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## **Towards peptide based therapeutics-applications in celiac disease and infectious diseases**

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## Chapter 5

### Adamantyl Amino Acid Modified Gramicidin S Analogs Combine more Potent and Broader Bactericidal Activity with Reduced Hemolytic Activity in Comparison with Natural Gramicidin S

*Patent filed:*

*Kapoerchan, V. V.; Van der Marel, G. A.; Noort, D.; De Neeling, A. J.; Overkleef, H. S.; Overhand, M. 'Antimicrobial cyclic peptides' GB0905821.5 (filing date: April 3rd, 2009).*

*Manuscript in preparation:*

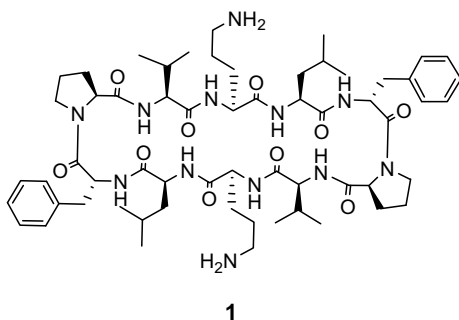
*Kapoerchan, V. V.; Spalburg, E.; De Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Van der Marel, G. A.; Overkleef, H. S.; Overhand, M. 'Adamantyl amino acids containing analogs of Gramicidin S display broader antibacterial activity and reduced hemolytic activity'.*

#### Introduction

The cyclic decapeptide Gramicidin S (GS, **1**, *cyclo*-(Pro-Val-Orn-Leu-<sup>D</sup>Phe)<sub>2</sub>, Figure 1), produced by *Bacillus Brevis*<sup>1</sup> belongs to the class of cationic antimicrobial peptides. The peptide is able to disrupt the bacterial cell membrane resulting in bacterial cell lysis and subsequent cell death. GS displays considerable activity against both Gram-positive and Gram-negative bacteria.<sup>2</sup> The ability of GS to disrupt the integrity of lipid bilayers is attributed<sup>3</sup> to both the basic and amphiphilic nature of its rigid cyclic  $\beta$ -hairpin molecular structure.<sup>4</sup> The fact that the enantiomer of GS is equally active<sup>5</sup> supports the current thinking that the cell membrane, and not a specific gene product,<sup>6</sup> is targeted by GS. The structure of GS is amphiphilic with the side chains of the two Orn residues on one side of the molecule and the hydrophobic side chains of the two Val and Leu residues residing on the opposing face (see Appendix, Figure 5). It has been suggested that the positively charged residues are needed for initial

interaction with the negatively charged phosphate groups on the surface of the lipid bilayer, and that the hydrophobic residues have interaction with the inner lipophilic part of the bilayer.<sup>7</sup>

**Figure 1** The structural formula of GS.



Drawback of GS is its toxicity toward human cells, limiting its antibiotic use to topical applications.<sup>8</sup> This toxicity is associated with the non-discriminative character of GS to disrupt both bacterial- and mammalian cell membranes. Since GS and similar cationic peptides do not target a specific bacterial gene product, antibiotic resistance against this type of compounds may not readily occur.<sup>9</sup> Therefore, modification of GS in order to arrive at compounds with an improved therapeutic profile appears to be an attractive strategy in the search for an efficient broad spectrum antibiotic.

In the 70s and 80s, GS has been the subject of extensive structure-activity relationship studies, in which amino acids throughout the molecule were systematically replaced with a variety of  $\alpha$ -amino acids. From these studies it followed that activity is highly dependent on the hydrophobic nature of the amino acids on the positions of the Val and Leu residues.<sup>10</sup> Furthermore, the Pro residue may be replaced with Gly or Leu without affecting the antibacterial activity.<sup>11</sup> Finally, replacement of the <sup>D</sup>Phe residue with other amino acids is allowed, provided that the side chains of these are bulky and of hydrophobic nature.<sup>12</sup> However, replacing D-phenylalanine with L-phenylalanine resulted in an inactive compound.<sup>12a</sup> Grotenbreg *et al.* focused on the design of GS analogs in which one or both  $\beta$ -turn regions were varied.<sup>13</sup> In a first series of analogs, the <sup>D</sup>Phe or Pro residues were modified by attachment of different groups to the phenyl of pyrrolidine ring. Alternatively, the <sup>D</sup>Phe-Pro motif was replaced with various sugar amino acids. In these cases, modification of the  $\beta$ -turn did not result in peptides with an improved biological profile. Antibacterial activity turned out to correlate hemolytic activity: when an analog with decreased hemolytic activity was identified, this analog also showed decreased antibacterial activity and *vice versa*.

The group of Izumiya, amongst others, has synthesized some GS analogs in which either the number of charges or the nature of the hydrophobic residues was altered.<sup>14</sup>

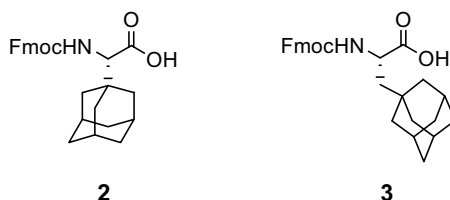
Unfortunately, these molecules were evaluated for their antibacterial activity only and thus nothing is known about the relationship between peptide hydrophobicity and toxicity for these molecules. Hodges and co-workers prepared various analogs of GS by systematically replacing an amino acid with its enantiomer, using either GS or ring analogs, both smaller and larger rings, as starting point.<sup>15</sup> Some of the extended GS analogs, having four positive charges,<sup>15a</sup> were found to possess significantly less hemolytic activity while retaining, to a certain extent, their antibacterial activity.

The aim of the research described in this Chapter is to correlate the amphiphilic character of GS-derived cyclic peptides with both hemolytic and antibacterial activities. Amino acid substitutions in the GS parent peptide were introduced such that a series of amphiphilic derivatives were obtained, with either two or four positive charges, and ranging between having very polar and very hydrophobic characteristics. Thus, eleven cyclic peptides were synthesized and investigated for their structural and biological properties in comparison with GS. The design of the peptides, their synthesis, structural and biological evaluation are discussed in detail in the following sections.

### Design of target compounds

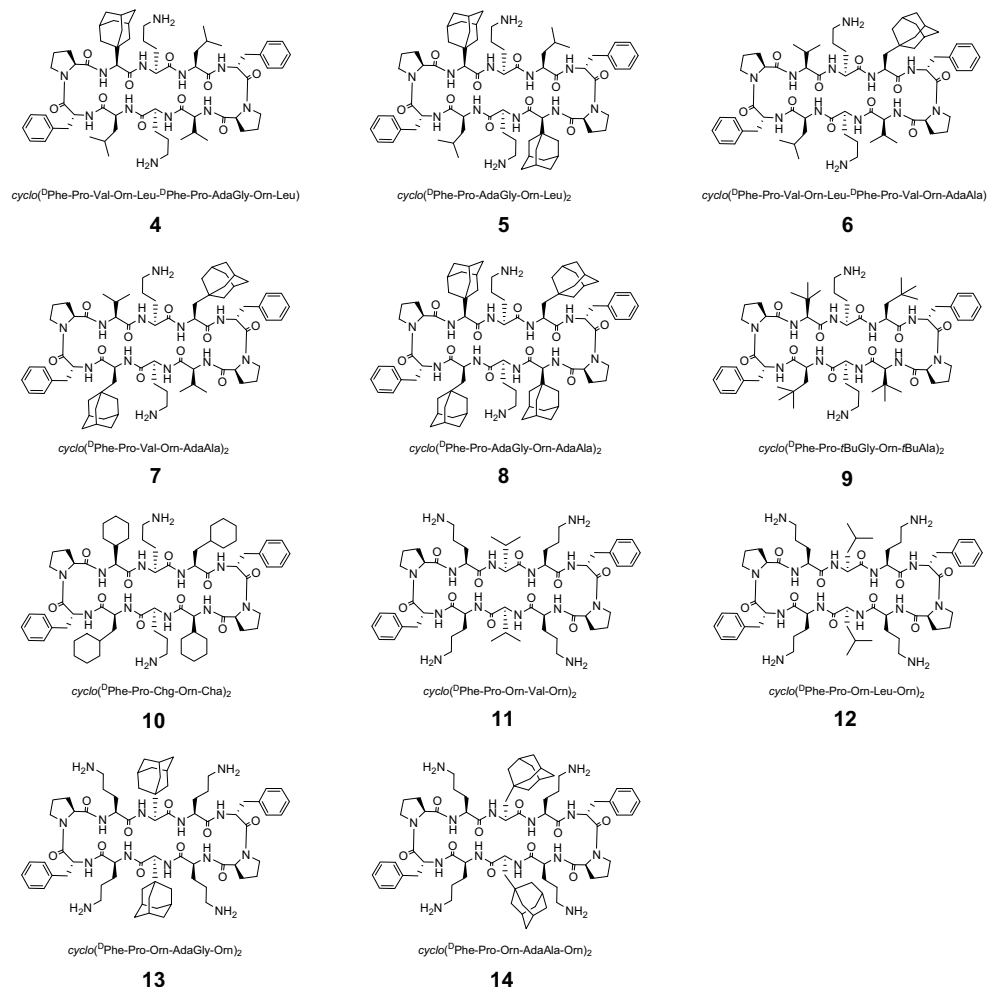
The adamantyl-amino acids<sup>16</sup> **2** and **3** (Figure 2) were chosen because adamantane moieties are hydrophobic and thought to be ideally suited for interaction with lipid bilayers.<sup>17</sup>

**Figure 2** Adamantyl amino acids used to incorporate in GS analogs.



Adamantyl-L-glycine functioned as replacement for valine residues, and adamantyl-L-alanine served as a replacement for leucine residues. A series of eleven GS analogs **4-14** (Figure 3) was designed, in which either one or more of the hydrophobic Val and Leu residues were replaced with the more hydrophobic adamantyl-, *t*-butyl-, or cyclohexyl amino acids (peptides **4-10**), or the hydrophobic residues were swapped for cationic residues and *vice versa* (peptides **11-14**, for similar approaches, see ref. 14b-e). The first series of modifications would result in peptides more hydrophobic than GS, the second in peptides more hydrophilic than GS. In all cases, the turn regions were as in the parent compound, GS (**1**).

**Figure 3** GS analogs discussed in this chapter: one or more hydrophobic residues replaced with adamantane residues (**4-8**), all residues replaced with either *tert*-butyl- or cyclohexyl amino acids (**9, 10**) and ‘inverted’ GS analogs, with either two valines, two leucines, two adamantylglycines or two adamantylalanines and four ornithines (**11-14**).

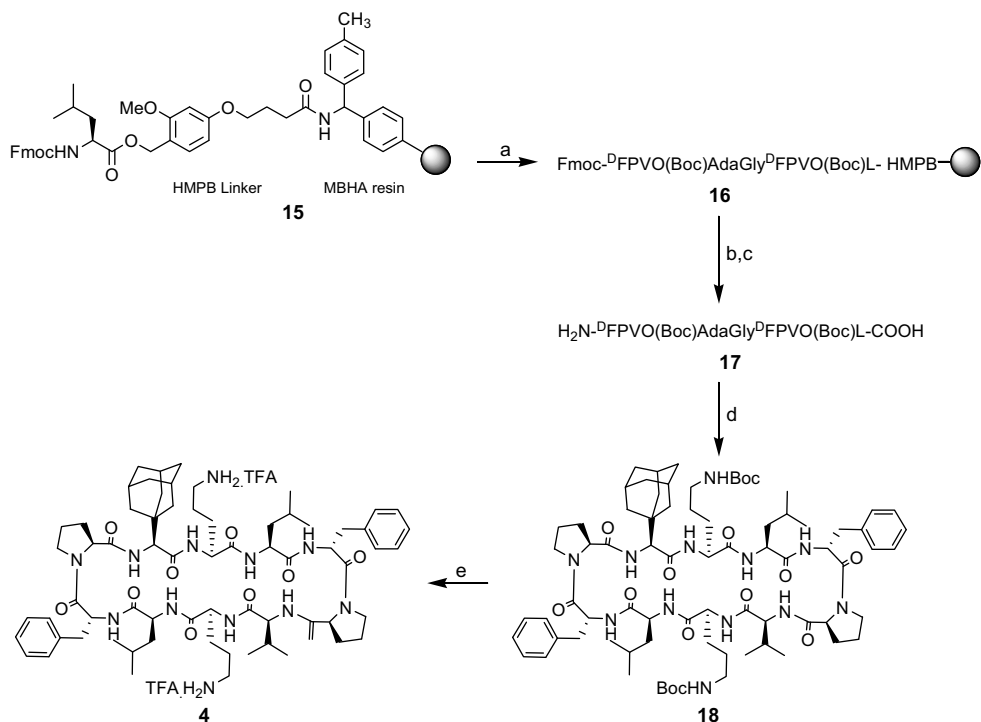


### Preparation of the GS analogs

Adamantyl amino acids **2** and **3** were synthesized as described in Chapter 3, using a slightly modified literature procedure.<sup>18</sup> Previous studies have shown that GS and its derivatives can be readily synthesized by solution phase cyclization of the corresponding linear precursor peptide(s) with protected ornithine side chains.<sup>19</sup> The efficiency of the cyclization reaction of an ester-activated linear GS precursor depends on its ability to preorganize into a  $\beta$ -hairpin conformation in solution.<sup>20</sup> This is facilitated by the presence of one  $\beta$ -turn in the middle of the sequence. With this in mind, decameric peptides were stepwise assembled on the solid support, with either

leucine (for peptide **4** and **5**), adamantyl-L-alanine (peptides **6-8**), *tert*-butyl-L-alanine (peptide **9**); cyclohexyl-L-alanine (peptide **10**) or ornithine (peptides **11-14**) being the first amino acid immobilized on the resin. A representative example of the synthesis of the GS analogs is given in Scheme 1.

**Scheme 1** Representative synthesis of peptide **4** using Fmoc SPPS methods.



**Reagents and conditions:** a) repeated coupling cycles consisting of: i. Fmoc deprotection (20% piperidine/NMP, 15 min). ii. Amino acid condensation (3 eq Fmoc-AA-OH, 3 eq HCTU, 3.6 eq DIPEA, NMP, 2 h). b) 20% piperidine/NMP, 15 min. c) 1% TFA/DCM, 4 x 10 min. d) 5 eq PyBOP, 5 eq HOBT, 15 eq DIPEA, DMF, 16 h. e) 50% TFA/DCM, 30 min, 27% after HPLC purification.

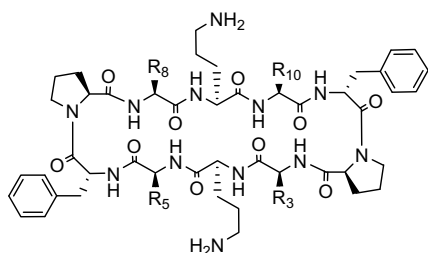
The elongation towards the immobilized decapeptides, cleavage from resin, cyclization, deprotection and purification of the peptides proceeded uneventfully and the target peptides were obtained as their TFA salts in high purity (>95 %) and in good yields.

### Structural analysis of the synthesized GS analogs

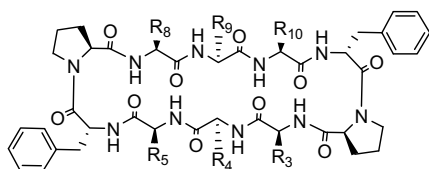
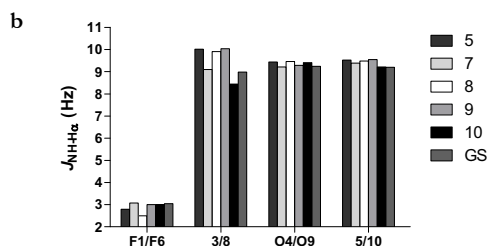
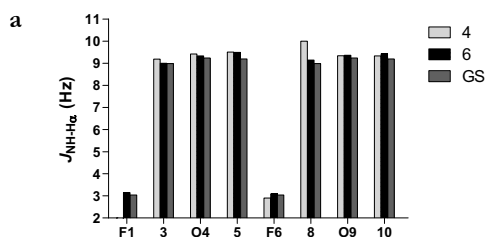
All peptides were analyzed by NMR at 298 K to evaluate their secondary structure. The general structural characteristics of peptides **5**, **7-11** and **13** as established in this way highly resemble those of GS (Figure 4). The four signals in the CONH region of

the  $^1\text{H}$  spectra showed that the molecules possess a C2-symmetric secondary structure, just like GS itself. Asymmetric peptides **4** and **6** have eight signals in the CONH region.

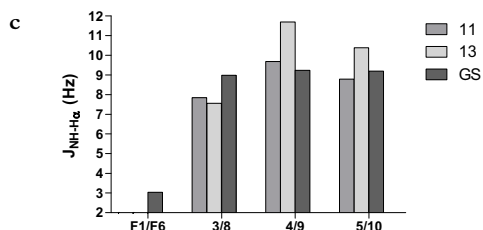
**Figure 4** Comparison of  $J_{\text{NH-H}\alpha}$  values to those of GS for GS analogs. a) Asymmetric peptides **4** and **6**. For **4**, no detectable  $J_{\text{NH-H}\alpha}$  was observed for F1. b) Symmetric peptides **5** and **7-10**. c) Inverted symmetric peptides **11** and **13**. For both analogs, no detectable  $J_{\text{NH-H}\alpha}$  was observed for both phenylalanine residues.



- 4**  $R_3 = \text{Val}, R_5 = R_{10} = \text{Leu}, R_8 = \text{AdaGly}$   
**5**  $R_3 = R_8 = \text{AdaGly}, R_5 = R_{10} = \text{Leu}$   
**6**  $R_3 = R_8 = \text{Val}, R_5 = \text{Leu}, R_{10} = \text{AdaAla}$   
**7**  $R_3 = R_8 = \text{Val}, R_5 = R_{10} = \text{AdaAla}$   
**8**  $R_3 = R_8 = \text{AdaGly}, R_5 = R_{10} = \text{AdaAla}$   
**9**  $R_3 = R_8 = \text{tBuGly}, R_5 = R_{10} = \text{tBuAla}$   
**10**  $R_3 = R_8 = \text{Chg}, R_5 = R_{10} = \text{Cha}$   
**GS**  $R_3 = R_8 = \text{Val}, R_5 = R_{10} = \text{Leu}$



- 11**  $R_3 = R_5 = R_8 = R_{10} = \text{Orn}, R_4 = R_9 = \text{Val}$   
**13**  $R_3 = R_5 = R_8 = R_{10} = \text{Orn}, R_4 = R_9 = \text{AdaGly}$   
**GS**  $R_3 = R_8 = \text{Val}, R_4 = R_9 = \text{Orn}, R_5 = R_{10} = \text{Leu}$



The  $J_{\text{NH-H}\alpha}$  values of the ornithine, adamantylglycine, adamantylalanine, leucine and valine residues were all between 8 and 12 Hz (Figure 4), which is a strong indication of a  $\beta$ -strand conformation.<sup>21</sup> The  $J_{\text{NH-H}\alpha}$  values of the  $^{\text{D}}\text{Phe}$  residues were in the range of 2-4 Hz, which is typical for an amino acid as part of a  $\beta$ -turn.<sup>21</sup> The NOE signals between the backbone amide NH protons were similar to the corresponding NOE crosspeaks of GS. Thus, the NMR data of these compounds indicate a rigid cyclic  $\beta$ -hairpin secondary structure in solution, which is also observed for GS. The NMR data

of compounds **12** and **14** were remarkably different in comparison to GS and the other compounds: no C2-symmetric conformer could be observed at 298 K (for details, see the end of this Chapter).

One of the synthesized analogs (**9**) provided crystals suitable for X-ray analysis. Compound **9** adopts in the solid state an amphiphilic  $\beta$ -hairpin structure (See appendix, Figure 6), agreeing with the NMR data of this compound in solution and closely resembling the X-ray structure of GS.

### Evaluation of antibacterial activity

The antibacterial activities of GS and analogs **4-14** against a variety of Gram-positive and Gram-negative bacteria, including several MRSA strains, are shown in Table 1.

GS shows, as expected,<sup>2</sup> strong activity against Gram-positive bacteria and, to a lesser extent, Gram-negative bacteria.

**Table 1** Antimicrobial properties of synthesized GS and analogs **4-14**. MIC-values (MIC = Minimal Inhibitory Concentration) are given in  $\mu\text{g}/\text{ml}$  and were measured after 24 hours of incubation.

	Gram+ <i>S. aureus</i>	Gram+ <i>S. epidermidis</i>	Gram+ <i>E. faecalis</i>	Gram+ <i>B. cereus</i>	Gram- <i>E. coli</i>	Gram- <i>P. aeruginosa</i>	MRSA-NT 1110301981H-T034- PVL+	MRSA-NT N133-T034-PVL-	MRSA-NT N229-T034-PVL-	MRSA-Cluster218 USA300-1110301146 PVL+
<b>GS</b>	8	4	8	8	32	64	8	16	8	8
<b>4</b>	8	4	8	8	>64	>64	--	--	--	--
<b>5</b>	16	16	16	16	64	64	--	--	--	--
<b>6</b>	8	4-8	8	4	>64	>64	--	--	--	--
<b>7</b>	>64	16	16	64	>64	>64	--	--	--	--
<b>8</b>	>64	>64	>64	>64	>64	>64	--	--	--	--
<b>9</b>	4	4	4	4	64	32	8	8	8	8
<b>10</b>	16	16	32	16	>64	>64	--	--	--	--
<b>11</b>	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
<b>12</b>	>64	64	>64	>64	>64	>64	--	--	--	--
<b>13</b>	8	4	8	8	8	16	8	8	8	8
<b>14</b>	8	4	8	8	8	16	8	8	8	8

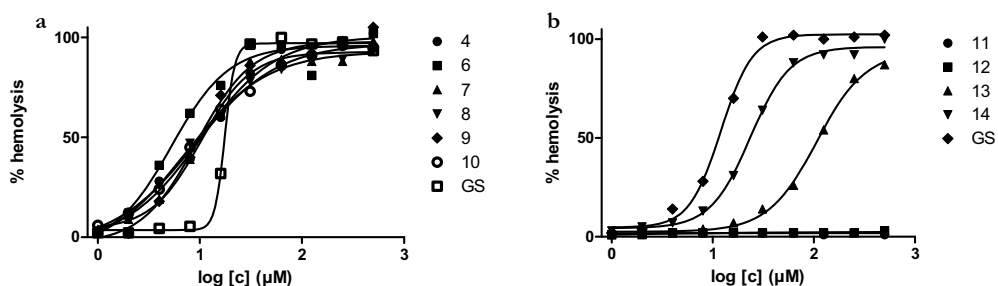


Also, it is very effective against different MRSA strains.<sup>22</sup> Compounds **4** and **6** are as active as GS against Gram-positive bacteria and less active against Gram-negative bacteria, but **5** and **7** show less activity against all bacteria assayed. Peptide **8** is completely inactive. Peptide **9** shows activity comparable to that of GS against Gram-positive bacteria, and performs well against the MRSA strains. Peptide **10** is less active against all bacteria. Of the inverted compounds **11** and **12** do not display any inhibition in bacterial growth, but peptides **13** and **14** are as active as GS towards Gram-positive bacteria. Furthermore, compared to GS, they show an increased activity against the two Gram-negative bacteria. The activity against the MRSA strains is comparable to that of GS.

### Hemolytic activity of GS analogs

A frequently used procedure to assess the toxicity of a compound towards mammalian cells is by measuring its hemolytic activity at various concentrations. Thus, the ability of the peptides **4-14** to lyse human red blood cells was determined. The results are depicted in Figure 5. As expected,<sup>2</sup> GS shows high hemolytic activity. Peptides **4** and **6-10** are more hemolytic than GS,<sup>23</sup> as can be judged from the lower concentrations needed to effect 50% hemolysis. Peptide **5** did not dissolve well in the buffers used for the assay and therefore no reliable measurements could be performed. The derivatives **11** and **12** are not toxic for human red blood cells. Interestingly, the peptides **13** and **14** show reduced hemolytic properties.

**Figure 5** Hemolytic activity of GS analogs **4-14** on human red blood cells, fitted in logarithmic dose-response curves. Experiments were carried out in triplicate. a) Peptides **4-10** compared to GS. Compound **5** did not dissolve well in the buffers used in the assay and is therefore not included in the graph. b) Inverted GS analogs **11-14** compared to GS.



Reversed phase LC-MS was used to determine the retention times of the cyclic peptides **4-14** to assess whether amphiphilic characteristics of these GS analogs can be correlated to their antimicrobial/hemolytic activity.<sup>24</sup>

**Table 2** LC-MS retention times and the number of charges of GS and analogs **4-14**, ranked from least to most hydrophobic. LC-MS spectra were recorded using a gradient of 10 → 90% MeCN in the presence of 0.1% TFA on a C<sub>18</sub> column and a run duration of 15 min.

Peptide	Number of charges	Retention time	Peptide	Number of charges	Retention time
<b>11</b>	4	4.70	<b>6</b>	2	9.72
<b>12</b>	4	4.97	<b>4</b>	2	9.77
<b>13</b>	4	6.39	<b>10</b>	2	10.43
<b>14</b>	4	6.66	<b>5</b>	2	11.01
<b>GS</b>	2	8.38	<b>7</b>	2	11.09
<b>9</b>	2	9.43	<b>8</b>	2	12.57

Using this empiric method, the cyclic peptides with the shortest retention times are termed hydrophilic and those with the longest retention time are termed hydrophobic. All peptides with longer retention times than GS display reduced antibacterial activity. It thus appears that hydrophobicity and antibacterial activity are inversely related, to a certain extent: upon increase of hydrophobicity, the antibacterial activity decreases with the most hydrophobic peptide **8** showing no activity at all. The most hydrophilic compounds **11** and **12** are also devoid of antimicrobial activity. Peptides **13** and **14**, with retention times close to that of GS, have a slightly improved antibacterial activity. The hemolytic activities of the analogs may be correlated to hydrophobicity as well: peptides more hydrophobic than GS show similar ability to lyse human red blood cells. Upon increase of hydrophilicity, the hemolytic activity decreases, with compounds **14** and especially **13** showing reduced toxicity and with **11** and **12** having no antibacterial and hemolytic activity at all. These findings may be explained by the fact that the composition of bacterial cell membranes differs from those of mammalian membranes. Bacterial cell membranes contain a high percentage of the negatively charged phospholipids such as phosphatidylglycerol, cardiolipin and phosphatidylserine. Mammalian cell membranes in contrast contain more zwitterionic and uncharged molecules such as phosphatidylethanolamine, phosphatidylcholine and cholesterol.<sup>25</sup> This hypothesis is supported by the fact that in model lipid bilayer systems, the activity of both GS itself and a larger ring size analog is strongly dependent on the lipid bilayer composition.<sup>7,26</sup>

Apparently, increasing the positive charge of a cationic antimicrobial peptide results in higher affinity for the bacterial membrane overall and less affinity for the red blood cells, provided that this additional charge is compensated for by incorporating hydrophobic amino acids in the peptide, such as the adamantyl amino acids. Only incorporating more hydrophobic residues in the peptide without increasing the charge

of the molecule, and thus making it more hydrophobic, decreases affinity towards bacterial membranes, while red blood cells are still targeted.

### Conclusion

In this Chapter the synthesis of eleven GS analogs, each with a different amphiphilic profile, is described. Their antibacterial and hemolytic activities were evaluated, and the results correlated. It was found that the amphiphilic peptides possessing four positive charges and two adamantane moieties show improved antibacterial activity and strongly reduced hemolytic activity. Peptides **13** and **14** emerged as the most promising compounds because of their ability to distinguish, to a certain extent, between bacterial- and mammalian cells. Apparently, there seems to be an optimal distribution between charge on one side of the molecule and hydrophobicity on the other side, which can be visualized by comparing LC-MS retention times. A similar observation was made in a recent literature report for  $\alpha$ -helical amphiphilic antimicrobial peptides.<sup>27</sup> Furthermore, it cannot be excluded that the compact and hydrophobic nature of the adamantane moiety is an important factor in the interaction of the GS analogs with bacterial cell membranes.

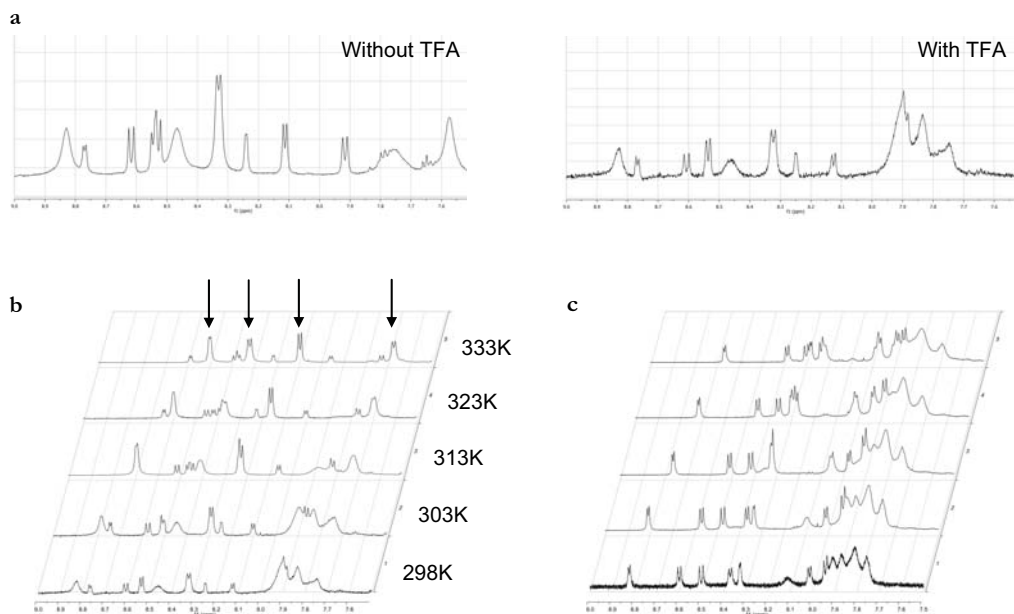
Compounds **13** and **14** are spectroscopically related, except for two additional methylene moieties in **14**. Both peptides show similar antibacterial activity, but **14** is more toxic against red blood cells and shows different structural characteristics as witnessed by NMR. This suggests that differences seen in hemolytic activity may not be solely due to alterations in the amphiphilic character.<sup>15a,15b</sup> However, it remains to be seen whether peptide **14** shows the same conformational change when present in a membrane environment *in vivo*. Based on their decreased hemolytic activity combined with enhanced antimicrobial activity and their action on a broader spectrum of bacteria as compared to GS, as well as their activity against MRSA strains, peptide **14** and especially peptide **13** are interesting lead structures for the development of novel broad applicable antibiotics against a wide spectrum of bacteria, including multi-drug resistant strains.

## NMR analysis of peptides **12** and **14**

In the NH region (9-7.5 ppm) of the NMR spectrum of compound **12** in CD<sub>3</sub>OH at 298 K broad and sharp signals were observed, but the number of NH signals did not indicate a GS-like C<sub>2</sub>-symmetric conformation. The same was the case for peptide **14**. Upon addition of trifluoroacetic acid to **12**, some peaks shifted (for instance, the peaks at 8.5 ppm and 7.6-7.8 ppm) and others broadened (7.5-7.8 ppm) (Figure 6a), indicating that the ornithine side chains were completely protonated and protons were exchanged with either the solvent or with another conformer. Upon heating to 333 K some broad peaks disappeared (those resulting from solvent exchange) and others sharpened (those resulting from exchange between conformers). Two conformers in a ~1:4 ratio were clearly observed (Figure 6b). The major conformer possessed the same C<sub>2</sub> symmetric secondary structure as GS and analogs **4-11** and **13**. The minor conformer did not show this symmetry.

For the symmetrical conformer of **12**, the  $J_{\text{NH-H}\alpha}$  coupling constants were similar to those observed in GS; for <sup>2</sup>D<sub>2</sub>Phe a coupling constant of ~4 Hz was observed; for the other residues  $J_{\text{NH-H}\alpha}$  values were between 7 and 9 Hz. The  $J_{\text{NH-H}\alpha}$  coupling constants of the unsymmetrical conformer of **12** showed a completely different picture: one <sup>2</sup>D<sub>2</sub>Phe had a  $J_{\text{NH-H}\alpha}$  value of 5.2 Hz and the other <sup>2</sup>D<sub>2</sub>Phe was not observed, while one of the ornithine NH signals showed a remarkably low coupling constant of 3.4 Hz. Also, both leucines displayed lowered  $J_{\text{NH-H}\alpha}$  values of ~6.8 Hz. For all the other residues,  $J_{\text{NH-H}\alpha}$  coupling constants varied from 8 to 9.5 Hz. Compound **14** showed behavior similar to **12** upon heating in the presence of a trace of TFA (Figure 6c), however at 333 K, no convergence to a symmetric secondary structure was seen, which made proton assignment quite complicated.

**Figure 6** Comparison of <sup>1</sup>H NMR spectra for compounds **12** and **14** in the range 9-7.5 ppm. a) The <sup>1</sup>H NMR spectrum of **12** in the absence (left) and presence (right) of TFA. b) Convergence to one major conformer is seen when peptide **12** is heated to 333K. The NH signals of the major symmetrical conformer are indicated by arrows. c) Heating of **14** does not have any effect on the ratio between the conformers. All spectra were recorded in CD<sub>3</sub>OH at 600 MHz.



The low  $J_{\text{NH-H}\alpha}$  for one of the ornithines suggests that the asymmetric conformer probably possesses an additional twist in the  $\beta$ -sheet region. To verify this, another NMR analysis method was used; namely the chemical shift perturbation method.<sup>28</sup> In this method, the chemical shift of the  $H_\alpha$  of an amino acid is compared to that of the same residue in a random coil. This was applied to the asymmetric conformer of **12** and the results are shown in Table 3.

Looking at the individual perturbations it becomes obvious that three ornithines indeed are part of a  $\beta$ -strand. The fourth, with  $\delta H_\alpha = 3.96$ , shows a perturbation of  $-0.40$  ppm, which indicates the presence of a turn. This is confirmed by the small  $J_{\text{NH-H}\alpha}$  value of 3.4 Hz. Although the NH-H $\alpha$  coupling constant for the <sup>15</sup>Phe residue was larger compared to the same residue in GS, the perturbation value indicates that this amino acid is part of a turn. The two leucine residues have roughly the same  $J_{\text{NH-H}\alpha}$  values (6.77 and 6.81 Hz), but the perturbation values show that while one leucine is part of a  $\beta$ -strand, the other exists in a random coil conformation. These results strongly support a conformation in which one Orn-Leu-Orn sequence forms a  $\beta$ -strand, and the opposing Orn-Leu-Orn sequence is twisted.

**Table 3** Chemical shift perturbation of  $H_\alpha$  protons in the asymmetric conformer of **12**. For ornithine residues, the reported<sup>28</sup>  $\delta(H_\alpha)$  random coil value of lysine was taken. The chemical shift perturbation was calculated as  $\Delta \delta H_\alpha = \delta H_\alpha(\text{observed}) - \delta H_\alpha(\text{random coil})$ .

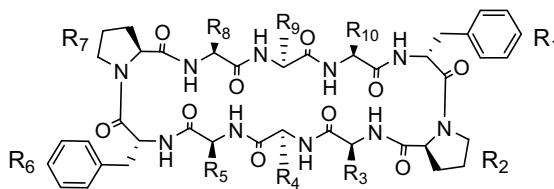
$\delta H_\alpha$ (ppm)	Residue	Reported value <sup>28</sup> (ppm)	$\Delta \delta H_\alpha$ (ppm)
4.81	Orn	4.36	0.45
4.65	Orn	4.36	0.29
4.60	Orn	4.36	0.24
4.45	<sup>15</sup> Phe	4.66	-0.21
4.28	Leu	4.17	0.11
4.17	Leu	4.17	0.00
3.96	Orn	4.36	-0.40

## Experimental Section

### General

Reagents and solvents were used as provided, unless stated otherwise. NMR spectra were recorded at 298K on a Bruker AV400 or a Bruker DMX600 using deuterated solvents. All carbon spectra are proton-decoupled. Chemical shifts ( $\delta$ ) are given in ppm, in <sup>13</sup>C spectra relative to the solvent peak of CD<sub>3</sub>OH (49.0 ppm), in <sup>1</sup>H spectra relative to the solvent peak of CD<sub>3</sub>OH (3.31 ppm). Coupling constants are given in Hz. IR spectra were recorded on a Perkin Elmer Paragon 1000 FT-IR Spectrometer. High resolution mass spectra were recorded by direct injection (2  $\mu$ L of a 2  $\mu$ M solution in H<sub>2</sub>O/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 250 °C) with resolution R = 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). Peptides were synthesized on solid support (pre-loaded Fmoc-Leu-HMPB-BHA resin, Novabiochem, 0.51 mmol g<sup>-1</sup>, 100-200 Mesh or MBHA resin HL.HCl, Novabiochem, 100-200 Mesh) and amino acids were coupled manually using Fmoc based peptide synthesis methods and commercially available Fmoc amino acids. Fmoc- $\alpha$ -(cyclohexyl)-L-glycine (Fmoc-Chg-OH) was purchased from ChemImpex. Fmoc- $\beta$ -cyclohexyl-L-alanine (Fmoc-Cha-OH) and Fmoc-L- $\alpha$ -*t*-butylglycine (Fmoc-*t*BuGly-OH) were purchased from Novabiochem. Fmoc-L- $\beta$ -*t*-butylalanine

(Fmoc-*t*BuAla-OH) was purchased from NeMPS. Fmoc-adamantyl-L-glycine and Fmoc-adamantyl-L-alanine were prepared as described in Chapter 3 using slightly modified literature procedures.<sup>18</sup> LC-MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electrospray Interface (ESI). An analytical Gemini C<sub>18</sub> column (Phenomenex, 50 x 4.60 mm, 3 micron) was used in combination with buffers A: H<sub>2</sub>O, B: MeCN and C: 1.0% aq. TFA. An analytical Alltima CN column (Alltech, 150 x 4.6 mm, 3 micron) was used for analysis of cyclized protected peptides and peptide **8**. Alternatively, a Finnigan Surveyor HPLC system with a Gemini C<sub>18</sub> 50 × 4.6 mm column (3 micron, Phenomenex, Torrance, CA, USA) (detection at 200-600 nm), coupled to a Thermo Finnigan LCQ Advantage Max mass spectrometer (Breda, The Netherlands) with electrospray ionization (ESI; system 1) was used, with the same buffers as described above. For RP-HPLC purification of the peptides, a BioCAD ‘Vision’ automated HPLC system (PerSeptiveBiosystems, inc.) supplied with a preparative Gemini C<sub>18</sub> column (Phenomenex, 150 x 21.2 mm, 5 micron) was used. For purification of peptide **8**, a preparative Alltima CN column (Alltech, 150 x 22 mm, 5 micron) was used. The applied buffers were A: H<sub>2</sub>O, B: MeCN and C: 1.0% aq. TFA. All NMR signals were assigned as much as possible. For the assignment of all GS analogs, residue numbering was used as depicted on the right. Graphs were made using Graphpad Prism 5, and data concerning the hemolytic activity of the peptides were fitted using the same program.



### Antibacterial Assays

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), and the MRSA strains MRSA-NT 1110301981H-T034-PVL+, MRSA-NT N133-T034-PVL-, MRSA-NT N229-T034-PVL- (cattle-related strains) and MRSA-Cluster218 USA300-1110301146 PVL+ (a MRSA strain well known in the USA). Bacteria were stored at -70 °C and grown at 35 °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 (100 x) with physiological saline, and 2 µl of this inoculum was added to 100 µl growth medium, Cation Adjusted Mueller Hinton II Broth (BBL ref. nr. 212322, lot nr. 7079753), in microtiter plates (96 wells). The peptides GS and **4-14** were dissolved in methanol (1 g/l) and two-fold diluted in the broth (64, 32, 16, 8, 4 and 1 mg/l). The plates were incubated at 35 °C (24 h) and the MIC was determined as the lowest concentration inhibiting bacterial growth.

### Hemolytic assays

Freshly drawn heparinized blood was centrifuged for 10 minutes at 1000g 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 (**4-14** and GS) 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. 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 of wells A10-C10. These steps were repeated for the other peptides. Subsequently, 50 µl of the red blood cell suspension was added to the wells and the plates were incubated at 37 °C for 4 hours. After incubation, the plates were centrifuged at 1000g 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.

## Peptide Synthesis

### a) Loading of the resin

GS and its analogs were synthesized as reported previously.<sup>13a</sup> The 'inverted' GS analogs **11-14** were synthesized starting from highly acid-labile HMPB-MBHA resin. To MBHA resin (Novabiochem, MBHA resin HL.HCl, 100-200 Mesh, theoretical load: 1.2 mmol g<sup>-1</sup>, 2 g) the HMPB linker was coupled as described hereafter. The resin was swollen in DCM for 2 h, after removal of the solvent a solution of HMBP (1.73 g, 7.2 mmol, 3 eq), PyBOP (3.04 g, 7.2 mmol, 3 eq) and DIPEA (2.5 ml, 14.4 mmol, 6 eq) in NMP was added to the dry resin and the mixture shaken overnight. When Kaiserstest indicated a complete coupling, the solvent was removed and the resin washed (NMP, DCM and Et<sub>2</sub>O) and air-dried.

Dry HMPB-MBHA resin (estimated load: 0.5 mmol g<sup>-1</sup>, 1 g, 500 μmol) was swollen in DCM, after removal of the solvent a solution of Fmoc-Orn(Boc)-OH (1.14 g, 2.5 mmol, 5 eq), HCTU (1.03 g, 2.5 mmol, 5 eq), DIPEA (871 μl, 5 mmol, 10 eq) in NMP/DCM (1:1 v/v) was added to the resin and the mixture was shaken overnight. Solvents were removed, the resin was washed (NMP, DCM and Et<sub>2</sub>O) and air-dried. Resin loading was determined using the Fmoc test to be 0.48 mmol g<sup>-1</sup>.

The resin needed for preparation of **8** was prepared from HMPB-MBHA resin, which was loaded with Fmoc-AdaAla-OH as described above.

### b) Synthesis of the linear decamer on resin, general procedure (Scheme 1)

Peptide synthesis was performed using standard Fmoc SPPS methods. Elongation of the peptide chain was performed using repeating coupling cycles containing the following steps: I) Fmoc deprotection (20% piperidine/NMP, 15 min). II) Peptide coupling (3 eq Fmoc-AA-OH, 3 eq HCTU, 3.6 eq DIPEA, NMP, 2h).

For the synthesis of **4-8**, amino acids were attached in the following order, starting from HMPB-MBHA resin: Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-<sup>D</sup>Phe-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-<sup>D</sup>Phe-OH. Where needed, Fmoc-Val-OH and Fmoc-Leu-OH were replaced with Fmoc-AdaGly-OH or Fmoc-AdaAla-OH, respectively. Peptides **9-10** were synthesized in the same manner, but with Fmoc-Val-OH and Fmoc-Leu-OH replaced with Fmoc-Chg-OH and Fmoc-<sup>t</sup>BuGly-OH or Fmoc-Cha-OH and Fmoc-<sup>t</sup>BuAla-OH, respectively.

For the inverted GS analogs **11-14**, synthesis was started from Fmoc-Orn(Boc)-HMPB-MBHA resin. The peptide chain was elongated in the usual manner, coupling amino acids in the following order: Fmoc-Val-OH, Fmoc-Orn(Boc)-OH, Fmoc-Pro-OH, Fmoc-<sup>D</sup>Phe-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Orn(Boc)-OH, Fmoc-Pro-OH, Fmoc-<sup>D</sup>Phe-OH. For the other inverted GS analogs, Fmoc-Val-OH was replaced with Fmoc-Leu-OH, Fmoc-AdaGly-OH or Fmoc-AdaAla-OH.

### c) Cleavage from resin, general procedure (Scheme 1)

After attachment of the final amino acid to the peptide (150 μmol), the Fmoc group was liberated and the peptide cleaved from the resin (1% TFA/DCM, 4 x 10 min, 4 x 15 ml). The washings were collected in a toluene-containing flask (20 ml toluene) and concentrated to ~10% of the volume, this to prevent the mixture from becoming too acidic, with undesired cleavage of side chain protecting groups as a consequence. Fresh toluene was added and the solution again concentrated to 10% of the volume. Finally, another batch of toluene was added and the mixture concentrated to dryness to yield the crude peptide as a white solid.

### d) Cyclization, general procedure (Scheme 1)

The crude linear peptide (150 μmol) was taken up in DMF (7.5 ml) and added dropwise during 1 h to a solution of PyBOP (0.39 g, 750 μmol, 5 eq), HOBT (0.10 g, 750 μmol, 5 eq) and DIPEA (0.39

ml, 2.25 mmol, 15 eq) in DMF (120 ml). After stirring overnight, the solvent was evaporated and the residue applied to a LH-20 size-exclusion column, using MeOH as eluent. The peptide was obtained as a yellowish solid.

### e) Deprotection, general procedure (Scheme 1)

The protected peptide was dissolved in DCM (11 ml) and cooled to 0° C. TFA (11 ml) was added slowly and the mixture warmed to rt. After stirring for 30 min, solvents were evaporated and the residue coevaporated with toluene (three times) to remove all traces of TFA. The crude peptide was obtained as a yellowish solid and purified by RP-HPLC, using gradients of H<sub>2</sub>O/MeCN/1% TFA. The fractions containing the pure product were pooled, concentrated and freeze-dried to yield the pure peptide.

### Analytical data for synthesized peptides

#### *Cyclo*(<sup>D</sup>Phe-Pro-Val-Orn-Leu-<sup>D</sup>Phe-Pro-AdaGly-Orn-Leu)<sub>2</sub>TFA (4)

Following the general procedures for peptide synthesis and cleavage, **4** was obtained from Fmoc-Leu-HMPB-MBHA resin (150 μmol) as a white powder (50.3 mg, 40.7 μmol, 27%) using an HPLC gradient of 40:50:10 → 0:90:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.92 (d, *J* = 2.9 Hz, 1H, NH <sup>D</sup>Phe<sub>6</sub>); 8.82-8.79 (m, 2H, NH Leu<sub>10</sub>, NH <sup>D</sup>Phe<sub>1</sub>); 8.76 (d, *J* = 9.4 Hz, 1H, NH Orn<sub>4</sub>); 8.60 (d, *J* = 9.5 Hz, 1H, NH Leu<sub>5</sub>); 8.53 (d, *J* = 9.3 Hz, 1H, NH Orn<sub>9</sub>); 7.87 (d, *J* = 18.5 Hz, 4H, 2 x NH Orn side chain); 7.68 (d, *J* = 9.2 Hz, 1H, NH Val<sub>3</sub>); 7.57 (d, *J* = 10.0 Hz, 1H, NH AdaGly<sub>8</sub>); 7.34-7.27 (m, 6H, CH <sup>D</sup>Phe); 7.26-7.23 (m, 4H, CH <sup>D</sup>Phe); ~4.90 (H<sub>α</sub> Orn, peak not visible due to solvent suppression); 4.71-4.65 (m, 2H, 2 x H<sub>α</sub> Leu<sub>5</sub>, Leu<sub>10</sub>); 4.52-4.47 (m, 2H, 2 x H<sub>α</sub> <sup>D</sup>Phe<sub>1</sub>, <sup>D</sup>Phe<sub>6</sub>); 4.38-4.31 (m, 3H, H<sub>α</sub> AdaGly, 2 x H<sub>α</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 4.22 (t, *J* = 8.8 Hz, 1H, H<sub>α</sub> Val<sub>3</sub>); 3.80 (t, *J* = 8.5 Hz, 1H, H<sub>δA</sub> Pro<sub>2</sub>); 3.73 (t, *J* = 7.9 Hz, 1H, H<sub>δA</sub> Pro<sub>7</sub>); 3.10 (t, *J* = 5.0 Hz, 1H, H<sub>βA</sub> <sup>D</sup>Phe<sub>6</sub>); 3.08 (t, *J* = 4.8 Hz, 1H, H<sub>βA</sub> <sup>D</sup>Phe<sub>1</sub>); 3.04-2.97 (m, 2H, H<sub>δA</sub> Orn<sub>4</sub>, Orn<sub>9</sub>); 2.96-2.90 (m, 2H, H<sub>βB</sub> <sup>D</sup>Phe<sub>6</sub>, <sup>D</sup>Phe<sub>1</sub>); 2.90-2.85 (m, 2H, H<sub>δB</sub> Orn<sub>4</sub>, Orn<sub>9</sub>); 2.55 (dd, *J* = 17.0 Hz, *J* = 9.6 Hz, 1H, H<sub>δB</sub> Pro<sub>2</sub>); 2.47 (dd, *J* = 17.5 Hz, *J* = 8.7 Hz, 1H, H<sub>δB</sub> Pro<sub>7</sub>); 2.28-2.20 (m, 1H, H<sub>β</sub> Val<sub>3</sub>); 2.06-1.98 (m, 4H, H<sub>β</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 1.94 (s, 4H, Ada side chain); 1.76-1.66 (m, 13H, H<sub>γ</sub> Orn<sub>4</sub>, Orn<sub>9</sub>, Ada side chain, H<sub>γA</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 1.64-1.55 (m, 10H, H<sub>β</sub> Orn<sub>4</sub>, Orn<sub>9</sub>, Ada side chain, H<sub>γB</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 1.55-1.50 (m, 4H, H<sub>γ</sub> Leu<sub>5</sub>, Leu<sub>10</sub>, H<sub>βA</sub> Leu<sub>5</sub>, Leu<sub>10</sub>); 1.48-1.40 (m, 2H, H<sub>βB</sub> Leu<sub>5</sub>, Leu<sub>10</sub>); 0.95 (d, *J* = 6.7 Hz, 3H, H<sub>γ</sub> Val<sub>3</sub>); 0.92-0.86 (m, 15H, H<sub>γ</sub> Val<sub>3</sub>, H<sub>δ</sub> Leu<sub>5</sub>, Leu<sub>10</sub>). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 173.6 (C=O amide); 173.5 (C=O amide); 173.5 (C=O amide); 173.5 (C=O amide); 173.4 (C=O amide); 173.2 (C=O amide); 172.6 (C=O amide); 172.4 (C=O amide); 172.3 (C=O amide); 170.4 (C=O amide); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 130.3 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 128.5 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 62.8 (C<sub>α</sub> AdaGly<sub>8</sub>); 62.1 (C<sub>α</sub> Pro<sub>7</sub>); 62.0 (C<sub>α</sub> Pro<sub>2</sub>); 60.1 (C<sub>α</sub> Val<sub>3</sub>); 55.9 (C<sub>α</sub> <sup>D</sup>Phe); 55.9 (C<sub>α</sub> <sup>D</sup>Phe); 52.7 (C<sub>α</sub> Orn); 52.4 (C<sub>α</sub> Orn); 51.3 (C<sub>α</sub> Leu<sub>5</sub>, Leu<sub>10</sub>); 48.1 (C<sub>δ</sub> Pro<sub>7</sub>); 47.9 (C<sub>δ</sub> Pro<sub>2</sub>); 42.3 (C<sub>β</sub> Leu); 41.6 (C<sub>β</sub> Leu); 40.7 (C<sub>δ</sub> Orn); 40.6 (C<sub>δ</sub> Orn); 40.3 (Ada side chain); 37.7 (Ada side chain); 37.6 (Ada side chain); 37.3 (C<sub>β</sub> <sup>D</sup>Phe); 32.3 (C<sub>β</sub> Val<sub>3</sub>); 30.9 (C<sub>β</sub> Orn); 30.8 (C<sub>β</sub> Orn); 30.7 (C<sub>β</sub> Pro); 30.6 (C<sub>β</sub> Pro); 29.9 (Ada side chain); 25.6 (C<sub>γ</sub> Leu); 25.6 (C<sub>γ</sub> Leu); 24.8 (C<sub>γ</sub> Orn); 24.7 (C<sub>γ</sub> Orn); 24.5 (C<sub>γ</sub> Pro); 24.4 (C<sub>γ</sub> Pro); 23.4 (C<sub>δ</sub> Leu); 23.2 (C<sub>δ</sub> Leu); 23.1 (C<sub>δ</sub> Leu); 23.0 (C<sub>δ</sub> Leu); 19.7 (C<sub>γ</sub> Val<sub>3</sub>); 19.3 (C<sub>γ</sub> Val<sub>3</sub>). IR (neat): 3405.3; 3270.2; 2959.2; 2927.9; 2904.3; 2340.1; 2359.6; 1683.7; 1682.9; 1674.4; 1668.5; 1661.7; 1659.2; 1651.7; 1644.8; 1639.7; 1634.0; 1628.2; 1622.4; 1616.0; 1575.0; 1568.3; 1563.7; 1553.7; 1557.7; 1549.3; 1538.2; 1532.0; 1526.7; 1520.5; 1516.0; 1505.9; 1471.3; 1463.3; 1455.7; 1452.2; 1447.8; 1435.9; 1344.0; 1200.5; 1134.0; 839.0; 800.2; 748.2; 722.3; 701.8; 667.9; 506.4. LC-MS retention time: 5.64 min (50 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 617.60 [M + 2H]<sup>2+</sup>; 1233.80 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>67</sub>H<sub>102</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 617.39155; [C<sub>67</sub>H<sub>101</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1233.7758. Found: 617.39130 [M + 2H]<sup>2+</sup>; 1233.77687 [M + H]<sup>+</sup>.



**Cyclo-(<sup>D</sup>Phe-Pro-AdaGly-Orn-Leu)<sub>2</sub>-2TFA (5)**

Following the general procedures for peptide synthesis and cleavage, **5** was obtained from Fmoc-Leu-HMPB-MBHA resin (156 μmol) as a white powder (99.4 mg, 75.0 μmol, 48%), using an HPLC gradient of 20:70:10 → 0:90:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.79 (d, *J* = 2.8 Hz, 2H, NH <sup>D</sup>Phe); 8.71 (d, *J* = 9.5 Hz, 2H, NH Leu); 8.62 (d, *J* = 9.4 Hz, 2H, NH Orn); 7.90 (s, 4H, NH Orn side chain); 7.57 (d, *J* = 10.0 Hz, 2H, NH AdaGly); 7.33-7.22 (m, 10H, CH <sup>D</sup>Phe); ~4.83 (H<sub>α</sub> Orn, not visible due to solvent suppression); 4.70 (dd, *J* = 16.8 Hz, *J* = 7.6 Hz, 2H, H<sub>α</sub> Leu); 4.52-4.47 (m, 2H, H<sub>α</sub> <sup>D</sup>Phe); 4.38 (d, *J* = 10.0 Hz, 2H, H<sub>α</sub> AdaGly); 4.33 (d, *J* = 7.9 Hz, 2H, H<sub>α</sub> Pro); 3.82 (t, *J* = 8.6 Hz, 2H, H<sub>δA</sub> Pro); 3.08 (dd, *J* = 12.4 Hz, *J* = 4.6 Hz, 2H, H<sub>βA</sub> <sup>D</sup>Phe); 2.96-2.85 (m, 6H, H<sub>βB</sub> <sup>D</sup>Phe, H<sub>δ</sub> Orn); 2.55 (dd, *J* = 16.9 Hz, *J* = 9.6 Hz, 2H, H<sub>δB</sub> Pro); 2.06-1.97 (m, 4H, H<sub>β</sub> Pro); 1.94 (s, 8H, Ada side chain); 1.76-1.67 (m, 18H, H<sub>γA</sub> Orn, Ada side chain, H<sub>γA</sub> Pro); 1.66-1.52 (m, 20H, H<sub>γB</sub> Orn, H<sub>β</sub> Orn, Ada side chain, H<sub>βA</sub> Leu, H<sub>γB</sub> Pro, H<sub>γ</sub> Leu); 1.50-1.45 (m, 2H, H<sub>βB</sub> Leu); 0.89 (d, *J* = 6.4 Hz, 12H, H<sub>δ</sub> Leu). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 173.6 (C=O amide); 173.4 (C=O amide); 173.1 (C=O amide); 172.2 (C=O amide); 170.3 (C=O amide); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 62.7 (C<sub>α</sub> AdaGly); 62.1 (C<sub>α</sub> Pro); 55.9 (C<sub>α</sub> <sup>D</sup>Phe); 52.6 (C<sub>α</sub> Orn); 51.1 (C<sub>α</sub> Leu); 48.2 (C<sub>δ</sub> Pro); 42.0 (C<sub>β</sub> Leu); 40.6 (C<sub>δ</sub> Orn); 40.3 (Ada side chain); 37.8 (Ada side chain); 37.8 (Ada side chain); 37.3 (C<sub>β</sub> <sup>D</sup>Phe); 30.9 (C<sub>β</sub> Pro); 30.7 (C<sub>β</sub> Orn); 29.8 (Ada side chain); 25.5 (C<sub>γ</sub> Leu); 24.8 (C<sub>γ</sub> Orn); 24.5 (C<sub>γ</sub> Pro); 23.4 (C<sub>δ</sub> Leu); 23.1 (C<sub>δ</sub> Leu). IR (neat): 3287.9; 2953.4; 2907.8; 2846.7; 1684.2; 1674.6; 1668.4; 1661.6; 1658.0; 1651.6; 1645.0; 1639.9; 1634.3; 1557.7; 1553.8; 1549.0; 1544.1; 1538.2; 1531.9; 1526.0; 1520.0; 1515.7; 1505.8; 1471.4; 1463.8; 1455.6; 1452.1; 1447.5; 1436.0; 1266.7; 1257.1; 1241.9; 1202.2; 1181.4; 1133.8; 1066.0; 872.1; 834.7; 799.5; 722.3; 702.4; 667.9; 508.1. LC-MS retention time: 7.85 min (50 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 664.00 [M + 2H]<sup>2+</sup>; 1326.93 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>74</sub>H<sub>110</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 663.42285; [C<sub>74</sub>H<sub>109</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1325.8384. Found: 663.42300 [M + 2H]<sup>2+</sup>; 1325.83955 [M + H]<sup>+</sup>.

**Cyclo-(<sup>D</sup>Phe-Pro-Val-Orn-Leu-<sup>D</sup>Phe-Pro-Val-Orn-AdaAla)<sub>2</sub>-2TFA (6)**

Following the general procedures for peptide synthesis and cleavage, **6** was obtained from Fmoc-Leu-HMPB-MBHA resin (150 μmol) as a white powder (94 mg, 76.2 μmol, 51%), using an HPLC gradient of 25:65:10 → 0:90:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.92 (d, *J* = 3.2 Hz, 1H, NH <sup>D</sup>Phe<sub>1</sub>); 8.89 (d, *J* = 3.1 Hz, 1H, NH <sup>D</sup>Phe<sub>6</sub>); 8.74 (d, *J* = 9.4 Hz, 1H, NH AdaAla<sub>10</sub>); 8.71 (d, *J* = 9.5 Hz, 1H, NH Leu<sub>5</sub>); 8.69 (d, *J* = 9.3 Hz, 1H, NH Orn<sub>4</sub>); 8.62 (d, *J* = 9.4 Hz, 1H, NH Orn<sub>9</sub>); 7.85 (s, 4H, NH Orn side chain); 7.71 (d, *J* = 9.0 Hz, 1H, NH Val<sub>3</sub>); 7.66 (d, *J* = 9.1 Hz, 1H, NH Val<sub>8</sub>); 7.33-7.23 (m, 10H, CH <sup>D</sup>Phe); ~4.97 (H<sub>α</sub> Orn, not visible due to solvent suppression); 4.71 (dt, *J* = 8.9 Hz, *J* = 4.3 Hz, 1H, H<sub>α</sub> AdaAla<sub>10</sub>); 4.66 (dd, *J* = 16.2 Hz, *J* = 7.5 Hz, 1H, H<sub>α</sub> Leu<sub>5</sub>); 4.52-4.44 (m, 2H, H<sub>α</sub> <sup>D</sup>Phe<sub>1</sub>, <sup>D</sup>Phe<sub>6</sub>); 4.34 (t, *J* = 6.0 Hz, 2H, H<sub>α</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 4.22 (t, *J* = 8.6 Hz, 1H, H<sub>α</sub> Val<sub>8</sub>); 4.14 (t, *J* = 8.8 Hz, 1H, H<sub>α</sub> Val<sub>3</sub>); 3.74 (q, *J* = 10.0 Hz, *J* = 9.7 Hz, 2H, H<sub>δA</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 3.11-3.06 (m, 2H, H<sub>βA</sub> <sup>D</sup>Phe<sub>1</sub>, <sup>D</sup>Phe<sub>6</sub>); 3.06-2.99 (m, 2H, H<sub>δA</sub> Orn<sub>4</sub>, Orn<sub>9</sub>); 2.94 (dt, *J* = 12.3 Hz, *J* = 3.9 Hz, 2H, H<sub>βB</sub> <sup>D</sup>Phe<sub>1</sub>, <sup>D</sup>Phe<sub>6</sub>); 2.91-2.84 (m, 2H, H<sub>δB</sub> Orn<sub>4</sub>, Orn<sub>9</sub>); 2.54 (q, *J* = 9.2 Hz, *J* = 9.1 Hz, 1H, H<sub>δB</sub> Pro<sub>2</sub>); 2.50-2.44 (m, 1H, H<sub>δB</sub> Pro<sub>7</sub>); 2.32-2.21 (m, 2H, H<sub>β</sub> Val<sub>3</sub>, Val<sub>8</sub>); 2.09-1.96 (m, 4H, H<sub>β</sub> Pro<sub>2</sub>, Pro<sub>7</sub>); 1.90 (s, 3H, Ada side chain); 1.83-1.66 (m, 14H, H<sub>γA</sub> AdaAla<sub>10</sub>, H<sub>γA</sub> Orn, Ada side chain, H<sub>γA</sub> Pro, H<sub>βA</sub> Orn); 1.66-1.57 (m, 7H, Ada side chain, H<sub>γB</sub> Orn, H<sub>γB</sub> Pro, H<sub>βB</sub> Orn); 1.56-1.49 (m, 5H, Ada side chain, H<sub>βA</sub> Leu, H<sub>γ</sub> Leu<sub>5</sub>); 1.47-1.39 (m, 5H, Ada side chain, H<sub>βB</sub> Leu<sub>5</sub>); 1.07 (dd, *J* = 14.0 Hz, *J* = 4.2 Hz, 1H, H<sub>βB</sub> AdaAla<sub>10</sub>); 0.96 (dd, *J* = 15.1 Hz, *J* = 6.7 Hz, 6H, H<sub>γ</sub> Val); 0.89 (dd, *J* = 14.5 Hz, *J* = 7.6 Hz, 12H, H<sub>γ</sub> Val, H<sub>δ</sub> Leu<sub>5</sub>). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 174.1 (C=O amide); 173.6 (C=O amide); 173.5 (C=O amide); 173.5 (C=O amide); 173.5 (C=O amide); 173.4 (C=O amide); 172.8 (C=O amide); 172.8 (C=O amide); 172.5 (C=O amide); 171.8 (C=O amide); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 129.6 (CH <sup>D</sup>Phe); 128.5 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 62.0 (C<sub>α</sub> Pro); 62.0 (C<sub>α</sub> Pro); 60.4 (C<sub>α</sub> Val<sub>3</sub>); 60.1 (C<sub>α</sub> Val<sub>8</sub>); 56.0 (C<sub>α</sub> <sup>D</sup>Phe); 55.9 (C<sub>α</sub> <sup>D</sup>Phe); 52.5 (C<sub>α</sub> Orn); 51.5 (C<sub>α</sub> Leu<sub>5</sub>); 48.8 (C<sub>α</sub> AdaAla<sub>10</sub>); 48.0 (C<sub>δ</sub> Pro<sub>2</sub>); 47.9 (C<sub>δ</sub> Pro<sub>7</sub>); 47.7 (C<sub>β</sub> AdaAla<sub>10</sub>); 43.4 (CH<sub>2</sub> Ada side chain); 41.9 (C<sub>β</sub> Leu<sub>5</sub>); 40.6 (C<sub>δ</sub> Orn); 40.6 (C<sub>δ</sub> Orn); 37.9 (CH<sub>2</sub> Ada side chain); 37.3 (C<sub>β</sub> <sup>D</sup>Phe); 37.2 (C<sub>β</sub> <sup>D</sup>Phe); 32.9 (C<sub>q</sub> Ada); 32.1 (C<sub>β</sub> Val<sub>8</sub>); 31.9 (C<sub>β</sub> Val<sub>3</sub>); 30.9 (C<sub>β</sub> Pro); 30.7 (C<sub>β</sub> Orn); 30.6 (C<sub>β</sub> Orn); 29.9 (Ada

side chain); 25.7 (C<sub>γ</sub> Leu<sub>5</sub>); 24.6 (C<sub>γ</sub> Orn); 24.6 (C<sub>γ</sub> Orn); 24.5 (C<sub>γ</sub> Pro); 24.4 (C<sub>γ</sub> Pro); 23.3 (C<sub>δ</sub> Leu); 23.1 (C<sub>δ</sub> Leu); 19.8 (C<sub>γ</sub> Val); 19.7 (C<sub>γ</sub> Val); 19.5 (C<sub>γ</sub> Val<sub>3</sub>); 19.2 (C<sub>γ</sub> Val<sub>8</sub>). IR (neat): 3271.6; 3063.6; 2962.9; 2930.1; 2848.3; 2902.7; 1704.1; 1699.8; 1694.4; 1688.2; 1683.7; 1680.0; 1674.0; 1667.9; 1661.8; 1658.1; 1651.7; 1645.4; 1639.9; 1634.1; 1628.2; 1622.1; 1616.0; 1574.8; 1568.0; 1563.7; 1557.7; 1553.8; 1549.1; 1543.8; 1538.1; 1531.9; 1526.1; 1520.0; 1515.9; 1505.8; 1471.3; 1463.6; 1455.7; 1452.0; 1447.2; 1435.9; 1343.9; 1202.7; 1179.3; 1134.1; 837.6; 799.5; 748.0; 722.2; 701.5; 667.9; 589.8; 559.8; 506.6. LC-MS retention time: 5.49 min (50 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 617.60 [M + 2H]<sup>2+</sup>; 1233.93 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>67</sub>H<sub>102</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 617.39155; [C<sub>67</sub>H<sub>101</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1233.77581. Found: 617.39156 [M + 2H]<sup>2+</sup>; 1233.77647 [M + H]<sup>+</sup>.

#### **Cyclo-(<sup>D</sup>Phe-Pro-Val-Orn-AdaAla)<sub>2</sub>. 2TFA (7)**

Following the general procedures for peptide synthesis and cleavage, **7** was obtained from Fmoc-Leu-HMPB-MBHA resin (183 μmol) as a white powder (100.9 mg, 76.7 μmol, 42%), using an HPLC gradient of 30:60:10 → 0:90:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.87 (d, *J* = 3.07 Hz, 2H, NH <sup>D</sup>Phe); 8.71 (d, *J* = 9.4 Hz, 2H, NH AdaAla); 8.61 (d, *J* = 9.2 Hz, 2H, NH Orn); 7.84 (s, 4H, NH Orn side chain); 7.67 (d, *J* = 9.1 Hz, 2H, NH Val); 7.33-7.23 (m, 10H, CH <sup>D</sup>Phe); ~ 4.9 (H<sub>x</sub> Orn, peak not visible due to solvent suppression); 4.73-4.68 (m, 2H, H<sub>x</sub> AdaAla); 4.49-4.44 (m, 2H, H<sub>x</sub> <sup>D</sup>Phe); 4.34 (d, *J* = 7.3 Hz, 2H, H<sub>x</sub> Pro); 4.22 (t, *J* = 8.5 Hz, 2H, H<sub>x</sub> Val); 3.77-3.73 (m, 2H, H<sub>δA</sub> Pro); 3.09 (dd, *J* = 12.6 Hz, *J* = 5.2 Hz, 2H, H<sub>βA</sub> <sup>D</sup>Phe); 3.06-2.99 (m, 2H, H<sub>δA</sub> Orn); 2.94 (t, *J* = 11.8 Hz, 2H, H<sub>βB</sub> <sup>D</sup>Phe); 2.91-2.86 (m, 2H, H<sub>δB</sub> Orn); 2.54 (dd, *J* = 17.4 Hz, *J* = 8.6 Hz, 2H, H<sub>δB</sub> Pro); 2.35 (t, *J* = 8.1 Hz, 1H, H<sub>βA</sub> Pro); 2.32-2.24 (m, 2H, H<sub>β</sub> Val); 2.06-1.97 (m, 4H, H<sub>β</sub> Pro); 1.90 (br s, 6H, Ada side chain); 1.81-1.68 (m, 16H, H<sub>βA</sub> AdaAla, H<sub>γA</sub> Orn, Ada side chain, H<sub>γA</sub> Pro, H<sub>βA</sub> Orn); 1.66-1.58 (m, 10H, Ada side chain, H<sub>γB</sub> Orn, H<sub>γB</sub> Pro, H<sub>βB</sub> AdaAla); 1.50 (dd, *J* = 54.0 Hz, *J* = 11.9 Hz, 12H, Ada side chain); 1.09 (dd, *J* = 14.1 Hz, *J* = 4.5 Hz, 2H, H<sub>βB</sub> AdaAla); 0.97 (d, *J* = 6.7 Hz, 6H, H<sub>γ</sub> Val); 0.89 (d, *J* = 6.7 Hz, 6H, H<sub>γ</sub> Val). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 174.0 (C=O amide); 173.6 (C=O amide); 173.5 (C=O amide); 172.8 (C=O amide); 171.9 (C=O amide); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 129.6 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 62.0 (C<sub>α</sub> Pro); 60.1 (C<sub>α</sub> Val); 56.0 (C<sub>α</sub> <sup>D</sup>Phe); 52.6 (C<sub>α</sub> Orn); 49.6 (C<sub>α</sub> AdaAla); 47.9 (C<sub>δ</sub> Pro); 47.6 (C<sub>β</sub> AdaAla); 43.5 (CH<sub>2</sub> Ada side chain); 40.6 (C<sub>δ</sub> Orn); 37.9 (CH<sub>2</sub> Ada side chain); 37.2 (C<sub>β</sub> <sup>D</sup>Phe); 32.9 (C<sub>q</sub> Ada side chain); 32.1 (C<sub>β</sub> Val); 30.7 (C<sub>γ</sub> Orn); 30.7 (C<sub>β</sub> Pro); 29.9 (CH Ada side chain); 24.6 (C<sub>β</sub> Orn); 24.5 (C<sub>γ</sub> Pro); 19.9 (C<sub>γ</sub> Val); 19.2 (C<sub>γ</sub> Val). IR (neat): 3409.9; 3268.1; 3070.5; 2963.1; 2902.2; 2848.6; 1684.0; 1675.3; 1668.7; 1659.0; 1652.5; 1640.3; 1633.9; 1628.9; 1624.0; 1618.3; 1563.2; 1557.7; 1553.7; 1548.7; 1543.2; 1538.1; 1532.0; 1520.4; 1516.1; 1506.0; 1470.3; 1453.9; 1451.3; 1435.5; 1344.1; 1319.6; 1311.4; 1200.7; 1185.3; 1172.2; 1163.9; 1138.4; 917.7; 838.5; 800.1; 747.9; 722.6; 701.7; 667.9; 601.8; 588.5; 556.3; 506.3. LC-MS retention time: 7.99 min (50 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 664.07 [M + 2H]<sup>2+</sup>; 1326.87 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>74</sub>H<sub>110</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 663.42285; [C<sub>74</sub>H<sub>109</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1325.83841. Found: 663.42303 [M + 2H]<sup>2+</sup>; 1325.83958 [M + H]<sup>+</sup>.

#### **Cyclo-(<sup>D</sup>Phe-Pro-AdaGly-Orn-AdaAla)<sub>2</sub>. 2TFA (8)**

Following the general procedures for peptide synthesis and cleavage, **8** was obtained from Fmoc-AdaAla-HMPB-MBHA resin (150 μmol) as a white powder (20.4 mg, 11.7 μmol, 8%), using an HPLC gradient of 80:10:10 → 0:90:10 H<sub>2</sub>O/MeCN/1% aq. TFA (CN column) for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.87 (d, *J* = 2.49 Hz, 2H, NH <sup>D</sup>Phe); 8.69 (d, *J* = 9.48 Hz, 2H, NH AdaAla); 8.52 (d, *J* = 9.46 Hz, 2H, NH Orn); 7.86 (s, 4H, NH Orn side chain); 7.64 (d, *J* = 9.91 Hz, 2H, NH AdaGly); 7.33-7.23 (m, 10H, CH <sup>D</sup>Phe); ~4.87 (H<sub>x</sub> Orn, peak not visible due to solvent suppression); 4.75 (t, *J* = 9.6 Hz, 2H, H<sub>x</sub> AdaAla); 4.49-4.44 (m, 2H, H<sub>x</sub> <sup>D</sup>Phe); 4.33 (d, *J* = 10.0 Hz, 2H, H<sub>x</sub> AdaGly); 4.31 (d, *J* = 7.6 Hz, 2H, H<sub>x</sub> Pro); 3.81 (t, *J* = 8.7 Hz, 2H, H<sub>δA</sub> Pro); 3.10 (dd, *J* = 12.5 Hz, *J* = 5.0 Hz, 2H, H<sub>βA</sub> <sup>D</sup>Phe); 3.01-2.90 (m, 4H, H<sub>δA</sub> Orn, H<sub>βB</sub> <sup>D</sup>Phe); 2.88-2.81 (m, 2H, H<sub>δB</sub> Orn); 2.62 (dd, *J* = 16.8 Hz, *J* = 9.4 Hz, 2H, H<sub>δB</sub> Pro); 2.12-2.00 (m, 6H, H<sub>βA</sub> AdaAla, H<sub>βA</sub> Orn, H<sub>βA</sub> Pro); 1.98 (s, 8H, Ada side chain); 1.91 (s, 8H, Ada side chain); 1.81-1.70 (m, 20H, H<sub>γ</sub> Orn, Ada side chain, H<sub>γA</sub> Pro, H<sub>βB</sub> Orn); 1.68-1.52 (m, 26H, H<sub>βB</sub> Pro, H<sub>γB</sub> Pro, Ada side chain); 1.44-1.39 (m, 8H, Ada side chain); 0.96 (d, *J* = 12.3 Hz, 2H, H<sub>βB</sub> AdaAla). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 173.7

(C=O); 173.6 (C=O); 173.6 (C=O); 171.3 (C=O); 170.7 (C=O); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.8 (CH <sup>D</sup>Phe); 130.7 (CH <sup>D</sup>Phe); 130.3 (CH <sup>D</sup>Phe); 129.6 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 62.9 (C<sub>α</sub> AdaGly); 62.1 (C<sub>α</sub> Pro); 56.0 (C<sub>α</sub> <sup>D</sup>Phe); 52.5 (C<sub>α</sub> Orn); 48.5 (C<sub>α</sub> AdaAla); 48.2 (C<sub>δ</sub> Pro); 47.8 (C<sub>β</sub> AdaAla); 43.7 (Ada side chain); 40.4 (C<sub>δ</sub> Orn); 37.9 (Ada side chain); 37.8 (Ada side chain); 37.2 (C<sub>β</sub> <sup>D</sup>Phe); 30.9 (C<sub>β</sub> Pro); 30.8 (C<sub>β</sub> Orn); 30.7 (C<sub>β</sub> Orn); 30.0 (Ada side chain); 29.9 (Ada side chain); 24.7 (C<sub>γ</sub> Orn); 24.5 (C<sub>γ</sub> Pro). IR (neat): 3278.0; 3059.7; 2902.3; 2848.6; 1683.6; 1674.0; 1667.7; 1656.4; 1651.6; 1639.7; 1634.1; 1557.6; 1553.1; 1543.8; 1537.9; 1531.8; 1526.0; 1506.1; 1450.4; 1436.1; 1344.5; 1312.4; 1201.6; 1181.7; 1135.8; 834.4; 799.2; 747.7; 722.0; 701.7; 667.8; 589.0; 507.5. LC-MS retention time: 12.57 min (10 → 90% MeCN, 15 min run). Mass (ESI):  $m/z$  755.53 [M + 2H]<sup>2+</sup>; 1511.00 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>88</sub>H<sub>126</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 755.98712; [C<sub>88</sub>H<sub>125</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1509.96361. Found: 755.98696 [M + 2H]<sup>2+</sup>; 1509.96469 [M + H]<sup>+</sup>.

### **Cyclo-(<sup>D</sup>Phe-Pro-*t*BuGly-Orn-*t*BuAla)<sub>2</sub>. 2TFA (9)**

Following the general procedures for peptide synthesis and cleavage, **9** was obtained from Fmoc-*t*BuAla-HMPB-MBHA resin (94.5 μmol) as a white powder (48.2 mg, 40.2 μmol, 27%), using an HPLC gradient of 40:50:10 → 20:70:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.78 (d, *J* = 9.6 Hz, 2H, NH *t*BuAla); 8.70 (d, *J* = 3.0 Hz, 2H, NH <sup>D</sup>Phe); 8.64 (d, *J* = 9.3 Hz, 2H, NH Orn); 7.89 (s, 4H, NH Orn side chain); 7.57 (d, *J* = 10.0 Hz, 2H, NH *t*BuGly); 7.33-7.22 (m, 10H, CH <sup>D</sup>Phe); 4.75 (dt, *J* = 10.1 Hz, *J* = 4.8 Hz, 2H, H<sub>α</sub> Orn); 4.69 (td, *J* = 9.5 Hz, *J* = 6.6 Hz, 1H, H<sub>α</sub> *t*BuAla); 4.51 (d, *J* = 10.1 Hz, 2H, H<sub>α</sub> *t*BuGly); 4.47-4.42 (m, 2H, H<sub>α</sub> <sup>D</sup>Phe); 4.35-4.30 (m, 2H, H<sub>α</sub> Pro); 3.77 (dd, *J* = 12.9 Hz, *J* = 5.4 Hz, 2H, H<sub>δA</sub> Pro); 3.08 (dd, *J* = 12.5 Hz, *J* = 4.8 Hz, 2H, H<sub>βA</sub> <sup>D</sup>Phe); 2.96-2.87 (m, 6H, H<sub>βB</sub> <sup>D</sup>Phe, H<sub>δ</sub> Orn); 2.56 (dd, *J* = 17.0 Hz, *J* = 9.3 Hz, 2H, H<sub>βB</sub> Pro); 2.04-1.96 (m, 4H, H<sub>βA</sub> Pro, H<sub>βA</sub> Orn); 1.88 (dd, *J* = 14.2 Hz, *J* = 6.9 Hz, 2H, H<sub>βA</sub> *t*BuAla); 1.78-1.69 (m, 4H, H<sub>γ</sub> Orn); 1.69-1.62 (m, 4H, H<sub>γA</sub> Pro, H<sub>βB</sub> Pro); 1.62-1.54 (m, 5H, H<sub>βB</sub> Orn, H<sub>γB</sub> Pro); 1.41 (dd, *J* = 14.3 Hz, *J* = 6.3 Hz, 2H, H<sub>βB</sub> *t*BuAla); 1.00 (s, 18H, CH<sub>3</sub> *t*Bu); 0.91 (s, 18H, CH<sub>3</sub> *t*Bu). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 173.6 (C=O amide); 173.4 (C=O amide); 171.8 (C=O amide); 170.9 (C=O amide); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.3 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 62.1 (C<sub>α</sub> Pro); 61.7 (C<sub>α</sub> *t*BuGly); 55.9 (C<sub>α</sub> <sup>D</sup>Phe); 53.0 (C<sub>α</sub> Orn); 50.3 (C<sub>α</sub> *t*BuAla); 48.1 (C<sub>δ</sub> Pro); 46.2 (C<sub>β</sub> *t*BuAla); 40.8 (C<sub>δ</sub> Orn); 37.3 (C<sub>β</sub> <sup>D</sup>Phe); 36.1 (C<sub>q</sub> *t*Bu); 30.9 (C<sub>β</sub> Pro); 30.8 (C<sub>β</sub> Pro); 30.5 (C<sub>β</sub> Orn); 30.3 (CH<sub>3</sub> *t*Bu); 27.6 (CH<sub>3</sub> *t*Bu); 24.8 (C<sub>γ</sub> Orn); 24.5 (C<sub>γ</sub> Pro). IR (neat): 3286.0; 3064.7; 2957.4; 2874.8; 1699.6; 1694.1; 1687.8; 1683.7; 1674.0; 1667.9; 1661.2; 1657.2; 1651.6; 1639.8; 1634.4; 1562.7; 1557.7; 1553.6; 1548.0; 1543.8; 1538.2; 1531.8; 1525.8; 1520.1; 1516.0; 1505.9; 1496.2; 1479.5; 1475.9; 1471.5; 1463.3; 1455.6; 1435.9; 1417.9; 1398.4; 1367.7; 1201.6; 1183.2; 1135.5; 836.0; 799.7; 748.0; 722.2; 701.9; 667.9; 589.6; 558.2; 515.8. LC-MS retention time: 5.37 min (50 → 90% MeCN, 15 min run). Mass (ESI):  $m/z$  599.53 [M + 2H]<sup>2+</sup>; 1197.80 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>64</sub>H<sub>102</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 599.39155; [C<sub>64</sub>H<sub>101</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1197.77581. Found: 599.39141 [M + 2H]<sup>2+</sup>; 1197.77661 [M + H]<sup>+</sup>.

### **Cyclo-(<sup>D</sup>Phe-Pro-Chg-Orn-Cha)<sub>2</sub>. 2TFA (10)**

Following the general procedures for peptide synthesis and cleavage, **10** was obtained from Fmoc-Cha-HMPB-MBHA resin (98 μmol) as a white powder (37.4 mg, 28.8 μmol, 29%), using an HPLC gradient of 40:50:10 → 5:85:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 8.99 (d, *J* = 3.0 Hz, 2H, NH <sup>D</sup>Phe); 8.73 (d, *J* = 9.2 Hz, 2H, NH Cha); 8.61 (d, *J* = 9.4 Hz, 2H, NH Orn); 7.87 (s, 4H, NH Orn side chain); 7.75 (d, *J* = 8.4 Hz, 2H, NH Chg); 7.34-7.23 (m, 10H, CH <sup>D</sup>Phe); 5.10-5.03 (m, 1H, H<sub>α</sub> Orn, reduced integral due to solvent suppression); 4.69 (td, *J* = 9.1 Hz, *J* = 6.4 Hz, 2H, H<sub>α</sub> Cha); 4.56-4.50 (m, 2H, H<sub>α</sub> <sup>D</sup>Phe); 4.31 (d, *J* = 6.5 Hz, 2H, H<sub>α</sub> Pro); 4.04 (t, *J* = 9.0 Hz, 2H, H<sub>α</sub> Chg); 3.75 (t, *J* = 7.8 Hz, 2H, H<sub>δA</sub> Pro); 3.10 (dd, *J* = 12.4 Hz, *J* = 4.7 Hz, 4H, H<sub>βA</sub> <sup>D</sup>Phe); 2.93 (t, *J* = 11.9 Hz, 2H, H<sub>βB</sub> <sup>D</sup>Phe); 2.90-2.84 (m, 2H, H<sub>βB</sub> Orn); 2.45 (q, *J* = 9.4 Hz, *J* = 9.2 Hz, 2H, H<sub>βB</sub> Pro); 2.12-2.00 (m, 6H, H<sub>β</sub> Chg, H<sub>β</sub> Orn); 2.00-1.94 (m, 2H, H<sub>βA</sub> Pro); 1.84-1.54 (m, 42H, H<sub>γ</sub> Orn, H<sub>βA</sub> Cha, H<sub>βB</sub> Pro, H<sub>γ</sub> Pro, cyclohexyl side chains); 1.48 (d, *J* = 12.0 Hz, 2H, H<sub>γ</sub> Cha); 1.36-1.27 (m, 8H, H<sub>βB</sub> Cha, cyclohexyl side chains); 1.27-1.13 (m, 18H, cyclohexyl side chains); 1.04-0.87 (m, 10H, cyclohexyl side chains). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 173.7 (C=O amide); 173.5 (C=O amide); 173.5 (C=O amide); 172.9 (C=O amide); 172.3

(C=O amide); 136.9 (C<sub>q</sub> <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 128.5 (CH <sup>D</sup>Phe); 62.0 (C<sub>α</sub> Pro); 60.0 (C<sub>α</sub> Chg); 56.0 (C<sub>α</sub> <sup>D</sup>Phe); 52.3 (C<sub>α</sub> Orn); 51.0 (C<sub>α</sub> Cha); 47.9 (C<sub>δ</sub> Pro); 41.0 (C<sub>β</sub> Cha); 40.5 (C<sub>β</sub> Chg); 40.5 (C<sub>δ</sub> Orn); 37.3 (C<sub>β</sub> <sup>D</sup>Phe); 35.3 (C<sub>γ</sub> Cha); 34.8 (cyclohexyl side chain); 34.2 (cyclohexyl side chain); 30.8; 30.8; 30.7; 30.6; 27.4; 27.2; 27.1; 24.7; 24.6; 24.5 (cyclohexyl side chains, C<sub>β</sub> and C<sub>γ</sub> of Orn and Pro). IR (neat): 3266.0; 3066.8; 2925.8; 2852.4; 1699.7; 1687.9; 1683.6; 1674.0; 1668.0; 1661.5; 1657.5; 1651.5; 1639.5; 1634.0; 1557.7; 1553.6; 1547.7; 1543.7; 1538.1; 1532.1; 1526.4; 1520.3; 1505.8; 1455.3; 1448.6; 1435.9; 1339.6; 1201.9; 1184.6; 1135.4; 835.9; 799.8; 748.0; 722.2; 701.9; 667.9; 589.9; 559.4; 507.7. LC-MS retention time: 7.36 min (50 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 652.00 [M + 2H]<sup>2+</sup>; 1301.87 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>72</sub>H<sub>110</sub>N<sub>12</sub>O<sub>10</sub>]<sup>2+</sup>: 651.42285; [C<sub>72</sub>H<sub>109</sub>N<sub>12</sub>O<sub>10</sub>]<sup>+</sup>: 1301.83841. Found: 651.42300 [M + 2H]<sup>2+</sup>; 1301.83950 [M + H]<sup>+</sup>.

### **Cyclo-(<sup>D</sup>Phe-Pro-Orn-Val-Orn)<sub>2</sub>. 4TFA (11)**

Following the general procedures for peptide synthesis and cleavage, **11** was obtained from Fmoc-Orn(Boc)-HMPB-MBHA resin (150 μmol) as a white powder (91.55 mg, 57.2 μmol, 38%), using an HPLC gradient of 70:20:10 → 30:60:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH, 600 MHz): δ 9.18 (s, 2H, NH <sup>D</sup>Phe); 8.73 (d, *J* = 7.85 Hz, 2H, NH Orn<sub>3</sub>, Orn<sub>8</sub>); 8.18 (d, *J* = 9.69 Hz, 2H, NH Val); 7.93 (s, 8H, NH Orn side chain); 7.81 (d, *J* = 8.79 Hz, 2H, NH Orn<sub>5</sub>, Orn<sub>10</sub>); 7.34-7.20 (m, 10H, CH <sup>D</sup>Phe); ~4.8 (H<sub>α</sub> Val, not visible due to solvent suppression); 4.50-4.38 (m, 7H, H<sub>α</sub> all other residues); 3.62 (t, *J* = 7.60 Hz, 2H, H<sub>δA</sub> Pro); 3.03 (dd, *J* = 12.50, 5.08 Hz, 2H, H<sub>βA</sub> <sup>D</sup>Phe); 3.00-2.92 (m, 13H, H<sub>βB</sub> <sup>D</sup>Phe, H<sub>δ</sub> Orn<sub>3</sub>, Orn<sub>5</sub>, Orn<sub>8</sub>, Orn<sub>10</sub>); 2.41-2.35 (m, 2H, H<sub>δB</sub> Pro); 2.04-1.98 (m, 2H, H<sub>βA</sub> Pro); 1.98-1.91 (m, 6H, H<sub>βB</sub> Pro, H<sub>β</sub> Val, H<sub>βA</sub> Orn<sub>5</sub>, Orn<sub>10</sub>); 1.85-1.74 (m, 4H, H<sub>βB</sub> Orn<sub>5</sub>, Orn<sub>10</sub>, H<sub>βA</sub> Orn<sub>3</sub>, Orn<sub>8</sub>); 1.73-1.56 (m, 12H, H<sub>γA</sub> Pro, H<sub>βB</sub> Orn<sub>3</sub>, Orn<sub>8</sub>, H<sub>γ</sub> Orn<sub>3</sub>, Orn<sub>5</sub>, Orn<sub>8</sub>, Orn<sub>10</sub>); 1.56-1.50 (m, 2H, H<sub>γA</sub> Pro); 1.03 (d, *J* = 6.73 Hz, 6H, H<sub>γ</sub> Val); 0.96 (d, *J* = 6.79 Hz, 6H, H<sub>γ</sub> Val). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 173.7 (C=O amide); 173.7 (C=O amide); 172.9 (C=O amide); 172.6 (C=O amide); 172.5 (C=O amide); 137.0 (C<sub>q</sub> <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 128.4 (CH <sup>D</sup>Phe); 61.5 (C<sub>α</sub> Orn<sub>5</sub>, Orn<sub>10</sub>); 59.1 (C<sub>α</sub> Val); 56.0 (C<sub>α</sub> Pro); 53.9 (C<sub>α</sub> Orn<sub>3</sub>, Orn<sub>8</sub>); 53.6 (C<sub>α</sub> <sup>D</sup>Phe); 47.5 (C<sub>δ</sub> Pro); 40.5 (C<sub>δ</sub> Orn); 40.4 (C<sub>δ</sub> Orn); 37.4 (C<sub>β</sub> <sup>D</sup>Phe); 33.6 (C<sub>β</sub> Val); 30.3 (C<sub>β</sub> Pro); 30.3 (C<sub>β</sub> Orn); 25.3 (C<sub>γ</sub> Orn<sub>5</sub>, Orn<sub>10</sub>); 25.0 (C<sub>γ</sub> Orn<sub>3</sub>, Orn<sub>8</sub>); 24.5 (C<sub>γ</sub> Pro); 19.8 (C<sub>γ</sub> Val); 19.5 (C<sub>γ</sub> Val). IR (neat): 3271.9; 3063.6; 2969.8; 1699.6; 1695.2; 1687.9; 1683.7; 1679.8; 1673.9; 1668.2; 1661.8; 1658.6; 1651.8; 1645.4; 1639.8; 1634.8; 1627.9; 1622.4; 1616.2; 1557.8; 1553.8; 1538.8; 1532.3; 1525.8; 1521.3; 1516.1; 1506.0; 1471.6; 1455.5; 1435.7; 1200.1; 1173.5; 1133.1; 873.3; 834.6; 798.8; 746.7; 722.0; 703.2; 667.9; 611.9; 605.4; 596.0; 590.1; 518.1; 507.6. LC-MS retention time: 4.70 min (10 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 573.00 [M + 2H]<sup>2+</sup>; 1143.80 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>58</sub>H<sub>92</sub>N<sub>14</sub>O<sub>10</sub>]<sup>2+</sup>: 572.35549, found: 572.35542 [M + 2H]<sup>2+</sup>.

### **Cyclo-(<sup>D</sup>Phe-Pro-Orn-Leu-Orn)<sub>2</sub>. 4TFA (12)**

Following the general procedures for peptide synthesis and cleavage **12** was obtained from Fmoc-Orn(Boc)-HMPB-MBHA resin (150 μmol) as a white powder (105 mg, 64.5 μmol, 43%), using an HPLC gradient of 70:20:10 → 30:60:10 H<sub>2</sub>O/MeCN/1% aq. TFA for purification.

<sup>1</sup>H NMR (CD<sub>3</sub>OH/trace TFA, 600 MHz, 323K): δ 8.64 (d, *J* = 5.2 Hz, 0.2H, NH <sup>D</sup>Phe minor); 8.62-8.58 (m, 0.8H, NH <sup>D</sup>Phe); 8.45 (d, *J* = 9.4 Hz, 0.2H, NH Orn minor); 8.42 (d, *J* = 6.8 Hz, 0.2H, NH Leu minor); 8.40-8.34 (m, 0.9H, NH Orn minor, Orn major); 8.22 (d, *J* = 3.4 Hz, 0.2H, NH Orn minor); 8.16 (d, *J* = 7.8 Hz, 0.8H, NH Leu major); 8.00 (d, *J* = 6.8 Hz, 0.2H, NH Leu minor); ~7.8 (Orn side chains); 7.76 (d, *J* = 8.6 Hz, 0.3H, NH Orn minor); 7.69 (d, *J* = 6.6 Hz, 1H, NH Orn major); 7.33-7.28 (m, 3H, CH <sup>D</sup>Phe); 7.28-7.22 (m, 4H, CH <sup>D</sup>Phe); 7.22-7.11 (m, 1H, CH <sup>D</sup>Phe); ~4.8 (H<sub>α</sub> Orn minor, not seen due to solvent suppression); 4.62-4.52 (m, 1H, H<sub>α</sub> <sup>D</sup>Phe major, Leu major, 2 x Orn minor); 4.49-4.37 (m, 2.5H, H<sub>α</sub> <sup>D</sup>Phe minor, 2 x Orn major); 4.33-4.26 (m, 0.2H, H<sub>α</sub> Leu minor); 4.24-4.19 (m, 0.2H, H<sub>α</sub> <sup>D</sup>Phe minor); 4.18-4.14 (m, 0.2H, H<sub>α</sub> Leu minor); 3.95 (d, *J* = 10.1 Hz, 0.2H, H<sub>α</sub> Orn minor); 3.64-3.57 (m, 1.2H, H<sub>δ</sub> Pro); 3.49 (dd, *J* = 19.5 Hz, *J* = 8.3 Hz, 0.2H, H<sub>δ</sub> Pro minor); 3.44-3.41 (m, 0.2H, H<sub>δ</sub> Pro minor); 3.20-3.11 (m, 0.5H, H<sub>δ</sub> Orn minor); 3.07-2.90 (m, 8H, H<sub>β</sub> <sup>D</sup>Phe major, <sup>D</sup>Phe minor, H<sub>δ</sub> Orn major); 2.83 (dd, *J* = 13.9 Hz, *J* = 8.9 Hz, 1H, H<sub>δ</sub> Orn minor); 2.71-2.62 (m, 1H); 2.59-2.52 (m, 0.1H); 2.49-2.40 (m, 0.2H); 2.27 (ddd, *J* = 10.4 Hz, *J* = 7.2

Hz,  $J = 3.3$  Hz, 0.2H); 2.21 (t,  $J = 7.6$  Hz, 2H); 2.13 (s, 0.3H); 2.05-1.99 (m, 2H); 1.98-1.91 (m, 1H, ); 1.88-1.79 (m, 4H,  $H_{\beta}$  Pro,  $H_{\beta}$  Orn); 1.77-1.53 (m, 20H,  $H_{\beta}$  Leu major,  $H_{\beta}$  Leu minor,  $H_{\gamma}$  Leu major,  $H_{\gamma}$  Leu minor,  $H_{\gamma}$  Pro major,  $H_{\gamma}$  Pro minor,  $H_{\gamma}$  Orn major,  $H_{\gamma}$  Orn minor); 1.53-1.45 (m, 2H); 1.38-1.25 (m, 13H); 0.97-0.84 (m, 12H,  $H_{\delta}$  Leu).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OH}$ , 150 MHz, 298K):  $\delta$  176.5; 175.5; 175.3; 175.2; 173.6; 173.6; 173.4; 173.1; 172.9; 172.7; 172.7; 172.0; 171.2; 162.8; 162.6; 162.3; 162.1; 139.4; 137.2; 137.1; 130.7; 130.4; 129.7; 129.6; 129.6; 129.1; 128.5; 128.4; 127.4; 68.1; 61.5; 61.0; 60.5; 57.2; 55.6; 55.6; 55.5; 54.4; 53.8; 53.3; 53.2; 53.2; 52.9; 48.0; 47.6; 42.7; 42.0; 41.4; 40.6; 40.5; 40.4; 40.3; 40.3; 40.2; 40.1; 39.8; 38.7; 37.9; 37.7; 34.5; 31.4; 30.3; 30.2; 30.1; 30.1; 29.0; 28.1; 28.1; 26.0; 25.8; 25.5; 25.2; 25.1; 25.1; 25.0; 24.9; 24.8; 24.6; 23.4; 23.4; 23.3; 23.1; 21.5; 20.9. IR (neat): 3277.2; 3049.3; 2958.2; 1703.8; 1699.7; 1693.8; 1688.0; 1683.6; 1673.8; 1667.9; 1661.7; 1651.7; 1645.6; 1640.2; 1634.1; 1628.0; 1622.2; 1615.9; 1575.4; 1567.6; 1563.8; 1557.7; 1553.7; 1548.0; 1543.9; 1526.0; 1519.9; 1515.8; 1505.8; 1496.1; 1471.6; 1463.7; 1455.7; 1435.8; 1175.5; 1200.1; 1132.8; 834.5; 798.9; 721.9; 703.3; 667.8; 597.9; 517.9. LC-MS retention time: 4.97 min (10  $\rightarrow$  90% MeCN, 15 min run). Mass (ESI):  $m/z$  586.47  $[\text{M} + 2\text{H}]^{2+}$ ; 1171.80  $[\text{M} + \text{H}]^+$ . Exact mass: calculated for  $[\text{C}_{60}\text{H}_{96}\text{N}_{14}\text{O}_{10}]^{2+}$ : 586.37114, found: 586.37259  $[\text{M} + 2\text{H}]^{2+}$ .

### **Cyclo-( $^{\text{D}}$ Phe-Pro-Orn-AdaGly-Orn) $_2$ . 4TFA (13)**

Following the general procedures for peptide synthesis and cleavage, **13** was obtained from Fmoc-Orn(Boc)-HMPB-MBHA resin (150  $\mu\text{mol}$ ) as a white powder (92 mg, 51.6  $\mu\text{mol}$ , 34%), using an HPLC gradient of 70:20:10  $\rightarrow$  10:80:10  $\text{H}_2\text{O}/\text{MeCN}/1\%$  aq. TFA for purification.

$^1\text{H}$  NMR ( $\text{CD}_3\text{OH}$ , 600 MHz):  $\delta$  9.23 (s, 2H, NH  $^{\text{D}}$ Phe); 8.66 (d,  $J = 7.6$  Hz, 2H, NH Orn $_3$ , Orn $_8$ ); 7.98-7.90 (m, 10H, NH Orn $_5$ , Orn $_{10}$ , NH AdaGly, NH Orn side chains); 7.33-7.20 (m, 10H, CH  $^{\text{D}}$ Phe); 4.74 (d,  $J = 10.0$  Hz, 1H,  $H_{\alpha}$  Orn $_5$ , Orn $_{10}$ , integral reduced due to solvent suppression); 4.51-4.39 (m, 8H,  $H_{\alpha}$  all other residues); 3.64-3.60 (m, 2H,  $H_{\delta\text{A}}$  Pro); 3.03 (dd,  $J = 12.4$  Hz,  $J = 4.7$  Hz, 2H,  $H_{\beta\text{A}}$   $^{\text{D}}$ Phe); 3.00-2.91 (m, 10H,  $H_{\beta\text{B}}$   $^{\text{D}}$ Phe,  $H_{\delta}$  Orn $_3$ , Orn $_5$ , Orn $_8$ , Orn $_{10}$ ); 2.39-2.32 (m, 2H,  $H_{\delta\text{B}}$  Pro); 2.03 (s, 7H, Ada side chain); 2.00-1.95 (m, 4H,  $H_{\beta\text{A}}$  Pro,  $H_{\beta\text{A}}$  Orn $_3$ , Orn $_8$ ); 1.86-1.78 (m, 4H,  $H_{\beta\text{B}}$  Orn $_3$ , Orn $_8$ ,  $H_{\beta\text{A}}$  Orn $_5$ , Orn $_{10}$ ); 1.78-1.67 (m, 27H,  $H_{\beta\text{B}}$  Orn $_5$ , Orn $_{10}$ ,  $H_{\gamma\text{A}}$  Orn $_5$ , Orn $_{10}$ , Ada side chain); 1.67-1.55 (m, 14H,  $H_{\beta\text{B}}$  Pro,  $H_{\gamma\text{B}}$  Orn $_5$ , Orn $_{10}$ ,  $H_{\gamma}$  Orn $_3$ , Orn $_8$ ,  $H_{\gamma\text{A}}$  Pro); 1.55-1.50 (m, 2H,  $H_{\gamma\text{B}}$  Pro).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OH}$ , 150 MHz):  $\delta$  173.9 (C=O amide); 172.7 (C=O amide); 172.5 (C=O amide); 172.4 (C=O amide); 172.1 (C=O amide); 137.0 ( $\text{C}_{\alpha}$   $^{\text{D}}$ Phe); 130.4 (CH  $^{\text{D}}$ Phe); 129.6 (CH  $^{\text{D}}$ Phe); 128.4 (CH  $^{\text{D}}$ Phe); 61.8 ( $\text{C}_{\alpha}$  Orn $_5$ , Orn $_{10}$ ); 61.3 ( $\text{C}_{\alpha}$  Pro); 56.1 ( $\text{C}_{\alpha}$   $^{\text{D}}$ Phe); 54.0 ( $\text{C}_{\alpha}$  Orn $_3$ , Orn $_8$ ); 53.9 ( $\text{C}_{\alpha}$  AdaGly); 47.4 ( $\text{C}_{\delta}$  Pro); 40.5 ( $\text{C}_{\delta}$  Orn $_3$ , Orn $_8$ ); 40.4 ( $\text{C}_{\delta}$  Orn $_5$ , Orn $_{10}$ ); 39.5 (Ada side chain); 38.3 ( $\text{C}_{\alpha}$  Ada side chain); 37.9 (Ada side chain); 37.4 ( $\text{C}_{\beta}$   $^{\text{D}}$ Phe); 30.3 ( $\text{C}_{\beta}$  Orn $_5$ , Orn $_{10}$ ); 30.2 ( $\text{C}_{\beta}$  Orn $_3$ , Orn $_8$ ); 30.1 ( $\text{C}_{\beta}$  Pro); 29.8 (Ada side chain); 25.4 ( $\text{C}_{\gamma}$  Orn $_5$ , Orn $_{10}$ ); 25.1 ( $\text{C}_{\gamma}$  Orn $_3$ , Orn $_8$ ); 24.5 ( $\text{C}_{\gamma}$  Pro). IR (neat): 3281.4; 3055.3; 2918.3; 2853.4; 1705.8; 1701.6; 1696.0; 1685.7; 1676.0; 1670.7; 1664.0; 1659.6; 1654.0; 1648.0; 1636.7; 1630.4; 1624.8; 1618.8; 1560.0; 1555.7; 1551.7; 1545.9; 1541.8; 1534.0; 1528.6; 1522.2; 1518.0; 1513.2; 1508.2; 1501.9; 1498.2; 1457.9; 1449.8; 1438.0; 1430.6; 1426.0; 1420.4; 1342.6; 1313.7; 1200.4; 1176.5; 1132.6; 834.2; 798.9; 749.9; 721.9; 701.8; 594.1; 504.7. LC-MS retention time: 6.66 min (10  $\rightarrow$  90% MeCN, 15 min run). Mass (ESI):  $m/z$  664.47  $[\text{M} + 2\text{H}]^{2+}$ ; 1327.67  $[\text{M} + \text{H}]^+$ . Exact mass: calculated for  $[\text{C}_{72}\text{H}_{108}\text{N}_{14}\text{O}_{10}]^{2+}$ : 664.41809;  $[\text{C}_{72}\text{H}_{107}\text{N}_{14}\text{O}_{10}]^+$ : 1327.82891. Found: 664.41794  $[\text{M} + 2\text{H}]^{2+}$ ; 1327.83005  $[\text{M} + \text{H}]^+$ .

### **Cyclo-( $^{\text{D}}$ Phe-Pro-Orn-AdaAla-Orn) $_2$ . 4TFA (14)**

Following the general procedures for peptide synthesis and cleavage, **14** was obtained from Fmoc-Orn(Boc)-HMPB-MBHA resin (150  $\mu\text{mol}$ ) as a white powder (46.7 mg, 25.8  $\mu\text{mol}$ , 17%), using an HPLC gradient of 55:35:10  $\rightarrow$  30:60:10  $\text{H}_2\text{O}/\text{MeCN}/1\%$  aq. TFA for purification.

$^1\text{H}$  NMR ( $\text{CD}_3\text{OH}$ , 600 MHz):  $\delta$  8.83 (d,  $J = 4.7$  Hz, 1H, NH  $^{\text{D}}$ Phe); 8.56 (dd,  $J = 17.0$  Hz,  $J = 8.5$  Hz, 3H, NH AdaAla, NH Orn, NH  $^{\text{D}}$ Phe); 8.40 (d,  $J = 3.1$  Hz, 1H, NH Orn); 8.14 (d,  $J = 4.6$  Hz, 1H, NH Orn); 8.07 (d,  $J = 7.2$  Hz, 1H, NH AdaAla); 7.98 (d,  $J = 8.6$  Hz, 1H, NH Orn); 7.96-7.82 (m, 5H, NH Orn side chain); 7.76 (s, 2H, NH Orn side chain); 7.39-7.09 (m, 11H, NH Orn side chain, CH  $^{\text{D}}$ Phe); 4.80-4.76 (m, 1H,  $H_{\alpha}$  Orn); 4.67-4.61 (m, 1H,  $H_{\alpha}$  Orn); 4.56 (dt,  $J = 9.4$  Hz,  $J = 5.1$  Hz, 1H,  $H_{\alpha}$   $^{\text{D}}$ Phe); 4.45 (dd,  $J = 7.9$  Hz,  $J = 2.6$  Hz, 1H,  $H_{\alpha}$  Orn); 4.40 (dd,  $J = 13.4$  Hz,  $J = 7.5$  Hz, 2H,  $H_{\alpha}$   $^{\text{D}}$ Phe,  $H_{\alpha}$  AdaAla); 4.33 (dd,  $J = 8.2$  Hz,  $J = 2.3$  Hz, 1H,  $H_{\alpha}$  Pro); 4.26 (dd,  $J = 12.1$  Hz,  $J = 4.7$  Hz, 2H,  $H_{\beta}$  Pro,  $H_{\beta}$  Orn); 3.03 (dd,  $J = 12.4$  Hz,  $J = 4.7$  Hz, 2H,  $H_{\beta\text{A}}$   $^{\text{D}}$ Phe); 3.00-2.91 (m, 10H,  $H_{\beta\text{B}}$   $^{\text{D}}$ Phe,  $H_{\delta}$  Orn $_3$ , Orn $_5$ , Orn $_8$ , Orn $_{10}$ ); 2.39-2.32 (m, 2H,  $H_{\delta\text{B}}$  Pro); 2.03 (s, 7H, Ada side chain); 2.00-1.95 (m, 4H,  $H_{\beta\text{A}}$  Pro,  $H_{\beta\text{A}}$  Orn $_3$ , Orn $_8$ ); 1.86-1.78 (m, 4H,  $H_{\beta\text{B}}$  Orn $_3$ , Orn $_8$ ,  $H_{\beta\text{A}}$  Orn $_5$ , Orn $_{10}$ ); 1.78-1.67 (m, 27H,  $H_{\beta\text{B}}$  Orn $_5$ , Orn $_{10}$ ,  $H_{\gamma\text{A}}$  Orn $_5$ , Orn $_{10}$ , Ada side chain); 1.67-1.55 (m, 14H,  $H_{\beta\text{B}}$  Pro,  $H_{\gamma\text{B}}$  Orn $_5$ , Orn $_{10}$ ,  $H_{\gamma}$  Orn $_3$ , Orn $_8$ ,  $H_{\gamma\text{A}}$  Pro); 1.55-1.50 (m, 2H,  $H_{\gamma\text{B}}$  Pro).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OH}$ , 150 MHz):  $\delta$  173.9 (C=O amide); 172.7 (C=O amide); 172.5 (C=O amide); 172.4 (C=O amide); 172.1 (C=O amide); 137.0 ( $\text{C}_{\alpha}$   $^{\text{D}}$ Phe); 130.4 (CH  $^{\text{D}}$ Phe); 129.6 (CH  $^{\text{D}}$ Phe); 128.4 (CH  $^{\text{D}}$ Phe); 61.8 ( $\text{C}_{\alpha}$  Orn $_5$ , Orn $_{10}$ ); 61.3 ( $\text{C}_{\alpha}$  Pro); 56.1 ( $\text{C}_{\alpha}$   $^{\text{D}}$ Phe); 54.0 ( $\text{C}_{\alpha}$  Orn $_3$ , Orn $_8$ ); 53.9 ( $\text{C}_{\alpha}$  AdaGly); 47.4 ( $\text{C}_{\delta}$  Pro); 40.5 ( $\text{C}_{\delta}$  Orn $_3$ , Orn $_8$ ); 40.4 ( $\text{C}_{\delta}$  Orn $_5$ , Orn $_{10}$ ); 39.5 (Ada side chain); 38.3 ( $\text{C}_{\alpha}$  Ada side chain); 37.9 (Ada side chain); 37.4 ( $\text{C}_{\beta}$   $^{\text{D}}$ Phe); 30.3 ( $\text{C}_{\beta}$  Orn $_5$ , Orn $_{10}$ ); 30.2 ( $\text{C}_{\beta}$  Orn $_3$ , Orn $_8$ ); 30.1 ( $\text{C}_{\beta}$  Pro); 29.8 (Ada side chain); 25.4 ( $\text{C}_{\gamma}$  Orn $_5$ , Orn $_{10}$ ); 25.1 ( $\text{C}_{\gamma}$  Orn $_3$ , Orn $_8$ ); 24.5 ( $\text{C}_{\gamma}$  Pro). IR (neat): 3281.4; 3055.3; 2918.3; 2853.4; 1705.8; 1701.6; 1696.0; 1685.7; 1676.0; 1670.7; 1664.0; 1659.6; 1654.0; 1648.0; 1636.7; 1630.4; 1624.8; 1618.8; 1560.0; 1555.7; 1551.7; 1545.9; 1541.8; 1534.0; 1528.6; 1522.2; 1518.0; 1513.2; 1508.2; 1501.9; 1498.2; 1457.9; 1449.8; 1438.0; 1430.6; 1426.0; 1420.4; 1342.6; 1313.7; 1200.4; 1176.5; 1132.6; 834.2; 798.9; 749.9; 721.9; 701.8; 594.1; 504.7. LC-MS retention time: 6.66 min (10  $\rightarrow$  90% MeCN, 15 min run). Mass (ESI):  $m/z$  664.47  $[\text{M} + 2\text{H}]^{2+}$ ; 1327.67  $[\text{M} + \text{H}]^+$ . Exact mass: calculated for  $[\text{C}_{72}\text{H}_{108}\text{N}_{14}\text{O}_{10}]^{2+}$ : 664.41809;  $[\text{C}_{72}\text{H}_{107}\text{N}_{14}\text{O}_{10}]^+$ : 1327.82891. Found: 664.41794  $[\text{M} + 2\text{H}]^{2+}$ ; 1327.83005  $[\text{M} + \text{H}]^+$ .

= 6.9 Hz, 1H, H<sub>z</sub> AdaAla); 4.24-4.19 (m, 1H, H<sub>z</sub> Pro); 3.87-3.80 (m, 1H, H<sub>z</sub> Orn); 3.62-3.56 (m, 1H, H<sub>δ</sub> Pro); 3.54-3.46 (m, 1H, H<sub>δ</sub> Pro); 3.41-3.35 (m, 1H, H<sub>δ</sub> Orn); 3.11 (dd, *J* = 13.9 Hz, *J* = 4.9 Hz, 1H, H<sub>βA</sub> <sup>D</sup>Phe); 3.08-2.95 (m, 10H, H<sub>β</sub> <sup>D</sup>Phe, H<sub>δA</sub> Orn); 2.95-2.87 (m, 6H, H<sub>δB</sub> Orn); 2.85-2.75 (m, 6H, H<sub>δA</sub> Orn, H<sub>βB</sub> <sup>D</sup>Phe); 2.68 (s, 1H, H<sub>δB</sub> Orn); 2.55 (q, *J* = 7.6 Hz, *J* = 7.5 Hz, 1H, H<sub>δA</sub> Orn); 2.51-2.44 (m, 1H, H<sub>βA</sub> Pro); 2.29-2.23 (m, 1H, H<sub>βB</sub> Pro); 2.16-2.02 (m, 4H); 2.02-1.90 (m, 16H); 1.87-1.79 (m, 7H); 1.77-1.69 (m, 23H); 1.69-1.50 (m, 50H); 1.49-1.42 (m, 13H); 1.34-1.27 (m, 3H). <sup>13</sup>C NMR (CD<sub>3</sub>OH, 150 MHz): δ 177.2 (C=O); 175.9 (C=O); 175.5 (C=O); 174.4 (C=O); 173.6 (C=O); 173.5 (C=O); 172.6 (C=O); 172.2 (C=O); 172.0 (C=O); 171.2 (C=O); 137.4 (C<sub>q</sub> <sup>D</sup>Phe); 137.0 (C<sub>q</sub> <sup>D</sup>Phe); 130.7 (CH <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 130.4 (CH <sup>D</sup>Phe); 129.7 (CH <sup>D</sup>Phe); 129.6 (CH <sup>D</sup>Phe); 129.1 (CH <sup>D</sup>Phe); 128.5 (CH <sup>D</sup>Phe); 127.4 (CH <sup>D</sup>Phe); 61.5 (C<sub>z</sub> <sup>D</sup>Phe); 61.0 (C<sub>z</sub> Pro); 60.6 (C<sub>z</sub> Orn); 57.1 (C<sub>z</sub> Orn); 53.6 (C<sub>z</sub> Orn); 53.3 (C<sub>z</sub> Pro, <sup>D</sup>Phe); 53.3 (C<sub>z</sub> Orn); 52.7 (C<sub>z</sub> AdaAla); 51.6 (C<sub>z</sub> AdaAla); 48.9 (C<sub>δ</sub> Pro) 48.0 (C<sub>δ</sub> Orn); 47.6 (C<sub>β</sub> AdaAla); 45.0 (C<sub>β</sub> AdaAla); 43.4 (Ada side chain); 43.3 (Ada side chain); 43.2 (Ada side chain); 41.5 (C<sub>δ</sub> Orn); 40.6, 40.5, 40.4 (C<sub>δ</sub> Orn); 40.3, 40.2 (C<sub>δ</sub> Orn); 38.8 (C<sub>β</sub> <sup>D</sup>Phe); 37.9 (C<sub>β</sub> <sup>D</sup>Phe); 37.8 (Ada side chain); 34.4 (C<sub>β</sub> Pro); 33.8; 33.6; 33.5; 31.1 (C<sub>γ</sub> Pro); 30.0 (Ada side chain); 29.9 (Ada side chain); 29.9 (Ada side chain); 28.4; 28.3; 28.2; 26.1; 25.5; 25.3; 24.6; 23.3; 23.2. IR (neat): 3053.5; 2912.4; 2901.8; 2853.4; 2849.8; 2846.3; 1704.1; 1699.5; 1694.1; 1688.2; 1683.6; 1679.7; 1673.8; 1668.0; 1661.7; 1651.7; 1645.7; 1640.2; 1634.2; 1627.9; 1622.1; 1615.9; 1574.2; 1568.1; 1563.3; 1557.7; 1553.8; 1549.1; 1543.8; 1538.2; 1531.8; 1525.9; 1520.1; 1515.6; 1505.7; 1495.9; 1471.2; 1463.1; 1455.6; 1447.6; 1435.6; 1201.3; 1186.2; 1179.1; 1133.7; 837.8; 799.5; 722.2; 702.1; 595.9; 518.0. LC-MS retention time: 6.39 min (10 → 90% MeCN, 15 min run). Mass (ESI): *m/z* 678.67 [M + 2H]<sup>2+</sup>; 1355.80 [M + H]<sup>+</sup>. Exact mass: calculated for [C<sub>74</sub>H<sub>112</sub>N<sub>14</sub>O<sub>10</sub>]<sup>2+</sup>: 678.43374; [C<sub>74</sub>H<sub>111</sub>N<sub>14</sub>O<sub>10</sub>]<sup>+</sup>: 1355.86021. Found: 678.43354 [M + 2H]<sup>2+</sup>; 1355.86148 [M + H]<sup>+</sup>.

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