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Graphical Abstract

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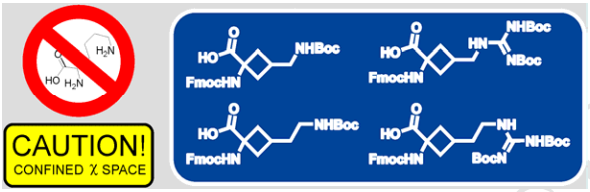

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Confining the χ space of basic natural amino acids: cyclobutane-derived χ_1,χ_2-constrained analogues of arginine, lysine and ornithine

Dmytro S. Radchenko,[a,b] Oleg M. Michurin,[a,b] Oleksandr O. Grygorenko,[*a,b] Kathi Scheinpflug,[c] Margitta Dathe,[c] Igor V. Komarov[a,b]

[a]*Kyiv National Taras Shevchenko University, Volodymyrska Street 64, Kyiv 01601, Ukraine*
[b]*Enamine Ltd., Alexandra Matrosova Street 23, Kyiv 01103, Ukraine*
[c]*Leibniz-Institut für Molekulare Pharmakologie im Forschungsverbund Berlin e.V., Robert-Roessle-Street 10, Berlin 13125,Germany*

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ABSTRACT

Four χ_1,χ_2-constrained cyclobutane-derived basic amino acids – conformationally restricted analogues of arginine, lysine and ornithine – were prepared as the derivatives properly protected for Fmoc-solid phase peptide synthesis. Compatibility of the synthesized arginine analogues with standard procedures of the Fmoc solid-phase peptide synthesis was demonstrated by incorporating these residues into the small cyclic antimicrobial peptide *c*-(RRRWFW) substituting the arginine residues. These replacements did not affect much the antimicrobial activity of the peptides.

1. Introduction

Side-chain flexibility is of fundamental importance for the function of proteins in nature. For example, conformational changes of enzymes during the catalytic cycles, antibodies on antigen recognition, or receptors during signal transduction involve long-range motions of the protein side chains, as well as the protein backbones.[1] On the other hand, binding of ligands, antigens, cofactors, or substrates by proteins decreases their conformational motions, and pre-organizations in the rigidified protein fragments might reduce the entropic cost of binding, enhancing the functional efficiency and selectivity. Therefore, subtle balance between molecular flexibility and rigidity of proteins is necessary for their overall functionality.[2] An instructive illustration is the rubisco enzyme (ribulose-1,5-bisphosphate carboxylase/oxygenase), the world's most abundant enzyme responsible for the CO_2 fixation in the biosphere.[3] The degree of the conformational motions of its Lys side chains in the active site and distant from it during the catalytic cycle and regulation of this enzyme by rubisco activase reflects the role of the residues in the corresponding reaction steps.[4]

Incorporation of conformationally restricted analogues of natural amino acids into peptides and proteins has long been a tool to probe the role of structural rigidity/flexibility in their function.[5] Such modifications might lead to an improvement in their functional characteristics which can be advantageously used in practice, for example, in medicinal chemistry.[6] While the site-directed mutagenesis of large proteins (like rubisco) by non-coded amino acids is very difficult though not impossible,[7] the same manipulation with small peptides, for example, antimicrobial, cell-penetrating, fusion peptides, and hormones is a trivial task due to the well-elaborated solid-phase peptide synthesis (SPPS) protocols.[8]

Of the natural proteinogenic amino acids, arginine (**1**) and lysine (**2**) possess the longest side chains with unrestricted rotation around the single bonds, and therefore, can be considered as the most flexible. Indeed, PDB analysis confirms that the side

*Corresponding author. Tel.: +38-044-239-33-15; fax: +38-044-502-48-32; e-mail: gregor@univ.kiev.ua

chains in these amino acid residues undergo the largest conformational changes in proteins upon ligand binding.[9] Conformationally restricted mimetics of arginine and lysine are therefore of theoretical and practical importance.[10] In this paper, we report on the design and synthesis of conformationally restricted mimetics of these amino acids, as well as their structurally related analogue, ornithine (**3**) – another natural, non-coded basic amino acid which also possess a flexible side chain.

One of the design principles leading to the conformationally restricted analogues of the amino acids with flexible side chains is insertion of the $–CH_2–$ bridges between the side chain atoms. Most of the non-natural, conformationally restricted arginine and lysine analogues described in the literature, the so-called methanologs of the amino acids, contain a cyclopropane ring.[11, 12] There is a known natural cyclopropane-derived arginine analogue, carnosadine (**4**), isolated from red algae (*Grateloupia carmosa*); chemical syntheses of carnosadine were reported.[12] Methanologs containing cyclobutane are much rarer. The cyclobutane derivatives **5** – **10** could be constructed by insertion of one or two $–CH_2–$ bridges between the every even atoms in the parent compounds **1**-**3**. The angles χ_1 and χ_2 are constrained in the molecules **5** – **7**, whereas the rigid molecules **8** – **10** are characterized by the restriction of all side chain torsion angles. To date, only the cyclobutane derivatives **5** – **7** and **9**, designed as arginine and ornithine analogues, were described in the literature.[13, 14]

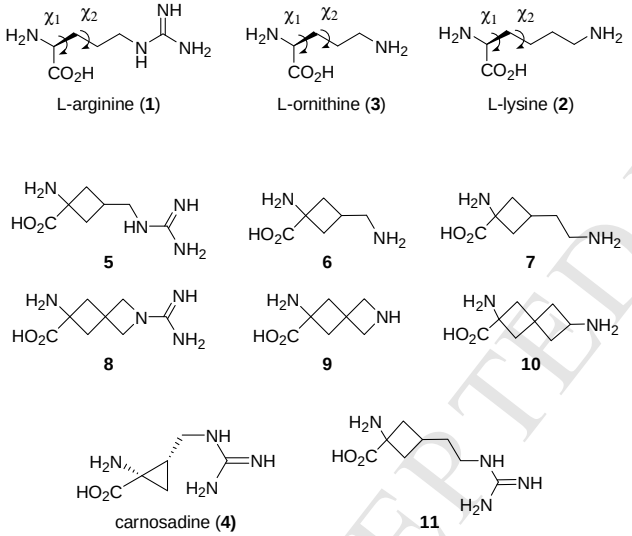


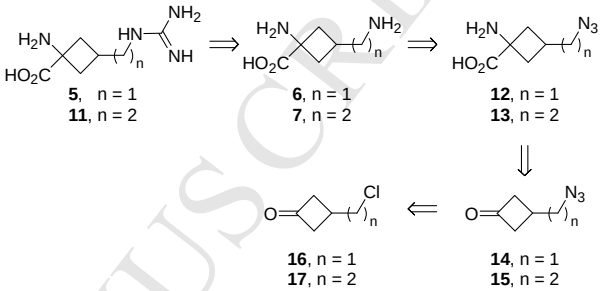
Figure 1. Basic amino acids and some of their conformationally restricted analogues

This paper describes our synthesis of the protected derivatives of the compounds **5** – **7**, and closely related extended arginine analogue **11**. The synthetic approach to **5** – **7** reported previously in the literature[13] allowed for the preparation of the unprotected amino acids. Our synthesis of the derivatives of **5** – **7** and **11** relied on well-known classical chemistry and yielded compounds with orthogonal protection of the α-amino and side chain functional groups. The Fmoc protection was chosen for the α-amino groups to ensure compatibility of the derivatives with common Fmoc SPPS procedures. To demonstrate this, residues of **5** and **11** were incorporated into a known small cyclic antimicrobial peptide *c*-WFW (*cyclo*-(RRRWFW)).[15]

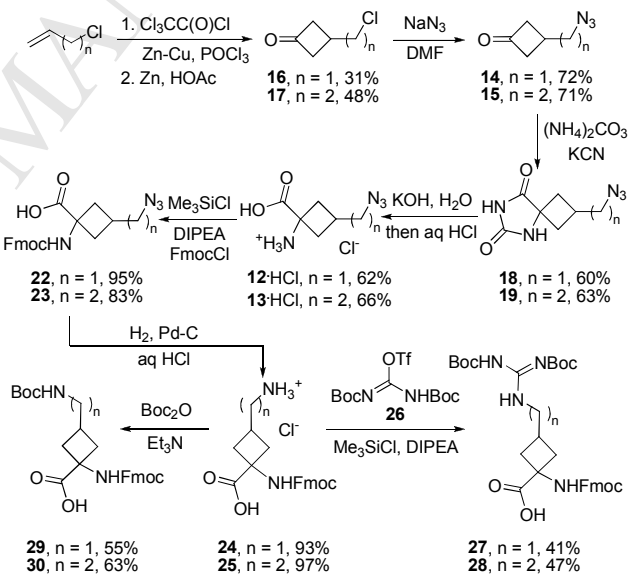
2. Results and discussion

2.1. Synthesis of protected amino acids

Our retrosynthetic analysis of the amino acids **5** – **7** and **11** (as well as their orthogonally protected derivatives) is shown in Scheme 1. Obviously, the reactive guanidine group should be introduced in the last steps of the synthesis; hence the first retrosynthetic disconnection of **5** and **11** led to the derivatives of **6** and **7**, respectively. To avoid excessive protective group manipulation, an azide was used as synthetic equivalent of the primary amino group. The azide function is stable towards many reagents and conditions and could be introduced at an early stage of the synthesis. The corresponding azides **12** and **13** could be obtained from ketones **14** and **15** using classical approaches used in amino acid synthesis, such as Strecker or Bucherer-Bergs reactions. The final step of the retrosynthetic analysis led to haloketones **16** and **17** as the starting compounds, their syntheses having been described previously.[16]



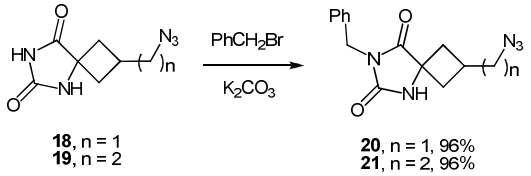
Scheme 1. Retrosynthetic analysis of amino acids **5** – **7** and **11**



Scheme 2. Synthesis of amino acid derivatives **27** – **30**

Implementation of the above retrosynthetic plan is shown in the Scheme 2. The haloketones **16** and **17** were obtained in two steps via [2+2] cycloaddition of *in situ* generated dichloroketene with allyl and homoallyl chloride, respectively.[16] Nucleophilic substitution of the chlorine atoms in **16** and **17** was achieved using sodium azide in DMF at 80 °C and led to **14** and **15** in 71–72% yields. In our hands, a modified Strecker reaction of **14**, using benzylamine, and TMSCN in methanol was unfruitful and led to a complex mixture of unidentified products. On the contrary, the Bucherer-Bergs conditions were successful and gave hydantoins **18** and **19** in good yields (60–63%). It should be noted that **18** and **19** were obtained as mixtures of diastereomers (*cis* : *trans* = 70 : 30 and 2 : 1, respectively). The relative stereo-

chemistry of **18** and **19** was established using NOESY experiments with their benzyl derivatives **20** and **21** (Scheme 3, Figures 2 and 3).



Scheme 3. Synthesis of derivatives **20** and **21**

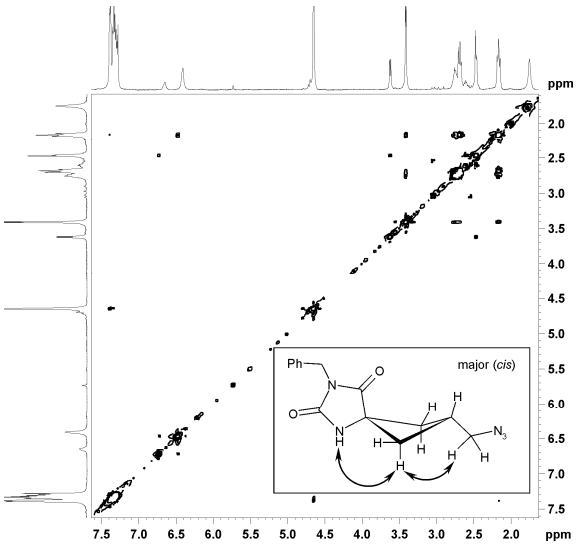


Figure 2. NOESY spectrum of **20**

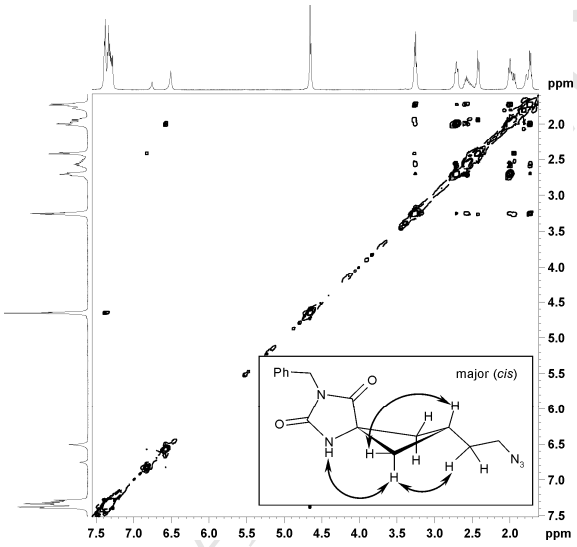


Figure 3. NOESY spectrum of **21**

Alkaline hydrolysis of **18** and **19** led to amino acids **12** and **13** which were isolated as hydrochlorides. Fmoc derivatization of **12** under classical Schotten-Baumann conditions (FmocCl, KOH) was not successful; therefore, we used another published procedure[17] relying on the use of a TMS group as dynamic protection for the carboxyl moiety. The corresponding Fmoc derivatives **22** and **23** were isolated in 83–95% yields and subjected to catalytic hydrogenation to give **24** and **25** (93–97%) which were used in the next steps without purification. In the final steps, the guanidine fragment was introduced into molecules **24** and **25** using *N,N'*-di-Boc-*N''*-triflylguanidine (**26**) as the guanidylation reagent.[18] In this case, we have also used the TMS group as dynamic protection for the carboxyl moiety to increase the reactivity of the amino acid derivatives **24** and **25**, and hence to improve the yields of the products **27** and **28** (41–47%). Boc derivatives of **24** and **25** (namely, compounds **29** and **30**) suitably protected for SPPS were also prepared from **24** and **25** in 55–63% yields.

2.2. Synthesis and biological evaluation of c-WFW analogues

To demonstrate the compatibility of the synthesized amino acid derivatives with standard Fmoc SPPS protocols, we have incorporated residues of **5** and **11** as substitute for the arginine residues in a small cyclic antimicrobial peptide *c*-WFW (*c*-(RRRWFW)). The artificial antimicrobial peptide *c*-WFW has a low molecular weight and showed high activity against bacterial cells.[15] Modification of the charged side chains in this peptide can be a tool for studying the mechanism of its antimicrobial action. Gratifyingly, the synthesized arginine derivatives **27** and **28** turned out to be fully compatible with the Fmoc SPPS protocols. Eight peptides, the *c*-WFW analogues with the residues of **5** or **11**, were synthesized using the standard procedures (Table 1).

The antimicrobial activity of the synthesized peptides was tested against Gram-negative bacteria *E. coli DH5α* and Gram-positive *Bacillus subtilis DSM 347*. The results are summarized in Table 1, together with the values of the retention time displayed by these peptides in reversed-phase HPLC (see experimental part for details).

The mode of action of most known antimicrobial peptides was suggested to be insertion into the lipid matrix and permeabilisation of the bacterial cell membrane, which finally leads to cell death.[19] Peptide charge and amphipaticity, reflected in the retention times (t_R) in reversed-phase HPLC, are important structural prerequisites of this type of action.

Table 1. Minimal inhibitory concentration (MIC) values for the peptides studied in this work, and their reversed-phase HPLC retention times (t_R).

Peptide	MIC, μM		t_R, min
	E. c. DH5α	*B. s. DSM 347*	
c-((**5**)RRWFW)	6.2	6.2	19.60, 20.12[a]
c-(R(**5**)RWFW)	6.2	3.1	21.19
c-(RR(**5**)WFW)	6.2	3.1	20.18, 20.58[a]
c-((**5**)(**5**)(**5**)WFW)	12.5	3.1	20.55
c-((**11**)RRWFW)	6.2	6.2	19.73, 20.92[a]
c-(R(**11**)RWFW)	6.2	3.1	21.19
c-(RR(**11**)WFW)	6.2	3.1	20.36, 20.63[a]
c-((**11**)(**11**)(**11**)WFW)	6.2	3.1	21.37, 21.90[a]
c-(RRRWFW) (*c*-WFW)	3.1	3.1	21.17

[a]Retention times of the two diastereomers

All peptides bearing the residues of **5** or **11** showed high antibacterial activity, which was almost identical to the activity of the parent *c*-WFW. Obviously, conformation constraints in the side chain did not influence the activity.

Previously we suggested[20] that the guanidine group plays a crucial role in the bioactivity of the cyclic hexapeptides.

However, conformational constraints in the arginine side chain seem to have minor influence. A correlation between peptide affinity towards hydrophobic surfaces (measured by t_R) and bioactivity is not obvious. These results support the previous hypothesis, that membrane permeabilisation is not the preferred mode of action of this RW-rich cyclic antimicrobial peptide.[21]

3. Experimental part

3.1. General

Solvents were purified according to the standard procedures. Compounds **16**,[16a] **17**,[16b] and **26**[18] were prepared using the procedures reported in the literature. All starting materials were purchased from Acros, Merck, Fluka, and UORSY. Analytical TLC was performed using Polychrom SI F254 plates. Column chromatography was performed using Kieselgel Merck 60 (230–400 mesh) as the stationary phase. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 170 Avance 500 spectrometer (at 500 MHz for Protons and 125 MHz for Carbon-13). Chemical shifts are reported in ppm downfield from TMS (^{1}H, ^{13}C) as internal standard. For the compounds obtained as the mixtures of *cis*- and *trans*-isomers, as well for those existing as mixtures of rotamers (*i. e.* containing Boc or Fmoc protective group), fractional integral intensities were reported where appropriate. IR spectra were obtained on Perkin Elmer BX II FT-IR spectrometer. ν_{max} (cm^{-1}) values in IR spectra are given for the main absorption bands. Elemental analyses were performed at the Laboratory of Organic Analysis, Department of Chemistry, Kyiv National Taras Shevchenko University. Mass spectra were recorded on an Agilent 1100 LCMSD SL instrument (chemical ionization (APCI), electrospray ionization (ESI)) and Agilent 5890 Series II 5972 GCMS instrument (electron impact ionization (EI)).

*3.2. 3-(Azidomethyl)cyclobutanone (**14**).*

Ketone **16** (8.49 g, 0.072 mol) and NaN_3 (13.9 g, 0.214 mol) were stirred in DMF (50 mL) at 80 °C for 24 h. The reaction mixture was cooled, diluted with H_2O (200 mL), and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with H_2O (2 × 100 mL) and brine (100 mL), dried over $MgSO_4$ and evaporated *in vacuo*. The product **14** (6.53 g, 72%) was pure enough to be used in the next step without additional purification. An analytical sample of **14** was prepared by distillation *in vacuo*. Colorless liquid. Bp 70–74 °C / 5 mmHg. Anal. Calcd for $C_5H_7N_3O$ C 47.99, H 5.64, N 33.58. Found C 47.62, H 5.87, N 33.21. ^{1}H NMR ($CDCl_3$), δ 2.68 (1H, sept, J = 6.6 Hz), 2.82–2.91 (2H, m), 3.13–3.23 (2H, m), 3.52 (2H, d, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$), δ 23.4 (*C*H), 50.5 (*C*H$_2$), 55.1 (*C*H$_2$), 205.4 (*C*=O).

*3.3. 3-(2-Azidoethyl)cyclobutanone (**15**).*

Compound **15** was prepared from ketone **17** analogously to **14**. Yield 11.4 g (71%). Colorless liquid. Bp 76–80 °C / 5 mmHg. Anal. Calcd for $C_6H_9N_3O$ C 51.79, H 6.52, N 30.20. Found C 51.83, H 6.35, N 30.57. MS (ESI, *m/z*): 140 (MH^+), 112 ($MH^+ - N_2$). ^{1}H NMR ($CDCl_3$), δ 1.89 (2H, q, J = 6.6 Hz), 2.49 (1H, sept, J = 7.3 Hz), 2.72–2.77 (2H, m), 3.18–3.23 (2H, m), 3.35 (2H, t, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$), δ 21.6 (*C*H), 35.1 (*C*H$_2$), 50.3 (*C*H$_2$), 52.5 (*C*H$_2$), 206.8 (*C*=O).

*3.4. 2-(Azidomethyl)-5,7-diazaspiro[3.4]octan-6,8-dione (**18**).*

Compound **14** (5.00 g, 36.0 mmol), KCN (3.90 g, 60.0 mol), and $(NH_4)_2CO_3$ (15.4 g, 0.160 mol) were stirred in H_2O (30 mL) and EtOH (30 mL) at 60 °C for 24 h. The reaction mixture was cooled and neutralized with 1 M aq HCl. The precipitate was filtered to give **18** (4.68 g, 60%, *cis* : *trans* = 70 : 30). Yellowish solid. Mp 196–198 °C (EtOH – Hexanes). Anal. Calcd for $C_7H_9N_5O_2$ C 43.08, H 4.65, N 35.88. Found C 42.75, H 4.61, N 35.59. IR (cm^{-1}): 3438, 3177, 2096, 1782, 1736. MS (CI, *m/z*): 196 (MH^+). ^{1}H NMR (CF_3COOD), δ, *cis*-isomer (major) 2.39–2.52 (2H, m), 2.70–2.92 (3H, m), 3.49 (2H, d, J = 5.6 Hz); *trans*-isomer (minor) 2.52–2.62 (2H, dd, J = 13.7 Hz and 6.3 Hz), 2.70–2.92 (3H, m), 3.49 (2H, d, J = 7.6 Hz). ^{13}C NMR (CF_3COOD), δ, *cis*-isomer (major) 26.2 (*C*H), 35.2 (*C*H$_2$), 55.1 (*C*H$_2$), 60.2 (*C*), 158.9 (*C*=O), 180.7 (*C*=O); *trans*-isomer (minor) 26.0 (*C*H), 34.3 (*C*H$_2$), 54.4 (*C*H$_2$), 61.4 (*C*), 158.9 (*C*=O), 179.8 (*C*=O).

*3.5. 2-(2-Azidoethyl)-5,7-diazaspiro[3.4]octan-6,8-dione (**19**).*

Compound **19** was prepared from **15** analogously to **18**. Yield 9.48 g (63%, *cis* : *trans* = 2 : 1). Yellowish solid. Mp 196–201 °C (EtOH–Hexanes). Anal. Calcd for $C_8H_{11}N_5O_2$ C 45.93, H 5.30, N 33.48. Found C 45.62, H 5.07, N 33.60. IR (cm^{-1}): 3432, 3184, 2100, 1779, 1733. MS (CI, *m/z*): 210 (MH^+), 182 ($MH^+ - N_2$). ^{1}H NMR (CF_3COOD), δ, 1.98–2.08 (1.4H, m), 2.10–2.19 (0.6H, m), 2.46–2.55 (1.4H, m), 2.63–2.71 (0.6H, m), 2.73–2.89 (1.6H, m), 2.90–2.98 (1.4H, m), 3.45–3.52 (2H, m). ^{13}C NMR (CF_3COOD), δ, *cis*-isomer (major) 24.6 (*C*H), 34.5 (*C*H$_2$), 37.4 (*C*H$_2$), 49.1 (*C*H$_2$), 60.4 (*C*), 158.8 (*C*=O), 180.9 (*C*=O); *trans*-isomer (minor) 24.8 (*C*H), 33.0 (*C*H$_2$), 36.1 (*C*H$_2$), 49.1 (*C*H$_2$), 61.8 (*C*), 158.8 (*C*=O), 179.6 (*C*=O).

*3.6. 2-(Azidomethyl)-7-benzyl-5,7-diazaspiro[3.4]octan-6,8-dione (**20**).*

DMF (0.5 mL), benzyl bromide (44 mg, 0.26 mmol), and K_2CO_3 (37 mg, 0.27 mmol) were added to **18** (50 mg, 0.26 mmol). The mixture was stirred at rt for 18 h and then partitioned between H_2O (2 mL) and EtOAc (2 mL). The organic phase was separated, and the aqueous layer was extracted with EtOAc (2 × 1 mL). The combined organic phases were washed with water (2 × 3 mL), brine (1 × 3 mL), dried over $MgSO_4$, and evaporated *in vacuo* to give **20**. Yield 70 mg (96%, *cis* : *trans* = 70 : 30). White solid. Mp 108–111 °C. Anal. Calcd for $C_{14}H_{15}N_5O_2$ C 58.94, H 5.30, N 24.55. Found C 59.28, H 5.09, N 24.74. IR (cm^{-1}): 3236, 2100, 1780, 1725, 1704. MS (CI, *m/z*): 286 (MH^+), 258 ($MH^+ - N_2$). ^{1}H NMR ($CDCl_3$), δ, *cis*-isomer (major) 2.15 (t, J = 10.0 Hz, 2H, 1- and 3-C*H*H), 2.67 (t, J = 10.0 Hz, 2H, 1- and 3-CH*H*), 2.70–2.77 (m, 1H, 2-C*H*), 3.39 (d, J = 4.9 Hz, 2H, C*H*$_2$N$_3$), 4.63 (s, 2H, C*H*$_2$C$_6$H$_5$), 6.39 (s, 1H, N*H*), 7.26–7.33 (m, 3H), 7.37 (d, J = 7.3 Hz, 2H, 2′-CH of C_6H_5); *trans*-isomer (minor) 2.43–2.47 (m, 4H, 1- and 3-C*H*$_2$), 2.55–2.63 (m, 1H, 2-C*H*), 3.60 (d, J = 8.0 Hz, 2H, C*H*$_2$N$_3$), 4.63 (s, 2H, C*H*$_2$C$_6$H$_5$), 6.63 (s, 1H, N*H*), 7.26–7.33 (m, 3H), 7.37 (d, J = 7.3 Hz, 2H, 2′-CH of C_6H_5). ^{13}C NMR ($CDCl_3$), δ, *cis*-isomer (major) 26.6 (2-*C*H), 36.1 (1- and 3-*C*H$_2$), 42.3 (*C*H$_2$C$_6$H$_5$), 55.2 (*C*H$_2$N$_3$), 57.5 (4-*C*), 128.0 (*C*H), 128.6 (*C*H), 128.8 (*C*H), 136.1 (1′-*C* of C_6H_5), 156.2 (*C*=O of Boc), 176.3 (*C*OOH); *trans*-isomer (minor) 26.6 (2-*C*H), 35.6 (1- and 3-*C*H$_2$), 42.5 (*C*H$_2$C$_6$H$_5$), 54.5 (*C*H$_2$N$_3$), 58.7 (4-*C*), 128.1 (*C*H), 128.6 (*C*H), 128.8 (*C*H), 136.0 (1′-*C* of C_6H_5), 156.5 (*C*=O of Boc), 175.7 (*C*OOH).

*3.7. 2-(2-Azidoethyl)-7-benzyl-5,7-diazaspiro[3.4]octan-6,8-dione (**21**).*

Compound **21** was prepared from **19** analogously to **20**. Yield 138 mg (96%, *cis* : *trans* = 2 : 1). White solid. Mp 106–109 °C. Anal. Calcd for $C_{15}H_{17}N_5O_2$ C 60.19, H 5.72, N 23.40. Found C 60.46, H 5.55, N 23.18. MS (CI, *m/z*): 300 (MH^+), 272 ($MH^+ - N_2$). ^{1}H NMR ($CDCl_3$), δ, *cis*-isomer (major) 1.64 (q, J = 7.0 Hz, 2H, C*H*$_2$CH$_2$N$_3$), 1.90 (td, J = 9.0 Hz and 2.6 Hz, 2H, 1- and 3-C*H*H) 2.49 (sept, J = 8.4 Hz, 1H, 2-C*H*), 2.62 (td, J = 9.0 Hz and

2.5 Hz, 2H, 1- and 3-CH*H*), 3.16 (t, *J* = 6.7 Hz, 2H, $CH_2CH_2N_3$), 4.57 (s, 2H, $CH_2C_6H_5$), 6.42 (s, 1H, 5-N*H*), 7.19–7.26 (m, 3H), 7.29 (d, *J* = 6.7 Hz, 2H, 2′-CH of C_6H_5); *trans*-isomer (minor) 1.85 (q, *J* = 6.8 Hz, 2H, $CH_2CH_2N_3$), 2.33 (d, *J* = 8.2 Hz, 4H, 1- and 3-C*H*), 2.44 (sept, *J* = 8.2 Hz, 1H, 2-C*H*), 3.17 (t, *J* = 6.7 Hz, 2H, $CH_2CH_2N_3$), 4.56 (s, 2H, $CH_2C_6H_5$), 6.67 (s, 1H, 5-N*H*), 7.19–7.26 (m, 3H), 7.29 (d, *J* = 6.7 Hz, 2H, 2′-CH of C_6H_5). ^{13}C NMR ($CDCl_3$), *δ*, *cis*-isomer (major) 24.0 (2-*C*H), 34.7 ($CH_2CH_2N_3$), 38.2 (1- and 3-CH_2), 41.1 ($CH_2C_6H_5$), 48.5 ($CH_2CH_2N_3$), 56.7 (4-*C*), 126.9 (*C*H of C_6H_5), 127.4 (*C*H of C_6H_5), 127.7 (*C*H of C_6H_5), 135.02 (1′-*C* of C_6H_5), 155.6 (*C*=O of Boc), 175.5 (*C*OOH); *trans*-isomer (minor) 23.97 (2-*C*H), 32.7 ($CH_2CH_2N_3$), 36.2 (1- and 3-CH_2), 41.2 ($CH_2C_6H_5$), 48.5 ($CH_2CH_2N_3$), 58.0 (4-*C*), 126.9 (*C*H of C_6H_5), 127.42 (*C*H of C_6H_5), 127.7 (*C*H of C_6H_5), 134.97 (1′-*C* of C_6H_5), 155.6 (*C*=O of Boc), 174.5 (*C*OOH).

*3.8. 1-Amino-3-(azidomethyl)cyclobutanecarboxylic acid, hydrochloride (**12**·HCl).*

Compound **18** (7.00 g, 35.9 mol) was dissolved in a solution of KOH (12.0 g, 0.216 mol) in H_2O (70 mL). The mixture was refluxed for 48 h, then cooled, acidified with 6 M aq HCl to pH = 3 and evaporated to dryness. The residue was dissolved in H_2O (100 mL), and the solution was evaporated to dryness again. The residue was extracted with EtOH (3 × 150 mL). Combined filtrates were evaporated to dryness to give the hydrochloride of **12** (4.6 g, 62%, *cis* : *trans* = 70 : 30). Yellowish solid. Mp 153–157 °C (EtOH–Et_2O). Anal. Calcd for $C_6H_{11}ClN_4O_2$ C 34.88, H 5.37, Cl 17.16, N 27.11. Found C 35.03, H 5.26, Cl 17.24, N 27.40. IR (cm^{-1}): 3403, 2998, 2095, 1742. MS (CI, *m/z*): 171 (MH^+). ^{1}H NMR (D_2O), *δ*, *cis*-isomer (major) 2.06–2.11 (2H, m), 2.52–2.57 (2H, m), 2.68–2.77 (1H, m), 3.37 (2H, d, *J* = 6.1 Hz); *trans*-isomer (minor) 2.29–2.34 (2H, m), 2.38–2.43 (2H, m), 2.68–2.77 (1H, m), 3.44 (2H, d, *J* = 7.1 Hz). ^{13}C NMR (D_2O), *δ*, *cis*-isomer (major) *δ* 27.6 (*C*H), 33.8 (CH_2), 54.8 (CH_2), 55.4 (*C*), 176.8 (*C*=O); *trans*-isomer (minor) 27.0 (*C*H), 32.9 (CH_2), 55.0 (CH_2), 57.2 (*C*), 176.3 (*C*=O).

*3.9. 1-Amino-3-(2-azidoethyl)cyclobutanecarboxylic acid, hydrochloride (**13**·HCl).*

Compound **13**·HCl was prepared from **19** analogously to **12**·HCl. Yield 1.39 g (66%, *cis* : *trans* = 2 : 1). Yellowish solid. Mp 164–168 °C (EtOH–Et_2O). Anal. Calcd for $C_7H_{13}ClN_4O_2$ C 38.10, H 5.94, Cl 16.07, N 25.39. Found C 37.86, H 6.27, Cl 16.31, N 25.35. IR (cm^{-1}): 3397, 2970, 2091, 1744. MS (CI, *m/z*): 185 (MH^+), 140 (MH^+–CO_2H). ^{1}H NMR (D_2O), *δ* 1.71 (1.3H, dd, *J* = 7.3 Hz and 6.9 Hz), 1.77 (0.7H, dd, *J* = 7.3 Hz and 6.9 Hz), 1.95–2.02 (1.3H, m), 2.28–2.36 (1.3H, m), 2.49 (0.7H, sept, *J* = 7.8 Hz), 2.55–2.62 (1.7H, m), 3.22 (d, *J* = 6.7 Hz, 0.7H), 3.24 (d, *J* = 6.7 Hz, 1.3H). ^{13}C NMR (D_2O), *δ*, *cis*-isomer (major) 25.8, 34.4, 35.8, 49.1, 54.0, 174.2; *trans*-isomer (minor) 25.3, 33.7, 34.9, 49.1, 55.8, 173.5.

*3.10. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(azidomethyl)cyclobutanecarboxylic acid (**22**).*

To a suspension of **12**·HCl (1.00 g, 4.84 mmol) in $CHCl_3$ (20 mL), Me_3SiCl (1.79 mL, 14.5 mmol) was added dropwise, followed by DIPEA (4.20 mL, 24.2 mmol). The resulting mixture was stirred for 30 min. To the clear solution formed, FmocCl (1.37 g, 4.8 mmol) was added, and the mixture was stirred at rt for 3 h. The solvent was removed *in vacuo*, saturated aq $NaHCO_3$ (50 mL) was added, and the mixture was washed with Et_2O (3 × 50 mL). The organic phases were washed with H_2O (100 mL). Combined aqueous phases were acidified to pH = 5 with 2 M HCl and extracted with EtOAc (3 × 50 mL). Combined organic extracts were dried over $MgSO_4$ and evaporated to dryness to give **22** (1.85 g, 95%, *cis* : *trans* = 70 : 30) which was pure enough to be used in the next step without additional purification. Yellowish solid. Mp 137–140 °C. Anal. Calcd for $C_{21}H_{20}N_4O_4$ C 64.28, H 5.14, N 14.28. Found C 64.01, H 5.50, N 14.49. MS (CI, *m/z*): 393 (MH^+), 365 ($MH^+ - N_2$). ^{1}H NMR ($CDCl_3$), *δ* 1.74 (br s, 0.2H), 1.97 (br s, 0.1H), 2.18 (br s, 0.2H), 2.29–2.60 (br m, 2.5H), 2.76 (br s, 2H), 3.14–3.43 (br m, 0.6H), 3.43 (br s, 1.4H), 4.16 and 4.23 (two br s, 1H), 4.43 (br s, 1.5H), 4.63 (br s, 0.5H), 5.55 (br s, 0.2H), 5.66 (br s, 0.5H), 6.28 (br s, 0.2H), 6.37 (br s, 0.1H), 7.32 (br s, 2H), 7.41 (br s, 2H), 7.53 (br s, 0.5H), 7.63 (br s, 1.5H), 7.78 (br d, *J* = 6.0 Hz, 2H). The COOH proton is not observed due to exchange with HDO. ^{13}C NMR ($CDCl_3$), *δ* 27.8 and 28.4, 34.4 and 35.0, 47.3, 54.9 and 55.4, 55.9, 67.0, 120.1, 124.6 and 125.1, 127.2, 127.8, 141.5, 143.8, 155.4, 178.4.

*3.11. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(2-azidoethyl)cyclobutanecarboxylic acid (**23**).*

Compound **23** was prepared from **13**·HCl analogously to **22**. Yield 4.53 g (83%, *cis* : *trans* = 2 : 1). Yellowish solid. Mp 142–144 °C. Anal. Calcd for $C_{22}H_{22}N_4O_4$ C 65.01, H 5.46, N 13.78. Found C 65.36, H 5.23, N 13.90. IR (cm^{-1}): 3368, 3306, 3262, 2098, 1718, 1697, 1675. MS (CI, *m/z*): 407 (MH^+), 379 ($MH^+ - N_2$). ^{1}H NMR ($CDCl_3$), *δ* 1.41–1.69 (br m, 0.5H), 1.72 (br s, 1.5H), 1.86 (br s, 0.2H), 1.98 (br s, 0.3H), 2.14 (br s, 1H), 2.32 (br s, 1.5H), 2.43 (br s, 0.6H), 2.53 (br s, 0.4H), 2.70 (br s, 1H), 2.99 (br s, 0.5H), 3.14 (1.5H), 4.05 (br s, 0.3H), 4.13 (br s, 0.7H), 4.32 (br s, 1.5H), 4.51 (br s, 0.5H), 5.38 (br s, 0.2H), 5.52 (br s, 0.5H), 6.16 (br s, 0.2H), 6.23 (br s, 0.1H), 7.19–7.24 (m, 2H), 7.27–7.32 (m, 2H), 7.44 (br s, 0.5H), 7.50 (br s, 1.5H), 7.66 (br d, *J* = 6.5 Hz, 2H), 8.91 (br s, 1H). ^{13}C NMR ($CDCl_3$), *δ* 26.0 and 26.9, 34.8 and 35.9, 36.3 and 37.4, 47.3, 49.5, 55.1 and 56.0, 67.0 and 67.1, 120.1, 124.6 and 125.2, 127.2, 127.8, 141.5, 143.8, 155.4, 177.9 and 178.8.

*3.12. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(aminomethyl)cyclobutanecarboxylic acid, hydrochloride(**24**).*

To a solution of **22** (3.20 g, 8.16 mmol) in 2-propanol (80 mL), 2 M aq HCl (16 mL) and 5% Pd-C (0.320 g) were added. A slow stream of hydrogen was passed through the mixture upon stirring for 2 h. The catalyst was filtered off, and the filtrate was evaporated *in vacuo*. Benzene (100 mL) was added and evaporated *in vacuo* to dryness to remove HCl. The residue was dried *in vacuo* over P_2O_5 to give **24** (3.10 g, 93%), which was used in the next step without any purification. MS (CI, *m/z*): 367 (MH^+).

*3.13. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(2-aminoethyl)cyclobutanecarboxylic acid, hydrochloride (**25**).*

Compound **25** was prepared from **23** analogously to **24** and used in the next step without purification. Yield 3.50 g (97%). MS (CI, *m/z*): 381 (MH^+).

*3.14. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-((2,3-bis(tert-butoxycarbonyl)guanidino)methyl)cyclobutanecarboxylic acid (**27**).*

To a solution of **24** (2.90 g, 7.20 mmol) in $CHCl_3$ (58 mL), Me_3SiCl (2.66 mL, 21.6 mmol) was added dropwise, followed by DIPEA (6.88 mL, 39.6 mmol). The resulting mixture was stirred for 30 min. To the clear solution formed, *N*,*N*′-di-Boc-*N*″-triflylguanidine (**26**) (2.81 g, 7.18 mmol) was added. The resulting mixture was stirred for 3 h, then washes with 10% aq citric acid (2 × 25 mL), brine (25 mL), dried over Na_2SO_4 and evaporated *in vacuo*. The residue was purified by column

chromatography (CH_2Cl_2 – MeOH (20 : 1) as an eluent) to give **27** (1.81 g, 41%, *cis* : *trans* = 70 : 30). White solid. Mp 115–117 °C. Anal. Calcd for $C_{32}H_{40}N_4O_8$ C 63.14, H 6.62, N 9.20. Found C 63.07, H 6.93, N 8.86. IR (cm^{-1}): 3409, 3330, 3288, 1727, 1643, 1616. MS (ESI, *m/z*): 609 (MH^+), 553 ($MH^+ – C_4H_8$). ^1H NMR ($CDCl_3$), δ 1.50 (18H, s), 2.20–2.26 (1H, m), 2.37–2.56 (1H, br m), 2.69 (0.7H, br s), 2.76–2.83 (1.3H, br s), 3.34–3.62 (2H, m), 2.23 (1H, m), 4.20–4.25 (1H, m), 4.38–4.44 (2H, m), 4.62 (1H, br s), 5.71 (0.3H, br s), 6.03 (0.7H, br s), 7.31 (2H, t, J = 7.6 Hz), 7.40 (2H, t, J = 7.3 Hz), 7.61 (2H, m), 7.76 (2H, d, J = 7.3 Hz), 8.46 (1H, br s), 11.50 (1H, br s). ^{13}C NMR ($CDCl_3$), δ 28.2, 28.4, 31.2 and 31.3, 34.6 and 35.0, 46.1, 47.2, 55.4 and 56.1, 67.0, 79.6, 83.4, 120.0, 125.3, 127.2, 127.8, 141.4, 143.9, 153.3, 155.8, 156.6, 163.4, 177.6.

*3.15. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(2-(2,3-bis(tert-butoxycarbonyl)guanidino)ethyl)cyclobutanecarboxylic acid (**28**).*

Compound **28** was prepared from **25** analogously to **27**. Yield 1.4 g (47%, *cis* : *trans* = 2 : 1). White solid. Mp 124–127 °C. Anal. Calcd for $C_{33}H_{42}N_4O_8$ C 63.65, H 6.80, N 9.00. Found C 63.20, H 7.12, N 8.77. IR (cm^{-1}): 3405, 3333, 3290, 1721, 1640, 1619. MS (ESI, *m/z*): 623 (MH^+), 523 ($MH^+ – C_4H_8 – CO_2$). ^1H NMR ($CDCl_3$), δ 1.48 (18H, s), 1.72–1.89 (2H, br m), 2.28–2.49 (3H, br m), 2.57 (br s, 0.5H), 2.63–2.75 (1.5H, m), 3.20–3.38 (2H, br m), 4.21 (1H, br s), 4.34–4.49 (2H, br m), 4.58 (1H, br s), 5.42 (0.3H, br s), 5.81 (0.7H, br s), 7,31 (2H, t, J = 7.6 Hz), 7.40 (2H, t, J = 7.3 Hz), 7.61 (2H, m), 7.76 (2H, d, J =7.3 Hz), 8.29 (0.3H, br s), 8.41 (0.7H, br s), 11.52 (1H, br s). ^{13}C NMR ($CDCl_3$), δ 25.9, 28.2, 28.3 and 28.4, 35.2 and 36.2, 36.5 and 37.0, 39.1 and 39.5, 47.3, 55.2 and 56.2, 66.7 and 67.0, 79.5 and 79.8, 83.3 and 83.4, 120.0, 125.3, 127.2, 127.8, 141.4, 144.0, 153.3 and 153.4, 155.4, 156.3, 163.0 and 163.4, 177.0.

*3.16. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-((tert-butoxycarbonylamino)methyl)cyclobutanecarboxylic acid (**29**).*

To a suspension of **24** (0.640 g, 1.59 mmol) in CH_2Cl_2 (13 mL), ethyl diisopropylamine (0.830 mL, 4.77 mmol) was added dropwise, followed by Boc_2O (0.523 g, 2.40 mmol). The resulting mixture was stirred for 12 h. The solvent was removed *in vacuo*, and saturated aq $NaHCO_3$ (50 mL) was added to the residue. The mixture was washed with Et_2O (3 × 50 mL). The organic phases were washed with H_2O (60 mL). The combined aqueous phases were acidified to pH = 5 with 1 M HCl and extracted with EtOAc (3 × 30 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by column chromatography (CH_2Cl_2 – MeOH (15 : 1) as an eluent) to give **29**. Yield 0.411 g (55%, *cis* : *trans* = 70 : 30). White solid. Mp 80–84 °C. Anal. Calcd for $C_{26}H_{30}N_2O_6$ C 66.94, H 6.48, N 6.00. Found C 66.73, H 6.71, N 6.28. IR (cm^{-1}): 3403, 3335, 1709. MS (ESI, *m/z*): 489 (MNa^+), 367 ($MH^+ – C_4H_8 – CO_2$). MS (CI, *m/z*, negative ion scan): 465 ($M–H^+$). ^1H NMR ($CDCl_3$), δ 1.48 (s, 9H), 2.22–2.55 (br m, 3H), 2.60–2.75 (br m, 2H), 3.04–3.32 (br m, 2H), 4.12–4.25 (br m, 1H), 4.38 (br s, 2H), 4.54 (br s, 0.5H), 4.76 (br s, 0.1H), 4.95 (br s, 0.1H), 5.15 (br s, 0.3H), 5.98–6.60 (br m, 1H), 7.30 (t, J = 6.5 Hz, 2H), 7.38 (t, J = 6.5 Hz, 2H), 7.53–7.65 (br m, 2H), 7.74 (d, J = 6.5 Hz, 2H), 10.46 (br s, 1H). ^{13}C NMR ($CDCl_3$), δ 28.0, 28.4, 34.1 and 34.7 and 35.6, 44.9 and 45.4 and 46.2, 47.1, 54.6 and 55.4, 66.8, 79.4 and 81.0, 119.9, 124.8 and 125.2, 127.1, 127.7, 141.2, 143.8, 155.4 and 155.9 and 156.4, 156.5 and 158.1, 177.0 and 177.6.

*3.17. 1-(((9H-Fluoren-9-yl)methoxy)carbonylamino)-3-(2-(tert-butoxycarbonylamino)ethyl)cyclobutanecarboxylic acid (**30**).*

Compound **30** was prepared from **25** analogously to **29**. Yield 0.385 g (63%, *cis* : *trans* = 2 : 1). White solid. Mp 78–80 °C. Anal. Calcd for $C_{27}H_{32}N_2O_6$ C 67.48, H 6.71, N 5.83. Found C 67.39, H 6.93, N 6.15. IR (cm^{-1}): 3406, 3337, 1708. MS (CI, *m/z*): 503 (MNa^+), 381 ($MH^+ – C_4H_8 – CO_2$). ^1H NMR ($CDCl_3$), δ 1.49 (s, 9H), 1.69 (br s, 2H), 1.96–2.58 (br m, 4H), 2.76 (br s, 1H), 3.06 (br s, 2H), 4.21 (br s, 1H), 4.37 (br s, 2H), 4.51 (br s, 0.4H), 4.64 (br s, 0.6H), 5.78–6.28 (br m, 1H), 7.30 (t, J = 6.9 Hz, 2H), 7.38 (t, J = 6.9 Hz, 2H), 7.60 (d, J = 6.9 Hz, 2H), 7.75 (d, J = 6.9 Hz, 2H), 10.00 (br s, 1H). ^{13}C NMR ($CDCl_3$), δ 26.0 and 27.0, 28.5, 36.1 and 36.4, 37.2 and 37.4, 38.6 and 39.5, 47.2, 55.1 and 56.1, 66.8 and 66.9, 79.4 and 80.9, 120.0, 124.9 and 125.2, 127.1, 127.7, 141.4, 143.9, 155.4 and 156.0, 156.2 and 158.0, 176.9 and 177.7.

3.18. Synthesis and determination of the minimal inhibitory concentration (MIC) of the peptides

Peptides were prepared manually or using automatic peptide synthesizer by solid-phase peptide synthesis (SPPS) using the standard Fmoc/*tert*-butyl (*t*-Bu) protocol.[8] Commercially avaiable Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH and Fmoc-Phe-OH were used for incorporation of amino acid residues into peptides. 2-Chlorotrityl chloride resin was used. The first Fmoc-protected amino acid (0.3 mmol per 1 g of resin) was introduced by treatment with ethyl diisopropylamine (0.6 mmol) in dry CH_2Cl_2 (10 mL) for 1 h, followed by capping of the resin with excess of ethyl diisopropylamine and MeOH. Further Fmoc-protected amino acids (4 eq) were activated in DMF by a mixture of *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate (HBTU, 3.9 eq) and ethyl diisopropylamine (DIPEA, 8 eq) before coupling. A solution of piperidine (20% in DMF) was used for Fmoc-deprotection (2 × 15 min). Cleavage of linear peptide was done in a TFA/TIS cleavage cocktail (95:5 v/v) at ambient temperature (3 h). The volatile products were removed *in vacuo* and the residue was triturated with ether, and the precipitate was subjected to centrifugation. The solvent was removed by decantation; the residues were dried on air and then lyophilized. Head-to-tail cyclization of linear precusor peptides (0.1% solution in dry DMF) was performed using PyBOP (0.3 mmol) and DIEA(0.6 mmol). Cyclic peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and then lyophilized.

Chromatographic characterization was performed on a Jasco HPLC system (Japan) using a diode array detector operating at 220 nm. Runs were carried out on a PolyEncap A 300 column (250 mm × 4.0 mm; Bischoff Analysentechnik, Germany). The sample concentration was 1 mg of peptide/ml in eluent A. The mobile phase A was 0.1% trifluoroacetic acid (TFA) in H_2O, and phase B was 0.1% TFA in 80% MeCN – 20% H_2O (v/v). The retention time (t_R) of the peptides was determined at rt using a linear gradient of 5 to 95% phase B during 40 min.

The MIC against Gram-positive *B. subtilis* (strain DSM 347) and Gram-negative *E. coli* (strain DH 5α) was determined in 96-well microtiter plates.[21] Cells were cultivated in lysogeny broth (LB) (Sigma-Aldrich, Germany). The inoculum was prepared from mid-logarithmic-phase cultures (OD_{600} = 0.4). The OD_{600} = 1.0 for *B. subtilis* and *E. coli* corresponds to 8.8×10^7 cells/mL and 2.2×10^8 cells/mL, respectively. Aliquots of the cell suspensions were added to the peptide-containing wells of a microtiter plate. The final peptide concentrations ranged between 100 μM and 0.1 μM in 2-fold dilution. The final number of bacterial cells per well was 5×10^5 cfu / 200 μL. Peptide concentrations were tested in triplicate. The test plates were incubated at 37 °C for 17 h whilst shaking at 180 rpm. The absorbance was read at 600 nm (Safire Microplate Reader;

Tecan, Germany). The MIC is defined as the lowest concentration of peptide at which there was no change in optical density.

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