Pergamon

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Bioorganic & Medicinal Chemistry 11 (2003) 2835–2841

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Biological Evaluation of Novel Turn-Modified Gramicidin S Analogues

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Received 3 January 2003; accepted 25 March 2003

Abstract—The synthesis of novel gramicidin S analogues having additional functionalities in the turn region by employing a biomimetic approach is described. The preservation of β-sheet character in all analogues was established by NMR and the biological activity was evaluated.

Introduction

The cationic antimicrobial peptide gramicidin S (GS, **1**, Fig. 1), isolated from *Bacillus brevis*,[1] is active against a wide range of bacteria and fungi.[2,3] The accumulation of GS in cytoplasmic membranes leads to loss of lipid bilayer integrity and increased cell permeability, ultimately resulting in bacterial cell death. GS resides in the polar–apolar interfacial regions of lipid bilayers.[4] Presumably, electrostatic and hydrophobic peptide–membrane interactions are predominantly responsible for antibacterial activity.

GS is a C_2-symmetric cyclic decamer that adopts a rigid β-sheet structure,[5–7] in which the Val-, Orn- and Leu-residues align to form the antiparallel β-strands and Pro-DPhe induce the type II' β-turns.[8] The side chains of the hydrophilic and hydrophobic residues are positioned on opposite faces of the molecule, thus creating the amphiphilic arrangement that is thought to be at the basis of the biological properties of GS.

There is a wide interest in the generation of new GS analogues with improved antimicrobial activities. In this respect, a plethora of GS derivatives, in which the β-strand region has been modified, has been described over the last decades.[2,9] These examples include modulation of the amino acid composition as well as enlarging the β-strand region (i.e., the synthesis of GS homologues).[10,11] Perusal of the latter studies revealed a number of factors influencing the bactericidal efficiency of β-strand modified GS analogues such as amphiphilicity, hydrophobicity, nature and number of cationic residues, backbone size and conformation. However, the wealth of information gathered has, to date, not resulted in the generation of a synthetic, clinically applicable antibiotic based on GS.

Modulation of the turn regions of GS is a relatively unexplored area of research. It is likely that substitution of the turn region amino acids, especially the proline residue, in most cases will lead to loss of β-sheet character and concomitant loss of antimicrobial activity.[3] We envisaged that the development of a strategy that allows the introduction of additional functionalities to the β-turn region of GS, without interfering with its intrinsic β-sheet character, would provide a potential entrance towards new GS-based antibiotics. Here, we report our results on the generation of turn-modified GS analogues, in which the two DPhe residues are replaced by benzylated D-tyrosine (i.e., DTyr(Bn), peptide **3**, Fig. 1), as well as derivatives where Pro is substituted for either 2*S*,4*R*-azidoproline (*R*-Azp, peptide **4**) or 2*S*,4*S*-azidoproline (*S*-Azp, peptide **5**). Furthermore, the transformation of the azide residues into secondary amines (**6** and **7**, having additional cationic

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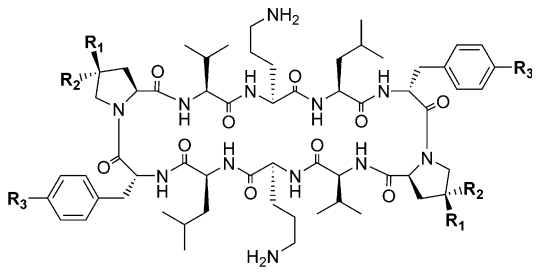
0968-0896/03/$ - see front matter
doi:10.1016/S0968-0896(03)00219-0



	R_1	R_2	R_3
1	H	H	H
2	H	H	OH
3	H	H	OBn
4	H	N_3	H
5	N_3	H	H
6	H	NH_2	H
7	NH_2	H	H
8	H	NH-Z	H
9	NH-Z	H	H
10	H	NH-CO$(CH_2)_2$COOH	H
11	NH-CO$(CH_2)_2$COOH	H	H

Figure 1. Gramicidin S and envisaged analogues.

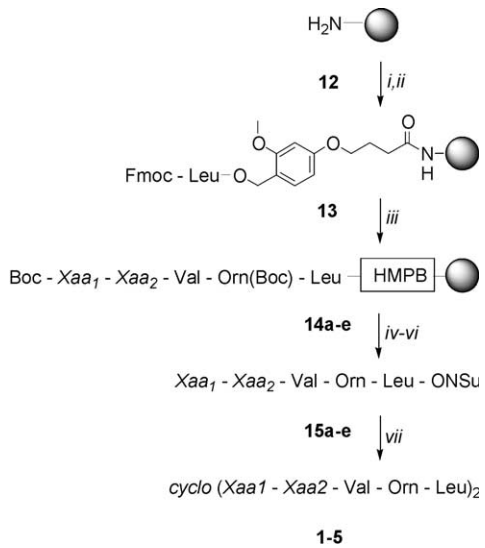
functionality in the turn region), benzyloxycarbamates (**8** and **9**, with bulky hydrophobic residues) and succinylamides (**10** and **11**, featuring carboxylic acid turn region elements) is presented. The secondary structure as well as the capacity to arrest proliferation of various Gram-positive and -negative bacterial strains of GS analogues **3**–**11** were compared with GS and the known GS analogue **2**.

Results and Discussion

Chemistry

Several different synthetic strategies towards GS and its analogues have been reported in literature.[12] Tamaki and coworkers followed a biomimetic approach in which the specific pentameric sequence H_2N-^{D}Phe-Pro-Val-Orn-Leu-ONSu was found to cyclodimerize and produce GS. The ability of this particular sequence to form GS was attributed to a preorganization of the activated decameric linear peptide forming a β-hairpin structure.[13] Importantly, the replacement of specific amino acid residues in the synthetic sequence (e.g., ^{D}Phe(4-Br) instead of ^{D}Phe) was shown not to interfere with the dimerization-cyclization reaction.[14] We therefore chose to study the efficiency of this biomimetic synthesis for the construction of GS analogues **2**–**5**.

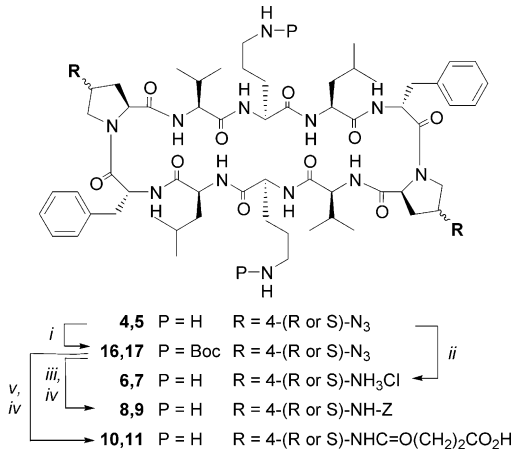
Commercially available 4-methylbenzhydrylamine (MBHA) resin (**12**, Scheme 1) was equipped with the acid-labile 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker[15] under the agency of Castro's reagent[16] and *N*,*N*′-diisopropylethylamine (DiPEA) and condensed with Fmoc-Leu-OH using *N*,*N*′-diisopropylcarbodiimide (DIC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) to give the functionalized resin **13**. The immobilized pentapeptide sequences **14a**–**e** were synthesized via standard peptide chemistry, employing, next to standard amino acid building blocks, the readily available Fmoc-2*S*,4*R*-azidoproline (in **4**) and Fmoc-2*S*,4*S*-azidoproline (in **5**), respectively.[17–19] After mild cleavage (1% TFA/DCM) from the solid



Scheme 1. Synthesis of GS derivatives; **a** Xaa_1 = ^{D}Phe, Xaa_2 = Pro; **b** Xaa_1 = ^{D}Tyr, Xaa_2 = Pro; **c** Xaa_1 = ^{D}Tyr(Bn), Xaa_2 = Pro; **d** Xaa_1 = ^{D}Phe, Xaa_2 = R-Azp; **e** Xaa_1 = ^{D}Phe, Xaa_2 = S-Azp; (i) HMPB, BOP, DiPEA, NMP; (ii) Fmoc-Leu-OH, DIC, DMAP (5 mol%), DCM (iii) Repetitive deprotect: 20% piperidine in NMP, condense; Fmoc-aa-OH or Boc-Xaa_1-OH, BOP, HOBt, DiPEA, NMP; (iv) 1% TFA in DCM; (v) HONSu, EDC, DCM; (vi) 50% TFA in DCM; (vii) **15a–e** in DMF, add to pyridine.

support the Boc-protected pentamers were condensed with HOSu under the agency of EDC to give their respective *N*-succinimic esters. After removal of the Boc-protective groups (TFA/DCM, 1/1, v/v) the activated pentapeptides **14a**–**e** were subjected to cyclodimerization by slow addition to a pyridine solution up to a final concentration of 3×10^{-3} M at 25 °C.[13] The resulting cyclic decamers were identified using LC/MS and purified by semi-preparative HPLC to give GS and **2**–**5** in yields of around 5%, based on the initial loading of resin **13**. The crude cyclodimerization mixture contained, besides the expected products (i.e., the cyclic monomer and dimer described by Tamaki et al.), several other unidentified fragments that displayed an identical ESI-MS profile. Apparently, the formation of cyclization products involving the ornithine side chain can also occur once the linear decameric active ester is formed. Notwithstanding the formation of these undesired side products, the biomimetic synthetic sequence provides an easy and rapid access to the construction of C_2-symmetric GS analogues **2**–**5**.

As the next research objective, we set out to functionalize *R*/*S*-Azp containing GS analogues **4** and **5**. Treatment of azides **4** and **5** (Scheme 2) with 10% Pd/C under hydrogen atmosphere in the presence of $CHCl_3$ directly furnished the positively charged aminoproline (Amp) derivatives **6** and **7** in a respective yield of 58 and 60%, after HPLC purification. Alternatively, protection of the ornithine side chains (Boc_2O, **4**–**16** and **5**–**17**, 89 and 86%, respectively) allowed the selective modification of the azidoproline derivatives, as follows. Staudinger reduction[20] of the azides in **16** and **17**, followed by condensation of the resulting secondary amines with either benzyl chloroformate or succinic anhydride, and final acidic removal of the Boc protective groups afforded



Scheme 2. Reaction conditions: (i) Boc_2O, DiPEA, MeCN; (ii) 10% Pd/C, H_2, $CHCl_3$/MeOH (1/1 v/v); (iii) (a) PMe_3, 1,4-dioxane/MeCN/H_2O (20/20/1 v/v/v); (b) Z-Cl, DiPEA, DMF; (vi) 50% TFA in DCM; (v) (a) PMe_3, 1,4-dioxane/MeCN/H_2O (20/20/1 v/v/v); (b) $(CH_2CO)_2O$, TEA, DMF.

target compounds **8**–**11** in good yields (**8**: 42%, **9**: 36%, **10**: 78%, **11**: 66%, respectively, after HPLC purification).

Having all desired cyclic peptides in hand, attention was focused on their structural evaluation by NMR. The resonance assignment of compounds **1**–**11** was unambiguously accomplished using two-dimensional NMR experiments (i.e., COSY, TOCSY). Several methods for the interpretation of the acquired ^{1}H NMR spectra can be applied to establish secondary structure elements in peptides.

The presence of the ^{D}Phe and ^{D}Tyr residues in the turn regions was indicated by the small vicinal spin-spin coupling constants ($^3J_{HN\alpha} < 4$ Hz), as was postulated by Ramachandran et al.[21] As can be seen by tabulating the coupling constants for peptides **1**–**11** (Fig. 2) the $^3J_{HN\alpha}$ of the Leu, Orn and Val (ranging between 8.5 and 9.0 Hz) corresponds to a β-sheet structure.[22] The value of the coupling constants for all residues are largely comparable with the corresponding values in GS.

The perturbation of chemical shift is defined by Wishart and co-workers[23] as the difference between the measured chemical shift for the H_α of an amino acid and the H_α chemical shift value of the same residue reported for a random coil peptide. The presence of three or more consecutive residues having $\Delta\delta H_\alpha > 0.1$ ppm signifies an extended β-strand conformation. As can been seen from the data displayed in Figure 3, the α-protons of the Leu-Orn-Val sequence of all presented peptides (i.e., **1**–**11**) clearly show idiosyncratic secondary chemical shifts.[24] The ^{D}Phe and ^{D}Tyr residues location (i.e., in the turn region) is illustrated by a negative value in these peptides. In summary, the vicinal coupling constants and perturbation of chemical shift of compounds **2**–**11** agree well with GS. Therefore, peptides **2**–**11** have a β-sheet structure which is most likely similar to that of GS.

Biological activity

The assessment of antibacterial activity of peptides **2**–**11** and GS was performed using a standard minimal inhibitory concentration (MIC) test employing several Gram-positive and Gram-negative bacterial strains. The results, listed in Table 1, show activity for GS and peptide **2** that are in agreement with the literature data.[3] Azides **4** and **5** as well as peptide **3**, containing benzylated ^{D}Tyr, have activity profiles comparable to gramicidin S. However, peptides **6**–**11** display a considerable loss of activity. The exocyclic amines of Amp, adding positive charge to the turn regions of **6**, **7** retain a small activity for *Staphylococcus epidermidis*. Supplementary negative charge, introduced by the succinyl-group, leads to some activity for peptide **10** against *S. epidermidis, Enterococcus faecalis* and *Escherichia coli* and for peptide **11** against *Pseudomonas aeruginosa*. In contrast to the introduction of hydrophobic moieties on the tyrosine residues, as in **3**, supplementary hydrophobicity on the proline residue (i.e., **8** and **9**) largely abolishes all activity. These findings underscore the speculation of Izuyama et al.,[2] that large groups on the ^{D}Phe-position have a stabilizing effect on the β-turn, thereby enhan-

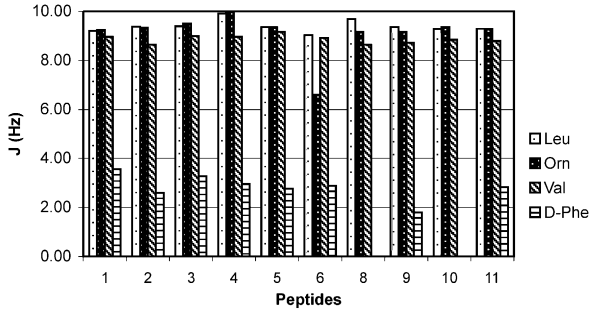


Figure 2. Coupling constants ($^3J_{HN\alpha}$) are given in Hertz (Hz). In the ^{1}H spectrum of peptide **7**, no splitting pattern of amide resonance signals could be observed and was therefore excluded. Peptides **8** and **10** showed no observable $^3J_{HN\alpha}$ for the ^{D}Phe residues.

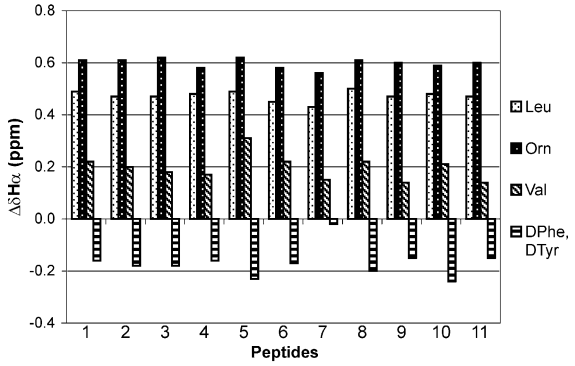


Figure 3. Chemical shift perturbation: $\Delta\delta H_\alpha$ = observed δH_α − random coil δH_α. For entries **2** and **3**, the random coil value for tyrosine was used. As the substitution pattern on the pyrrolidine moiety influences the chemical shift of the H_α, no reference values for the Azp residues were available and were therefore omitted. The reported random coil value for lysine was used for ornithine.[25]

Table 1. Antimicrobial activity (MIC in μg/mL)

Compds	*S. aureus*[a]		*S. epidermidis*[a]		*E. faecalis*[a]		*E. coli*[b]		*P. aeruginosa*[b]		*B. cereus*[a]	
	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]	25W[c]	MT[d]
1	4	8	4	4	8	16	32	32	—	>64	—	8
2	32	32	8	8	32	32	64	>64	—	>64	—	8
3	8	16	4	8	8	32	>64	>64	—	>64	—	16
4	4	8	2	4	8	8	32	32	—	>64	—	>64
5	4	8	4	8	16	16	>64	>64	—	>64	—	16
6	>64	>64	32	32	>64	>64	>64	>64	>64	>64	>64	>64
7	>64	>64	64	64	64	>64	>64	>64	>64	>64	>64	>64
8	>64	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
9	>64	>64	32	64	64	64	>64	64	>64	>64	>64	>64
10	>64	>64	>64	32	32	32	>64	32	>64	>64	>64	64
11	>64	>64	64	>64	64	>64	64	>64	64	32	64	64

Measurements were executed using standard agar dilution techniques (— = not determined).
[a]Gram-positive.
[b]Gram-negative.
[c]3 mL/25 well plates.
[d]100 μL/96 microtiter plates.

cing the antimicrobial activity. However, as the exact process of membrane disruption is not fully understood,[4] the existence of β-sheet structure alone in the presented GS analogues can not be used for the prediction of potential antimicrobial activity.

Conclusions

The biomimetic synthesis of GS and analogues **2**–**5** was successfully employed. Modification of the fully assembled azide containing peptides **4** and **5** led to several GS analogues (**6**–**11**) having hydrophobic and hydrophilic functionalities in the turn region. The conservation of β-sheet character was confirmed for all peptides (**1**–**11**) utilizing several NMR techniques. Examination of the antimicrobial activity of the aforementioned peptides showed a lowered bactericidal effect for compounds **6**–**11**. It may be concluded that modification of the proline residue is counterproductive with respect to antibacterial activity even when the β-sheet character is preserved. The highest antimicrobial activity was for azides **4** and **5**, reflecting the small tolerance for turn region modifications. Surprisingly, the benzylated DTyr analogue **3** is more active than its unprotected counterpart **2**. Thus, the introduction of large hydrophobic entities in the DPhe region holds promise for the future development of GS-based antibiotics.

Experimental

Chemistry

The SPPS was performed on an ABI 433A (Applied Biosystems) automated peptide synthesizer supplied with the FastMoc® peptide synthesis protocol. ^{1}H NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer at 298 K. Chemical shifts (δ) are tabulated in ppm, relative to the solvent peak of CD_3OH (3.30 ppm), unless stated otherwise. LC/MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electronspray Interface (ESI). An analytical Alltima C_{18} colomn (Alltech, 4.6 mmD × 250 mmL, 5 μ particle size) was used in combination with buffers A: H_2O, B: MeCN and C: 0.5% aq TFA. For RP-HPLC purification of the peptides, a BioCAD 'Vision' automated HPLC system (PerSeptiveBiosystems, inc.) supplied with a semi-preperative Alltima C_{18} column (Alltech, 10.0 mmD × 250 mmL, 5 μ particle size) was used. The applied buffers were A: H_2O, B: MeCN and C: 1.0% aq TFA.

***cyclo*(DPhe-Pro-Val-Orn-Leu)$_2$ (1).** RP-HPLC purification (linear gradient of 3.5 CV; 40→75% B; R_t 3.2 CV) followed by lyophilization gave GS in a yield of 5.9 mg (5.2 μmol, 5%). LC/MS analysis: R_t 20.48 min (linear gradient 50→90% B in 20 min); m/z = 1142.0 $[M+H]^+$, 571.6 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DTyr-Pro-Val-Orn-Leu)$_2$ (2).** RP-HPLC purification (linear gradient of 4.0 CV; 30→65% B; R_t 3.5 CV) furnished, after lyophilization, unprotected peptide **2** in a yield of 3.8 mg (3.2 μmol, 3%). LC/MS analysis: R_t 10.15 min (linear gradient 25→90% B in 20 min); m/z = 1173.8 $[M+H]^+$, 587.5 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DTyr(Bn)-Pro-Val-Orn-Leu)$_2$ (3).** RP-HPLC purification (linear gradient of 4.0 CV; 40→85% B; R_t 4.0 CV) furnished, after lyophilization, benzyl protected peptide **3** in a yield of 2.4 mg (1.7 μmol, 2%). LC/MS analysis: R_t 23.73 min (linear gradient 50→90% B in 20 min); m/z = 1354.1 $[M+H]^+$, 677.8 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DPhe-2S,4R-Azp-Val-Orn-Leu)$_2$ (4).** RP-HPLC purification (linear gradient of 3.5 CV; 50→70% B; R_t 2.9 CV) gave, after lyophilization, azide **4** in a yield of 3.8 mg (3.1 μmol, 3%). LC/MS analysis: R_t 20.43 min

Table 2. Chemical shift (δ in ppm)

	αNH	α	β_d	β_u	γ_d	γ_u	δ_d	δ_u	NH
GS									
Leu	8.80	4.66	1.55	1.41	1.50	—	0.87	0.87	—
Orn	8.70	4.97	2.05	1.64	1.79	1.79	3.05	2.91	7.80
Val	7.73	4.17	2.26	—	0.96	0.86	—	—	—
Pro	—	4.35	2.00	1.67	1.71	1.59	3.73	2.48	—
DPhe	8.90	4.50	3.10	2.96			7.33–7.24		
Peptide 2									
Leu	8.72	4.65	1.53	1.39	1.53	—	0.88	0.88	—
Orn	8.68	4.97	2.01	1.64	1.74	1.74	2.99	2.81	7.81
Val	7.71	4.15	2.26	—	0.95	0.88	—	—	—
Pro	—	4.36	1.98	1.54	1.64	1.52	3.74	2.56	—
DTyr	8.86	4.42	3.02	2.86			7.06–6.71		
Peptide 3									
Leu	8.70	4.64	1.52	1.39	1.52	—	0.88	0.88	—
Orn	8.67	4.98	2.02	1.60	1.74	1.74	3.01	2.84	7.81
Val	7.68	4.13	2.23	—	0.93	0.86	—	—	—
Pro	—	4.28	1.92	1.58	1.67	1.52	3.68	2.40	—
DTyr	8.87	4.42	3.02	2.87			7.12–6.90		
Bn	—	4.87	—	—			7.42–7.25		
Peptide 4									
Leu	8.74	4.65	1.51	1.38	1.51	—	0.87	0.87	—
Orn	8.72	4.94	2.06	1.65	1.75	1.75	3.02	2.90	7.80
Val	7.67	4.12	2.25	—	0.96	0.87	—	—	—
Azp	—	4.41	2.25	1.88	3.95	—	3.95	2.54	—
DPhe	8.92	4.50	3.13	2.96			7.34–7.24		
Peptide 5									
Leu	8.68	4.66	1.52	1.39	1.52	—	1.01	1.01	—
Orn	8.64	4.98	2.04	1.61	1.77	1.77	3.04	2.89	7.82
Val	7.72	4.26	2.24	—	1.01	0.89	—	—	—
Azp	—	4.48	2.25	1.87	3.98	—	3.56	2.53	—
DPhe	8.86	4.43	3.09	2.94			7.31–7.23		
Peptide 6									
Leu	8.72	4.62	1.47	1.37	1.47	—	0.85	0.85	—
Orn	8.70	4.94	2.02	1.65	1.74	1.74	3.03	2.91	7.81
Val	7.68	4.17	2.25	—	0.97	0.88	—	—	—
Amp	—	4.57	2.48	1.99	3.76	—	4.31	2.84	—
DPhe	8.86	4.49	3.03	3.03			7.35–7.26		
Peptide 7									
Leu	8.69	4.60	1.50	1.37	1.50	—	0.87	0.87	—
Orn	8.64	4.92	2.03	1.68	1.77	1.77	3.02	2.94	7.81
Val	7.71	4.10	2.24	—	1.00	0.89	—	—	—
Amp	—	4.38	2.67	1.77	3.50	—	3.34	3.34	—
DPhe	8.96	4.64	3.12	2.94			7.38–7.16		
Peptide 8									
Leu	8.73	4.67	1.56	1.41	1.56	—	0.91	0.91	—
Orn	8.68	4.97	2.04	1.63	1.76	1.76	3.05	2.90	7.80
Val	7.72	4.17	2.29	—	1.01	0.91	—	—	—
Amp	—	4.40	2.29	1.75	4.06	—	4.13	2.29	7.03
DPhe	8.88	4.46	3.08	2.93			7.35–7.22		
Z	—	4.90	—	—			7.35–7.22		
Peptide 9									
Leu	8.72	4.64	1.52	1.38	1.52	—	0.88	0.88	—
Orn	8.63	4.96	2.06	1.62	1.76	1.76	3.06	2.88	7.79
Val	7.64	4.09	2.22	—	0.93	0.85	—	—	—
Amp	—	4.31	2.24	1.90	3.37	—	3.37	3.02	6.99
DPhe	8.87	4.51	3.10	2.92			7.42–7.16		
Z	—	4.88	—	—			7.42–7.16		
Peptide 10									
Leu	8.70	4.65	1.46	1.38	1.46	—	0.88	0.88	—
Orn	8.66	4.95	2.02	1.61	1.74	1.74	3.04	2.89	7.82
Val	7.74	4.16	2.25	—	0.98	0.88	—	—	—
Amp	—	4.42	2.27	1.69	4.20	—	4.20	2.27	7.96
DPhe	8.88	4.42	3.08	2.92			7.32–7.23		
Succinyl	—	—	2.52	2.52	2.36	2.36	—	—	—
Peptide 11									
Leu	8.74	4.64	1.51	1.38	1.51	—	0.89	0.89	—
Orn	8.64	4.96	2.05	1.63	1.76	1.76	3.04	2.88	7.79
Val	7.64	4.09	2.25	—	1.00	0.87	—	—	—
Amp	—	4.30	2.40	1.76	3.47	—	3.23	3.23	7.95
DPhe	8.86	4.51	3.10	2.91			7.35–7.21		
Succinyl	—	—	2.52	2.52	2.37	2.37	—	—	—

(linear gradient 50→90% B in 20 min); $m/z = 1223.9$ $[M+H]^+$, 612.6 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DPhe-2*S*,4*S*-Azp-Val-Orn-Leu)$_2$ (5).** RP-HPLC purification (linear gradient of 4.0 CV; 40→75% B; R_t 3.8 CV) gave, after lyophilization, azide **5** in a yield of 5.1 mg (4.2 μmol, 4%). LC/MS analysis: R_t 21.15 min (linear gradient 50→90% B in 20 min); $m/z = 1224.0$ $[M+H]^+$, 612.6 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DPhe-2*S*,4*R*-Amp-Val-Orn-Leu)$_2$ (6).** The unprotected azide **4** (23 mg, 19 μmol) was dissolved in chloroform (2 mL) and methanol (2 mL) and a catalytic amount of 10% Pd/C was added. The resulting mixture was placed under an atmosphere of hydrogen and stirred 16 h. The suspension was filtered over a plug of Hyflow Super Gel® and concentrated in vacuo. RP-HPLC purification (linear gradient of 3.5 CV; 30→55% B; R_t 1.6 CV) and freeze-drying of the combined collected fractions, furnished 12.93 mg of peptide **6** (11 μmol, 58%). LC/MS analysis: R_t 13.64 min (linear gradient 20→60% B in 20 min); $m/z = 1172.0$ $[M+H]^+$, 586.6 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DPhe-2*S*,4*S*-Amp-Val-Orn-Leu)$_2$ (7).** The unprotected azide **5** (17 mg, 14 μmol) was treated similarly to **4**, to give, after RP-HPLC purification (linear gradient of 3.5 CV; 30→55% B; R_t 1.4 CV) and freeze-drying of all collected fractions, 9.89 mg of peptide **7** (8.4 μmol, 60%). LC/MS analysis: R_t 12.85 min (linear gradient 20→60% B in 20 min); $m/z = 1172.0$ $[M+H]^+$, 586.6 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DPhe-2*S*,4*R*-Amp(*Z*)-Val-Orn-Leu)$_2$ (8).** To a solution of peptide **16** (17 mg, 12 μmol) in 1,4-dioxane (2 mL) and MeCN (2 mL) was added 100 μL (100 μmol) of PMe_3 (1 M in toluene). The mixture was stirred for 3 h after which H_2O (200 μL) was added and allowed to stir 16 h. All volatiles were evaporated under reduced pressure, followed by addition of benzylchoroformate (7 μL, 48 μmol) and *N*,*N*′-diisopropylethylamine (12 μL, 72 μmol) in DMF (2 mL). The solution was stirred 6 h, concentrated, redissolved in DCM (2 mL) and cooled to 0 °C after which TFA (2 mL) was added. The resulting mixture was warmed to room temperature over a period of 30 min. Evaporation of all solvents and RP-HPLC purification (linear gradient of 3.0 CV; 60→90% B; R_t 1.9 CV) gave, after lyophilization, the Z-protected peptide **8** in 7.2 mg (5.0 μmol, 42%). LC/MS analysis: R_t 13.96 min (linear gradient 50→90% B in 20 min); $m/z = 1440.2$ $[M+H]^+$, 720.7 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(DPhe-2*S*,4*S*-Amp(*Z*)-Val-Orn-Leu)$_2$ (9).** To a solution of peptide **17** (4 mg, 2.8 μmol) in 1,4-dioxane (2 mL) and MeCN (2 mL) was added 23 μL (23 μmol) of PMe_3 (1 M in toluene). The mixture was stirred for 3 h after which H_2O (200 μL) was added and allowed to stir 16 h. All volatiles were evaporated under reduced pressure followed by addition of benzylchoroformate (2 μL, 12 μmol) and *N*,*N*′-diisopropylethylamine (3 μL, 17 μmol) in DMF (2 mL). The solution was stirred 6 h, concentrated, redissolved DCM (2 mL) and cooled to 0 °C after which TFA (2 mL) was added. The resulting mixture

was warmed to room temperature over a period of 30 min. Evaporation of all solvents and RP-HPLC purification (linear gradient of 3.0 CV; 50→90% B; R_t 2.4 CV) gave, after lyophilization, the Z-protected peptide **9** in 1.5 mg, (1.0 μmol, 36%). LC/MS analysis: R_t 16.02 min (linear gradient 50→90% B in 20 min); $m/z = 1440.0$ $[M+H]^+$, 720.7 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(ᴰPhe-2*S*,4*R*-Amp(Su)-Val-Orn-Leu)$_2$ (10).** Azide **16** (17 mg, 12 μmol) was dissolved in 1,4-dioxane (2 mL) and MeCN (2 mL) and to the solution was added trimethylphosphine (100 μL, 1.0 M in toluene). The resulting mixture was stirred for 3 h, followed by addition of H_2O (200 μL) and stirred 16 h. All volatiles were removed by evaporation and succinic anhydride (4.8 mg, 48 μmol) and triethylamine (6.8 μL, 48 μmol) in *N*,*N*′-dimethylformamide (4 mL) were added. After stirring for 2 h, the reaction was quenched with H_2O (200 μL) and concentrated. The resulting peptides were dissolved in DCM (5 mL) and cooled to 0 °C, after which TFA (5 mL) was added. The mixture was allowed to warm to room temperature, stirred for 30 min and concentrated in vacuo. RP-HPLC purification (linear gradient of 3.0 CV; 30→50% B; R_t 2.7 CV) followed by lyophilization furnished 12.8 mg of **10** (9.3 μmol, 78%). LC/MS analysis: R_t 15.39 min (linear gradient 20→60% B in 20 min.); $m/z = 1371.9$ $[M+H]^+$, 686.6 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

***cyclo*(ᴰPhe-2S,4S-Amp(Su)-Val-Orn-Leu)$_2$ (11).** Azide **17** (15 mg, 11 μmol) was subjected to the same reaction conditions as described for peptide **10**. RP-HPLC purification (linear gradient of 3.0 CV; 30→50% B; R_t 2.8 CV) followed by lyophilization furnished 9.4 mg of **11** (6.9 μmol, 66%). LC/MS analysis: R_t 14.30 min (linear gradient 20→60% B in 20 min); $m/z = 1372.0$ $[M+H]^+$, 686.7 $[M+H]^{2+}$. For ^{1}H NMR, see Table 2.

Fmoc-Leu-HMPB-MBHA resin (13). 4-Methylbenzhydrylamine resin **12** (806 mg, 0.5 mmol) was suspended in 1,4-dioxane, evaporated to dryness (3 × 50 mL) and resuspended in NMP (25 mL). To the mixture, HMPB (360 mg, 1.5 mmol), BOP (663 mg, 1.5 mmol) and DiPEA (0.523 mL, 3.0 mmol) were added. The suspension was shaken 16 h, filtered and the resin was consecutively washed with DCM (2 × 20 mL), MeOH (20 mL) and DCM (2 × 20 mL). The resin was suspended in 1,4-dioxane, evaporated to dryness (3 × 50 mL) and resuspended in DCM (25 mL). Subsequent condensation of the first amino acid was effected by addition of Fmoc-Leu-OH (530 mg, 1.5 mmol), DIC (0.258 mL, 1.65 mmol) and DMAP (10 mg, 82 μmol) after which the reaction mixture was shaken for 2 h. Washing of the resin and a second esterification cycle was performed as described above. The loading of the resin was determined to be 0.48 mmol × g^{-1}.

General procedure for peptide synthesis

Stepwise elongation. The immoblized peptides (**14a–e**) were synthesized using 210 mg (0.1 mmol) of resin **13**. The consecutive steps in each coupling cycle were: (i) Deprotection: 20% piperidine in NMP (2 mL) 5 × 1 min; (ii) Coupling: the appropriate amino acid (0.5 mmol) was dissolved in NMP (1 mL) and subsequently 0.5 mmol (0.5 M BOP/0.5 M HOBt in NMP/DMF 1/1, v/v) and 1.5 mmol of DiPEA (1.25 M in NMP) were added. The resulting mixture was transferred to the reaction vessel and shaken for 90 min; (iii) Capping: the resin was subjected to 1 min of shaking in a solution of 0.5 M acetic anhydride, 0.125 M DiPEA and 0.015 M HOBt in NMP (2 mL). The applied amino acids were Boc-ᴰPhe-OH, Boc-ᴰTyr-OH, Fmoc-2*S*,4*R*-Azp-OH, Fmoc-2*S*,4*S*-Azp-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Pro-OH and Fmoc-Val-OH. Double couplings were executed for Val.

Cleavage from the resin. The resin (**14a–e**) was treated with 1% TFA in DCM (4 × 10 mL). All fractions were collected and after addition of toluene (50 mL) concentrated under reduced pressure. The mass of the fully protected peptides was established by mass spectroscopy (ESI-MS): **a** $m/z = 830.4$ $[M+H]^+$, 852.6 $[M+Na]^+$ **b** $m/z = 830.7$ $[M+H]^+$, 852.6 $[M+Na]^+$ **c** $m/z = 895.7$ $[M+H]^+$, 917.7 $[M+Na]^+$ **d** $m/z = 805.7$ $[M+H]^+$, 827.5 $[M+Na]^+$ **e** $m/z = 789.9$ $[M+H]^+$, 811.7 $[M+Na]^+$

Activation of pentapeptides. The crude pentamers (100 μmol) were dissolved in DMF (2 mL) and cooled to 0 °C. To this mixture were added *N*-hydroxy succinimide (23 mg, 200 μmol) in DCM (1 mL) and *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (38 mg, 200 μmol). The reaction mixture was then allowed to warm to room temperature and stirred 16 h. The resulting solution was concentrated and partitioned between DCM (50 mL) and H_2O (10 mL). The organic layer was dried ($MgSO_4$) and concentrated.

Deprotection. The Boc-protection groups were removed by dissolving the peptide active esters in DCM (2 mL) followed by addition of TFA (2 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature, stirred for 30 min after which toluene (10 mL) was added and the solvents were removed in vacuo. The crude peptides (**15a–e**) were used in the following cyclodimerization reaction without further purification.

General cyclodimerization procedure

The crude active esters **15a–e** were taken up in DMF (2 mL) and added dropwise to pyridine (30 mL). After stirring for 24 h, the resulting mixture was concentrated, analysed by LC/MS and purified by RP-HPLC to give peptides **1–5**.

***cyclo*(ᴰPhe-2*S*,4*R*-Azp-Val-Orn(Boc)-Leu)$_2$ (16).** To a solution of cyclic decamer **4** (53 mg, 37 μmol) in MeCN (5 mL) were added DiPEA (34 μL, 194 μmol) in $CHCl_3$ (1 mL) and di-*tert*-butyl dicarbonate (21 mg, 97 μmol). After stirring for 3 h, the solvents were removed in vacuo and the resulting residue directly subjected to silica gel colomn chromatography (0→5% MeOH in EtOAc) to furnish **16** as a white amorphous solid (47 mg, 33 μmol, 89%). MS (ESI): $m/z = 1424.2$

$[M+H]^+$, 1445.9 $[M+Na]^+$ ^1H NMR (DMSO-d_6): δ 8.94 (d, 1H, NH ^{D}Phe, $J=2.6$ Hz), 8.55 (d, 1H, NH Orn, $J=8.8$ Hz), 8.36 (d, 1H, NH Leu, $J=8.6$ Hz), 7.26 (bs, 6H, H_{arom} ^{D}Phe, NH Val), 6.77 (m, 1H, δNH Orn), 4.70 (m, 1H, H_α Orn), 4.54 (m, 2H, H_α Leu, Amp), 4.39 (m, 2H, H_α ^{D}Phe, Val), 4.00 (m, 1H, H_γ Amp), 3.82 (m, 1H, H_δ Amp), 2.99 (m, 1H, H_β ^{D}Phe), 2.88 (m, 3H, H_δ Orn, H_β ^{D}Phe), 2.48 (m, 1H, H_δ Amp), 2.30 (m, 1H, H_β Amp), 2.01 (m, 1H, H_β Val), 1.67 (m, 1H, H_β Orn), 1.59 (m, 1H, H_β Amp), 1.43–1.15 (m, 15H, 3 × CH_3 Boc, 1 × H_β Orn, 2 × H_γ Orn, 2 × H_β Leu, 1 × H_γ Leu), 0.89–0.77 (m, 12H, 3 × H_δ Leu, 3 × H_γ Val).

***cyclo*(D-Phe-2*S*,4*S*-Azp-Val-Orn(Boc)-Leu)$_2$ (17).** Starting from **5** (51 mg, 35 μmol) peptide **17** was obtained as described for **16**, as a white amorphous solid (42 mg, 30 μmol, 86%). MS (ESI): $m/z=1424.2$ $[M+H]^+$, 1445.9 $[M+Na]^+$ ^1H NMR (DMSO-d_6): δ 8.81 (d, 1H, NH ^{D}Phe, $J=2.8$ Hz), 8.57 (d, 1H, NH Orn, $J=8.9$ Hz), 8.34 (d, 1H, NH Leu, $J=8.9$ Hz), 7.27 (bs, 5H, H_{arom} ^{D}Phe), 7.18 (d, 1H, NH Val, $J=8.9$ Hz), 6.80 (m, 1H, δNH Orn), 4.69 (m, 1H, H_α Orn), 4.55 (m, 3H, H_α Leu, H_α Val, H_α Amp), 4.35 (m, 1H, H_α ^{D}Phe), 4.11 (m, 1H, H_γ Amp), 3.41 (d, 1H, 1 × H_δ Amp, $J=11.5$ Hz), 2.92 (m, 4H, 2 × H_δ Orn, 2 × H_β ^{D}Phe), 2.73 (m, 1H, 1 × H_δ Amp), 2.20 (d, 1H, 1 × H_β Amp, $J=13.3$ Hz), 1.96 (m, 1H, H_β Val), 1.71 (m, 2H, 1 × H_β Amp, 1 × H_β Orn), 1.48–1.16 (m, 15H, 3 × CH_3 Boc, 1 × H_β Orn, 2 × H_γ Orn, 2 × H_β Leu, 1 × H_γ Leu), 0.87–0.80 (m, 12H, 3 × H_δ Leu, 3 × H_γ Val).

Biological activity

The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *Bacillus cereus* (ATCC 11778). Bacteria were stored at −70 °C and grown at 35 °C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) overnight and diluted in 0.9% NaCl. Large plates (25 wells of 3 mL) as well as microtitre plates (96 wells of 100 μL) were filled with Mueller Hinton II Agar (Becton Dickinson, Cockeysvill, USA) containing serial 2-fold dilutions of peptides **1**–**11**. To the wells was added 3 μL of bacteria, to give a final inoculum of 10^4 colony forming units (CFU) per well. The plates were incubated overnight at 35 °C and the MIC was determined as the lowest concentration inhibiting bacterial growth.

Acknowledgements

This work was financially supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO), the Netherlands Technology Foundation (STW) and DSM Research. We thank Nico Meeuwenoord and Hans van der Elst for their technical assistance and are grateful to Kees Erkelens and Fons Lefeber for their help with recording the NMR spectra.

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