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# Structural and biological evaluation of some loloatin C analogues

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ABSTRACT

Loloatin C is a cyclic cationic antimicrobial peptide which is active against Gram positive as well as certain Gram negative bacteria. Unfortunately, it is equally potent against human erythrocytes. To probe the structure–activity relationship of this promising antibiotic peptide, amino acid substitution and/or incorporation of a constraint sugar amino acid dipeptide isoster has been applied. Six new derivatives have been synthesized using SPPS and their solution structure investigated using NMR studies. Finally, the antimicrobial and the hemolytic activities have been determined.



## 1. Introduction

The loloatins are cationic antimicrobial peptides (CAPs) isolated from laboratory cultures of bacteria collected from the reefs of Loloata Island, Papua New Guinea.[1] The loloatins comprise four cyclic peptides that are named loloatin A, B, C and D (Fig. 1). Loloatins A–D are active against Gram positive bacteria with MIC values between 1 and 8 μg/mL against a panel of eight bacterial strains tested.[2] Besides being the most potent of the four family members, loloatin C is also the only one active against the Gram negative strain *Escherichia coli*, making it an interesting lead compound for further development towards broad spectrum antibiotics.

Loloatin C has a remarkable structural resemblance to Gramicidin S (GS). Both are cyclic decapeptides, and share 4 out of the 10 amino acids in their primary sequence. GS is composed of two identical pentapeptides, each consisting of a β-strand (Val-Orn-Leu) connected to a type II' β-turn motif (ᴰPhe-Pro). Loloatin C has one closely related pentapeptide motif (with a ᴰTyr instead of a ᴰPhe) connected to a Trp-ᴰPhe-Asn-Asp-Trp sequence. Whereas GS is likely to carry two positive charges in biological surroundings, loloatin C has a zwitterionic character. The solution structure of GS has been extensively studied in a variety of solvents.[3] In each of these solvents GS adopts a rigid cyclic β-hairpin conformation that is stabilized by four interstrand hydrogen bonds. This structure is characterized by an amphiphilic orientation of the individual side chains, with the hydrophobic Val and Leu residues on one side of the molecule and the hydrophilic Orn side chains on the other. The solution structure of loloatin C is much less well defined,[4] and is found to be solvent dependent. In DMSO, a hydrogen bond accepting solvent, the secondary structure of loloatin C was analyzed by means of chemical shift perturbation and NOESY analysis. A β-strand composed of Leu-Orn-Val and an opposing α-helical[5] Trp-ᴰPhe-Asn strand are interconnected via two turn motifs composed of Pro-ᴰTyr and Asp-Trp. The lack of chemical shift reference data in water/trifluoroethanol (TFE, a hydrogen bond donating solvent known to stabilize secondary structural elements)[6] defies conclusions about the conformation of loloatin C. In a 30% TFE/$H_2O$ mixture, NOESY analysis revealed a less defined structure with only one secondary structural element, namely, an inverse γ-turn around the ᴰTyr-Pro-Trp sequence. Increasing the TFE content in the solvent mixture to 70% induced a remarkable structural change. A dumbell-like conformation was identified centred around the Orn and ᴰPhe residues. Interestingly, this conformation orientates the hydrophilic sidechains (Orn, Asn and Asp) to one side of the molecule and the remaining hydrophobic side chains to the other, resulting in an amphiphilic overall structure.

We have previously reported on GS analogue **7** that contains a sugar amino acid (SAA) dipeptide isoster[7] derived from **6** as replacement of one of the two Pro-ᴰPhe β-turns.[8] We found that the secondary structure of this derivative differs slightly from that of GS, but that the biological activities of the two compounds were closely related. We here report on the synthesis and evaluation of a

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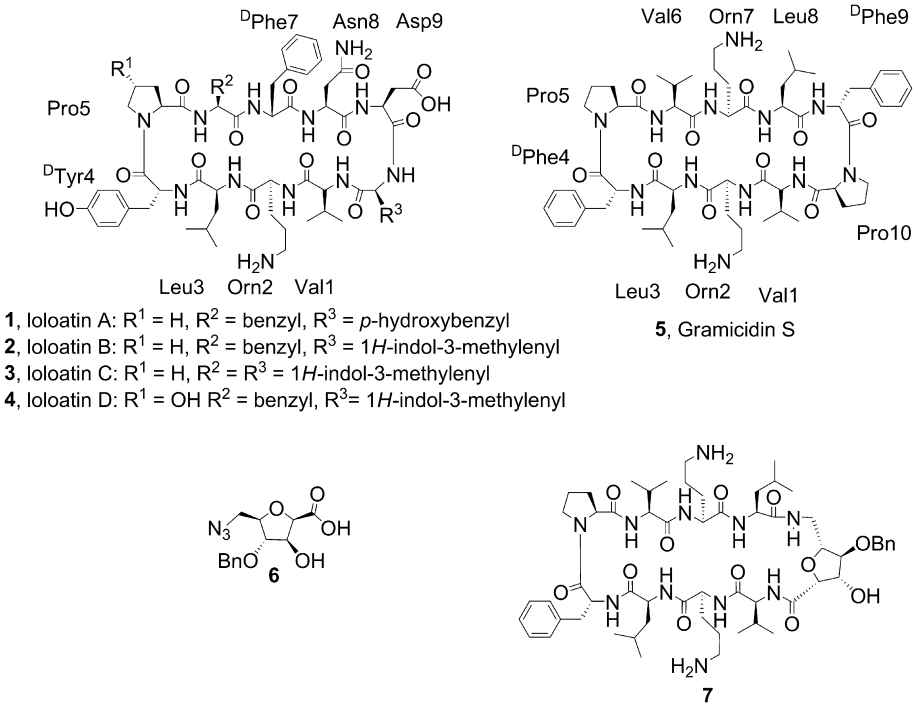



**Figure 1.** Structures of loloatins A–D, GS and sugar amino acid **6** containing GS derivative **7**.

set of loloatin C analogues modified in both strand and turn regions, and that include derivatives containing SAA **6** as a replacement of the turn region.

## 2. Results and discussion

The target molecules are depicted in Figure 2. In compound **8**, SAA **6** replaces the Pro-$^{D}$Tyr motif (Fig. 2). In compound **9** the

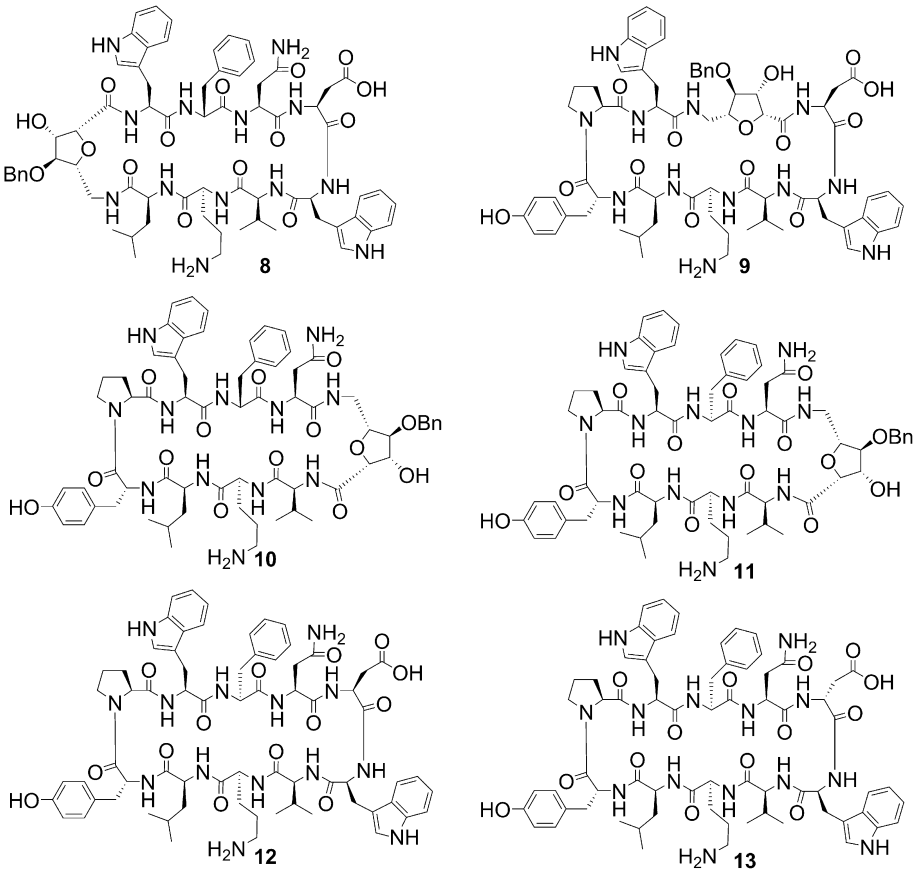



**Figure 2.** Loloatin C derivatives **8**–**13** that are the subject of the research described here.

Asn-$^{D}$Phe sequence is replaced (Fig. 2). Replacing the Asp-Trp motif with SAA **6** leads to compound **10**, whereas analogue **11** (Fig. 2) SAA **6** replaces the Asp-Trp motif and the $^{D}$Phe is replaced by $^{L}$Phe. The influence of using $^{L}$Phe instead of $^{D}$Phe is further studied in **12**. Compound **13** is analogous to **12**, but with $^{L}$Asp replaced by $^{D}$Asp (Fig. 2).

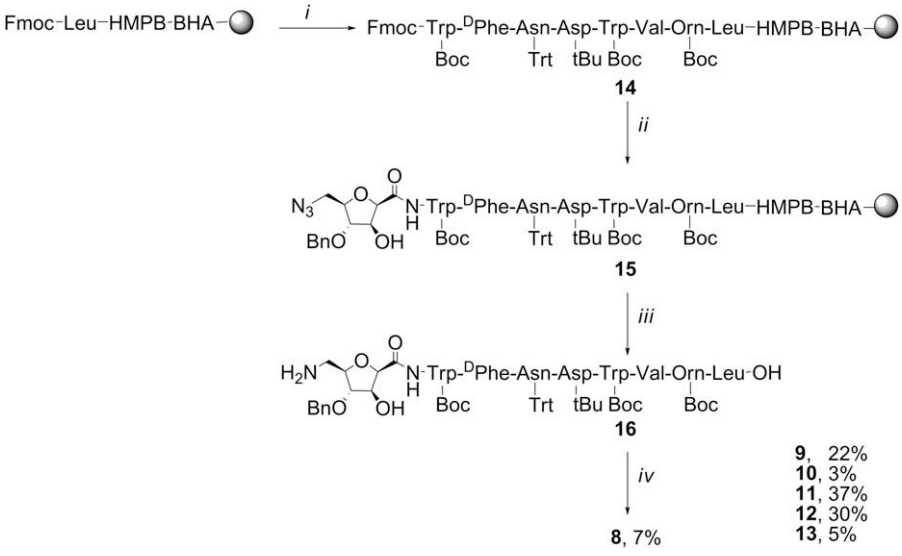
The synthesis of the loloatin C analogues is exemplified in Scheme 1 for the construction of compound **8**. Standard Fmoc based solid-phase peptide synthesis (SPPS) starting from commercially available HMPB-BHA resin preloaded with Fmoc-Leu is deprotected using 20% piperidine in NMP and subjected to seven coupling cycles under the agency of HCTU en DIPEA affording linear octapeptide **14**. Sugar amino acid **6** was coupled to deprotected **14** using 1.5 equiv of **6**, with prolonged coupling time. Staudinger reduction of the terminal azide functionality in **15** using $PMe_3$ in wet THF followed by acidic cleavage from the resin afforded **16** which was subsequently cyclized upon PyBOP/HOBt/DIPEA treatment in DMF under dilute conditions, deprotected using 50%TFA/DCM. HPLC purification yielded the target compound **8** in 7% yield. Compounds **9**–**13** were prepared following the same strategy in yields ranging from 3% to 37% after purification.

## 3. NMR studies of loloatin C (3) and derivatives 8–13

Next, attention was focused on the structural analysis of loloatin C (**3**) and the newly designed analogues **8**–**13**. The NMR spectra of our previously reported[7] GS derivatives were measured using $CD_3OH$ as solvent. Therefore, the structure of each of the loloatine analogues was assessed in methanol by the analysis of the vicinal $^{3}J_{NH\text{-}H\alpha}$ coupling constant[9] and the chemical shift perturbation.[10] The vicinal $^{3}J_{NH\text{-}H\alpha}$ coupling constant correlates with the dihedral angle within an amino acid residue. It can be used in a qualitative manner to identify secondary structural elements within a peptide. Consecutive large values (8.5–9.5 Hz) correspond to dihedral angles commonly found in β-sheets, small values for $^{3}J_{NH\text{-}H\alpha}$ (3.5–4.5 Hz) correspond to those found in an α-helix/turns. Isolated occurrences of small values for $^{3}J_{NH\text{-}H\alpha}$ (<4 Hz) indicate the presence of a turn within the peptide chain. Another qualitative method to distinguish between secondary structural elements is the chemical shift perturbation. This method relies of the comparison of the measured chemical shift of the α-proton of a certain amino acid and the standard value of that same amino acid in a random coil configuration ($\Delta\delta H_\alpha$ = observed $\delta H_\alpha$ − random coil $\delta H_\alpha$). This is based on the observation that α-protons of amino acids within an α-helix experience an upfield shift and residues within a β-sheet experience a downfield shift compared to the chemical shift of that residue in a random coil configuration.

The observed values for the vicinal coupling constants $^{3}J_{NH\text{-}H\alpha}$ for the loloatin C (**3**) and analogues are depicted in Figure 3A and the chemical shift perturbations are depicted in Figure 3B. The secondary structure of all derivatives contain a turn motif around $^{D}$Tyr ($^{3}J_{NH\text{-}H\alpha}$ <4 Hz), flanked by two strand regions (8.5 < $^{3}J_{NH\text{-}H\alpha}$ < 9.5 Hz). The Leu-Orn-Val sequence forms a β-strand in all analogues. Interpretation of the chemical shift perturbation underlines these findings. The residues in the Trp$_6$-$^{D/L}$Phe-Asn motif show both positive and negative values for the perturbation among the different compounds and no general trend is observed. In compound **10**, the $^{D}$Tyr, Trp$_6$ and $^{D}$Phe residues appear to form a helical region/turn, as evidenced by their negative chemical shift perturbation values. The position of residues 9 and 10 in these analogues are connected either by SAA **6** (in **11**) or by $^{(D\ or\ L)}$Asp-Trp motif (in **12** and **13,** respectively). On the basis of these 1D NMR analyses the structures of compounds **11** and **12** seem remarkably similar except for the regions round the $^{L}$Phe residues. The $^{1}$H NMR spectra of **9** and **13** show severe peak broadening indicating conformational flexibility, preventing complete assignment of their spectra.

To obtain more details concerning the structural properties of loloatin C and derivatives NOESY spectra were recorded. For this purpose NOESY spectra of loloatin C (**3**) in $CD_3OH$ and in TFE/$H_2O$ 7/3 were compared. At 500 MHz the amide region of compound **3** in TFE/$H_2O$ showed only poor resolution at ambient temperature hampering the assignment of the 2D spectra. Increasing the temperature resulted in an improved resolution with an optimum at 305 K (Fig. 4A). Despite the elevated temperature four NOE signals could be identified in the NH–NH region (Fig. 4B). In methanol, well resolved spectra at ambient temperature were obtained (Fig. 4C). Two important long range NOE's are observed in both solvents, with the NH–NH NOE between $^{D}$Phe and Val (B in Fig 4B and C) being the most prominent. Besides minor differences in these two solvent systems, loloatin C is folded back onto itself in




**Scheme 1.** Synthesis of lolatin C derivatives using SPPS. Reagents and conditions: 7 cycles of (a) 20% pip/NMP, 3 × 5 min; (b) Fmoc-AA-OH (5 equiv), HCTU (5 equiv), DIPEA (10 equiv) 90 min; (ii) **6** (1.5 equiv), HCTU (1.5 equiv), DIPEA (3 equiv), 16 h; (iii) (a) $PMe_3$ (1 M in 9:1 THF/$H_2O$, 25 equiv), 16 h; (b) 1% TFA/DCM; (iv) (a) PyBOP (5 equiv), HOBt (5 equiv), DIPEA (10 equiv), 16 h; (b) 50% TFA/DCM, 1 h.

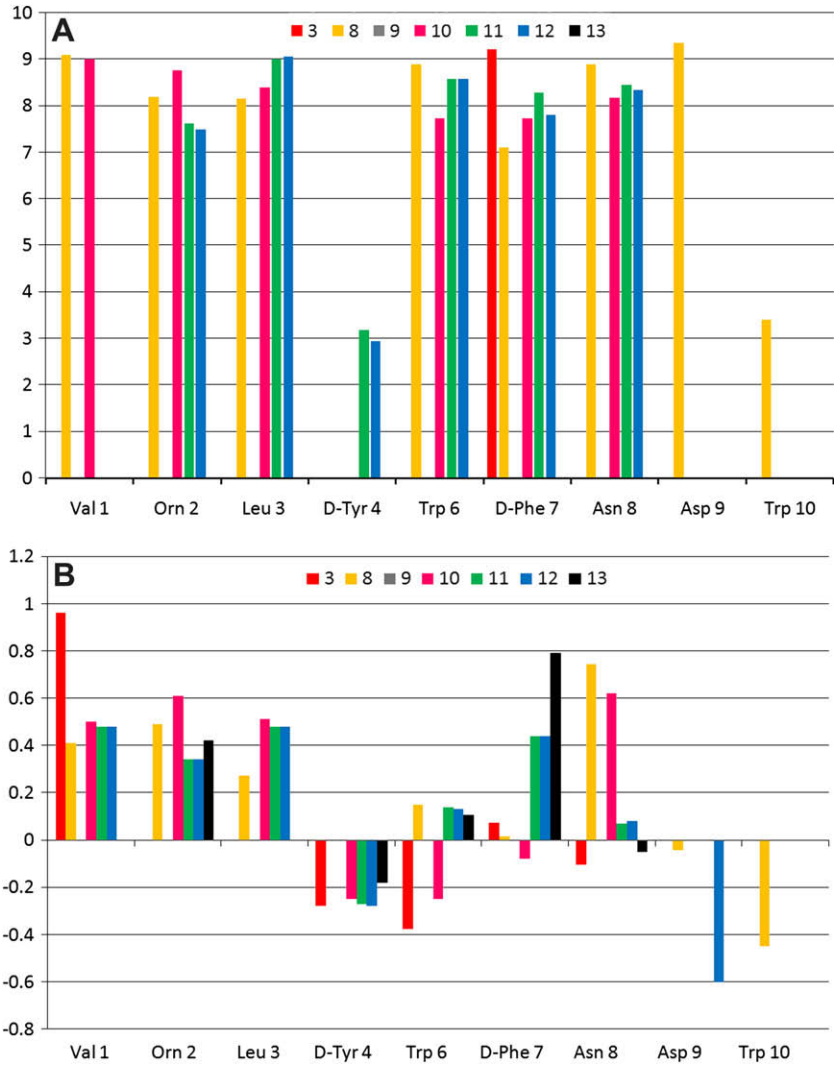



**Figure 3.** Values of the vicinal $^{3}J_{NH\text{-}H\alpha}$ (A) and of the chemical shift perturbation (B) for loloatin C (**3**) and derivatives **8**–**13** in $CD_3OH$. For some residues, amide protons appeared as broad singlets, α-protons were buried under other peaks or residues were replaced by **6** resulting in their omission.

agreement with adopting a dumbbell-like structure as described in the literature.[4]

Because of the ease of recording NMR spectra at ambient temperature the NOESY spectra of compounds **8**, **10**, **11** and **12** were for comparison also recorded in methanol. In Figure 4D, E and F parts of the NH–NH regions of compounds **8**, **11** and **12** are depicted. Compound **8** has a distorted turn region, presumably due to the presences of the sugar amino acid moiety and the $^{D}$Phe residue (Fig. 4D). The NOE's of compound **11** and **12** indicate beta-hairpin structures similar to gramicidine S (Fig. 4E and F). Compound **10** does not have long range NH–NH NOE's. Sharp NH signals in the proton spectrum, however together with several other NOE correlations are indicative of the presence of a secondary structure.

## 4. Biological evaluation

The antimicrobial and hemolytical potencies of the novel loloatin C derivatives were determined (Table 1 and Fig. 5). Whereas the native loloatin C (**3**) shows some antimicrobial potency against gram positive strains, it is inactive against the gram negative strains used in this assay. In addition, loloatin C is also hemolytically active.[11] As for the analogues **8**, **9**, **10** and **13** both the antimicrobial and hemolytical[12] activities are highly reduced compared to loloatin C. Only analogues **11** and **12** are marginally active in both assays.

## 5. Conclusion and discussion

Six novel analogues of loloatin C were synthesized and analyzed by means of NMR spectroscopy. The unexpectedly low yield for some of the derivatives can be explained by a difficult macrocyclization and a laborious HPLC purification. The secondary structures of these analogous were analyzed using 1D- and 2D-NMR spectroscopy. Analogues **3**, **8**, **10**, **11** and **12** adopt a secondary structure in solution (methanol), compounds **9** and **13** proved to have multiple conformations in the same solvent. The peak broadening of certain NH signals observed in the NMR spectrum of loloatin C (**3**) indicate partial conformational flexibility, however, a dumbbell-like conformation[4] is apparent as judged by the observed long range NOE's. The secondary structures of compounds **8**, **10**, **11** and **12** as judged by the NMR experiments differs substantially from the loloatin C structure.

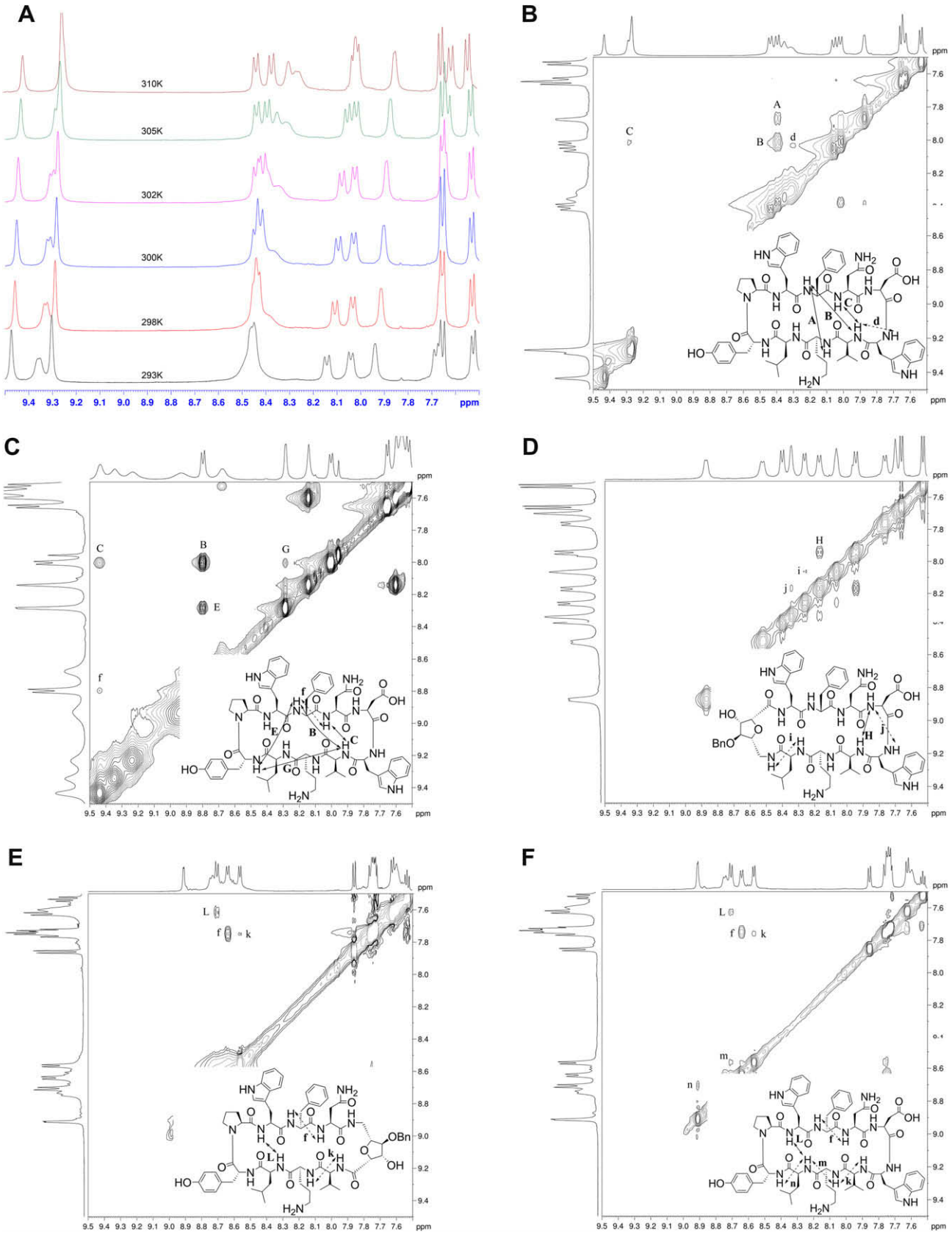



**Figure 4.** (A) The amide region of the 1D proton spectrum (500 MHz) of Loloatin C in TFE/$H_2O$ 7/3 at different temperatures. (B) A close-up of the NH–NH region of Loloatin C in TFE/$H_2O$ 7/3 at 305 K and assigned correlations. (C–F) Close-ups of the NH–NH region of the NOE spectra (600 MHz) in $CD_3OH$ of Loloatin C (C), **8** (D), **11** (E) and **12** (F) and the assigned correlations. Dashed arrows and lowercase signals indicate sequential NOEs, solid lines and uppercase are long range NOEs.

**Table 1**
MIC values for compounds **3** and **8–13**

| | Gram pos. *Staph. aureus* | Gram pos. *Staph. epidermis* | Gram pos. *Enterococ faecalis* | Gram. neg. *E. coli* | Gram neg. *P. auriginosa* | Gram pos. *Bacillus cereus* |
|---|---|---|---|---|---|---|
| **3** | 8 | 8 | 8 | >64 | >64 | 8 |
| **8** | >64 | >64 | >64 | >64 | >64 | >64 |
| **9** | >64 | >64 | >64 | >64 | >64 | >64 |
| **10** | >64 | >64 | >64 | >64 | >64 | >64 |
| **11** | >64 | 32 | >64 | >64 | >64 | 64 |
| **12** | 64 | 32 | >64 | >64 | >64 | 32 |
| **13** | >64 | >64 | >64 | >64 | >64 | >64 |

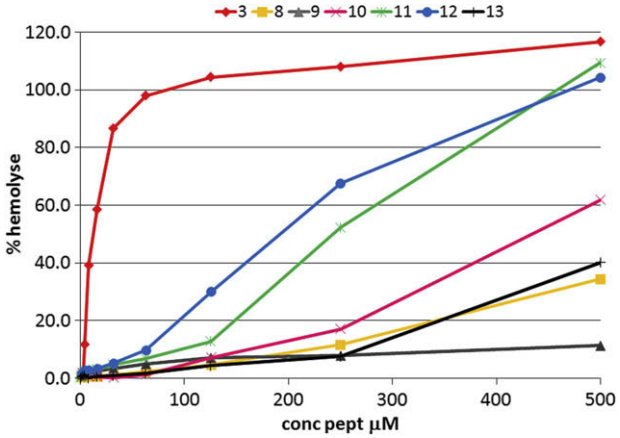



**Figure 5.** Hemolytic activity of loloatin C and derivatives **8–13**.

The biological activities of all analogues were rather low as compared with loloatin C with only marginal activities of analogues **11** and **12** in both the antimicrobial and hemolytical assay.

The reduced activity of analogues **8–13** are likely caused by their inability to adopt a similar amphiphilic biologically active conformation as the parent compound loloatin C. Thus, the strategy of replacing particular dipeptide sequences with the constraint sugar amino acid **6**, as successfully applied in the case of the cationic cyclic decapeptide GS, does not work in the case of the related zwitterionic peptide loloatin C.

## 6. Experimental section

For LC/MS analysis, an JASCO HPLC system (detection simultaneously at 214 and 254 nm) equipped with an analytical $C_{18}$ column (4.6 mmD × 250 mmL, 5µ particle size) in combination with buffers A: $H_2O$, B: MeCN and C: 0.5% aq TFA and coupled to a mass instrument with a custom-made Electronspray Interface (ESI) was used. For reversed-phase HPLC purification of the final compounds, an automated HPLC system supplied with a semi-preparative $C_{18}$ column (10.0 mmD × 250 mmL, 5µ particle size) was used. The applied buffers were A: $H_2O$, B: MeCN and C: 1.0% aq TFA. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution $R = 60{,}000$ at $m/z$ 400 (mass range $m/z = 150$–2000) and dioctylpthalate ($m/z = 391.28428$) as a 'lock mass'.[13] The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). $^1H$ and $^{13}C$ NMR spectra were measured on a Bruker AV-500 (500/125 MHz) or a Bruker DMX-600 (600/125 MHz). Chemical shifts are given in ppm ($\delta$) relative to TMS (0 ppm) or MeOD (3.30 ppm) and coupling constants are given in Hz.

### 6.1. General procedure for peptide synthesis

(a) *Stepwise elongation*: Fmoc-Leu-HMPB-BHA resin (196 mg, 0.51 mmol/g, 0.1 mmol) was submitted to seven cycles of Fmoc solid-phase synthesis with the appropriate commercially available amino acid building blocks Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Pro-OH, Fmoc-$^D$Phe-OH, Fmoc-Phe-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Asn(Trt)-OH and Fmoc-Trp(Boc)-OH as follows: (a) deprotection with piperidine/NMP (1/4, v/v, 5 mL, 15 min); (b) wash with NMP (5 mL, 3×, 3 min); (c) coupling of the appropriate Fmoc amino acid (5 equiv, 0.5 mmol) in the presence of HCTU (5 equiv, 0.5 mmol, 206 mg) and DIPEA (10 equiv, 1 mmol, 162 µL) which was preactivated for 2 min in NMP (5 mL) and shaken for 90 min; (d) wash with NMP (5 mL, 3×, 3 min). Couplings were monitored for completion by the Kaiser test.[14] Finally, the N-terminal amine was liberated by Fmoc-deprotection with piperidine/NMP (1/4, v/v, 5 mL, 15 min) followed by washing with NMP (5 mL, 3×, 3 min). Coupling of SAA **6** was performed as follows: To the resin-bound peptide, a preactivated solution of SAA **6** (1.5 equiv, 44 mg, 0.150 mmol), HCTU (1.5 equiv, 62 mg, 0.150 mmol) and DIPEA (3.0 equiv, 74 µL, 0.45 mmol) in NMP (3 mL) was added and the resulting suspension was shaken for 16 h. The resin was finally washed with NMP (5 mL, 3×, 3 min) to give the cyclized peptide.

(b) *On-resin Staudinger reduction*: The appropriate resin-bound azide was treated with a pre-mixed cocktail of $H_2O$ (0.5 mL) and $PMe_3$ (3.5 mL, 1 M in THF) and shaken for 16 h. The resin was washed with methanol (4 mL, 3×, 3 min) and DMF (4 mL, 3×, 3 min).

(c) *Cleavage from the resin*: Resin-bound nonapeptide was repeatedly treated with 1% TFA/DCM (4 mL). The filtrate was coevaporated with toluene (3×) and the residue was used as such in the cyclization step.

(d) *Cyclization*: The linear nonapeptide was taken up in DMF (5 mL) and added dropwise over the course of an hour to a solution of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (5 equiv, 270 mg, 0.5 mmol), HOBt (5 equiv, 67 mg, 0.5 mmol) and DIPEA (15 equiv, 254 µL, 1.5 mmol) in DMF (70 mL) and allowed to stir for 16 h. The solvent was removed in vacuo and the resulting mixture was used without further purification in the deprotection step.

(e) *Deprotection*: The crude cyclized peptide was treated with 50% TFA/DCM (10 mL) for 1 h, before it was concentrated and purified by HPLC purification.

### 6.2. Antibacterial assays

The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at −70 °C and grown at 30 °C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) suspended in physiological saline until an optical density of 0.1 AU (at 595 nm, 1 cm cuvette). The

suspension was diluted (10×) with physiological saline, and 2 μL of this inoculum was added to 100 μL growth medium, Nutrient Broth from Difco (ref. nr. 234000, lot nr. 6194895) with yeast extract (Oxoid LP 0021, lot nr. 900711, 2 g/400 mL broth) in microtiter plates (96 wells). The peptides were dissolved in ethanol (4 g/L) and diluted in distilled water (1000 mg/L), and twofold diluted in the broth (64, 32, 16, 8, 4 and 1 mg/L). Incubation at 30 °C (24–96 h) and the MIC was determined as the lowest concentration inhibiting bacterial growth.

### 6.3. Hemolytic assays

Freshly drawn heparinized blood was centrifuged for 10 min at 1000*g* at 10 °C. Subsequently, the erythrocyte pellet was washed three times with 0.85% saline solution 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 was dispensed in columns 1–11 of a microtiter plate, and 100 μL of 1% Triton solution was dispensed in column 12. To wells A1-C1, 100 μL of the peptide was added and mixed properly. Hundred microlitres 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 was 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 microtitre plate, 50 μL of the supernatant of each well was dispensed into a corresponding well. The absorbance at 405 nm was measured and the percentage of hemolysis was determined.

#### 6.3.1. *cyclo*-[Val-Orn-Leu-$^{D}$Tyr-Pro-Trp-$^{D}$Phe-Asn-Asp-Trp] 3

Prepared according to the general procedure. Yield: 19.7 mg, 13.5 μmol, 14%. LC–MS: *t*R 7.26 min (linear gradient 10→90% B in 13.5 min), *m/z* = 1335.6 $[M+H]^+$, 668.5 $[2M+H]^+$. $^1$H NMR (500 MHz, $CD_3OH$) $\delta$ 10.25 (s, 1H), 10.22 (s, 1H), 9.43 (bs, 1H), 9.34 (br s, 1H), 9.23 (br s, 1H), 8.93 (br s, 1H), 8.80 (d, *J* = 9.3, 1H), 8.68 (br s, 1H), 8.28 (d, *J* = 2.6, 1H), 8.14 (s, 1H), 8.00 (d, *J* = 8.6, 1H), 7.65 (d, *J* = 7.7, 1H), 7.55 (dd, *J* = 13.3, 29.2, 4H), 7.43–7.32 (m, 3H), 7.28–7.13 (m, 5H), 7.09–6.89 (m, 6H), 6.84 (s, 1H), 6.72 (br s, 1H), 6.63 (d, *J* = 8.1, 2H), 5.94–5.84 (m, 1H), 5.52 (d, *J* = 5.9, 1H), 4.78–4.68 (m, 1H), 4.68–4.60 (m, 2H), 4.32 (br s, 2H), 4.06 (d, *J* = 7.1, 1H), 3.65–3.63 (m, 1H), 3.45–3.35 (m, 2H), 3.22–3.00 (m, 4H), 3.00–2.79 (m, 4H), 2.64 (t, *J* = 13.5, 1H), 2.40–2.14 (m, 5H), 2.14–2.05 (m, 1H), 2.03–1.93 (m, 2H), 1.93–1.84 (m, 2H), 1.83–1.75 (m, 4H), 1.74–1.65 (m, 2H), 1.61–1.48 (m, 2H), 1.39–1.23 (m, 3H), 1.20 (s, 3H), 1.16 (m, 6H), 1.12–1.04 (m, 6H), 0.94–0.82 (m, 1H), 0.19 (s, 1H).

#### 6.3.2. *cyclo*-[Val-Orn-Leu-SAA-Trp-$^{D}$Phe-Asn-Asp-Trp] 8

Prepared according to the general procedure. Yield: 9.73 mg, 6.76 μmol, 7%. LC–MS: *t*R 5.60 min (linear gradient 10→90% B in 13.5 min), *m/z* = 1324.7 $[M+H]^+$, 663.1 $[2M+H]^+$. $^1$H NMR (500 MHz, $CD_3OH$) $\delta$ 10.39 (s, 1H), 10.33 (s, 1H), 8.88 (d, *J* = 7.1, 1H), 8.53 (d, *J* = 8.3, 1H), 8.39 (d, *J* = 8.9, 1H), 8.34 (d, *J* = 3.4, 1H), 8.25 (d, *J* = 8.2, 1H), 8.19 (d, *J* = 9.3, 1H), 8.08–8.03 (m, 1H), 7.98–7.93 (m, 1H), 7.75 (d, *J* = 8.9, 1H), 7.73 (s, 1H), 7.66 (d, *J* = 7.8, 1H), 7.52 (d, *J* = 7.9, 1H), 7.38 (br s, 1H), 7.34–6.94 (m, 20H), 5.48 (d, *J* = 7.7, 1H), 4.82 (dd, *J* = 8.1, 13.8, 1H), 4.71 (dt, *J* = 4.8, 10.2, 1H), 4.67–4.62 (m, 1H), 4.56 (d, *J* = 11.7, 1H), 4.52 (d, *J* = 11.6, 1H), 4.46–4.39 (m, 1H), 4.38–4.30 (m, 3H), 4.21 (d, *J* = 10.0, 2H), 3.84 (s, 1H), 3.76–3.68 (m, 1H), 3.34 (s, 1H), 3.24–3.15 (m, 3H), 3.11–3.04 (m, 3H), 2.99–2.92 (m, 1H), 2.91–2.79 (m, 3H), 2.73 (br s, 1H), 2.69 (dd, *J* = 3.9, 17.4, 2H), 2.30–2.22 (m, 1H), 2.06–1.94 (m, 2H), 1.85–1.76 (m, 1H), 1.72–1.57 (m, 4H), 1.57–1.46 (m, 2H), 1.36–1.34 (m, 1H), 1.32–1.25 (m, 2H), 1.25–1.16 (m, 2H), 1.01 (d, *J* = 6.7, 3H), 0.97 (d, *J* = 6.6, 3H), 0.85 (d, *J* = 5.9, 3H), 0.79 (d, *J* = 5.9, 3H).

#### 6.3.3. *cyclo*-[Val-Orn-Leu-$^{D}$Tyr-Pro-Trp-SAA-Asp-Trp] 9

Prepared according to the general procedure. Yield: 29.8 mg, 22.5 μmol, 23%. LC–MS: *t*R 6.04 min (linear gradient 10→90% B in 13.5 min), *m/z* = 1324.0 $[M+H]^+$, 662.8 $[2M+H]^+$. $^1$H NMR (600 MHz, MeOD) $\delta$ 10.67 (s, 1H), 10.65 (s, 1H), 10.32 (s, 1H), 8.99 (s, 1H), 8.38 (d, *J* = 9.5, 1H), 7.85 (br s, 1H), 7.82 (br s, 1H), 7.74 (d, *J* = 7.7, 2H), 7.57 (d, *J* = 8.1, 1H), 7.55 (br s, 1H), 7.49 (d, *J* = 7.8, 1H), 7.45 (s, 1H), 7.38 (d, *J* = 7.6, 3H), 7.32 (t, *J* = 7.5, 2H), 7.27 (t, *J* = 7.9, 2H), 7.22–7.14 (m, 3H), 7.08 (s, 1H), 7.03 (dd, *J* = 7.2, 12.4, 2H), 6.96 (t, *J* = 7.4, 1H), 6.92 (d, *J* = 8.3, 2H), 6.83–6.73 (m, 1H), 6.65 (d, *J* = 8.4, 2H), 4.65–4.59 (m, 2H), 4.59–4.50 (m, 3H), 4.33 (d, *J* = 14.4, 1H), 4.29 (d, *J* = 13.2, 2H), 4.19 (t, *J* = 5.0, 1H), 4.15 (br s, 1H), 4.11–4.06 (m, 2H), 4.06–4.00 (m, 1H), 3.90 (d, *J* = 8.1, 1H), 3.88 (br s, 1H), 3.72 (s, 1H), 3.52–3.45 (m, 2H), 3.45–3.35 (m, 1H), 3.34 (s, 1H), 3.28–3.16 (m, 4H), 2.90 (t, *J* = 12.6, 1H), 2.77 (dt, *J* = 5.4, 17.6, 1H), 2.77 (br s, 1H), 2.61 (br s, 1H), 2.19–2.06 (m, 2H), 2.01–1.87 (m, 2H), 1.78–1.69 (m, 1H), 1.63 (dd, *J* = 11.0, 22.2, 2H), 1.60–1.53 (m, 2H), 1.47–1.38 (m, 2H), 1.38–1.25 (m, 4H), 1.25–1.17 (m, 7H), 1.15–1.06 (m, 2H), 1.06–0.98 (m, 5H), 0.98–0.81 (m, 6H), 0.74–0.62 (m, 1H), 0.61–0.47 (m, 1H), 0.44–0.29 (m, 1H).

#### 6.3.4. *cyclo*-[Val-Orn-Leu-$^{D}$Tyr-Pro-Trp-$^{D}$Phe-Asn-SAA] 10

Prepared according to the general procedure. Yield: 4.0 mg, 3.0 μmol, 3%. LC–MS: *t*R 6.14 min (linear gradient 10→90% B in 13.5 min), *m/z* = 1336.0 $[M+H]^+$, 668.7 $[2M+H]^+$. $^1$H NMR (600 MHz, $CD_3OH$) $\delta$ 10.30 (s, 2H), 8.95 (s, 1H), 8.79 (d, *J* = 8.4, 1H), 8.59 (d, *J* = 8.1, 1H), 8.55 (d, *J* = 7.7, 1H), 8.49 (d, *J* = 8.7, 1H), 8.18 (br s, 1H), 8.14 (d, *J* = 7.7, 1H), 8.02 (d, *J* = 9.0, 1H), 7.78 (d, *J* = 9.2, 2H), 7.73 (d, *J* = 7.9, 1H), 7.55 (d, *J* = 7.9, 1H), 7.32 (d, *J* = 8.1, 1H), 7.27 (d, *J* = 8.2, 1H), 7.21–7.14 (m, 4H), 7.14–7.09 (m, 4H), 7.09–6.96 (m, 10H), 6.94 (t, *J* = 7.4, 2H), 6.66 (d, *J* = 8.4, 2H), 5.41–5.31 (m, 1H), 4.82–4.75 (m, 1H), 4.69 (dd, *J* = 7.9, 15.5, 1H), 4.60–4.54 (m, 1H), 4.46 (t, *J* = 8.0, 1H), 4.39–4.33 (m, 1H), 4.16 (dd, *J* = 2.4, 7.5, 1H), 4.12–4.07 (m, 1H), 3.37 (d, *J* = 7.6, 3H), 3.26 (d, *J* = 9.3, 1H), 3.18 (dd, *J* = 4.4, 14.4, 1H), 3.07 (dd, *J* = 6.8, 13.8, 1H), 3.05–2.98 (m, 2H), 2.96 (dd, *J* = 4.7, 12.7, 2H), 2.91–2.74 (m, 5H), 2.72–2.55 (m, 3H), 2.27 (dd, *J* = 9.5, 17.2, 1H), 2.20 (dd, *J* = 9.3, 17.1, 1H), 2.13 (dd, *J* = 6.8, 13.7, 1H), 1.96–1.88 (m, 1H), 1.79–1.72 (m, 1H), 1.72–1.61 (m, 6H), 1.61–1.54 (m, 2H), 1.44–1.33 (m, 3H), 1.31–1.24 (m, 1H), 1.02–0.91 (m, 18H), 0.43–0.32 (m, 1H).

#### 6.3.5. *cyclo*-[Val-Orn-Leu-$^{D}$Tyr-Pro-Trp-Phe-Asn-SAA] 11

Prepared according to the general procedure. Yield: 46.6 mg, 36.6 μmol, 37%. LC–MS: *t*R 6.34 min (linear gradient 10→90% B in 13.5 min), *m/z* = 1283.9 $[M+H]^+$, 642.8 $[2M+H]^+$. $^1$H NMR (600 MHz, MeOD) $\delta$ 10.41 (br s, 1H), 8.99 (d, *J* = 3.1, 1H), 8.82 (d, *J* = 8.4, 1H), 8.78 (d, *J* = 9.1, 2H), 8.72 (d, *J* = 8.4, 2H), 8.64 (d, *J* = 7.6, 2H), 7.94 (d, *J* = 8.4, 1H), 7.85–7.79 (m, 5H), 7.70 (d, *J* = 8.6, 2H), 7.69–7.65 (m, 2H), 7.63–7.60 (m, 1H), 7.57–7.53 (m, 1H), 7.51–7.48 (m, *J* = 12.5, 1H), 7.40–7.37 (m, 5H), 7.37–7.30 (m, 7H), 7.29–7.22 (m, 7H), 7.18–7.14 (m, 1H), 7.12 (t, *J* = 7.3, 2H), 7.07–6.99 (m, 5H), 5.18 (q, *J* = 7.6, 1H), 5.02 (s,1H), 4.94–4.85 (m, 1H), 4.82–4.76 (m, 1H), 4.76–4.71 (m, 1H), 4.71–4.67 (m, 2H), 4.62 (d, *J* = 11.7, 1H), 4.57–4.47 (m, 5H), 4.43–4.36 (m, 2H), 4.28–4.25 (m, 1H), 4.24 (d, *J* = 3.0, 1H), 4.17 (dd, *J* = 1.7, 8.2, 1H), 4.12 (t, *J* = 10.6, 1H), 3.92 (d, *J* = 2.8, 2H), 3.81 (d, *J* = 16.7, 1H), 3.76–3.70 (m, 1H), 3.65 (s, 1H), 3.31 (dd, *J* = 9.1, 14.7, 18H), 3.23–3.18 (m, 25H), 3.03–2.96 (m, 3H), 2.89–2.81 (m, 3H), 2.80–2.73 (m,

1H), 2.70 (dd, $J$ = 5.7, 15.5, 1H), 2.55–2.47 (m, 1H), 2.31–2.20 (m, 3H), 2.09–2.02 (m, 11H), 2.01–1.93 (m, 2H), 1.93–1.87 (m, 21H), 1.84–1.76 (m, 2H), 1.76–1.67 (m, 2H), 1.67–1.52 (m, 7H), 1.52–1.38 (m, 4H), 1.38–1.30 (m, 2H), 1.28 (s, 1H), 1.11–1.03 (m, 20H), 1.03–0.93 (m, 5H), 0.42–0.30 (m, 1H).

### 6.3.6. *cyclo*-[Val-Orn-Leu-$^{D}$Tyr-Pro-Trp-Phe-Asn-Asp-Trp] 12

Prepared according to the general procedure. Yield: 39 mg, 30 μmol, 30%. LC–MS: *t*R 6.40 min (linear gradient 10→90% B in 13.5 min), $m/z$ = 1283.9 $[M+H]^+$, 642.7 $[2M+H]^+$. $^1$H NMR (600 MHz, MeOD) $\delta$ 10.32 (s, 1H), 8.91 (d, $J$ = 2.9, 1H), 8.75 (d, $J$ = 7.9, 1H), 8.71 (d, $J$ = 9.0, 1H), 8.64 (d, $J$ = 8.4, 1H), 8.57 (d, $J$ = 7.5, 1H), 7.86 (d, $J$ = 8.4, 1H), 7.78–7.71 (m, 3H), 7.62 (d, $J$ = 8.6, 1H), 7.60 (br s, 1H), 7.55–7.50 (m, 1H), 7.47 (t, $J$ = 7.6, 1H), 7.42 (s, 1H), 7.33–7.22 (m, 9H), 7.21–7.14 (m, 5H), 7.04 (t, $J$ = 7.5, 1H), 6.98–6.93 (m, 3H), 6.67 (s, 1H), 6.64 (d, $J$ = 8.4, 2H), 5.10 (d, $J$ = 5.7, 1H), 4.74–4.69 (m, 1H), 4.66 (dd, $J$ = 7.8, 16.2, 1H), 4.61 (d, $J$ = 11.7, 1H), 4.54 (d, $J$ = 11.7, 1H), 4.47 (d, $J$ = 3.9, 1H), 4.45–4.40 (m, 2H), 4.34–4.29 (m, 1H), 4.16 (d, $J$ = 2.9, 1H), 4.09 (d, $J$ = 8.3, 1H), 3.85 (d, $J$ = 2.6, 1H), 3.73 (d, $J$ = 14.3, 1H), 3.62 (d, $J$ = 11.1, 1H), 3.58–3.54 (m, 1H), 3.29–3.17 (m, 8H), 3.17–3.11 (m, 2H), 2.95–2.88 (m, 2H), 2.81–2.73 (m, 2H), 2.73–2.66 (m, 1H), 2.62 (dd, $J$ = 5.6, 15.4, 1H), 2.47–2.38 (m, 1H), 2.23–2.13 (m, 2H), 2.01–1.93 (m, 4H), 1.92–1.80 (m, 1H), 1.77–1.68 (m, 1H), 1.68–1.59 (m, 1H), 1.59–1.45 (m, 4H), 1.45–1.31 (m, 2H), 1.30–1.24 (m, 1H), 1.02–0.95 (m, 14H), 0.94–0.85 (m, 2H), 0.33–0.24 (m, 1H).

### 6.3.7. *cyclo*-Val-Orn-Leu-$^{D}$Tyr-Pro-Trp-Phe-Asn-DAsp-Trp] 13

Prepared according to the general procedure. Yield: 7.2 mg 5.4 μmol, 5%. LC–MS: *t*R 6.16 min (linear gradient 10→90% B in 13.5 min), $m/z$ = 1335.9 $[M+H]^+$, 668.8 $[2M+H]^+$. $^1$H NMR (600 MHz, $CD_3OH$) $\delta$ 10.32 (s, 2H), 10.30 (s, 2H), 9.05 (br s, 2H), 8.72 (br s, 2H), 8.61 (d, $J$ = 8.6, 2H), 8.53 (d, $J$ = 6.6, 2H), 8.45 (br s, 3H), 7.95 (d, $J$ = 10.2, 2H), 7.78 (br s, 2H), 7.71 (d, $J$ = 7.9, 3H), 7.66 (d, $J$ = 8.8, 2H), 7.47 (br s, 2H), 7.28 (d, $J$ = 8.0, 7H), 7.25–7.11 (m, 18H), 7.10–6.96 (m, 20H), 6.93 (t, $J$ = 7.5, 3H), 6.69 (d, $J$ = 8.2, 5H), 5.45 (s, 1H), 4.84–4.57 (m, 2H), 4.49–4.24 (m, 3H), 4.19 (s, 2H), 3.45–3.32 (m, 6H), 3.27–3.22 (m, 4H), 3.10 (s, 9H), 3.03–2.83 (m, 6H), 2.74 (dd, $J$ = 4.2, 17.3, 4H), 2.67 (dd, $J$ = 5.2, 16.7, 5H), 2.30–2.04 (m, 6H), 1.88–1.52 (m, 21H), 1.42 (s, 7H), 1.35–1.21 (m, 4H), 1.13–0.74 (m, 43H), 0.50 (s, 2H).

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.07.049.

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