Synthetic modifications of the antibiotic peptide gramicidin S : conformational and biological aspects

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6

"Inverted" gramicidin S derivatives with combined adamantyl amino acids and sugar amino acids with varying ring-size*

Introduction

The antimicrobial peptide gramicidin S (GS, Figure 1) is a metabolite of the bacteria *Aneurinibacillus Migulans* and is a part of its defence mechanism to kill invading pathogens.[1] The secondary structure of GS, an antiparallel β-sheet enclosed by two type II' β-turns, ensues its amphipathic character: the cationic side chains (two ornithines) are directed opposite to the apolar side chains (two valines and two leucines) in the β-sheet region.[2] A strategy to diminish the toxicity of GS is to modulate this amphipathicity by changing the amino acid composition of the decameric peptide such as in compound **1** (Figure 1). This "inverted" GS derivative contains four cationic amino acids (ornithines) and two apolar residues (adamantyl-L-glycine and adamantyl-L-alanine). Compound **1** possesses the same

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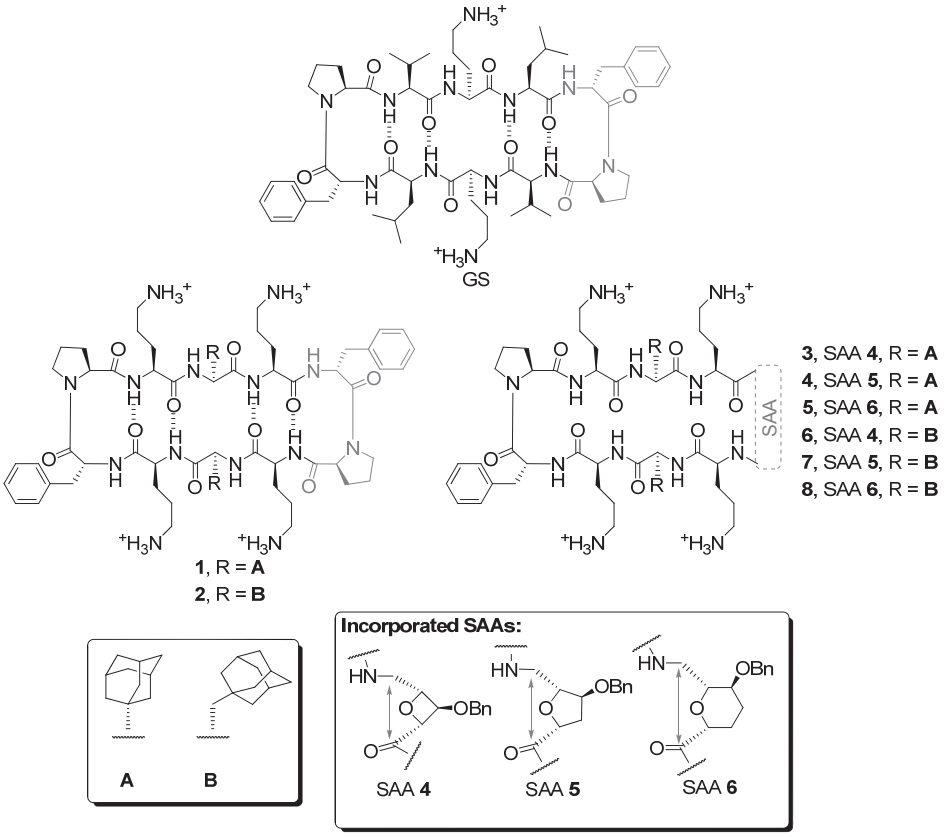


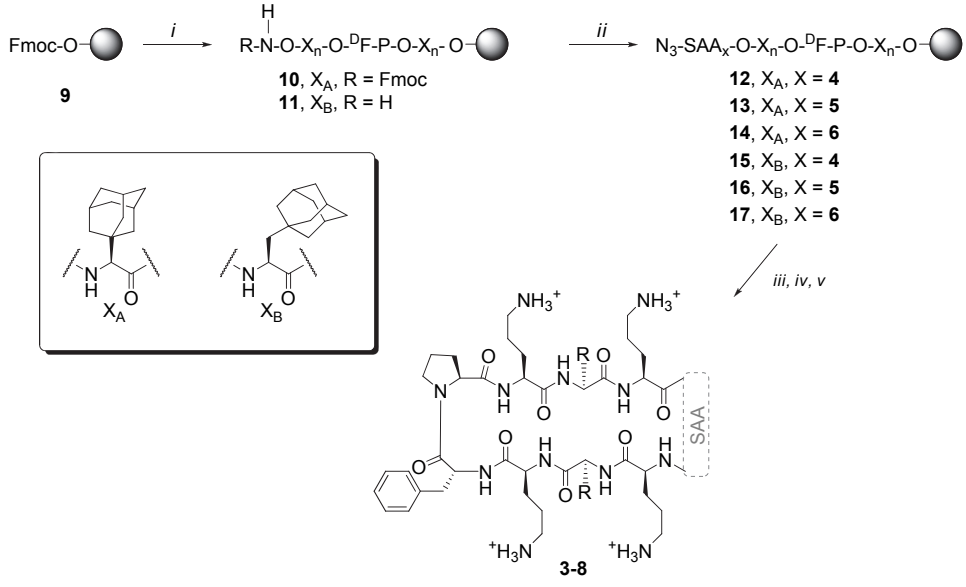
Figure 1. "Inverted" GS derivates **1**, **2** with adamantyl-glycine (**A**) and -alanine (**B**) amino acids and β-turn modified GS "inverted" derivatives **3**-**8** with SAA **4**, **5** and **6**.

antimicrobial activity as GS against Gram-positive bacteria and even higher activity against some Gram-negative bacteria.[3] In addition, analog **1** shows a diminished activity against mammalian cell membranes in terms of hemolysis. In contrast, homolog **2,** having adamantyl-l-alanine as apolar amino acid, retains the same antimicrobial activity as **1**, but has only marginal lower hemolytic activity than GS.[4] Intrigued by these results these molecules were used as templates to incorporate sugar amino acids (SAAs) with an oxetanoid- (**4**), a furanoid- (**5**), and a pyranoid-ring (**6**). As described in Chapter 2 these SAAs are useful as dipeptide isosteres for type II' β-turns. By varying the ring-size of the carbohydrate ring, the span of the turn and the hydrophobicity of the SAA is step by step increased. Subtle changes in hydrophobicity and in the conformation can lead to molecules with a better therapeutic profile.[5] This chapter describes the synthesis of the peptide series **3**-**8**. NMR and CD analysis were used to study their conformational behavior. The compounds were screened for bactericidal activity and tested on hemolytic activity to determine their biological activity.

Results and discussion

Synthesis. The peptides **3**-**8** were synthesized according to the procedure described in Chapter 2.[5] Acid labile HMPB-BHA resin was preloaded with Fmoc-ornithine (Scheme 1, **9**) and subsequently the peptide chain was elongated by SPPS to give octamers **10** and **11**, containing two adamantyl-L-glycine or two adamantyl-L-alanine residues, respectively. Both resins were coupled to SAA **4**-**6** decorated with an azide, which masked the amine, resulting in peptides **12**-**17**. The azide was reduced towards the amine by aq. trimethylphosphine and the linear peptides were released from the resin by mild acidic cleavage. Cyclization under highly dilute conditions and final deprotection with strong acid gave **3**-**8** in moderate overall yield ranging from 5-20%.

NMR and CD analysis. Peptides **3**-**8** were subjected to conformational analysis by NMR and CD in methanol. The NMR spectra recorded at room temperature of compounds **2**, **3** and **6**-**8** show broad signals indicating the absence of stable secondary structures under these conditions. Only two peptides give well-resolved NMR spectra, that is, peptides **4** and **5**. The amide coupling constants ($^3J_{HN\alpha}$) and chemical shift perturbations ($\Delta\delta H_\alpha$) are given in Figure 2. The $^3J_{HN\alpha}$ of **4** and **5** both exhibit the typical β-sheet structure ($^3J_{HN\alpha}$ > 6.5 Hz, Figure 2A), however no amide coupling signal could be assigned for the turn residue D-Phe. Based on the



Scheme 1: Reagents and conditions: (*i*) SPPS; (*ii*) SAA_x, HCTU, DiPEA; (*iii*) PMe_3, 1,4-dioxane/THF/H_2O; (*iv*) 1% TFA in DCM; (*v*) 1. pyBOP, HOBt, DiPEA, DMF; 2. 95/2.5/2.5 TFA/TIS/H_2O.

observation of the chemical shift perturbations of peptide **4**, the β-sheet structure is more distorted than compound **5:** the ornithines of **4** have $\Delta\delta H_\alpha$ smaller than 0.1 (Figure 2B). Peptide **5** shows higher $\Delta\delta H_\alpha$ for the ornithine residues and thus a more pronounced β-sheet. The $\Delta\delta H_\alpha$ data does also not provide direct evidence of a well-defined turn region for peptide **4** and **5** although the $\Delta\delta H_\alpha$ of D-Phe is smaller than -0.1, the $\Delta\delta H_\alpha$ of Pro is positive (Figure 2B).[6] To further verify the secondary structure of the peptides, CD spectra were measured of the peptide series **1**-**8** (Figure 3A and 3B). Template **1** shows the strongest β-sheet/β-turn structure in the CD spectra unlike template **2**, which exhibits a less structured β-hairpin. CD corroborates the trend observed in NMR for peptides **4** and **5** (Figure 3A). Peptide **5** show a higher cotton effect than **4** at 220 nm, which is the typical wavelength for a β-sheet conformation. Peptides **3** and **4** are less structured compared to **1** and **5.** SAA modified peptides **6**-**8** have a less pronounced and more distorted secondary structure (in the decreasing order **8** > **7** > **6**) compared to **2** (Figure 3B).[7]

Physical and biological properties. Peptides **3**-**8** were tested against a standard panel of Gram-positive and Gram-negative bacteria. As controls, peptide **1** and GS were screened in the same experiment (Table 1). All peptides show high antimicrobial activity against Gram-positive bacteria to the same extent as GS. With the possible exception of **6**, peptides **3**-**8** outperform GS in terms of activity against Gram-negative bacteria.

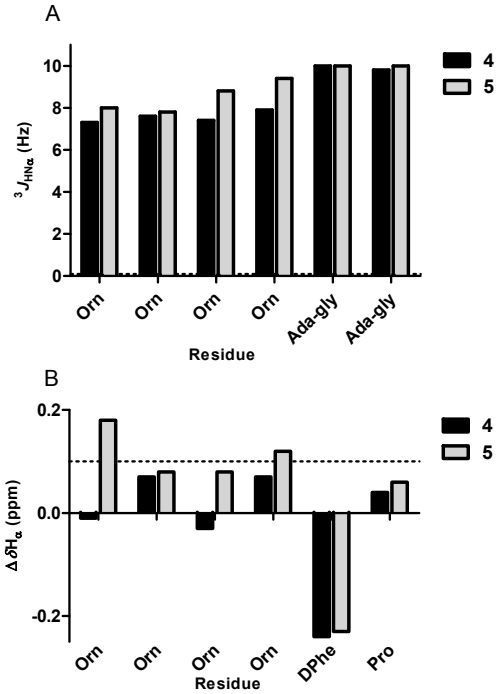


Figure 2: [A] $^3J_{HN\alpha}$ in Hz of peptides **4** and **5** in methanol; [B] $\Delta\delta H_\alpha$ in ppm of **4** and **5** ($\Delta\delta H_\alpha = \delta H_\alpha - \delta H_{\alpha\ random\ coil}$) in methanol.

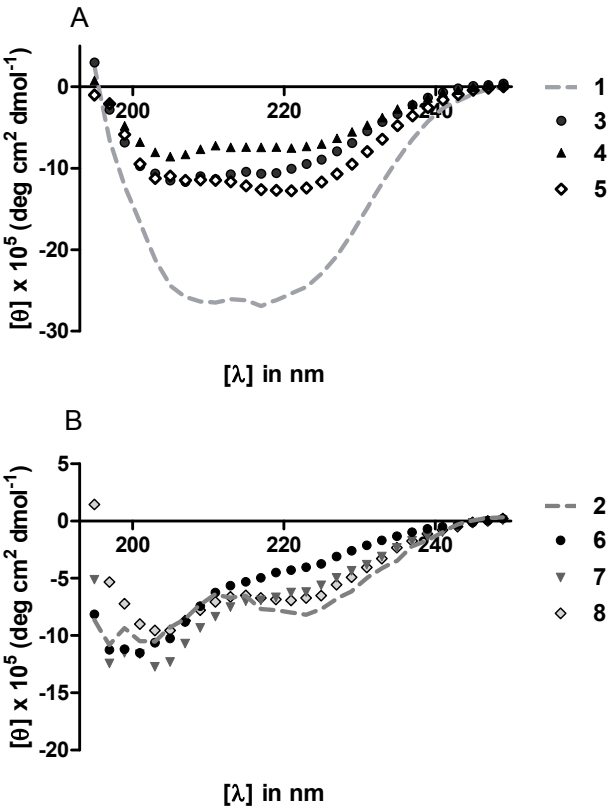


Figure 3: CD spectra of [A] **1**, **3-5** and [B] **2**, **6**-**8** recorded in 0.1 mM in methanol.

Table 1: Antimicrobial Activity (MIC) and peptide hydrophobicity of GS, **1**, **3**-**8**

Analogs[a]	HPLC retention time	*S. aureus*[b]	*S. epidermidis*[b]	*E. faecalis*[b]	*B. cereus*[b]	*P. aeruginosa*[c]	*E. coli*[c]
GS	8.38[7]	4	2	8	4	64	32
1	6.39[7]	8	2	16	8	8	8
3	6.48	8	2	8	8	8	8
4	6.72	8	2	16	4	4	8
5	6.79	4	2	8	4	8	8
6	6.22	16	4	32	16	32	16
7	6.41	4	2	8	4	8	8
8	6.45	16	1	8	4	8	8

[a] Molecular weight GS: 1369.49; **1**: 1783.79; **3**: 1758.74; **4**: 1772.77; **5**: 1786.79; **6**: 1786.79; **7**:1800.82; **8**: 1814.59. [b] Gram-positive bacteria, MIC in mg/L. For detailed experimental set up: see experimental section [c] Gram-negative bacteria, MIC in mg/L.

The hemolytic activity of **3**-**8** is reported in Figure 4. Compounds **3**-**8** are less hemolytic than GS except for **5** at low concentrations (Figure 4). Peptide **6** has a decreased activity and even at 500 μM does not induce 100% hemolysis. Peptides **3**, **4**, **7** and **8** show a slightly higher or the same activity compared to template **1**.

As a measure of peptide hydrophobicity the retention times on a reversed phase HPLC column were determined (Table 1).[8] With increasing ring-size of the SAA (oxetane **3**, **6**; furanoid **4**, **7** and pyranoid **5**, **8**) the hydrophobicity of the peptide is increased. All analogs except **6** are more hydrophobic than template **1**.

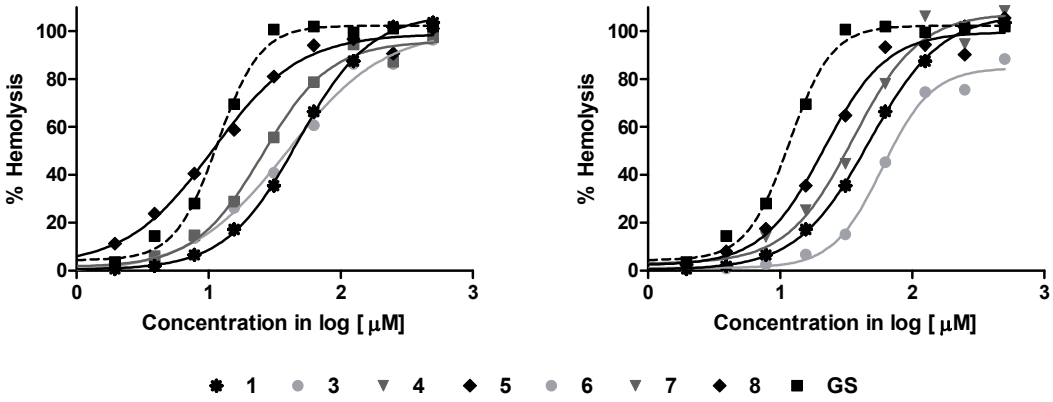


Figure 4: Hemolytic curves of GS, **1** and **3**-**8**.

Conclusion

A peptide series (**3**-**8**) with SAAs of varying ring-size in the turn region was synthesized based on templates **1** and **2**; GS analogs containing four cationic charges and two adamantyl-L-glycine or two adamantyl-L-alanine residues, respectively. Conformational analysis shows that the pyranoid SAA incorporated in peptide **5** has the most pronounced secondary structure of the series **3**-**8**. This trend is also observed for the peptide series reported in Chapter 2, where the same SAAs series were incorporated in GS. In contrast with the findings in Chapter 2 where the incorporation of SAA **6** results in a better therapeutic profile, peptide **5** is the most hemolytic of the peptide series **3**-**8**. The reason for this might be that compound **5** is the most hydrophobic compound of this series and fits the general trend that with increasing hydrophobicity, the hemolytic activity is also augmented.[3,9] In terms of improvement of therapeutic profile peptide **6** stands out: despite its slightly decreased antimicrobial activity compared to GS, this molecule has a very low hemolytic activity. Although none of the novel

compounds reported here have a combined good antimicrobial activity and lower hemolytic activity, it should be noted that peptides **3**-**8** are all less toxic than GS and especially compounds **3** and **7** are more potent against the two Gram negative strains tested. In addition. peptides **3**, **4**, **7** and **8** show the same antimicrobial activity as template **1**, however do not outperform **1** in terms of hemolytic activity.

Experimental section

General:

Light petroleum ether with a boiling range of 40-60 ºC was used. All other solvents used under anhydrous conditions were stored over 4Å molecular sieves except for methanol which was stored over 3Å molecular sieves. Solvents used for work-up and silica gel column chromatography were of technical grade and distilled before use. All other solvents were used without further purification. Reactions were monitored by TLC-analysis. The linear peptides were cleaved from resin, cyclized and purified by RP-HPLC (Gilson GX-281) with a preparative Gemini C18 column (Phenomenex 150 x 21.2 mm, 5µg particle size) or semi-preparative (250 x 21.2 mm, 5µg particle size). The applied eluents were A: 0.1 % aq. TFA, B: MeCN. The linear peptides and cyclized peptides were analyzed with LC/MS (detection simultaneously at 214 and 254 nm) equipped with an analytical C18 column (4.6 mmD x 250 mmL, 5µg particle size). The applied eluents 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 H_2O/MeCN; 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 523 K) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as lock mass. CD and hemolytic curves were analyzed with Graphpad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA.

NMR spectroscopy:

The spectra of the peptides (GS**3-8**) were recorded on a Bruker DMX 600 equipped with a pulsed field gradient accessory and a cryo-probe. For the 2D cROESY spectra (200 msec mixing time) the peptides were dissolved in CD_3OH. Standard DQF-COSY (512c x 2084c) and TOCSY (400c x 2048c) spectra were recorded using presaturation for solvent suppression. cROESY[13] spectra (400c x 2048c, τ_{mix} = 180 ms) were recorded using the presat solvent suppression. All spectra were recorded in phase-sensitive mode, using either the TPPI or states-TPPI for quadrature detection in the indirect dimension. Homonuclear coupling constants were determined from the corresponding ^1H spectra.

CD spectroscopy:

CD spectra were recorded at 298 K on a Jasco J-815 spectropolarimeter using 0.1 cm path length quartz cells. The CD spectra are averages of four scans, collected at 0.1 nm intervals between 190 and 250 nm with scanning speed 50 nm/min. The peptides were prepared at concentrations of 0.1 mM in CH_3OH. Ellipticity is reported as mean residue ellipticity [θ], with approximate errors of ± 10% at 220 nm.

Antimicrobial assay

The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at –70

ºC and grown at 30 ºC 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 x) with physiological saline and 2 µL of this inoculum was added to 100 µL of growth medium, [Nutrient Broth from Difco (ref. nr. 234000, lot nr. 6194895) with yeastextract (Oxoid LP 0021, lot nr. 900711, 2 g/400 mL of broth)] in microtiter plates (96 wells). The peptides (**1**, **3-8** and GS) were dissolved in ethanol (4 g/L) and further diluted in distilled water (1 g/L), and two-fold diluted in the broth (64, 32, 16, 8, 4 and 1 mg/L). The plates were incubated at 30 °C (24-96 h) and the MIC was determined as the lowest concentration inhibiting bacterial growth at 24h. The experiments were conducted twice, the experimental error is one MIC interval (a factor two).

Hemolytic assays

Freshly drawn heparinized blood was centrifuged for 10 minutes 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 (**1**, **3**-**8** and GS) were dissolved in a 30% DMSO/0.5 mM saline to a stock of 1.5 mM. If required (suspension formed) the stock solution was sonicated for a few seconds. prior to use. As a positive control a 1% Triton-X100 solution was prepared. For the assay 100 µL of saline was dispensed in columns 1-11 of a microtiter plate and 100 µL of 1% Triton solution in column 12. To wells A1-C1, 100 µL of the peptide was added and mixed properly. Next, 100 µL of wells A1-C1 was dispensed into wells A2-C2. This process was repeated until wells A10-C10, followed by discarding 100 µL from 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 hours. After incubation, the plates were centrifuged at 1000*g* at RT for 5 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. The experiment was conducted once and carried out in triplicate. A maximum of 10% experimental error was found.

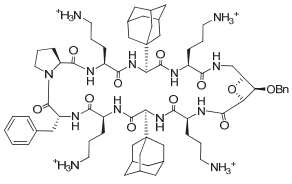
General Peptide Synthesis

*(a)Stepwise elongation for octamer **10**:* Preloaded resin with Fmoc-Ornithine (3.16 g, 0.57 mmol/g, 1.8 mmol) was submitted to 8 cycles of Fmoc solid-phase synthesis with use of the building blocks in the order: Fmoc-Adamantyl-L-glycine-OH, Fmoc-Orn(Boc)-OH, Fmoc-Pro-OH, Fmoc-D-Phe-OH, Fmoc-Orn(Boc)-OH, Fmoc-Adamantyl-L-glycine-OH, Fmoc-Orn(Boc)-OH. (a) deprotection with 20% piperidine in NMP (3x 50 mL, 15 min); (b) washing with NMP (2 x 50 ml) and DCM (2 x 50 mL); (c) pre-activation of the next building block (3 eq. for Orn, Pro, D-Phe; 1.5 eq. for Adamantyl-L-glycine) in 50 mL NMP, HCTU (3 eq. 2.23 g; 1.5 eq. 1.11 g) in NMP and DiPEA (3.6 eq, 1.13 mL; 1.8 eq 564 µL) and subsequent coupling with the resin for 4 hours; (d) washing with NMP (2 x 50 mL) and DCM (2 x 50 mL). Couplings were monitored for completion by the Kaiser test. (f) The resin was washed with MeOH (2 x 50 mL), NMP (2 x 50 mL), DCM (2 x 50 mL). Loading tested by Fmoc test: 0.21 mmol/g. LC/MS: R_t 6.41 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1323.67 $[M+H]^+$.

*(a)Stepwise elongation for octamer **11**:* Preloaded resin with Fmoc-Ornithine (400 mg, 0.75 mmol/g, 0.3 mmol) was submitted to 8 cycles of automated Fmoc solid-phase synthesis using the Fmoc based solid phase peptide synthesis protocols with the building blocks in the order: Fmoc-Adamantyl-L-alanine-OH, Fmoc-Orn(Boc)-OH, Fmoc-Pro-OH, Fmoc-D-Phe-OH, Fmoc-Orn(Boc)-OH, Fmoc-Adamantyl-L-alanine-OH, Fmoc-Orn(Boc)-OH. Orn, Pro, D-Phe were coupled with 90% HATU with respect to 3.3 equivalents of amino acid in 30 minutes. Fmoc adamantyl-L-alanine was coupled with 90% HATU coupling reagent with respect to 1.5 equivalent amino acid in 30 minutes. The resin was air-dried (621 mg; loading 0.48 mmol/g). LC/MS: R_t 5.59 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1130.6 $[M+H]^+$.

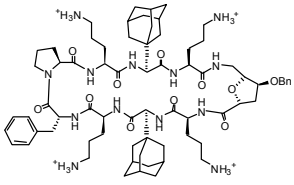
(b) General incorporation of SAA: (a) The resin was washed with MeOH (2 x 10 mL), NMP (2 x 10 mL), DCM (2 x 10 mL); (b) deprotection with 20% piperidine in NMP (3 x 10 mL); (c) coupling of SAA to dried resin (for conditions: see experimental procedure analogs **3**-**8**) (d) washing with NMP (2 x 10 mL) and DCM (2 x 10 mL).
(c) Azide reduction: The resin was washed with 1,4-dioxane (3 x 10 mL), and taken up in 1,4-dioxane (10 mL) to which trimethylphosphine (16 eq, 1M in THF) pre-mixed with H_2O (0.6 eq.) was added. The resin was shaken for 24 hours; Saa4-adagly LC/MS: R_t 5.67 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1320.67 $[M+H]^+$. SAA6-adagly: LC/MS: R_t 5.74 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1348.60 $[M+H]^+$.
(d) Cleavage from resin: The peptide was released from the resin by mild acidic cleavage (4 x 10 min, 10 mL 1% TFA in DCM). The fractions were collected and coevaporated with toluene (3 x 50 mL) to give the crude linear peptide which was immediately cyclized without further purification.
(e) Cyclization: To solution of HOBt (5 eq.), pyBOP (5 eq.) and DIPEA (15 eq.) in DMF (80 mL) was overnight dropwise added the crude peptide in DMF (20 mL). The solvent was evaporated and the residue was subjected to a Sephadex® size exclusion column (50.0 mmD x 1500 mmL) eluting with MeOH.
(f) Deprotection: The Boc-protection groups were removed by addition of TFA/TIS/H_2O mixture(10 mL, 95/2.5/2.5) and subsequently the peptide was purified by preparative RP-HPLC

cyclo-[SAA_4-$OX_{gly}O^DFPOX_{gly}O$] · 4TFA (3)



SAA **4** (2 eq, 79 mg) was pre-activated with HBTU (3 eq, 2.25 mL, 0.2 M HBTU in NMP), DiPEA (6 eq, 0.15 mL) in 10 mL NMP and subsequent coupling with the immobilized peptide **10** (714 mg, 0.15 mmol, 0.21 mmol/g) for 4 hours; **12**: LC/MS: R_t 6.23 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1346.67 $[M+H]^+$. After general peptide synthesis steps *c-f* the deprotected peptide was RP-HPLC purified (linear gradient of 35-44%, 3 CV) and yielded **3** as white powder (62 mg, 35.3 µmol, 24%). ^{1}H NMR (600 MHz, CD_3OH) δ 9.12 (br. s, 1H), 9.03 (br. s, 1H), 8.53 (br. s, 2H), 8.41 (br. s, 2H), 8.15 (br. s, 2H), 8.03 (br. s, 1H), 7.94 (br. s, 3H), 7.39 – 7.20 (m, 10H), 4.87 – 4.83 (m, 2H), 4.56 (d, J = 11.4 Hz, 2H), 4.55 – 4.49 (m, 2H), 4.49 – 4.44 (m, 2H), 4.42 – 4.38 (m, 2H), 4.33 – 4.24 (m, 3H), 3.81 – 3.69 (m, 1H), 3.66-3.52 (m, 1H), 3.42-3.36 (m, 1H), 3.10 – 2.87 (m, 10H), 2.53-2.45 (m, 1H), 2.07 – 1.46 (m, 50H). ^{13}C NMR (151 MHz, CD_3OH) δ 174.23, 174.05, 173.00, 172.32, 172.16, 162.74, 162.51, 138.50, 137.27, 130.58, 129.74, 129.45, 128.98, 128.44, 128.27, 86.15, 83.99, 77.48, 72.33, 62.44, 61.48, 54.30, 54.16, 53.90, 49.58, 49.43, 49.29, 49.15, 49.09, 49.08, 49.01, 48.87, 48.73, 47.71, 42.93, 40.56, 40.53, 40.43, 39.60, 39.54, 37.90, 37.67, 30.43, 30.00, 29.77, 25.56, 25.49, 25.32, 25.14. HRMS (ESI) m/z 1302.79788 $[M + H]^+$, 1302.79728 calcd. for $C_{70}H_{104}N_{13}O_{11}$ LC/MS: Rf 6.48 min, linear gradient 10→90% B in 13.5 min.; m/z = 1302.60 $[M+H]^+$.

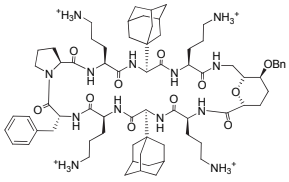
cyclo-[SAA_5-$OX_{gly}O^DFPOX_{gly}O$] · 4TFA (4)



SAA **5** was coupled twice to resin **10**: SAA **5** (1. 42 mg, 1,5 eq.; 2. 53 mg, 1.9 eq.) was pre-activated with HCTU (1. 1.5 eq, 62 mg; 2. 1.9, 79 mg), DIPEA (1. 3 eq, 50 µL; 2. 0.38 eq. 63 µL) in 10 mL NMP and subsequent coupling with the immobilized peptide **10** (714 mg, 0.15 mmol, 0.21 mmol/g) for 4 hours; **13**: LC/MS: R_t 6.26 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1360.6 $[M+H]^+$. After general peptide synthesis steps *c-f* the deprotected peptide was RP-HPLC purified (linear gradient of 37-41%, 3 CV) and yielded **4** as white powder (16.2 mg, 9.14 µmol, 9%). ^{1}H NMR (600 MHz, CD_3OH) δ 9.23 (br. s, 1H, N*H* D-Phe_5), 9.04 (d, *J* = 7.3 Hz, 1H, N*H* Orn), 8.79 (br. s, 1H, N*H* SAA), 8.56 (d, *J* = 7.6 Hz, 1H, N*H* Orn), 8.12 (d, *J* = 9.8 Hz, 1H, N*H* Ada-gly), 8.03

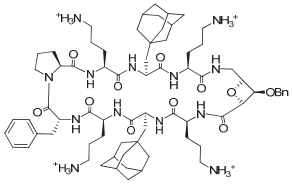
(d, *J* = 7.4 Hz, 1H, N*H* Orn), 7.95 (d, *J* = 10.0 Hz, 1H, N*H* Adagly), 7.89 (d, *J* = 8.9 Hz, 1H, N*H* Orn), 7.95-7.89 (br. s., 4H, NH_2 Orn), 7.37 – 7.18 (m, 10H), 4.82-4.79 (m, 2H), 4.54 (d, 1H, *J* = 12.0 Hz), 4.48 (d, 1H, *J* = 12.1 Hz) , 4.49-4.47 (m, 1H), 4.45 – 4.39 (m, 3H), 4.39 – 4.29 (m, 3H), 4.23 (s, 1H), 4.00 (d, *J* = 5.5 Hz, 1H), 3.93 (dd, *J* = 14.7, 6.2 Hz, 1H), 3.65-3.59 (m, 1H), 3.27 – 3.21 (m, 1H), 3.07 – 2.87 (m, 10H), 2.39-2.37 (m, 1H), 2.32 (dd, *J* = 13.0, 5.2 Hz, 1H), 2.11 – 1.49 (m, 51H). ^{13}C NMR (151 MHz, CD_3OH) δ 174.99, 174.09, 173.90, 173.77, 172.94, 172.80, 172.42, 172.31, 172.29, 139.15, 137.12, 130.54, 129.78, 129.61, 128.93, 128.70, 128.52, 87.56, 80.89, 79.27, 72.11, 68.17, 62.25, 61.74, 61.44, 56.17, 55.10, 54.14, 54.03, 53.87, 49.72, 47.52, 47.43, 47.40, 43.20, 40.61, 40.56, 40.48, 40.45, 39.73, 39.54, 39.16, 38.57, 38.32, 38.01, 37.80, 37.57, 30.93, 30.41, 30.31, 30.24, 30.01, 29.96, 29.26, 27.42, 27.37, 25.70, 25.51, 25.19, 24.68. HRMS (ESI) m/z 1316.81462 $[M + H]^+$, 1316.81293 calcd. for $C_{71}H_{106}N_{13}O_{11}$ LC/MS: R_t 6.72 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1316.67 $[M+H]^+$.

cyclo-[SAA$_6$-OX$_{gly}$O^{D}FPOX$_{gly}$O] · 4TFA (5)



SAA **6** (1.2 eq, 52 mg) was pre-activated with HCTU (1.2 eq, 74 mg), DIPEA (2.4 eq, 60 µL) in 10 mL NMP and subsequent coupling with the immobilized peptide **10** (714 mg, 0.15 mmol, 0.21 mmol/g) for 4 hours; **14**: LC/MS: R_t 6.41 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1374.60 $[M+H]^+$. After general peptide synthesis steps *c-f* the deprotected peptide was RP-HPLC purified (linear gradient of 38-47%, 3 CV) and yielded 5 as white powder (19.3 mg, 10.7 µmol, 7%). ^{1}H NMR (600 MHz, CD_3OH) δ 9.21 (s, 1H, N*H* D-Phe$_5$), 8.68 (d, *J* = 8.0 Hz, 1H, N*H* Orn$_9$), 8.58 (d, *J* = 7.8 Hz, 1H, N*H* Orn$_4$), 8.51 (t, *J* = 5.9 Hz, 1H, N*H* SAA), 8.15 (d, *J* = 10.0 Hz, 1H, N*H* Ada-gly), 8.11 (d, *J* = 9.4 Hz, 1H, N*H* Orn$_2$), 7.97 (d, *J* = 10.0 Hz, 1H, N*H* Ada-gly), 7.90 (br. s, 4H, NH_2 Orn), 7.88 (d, *J* = 8.8 Hz, 1H, NH Orn$_7$), 7.40 – 7.18 (m, 10H), 4.86 – 4.84 (m, 2H, *H*α Ada-gly), 4.64 (d, *J* = 11.0 Hz, 1H), 4.56-4.53 (m, 1H, *H*α Orn$_2$), 4.50 (d, *J* = 8.1 Hz, 1H), 4.49-4.43(m, 5H, *H*α Pro$_6$, *H*α Orn$_{4,7,9}$, *H*α D-Phe$_5$), 3.93-3.87 (m, 2H), 3.68 – 3.55 (m, 1H), 3.55 – 3.46 (m, 1H), 3.29 – 3.19 (m, 2H), 3.13 – 2.83 (m, 10H), 2.41– 2.35 (m, 1H), 2.35 – 2.18 (m, 1H), 2.16 – 2.06 (m, 1H), 2.06 – 1.15 (m, 52H). ^{13}C NMR (151 MHz, CD_3OH) δ 174.27, 174.10, 173.93, 173.85, 173.00, 172.83, 172.44, 172.26, 172.22, 139.91, 137.15, 130.55, 129.79, 129.50, 128.82, 128.67, 128.53, 79.51, 75.63, 74.05, 73.74, 71.70, 64.59, 62.20, 61.69, 61.45, 56.19, 55.19, 54.05, 53.99, 52.92, 49.72, 47.50, 45.24, 40.63, 40.49, 39.83, 39.56, 38.35, 38.30, 37.98, 37.79, 30.94, 30.40, 30.01, 29.95, 29.06, 28.87, 25.84, 25.50, 25.25, 25.18, 24.68, 24.00. HRMS (ESI) *m/z* 1330.83015 $[M + H]^+$, 1330.82858 calcd. for $C_{72}H_{108}N_{13}O_{11}$; LC/MS: R_t 6.79 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1330.73 $[M+H]^+$.

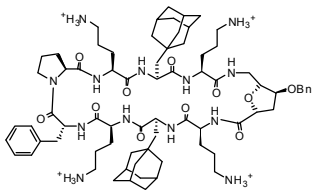
cyclo-[SAA$_4$-OX$_{ala}$O^{D}FPOX$_{ala}$O] · 4TFA (6)



SAA **4** was coupled twice to resin **11**: SAA **4** (37 mg, 1,4 eq.) was pre-activated with HCTU (1.4 eq, 58 mg), DIPEA (2.8 eq, 46 µL) in 10 mL NMP and subsequent coupling with the immobilized peptide **11** (207 mg, 0.1 mmol, 0.483 mmol/g) for 4 hours; **15**: LC/MS: R_t 6.52 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1374.7 $[M+H]^+$. After general peptide synthesis steps *c-f* the deprotected peptide was RP-HPLC purified (linear gradient of 31-40%, 3 CV) and yielded **6** as white powder (7.18 mg, 4.02 µmol, 4%). ^{1}H NMR (600 MHz, CD_3OH) δ 8.60 (d, *J* = 6.4 Hz, 1H), 8.52 4.49 (m, 1H), 8.40 – 8.38 (m, 1H), 8.30 -8.28 (m, 2H), 8.21-7.79 (m, 7H), 7.38 – 7.20 (m, 10H), 4.99 – 4.58 (m, 6H), 4.49 – 4.24 (m, 6H), 4.06 (t, *J* = 4.9 Hz, 1H), 3.90 – 3.75 (m, 1H), 3.54 – 3.52 (m, 1H), 3.42-3.37 (m, 1H), 3.17-3.09 (m, 2H), 3.08-2.83 (m, 8H), 2.03-1.42 (m, 55H). ^{13}C NMR (151 MHz, CD_3OH) δ 176.01, 175.20, 174.67, 173.96, 173.50, 173.12, 172.65, 171.89, 162.84, 138.54, 137.69, 130.55, 129.72, 129.55, 129.14, 128.95, 128.51, 128.35, 86.57, 84.17, 78.96, 72.67, 68.17, 64.48, 61.47, 54.60, 54.16, 53.67, 52.31, 52.10, 49.72, 48.45, 47.72, 47.43, 43.62, 43.55, 43.52, 43.00, 40.89, 40.53,

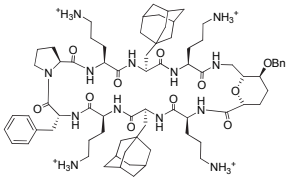
38.36, 37.98, 37.91, 33.80, 33.55, 30.89, 30.22, 30.17, 30.08, 29.43, 28.98, 27.36, 25.80, 25.49, 25.12, 24.89, 24.76. HRMS (ESI) m/z 444.28005 $[M + H]^{3+}$, 444.28104 calcd. for $C_{72}H_{110}N_{13}O_{11}$ LC/MS: R_t 6.22 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1330.87 $[M+H]^+$.

cyclo-[SAA_5-$OX_{ala}O^DFPOX_{ala}O$] · 4TFA (7)



SAA **5** was coupled to resin **11**: SAA **5** (50 mg, 1,8 eq.) was pre-activated with HCTU (1.8 eq, 74 mg), DIPEA (3.6 eq, 60 µL) in 10 mL NMP and subsequent coupling with the immobilized peptide **11** (207 mg, 0.1 mmol, 0.483 mmol/g) for 4 hours; **16**: LC/MS: R_t 6.58 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1388.7 $[M+H]^+$. After general peptide synthesis steps *c-f* the deprotected peptide was RP-HPLC purified (linear gradient of 32-41%, 3 CV) and yielded **7** as white powder (12.5 mg, 6.94 µmol, 7%). ^{1}H NMR (600 MHz, CD_3OH) δ 8.75 (d, *J* = 3.3 Hz, 1H), 8.44 – 8.42 (m, 2H), 8.18-8.05 (m, 2H), 7.96-7.79 (m, 7H), 7.39 – 7.17 (m, 10H), 4.87 – 4.84 (m, 2H), 4.65- 4.63 (m, 1H), 4.59 – 4.44 (m, 5H), 4.44 – 4.31 (m, 3H), 4.12 – 4.09 (m, 1H), 4.03 – 3.97 (m, 1H), 3.55 – 3.49 (m, 1H), 3.50 – 3.44 (m, 1H), 3.43 – 3.37 (m, 1H), 3.18 – 2.81 (m, 10H), 2.67 (s, 1H), 2.38- 2.36 (m, 1H), 2.15 – 1.38 (m, 55H). ^{13}C NMR (151 MHz, CD_3OH) δ 176.00, 175.38, 174.52, 173.76, 173.29, 172.27, 163.06, 162.83, 162.60, 139.26, 137.57, 130.54, 129.75, 129.52, 128.94, 128.85, 128.41, 86.48, 81.29, 79.29, 72.64, 68.17, 61.51, 55.06, 54.11, 53.72, 52.01, 51.73, 49.72, 48.13, 47.52, 43.58, 43.46, 42.99, 40.63, 40.53, 38.15, 38.00, 37.91, 33.79, 33.57, 30.42, 30.22, 30.16, 30.09, 30.03, 25.53, 25.40, 25.15, 25.03, 24.77. HRMS (ESI) *m/z* 1344.84641 $[M + H]^+$, 1344.84423 calcd. for $C_{73}H_{110}N_{13}O_{11}$; LC/MS: R_t 6.41 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1344.73 $[M+H]^+$.

cyclo-[SAA_6-$OX_{ala}O^DFPOXalaO$] · 4TFA (8)



SAA **6** (35 mg, 1,2 eq.) was pre-activated with HCTU (1.2 eq, 74 mg), DIPEA (2.4 eq, 40 µL) in 10 mL NMP and subsequent coupling with the immobilized peptide **11** (207 mg, 0.1 mmol, 0.483 mmol/g) for 4 hours; **17**: LC/MS: R_t 6.71 min, linear gradient 10→90% B in 13.5 min.; *m/z* = 1403.7 $[M+H]^+$. After general peptide synthesis steps *c-f* the deprotected peptide was RP-HPLC purified (linear gradient of 35-44%, 3 CV) and yielded **8** as white powder (5.1 mg, 2.8 µmol, 3%).^{1}H NMR (600 MHz, CD_3OH) δ 8.87 (br. s, 1H), 8.41 – 8.39 (m, 2H), 8.24 – 8.22 (m, 2H), 8.14 – 8.12 (m, 1H), 7.92 – 7.90 (m, 2H), 7.87 – 7.85 (m, 3H), 7.79 - 7.76 (m, 1H), 7.44 – 7.16 (m, 10H), 4.86 – 4.84 (m, 2H), 4.66 (d, *J* = 11.3 Hz, 1H), 4.63 – 4.19 (m, 7H), 4.01 – 3.93 (m, 1H), 3.86 – 3.48 (m, 3H), 3.42 – 3.40 (m, 1H), 3.27 – 3.25 (m, 1H), 3.12 – 2.79 (m, 10H), 2.67 (s, 1H), 2.48 – 2.37 (m, 1H), 2.26 – 2.09 (m, 1H), 2.09 – 1.28 (m, 64H).^{13}C NMR (151 MHz, CD_3OH) δ 176.16, 175.62, 173.55, 173.02, 172.75, 139.84, 137.51, 130.52, 129.81, 129.48, 128.99, 128.85, 128.48, 80.56, 71.51, 61.60, 53.61, 52.05, 51.37, 43.61, 43.51, 43.37, 40.56, 38.01, 37.93, 33.75, 33.50, 30.30, 30.08, 29.95, 29.47, 25.19. HRMS (ESI) m/z 1358.86244 $[M + H]^+$, 1358.85988 calcd. for $C_{74}H_{112}N_{13}O_{11}$ LC/MS: R_t 6.45 min, linear gradient 10→90% B in 13.5 min.; m/z = 1358.73 $[M+H]^+$.

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