Bioorganic & Medicinal Chemistry 19 (2011) 3402–3409

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and evaluation of strand and turn modified ring-extended gramicidin S derivatives

Annemiek D. Knijnenburg [a], Varsha V. Kapoerchan [a], Gijsbert M. Grotenbreg [a,†], Emile Spalburg [b], Albert J. de Neeling [b], Roos H. Mars-Groenendijk [c], Daan Noort [c], José M. Otero [d,e], Antonio L. Llamas-Saiz [f], Mark J. van Raaij [d,g], Bep Ravensbergen [h], Peter H. Nibbering [h], Gijs A. van der Marel [a], Herman S. Overkleeft [a], Mark Overhand [a,*]

[a] Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands
[b] National Institute of Public Health and the Environment, Laboratory for Infectious Diseases, PO Box 1, 3720 BA Bilthoven, The Netherlands
[c] TNO, Prins Maurits Laboratory, PO Box 45, 2280 AA Rijswijk, The Netherlands
[d] Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Campus Vida, Universidad de Santiago, 15782 Santiago de Compostela, Spain
[e] Laboratoire des Proteines Membranaires, Institut de Biologie Structurale J. P. Ebel, 41 Rue Jules Horowitz, F-38027 Grenoble, France
[f] Unidad de Rayos X (RIAIDT), Laboratorio Integral de Dinámica y Estructura de Biomoléculas 'José R. Carracido', Edificio CACTUS, Campus Vida, Universidad de Santiago, 15782 Santiago de Compostela, Spain
[g] Laboratorio M-4, Departamento de Estructura de Macromoleculas, Centro Nacional de Biotecnologia, c/Darwin 3, Campus Cantoblanco, E-28049 Madrid, Spain
[h] Center for Infectious Diseases, Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 25 February 2011
Revised 5 April 2011
Accepted 13 April 2011
Available online 22 April 2011

Keywords:
Cationic antimicrobial peptides
Gramicidin S, GS14K4
Antibiotics
Secondary structure
Amphipathic peptides
Dipeptide isostere

ABSTRACT

In this paper, we describe the crystal structure of previously reported ring-extended gramicidin S (GS) derivative **2** (GS14K4), containing a D-amino acid residue in one of the β-strand regions. This structure is in agreement with a previously reported modeling study of the same molecule. The polar side chain of the additional D-amino acid residue is positioned at the same face of the molecule as the hydrophobic side chains, and we believe that because of this compound **2** is considerably less hydrophobic than extended GS derivatives in which the strand regions are exclusively composed of L-amino acids. Using this backbone structure as our benchmark we prepared a small series of ring-extended GS analogues featuring sugar amino acid dipeptide isosteres of varied hydrophobicity at the turn region. We show that via this approach hydrophobicity of extended GS analogues can be tuned without affecting the secondary structure (as observed from NMR and CD spectra). Biological evaluation reveals that hydrophobicity correlates to cell toxicity, but still bacteriolysis is induced with GS analogues that are too hydrophilic to efficiently lyse human red blood cells.

1. Introduction

The increasing number of pathogenic bacteria that are resistant to clinically applied antibiotics has led to a search for potent new antibiotics with alternative targets.[1] Cationic antimicrobial peptides (CAPs) comprise a broad class of molecules that target the lipid bilayer of bacteria. Bacteria do not easily build up resistance against such peptides,[2] and therefore CAPs are considered as promising leads for the development of new antibiotics. A prominent member of the CAP family is the *cyclo*-decapeptide gramicidin S (GS) isolated from *Aneurinibacillus migulans*.[3] Its secondary structure consists of a short antiparallel β-sheet interconnected by two type II' β-turns. The amino acid sequence and its secondary structure render the molecule amphipathic with the cationic side chains oriented on one face of the β-sheet and the hydrophobic side chains on the opposing face.[4] The cationic amphipathic characteristics of the peptide are responsible for its membrane disrupting properties, and GS is active against both Gram-positive and to a lesser extent against Gram-negative bacteria.[5] In combination with the antibiotic agent framycetin, which targets Gram-negative bacteria, GS has proven to be an effective antibiotic against external ear infections.[6] The major drawback of GS, however, is that it is hemolytic and therefore it is only used to treat topical infections. Over the years many GS derivatives have been designed with the aim to improve the therapeutic index, that is, decreasing hemolytic activity while retaining potent antimicrobial activity.[7] One promising strategy is modification of the β-turn region of the peptide with unnatural amino acids[8] or dipeptide isosteres.[9] In this context, sugar amino acids (SAAs) featuring a carboxylate and an amine[10]

* Corresponding author. Tel.: +31 715274483; fax: +31 715274307.
E-mail address: overhand@chem.leidenuniv.nl (M. Overhand).
† Present address: Department of Microbiology, National University of Singapore CeLs, 28 Medical Drive, Singapore 117456, Singapore.

0968-0896/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2011.04.031

can be used to influence conformational behavior, and at the same time allow the introduction of additional functionalities and thus modulation of the overall physical properties. An alternative strategy to improve the therapeutic index of GS is to adjust the amphipathic nature by modifying the β-strand regions.[11] In this context, the design and evaluation of tetradecameric GS analogues have been reported, including derivatives containing six apolar and four cationic residues. These appeared to behave as ring-extended GS analogues in that they adopt stable cyclic β-hairpin conformations in solution with altered amphipathicity.[12] Kondejewski and coworkers reported the substitution of several amino acids in both the β-turn and β-strand regions without significantly changing secondary structure characteristics.[13] Single D-amino acid substitution of the fourth position in the β-strand of tetradecameric GS analogues such as in **2** (GS14K4[13a], Figure 1), led to a compound with a reported improved therapeutic index compared to the L-amino acid substituted **1** (GS14, Fig. 1). Molecular modeling studies on **2** indicate that the inserted D-amino acid in the sequence has no major influence on the cyclic β-hairpin secondary structure.

Recently, we reported the incorporation of furanoid SAAs as β-turn mimetic in tetradecameric GS derivatives (**3** and **5**–**7**, Fig. 1).[14] NMR- and CD spectroscopy showed that these peptides adopt cyclic β-hairpin structures. Variation of the substitution pattern on the furanoid core provided derivatives that displayed an increased antibacterial activity and reduced hemolytic activity compared to **3**. Compared to GS and **3**, these analogues display enhanced antimicrobial activity against Gram-negative bacterial strains. Because of these promising results we decided to synthesize tetradecameric GS parent compound **4** that contains an additional D-amino acid in the sequence and the furanoid SAAs β-turn modified derivatives **8**–**10** (Fig. 1). The new peptides were subjected to a detailed study of their structural features and these were compared with those of **1**–**3** and **5**–**7**. The entire series **1**–**10** was evaluated for their bactericidal properties against an extended panel of bacterial strains, as well as their hemolytic properties.

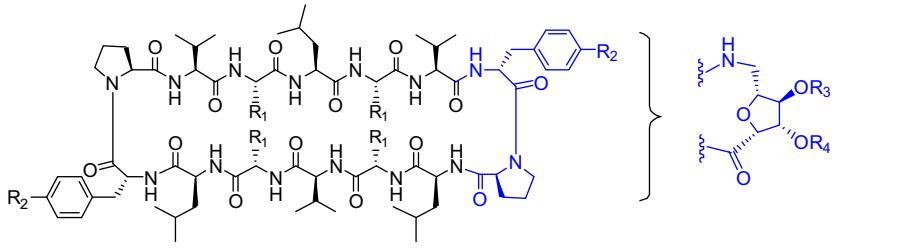
2. Results and discussion

2.1. Synthesis

The precursors of peptides **2**[13a] and **4** were assembled on the highly acid sensitive HMPB–BHA-resin using a standard step-wise solid phase Fmoc-based peptide synthesis protocol.[15] After mild acidic cleavage from the solid support, the linear peptides having the basic side chains protected with Boc-groups were cyclized under high dilution and purified using gel filtration. Boc deprotection under strong acidic conditions and purification by preparative HPLC afforded peptides **2** and **4**. Peptides **8**–**10** were synthesized according to a previous described procedure[14] using the appropriately functionalized SAA building blocks **11**[16] and **12** (Scheme 1). SAA **12** was prepared by treating known SAA **11** with excess sodium hydride and 1.2 equiv of benzyl bromide (Scheme 1). SAAs **11** and **12** were coupled to preloaded HMPB-BHA-resin (**13**) yielding peptide **14** and **15**, respectively. After reducing the azide functionalities using aqueous trimethylphosphine the sequences were elongated by solid phase peptide synthesis after which the ensuing linear peptides **16** and **17** were cleaved from resin and converted to the cyclic peptides **18** and **19**. The benzyl group in Boc-protected peptide **19** were removed using 10% palladium hydroxide on charcoal in a hydrogen gas atmosphere yielding Boc protected cyclic peptide **20**. Removal of the Boc protective groups of peptides **18**–**20** was accomplished using trifluoroacetic acid in the presence of triethylsilane and the target peptides were purified using preparative HPLC. Compounds **2**, **4** and **8**–**10** were obtained in 25–40% overall yield. Peptides **1**, **3** and **5**–**7** were prepared as reported previously.[14]

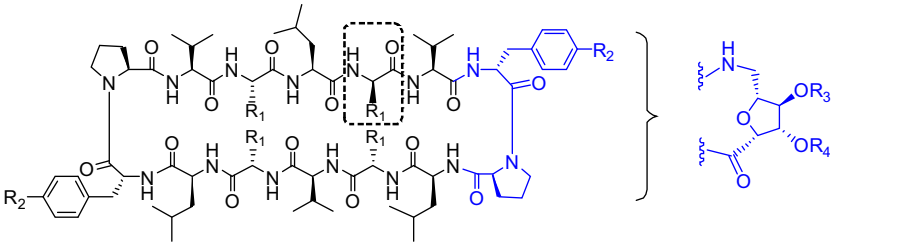
2.2. X-ray crystallography

Thus far no crystal structure of any cyclic tetradecameric GS analogue has been reported. We were therefore pleased to see that compound **2** gave crystals, which was used to determine the atomic structure by high resolution X-ray crystallography. The side chain of the D-lysine residue situated at the 4-position cleary re-



1, GS14: $R_1 = (CH_2)_4NH_2$, R_2 = OH
3, $R_1 = (CH_2)_3NH_2$, R_2 = H

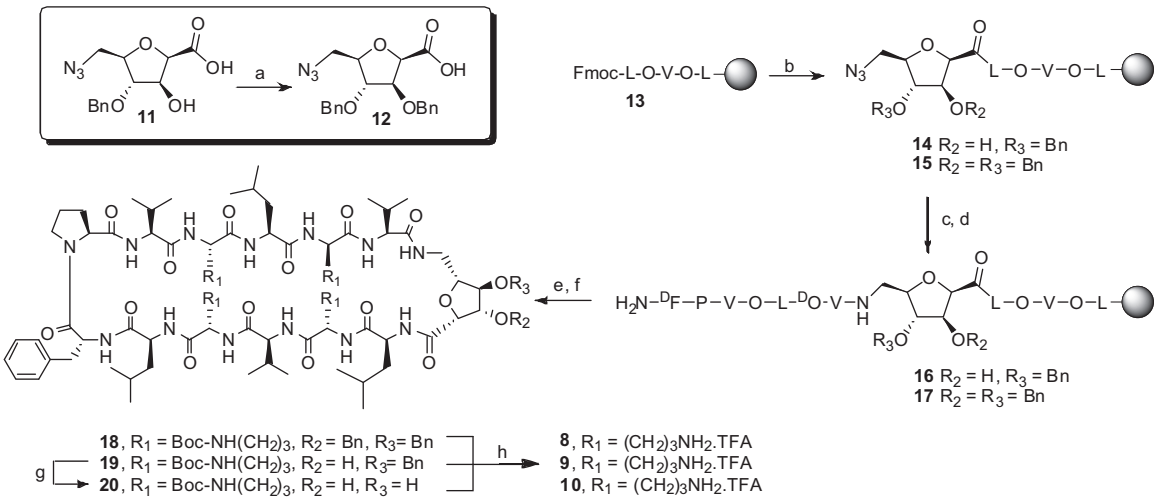
5, $R_1 = (CH_2)_3NH_2$, R_2 = H, $R_3 = R_4$ = Bn
6, $R_1 = (CH_2)_3NH_2$, $R_2 = R_4$ = H, R_3 = Bn
7, $R_1 = (CH_2)_3NH_2$, $R_2 = R_3 = R_4$ = H



2, GS14K4: $R_1 = (CH_2)_4NH_2$, R_2 = OH
4, $R_1 = (CH_2)_3NH_2$, R_2 = H

8, $R_1 = (CH_2)_3NH_2$, R_2 = H, $R_3 = R_4$ = Bn
9, $R_1 = (CH_2)_3NH_2$, $R_2 = R_4$ = H, R_3 = Bn
10, $R_1 = (CH_2)_3NH_2$, $R_2 = R_3 = R_4$ = H

Figure 1. Structures of the tetradecameric Gramicidin S derivatives **1**–**4** and analogues with a SAA modified β-turn (**5**–**10**). Numbering of amino acids N → C: *cyclo*-(Val1-Orn2-Leu3-D-Orn4-Val5-D-Phe6-Pro7/SAA-Leu8-Orn9-Val10-Orn11-Leu12-D-Phe13-Pro14).



Scheme 1. Reagents and conditions: (a) NaH (2.5 equiv), BnBr (1.2 equiv), DMF, 0 °C, 5 h, 71%.; (b) (i) 20% piperidine in NMP. (ii) using SAA **11** and **12** (1.5 equiv), HBTU (3 equiv), DiPEA (6 equiv), DMF, 16 h; (c) PMe_3 (1 M in 9:1 THF/H_2O, 25 equiv), 16 h; (d) standard sequential Fmoc SPPS; (e) 1% TFA in DCM; (f) pyBOP (5 equiv), HOBt (5 equiv), DiPEA (15 equiv), DMF; (g) H_2, 10% $Pd(OH)_2$/C in MeOH, 5 h; (h) 95/2.5/2.5 TFA/Tis/H_2O.

sides on the same face as the hydrophobic side chains of the valine and leucine residues (Fig. 2A). Because of this it is likely that compound **2** possesses amphipathic characteristics rather different from those of the lead compound **1**. A small distortion is seen in the pleated sheet structure of the molecule at the position of the D-lysine substitution and the β-sheet is slightly curved (Fig. 2A). The presence of the D-amino acid residue however does not preclude the formation of the cyclic β-hairpin conformation in which the distances between specific amides and carbonyl functionalities of opposing strands clearly indicate the presence of six intramolecular hydrogen bonding interactions (Fig. 2B). Notable is the great similarity with the reported molecular modeling structure of **2**[17] in which the same arrangement of the side chain functionalities was observed. In the crystal structure the molecules are packed in a roof-tile shape with the hydrophilic residues at the concave face and the hydrophobic residues at the convex face (Fig. 2C). One of each D-Tyr-Pro sequences of alternate molecules are placed such that they close the concave face like a zipper forming an asymmetric channel with a hydrophilic core (Fig. 2D and E). This crystal packing differs from the crystal lattice reported for GS, which is reported to line up into double stranded helical channels.[18]

2.3. NMR and CD analysis

Compounds **4** and **8**–**10** give well-resolved ^{1}H NMR spectra in CD_3OH, which allowed the assessment of their secondary structure in solution. To enable comparison between L- and D-ornithine substituted analogues and the influence of the furanoid SAA in the type II' β-turn, all amide coupling constants and chemical shift perturbations of peptides **1**–**10** were determined (Table S1 in Supplementary data). In all cases the peptides appear to possess the typical β-sheet/β-hairpin character as observed from their amide coupling constants and chemical shift perturbations ($^3J_{HN\alpha}$: 7–9 Hz for Val, Leu and Orn residues; 4 Hz for D-Phe and $\Delta\delta H_\alpha$: >0.1 for Val, Leu, and Orn; <0 for D-Phe and Pro).[19] In general, peptides **8**–**10** exhibit lower values in chemical shift perturbation and amide coupling constants than the parent peptide **4**. The same trend was observed for the L-ornithine substituted analogues **5**–**7** compared to **3**. This indicates that the presence of the SAA turn mimetic results in a less pronounced secondary structure. Interestingly, no major differences in coupling constants and chemical shift perturbation were observed between peptides **5**–**7** and **8**–**10**. This finding suggests that the incorporation of D-ornithine

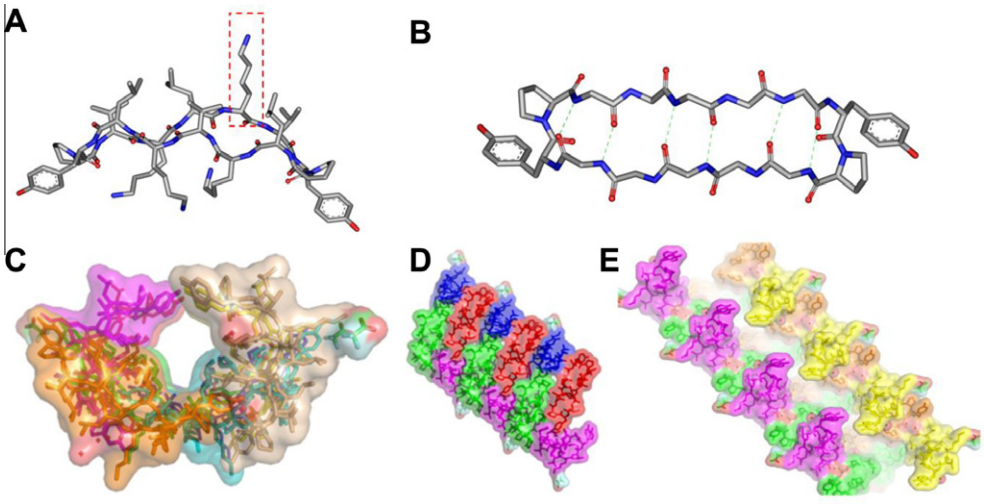


Figure 2. (A) Side view of the crystal structure of **2**; (B) top view of (side chains are omitted for clarity); (C) top view of roof-tile shaped crystal lattice; (D) Side view of the 'zipper' forming an asymmetric channel; (E) cross-cut through the asymmetric channel.

has little influence on the stability of the cyclic β-hairpin structure in CD_3OH.

CD was used to study the conformational behavior of the secondary structures of the peptides in solvent systems of different polarity. The CD spectra measured in methanol are depicted in Figure 3A. Peptides **3** and **4** show the typical GS curve with negative ellipticities at 220 and 205 nm confirming the β-sheet/β-hairpin character.[20] CD corroborates the similarities in secondary structure in the series **5**–**7** and **8**–**10** (Fig. 3A) observed in the NMR measurements. The molar ellipticity of compounds **5–7** in TFE/H_2O (Figure 3B) is slightly enhanced compared to **8**–**10** at 220 nm which suggests that these former peptides (**5**–**7**) have a more pronounced β-sheet/β-hairpin structure in this solvent system.[21,22] In buffered aqueous environments somewhat more disordered curves were observed for several analogues. For instance, peptide **3** exhibits β-sheet/β-hairpin character although less pronounced than in TFE/H_2O. Peptide **4** has a high molar ellipticity at 220 nm, but also shows a wavelength shift (towards 200 nm), which can indicate a different backbone conformation.[13] The CD curves of **5**–**10** in water exhibit less pronounced negative molar ellipticities at 220 nm combined with a high negative molar ellipticity at 195 nm, suggesting that these peptides have a more disordered structure with less β-sheet character.

2.4. Biological evaluation

The peptides **1**–**10** and **GS** were screened for their hemolytic activity and antibacterial activity against a panel of Gram-positive and Gram-negative bacteria (Table 1, Fig. 4). Compounds **5**–**7** are the most potent bactericidals of the series presented here and are at least as active as the parent compound, GS. From a therapeutic point of view however, compound **8** might be the most interesting of the series. Although slightly less potent than GS against most of the bacterial strains assayed, this compound is considerably less hemolytic, and complete hemolysis is achieved only at 500 μM (compare the value for GS, being 62.5 μM). To a lesser extend the same trend is observed for compound **9**. This in contrast to the hemolytic properties of the most potent bactericidal compounds of the series, namely **5**–**7**, which with values between 15 and 30 μM appear to be at least tenfold more toxic (Fig. 4).

2.5. Correlation of hydrophobicity and biological activity

With the aim to get insight into the hydrophobicity of the peptides we established their HPLC retention times under controlled conditions (Table 1).[23,24] The analogues containing a D-ornithine residue (**2**, **4**, **8**–**10**) are significantly less hydrophobic than their L-ornithine counterparts (**1**, **3**, **5**–**7**). This result matches the trend previously observed for **1** and **2**.[13] As expected, the GS derivatives featuring benzylated SAA turn regions are more hydrophobic than their non-benzylated counterparts. The most hydrophilic compound of the series, compound **10**, appeared to be inactive in both biological assays, that is, it is neither antibacterial nor hemolytic. At the opposite site of the hydrophobicity spectrum is compound **5**, one of the most active compounds (both bactericidal and hemolytic) that has the longest retention time on the reverse phase HPLC. Interestingly, compounds **8** and **9**, which we identified as the most promising compounds that combine bactericidal activity

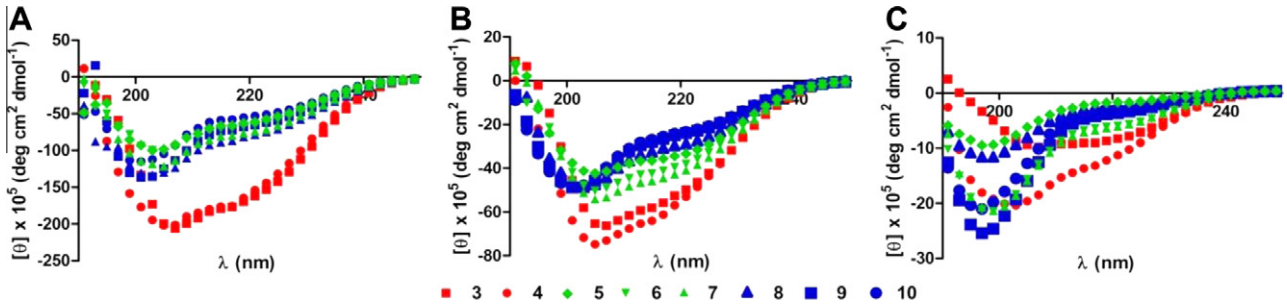


Figure 3. (A) CD spectra measured in CH_3OH; (B) CD spectra measured in 50% TFE/0.01 M aq NaOAc (pH 5.3); (C) CD spectra measured in aq NaOAc (pH 5.3). All peptides were measured at 0.1 mM concentration.

Table 1
Hemolytic (mg/L), antimicrobial activity (MIC, mg/L) and HPLC retention times of GS and ring-extended derivatives **1**–**10**

Peptide	Hemolysis[a]	*S. aureus*[b]	*S. epider midis*[b]	*E. faecalis*[b]	*B. cereus*[b]	*P. aeruginosa*[c]	*E. coli*[c]	*K. pneumoniae*[c]	*E. cloacae*[c]	*P. mirabilis*[c]	*HPLC retention time*[d]
GS	62.5	4	4	8	4	64	32	64	64	>64	8.38
1	3.9	>64	64	32	32	>64	>64	>64	>64	>64	7.26
2	500	64	8	64	16	>64	64	64	64	>64	5.95
3	3.9	16	8	8	16	64	32	64	64	>64	8.00
4	125	8	8	16	16	8	64	32	32	>64	6.48
5	15.6	4	4	8	2	16	16	32	32	>64	6.32
6	15.6	4	4	8	4	16	16	16	16	>64	6.28
7	31.3	8	4	8	4	16	8	16	16	>64	5.70
8	500	8	8	16	8	64	32	32	32	>64	5.67
9	500	16	8	32	8	64	32	32	32	>64	5.33
10	>500	>64	16	>64	64	>64	64	>64	64	>64	4.98

MW **GS**: 1369,49; MW **1** (GS14), **2** (GS14K4): 2126.23; MW **3,4**: 2038.12; MW **5,8**: 2133,22; MW **6,9**: 2043,09; MW **7,10**: 1952,97.
[a] Hemolytic activity (μM) in which 100% of the erythrocytes are lysed, hemolytic curves are shown in supporting information.
[b] Gram-positive bacteria, MIC in mg/L.
[c] Gram-negative bacteria, MIC mg/L.
[d] HPLC retention time in minutes. For conditions see experimental section.

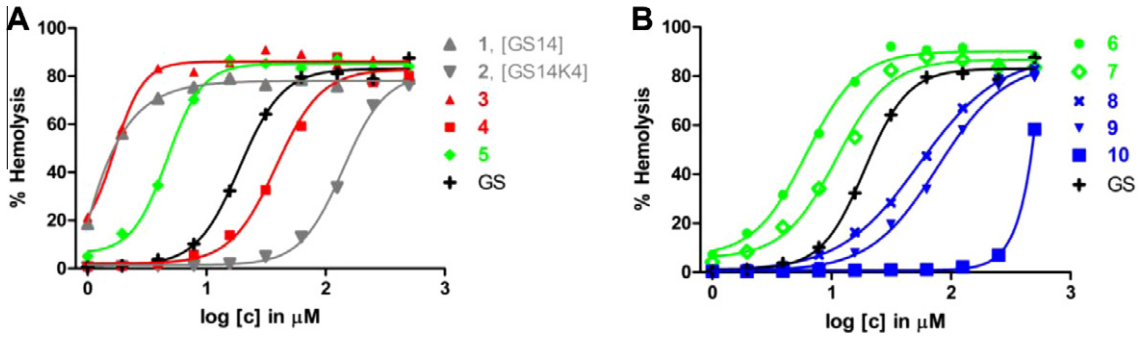


Figure 4. (A) Hemolytic activity of analogue **1**–**5** and GS; (B) hemolytic activity of analogue **6**–**10** and GS.

with limited hemolytic properties, appear to be in the middle of the hydrophobic range of the series. This observation underscores previous findings from our and other laboratories[8,9,11–14] with related molecules and suggests that there is an optimum in hydrophobicity in a series of amphiphilic peptides with otherwise unaltered secondary structure with respect to an optimal bactericidal/hemolytic ratio.

3. Conclusions

In conclusion, we have for the first time obtained a crystal structure of an extended gramicidin S analogue, the known compound **2** (GS14K4). This structure unambiguously demonstrates that insertion of an additional D-amino acid in a cyclic β-hairpin structure does not necessarily lead to a drastically altered secondary structure, and compound **2** appears to closely resemble the β-sheet/β-hairpin structure of **1** (GS14). As a result, the basic side chain of this D-amino acid residue points towards the same direction as the aliphatic side chains of the Leu/Val residues and thus whereas the secondary structure of the two compounds is much the same, the amphiphilic characteristics are rather more different. Building on these results and based on our previous findings,[14] we proceeded to generate analogues in which one of the turn regions contains a furanoid SAA of increasing hydrophobicity. NMR- and CD spectroscopy indicate that these analogues are all capable of adopting a cyclic β-hairpin structure. Notable is the observation in CD that compounds **5**–**10** are less structured under physiological conditions (aqueous buffer). Although it is difficult to link conformational properties directly to biological activity, compound **8**–**10** are less hemolytic.

The hydrophobic character of extended GS can be tuned by introduction at the appropriate site of a D-amino acid residue, by varying the nature of one of the β-turn regions, or a combination thereof. We have evaluated the consequences of these modifications on biological activity and have strong indications (compound **8**, to a lesser extend compound **9**) that there may be an optimal hydrophobicity in a series of closely related compounds (especially with respect to the secondary structure) with cell disrupting properties, in that bacteriolytic activity might be separated from hemolytic properties.

4. Experimental section

4.1. NMR spectroscopy

The peptides were recorded on a Bruker DMX 600 equipped with a pulsed field gradient accessory and a cryo-probe. For the 2D cROESY[25] spectra (τ_{mix} = 200 msec) the peptides were dissolved in CD_3OH. Standard DGF-COSY (512c × 2084c) and TOCSY (400c × 2048c) spectra were recorded using presaturation for solvent suppression. cROESY spectra (400c × 2048c, τ_{mix} = 180 ms) were recorded using the presaturation solvent suppression. All spectra were recorded in phase-sensitive mode using either the TPPI or states-TPPI for quadrature detection in the indirect dimension.

4.2. 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 an average 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 0.1 mM in MeOH, 0.1 mM in 50% TFE/0.01 M NaOAc (pH 5.3) or 0.1 mM in 0.01 M NaOAc (pH 5.3). Ellipticity is reported as mean residue ellipticity [θ], with approximate errors of ±10% at 220 nm.

4.3. Crystallization and crystal structure determination of 2

Colorless plate-shaped crystals were obtained after slow evaporation of 2 μL droplets of 9 mg/mL peptide in 80% solution of MeOH in H_2O plus 2 μL of 0.2 M HCl in MeOH under paraffin oil in Terasaki plates. A 0.20 × 0.05 × 0.05 mm crystal was mounted in air and directly flash-frozen in liquid nitrogen. Synchrotron data were collected at beamline BM14 at the ESRF (Grenoble, France). Images were collected with DNA software,[26] processed with MOSFLM[27] and scaled with POINTLESS and SCALA[28] to 1.00 Å resolution. The structure was solved by direct methods using the program ilMilione[29] and refined with no intensity cutoff using the full-matrix least-squares methods on F^2 refinement implemented in SHELXH[30] included in the WinGX package.[31] Throughout the refinement, bond-length, bond-angle and planarity restraints were imposed. All non-H atoms were refined anisotropically with suitable rigid-bond and similarity restraints. All hydrogen positions were calculated and refined using a riding atom model. The asymmetric unit contains two crystallographically independent molecules of **2** with several disordered amino acids, plus one molecule of trifluoroacetate and twelve point five molecules of water. Selected crystallographic data is reported in Table S2 in Supplementary data. Final figures were created using PyMOL (Delano Scientific, Palo Alto CA, USA). *CCDC-814883* contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/data_request/cif.

4.4. 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), *Klebsiella pneumoniae* 1190901525, *Enterobacter cloacae* 1190900252, *Pro-*

teus mirabilis 1190901236. Bacteria were stored at −70 °C and grown at 30 °C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) suspended in physiological saline until an optical density of 0.1 AU (at 595 nm, 1 cm cuvette). The suspension was diluted (10×) with physiological saline and 2 μL of this inoculum were 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**–**10** 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 24 h. The experiments were conducted once, the experimental error is one MIC interval (a factor two).

4.5. Hemolytic assays

Freshly drawn heparinized blood was centrifuged for 10 min at 1000 g at 10 °C. Subsequently, the erythrocyte pellet was washed three times with 0.85% saline solution and diluted with saline to a 1/25 packed volume of red blood cells. The peptides (**1**–**10** 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 possitive control a 1% Triton-X100 solution was prepared. For the assay 100 μL of saline were 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 were added and mixed properly. Next, 100 μL of wells A1–C1 were dispensed into wells A2–C2. This process was repeated until wells A10–C10, followed by discarding 100 μL 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 h. 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 were 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.

4.6. Synthesis peptides 2,4,8–10

4.6.1. General procedure GS analogues 8–10

Stepwise elongation: Preloaded resin with Fmoc-Leucine (2.8 g, 0.53 mmol/g, 1.5 mmol) was submitted to 4 cycles of Fmoc solid-phase synthesis with use of commercially available building blocks in the order: Fmoc-Orn(Boc)-OH, Fmoc-Val-OH, Fmoc-Orn(Boc)-OH, Fmoc-Val-OH. (a) deprotection with 20% piperidine in NMP (3 × 50 mL, 15 min); (b) washing with NMP (2 × 50 mL) and DCM (2 × 50 mL). (c) pre-activation of the up-following building block (3 equiv) in 50 mL of NMP, 0.2 M HBTU in NMP and DIPEA (6 equiv) and subsequent coupling with the resin and shaken for 4 h. (d) washing with NMP (2 × 50 mL) and DCM (2 × 50 mL). Couplings were monitored for completion by the Kaiser test and LC/MS. (e) The resin was capped with 0.45 M Ac_2O in NMP (5 equiv) and DIPEA (1 equiv). (f) The resin was washed with MeOH (2 × 50 mL), NMP (2 × 50 mL), DCM (2 × 50 mL). Dry resin **13**: 3.73 g, 0.4 mmol/g. LC/MS: R_f 5.61 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 794.7 $[M+H]^+$.

Incorporation of SAA building blocks: (a) The resin **13** (498 mg) was washed with MeOH (2 × 50 mL), NMP (2 × 50 mL), DCM (2 × 50 mL); (b) deprotection with 20% piperidine in NMP (3 × 10 mL); (c) pre-activation of 11 (1.75 equiv, 0.7 mmol) and 12 (1.5 equiv, 0.3 mmol), respectively with HBTU (3 equiv, 0.2 M HBTU in NMP), DIPEA (6 eq) in 10 mL of NMP and subsequent coupling with the resin and shaken for 4 h; (d) washing with NMP (2 × 50 mL) and DCM (2 × 50 mL); mini cleavage: LCMS **14**: R_f 5.69 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 847.0 $[M+H]^+$. LCMS **15**: R_f 7.05 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 937.4 $[M+H]^+$.

Azide reduction: Resin **14** and **15**, respectively were washed with 1,4-dioxane (3 × 10 mL), and taken up in 1,4-dioxane (10 mL) to which trimethylphosphine (16 equiv, 1 M in THF) pre-mixed with H_2O (0.6 equiv) was added. The resin was shaken for 24 h; the reduction of the azide functionality was monitored with the Kaiser test.

Automated SPPS elongation: Azide reduced resins **14** (0.2 mmol) and **15** (0.1 mmol), respectively were subjected to 7 cycles of SPPS with the use of commercially available building blocks in the following order: Fmoc-Val-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-Leu-OH, Fmoc-Orn(Boc)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-d-Phe-OH and subsequent Fmoc deprotection. Minicleavage LCMS **16**: R_f 4.84 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1607.2 $[M+H]^+$. LCMS **17**: R_f 5.18 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1695.5 $[M+H]^+$.

Cleavage from resin: The peptides were released from the resin by mild acidic cleavage (4 × 10 min, 10 mL 1% TFA in DCM). The fractions were collected and coevaporated with toluene (3 × 50 mL) to give the crude linear peptides which were immediately cyclized without further purification.

Cyclization: To a solution of HOBt (5 equiv), pyBOP (5 equiv) and DIPEA (15 equiv) in DMF (160 mL) were dropwise added the crude peptides in DMF (20 mL) over a periode of 16 h using the syringe-pump. The solvent was removed under reduced pressure and the resulting mixture was applied to a Sephadex® size exclusion column (50.0 mmD × 1500 mmL) and eluted with MeOH. LCMS **18** (deprotected in vial): R_f 5.12 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1588.2 $[M+H]^+$; HRMS (ESI) m/z 1987.21414 $[M+H]^+$, Calcd 1987.21296 for $C_{100}H_{164}N_{17}O_{24}$. LCMS **19** (deprotected in vial): R_f 5.46 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1678.6 $[M+H]^+$; HRMS (ESI) m/z 1039.13508 [½ $M+H]^+$, Calcd 1039.13360 for $C_{107}H_{170}N_{17}O_{24}$

Hydrogenation: Protected peptide **19** (121 mg, 60.9 μmol) was hydrogenated under a hydrogen atmosphere in MeOH (30 mL) with 10% $Pd(OH)_2/C$ (40 mg). The mixture was filtered over celite yielding the crude peptide **20** (99 mg, 52.1 μmol); LC/MS **20** (deprotected in vial): R_f 4.79 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1497.1 $[M+H]^+$; HRMS (ESI) m/z 1898.17050 $[M+H]^+$, Calcd 1898.16937 for $C_{93}H_{159}N_{17}O_{24}$

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. Yield of peptides **8**–**10** after HPLC is based on purified protected state.

4.6.2. *cyclo*-[Leu-Lys-Val-Lys-Leu-D-Tyr-Pro-Val- Lys-Leu-D-Lys-Val-D-Tyr-Pro] (2)

HMPB-BHA-Fmoc-Orn(Boc) preloaded resin was subjected to 13 cycles of SPPS; cleaved from resin; cyclized; washed; deprotected; purified by semi-preparative RP-HPLC (linear gradient of 29–38%, 3 CV) and lipophilization of the combined fractions furnished the peptide (71.7 mg, 33.7 μmol, 34%); LC/MS R_f 5.95 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1669.9 $[M+H]^+$; HRMS (ESI) m/z 835.54176 [½ $M+H]^+$, Calcd 835.54201 for $C_{85}H_{141}N_{18}O_{16}$; IR 3268.1, 2958.1, 1668.0, 1621.9, 1538.5, 1455.8, 1200.6, 1132.7, 839.1, 800.0, 722.4, 599.7; ^1H NMR (600 MHz, CD_3OH) δ 8.92 (d, J = 2.3, 1H, N*H* DTyr_6), 8.81 (d, J = 3.9, 1H, N*H* DTyr_{13}), 8.81 (d, J = 7.8, 1H, N*H* Val_{10}), 8.76 (d, J = 9.1, 1H, N*H* Leu_{12}), 8.65(d, J = 9.3, 1H, N*H* Leu_3), 8.59 (d, J = 8.6, 1H, N*H* Lys_{11}), 8.40 (d, J = 9.7, 1H, N*H* Lys_4), 8.38 (d, J = 10.5, 1H, N*H* Lys_2), 7.99 (d, J = 8.4, 1H, N*H* Val_5), 7.97 (d, J = 9.4, 1H, N*H* Leu_8), 7.81 (br s, NH_2 Lys), 7.64 (d, J = 9.2, 1H, N*H* Val_1), 7.55 (d, J = 9.3, 1H, N*H* Lys_9),

7.04 (dd, J = 2.0, 8.4, 4H, $H_{2,6}$ DTyr), 6.72 (t, J = 7.8, 4H, $H_{3,5}$ DTyr), 5.46 (m, 1H, H_α Lys_4), 5.19 (m, 1H, H_α Lys_9), 5.14 (m, 1H, H_α Lys_2), 4.97 (m, 1H, H_α Lys_{11}), 4.80 (m, 1H, H_α Leu_3), 4.61 (q, J = 7.6, 1H, H_α Leu_{12}), 4.52–4.34 (m, 7H, H_α Leu_8, $Val_{10,5}$, $DTyr_{13,6}$, $Pro_{7,14}$), 4.31 (t, J = 8.4, 1H, $H\alpha$ Val_1), 3.71 (m, 1H, $H_{\delta d}$ Pro_{14}), 3.62 (m, 1H, $H_{\delta d}$ Pro_7), 3.04-2.77 (m, 12H, $H_{\varepsilon d,u}$ Lys, $H_{\beta d,u}$ DTyr), 2.53 (m, 1H, $H_{\delta u}$ Pro_{14}), 2.49 (m, 1H, $H_{\delta u}$ Pro_7), 2.23 (dd, J = 6.8, 13.6, 1H, H_β Val_1), 2.13–1.89 (m, 8H, H_β $Val_{10,5}$, $H_{\beta d}$ Pro, $H_{\beta d}$ Lys), 1.86 (m, 3H, $H_{\beta d}$ Leu), 1.80–1.30 (m, 30H, $H_{\beta u,\gamma d}$ Leu, $H_{\beta u,\gamma d,u\delta d,u}$ Lys, $H_{\beta u,\gamma d,u}$ Pro), 1.28 (m, 1H, $H_{\gamma u}$ Lys_2), 1.10 (m, 1H, $H_{\gamma u}$ Lys_9) 1.07–0.80 (m, 36H, CH_3 Val, CH_3 Leu); ^{13}C NMR (151 DMHz, CD_3OH) δ 173.99, 173.86, 173.78, 173.64, 173.47, 173.42, 173.35, 173.17, 173.15, 172.76, 172.61, 172.37, 172.31, 163.02, 162.79, 158.14, 158.08, 131.45, 131.37, 127.37, 126.95, 117.13, 116.37, 116.33, 61.81, 61.71, 59.79, 58.67, 58.38, 56.09, 55.87, 53.82, 53.57, 53.20, 52.70, 52.14, 52.01, 51.76, 47.89, 47.59, 44.76, 42.10, 41.00, 40.91, 40.68, 40.54, 39.32, 36.77, 36.58, 35.77, 35.46, 33.73, 33.56, 33.37, 33.10, 32.59, 31.05, 30.77, 30.35, 28.73, 28.47, 28.33, 25.84, 25.63, 24.53, 24.39, 24.30, 24.04, 23.76, 23.63, 23.30, 23.20, 23.17, 23.00, 22.39, 22.03, 19.95, 19.51, 19.06, 18.86, 18.76, 18.36.

4.6.3. *cyclo*-[Leu-Orn-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Orn-Val-D-Phe-Pro] (4)

HMPB-BHA-Fmoc-Orn(Boc) preloaded resin was subjected to 13 cycles of SPPS; cleaved from resin; cyclized; washed; deprotected; purified by semi-preparative RP–HPLC (linear gradient of 29–38%, 3 CV) and lipophilization of the combined fractions furnished the peptide (53.3 mg, 26.1 μmol, 26%); LCMS R_f 6.48 min, linear gradient 10 → 90% B in 13.5 min; m/z = 1582.9 $[M+H]^+$; HRMS (ESI) m/z 1582.02418 $[M+H]^+$, Calcd 1582.02432 for $C_{81}H_{132}N_{18}O_{14}$; IR 3273.4, 2920.1, 1683.6, 1633.9, 1538.6, 1455.7, 1202.5, 1131.5, 1008.7, 837.1, 800.1, 721.4, 612.0; ^{1}H NMR (600 MHz, CD_3OH) δ 9.06 (d, J = 2.1, 1H, NH $^DPhe_6$), 8.98 (d, J = 2.9, 1H, NH $_dPhe_{13}$), 8.67 (d, J = 8.6, 3H, NH Val_{10}, Orn_{11}, Leu_{12}), 8.58 (d, J = 8.9, 2H, NH Leu_3, Orn_4), 8.48 (d, J = 9.5, 1H, NH Orn_2), 8.02 (d, J = 7.8, 1H, NH Val_5), 7.88 (d, J = 9.1, 1H, NH Leu_8), 7.69 (d, J = 8.9, 1H, NH Val_1), 7.58 (d, J = 9.7, 1H, NH Orn_9), 7.33–7.25 (m, 10H, CH Ar), 5.45 (m, 1H, H_α Orn_4), 5.34 (m, 1H, H_α Orn_9), 5.12 (m, 1H, H_α Orn_2), 5.02–4.68 (m, 3H, H_α Orn_{11}, $Leu_{12,3}$), 4.57–4.50 (m, 2H, H_α Leu_8, $^DPhe_{13}$), 4.46 (m, 2H, H_α Val_{10}, $^DPhe_6$), 4.38 (m, 2H, H_α Val_5, Pro_7), 4.36 (m, 1H, H_α Pro_{14}), 4.27 (t, J = 8.2, 1H, H_α Val_1), 3.73 (m, 1H, $H_{\delta d}$ Pro_7), 3.62 (m, 1H, $H_{\delta d}$ Pro_{14}), 3.10 (m, 2H, $H_{\beta d}$ ^DPhe), 3.04 (m, 1H, $H_{\delta d}$ Orn_{11}), 2.92 (m, 5H, $H_{\beta u}$ ^DPhe, $H_{\delta d,u}$ $Orn_{2,4,11}$), 2.80 (m, 1H, $H_{\delta d}$ Orn_9), 2.71 (m, 1H, $H_{\delta u}$ Orn_9), 2.47 (m, 1H, $H_{\delta u}$ $Pro_{7,14}$), 2.28 (dd, J = 6.9, 13.7, 1H, H_β Val_1), 2.12–1.84 (m, 11H, $H_{\beta d}$ Pro, $H_{\beta d}$ Orn, $H_{\beta d}$ Leu, H_β $Val_{5,10}$), 1.85–1.46 (m, 27H, $H_{\beta u,\gamma d,u}$ Leu, $H_{\beta u,\gamma d,u}$ Orn, $H_{\beta u,\gamma d,u}$ Pro), 1.44 (m, $H_{\gamma u}$ Leu_8), 1.35 (m, 1H, $H_{\gamma u}$ Leu_{12}), 1.03–0.80 (m, 36H, CH_3 Val, CH_3 Leu); ^{13}C NMR (151 MHz, CD_3OH) δ 174.46, 173.90, 173.72, 173.61, 173.58, 173.47, 173.22, 173.07, 172.97, 172.83, 172.70, 172.48, 172.40, 162.96, 162.72, 136.86, 136.33, 130.43, 130.36, 129.65, 128.57, 128.46, 101.28, 61.89, 60.08, 58.83, 58.34, 55.92, 55.61, 53.05, 52.78, 52.65, 52.16, 51.97, 51.83, 48.05, 47.73, 44.73, 42.08, 41.05, 40.76, 40.57, 40.51, 38.99, 37.56, 37.26, 33.82, 33.39, 33.29, 33.07, 32.28, 31.05, 30.94, 30.59, 30.45, 30.32, 25.90, 25.75, 25.65, 25.07, 24.96, 24.69, 24.54, 24.45, 24.35, 24.29, 23.41, 23.22, 22.91, 22.20, 21.90, 20.70, 19.93, 19.53, 19.19, 19.07, 18.72, 18.32.

4.6.4. *cyclo*-[(2*R*,3*S*,4*R*,5*R*)-5-aminomethyl-3,4-dibenzyloxy-tetrahydrofuran-2-carboxyl-Leu-Orn-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Orn-Val] (8)

Peptide **18** (116 mg, 55.8 μmol), as prepared described above, was deprotected using TFA/Tis/H_2O (95/2.5/2.5) and subsequently purified by semi-preparative RP–HPLC (linear gradient of 37–46, 3 CV and yielded 49.4 mg, 23.1 μmol, 42%; LCMS R_f 5.67 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1677.6 $[M+H]^+$; HRMS (ESI) m/z 1677.04996 $[M+H]^+$, Calcd 1677.05020 for $C_{87}H_{137}N_{17}O_{16}$; IR 3281.9, 2962.4, 1667.9, 1633.9, 1538.2, 1455.7, 1201.0, 1130.0, 835.3, 798.8, 721.5, 608.1; ^{1}H NMR (600 MHz, CD_3OH) δ 8.84 (d, J = 3.2, 1H, NH $^DPhe_{13}$), 8.57 (d, J = 7.5, 1H, NH Orn_2), 8.52 (d, J = 8.1, 1H, NH Orn_9), 8.51 (m, 1H, NH SAA_{12}), 8.50 (d, J = 9.3, 1H, NH Leu_{12}), 8.48 (d, J = 8.6, 1H, NH Orn_4), 8.31 (d, J = 7.0, 1H, NH Orn_{11}), 8.28 (d, J = 6.7, 1H, NH Leu_3), 8.27 (d, J = 6.9, 1H, NH Leu_8), 8.20 (d, J = 6.9, 1H, NH Val_5), 8.06 (d, J = 8.0, 1H, NH Val_{10}), 7.90 (br s, NH_2 Orn), 7.79 (d, J = 8.4, 1H, NH Val_1), 7.45–7.12 (m, 15H, CH Ar), 4.77 (m, 1H, H_α Orn_4), 4.71–4.52 (m, 6H, H_α $Orn_{2,9,11}$, $Leu_{3,9,12}$, $^DPhe_{13}$, H_1 SAA_{12}), 4.45 (m, 10H, CH_2 Bn) 4.37 (m, 1H, H_α Pro_{14}), 4.21 (d, J = 3.6, 1H, H_2 SAA_{12}), 4.12 (m, 3H, H_4 SAA_{12}, H_α $Val_{1,5}$), 4.04 (m, 1H, H_{5d} SAA_{12}), 3.88 (s, 1H, H_3 SAA_{12}), 3.69 (m, 1H, $H_{\delta d}$ Pro_{14}), 3.06 (m, 1H, $H_{\beta d,u}$ $^DPhe_{13}$), 2.95 (m, 7H, $H_{\delta d,u}$ Orn), 2.79 (m, 1H, $H_{\delta u}$ Orn_9), 2.58 (m, 1H, $H_{\delta u}$ Pro_{14}), 2.23 (dd, J = 6.9, 14.7, 1H, H_β Val_1), 2.17 (dd, J = 6.7, 13.3, 1H, H_β Val_5), 1.99 (m, 1H, H_β Val_{10}, $H_{\beta d}$ Pro_{14}), 1.90 (m, 2H, $H_{\beta d}$, $Orn_{4,11}$), 1.83–1.48 (m, 23H, $H_{\beta d,\gamma}$ Leu, $H_{\beta u,\gamma d,u}$ Orn), 1.39 (m, 1H, H_γ Leu_{12}), 1.05–0.82 (m, 36H, CH_3 Val, CH_3 Leu); ^{13}C NMR (151 MHz, CD_3OH) δ 175.12, 174.64, 173.85, 173.80, 173.67, 173.41, 173.31, 173.22, 173.16, 172.99, 172.68, 171.36, 138.71, 138.68, 137.02, 130.39, 129.63, 129.60, 129.46, 129.16, 129.05, 128.93, 128.76, 128.40, 87.19, 84.05, 83.82, 82.96, 73.41, 72.59, 61.81, 60.68, 59.77, 55.64, 54.01, 53.60, 53.32, 53.23, 52.46, 52.03, 49.57, 49.42, 49.28, 49.14, 49.08, 49.07, 49.00, 48.86, 48.80, 48.78, 48.72, 48.57, 47.85, 43.41, 42.47, 42.17, 40.74, 40.50, 40.40, 40.12, 37.45, 32.40, 32.18, 31.78, 31.38, 30.72, 30.55, 29.92, 25.98, 25.76, 25.71, 25.20, 24.71, 24.66, 24.55, 23.72, 23.26, 22.91, 22.86, 22.00, 21.81, 19.78, 19.71, 19.39, 18.67, 18.56.

4.6.5. *cyclo*-[(2*R*,3*S*,4*R*,5*R*)-5-aminomethyl-4-benzyloxy-3-hydroxytetrahydrofuran-2-carboxyl-Leu-Orn-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Orn-Val] (9)

Peptide **19** (125 mg, 62.9 μmol), as prepared described above, was deprotected using TFA/Tis/H_2O (95/2.5/2.5) and subsequently the peptide was purified by preparative RP-HPLC (linear gradient of 33-42%, 3CV and yielded 31.7 mg, 15.5 μmol, 25%; LCMS R_f 5.33 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1587.2 $[M+H]^+$; HRMS (ESI) m/z 1587.00388 $[M+H]^+$, Calcd 1587.00325 for $C_{80}H_{131}N_{17}O_{16}$; IR 3269.8, 2962.4, 1667.9, 1634.1, 1538.9, 1455.8, 1201.5, 1132.0, 837.3, 799.8, 722.1, 616.0; ^{1}H NMR (600 MHz, CD_3OH) δ 8.72 (d, J = 3.8, 1H, NH $^DPhe_{13}$), 8.50 (t, J = 5.2, 1H, NH SAA_{11}), 8.49 (d, J = 7.9, 1H, NH Orn_2), 8.43 (d, J = 5.7, 1H, NH Orn_9), 8.40 (d, J = 8.2, 1H, NH Orn_4), 8.38 (d, J = 7.6, 1H, NH Leu_{12}), 8.26 (d, J = 6.8, 2H, NH Orn_{11}, Leu_8), 8.07 (d, J = 7.5, 1H, NH Leu_3, Val_5), 7.98 (d, J = 6.3, 1H, NH Val_{10}), 7.88 (br s, 1H, NH_2 Orn), 7.83 (d, J = 7.9, 1H, NH Val_1), 7.73 (br s, 1H, NH_2 Orn), 7.39–7.20 (m, 10H, CH Ar), 4.76 (m, 1H, H_α Orn_{11}), 4.65–4.59 (m, 5H, H_α $Orn_{2,4}$, Leu_3, $^DPhe_{13}$, CH_2 Bn), 4.58 (d, J = 3.6, 1H, H_1 SAA_{11}), 4.56 (m, 1H, H_α Orn_9), 4.51 (m, 2H, H_α $Leu_{8,12}$, H_2 SAA_{11}), 4.37 (d, J = 5.9, 1H, H_α Pro_{14}), 4.25 (t, J = 7.0, 1H, H_α Val_{10}), 4.15 (m, 2H, H_α Val_5, H_4 SAA_{11}), 4.06 (t, J = 8.1, 1H, H_α Val_1), 4.01 (m, 1H, H_{5d} SAA_{11}), 3.84 (s, 1H, H_3 SAA_{11}), 3.71 (m, 1H, $H_{\delta d}$ Pro_{14}), 3.12–3.02 (m, 3H, H_{5u} SAA_{11}, $H_{\beta d,u}$ $^DPhe_{13}$), 3.02–2.85 (m, 9H, $H_{\delta d,u}$ Orn, H_{5u} SAA_{11}), 2.69 (m, 1H, $H_{\delta u}$ Pro_{14}), 2.21 (dd, J = 7.3, 14.3, 1H, H_β Val_1), 2.16 (dd, J = 6.7, 13.5, 1H, H_β Val_5), 2.08 (dd, J = 6.8, 13.6, 1H, H_β Val_{10}), 2.00 (m, 1H, $H_{\beta d}$ Pro_{14}), 1.90 (m, 3H, $H_{\beta d}$ $Orn_{4,9,11}$), 1.84–1.46 (m, 23H, $H_{\beta d,\gamma}$ Leu, $H_{\beta u,\gamma d,u}$ Orn), 1.40 (m, 1H, H_γ Leu_{12}) 1.11–0.82 (m, 36H, CH_3 Val, CH_3 Leu); ^{13}C NMR (151 MHz, CD_3OH) δ 174.55, 174.40, 173.97, 173.95, 173.66, 173.42, 173.31, 173.00, 172.97, 172.35, 138.89, 137.16, 130.41, 129.62, 129.45, 128.90, 128.79, 128.34, 86.86, 86.78, 84.44, 76.72, 72.59, 61.81, 60.91, 60.42, 55.49, 54.27, 53.88, 53.56, 53.31, 52.49, 52.38, 47.99, 43.52, 43.25, 42.14, 41.18, 40.53, 40.43, 40.33, 37.67, 32.27, 32.16, 31.49, 31.41, 30.64, 30.37, 29.86,

29.11, 25.95, 25.84, 25.72, 25.26, 24.82, 24.68, 23.78, 23.30, 23.06, 22.60, 21.97, 21.90, 19.91, 19.86, 19.76, 19.44, 18.89, 18.54.

4.6.6. *cyclo*-[(2*R*,3*S*,4*R*,5*R*)-5-aminomethyl-3,4-dihydroxytetrahydrofuran-2-carboxyl-Leu-Orn-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Orn-Val] (10)

Peptide **19** (99 mg, 52.1 μmol) was hydrogenated as described above. Subsequently **20** was deprotected using TFA/Tis/H_2O (95/2.5/2.5) and purified by preparative RP–HPLC (linear gradient of 31–40%, 3 CV and yielded 38.9 mg, 19.9 μmol, 38%; LCMS R_f 4.98 min, linear gradient 10 → 90% B in 13.5 min.; m/z = 1497.1 $[M+H]^+$; HRMS (ESI) m/z 1496.95527 $[M+H]^+$, Calcd 1496.95630 for $C_{73}H_{125}N_{17}O_{16}$; IR 3278.2, 2962.0, 1668.1, 1627.8, 1531.9, 1455.8, 1201.0, 1132.8, 836.6, 799.4, 721.8, 620.1; ^{1}H NMR (600 MHz, CD_3OD) δ 8.70 (d, J = 3.4, 1H, N*H* ^{D}Phe$_{13}$), 8.51 (t, J = 5.9, 1H, N*H* SAA), 8.49 (d, J = 8.4, 1H, N*H* Orn$_2$), 8.39 (d, J = 7.8, 2H, N*H* Orn$_{4,9}$), 8.37 (d, J = 8.9, 1H, N*H* Leu$_{12}$), 8.31 (d, J = 6.8, 1H, N*H* Leu$_8$), 8.24 (d, J = 6.8, 1H, N*H* Orn$_{11}$), 8.08 (d, J = 7.3, 2H, N*H* Leu$_3$ Val$_5$), 7.98 (d, J = 7.2, 1H, N*H* Val$_{10}$), 7.88 (br s, 2H, NH_2 Orn), 7.84 (d, J = 7.8, 1H, N*H* Val$_1$), 7.73 (br s, 2H, NH_2 Orn), 7.28 (m, 10H, C*H* Ar), 4.74 (m, 1H, H_α Orn$_{11}$), 4.65, (m, 1H, H_α Orn$_4$), 4.63 (m, 1H, H_α Orn$_2$), 4.62 (m, 1H, H_α Leu$_3$), 4.62 (d, J = 3.4, 1H, H_1 SAA), 4.60 (m, 1H, H_α ^{D}PHe$_{13}$), 4.55 (m, 1H, H_α Orn$_9$), 4.51 (m, 1H, H_α Leu$_{12}$), 4.50 (m, 1H, H_α Leu$_8$), 4.37 (d, J = 5.9, 1H, H_α Pro$_{14}$), 4.30 (d, J = 3.2, 1H, H_2 SAA), 4.23 (t, J = 7.4, 1H, H_α Val$_{10}$), 4.13 (t, J = 6.7, 1H, H_α Val$_5$), 4.06 (t, J = 8.0, 1H, H_α Val$_1$), 4.06 (m, 1H, H_4 SAA), 4.01 (m, 2H, H_3, H_{5d} SAA), 3.71 (m, 1H, $H_{\delta d}$ Pro$_{14}$), 3.06 (m, 3H, H_{5u} SAA, $H_{\beta d,\beta u}$ ^{D}Phe$_{13}$), 3.03–2.89 (m, 8H, $H_{\delta d,\delta u}$ Orn), 2.70 (m, 1H, $H_{\delta u}$ Pro$_{14}$), 2.21 (dd, J = 7.1, 14.3, 1H, H_β Val$_1$), 2.16 (dd, J = 6.8, 13.4, 1H, H_β Val$_5$), 2.09 (dd, J = 6.8, 13.6, 1H,H_β Val$_5$), 2.01 (m, 1H, $H_{\beta d}$ Pro) 1.91 (m, 4H $H_{\beta d}$ Orn), 1.83–1.44 (m, 23H, H$_{\beta d,u,\gamma}$ Leu, H$_{\beta u,,\gamma d,u}$ Orn), 1.41 (m, 1H, H_γ Leu$_{12}$), 1.13–0.77 (m, 36H, CH_3 Val, CH_3 Leu); ^{13}C NMR (151 MHz, CD_3OD) δ 174.58, 174.37, 173.92, 173.61, 173.49, 173.40, 172.93, 172.77, 172.73, 172.56, 162.92, 162.69, 138.38, 136.93, 130.38, 129.64, 129.52, 129.11, 128.78, 128.42, 86.16, 83.02, 77.26, 72.21, 61.87, 60.94, 60.77, 59.42, 55.73, 54.05, 53.63, 53.45, 53.19, 52.76, 51.79, 47.90, 43.42, 42.15, 42.07, 41.47, 40.75, 40.71, 40.60, 40.43, 37.47, 33.56, 31.47, 30.71, 30.64, 30.51, 30.40, 30.26, 29.82, 25.78, 25.75, 25.63, 25.16, 25.00, 24.68, 24.55, 23.90, 23.31, 23.02, 22.88, 22.29, 21.62, 19.88, 19.83, 19.68, 19.54, 19.37, 18.95.

Acknowledgements

This work was supported by the Leiden Institute of Chemistry and grant BFU2008-01588 from the Spanish Ministry of Science and Innovation. Assistance in performing NMR measurements by C. Erkelens, A. W. M. Lefeber and K. Babu is kindly acknowledged. We thank Hans van den Elst for recording HRMS data and maintaining LC/MS systems. We thank ESRF-Grenoble for provision of beamtime on beamline BM14. J.M.O. thanks the Xunta de Galicia and Spanish Ministry of Science and Innovation for 'Ángeles Alvariño' and 'José Castillejo' fellowships, respectively.

Supplementary data

Supplementary data (Table S1 (Amide coupling constants and chemical shift pertubations) and table S2 (selected crystallographic data of **2**)can be found in the supporting information. In addition 1H spectra of **2**,**4**, **8**–**10** are given.) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.031.

References and notes

1. Hulscher, M.; Grol, R.; van der Meer, J. W. M. *Lancet Inf. Dis.* **2010**, *10*, 167.
2. (a) Hancock, R. E. W.; Lehrer, R. *Trends Biotechnol.* **1998**, *16*, 82; (b) Peschel, A.; Sahl, H. G. *Nat. Rev.Microbiol.* **2006**, *4*, 529.
3. (a) Berditsch, M.; Afonin, S.; Ulrich, A. S. *Appl. Environ. Microbiol.* **2007**, *73*, 6620; (b) Gause, G. F.; Brazhnikova, M. G. *Nature* **1944**, *154*, 703.
4. (a) Schmidt, G. M. J.; Hodgkin, D. C.; Oughton, B. M. *Biochem. J.* **1957**, *65*, 744; (b) Hodgkin, D. C.; Oughton, B. M. *Biochem. J.* **1957**, *65*, 752.
5. Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Hancock, R. E. W.; Hodges, R. S. *Int. J. Pept. Prot. Res.* **1996**, *47*, 460.
6. (a) Wadsten, C. J.; Bertilsson, C. A.; Sieradzki, H.; Edstrom, S. *Arch.Oto-Rhino-Laryngology-Archiv Fur Ohren-Nasen-Und Kehlkopfheilkunde* **1985**, *242*, 135; (b) Smith, R. B.; Moodie, J. *Curr. Res. Med. Opin.* **1990**, *12*, 12.
7. (a)*Synthetic Aspects of Biologically Active Cyclic Peptides: Gramicidin S and Tyrocidines*; Izumiya, N., Kato, T., Aoyaga, H., Waki, M., Kondo, M., Eds.; Halsted Press: New York, 1979; (b) Ando, S.; Aoyagi, H.; Waki, M.; Kato, T.; Izumiya, N. *Tetrahedron Lett.* **1982**, *23*, 2195; (c) Ando, S.; Kato, T.; Izumiya, N. *Int. J. Pept. Prot. Res.* **1985**, *25*, 15; (d) Sato, K.; Nagai, U. *J. Chem. Soc. Perkin Trans. 1* **1986**, 1231; (e) Aimoto, S. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 2220; (f) Tamaki, M.; Takimoto, M.; Muramatsu, I. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 3925; (g) Bach, A. C.; Markwalder, J. A.; Ripka, W. C. *Int. J. Pept. Prot. Res.* **1991**, *38*, 314; (h) Ripka, W. C.; de Lucca, G. V.; Bach, A. C.; Pottorf, R. S.; Blaney, J. M. *Tetrahedron* **1993**, *49*, 3609; de La (i) Figuera, N.; Alkorta, I.; Garcia-Lopez, M. T.; Herranz, R.; Gonzalez-Muniz, R. *Tetrahedron* **1995**, *51*, 7841; (j) Xiao, J.; Weisblum, B.; Wipf, P. *J. Am. Chem. Soc.* **2005**, *127*, 5742.
8. (a) Solanas, C.; de la Torre, B. G.; Fernandez-Reyes, M.; Santiveri, C. M.; Jimenez, M. A.; Rivas, L.; Jimenez, A. I.; Andreu, D.; Cativiela, C. *J. Med. Chem.* **2010**, *53*, 4119; (b) Solanas, C.; de la Torre, B. G.; Fernandez-Reyes, M.; Santiveri, C. M.; Jimenez, M. A.; Rivas, L.; Jimenez, A. I.; Andreu, D.; Cativiela, C. *J. Med. Chem.* **2009**, *52*, 664.
9. (a) Knijnenburg, A. D.; Tuin, A. W.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. *Chem. Eur. J.* **2011**, *17*, 3995; (b) Tuin, A. W.; Palachanis, D. K.; Buizert, A. E. M.; Grotenbreg, G. M.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. *Eur. J. Org. Chem.* **2009**, 4231; (c) Grotenbreg, G. M.; Buizert, A. E. M.; Llamas-Saiz, A. L.; Spalburg, E.; van Hooft, P. A. V.; de Neeling, A. J.; Noort, D.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. *J. Am. Chem. Soc.* **2006**, *128*, 7559.
10. (a) Vonroedern, E. G.; Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 687; (b) Schweizer, F. *Angew. Chem., Int. Ed.* **2002**, *41*, 230; Risseeuw, M. D. P.; Overhand, M.; Fleet, G. W. J.; Simone, M. I. *Tetrahedron: Asymmetry* **2001**, *2007*, 18.
11. Kapoerchan, V. V.; Knijnenburg, A. D.; Niamat, M.; Spalburg, E.; de Neeling, A. J.; Nibbering, P. H.; Mars-Groenendijk, R. H.; Noort, D.; Otero, J. M.; Llamas-Saiz, A. L.; van Raaij, M. J.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. *Chem. Eur. J.* **2010**, *16*, 12174.
12. (a) Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Kay, C. M.; Hancock, R. E. W.; Hodges, R. S. *J. Biol. Chem.* **1996**, *271*, 25261; (b) Gibbs, A. C.; Kondejewski, L. H.; Gronwald, W.; Nip, A. M.; Hodges, R. S.; Sykes, B. D.; Wishart, D. S. *Nat. Struct. Biol.* **1998**, *5*, 284; (c) Ando, S.; Takiguchi, H.; Izumiya, N. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3781.
13. (a) Kondejewski, L. H.; Jelokhani-Niaraki, M.; Farmer, S. W.; Lix, B.; Kay, C. M.; Sykes, B. D.; Hancock, R. E. W.; Hodges, R. S. *J. Biol. Chem.* **1999**, *274*, 13181; (b) Lee, D. L.; Powers, J. P. S.; Pflegerl, K.; Vasil, M. L.; Hancock, R. E. W.; Hodges, R. S. *J. Pept. Res.* **2004**, *63*, 69.
14. Knijnenburg, A. D.; Spalburg, E.; de Neeling, A. J.; Mars-Groenendijk, R. H.; Noort, D.; Grotenbreg, G. M.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. *ChemMedChem* **2009**, *4*, 1976.
15. Grotenbreg, G. M.; Kronemeijer, M.; Timmer, M. S. M.; El Oualid, F.; van Well, R. M.; Verdoes, M.; Spalburg, E.; van Hooft, P. A. V.; de Neeling, A. J.; Noort, D.; van Boom, J. H.; van der Marel, G. A.; Overkleeft, H. S.; Overhand, M. *J. Org. Chem.* **2004**, *69*, 7851.
16. Timmer, M. S. M.; Verdoes, M.; Sliedregt, L.; van der Marel, G. A.; van Boom, J. H.; Overkleeft, H. S. *J. Org. Chem.* **2003**, *68*, 9406.
17. Jelokhani-Niaraki, M.; Prenner, E. J.; Kondejewski, L. H.; Kay, C. M.; McElhaney, R. N.; Hodges, R. S. *J. Pep. Res.* **2001**, *58*, 293.
18. Llamas-Saiz, A. L.; Grotenbreg, G. M.; Overhand, M.; van Raaij, M. J. *Acta Crystallogr.* **2007**, *D63*, 401.
19. (a) Pardi, A.; Billeter, M.; Wuthrich, K. *J. Mol. Biol.* **1984**, *180*, 741; (b) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
20. Wishart, D. S.; Kondejewski, L. H.; Semchuk, P. D.; Kay, C. M.; Hodges, R. S.; Sykes, B. D. In *Techniques in Protein Chemistry VI*; Crabb, J. W., Ed.; Academic Press: Orlando, 1995; pp 451–458.
21. Blanco, F. J.; Jimenez, M. A.; Pineda, A.; Rico, M.; Santoro, J.; Nieto, J. L. *Biochemistry* **1994**, *33*, 6004.
22. Cagas, P. M.; Corden, J. L. *Prot-Struct. Funct. Gen.* **1995**, *21*, 149.
23. Parker, J. M. R.; Guo, D.; Hodges, R. S. *Biochemistry* **1986**, *25*, 5425.
24. Guo, D. C.; Mant, C. T.; Taneja, A. L.; Parker, J. M. R.; Hodges, R. S. *J. Chromatogr.* **1986**, *359*, 499.
25. Griesinger, C.; Ernst, R. R. *J. Magn. Reson.* **1987**, *75*, 261.
26. Leslie, A. G. W.; Powell, H. R.; Winter, G.; Svensson, O.; Spruce, D.; McSweeney, S.; Love, D.; Kinder, S.; Duke, E.; Nave, C. *Acta Crystallogr.* **2002**, *D58*, 1924.
27. Leslie, A. G. W. *Acta Crystallogr.* **2006**, *D62*, 48.
28. Evans, P. *Acta Crystallogr.* **2006**, *D62*, 72.
29. Burla, M. C.; Caliandro, R.; Camalli, M.; Carrozzini, B.; Cascarano, G. L.; De Caro, L.; Giacovazzo, C.; Polidori, G.; Siliqi, D.; Spagna, R. J. *J. Appl. Crystallogr.* **2007**, 609.
30. Sheldrick, G. M. *Acta Crystallogr.* **2008**, *D64*, 112.
31. Farrugia, L. J. *J. Appl. Crystallogr.* **1999**, *32*, 837.