B) synthesis of fragment B.

Tetrapeptide **31** was also treated with Et_2NH to remove the Fmoc group, and the resulting amine was coupled to either Fmoc-D-Alg-OH or Fmoc-D-Alg(βMe)-OH[16] with BOP/DIPEA to obtain RCM-precursor peptides **32** and **33,** respectively, in 74 to 75% yield. Both linear peptides were subjected to RCM in the presence of second generation Grubbs' catalyst.[19] Peptide **32**, which contained D-allylglycine (D-Alg), cyclized efficiently (76%) to **34** in 5 h at 60 °C. However, peptide **33**, which contained D-Alg(βMe), was difficult to cyclize—after 16 h in refluxing DCE **35** was obtained in only 29% yield. However, it should be emphasized that the presence of the β-methyl substituent was probably responsible for significant steric hindrance, which hampered ring-closure. Moreover, there are very few reports that describe successful ring-closure in the presence of such sterically hindered alkenes.[33]

The assembly of **29** with **34** into the AB fragment mimic **4**, and **29** with **35** into mimic **6** is depicted in Scheme 10. Peptide ester **29** was saponified with LiOH in THF/H_2O, and peptide acid **36** was obtained in a quantitative yield. Fmoc-protected peptides **34** and **35** were treated with diethylamine to remove the Fmoc group. The resulting amines, **37** and **38**, respectively, were subsequently coupled to **36** by the EDCI/HOBt coupling method, as described above, to minimize racemization of the activated amino-acid residue. The protected bicyclic intermediates, **39** and **40**, respectively, were used without further purification in the subsequent synthesis steps. These steps firstly involved saponification of the methyl ester (LiOH in THF/H_2O), and secondly, treatment with TFA in the presence of TIS/H_2O as scavengers to remove both Boc-protecting groups. After purification by HPLC, peptides **4** and **6** were obtained in 26 and 35% yield, respectively. Both peptides were analyzed and characterized by using LC-ES-MS, MALDI-TOF, and LC-MS/MS. In separate experiments, AB fragment mimics **4** and **6** were reduced by Pd/H_2 to their corresponding alkane-bridged counterparts **5** and **7**, respectively, in yields of 33%.

Biochemical evaluation

The alkene- and alkane-bridged nisin AB(C)-fragment mimics **1**–**7** were tested for their lipid II binding affinities by evaluating their ability to inhibit nisin-induced carboxyfluorescein (CF) release from large unilamellar vesicles (LUVs). These vesicles contained DOPC and were loaded with lipid II (Figure 1).This assay was originally developed to investigate vancomycin and its derivatives for their affinity toward lipid II.[10a] Breukink and coworkers recently found that the mechanism of nisin-induced



Scheme 10. Assembly of bicyclic peptides **4** and **6** from their respective monocyclic fragments.

pore formation is initiated by binding of the N-terminal AB(C) fragment to lipid II. This enables the C-terminal DE fragment to interact with the phospholipid membrane and ultimately leads to pore formation and cell leakage.[34,35] In the present study, the lipid II binding affinities of mimics **1**–**7** were evaluated and compared with those of the native AB and ABC fragments **41** and **42**, respectively. The native AB and ABC fragments were obtained by trypsin and chymotrypsin digestion of nisin A, respectively, according to Chan et al.[36]

In a typical experiment, preincubation of CF-loaded DOPC–lipid II vesicles with AB(C) fragments **1**–**7**, **41**, and **42** should result in the occupation of nisin-binding sites of lipid II. Depending on the affinity of the synthetic AB(C) fragments toward lipid II, subsequent addition of full-length nisin will result in a reduced amount of pore formation and reduced fluorescence.

Nisin-induced pore formation was measured by the amount of CF release by using fluorescence spectroscopy, and was correlated to the maximum CF leakage, which was induced by the addition of Triton X-100. In a typical experiment, the synthetic AB(C) fragment was added at $t = 50$ s, and the fluorescence signal was measured at $t = 70$ s. (The fluorescence was monitored continuously; however, the fluorescence values at the given time points were used for the calculations.) Nisin was added at $t = 100$ s, and the increased fluorescence was measured at $t = 140$ s. Finally, Triton X-100 was added at $t = 150$ s, and the end level of the fluorescence was determined at $t = 190$ s. The leakage capacity was calculated by using the formula:

$$\%\,\text{CF leakage} = \frac{A_{t=140} - A_{t=70}}{A_{t=190} - A_{t=70}} \times 100$$

Compared to Triton X-100, the maximum nisin-induced leakage percentage was found to be 73%. The nisin-induced leakage percentages in the presence of the different AB(C) fragments were correlated to this maximum value (Table 1). The time courses of the fluorescence signals of nisin-induced CF-leakage in the presence of **1**, **5**, and **41** are given in Figure 1.

As expected, the natural AB(C) fragments (**41** and **42**) as well as the synthetic AB(C) fragment mimics (**1**–**7**) were not able to form pores in the phospholipid membrane since the C-terminal DE fragment of nisin, which is necessary for leakage, was absent. In the presence of these AB(C) fragment mimics, nisin-induced carboxyfluorescein release was reduced; this indicated that these compounds were capable of competing with the nisin ABC-ring system for the pyrophosphate binding site. The natural AB fragment **41** and the AB(C) fragment **42** were found to be effective inhibitors of nisin-induced CF-leak-

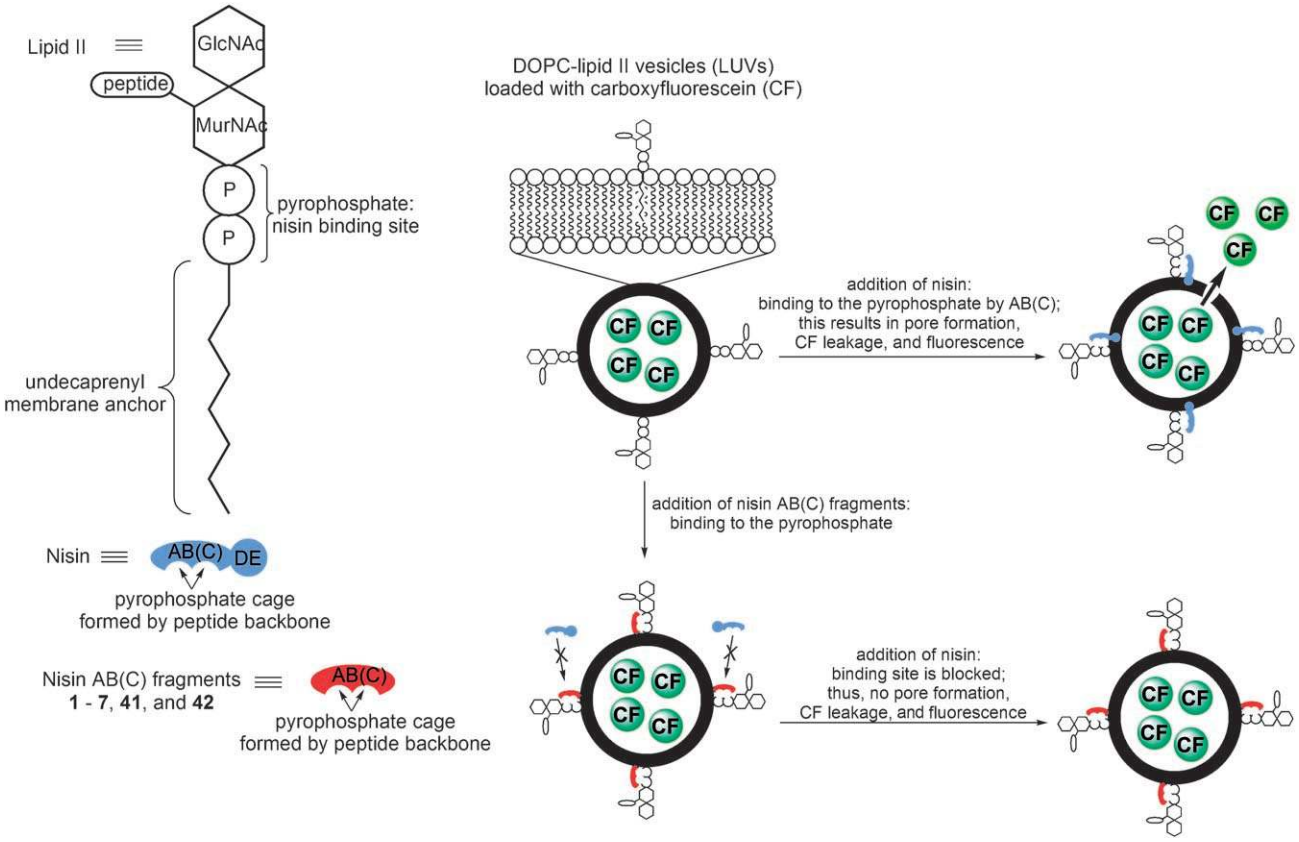


Figure 1. Schematic representation of the nisin-induced carboxyfluorescein (CF) release assay. Adapted from ref. [10a]; lipid II is shown only at the outer monolayer for clarity.

Table 1. Nisin-induced CF-leakage percentages in the presence of the AB(C) fragments.

	Compound	Conc. [μM]	Nisin-induced CF leakage [%]	Inhibition of CF leakage [%]
1	**1**	50	96	4
2	**1**	100	77	23
3	**2**	50	100	0
4	**2**	100	86	14
5	**2**	200	66	34
6	**3**	50	79	21
7	**3**	100	64	36
8	**4**	50	90	10
9	**4**	100	92	8
10	**4**	200	86	14
11	**4**	400	27	73
12	**5**	25	100	0
13	**5**	50	78	22
14	**5**	100	56	44
15	**6**	50	99	1
16	**6**	100	100	0
17	**6**	200	96	4
18	**7**	50	100	0
19	**7**	200	100	0
20	**41**	10	86	14
21	**41**	20	63	37
22	**41**	28	41	59
23	**41**	40	18	72
24	**42**	0.2	100	0
25	**42**	1	93	7
26	**42**	2.5	52	48
27	**42**	25	6	93

age, and were active in the low μM range (Table 1 and Figure 2).

Generally, the alkene- and alkane-bridged nisin AB(C)-mimics were found to be less active than their natural counterparts. However, subtle variations in activity were observed. The alkane-bridged derivatives showed at least a comparable activity, but were generally more active than their alkene-bridged congeners (compare entries 9 and 14 in Table 1). Also, the alkene-bridge ABC-ring fragment **3** was more active than the AB-ring mimic **1** (compare entries 2 and 7). Moreover, a clear improvement of lipid II-binding affinity was observed with these mimics when there was an identical stereochemistry to the native nisin fragments (compare entries 4 and 14). Remarkably, compounds **6** and **7**, which had an even closer resemblance to nisin, and had the 3-methyllanthionine mimic, were completely inactive in this assay. The highest affinity towards lipid II was found with the alkane-bridged compound **5**. These binding data imply that an alkane moiety is a suitable sulfide-bridge (thioether) mimic, and that compounds with the same backbone stereochemistry as nisin are considerably more active.

Computational modeling

Modeling studies on **1**, **2**, **4**–**7**, and **41** were performed with MacroModel[37] in order to find the energy and structure of the global minimum. To obtain the global minimum, conforma-

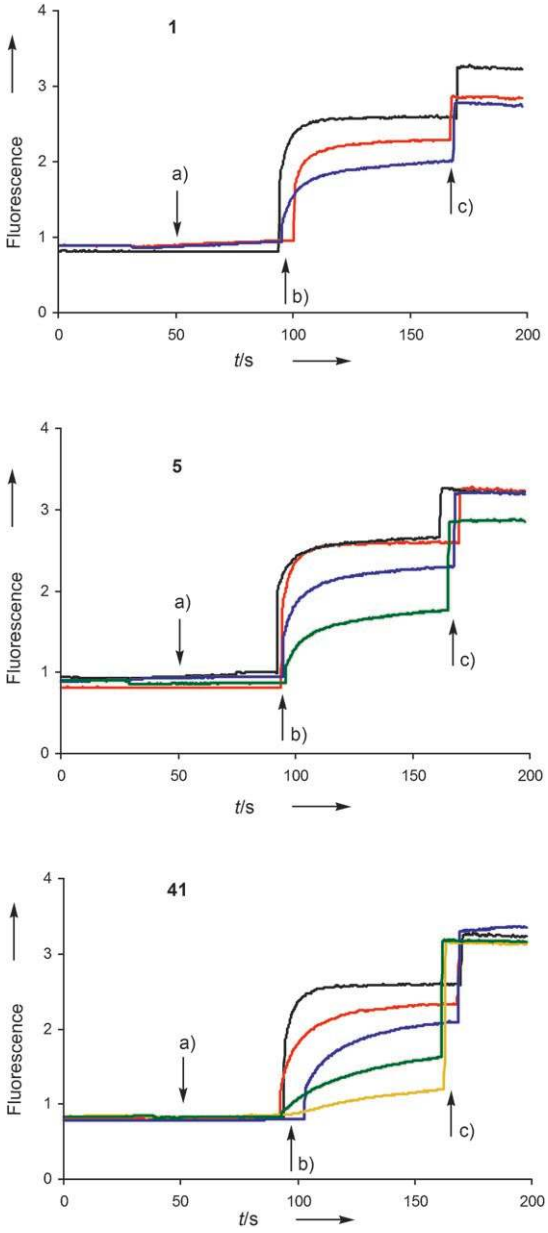


Figure 2. Time course of nisin-induced leakage of carboxyfluorescein (CF) from DOPC vesicles with lipid II (black line). After ~100 s (b), full-length nisin was added and CF release was monitored. The 100% leakage level was determined by addition of Triton X-100 after ~180 s (c). At t~50 s (a), a nisin AB(C) fragment (**1**, **5**, or **41**) was added as potential lipid II binder; this resulted in reduced CF efflux and thus lower fluorescence signals. The red, blue, green, and yellow traces indicate the increasing concentration of the AB(C) fragments: **1** (50 and 100 μM, respectively); **5** (25, 50, and 100 μM, respectively); **41** (10, 20, 28, and 40 μM, respectively).

tional searches were performed with H_2O as solvent since the binding studies were performed in aqueous solution. From each structure the molecular mechanics energy (kJ mol^{-1}) of the global minimum was calculated with *E*,*E*, *E*,*Z*, *Z*,*E*, and *Z*,*Z* geometries of the double bond (Table 2).

For compound **1** (all-L stereochemistry of the peptide backbone) the order of molecular mechanics energy value was: $Z,E \ll Z,Z \approx E,Z \ll E,E$. In the case of **4**, which had the same backbone stereochemistry as nisin, the order was found to be: $Z,Z \ll E,Z \approx E,E < Z,E$. However, the absolute value of the molecular mechanics energy was significantly lower for **1** than for **4**. This is in contrast to the alkane series for which the energy of **5** (derived from alkene **4**) was found to be much lower than **2** (derived from **1**).

Table 2. Modeling studies of the alkene- and alkane-bridged AB-ring mimics of nisin by using MacroModel.

Compound[a]	Double-bond geometry	Energy [kJ mol^{-1}]	RMS [Å][b]
1	*E*,*E*	286.6	–
1	*E*,*Z*	279.4	–
1	*Z*,*E*	268.1	2.24
1	*Z*,*Z*	278.9	–
2	–	153.7	1.11
4	*E*,*E*	316.1	–
4	*E*,*Z*	315.2	–
4	*Z*,*E*	318.6	–
4	*Z*,*Z*	308.1	1.64
5	–	130.4	1.73
6	*E*,*E*	332.6	1.26
6	*E*,*Z*	332.0	–
6	*Z*,*E*	339.8	–
6	*Z*,*Z*	337.9	–
7	–	157.8	1.54
nisin AB (**41**)	–	372.5	–

[a] *N*-acetylated and NHMe-amidated bicyclic compounds were used in the modeling experiments. [b] RMS values were calculated based on the superimposition of carbon atoms αC1, αC5, αC6, and αC9 onto **1**, **2**, **4**, **5**, **6**, **7**, and native nisin AB (**41**).

Superimposed images of the lowest energy conformers of **2** or **5** and native nisin AB (**41**) are shown in Figure 3. The root mean square (RMS) values were calculated based on the superimposition of carbon atoms αC1, αC5, αC6, and αC9 in these derivatives with native nisin AB (Table 2). As can be seen from Figure 3 the superimposition of the A ring is quite good compared to the B ring, which can be explained by the fact that each ring fragment can rotate independently. In this case, the lowest RMS value (1.1 Å) was observed with the dialkane-bridged compound **2**. Based on these modeling data, it was not possible to draw any conclusions, or to observe a correlation with respect to the measured lipid II binding affinities.

Conclusions

A series of alkene- and alkane-bridged AB(C)-ring fragments of the lantibiotic nisin were synthesized by ring-closing metathesis, to mimic the natural thioether covalent constraint. These compounds were tested for their binding affinities toward lipid II, and depending on the stereochemistry of the peptide backbone, were found to be active, although with a reduced affinity compared to their natural counterparts. The most active compound, **5**, resembled the native backbone stereochemistry, but the thioether was mimicked by an alkane bridge. These compounds comprise a first generation lead set for the design of novel peptide-based antibiotics based on nisin.

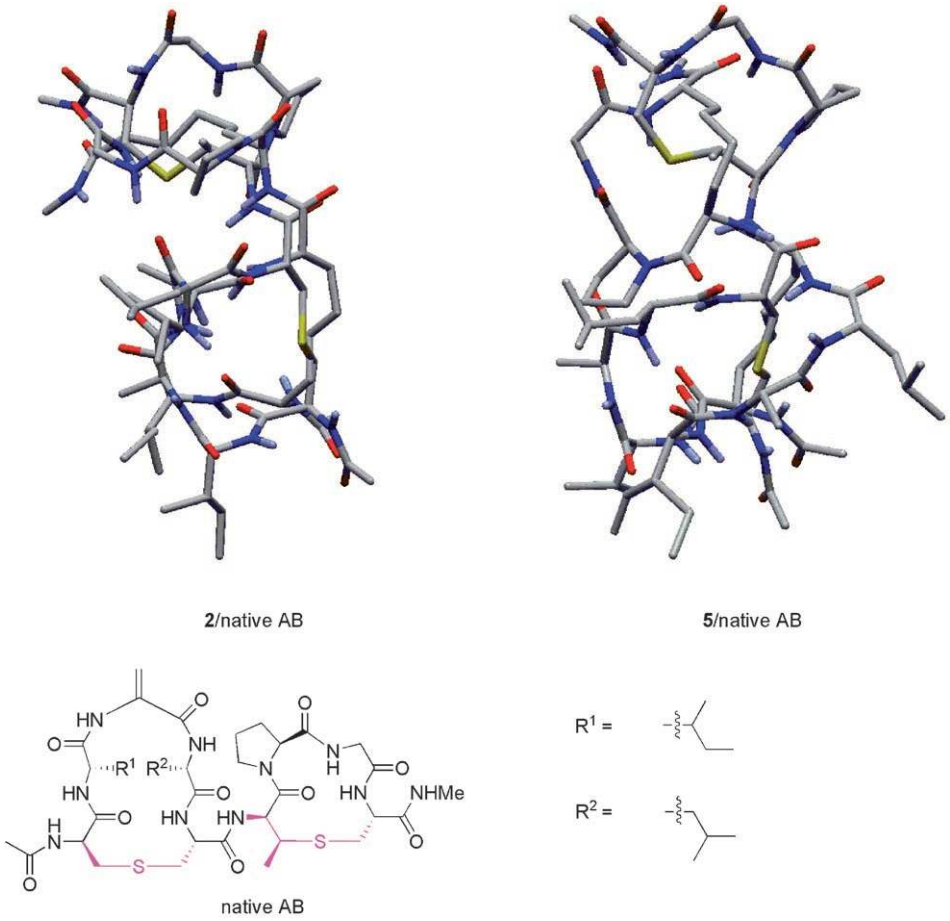


Figure 3. Superimposed images of the lowest energy conformations of **2** and **5** with native nisin AB fragment.

Experimental Section

General: Analytical HPLC was performed by using a Shimadzu automated HPLC system equipped with an evaporative light scattering detector (PL-ELS 1000, Polymer Laboratories) and a UV/Vis detector operated at 220/254 nm. Preparative HPLC was performed by using a Gilson HPLC workstation. Liquid chromatography electrospray ionization mass spectrometry (ES-MS) was measured by using a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer, which was operated in the positive-ionization mode. MALDI-TOF analysis was performed by using a Kratos Axima CFR apparatus, with bradykinin(1–7) as an external reference and α-cyano-4-hydroxycinnamic acid as matrix. MS/MS spectra were analyzed by using a Micromass Quattro Ultima or a Micromass Q-TOF mass spectrometer. ^{1}H NMR spectra were recorded by using a Varian G-300 (300 MHz) spectrometer or a Varian INOVA-500 spectrometer (500 MHz); chemical shifts (δ) were obtained in ppm relative to TMS. ^{13}C NMR spectra were recorded on a Varian G-300 spectrometer (75.5 MHz) and the chemical shifts were obtained in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded by using the attached proton test (APT) sequence. Retention factor (R_f) values were determined with thin-layer chromatography (TLC) by using Merck precoated silica gel 60F_{254} plates. Spots were visualized by UV-quenching, ninhydrin, or Cl_2/*N*,*N*,*N*′,*N*′-tetramethyl-4,4′-diaminodiphenylmethane (TDM).[38] The numbering of the amino acids listed in the NMR characterizations are given from the N to C terminus. Where appropriate, this numbering is indicated as superscripts in the compound name for clarity.

Chemicals and reagents: ArgoGel™ resin with a free hydroxyl moiety was used in all synthesis procedures. The coupling reagents 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[39] and benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP),[40] *N*-hydroxybenzotriazole (HOBt), and N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids were obtained from GL Biochem Ltd. (Shanghai, China). The Fmoc-protected L- and D-allylglycine (Alg) were purchased from Neosystem Laboratoire (Strasbourg, France). Peptide grade 1,2-dichloroethane (DCE), dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), *tert*-butyl methylether (MTBE), trifluoroacetic acid (TFA), and HPLC grade acetronitrile were purchased from Biosolve (Valkenswaard, The Netherlands). Piperidine, *N*,*N*-diisopropylethylamine (DIPEA) and 4-dimethylaminopyridine (DMAP) were obtained from Acros Organics (Geel, Belgium). Triisopropylsilane (TIS) and HPLC grade TFA were obtained from Merck. *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDCI) and second generation Grubbs' catalyst (Ru^{II}) were obtained from Aldrich. Nisin A, trypsin, and chymotrypsin were obtained from Sigma.

Solid-phase peptide synthesis: Peptides were synthesized manually at the 0.25 mmol scale on Argogel resin. Each synthetic cycle consisted of N^{α}-Fmoc removal by treatment with piperidine (20%) in DMF (3×10 mL, 8 min), a wash step (DMF: 3×10 mL, 2 min; DCM: 3×10 mL, 2 min; and DMF: 3×10 mL, 2 min), a coupling step (60 min, 1.0 mmol of preactivated Fmoc amino acid in the presence of 2 equiv DIPEA in 10 mL DMF), and a final wash step (DMF: 3×10 mL, 2 min; DCM: 3×10 mL, 2 min; and DMF: 3×10 mL, 2 min). N^{α}-Fmoc amino acids (1 mmol) were activated in situ with BOP (1 mmol) in the presence of DIPEA (2 mmol). Fmoc-removal and -coupling reactions were monitored by using the Kaiser test.[41] Peptides were cleaved from the resin by treatment with a catalytic amount of KCN in MeOH (15 mL) for 16 h. The resin was filtered and washed with MeOH (3×10 mL), and the filtrate was concentrated in vacuo to yield the crude peptide.

Solution-phase peptide synthesis

Coupling reaction: The carboxylic acid moiety (1 equiv) was coupled to the amine derivative (or its TFA salt, 1 equiv) in the presence of BOP (1 equiv) and DIPEA (2 equiv; 3 equiv when the amine was protonated) as coupling reagents in DCM (10 mL mmol^{-1}) as solvent (16 h, room temperature). After completion of the reaction, DCM was removed under reduced pressure, and the residue was dissolved in EtOAc (25 mL mmol^{-1}). The solution was washed with $KHSO_4$ (1 N, 3×25 mL), 10% Na_2CO_3 (3×25 mL), and brine (1×25 mL), then dried (Na_2SO_4), filtrated, and evaporated in vacuo. The

obtained crude product was analyzed by TLC, ^{1}H NMR spectroscopy, and ESI-MS, and in general proved pure enough to be used in subsequent synthesis steps.

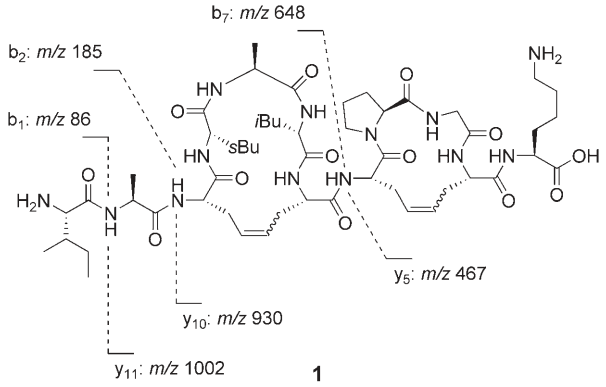
Boc removal: A Boc-protected intermediate was dissolved in TFA/DCM (1:1, *v/v*; 4 mL mmol^{-1}) and stirred at room temperature for 1 h. Then the solvents were removed under reduced pressure and the residue was coevaporated with toluene (2×25 mL), CH_3CN (2×25 mL), and DCM (2×25 mL) to remove residual TFA. The obtained TFA salt was used without further purification in the next synthesis step.

Peptide purification: The crude lyophilized peptides (30–60 mg) were dissolved in a minimum amount of TFA (0.1%) in CH_3CN/H_2O (8:2, *v/v*) and loaded onto an Adsorbosphere XL C8 HPLC column (90 Å pore size, 10 μm particle size, 2.2×25 cm). The peptides were eluted at a 10.0 mL min^{-1} over 60 min by using a linear gradient from 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*). The peptide purity and retention time (t_R) values were evaluated with analytical HPLC by using an Adsorbosphere XL C8 column (90 Å pore size, 5 μm particle size, 0.46×25 cm) at 1 mL min^{-1} over 30 min by using a linear gradient of buffers A and B as above.

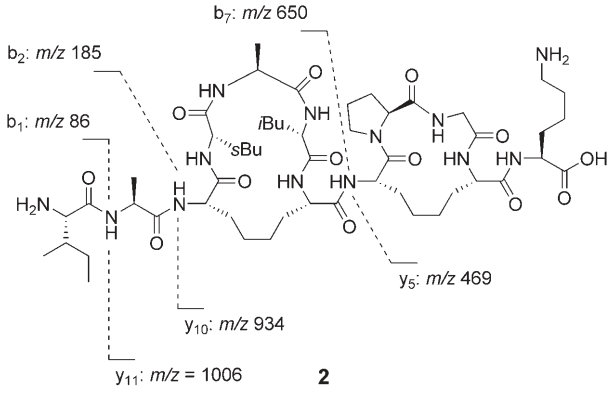
Peptide characterization: Peptides were characterized by mass spectrometry and ^{1}H NMR spectroscopy (300 or 500 MHz). The mass of each analogue was measured, and the observed monoisotopic $[M+H]^+$ values were correlated with the calculated $[M+H]^+$ values by using MacBioSpec (Perkin–Elmer Sciex Instruments, Thornhill, Ontario, Canada). Peak assignments were based on ^{1}H NMR COSY, TOCSY, and/or ROESY spectra.

Synthesis: Experimental details of all synthetic procedures and characterization data of all intermediate compounds are described in the Supporting Information.

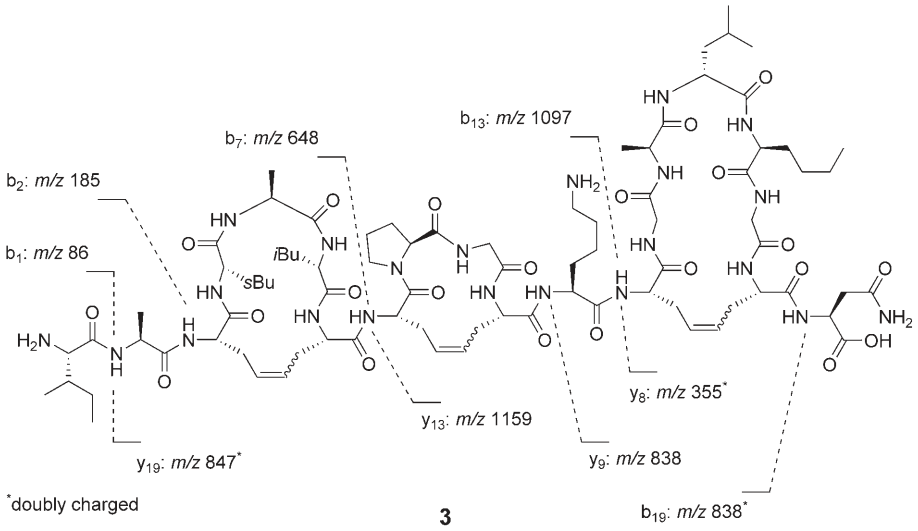
*2 TFA·H-Ile^1-Ala^2-cyclo[Alg^3-Ile^4-Ala^5-Leu^6-Alg^7]-cyclo[Alg^8-Pro^9-Gly^{10}-Alg^{11}]-Lys^{12}-OH (**1**)*: t_R: 14.2 min; ES-MS: calcd for $C_{53}H_{88}N_{13}O_{13}$: 1114.70, found: *m/z* $[M+H]^+$ 1114.75; MALDI-TOF: $[M+H]^+$ 1114.97, $[M+Na]^+$ 1136.92; MS/MS analysis:



*Bicyclic alkane-bridged compound **2***: t_R: 14.7 min; ES-MS: calcd for $C_{53}H_{92}N_{13}O_{13}$: 1118.70, found: *m/z* $[M+H]^+$ 1118.8, $[M+2H]^{2+}$ 560.1; MALDI-TOF: $[M+H]^+$ 1119.0, $[M+Na]^+$ 1141.0; MS/MS analysis:



*2 HCl·H-Ile^1-Ala^2-cyclo[Alg^3-Ile^4-Ala^5-Leu^6-Alg^7]-cyclo[Alg^8-Pro^9-Gly^{10}-Alg^{11}]-Lys^{12}-cyclo[Alg^{13}-Gly^{14}-Ala^{15}-d-Leu^{16}-Nle^{17}-Gly^{18}-Alg^{19}]-Asn^{20}-OH (**3**)*: t_R: 14.7 min; ES-MS: calcd for $C_{84}H_{137}N_{22}O_{22}$: 1807.1, found: *m/z* MALDI-TOF: $[M+H]^+$ 1807.13, $[M+Na]^+$ 1829.1; MS/MS analysis:

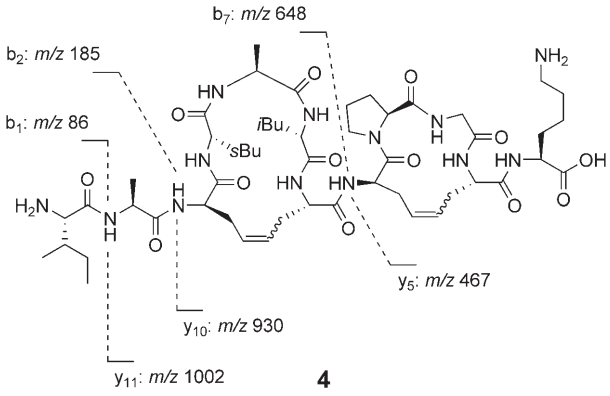


*2 TFA·H-Ile^1-Ala^2-cyclo[D-Alg^3-Ile^4-Ala^5-Leu^6-Alg^7]-cyclo[D-Alg^8-Pro^9-Gly^{10}-Alg^{11}]-Lys^{12}-OH (**4**)*: t_R: 14.2 min; ES-MS: calcd for $C_{53}H_{88}N_{13}O_{13}$: 1114.70, found: *m/z* $[M+H]^+$ 1115.25, $[M+2H]^{2+}$ 558.33; MALDI-TOF: $[M+H]^+$ 1114.65, $[M+Na]^+$ 1136.58; MS/MS analysis:

*Bicyclic alkane-bridged compound **5***: t_R: 16.2 min; ES-MS: calcd for $C_{53}H_{92}N_{13}O_{13}$: 1118.70, found: *m/z* $[M+H]^+$ 1118.90, $[M+2H]^{2+}$ 560.40; MALDI-TOF: $[M+H]^+$ 1118.6, $[M+Na]^+$ 1140.5; MS/MS analysis:

*2 TFA·H-Ile^1-Ala^2-cyclo[D-Alg^3-Ile^4-Ala^5-Leu^6-Alg^7]-cyclo[D-Alg(βMe)^8-Pro^9-Gly^{10}-Alg^{11}]-Lys^{12}-OH (**6**)*: t_R: 17.8 min; ES-MS: calcd for $C_{54}H_{90}N_{13}O_{13}$: 1128.67, found: *m/z* $[M+H]^+$ 1128.95, $[M+2H]^{2+}$ 565.35; MALDI-TOF: $[M+H]^+$ 1128.89, $[M+Na]^+$ 1150.85; $[M+K]^+$ 1166.81; MS/MS analysis:

*Bicyclic alkane-bridged compound **7***: t_R: 12.8 min; ES-MS: calcd for $C_{54}H_{94}N_{13}O_{13}$: 1132.67, found: *m/z* $[M+H]^+$ 1132.70, $[M+2H]^{2+}$



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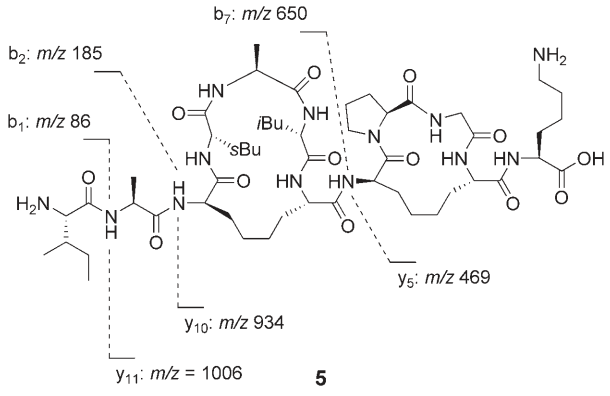

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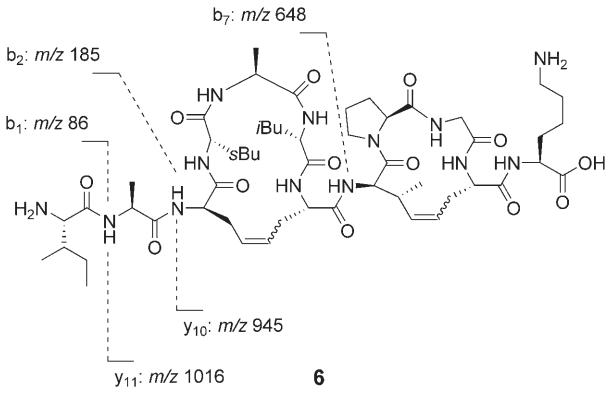

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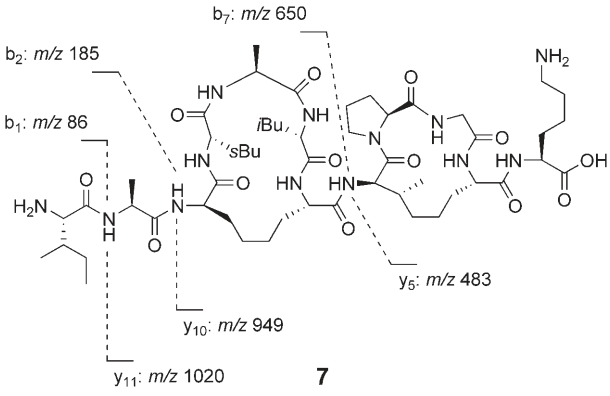
567.5; MALDI-TOF: $[M+H]^+$ 1132.80, $[M+Na]^+$ 1154.70; $[M+K]^+$ 1170.70; MS/MS analysis:



7

Biological evaluation: The method described by Wiedemann et al. was used.[10a] Briefly, large unilamellar vesicles (LUVs) for carboxyfluorescein (CF) efflux were prepared and treated as described by Breukink et al.[3a] Vesicles were made from 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) which contained lipid II (0.1%). CF-loaded vesicles were prepared with CF (50 mm) and then diluted in K^+ buffer (1.5 mL, containing 25 mM 4-morpholineethanesulfonic acid (MES)-KOH, pH 6.0, 75 mM K_2SO_4) at a final phospholipid concentration of 25 μM. After the addition of nisin, induced leakage was monitored for 3 min. The increase of fluorescence was measured at 515 nm (excitation at 492 nm) by using a SPF 500C spectrophotometer (SLM Instruments Inc.) at 20 °C. The nisin-induced CF leakage was expressed relative to the total amount of CF released after lysis of the vesicles by addition of Triton X-100 (20%, 10 μL).

Lipid II-binding affinity of the different AB(C) fragments **1**–**7**, **41**, and **42** was assayed by measuring the reduced induction of CF leakage in response to nisin (8 nm). The compounds were preincubated with CF-loaded vesicles for 50 s. None of the tested compounds induced CF leakage during this preincubation period. Then nisin was added, and an immediate increase in absorption was observed in cases where the AB(C) fragment did not bind lipid II. A reduced fluorescence signal as function of concentration was observed in cases where the AB(C) fragments showed an affinity toward lipid II. Finally, treatment with Triton X-100 induced total CF release; this value was used to calculate the relative potency of the AB(C) fragments to binding lipid II.

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Keywords: antibiotics · bioorganic chemistry · medicinal chemistry · membrane–peptide interactions · ring-closing metathesis

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