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Evaluation of Readily Accessible Azoles as Mimics of the Aromatic Ring of D-Phenylalanine in the Turn Region of Gramicidin S

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The influence of replacing the D-phenylalanine residue with substituted and unsubstituted azoles on the structure and biological activity of the antibiotic gramicidin S was investigated against a representative panel of Gram-positive and Gram-negative bacteria strains. Substituted triazole derivatives, obtained using a convergent synthetic strategy, are as active as gramicidin S, provided that any substituent on the triazole moiety is not too large. The unsubstituted triazole derivative was biologically less active than the parent natural product, gramicidin S. In general for the triazole series, the hemolytic activity could be correlated with the antibacterial activity, that is, the higher the antibacterial activity, the higher the toxicity towards blood cells. Interestingly, its imidazole counterpart showed high antibacterial activity, combined with significantly diminished hemolytic activity.

Introduction

The cyclic decapeptide gramicidin S (GS, **1**)[1] is a highly potent biocidal natural product, active against a broad range of Gram-positive and certain Gram-negative bacteria.[2] It is comprised of two type II′ β-turns and two β-strands, stabilised by four interstrand hydrogen bonds (Figure 1).[3] Consequently, the hydrophobic and hydrophilic side chains in the strand regions are on opposite faces of the molecule, rendering the peptide amphiphilic, which is a prerequisite for the antibacterial activity of the peptide. Though disruption of the membrane integrity appears to be the main mode of action of **1**, the *bd*-type quinol oxidase has recently been identified as a possible additional target.[4] The membrane lytic properties of **1** are not restricted to bacteria and other life-threatening pathogens, thus confining its use to topical applications.[5] As bacteria do not easily build up resistance against this type of antibiotic,[6] **1** is an attractive starting point for the development of compounds with improved biological profile, i.e., high bactericidal activity, combined with lower toxicity. Many derivatives of **1** have been designed and synthesised towards this goal.[7]

One approach that has been exploited to arrive at compounds with an improved biological profile is the introduction of alternative amino acids in the strands of the peptide in order to modulate the hydrophobicity/hydrophilicity ratio. Hodges et al. reported ring-extended GS analogues in which four lysine and four hydrophobic residues were included in the strand.[8] A decameric GS analogue with four ornithines and two adamantane-derived amino acids showed high antibacterial activity, coupled with diminished hemolytic activity.[9] Alternatively, slight alterations in one or both β-turns have been introduced; for example, it was found that the D-phenylalanine residue may be substituted by a hydrophobic D-amino acid[10] or a dehydroamino acid[11] in order to retain the cyclic β-hairpin structure and hence the antibacterial activity. Incorporation of D-*cyclo*-hexylalanine,[12] however, led to an almost complete loss of antibacterial activity. The latter finding strongly indicates that aromaticity at this position is vital for membrane

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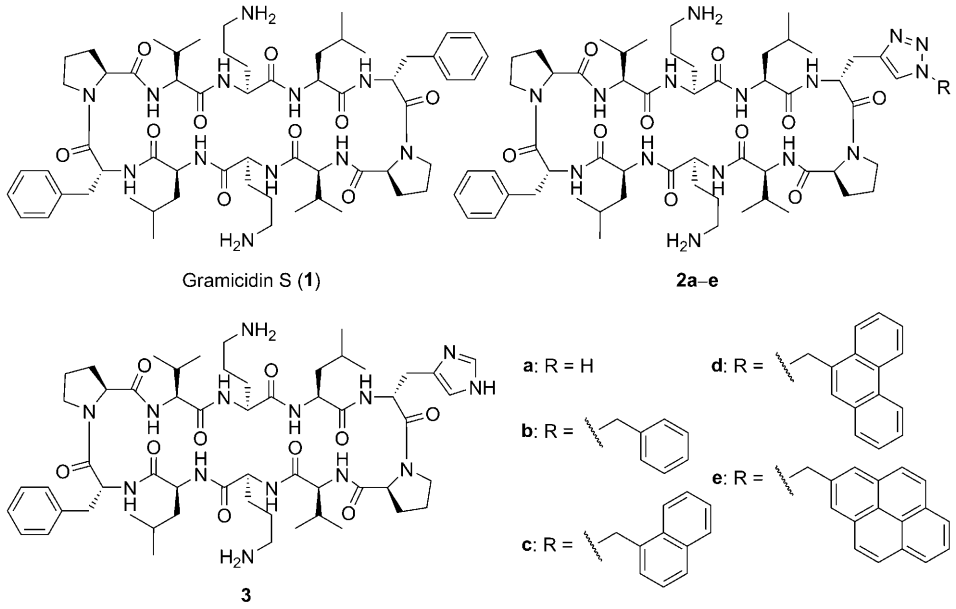


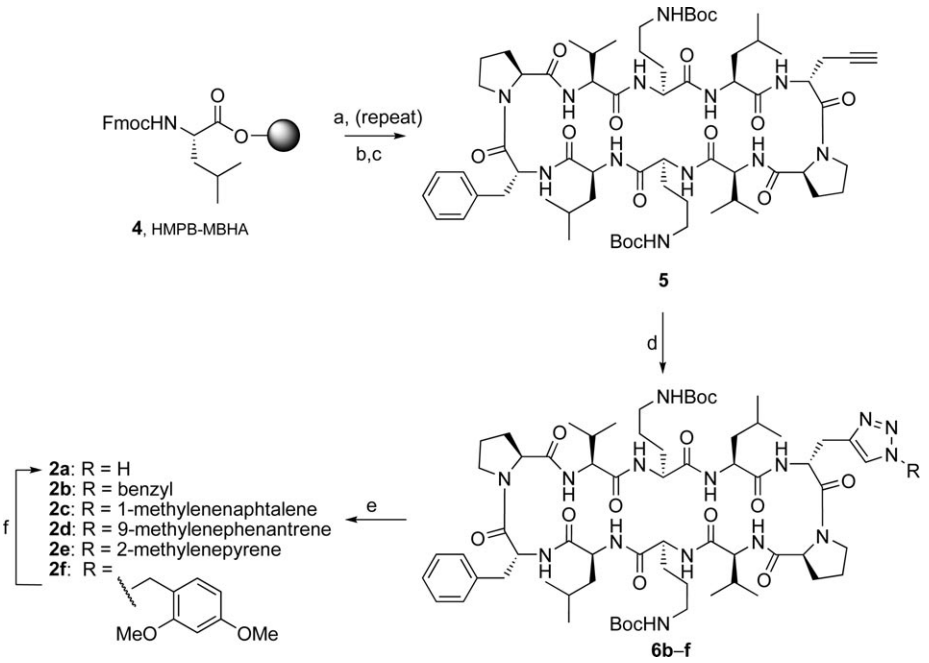
Figure 1. Structure of gramicidin S (GS, **1**) and the novel triazole and imidazole derivatives.

lytic activity.[13] Although proline may be replaced by other N-alkylated amino acids,[14] the possibilities for modifications at this residue in the β-turn appear to be limited.[13a]

With these considerations in mind, a series of azole derivatives of **1** was envisioned. The azoles are heterocycles containing at least one nitrogen atom, with aromatic properties and may, therefore, be appropriate mimics for the phenyl ring of D-phenylalanine. We anticipated straightforward access to the triazole derivatives by following a synthetic strategy in which a GS derivative containing one D-propargylglycine residue reacts with aryl azides in the presence of catalytic amounts of copper(I).[15] Here, we present a convergent synthetic route towards functionalised GS-derived triazoles **2b–e**, unsubstituted triazole **2a**, and an imidazole analogue **3**, in which one D-phenylalanine is replaced by one D-histidine residue.[8a,16] All compounds were evaluated against several bacterial strains and erythrocytes to determine their hemolytic properties.

Results and Discussion

Key intermediate **5** was prepared by solid-phase peptide synthesis[17] on a 1.5 mmol scale using commercially available Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids. The hyper-acid-labile 4-hydroxymethyl-3-methoxyphenoxybutyric acid (HMPB)-MBHA resin, loaded with Fmoc-leucine (**4**), was exposed to repeated cycles of Fmoc deprotection with 20% piperidine (pip) in *N*-methyl-pyrrolidone (NMP) and coupling with the appropriate Fmoc-amino acid (Fmoc-aa-OH), using 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) as an activator and *N*,*N*-diisopropylethylamine (DIPEA) as the base (Scheme 1). Commercially available *N*-Fmoc-D-propargylglycine was incorporated as the final amino acid of the immobilised sequence. After assembly of the linear decamer, the N-terminal Fmoc group was removed, and the peptide was subsequently liberated from the solid support under mildly acidic conditions, leaving the (*tert*-butoxy)carbamate (Boc) protection on the δ amine groups of ornithine residues in place. Cyclisation was performed under dilute conditions with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP)/hydroxybenzotriazole (HOBt) as an activator in *N*,*N*-dimethylformaldehyde (DMF), followed by LH-20 size-exclusion column chromatography, to give protected **5**.



Scheme 1. *Reagents and conditions*: a) 1. 20% pip/NMP (2×10 min); 2. Fmoc-aa-OH (2.5 equiv), HCTU (2.5 equiv), DIPEA (3 equiv), NMP, 1.5 h; b) 1% TFA/CH_2Cl_2 (6×10 min); c) PyBOP (5 equiv), HOBt (5 equiv), DIPEA (15 equiv), 0.01 M DMF; d) $CuSO_4$, sodium ascorbate, RN_3 (**7b–f**), DMF; e) TFA/CH_2Cl_2; f) 50% TFA/CH_2Cl_2, microwave.

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The alkyne functionality of compound **5** was used as a handle to readily construct the triazole ring by reaction with 2,4-dimethoxybenzyl azide using copper-catalysed 1,3-dipolar cycloaddition reactions. For this purpose, 2,4-dimethoxybenzyl azide **7f** was prepared from the corresponding alcohol by treatment with diphenylphosphoryl azide and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in toluene.[18] Using three equivalents of the organic azide in the presence of copper(I) generated in situ, the fully protected GS-derived triazole **6f** was obtained. Standard Boc deprotection using trifluoroacetic acid (TFA) gave **2f**, which was used without isolation to give 1*H*-triazole **2a** after microwave irradiation under the Boc removal conditions.

In addition, a series of other aromatic azides with an increasing number of aromatic rings were selected. Benzyl azide **7b** is commercially available, while the other azides were synthesised from their corresponding alcohols by treatment with diphenylphosphoryl azide in excellent yields (**7c**–**e**, 80–100%). Reaction of **5** with the respective azides as described above went to completion, giving the Boc-protected GS derivatives containing a 1,4-triazole (**6a**–**e**, Scheme 1). Simple treatment with TFA in dichloromethane at room temperature yielded the unprotected peptides **2b**–**e**.

Using a similar solid-phase strategy and cyclisation/deprotection approach, peptide **3** was prepared. All peptides (**2a**–**e** and **3**) were purified by preparative reverse-phase (RP) HPLC and characterised by high-resolution mass spectrometry (HRMS), ^{1}H NMR (1D and 2D) and ^{13}C NMR (see Experimental Section). The NMR signals were unambiguously assigned by a combination of ^{1}H NMR, COSY and TOCSY spectra. The spectra of the substituted 1,4-triazoles **2b**–**e**, the 1*H*-triazole **2a** and the histidine analogue **3** showed the characteristics of two type II′ β-turns connected by two β-sheets. That is, small coupling constants ($2.5 < J_{NH-H\alpha} < 3.5$ Hz) for the amide protons of D-phenylalanine, D-histidine and D-triazolalanine were observed. The $J_{NH-H\alpha}$ of the strand amino acid residues, on the other hand, showed large coupling constants ($J_{NH-H\alpha} > 8$ Hz). Both values support the cyclic β-hairpin structure.[19] In addition, crystals suitable for X-ray diffraction were obtained from peptides **2b** and **3** (Figure 2). The X-ray structures are in agreement with the NMR data and clearly show the anticipated secondary structure. However, what is remarkable is the considerable disorder of the β-hairpin structures, caused by the simple substitution of one phenyl ring by an azolic ring. This structural disorder is likely caused by the packing of the peptides, allowing the β-hairpin to adopt several different conformations in which main-chain hydrogen bonding is conserved. Peptide **2b** crystallises with little side chain disorder, whereas peptide **3** crystallises with multiple side chains disordered and the β-sheet adopting several conformations in the crystal packing. We found that peptide **2b** forms small amphiphilic channels in

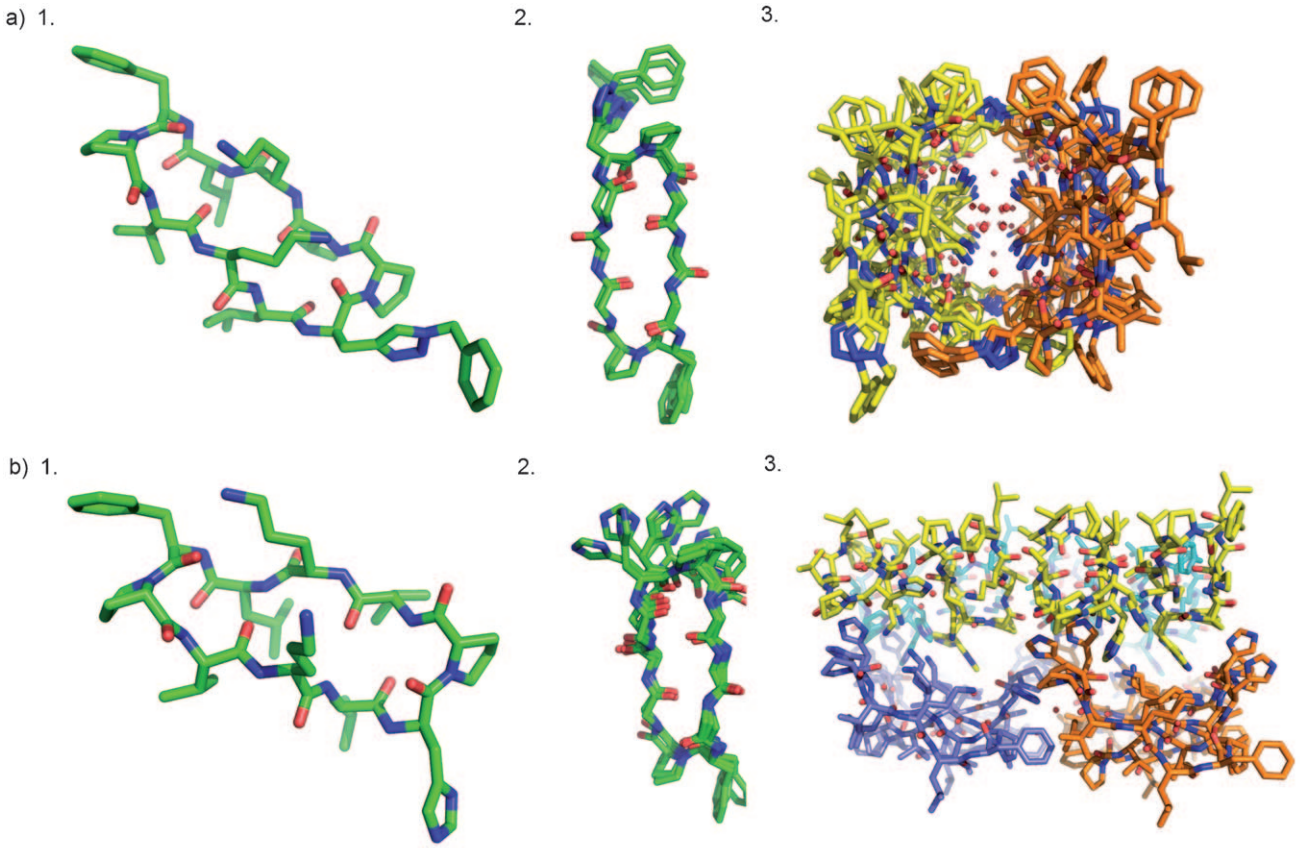

Figure 2. Crystallographic structures of GS derivatives a) **2b** and b) **3**. 1) Crystallographic structure of one selected peptide molecule. Hydrogen atoms and disordered side chains are omitted for clarity. 2) Overlay of all β-sheets present in the asymmetric crystallographic unit. Valine, ornithine and leucine side chains are omitted for clarity. 3) Detail of the crystallographic packing.

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the crystal with the hydrophobic residues in the outer surface and the hydrophilic residues in the inner (Figure 2a part 3). Peptide **3** shows a crystal packing similar to **2b** with the β-sheets extended along one direction, but in this case the amphiphilic channels are not formed in the crystallographic lattice since the rows of β-sheets formed are almost perpendicular to each other (Figure 2b part 3).

The antibacterial activities of triazoles **2a**–**e** and imidazole **3** were evaluated against a representative series of Gram-negative and- positive bacteria (Table 1). Gram-negative bacteria are quite insensitive towards the triazole derivatives **2a**–**e**, whereas histidine analogue **3** actively kills Gram-negative bacteria. Going from **2a** to **2b** to **2c**, the antibacterial activity increases, but increasing the number of aromatic rings even further (**2d**) lowers the activity, indicating that a steric optimum has been reached. What is remarkable is that compound **3** is a potent and broadly active antimicrobial agent, while compound **2a** is not. The hemolytic activities of triazoles **2a**–**e** and histidine derivative **3** were also determined (Figure 3). The most antibacterial compounds (**2c**–**e**) are all more toxic than GS (**1**). The benzyl-substituted derivatives **2b** and **2a** both show lower hemolytic activity, which correlates with a diminished antibacterial activity. The potent antibacterial agent, histidine analogue **3**, displays the lowest hemolytic activity of all peptides tested.

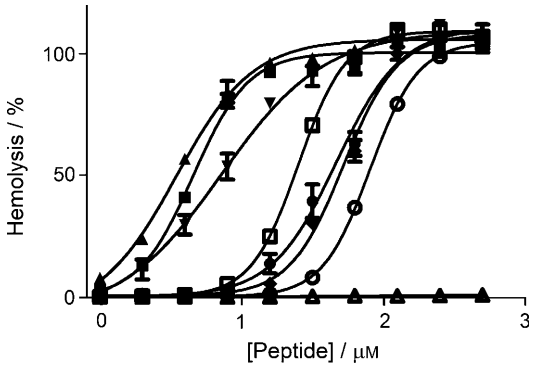

Figure 3. Hemolytic activity of peptides **2a** (◆), **2b** (●), **2c** (■), **2d** (▲), **2e** (▼) and **3** (○); GS (□) and DMSO (△) were used as controls.

Table 1. Antibacterial activities (μg mL^{-1}) of **2a**–**e** and **3** compared to GS (**1**) after 24 h.[a]

Bacterial strain[b]		GS (**1**)	**2a**	**2b**	**2c**	**2d**	**2e**	**3**
S. aureus 7323	(+)	8	16	8	4	4	8	4
S. aureus 7388	(+)	8	32	4	4	4	4	4
CNS 5277	(+)	4	16	8	4	4	4	4
CNS 5115	(+)	16	32	8	4	4	8	4
CNS 7368	(+)	8	8	8	4	4	4	4
E. faecalis 1131	(+)	8	64	16	4	4	16	16
E. coli ATCC 25922	(−)	32	32	32	64	64	>64	8
P. aeruginosa AK1	(−)	16	64	64	>64	64	8	4
P. aeruginosa ATCC19582	(−)	8	>64	64	>64	64	>64	16
S. mitis BMS	(+)	8	32	16	4	4	4	4
S. mitis ATCC 33399	(+)	4	64	32	4	8	16	16

[a] Experiments were conducted once, and the experimental error is one MIC interval. [b] Gram-positive (+); Gram-negative (−).

Conclusions

A series of heteroaromatic derivatives of gramicidin S (GS, **1**) was prepared. Of these, the methylarylated 1,4-triazoles **2b**–**d** exhibited highly potent antibacterial activities, showing that substituted triazoles may serve as mimics of the phenyl ring in the turn region of GS. Derivative **2e** containing the sterically bulky pyrene moiety showed weaker antibacterial activity compared to the less bulkier analogues **2b**–**d**, indicating that there is a limit to the number of aromatic rings that may be introduced without loss of activity, which agrees well with our previous findings.[10b] The unsubstituted triazole **2a** was less potent than the benzyl-substituted **2b**, underlining the importance of hydrophobic aromaticity in this region. In general for the triazole series, the hemolytic activity could be correlated with the antibacterial activity, that is, the higher the antibacterial activity, the higher the toxicity towards blood cells. Rather surprisingly, given the trend observed for the triazoles, histidine analogue **3** is the most promising compound of the series described here, with strong activity against both Gram-positive and Gram-negative bacteria. In fact, this compound is slightly more potent than the parent compound GS, which is only weakly active against the Gram-negative bacterial strains in our assays. The high potency of derivative **3** may arise from the higher basicity of the imidazole functionality (pK_a = ~7.0) compared with the 1*H*-triazole moiety of compound **2a** (pK_a = ~1.2).[8] Although we do not have a fitting explanation[11b] for this, to our satisfaction we found that peptide **3** displays also the lowest hemolytic activity of this series. Comparing the activity of the unsubstituted imidazole analogue **3** with the substituted triazole series, it would be of interest to find out whether the biological profiles of the latter can be improved by substituting the imidazole ring, either on the nitrogen or on the carbon atoms, with medium-sized aromatic groups. Several—transition metal-catalysed—methods are available to construct suitably substituted imidazole derivatives.[20]

Experimental Section

Chemistry

General methods: Solvents and chemicals were used as received from their supplier. Solvents were stored over 4 Å molecular sieves (or 3 Å MS for MeOH). Solvents for extractions and silica gel chromatography were of technical grade and distilled before use. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AV-400 (400/100 MHz), or Bruker DMX-600 spectrometer (600/150 MHz). Chem-

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ical shifts (δ) are given in parts per million (ppm) relative to tetramethylsilane (0 ppm) or CD_3OH (3.31 ppm) as the internal standard. High-resolution mass spectra (HRMS) were recorded by direct injection (2 μL of a 2 μM solution in H_2O/CH_3CN, 50:50 *v/v* and 0.1% formic acid) on a mass spectrometer (Termo Finnigan LTQ Orbitrap) equipped with an electrospray ion (ESI) source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with a resolution of 60000 at $m/z = 400$ (mass range $m/z =$ 150–2000) and dioctylphthalate ($m/z = 391.28428$) as a lock mass. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC/MS analyses were performed on a LCQ Adventage Max (Thermo Finnigan) equipped with a Gemini C18 column (Phenomenex). The applied buffers were A: H_2O, B: CH_3CN and C: 1% aq TFA. HPLC purifications were performed on a Gilson GX-281 automated HPLC system, equipped with a preparative Gemini C18 column (150× 21.20 mm, 5 μ). The applied buffers were: A: 0.2% aq TFA, B: CH_3CN.

Preparation of azides 7c–f: The alcohol (5 mmol) was dissolved in dry toluene (9 mL) and cooled to 0 °C under argon. Diphenylphosphoryl azide (DPPA, 1.2 equiv, 6 mmol, 1.32 mL) and DBU (1.2 equiv, 6 mmol, 0.92 mL) were subsequently added. When TLC indicated completion of the reaction, the mixture was diluted with Et_2O and washed with H_2O. The organic fractions were dried over $MgSO_4$, filtered and evaporated. The pure compound was obtained by silica gel column chromatography [10% toluene in petroleum ether (PE)].

1-(Azidomethyl)pyrene (7e): Compound was obtained as a yellow solid (97%, 4.85 mmol): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.13$–7.92 (m, 7H), 7.83 (d, $J = 8$ Hz, 2H), 4.89 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 131.6$, 131.1, 130.6, 129.1, 128.2, 128.1, 127.3, 127.2, 126.1, 125.5, 125.4, 124.9, 124.5, 122.5, 53.0 ppm; IR (neat): $\tilde{\nu} = 3042.0$, 2099.7, 1593.6, 1249.8, 1225.8, 894.6, 818.5, 752.0, 699.0, 663.7 cm^{-1}.

2,4-dimethoxybenzyl azide (7f): Column chromatography (5% Et_2O/PE + 0.1% Et_3N) gave the title compound as a colourless oil (80%, 4 mmol): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.14$ (d, $J = 10.4$ Hz, 1H), 6.49–6.44 (m, 2H), 4.27 (s, 2H), 3.83 (s, 3H), 3.81 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 161.2$, 158.8, 131.0, 116.2, 104.0, 98.6, 55.3, 49.8 ppm; IR (neat): $\tilde{\nu} = 2090.6$, 1611.6, 1587.7, 1507.9, 1288.2, 1265.9, 1156.9, 1128.6, 1032.3 cm^{-1}.

General procedure for the synthesis of GS derivatives 2–3: *Stepwise Elongation*: Fmoc-Leu-HMPB-MBHA resin **4** (resin loading = 0.56 mmol g^{-1}, 1.5 mmol) was submitted to nine cycles of Fmoc solid-phase synthesis with the appropriate commercial amino acids. The amino group on the side chain of ornithine was protected with a Boc group. Fmoc removal was effected by treatment with 20% piperidine (pip) in NMP for 2×10 min. The resin was subsequently washed with NMP, CH_2Cl_2, MeOH, and finally NMP. The Fmoc-aa-OH (3.75 mmol, 2.5 equiv), HCTU (3.75 mmol, 2.5 eq) in NMP was pre-activated for 1 min after the addition of DIPEA (7.5 mmol, 5 equiv) and then added to the resin. The suspension was shaken for 1.5 h. The resin was washed with NMP, CH_2Cl_2, MeOH and NMP.

Cleavage from the resin: After the final Fmoc deprotection, the resin was washed with NMP, CH_2Cl_2 and treated with 1% TFA in CH_2Cl_2 (50 mL, 6×10 min). The filtrates were collected and co-evaporated with toluene (3×100 mL).

Cyclisation: PyBOP (7.5 mmol, 5 equiv), HOBt (7.5 mmol, 5 equiv), and DIPEA (22.5 mmol, 15 equiv) were dissolved in DMF (1.2 L), and a solution of the linear decapeptide (1.5 mmol) in DMF (15 mL) was added dropwise over 1 h. After addition, the mixture was stirred for 16 h. The reaction was concentrated in vacuo, and the crude mixture was subjected to LH-20 size-exclusion chromatography.

cyclo-(DPhe-Pro-Val-Orn(Boc)-Leu-DPrg-Pro-Val-Orn(Boc)-Leu) (5): LC/MS: $t_R = 4.75$ min (50→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1289.67 $[M+H]^+$; HRMS calculated for $C_{66}H_{105}N_{12}O_{14}$ $[M+H]^+$: 1289.78677, found: 1289.78814.

General procedure for Cu^I-catalysed 1,3-dipolar cycloadditions: Alkyne **5** (50 mg, 39 μmol) and the appropriate azide **7b–f** (3 equiv) were dissolved in DMF (750 μL), and aq $CuSO_4$ was added (1 M, 0.1 equiv, 39 μL) followed by aq sodium ascorbate (1 M, 0.15 equiv, 59 μL). When TLC ($CHCl_3$/MeOH, 9:1 *v/v*) indicated completion, DMF was evaporated and the residue was redissolved in CH_2Cl_2, and washed with HCl (1 N) and aq $NaHCO_3$. The organic layer was dried ($MgSO_4$), filtered and concentrated.

General procedure for Boc deprotection: A solution of peptide **6** in CH_2Cl_2 (2 mL) was treated with TFA (2 mL) and stirred for 4 h. The reaction was concentrated in vacuo and coevaporated with toluene (3×10 mL), then the crude was purified by preparative RP-HPLC.

***cyclo*-(DPhe-Pro-Val-Orn-Leu-DTrA-Pro-Val-Orn-Leu)·2TFA (2a)**: The Boc-protected peptide **6a** was dissolved in CH_2Cl_2 (2 mL) and treated with TFA (2 mL). The mixture was heated under microwave irradiation for 5 min. The crude product was concentrated in vacuo and coevaporated with toluene (3×10 mL) to remove traces of TFA. The pure product was obtained by preparative RP-HPLC (27%, 11 μmol, 12 mg): ^{1}H NMR (600 MHz, CD_3OH): $\delta = 8.93$ (NH Phe, d, $J = 2.98$ Hz, 1H), 8.87 (NH TrA, d, $J = 2.86$ Hz, 1H), 8.77 (NH Leu, d, $J = 9.43$ Hz, 1H), 8.73 (NH Leu, d, $J = 9.40$ Hz, 1H), 8.73 (NH Orn, d, $J = 9.52$ Hz, 1H), 8.72 (triazole, s, 1H), 8.71 (NH Orn, d, $J = 10.04$, 1H), 7.71 (NH Val, d, $J = 9.01$ Hz, 2H), 7.33–7.24 (Ar Phe, m, 5H), 4.97 (H$^\alpha$ Orn, 2H), 4.67 (H$^\alpha$ Leu, 1H), 4.66 (H$^\alpha$ Leu, 1H), 4.61 (H$^\alpha$ TrA, 1H), 4.57 (H$^\alpha$ Pro, 2H), 4.49 (H$^\alpha$ Phe, 1H), 4.15 (H$^\alpha$ Val, 2H), 3.73 (H$^\delta$ Pro, 2H), 3.14 (H$^\beta$ TrA, 2H), 3.09 (H$^\beta$ Phe, 1H), 3.03 (H$^\delta$ Orn, 2H), 2.93 (H$^\beta$ Phe, 1H), 2.84 (H$^\delta$ Orn, 2H), 2.47 (H$^\delta$ Pro, 2H), 2.27 (H$^\beta$ Val, 2H), 2.05 (H$^\beta$ Orn, 2H), 2.00 (H$^\beta$ Pro, 2H), 1.77 (H$^\gamma$ Orn, 4H), 1.69 (H$^\beta$ Pro, 2H), 1.69 (H$^\gamma$ Pro, 2H), 1.59 (H$^\beta$ Orn, 2H), 1.58 (H$^\gamma$ Pro, 2H), 1.54 (H$^\beta$ Leu, 2H), 1.51 (H$^\gamma$ Leu, 2H), 1.41 (H$^\beta$ Leu, 2H), 0.97 (H$^\gamma$ Val, 6H), 0.89 (H$^\gamma$ Val, 6H), 0.89 ppm (H$^\delta$ Leu, 12H); ^{13}C NMR (150 MHz, CD_3OH): $\delta = 173.6$, 173.5, 173.4, 172.8, 172.7, 172.4, 136.8, 130.4, 129.7, 128.5, 62.2, 62.0, 60.4, 60.4, 55.9, 52.4, 51.4, 51.3, 48.2, 47.9, 42.0, 41.5, 40.5, 31.9, 30.8, 30.6, 25.6, 25.6, 24.6, 23.2, 23.2, 23.0, 22.9, 19.6, 19.5, 19.4 ppm; LC/MS: $t_R = 7.55$ min (10→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1132.60 $[M+H]^+$; HRMS calculated for $C_{56}H_{90}N_{15}O_{10}$ $[M+H]^+$: 1132.69896, found: 1132.69989.

Peptide 2b: Yield: 45% (18 μmol, 21 mg): ^{1}H NMR (600 MHz, CD_3OH): $\delta = 8.93$ (NH DPhe, d, $J = 2.99$ Hz, 1H), 8.87 (NH DTrA, d, $J =$ 3.05 Hz, 1H), 8.74 (NH Leu, 1H), 8.72 (NH Leu, 1H), 8.70 (NH Orn, 2H), 7.93 (triazole, s, 1H), 7.83 (NH_2 Orn, brs, 2H), 7.73 (NH_2 Orn, brs, 2H), 7.70 (NH Val, d, $J = 8.99$ Hz, 1H), 7.64 (NH Val, d, $J =$ 8.92 Hz, 1H), 7.39–7.35 (Ar Bn, m, 5H), 7.33–7.24 (Ar Phe, m, 5H), 5.57 (CH_2Ph, s, 2H), 4.99 (H$^\alpha$ Orn, m, 2H), 4.67 (H$^\alpha$ Leu, m, 2H), 4.51 (H$^\alpha$ Phe, Hα TrA, m, 2H), 4.33 (H$^\alpha$ Pro, m, 2H), 4.13 (H$^\alpha$ Val,1H), 4.11 (H$^\alpha$ Val,1H), 3.87 (H$^\delta$ Pro, m, 1H), 3.73 (H$^\delta$ Pro, m, 1H), 3.08 (H$^\beta$ TrA, m, 2H), 3.03 (H$^\delta$ Orn, 1H), 2.99 (H$^\delta$ Orn, 1H), 2.95 (H$^\beta$ Phe, m, 2H), 2.86 (H$^\delta$ Orn, 1H), 2.81 (H$^\delta$ Orn, 1H), 2,67 (H$^\delta$ Pro, 1H), 2.46 (H$^\delta$, 1H), 2.26 (H$^\beta$ Val, 2H), 2.04 (H$^\beta$ Pro, 1H), 2.03 (H$^\beta$ Orn, 4H), 1.98 (H$^\beta$ Pro, 1H), 1.75 (H$^\gamma$ Orn, 2H), 1.69 (H$^\gamma$ Pro, 2H), 1.68 (H$^\beta$ Pro, 1H), 1.59 (H$^\beta$ Pro, 2H), 1.56 (H$^\gamma$ Orn, 2H), 1.55 (H$^\gamma$ Pro, 2xH), 1.50 (H$^\beta$

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Leu, 4H), 1.40 (H^{γ} Leu, 2H), 0.96 (H^{γ} Val, 6H), 0.89 (H^{δ} Leu, 12H), 0.87 ppm (H^{γ} Val, 6H); ^{13}C NMR (150 MHz, CD_3OH): δ = 173.5, 172.4, 172.4, 136.8, 136.8, 130.4, 130.0, 129.7, 129.7, 129.2, 128.5, 124.5, 62.1, 62.0, 60.4, 60.4, 55.9, 54.9, 54.1, 52.4, 51.4, 51.3, 47.9, 40.6, 31.9, 31.9, 25.6, 25.6, 24.6, 23.2, 23.2, 23.0, 22.9, 19.6, 19.5 ppm; LC/MS: t_R = 8.06 min (10→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1222.73 $[M+H]^+$; HRMS calculated for $C_{63}H_{96}N_{15}O_{10}$ $[M+H]^+$: 1222.74591, found: 1222.74704.

Peptide 2c: Yield: 37% (14 μmol, 18 mg): ^{1}H NMR (600 MHz, CD_3OH): δ = 8.92 (NH Phe, d, *J* = 3.30 Hz, 1H), 8.82 (NH TrA, d, *J* = 3.32 Hz, 1H), 8.71 (NH Leu, d, *J* = 9.37 Hz, 1H), 8.70 (NH Leu, d, *J* = 9.43, 1H), 8.67 (NH Orn, d, *J* = 9.42 Hz, 2H), 8.25–7.51 (naph, m, 7H), 7.85 (triazole, s, 1H), 7.82 (NH_2 Orn, brs, 2H), 7.70 (NH_2 Orn, brs, 2H), 7.69 (NH Val, d, *J* = 8.97 Hz, 1H), 7.54 (NH Val, 1H), 7.33–7.23 (Ar Phe, m, 5H), 6.07 (CH_2Naph, dd, *J* = 14.4 Hz, *J* = 41.4 Hz, 2H), 4.95 (H^{α} Orn, 2H), 4.64 (H^{α} Leu, 1H), 4.59 (H^{α} Leu, 1H), 4.49 (H^{α} Phe, 1H), 4.41 (H^{α} TrA, 1H), 4.33 (H^{α} Pro, 1H), 4.15 (H^{α} Pro, 1H), 4.13 (H^{α} Val, 1H), 4.07 (H^{α} Val, 1H), 3.72 (H^{δ} Pro, 1H), 3.61 (H^{δ} Pro, 1H), 3.09 (H^{β} TrA, 1H), 3.08 (H^{β} Phe, 1H), 3.02 (H^{δ} Orn, 1H), 3.01 (H^{β} TrA, 1H), 2.96 (H^{δ} Orn, 1H), 2.92 (H^{β} Phe, 1H), 2.84 (H^{δ} Orn, 1H), 2.79 (H^{δ} Orn, 1H), 2.46 (H^{δ} Pro, 1H), 2.32 (H^{δ} Pro, 1H), 2.24 (H^{β} Val, 1H), 2.20 (H^{β} Val, 1H), 2.03 (H^{β} Orn, 2H), 2.01 (H^{β} Orn, 2H), 1.99 (H^{β} Pro, 1H), 1.75 (H^{γ} Orn, 1H), 1.73 (H^{β} Pro, 1H), 1.70 (H^{γ} Orn, 1H), 1.68 (H^{γ} Pro, 1H), 1.68 (H^{β} Pro, 1H), 1.57 (H^{γ} Pro, 1H), 1.56 (H^{γ} Orn, 1H), 1.54 (H^{γ} Orn, 1H), 1.52 (H^{β} Leu, 2H), 1.47 (H^{γ} Pro, 1H), 1.46 (H^{β} Leu, 2H), 1.37 (H^{γ} Leu, 1H), 1.36 (H^{γ} Leu, 1H), 1.09 (H^{β} Pro, 1H), 1.07 (H^{γ} Pro, 1H), 0.94 (H^{γ} Val, 3H), 0.88 (H^{δ} Leu, 12H), 0.86 (H^{γ} Val, 6H), 0.85 ppm (H^{γ} Val, 3H); ^{13}C NMR (150 MHz, CD_3OH): δ = 173.6, 173.5, 173.4, 173.3, 172.8, 172.7, 172.4, 172.3, 161.2, 143.3, 136.8, 135.5, 132.4, 132.0, 131.0, 130.4, 130.0, 129.7, 129.4, 128.5, 128.0, 127.4, 126.6, 124.4, 124.1, 105.4, 67.9, 61.9, 60.4, 55.9, 54.0, 52.9, 52.4, 51.4, 51.3, 47.9, 41.9, 41.7, 40.5, 40.5, 37.2, 31.9, 31.8, 30.8, 30.7, 30.6, 30.4, 27.0, 25.6, 25.5, 24.6, 24.4, 24.2, 23.2, 23.2, 23.0, 22.9 ppm; LC/MS: t_R = 7.66 min (10→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1273.1 $[M+H]^+$; HRMS calculated for $C_{67}H_{98}N_{15}O_{10}$ $[M+H]^+$: 1272.76156, found: 1272.76242.

Peptide 2d: Yield: 31% (12 μmol, 16 mg): ^{1}H NMR (600 MHz, CD_3OH): δ = 8.92 (NH Phe, d, *J* = 3.30 Hz, 1H), 8.85–7.64 (phen, m, 9H), 8.82 (NH TrA, d, *J* = 3.32 Hz, 1H), 8.71 (NH Leu, d, *J* = 9.37 Hz, 1H), 8.70 (NH Leu, d, *J* = 9.43 Hz, 1H), 8.67 (NH Orn, d, *J* = 9.42 Hz, 2H), 7.89 (triazole, s, 1H), 7.82 (NH_2 Orn, brs, 2H), 7.70 (NH_2 Orn, brs, 2H), 7.69 (NH Val, d, *J* = 8.97 Hz, 1H), 7.54 (NH Val, 1H), 7.33–7.23 (Ar Phe, m, 5H), 6.12 (CH_2Phen, dd, *J* = 14.77 Hz, *J* = 38.75 Hz, 2H), 4.95 (H^{α} Orn, 2H), 4.64 (H^{α} Leu, 1H), 4.59 (H^{α} Leu, 1H), 4.49 (H^{α} Phe, 1H), 4.41 (H^{α} TrA, 1H), 4.33 (H^{α} Pro, 1H), 4.15 (H^{α} Pro, 1H), 4.13 (H^{α} Val, 1H), 4.07 (H^{α} Val, 1H), 3.72 (H^{δ} Pro, 1H), 3.61 (H^{δ} Pro, 1H), 3.09 (H^{β} Phe, 1H), 3.08 (H^{β} Phe, 1H), 3.02 (H^{δ} Orn, 1H), 3.01 (H^{β} Phe, 1H), 2.92 (H^{β} Phe, 1H), 2.96 (H^{δ} Orn, 1H), 2.84 (H^{δ} Orn, 1H), 2.79 (H^{δ} Orn, 1H), 2.46 (H^{δ} Pro, 1H), 2.32 (H^{δ} Pro, 1H), 2.24 (H^{β} Val, 1H), 2.20 (H^{β} Val, 1H), 2.03 (H^{β} Orn, 2H), 2.01 (H^{β} Orn, 2H), 1.99 (H^{β} Pro, 1H), 1.75 (H^{γ} Orn, 1H), 1.73 (H^{β} Pro, 1H), 1.70 (H^{γ} Orn, 1H), 1.68 (H^{β} Pro, 1H), 1.68 (H^{γ} Pro, 1H), 1.57 (H^{γ} Pro, 1H), 1.56 (H^{γ} Orn, 1H), 1.54 (H^{γ} Orn, 1H), 1.52 (H^{β} Leu, 2H), 1.47 (H^{γ} Pro, 1H), 1.46 (H^{β} Leu, 2H), 1.37 (H^{γ} Leu, 1H), 1.36 (H^{γ} Leu, 1H), 1.09 (H^{β} Pro, 1H), 1.07 (H^{γ} Pro, 1H), 0.94 (H^{γ} Val, 3H), 0.88 (H^{δ} Leu, 12H),0.86 (H^{γ} Val, 6H), 0.85 ppm (H^{γ} Val, 3H); ^{13}C NMR (150 MHz, CD_3OH): δ = 173.5, 173.4, 172.7, 172.4, 172.3, 143.4, 136.8, 132.4, 132.3, 132.1, 131.0, 130.9, 130.3, 129.9, 129.6, 128.9, 128.5, 128.3, 128.3, 128.2, 125.0, 124.6, 124.4, 123.7, 61.9, 61.9, 60.4, 55.9, 54.0, 53.5, 52.3, 51.4, 51.2, 47.9, 47.8, 41.9, 40.5, 31.9, 31.8, 30.6, 25.6, 25.5, 24.5, 23.2, 23.1, 23.0, 22.9, 19.6, 19.5, 19.4 ppm; LC/MS: t_R = 8.36 min (10→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1323.1 $[M+H]^+$; HRMS calculated for $C_{71}H_{100}N_{15}O_{10}$ $[M+H]^+$: 1322.77721, found: 1322.77827.

Peptide 2e: Yield: 36% (14 μmol, 19 mg): ^{1}H NMR (750 MHz, CD_3OH): δ = 8.92–8.07 (Ar pyr, m, 9H), 8.88 (NH Phe, d, *J* = 2.53 Hz, 1H), 8.77 (NH TrA, d, *J* = 2.40 Hz, 1H), 8.67 (NH Leu, d, *J* = 9.17 Hz, 1H), 8.66 (NH Leu, d, *J* = 9.72 Hz, 1H), 8.65 (NH Orn, d, *J* = 9.82 Hz, 1H), 8.62 (NH Orn, d, *J* = 9.29 Hz, 1H), 7.90 (triazole, s, 1H), 7.80 (NH_2 Orn, brs, 2H), 7.68 (NH Val, d, *J* = 8.90 Hz, 1H), 7.64 (NH_2 Orn, brs, 2H), 7.44 (NH Val, d, *J* = 8.86 Hz, 1H), 7.31–7.23 (Ar Phe, m, 5H), 6.36 (CH_2pyr, dd, *J* = 14.89 Hz, *J* = 72.78 Hz, 2H), 4.90 (H^{α} Orn, 1H), 4.88 (H^{α} Orn, 1H), 4.62 (H^{α} Leu, 1H), 4.55 (H^{α} Leu, 1H), 4.48 (H^{α} Phe, 1H), 4.36 (H^{α} TrA, 1H), 4.32 (H^{α} Pro, 1H), 4.11 (H^{α} Val, 1H), 4.01 (H^{α} Pro, 1H), 3.98 (H^{α} Val, 1H), 3.72 (H^{δ} Pro, 1H), 3.47 (H^{δ} Pro, 1H), 3.10 (H^{β} Phe, 1H), 3.08 (H^{β} Phe, 1H), 3.00 (H^{β} Phe, 1H), 2.99 (H^{δ} Orn, 1H), 2.92 (H^{δ} Orn, 1H), 2.92 (H^{β} Phe, 1H), 2.81 (H^{δ} Orn, 1H), 2.76 (H^{δ} Orn, 1H), 2.47 (H^{δ} Pro, 1H), 2.23 (H^{β} Val, 1H), 2.17 (H^{δ} Pro, 1H), 2.13 (H^{β} Val, 1H), 2.00 (H^{β} Orn, 1H), 1.98 (H^{β} Orn, 1H), 1.97 (H^{β} Pro, 1H), 1.72 (H^{γ} Orn, 2H), 1.68 (H^{γ} Pro, 1H), 1.68 (H^{γ} Orn, 2H), 1.66 (H^{β} Pro, 1H), 1.56 (H^{γ} Pro, 1H), 1.53 (H^{β} Orn, 1H), 1.52 (H^{β} Orn, 1H), 1.50 (H^{β} Leu, 2H), 1.42 (H^{γ} Leu, 1H), 1.35 (H^{β} Leu, 2H), 1.31 (H^{γ} Leu, 1H), 1.30 (H^{γ} Pro, 1H), 1.15 (H^{γ} Pro, 1H), 0.93 (H^{γ} Val, 3H), 0.85 (H^{δ} Leu, 6H), 0.84 (H^{γ} Val, 3H), 0.80 (H^{δ} Leu, 6H), 0.80 (H^{γ} Val, 6H), 0.72 (H^{β} Pro, 1H), 0.59 ppm (H^{β} Pro, 1H); ^{13}C NMR (187 MHz, CD_3OH): δ = 173.5, 173.4, 173.4, 172.7, 172.7, 172.3, 143.4, 136.8, 133.5, 132.5, 131.9, 130.3, 129.7, 129.6, 129.4, 129.2, 128.5, 128.2, 127.6, 127.0, 126.8, 126.1, 125.5, 124.3, 123.4, 61.9, 61.7, 60.4, 60.3, 55.9, 54.0, 53.0, 52.3, 51.4, 51.2, 49.9, 47.9, 47.7, 41.9, 41.6, 40.5, 31.9, 31.7, 30.6, 25.6, 25.5, 24.5, 23.1, 23.0, 22.8, 19.5, 19.4, 19.4 ppm; LC/MS: t_R = 9.21 min (10→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1346.80 $[M+H]^+$; HRMS calculated for $C_{73}H_{100}N_{15}O_{10}$ $[M+H]^+$: 1346.77721, found: 1346.77851.

***cyclo*-(^{D}Phe-Pro-Val-Orn-Leu-^{D}His-Pro-Val-Orn-Leu) (3)**: Yield: 43% (17 μmol, 19 mg): ^{1}H NMR (600 MHz, CD_3OH): δ = 8.94 (NH, Phe, d, *J* = 3.12 Hz, 1H), 8.86 (NH His, d, *J* = 3.60 Hz, 1H), 8.82 (2-CH im, s, 1H), 8.74 (NH Leu, d, *J* = 8.77 Hz, 2H), 8.74 (NH Orn, d, *J* = 8.88 Hz, 2H), 7.70 (NH Val, d, *J* = 8.97 Hz, 1H), 7.63 (NH Val, d, *J* = 8.97 Hz, 1H), 7.45 (5-CH im, s, 1H), 7.32–7.24 (Ar Phe, m, 5H), 4.98 (H^{α} Orn, 2H), 4.71 (H^{α} His, 1H), 4.63 (H^{α} Leu, 2H), 4.51 (H^{α} Pro, 1H), 4.49 (H^{α} Phe, 1H), 4.34 (H^{α} Pro, 1H), 4.19 (H^{α} Val, 1H), 4.14 (H^{α} Val, 1H), 4.09 (H^{δ} Pro, 1H), 3.74 (H^{δ} Pro, 1H), 3.37 (H^{δ} Pro, 1H), 3.18 (H^{β} His, 2H), 3.10 (H^{β} Phe, 1H), 3.02 (H^{δ} Orn, 2H), 2.95 (H^{β} Phe, 1H), 2.88 (H^{δ} Orn, 2H), 2.48 (H^{δ} Pro, 1H), 2.29 (H^{δ} Val, 1H), 2.26 (H^{β} Val, 1H), 2.17 (H^{β} Pro, 1H), 2.06 (H^{β} Pro, 1H), 2.06 (H^{β} Orn, 2H), 2.01 (H^{β} Pro, 1H), 2.00 (H^{γ} Pro, 1H), 1.92 (H^{γ} Pro, 1H), 1.73 (H^{γ} Orn, 4H), 1.69 (H^{γ} Pro, 1H), 1.69 (H^{β} Pro, 1H), 1.63 (H^{β} Orn, 2H), 1.57 (H^{γ} Pro, 1H), 1.53 (H^{β} Leu, 2H), 1.49 (H^{γ} Leu, 2H), 1.42 (H^{β} Leu, 2H), 0.99 (H^{γ} Val, 3H), 0.95 (H^{γ} Val, 3H), 0.91 (H^{γ} Val, 3H), 0.88 (H^{δ} Leu, 12H), 0.86 ppm (H^{γ} Val, 3H); ^{13}C NMR (150 MHz, CD_3OH): δ = 173.5, 173.5, 173.4, 172.7, 172.5, 172.4, 172.3, 136.9, 130.4, 130.1, 129.6, 128.4, 118.9, 62.3, 61.9, 60.4, 60.4, 55.9, 53.2, 52.4, 51.4, 51.3, 48.4, 47.9, 42.0, 41.7, 40.5, 37.2, 31.9, 31.9, 30.9, 30.7, 30.7, 30.6, 25.7, 25.6, 25.5, 24.7, 24.6, 24.4, 23.2, 23.1, 23.0, 22.9, 19.6 ppm; LC/MS: t_R = 6.81 min (10→90% CH_3CN, 15 min run); MS (ESI): *m/z*: 1131.53 $[M+H]^+$; HRMS calculated for $C_{57}H_{91}N_{14}O_{10}$ $[M+H]^+$: 1131.70371, found: 1131.70509.

Biology

Antibacterial screening: Bacteria were stored at −70 °C and grown at 30 °C on Columbia agar with sheep blood (Oxoid, Wesel, Germany) suspended in physiological saline solution (PSS) until an optical density of 0.1 AU was reached (at 595 nm, 1 cm cuvette). The sus-

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pension was diluted (10×) with PSS, and 2 μL of this inoculum was added to 100 μL growth medium, nutrient broth (Difco, ref. 234000, lot 6194895) with yeast extract (Oxoid, LP 0021, lot 900711, 2 g per 400 mL broth) in microtiter 96-well plates. The peptides GS (**1**) and **2a**–**e** and **3** were dissolved in EtOH (4 g L^{-1}) and diluted in distilled H_2O (1000 mg L^{-1}); dilution in the broth achieved the appropriate concentration (64, 32, 16, 8, 4 and 1 mg L^{-1}). The bacteria were incubated at 30 °C (24–96 h) and the minimum inhibitory concentration (MIC), the lowest concentration inhibiting bacterial growth, was determined.

Hemolytic assay: Freshly drawn heparinised blood was centrifuged for 10 min at 1000 *g* at 10 °C. Subsequently, the erythrocyte pellet was washed 0.85% saline solution (3×) and diluted with saline to a 1/25 packed volume of red blood cells. The peptides to be evaluated were dissolved in a 30% DMSO/0.5 mM saline solution to give a 1.5 mM solution of peptide. If a suspension was formed, the suspension was sonicated for a few seconds. A 1% Triton-X solution was prepared. Subsequently, 100 μL of saline solution were dispensed in columns 1–11 of a microtiter plate, and 100 μL of 1% Triton solution were dispensed in column 12. To wells A1–C1, 100 μL of the peptide were added and mixed properly. Aliquots (100 μL) of wells A1–C1 were dispensed into wells A2–C2. This process was repeated until wells A10–C10, followed by discarding 100 μL of wells A10–C10. These steps were repeated for the other peptides. Subsequently, 50 μL of the red-blood-cell solution were added to the wells and the plates were incubated at 37 °C for 4 h. After incubation, the plates were centrifuged at 1000 *g* at 10 °C for 4 min. In a new microtiter plate, 50 μL of the supernatant of each well were dispensed into a corresponding well. The absorbance at 405 nm was measured and the percentage of hemolysis was determined.

X-ray crystallography

Crystallization: **Peptide 2b**: Suitable colourless prism-shaped crystals were obtained after slow evaporation at 295 K of 2 μL droplets of 10 mg mL^{-1} peptide in 33% (*v*/*v*) solution of MeOH in H_2O plus 2 μL of 0.05 M HCl in MeOH under paraffin oil in a Terasaki plate.

Cyclo-(D-Phe-Pro-Val-Orn-Leu-D-His-Pro-Val-Orn-Leu) (3): Suitable colourless prism-shaped crystals from peptide **3** were obtained after slow evaporation at 295 K of 2 μL droplets of 28 mg mL^{-1} peptide in H_2O plus 2 μL of 40% (*v*/*v*) DMSO and 0.1 M Tris·HCl in MeOH under paraffin oil in a Terasaki plate.

*Crystal structure determination of **2b** and **3***: Crystals were mounted into cryoloops and directly flash-frozen in liquid nitrogen. Preliminary diffraction tests were performed at beamline BM30 A and high- and low-resolution data sets were collected at beamline ID23–2 at the ESRF (Grenoble, France). Images were collected with DNA software[21] and processed to 1.02 Å with MOSFLM[22] for peptide **2b** or to 0.97 Å with XDS[23] for peptide **3**. Low- and high-resolution data sets were scaled and merged with POINTLESS and SCALA.[24] Both structures could be solved by direct methods using the SHELXD[25] program and were refined with no intensity cutoff using the full-matrix least-squares methods on F^2 with SHELXH[25] included in the WinGX[26] package. Throughout the refinement, bond length, bond angle and planarity restraints were imposed. All hydrogen positions were calculated and refined using a riding atom model. There are two and four crystallographically independent molecules per asymmetric unit in **2b** and **3** crystal structures, respectively, and there are several disordered parts in all molecules. All nonhydrogen atoms were refined using an anisotropic model. Selected crystallographic data is reported in the Supporting Information. Final figures were created using PyMOL software (PyMOL Molecular Graphics System, Schrödinger, LLC). CCDC 804272 and CCDC 804273 contain the supplementary crystallographic data for compounds **2b** and **3**, respectively. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/data_request/cif.

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