A Practical Large-Scale Synthesis of Cyclic RGD Pentapeptides Suitable for Further Functionalization through 'Click' Chemistry

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Abstract: A multigram batch of the *cyclo*[Arg-Gly-Asp-D-Phe-Lys] and its *N*-ε-azido derivative was accomplished via solution-phase synthesis using an epimerization-free fragment condensation. The *C*-terminus of D-Phe was protected as its *tert*-butyl ester. Fmoc (Arg, Gly, Asp, D-Phe) and Boc (Lys) groups were used to protect all *N*-α-termini. The Ts and NO_2 groups, respectively were chosen to protect the guanidine group. The macrocyclization step (between D-Phe and L-Lys) was carried out under TBTU/HOBt or DPPA condensation conditions. Finally, the ε-amino group of the lysine residue was selectively converted into the azido group by a diazo-transfer reaction.

Key words: RGD-peptides, solution-phase synthesis, amino acids, cyclizations, diazo compounds

Cyclic pentapeptides containing the RGD (Arg-Gly-Asp) motif were developed as highly active and selective antagonists for the $\alpha_v\beta_3$ integrin receptor.[1] A class of this heterodimeric transmembrane protein[2] exerts an important role in cell signaling and cell–cell and cell–matrix interactions and recognitions.[3] The *cyclo*-RGDfK,[4] its methylated analogues[5] and other related cyclic RGD-peptides[6] were designed, synthesized and frequently tested for their crucial location in tumor angiogenesis and metastasis,[3,7] as well as for the stimulation of cell adhesion.[6b,8]

Cyclic RGD pentapeptides mentioned above have typically been prepared by solid-phase synthesis.[4–6] Commonly, the linear-protected pentapeptide was prepared first, followed by cleavage from the polymeric resin. Cyclization and removal of the protecting groups finalized the synthesis according to the original or improved protocols of Kessler et al.[4a]

Importantly, the ε-amino lysine (K) moiety of the *c*RGDfK peptide can be readily modified and used for further functionalization by means of a 1,3-dipolar cycloaddition ('click' chemistry)[9] between alkynes and organic azides to afford the corresponding 1,4-disubstituted 1,2,3-triazoles. In many examples the method was applied in the synthesis of glycoconjugates, oligosaccharides, and glycopeptides.[10]

N-ε-Azido derivative of cyclic RGD peptides[4e,6e,11] were recently employed in the 'click' reaction with dendrimeric alkynes[4e,11] or under metal-free conditions to afford CF_3-triazole formation (tandem cycloaddition–retro-Diels–Alder reaction).[11]

In view of the importance of *cyclo*-RGDfK, there is a quest to develop a synthesis which can easily be upscaled, particularly as solid-phase peptide synthesis is of reduced practicability if gram amounts of a target peptide are required. In the present publication we, therefore, report the first approach towards *cyclo*[Arg-Gly-Asp-D-Phe-Lys] peptide (**1**) and its *N*-ε-azido derivative **2** solely based on solution-phase synthesis (Figure 1).

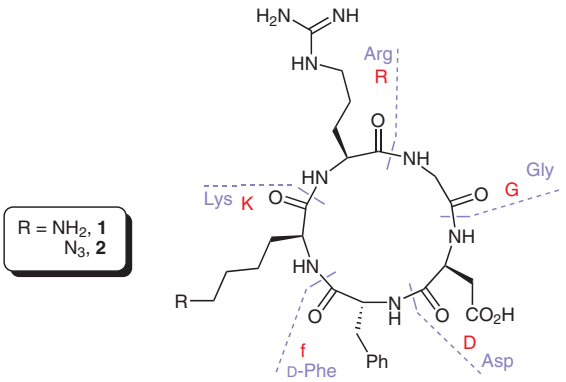

Figure 1 *cyclo*-RGDfK peptide (**1**) and *N*-ε-azido *cyclo*-RGDfK peptide (**2**)

In planning the synthesis a large variety of alternative coupling strategies for the various protected R-G-f-K amino acid fragments could be envisaged.[12] Retrosynthetically, the synthesis of title cyclic pentapeptide **1** and **2** (Scheme 1) should by achieved by macrolactamization of the linear pentapeptide [Lys(PG)-Arg(PG)-Gly-Asp(OPG)-D-Phe-OH] via a one-pot acidic deprotection of the terminal *N*-α-Boc-lysine and *tert*-butyl ester of D-phenylalanine [Boc-Lys(PG)-Arg(PG)-Gly-Asp(OPG)-D-Phe-O*t*-Bu]. The 4-toluenesulfonyl (Ts) group and alternatively the nitro (NO_2) group were chosen to protect the guanidine unit of arginine. The Fmoc group was chosen as the protecting group for the R-G-f amino acids at the *N*-α-termini. The ε-amino group of lysine should be selectively converted into the azido group by a diazo-transfer reaction in the last step.

Our solution-phase RGD protocol started with D-phenylalanine (Scheme 2), which was converted into its *tert*-butyl ester **3** by reaction with isobutene in a mixture of dioxane and sulfuric acid (82%).[13]

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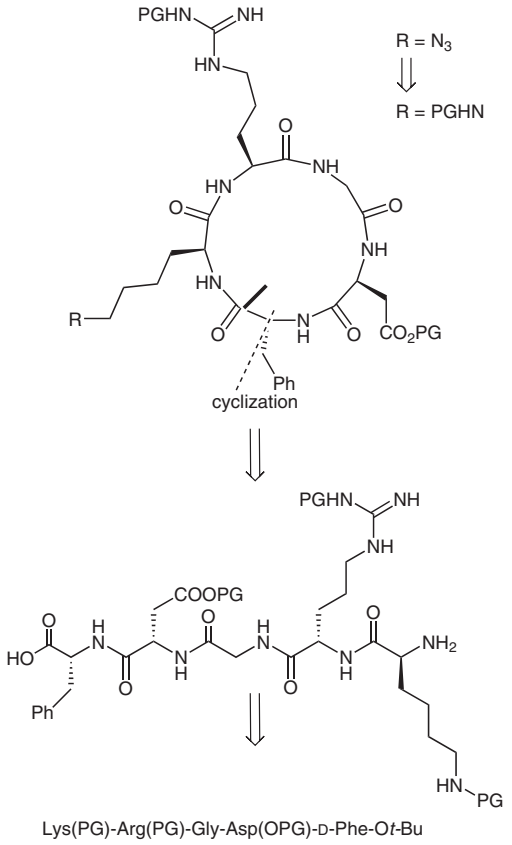
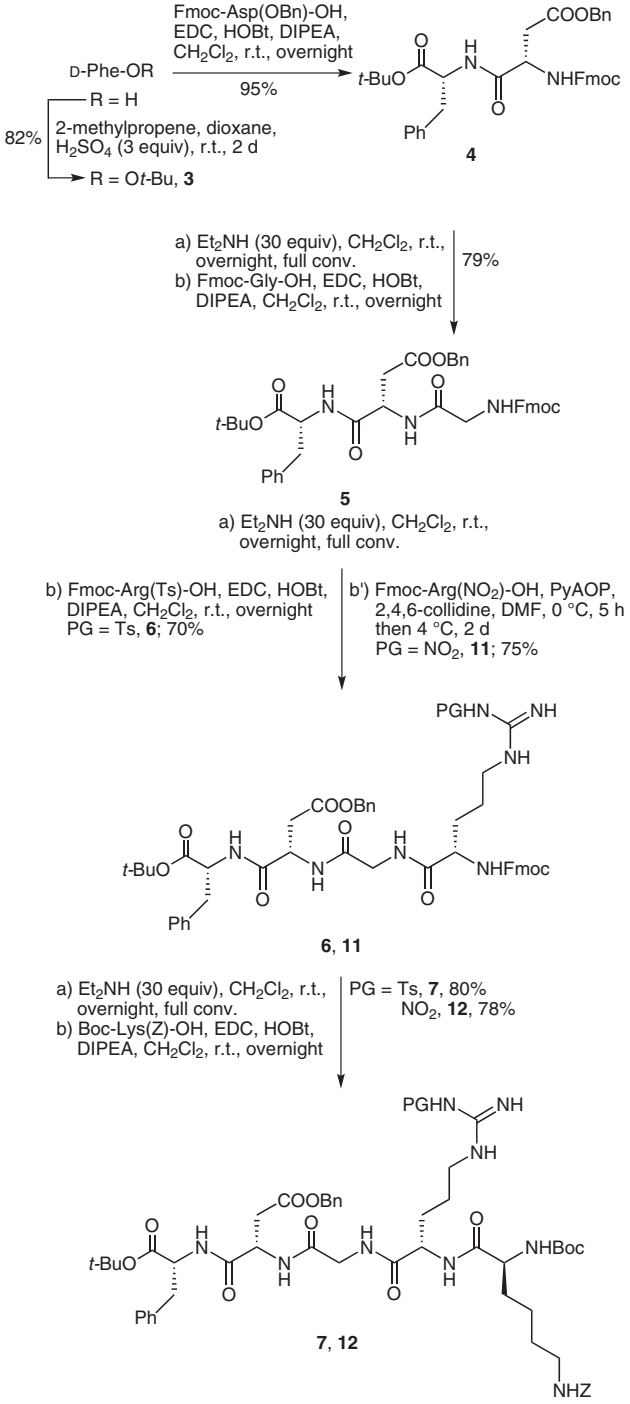


Scheme 1 Retrosynthetic analysis of the *cyclo*-RGDfK peptide (**1** and **2**)

The linear peptide fragments were synthesized using the standard epimerization-free condensation conditions (EDC and HOBt) according to Ley's protocol.[12b] Firstly, D-Phe-O*t*-Bu (**3**) was reacted with Fmoc-Asp(OBn)-OH to afford the corresponding dipeptide Fmoc-Asp(OBn)-D-Phe-O*t*-Bu (**4**) (95%). Next, the Fmoc group was removed[12c,h,14] from dipeptide **4**. The reaction was carried out with diethylamine in dichloromethane and subsequent coupling with Fmoc-Gly-OH gave the protected tripeptide Fmoc-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**5**) in 79% yield over two steps.

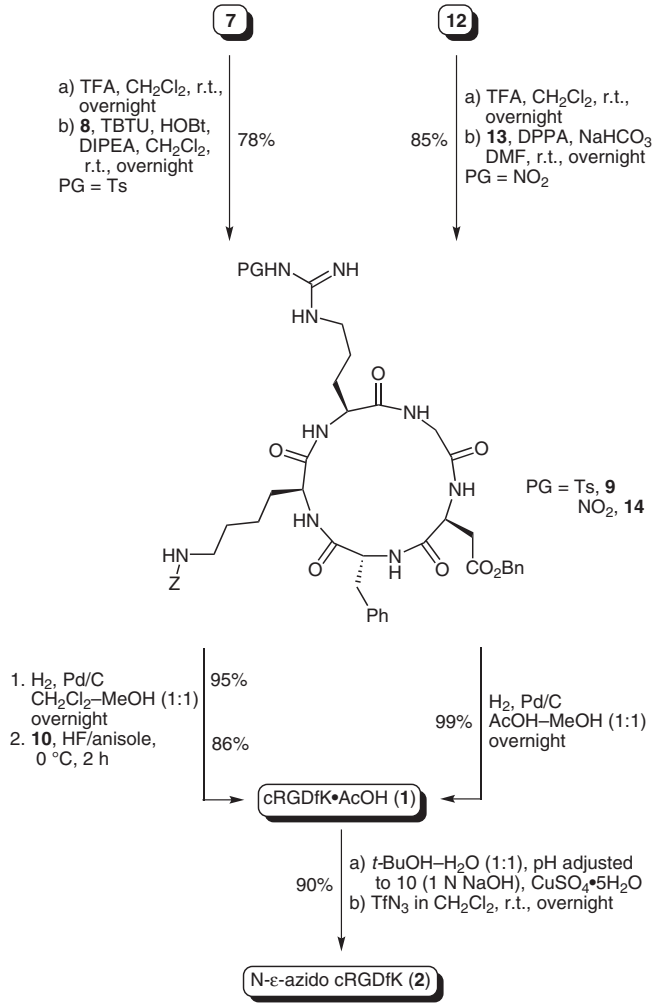
Then, the synthesis of peptides **1** and **2** progressed via the tripeptide **5** following two alternative routes that are based on two different protecting group strategies for the guanidine moiety, namely the 4-toluenesulfonyl and nitro group protection, respectively.

Via the 'tosyl' route, the linear tetrapeptide Fmoc-Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**6**) was formed after removal of the Fmoc group from the tripeptide **5** and the subsequent reaction with Fmoc-Arg(Ts)-OH (70% yield over two steps). Instead of the Fmoc functionalized lysine, Boc-protected lysine was selected for the last linear coupling step. The linear pentapeptide Boc-Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**7**) was prepared again via a two step sequence: (a) Fmoc deprotection of tetrapeptide **6** and (b) condensation with Boc-Lys(Z)-OH (80% over two steps).



Scheme 2 The 'tosyl' and 'nitro' routes: Synthesis of the linear pentapeptides: Boc-Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**7**) and Boc-Lys(Z)-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**12**).

Treatment of the pentapeptide **7** with a mixture of trifluoroacetic acid/dichloromethane[4b,12g,h,15] led to quantitative deprotection of its terminal carboxylate and amino groups (Scheme 3) to give the ammonium salt of pentapeptide Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-OH·TFA (**8**). The crucial macrolactamization step was performed with TBTU/HOBt[12b,16] conditions and afforded the cyclic pentapeptide *cyclo*[Arg(Ts)-Gly-Asp(OBn)-D-Phe-Lys(*Z*)] (**9**) in 78% yield over two steps.



Scheme 3 'Cyclization via the 'tosyl' and 'nitro' routes, respectively, deprotection steps: *cyclo*[Arg-Gly-Asp-D-Phe-Lys] (**1**) and 'diazo-transfer': *N*-ε-azido *cyclo*[Arg-Gly-Asp-D-Phe-Lys] (**2**)

For the alternative 'nitro' route, the protected cyclic pentapeptide *cyclo*[Arg(NO_2)-Gly-Asp(OBn)-D-Phe-Lys(Z)] (**14**) (Scheme 3) was synthesized again from tripeptide **5** via the nitro-guanidine-protected linear tetrapeptide **11** and pentapeptide **12** (Scheme 2) under similar conditions described above for the 'tosyl' route.

Coupling of Fmoc-deprotected tripeptide **5** with Fmoc-Arg(NO_2)-OH using EDC/HOBt unexpectedly gave a low yield (30%) of the corresponding tetrapeptide Fmoc-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**11**). Therefore, we had to employ different conditions for the coupling with the protected arginine.[17] In fact, minimization of the intramolecular δ-lactam formation of Fmoc-arginine had to be achieved.[17f–h] After substantial optimization, in order to suppress δ-lactam formation (Figure 2) peptide **11** was obtained in good yield (75%) using the reagent system PyAOP in *N*,*N*-dimethylformamide and 2,4,6-collidine as base. Then, the pentapeptide Boc-Lys(Z)-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (**12**) was again synthesized under the typical EDC/HOBt condition (78% over two steps).

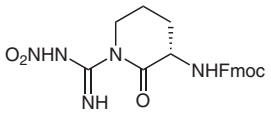


Figure 2 The structure of intramolecular δ-lactam formation of Fmoc-arginine(NO_2)

For the cyclization, the ammonium salt of pentapeptide Lys(Z)-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu·TFA (**13**) (Scheme 3) was quantitatively formed from peptide **12** in the presence of trifluoroacetic acid in dichloromethane. Then, the cyclization of pentapeptide **13** was repeated with TBTU/HOBt leading to full conversion of peptide **13** into cyclic peptide **14** as judged by thin layer chromatography (CH_2Cl_2–MeOH, 9:1). Purification of peptide **14** by column chromatography on silica gel turned out to be very problematic as elution was difficult due to its low solubility in organic solvents.

Therefore, we added liquid reagent DPPA/$NaHCO_3$ in *N*,*N*-dimethylformamide to the cyclization mixture.[18] The pure cyclic peptide **14** was obtained (85%) after filtration of solid sodium hydrogen carbonate, extraction (side products of coupling reagents were removed), and crystallization from methanol (traces of DPPA were removed).

The synthesis of *c*RGDfK peptide (**1**) was achieved via both routes after removal of the three remaining protecting groups (Scheme 3). For the 'tosyl' route, peptide **9** was transformed to the RGD peptide **1** in two steps. First, the benzyl and benzyloxycarbonyl groups were cleaved by hydrogenation[19] in methanol (95%). The final deprotection step required detosylation of the *N*-tosyl guanidine group in **10** with an excess of anhydrous hydrogen fluoride in the presence of anisole as an electrophilic scavenger using a Teflon flask.[12d,e,20] After treatment of the reaction mixture, the residue was dissolved in a 5% aqueous acetic acid solution (peptide **1** was transferred from the Teflon flask into a glass flask) and lyophilized, and the pure title peptide (monoacetate salt) **1** was obtained by crystallization from a mixture of diethyl ether–methanol (1:1) (yield 86%, purity >95% according to NMR spectroscopy).

The alternate 'nitro' route (Scheme 3), allowed all three protecting groups (Bn, Z and *N*-nitro guanidine[21]) in **14** to be cleaved simultaneously by catalytic hydrogenation in a mixture of acetic acid–methanol. We had noted that the presence of acetic acid plays an important role for accelerating the hydrogenation. Pure monoacetic acid salt of *c*RGDfK (**1**) was quantitatively isolated after simple filtration through a short pad of Celite (purity was >95% according to NMR analysis).

Finally, the ε-amino group of lysine was selectively converted into the corresponding azide by a diazo-transfer reaction (Scheme 3).[4e,6e,22] Pure azido-RGD peptide **2** was obtained after column chromatography on Sephadex G-25[12e,f,23] (yield 90% for 0.3 mmol scale, >95% purity according to NMR analysis). Noteworthy, the synthesis can be carried out on a multigram scale.

In summary, we describe a practical and scalable solution-phase synthesis of *cyclo*-RGDfK (**1**) and *N*-e-azido *cyclo*-RGDfK (**2**) peptides. The ε-amino or ε-azido group in the lysine moiety of RGD peptides **1** and **2** are suitable materials for further elaboration in various field of application. For example the fusion of peptide **2** with biomedical materials through 'click' cycloaddition has great potential for applications in the field of tissue engineering. These lines of research are currently pursued in our laboratories.

All solvents were dried by conventional methods. Starting materials and reagents were purchased from commercial suppliers and used without further purification. Preparative column chromatography was performed using silica gel 60, particle size 0.040–0.063 mm (230–240 mesh, flash). The azido RGD-peptide 2 was purified using Sephadex G-25. Analytical TLC was carried out employing silica gel 60 F254 plates from Macherey&Nagel. Visualization of the chromatograms was achieved by UV detection (254 nm), by coloration with a phosphomolybdic acid soln in EtOH or ninhydrin soln in EtOH. NMR spectra were recorded on Bruker ARX-400 or 500 spectrometers (^{1}H, 400 MHz or 500 MHz; ^{13}C, 100 MHz or 125 MHz). All spectra were measured using standard Bruker pulse sequences. 2D NMR spectroscopy (COSY, HSQC and HMBC) was used for the assignment of signals in the ^{1}H and ^{13}C NMR spectra. Mass spectra and HRMS data were recorded on a QTof Premier equipped with an Acquity UPLC (both Waters). Melting points were measured on a SRS OptiMelt apparatus and are uncorrected. The optical rotation of D-Phe-O*t*-Bu (**3**) was measured with a Perkin Elmer 341 polarimeter. The IR spectrum of azido peptide **2** was recorded with a Bruker Vektor 22 FT-IR spectrophotometer (Golden-Gate ATR unit).

Abbreviations: Arg (*R*): arginine; Asp (D): aspartate; D-Phe (f): D-phenylalanine; DPPA: diphenylphosphoryl azide; EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; Gdn: guanidine; Gly (G): glycine; HOBt: 1-hydroxybenzotriazole; Lys (K): lysine; PyAOP: (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

D-Phe-O*t*-Bu (3)

A mixture of D-phenylalanine (5 g, 30.3 mmol, 1 equiv) and concd H_2SO_4 (8.9 g, 90.8 mmol, 3 equiv) in anhyd 1,4-dioxane (60 mL) was cooled to –78 °C in a 2-neck round-bottomed flask (500 mL). 2-Methylpropene (50 g, 891 mmol, 29 equiv) was slowly bubbled and condensed into the flask. The mixture was warmed to r.t. and stirred for 2 d. Then the mixture was diluted with 1 M NaOH soln (500 mL) and Et_2O (200 mL). The phases were separated and the aqueous phase was extracted with Et_2O (3 × 200 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography (silica gel, EtOAc; R_f = 0.36) to afford **3** (5.5 g, 24.9 mmol; 82%) as a yellow oil.

$[\alpha]_D^{20}$ –24.4 (*c* 1.12, EtOAc) [Lit.[24a] $[\alpha]_D^{24}$ –22.4 (*c* 10.0 EtOH); Lit.[24b] $[\alpha]_D^{20}$ +25.1 (*c* 2.8, EtOAc) for L-Phe-O*t*-Bu].

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.46 [s, 9 H, $C(CH_3)_3$], 1.48 (s, 2 H, NH_2), 2.87 (dd, ^3J = 7.8 Hz, ^2J = 13.6 Hz, 1 H, CH_2), 3.07 (dd, ^3J = 5.7 Hz, ^2J = 13.6 Hz, 1 H, CH_2), 3.64 (dd, ^3J = 5.7 Hz, ^3J = 7.8 Hz, 1 H, CH), 7.21–7.38 (m, 5 H, ArH).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 27.9 [$C(CH_3)_3$], 41.2 (CH_2), 56.3 (CH), 81.1 [$C(CH_3)_3$], 126.6 (CH_{Ar}), 128.3 (CH_{Ar}), 129.3 (CH_{Ar}), 137.5 (C_{Ar}), 174.3 (*C*OO*t*-Bu).

HRMS (ESI+): *m*/*z* $[M + H]^+$ calcd for $C_{13}H_{20}NO_2$: 222.1494; found: 222.1493.

The spectroscopic data of D-Phe-O*t*-Bu (**3**) were in full agreement with those reported in the literature.[24]

Fmoc-Asp(OBn)-D-Phe-O*t*-Bu (4)

DIPEA (5.9 mL, 33.9 mmol, 1.5 equiv) and EDC (5.42 g, 28.3 mmol, 1.25 equiv) were added successively to a mixture of D-Phe-O*t*-Bu (**3**, 5 g, 22.6 mmol, 1 equiv), Fmoc-Asp(OBn)-OH (10.6 g, 23.7 mmol, 1.05 equiv), and HOBt (4.58 g, 33.9 mmol, 1.5 equiv) in CH_2Cl_2 (500 mL) at 0 °C. The mixture was warmed to r.t. and stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 99:1). Then, the mixture was concentrated under reduced pressure and purified by column chromatography (silica gel, CH_2Cl_2–MeOH, 99:1; R_f = 0.47) to afford **4** (13.9 g, 21.4 mmol; 95%) as colorless crystals; mp 42–44 °C.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.43 [s, 9 H, $C(CH_3)_3$], 2.66 (dd, ^3J = 6.5 Hz, ^2J = 17.1 Hz, 1 H, $\beta CH_{2\ Asp}$), 3.05 (m, 1 H, $\beta CH_{2\ Asp}$, 1 H, $\beta CH_{2\ Phe}$), 3.11 (dd, ^3J = 6.1 Hz, ^2J = 13.7 Hz, 1 H, $\beta CH_{2\ Phe}$), 4.22 (t, ^3J = 7.2 Hz, 1 H, OCH_2CH_{Fmoc}), 4.40 (m, 2 H, OCH_2CH_{Fmoc}), 4.63 (m, 1 H, αCH_{Asp}), 4.73 (m, 1 H, αCH_{Phe}), 5.15 (s, 2 H, $COOCH_2Ph$), 5.95 (d, ^3J = 8.2 Hz, 1 H, αNH_{Asp}), 6.99 (d, ^3J = 7.5 Hz, 1 H, αNH_{Phe}), 7.11–7.44 (m, 14 H, ArH, Fmoc), 7.59 (t, ^3J = 6.8 Hz, 2 H, Fmoc), 7.79 (d, ^3J = 7.5 Hz, 2 H, Fmoc).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 27.9 [$C(CH_3)_3$], 36.3 ($\beta CH_{2\ Asp}$), 38.0 ($\beta CH_{2\ Phe}$), 47.1 (OCH_2CH_{Fmoc}), 50.9 (αCH_{Asp}), 53.8 (αCH_{Phe}), 66.9 ($COOCH_2Ph$), 67.4 (OCH_2CH_{Fmoc}), 82.5 [$C(CH_3)_3$], 120.0 (CH_{Fmoc}), 125.1 (CH_{Fmoc}), 126.9 (CH_{Ar}), 127.1 (CH_{Ar} and CH_{Fmoc}), 127.7 (CH_{Fmoc}), 128.3 (CH_{Ar}), 128.4 (CH_{Ar}), 128.6 (CH_{Ar}), 129.4 (CH_{Ar}), 135.3 (C_{Ar}), 136.0 (C_{Ar}), 141.3 (C_{Fmoc}), 143.6 (C_{Fmoc}), 155.9 ($NHCOO_{Fmoc}$), 169.5 (NHCO), 170.0 (*C*OO*t*-Bu), 171.5 (*C*$OOCH_2Ph$).

HRMS (ESI+): *m*/*z* $[M + Na]^+$ calcd for: $C_{39}H_{40}N_2O_7Na$: 671.2733; found: 671.2733.

Fmoc-Gly-Asp(OBn)-D-Phe-O*t*-Bu (5)

Et_2NH (31 mL, 296 mmol, 30 equiv) was added dropwise to a stirred mixture of **4** (6.4 g, 9.87 mmol, 1 equiv) in CH_2Cl_2 (400 mL) at r.t. and stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 99:1 and 95:5). Then, the mixture was evaporated and dried overnight under vacuum. The nonpolar fluorenyl side product was removed by flash chromatography (silica gel, CH_2Cl_2–MeOH, 95:5 to 9:1). Then the crude Asp(OBn)-D-Phe-O*t*-Bu was directly employed in the second peptide coupling step.

DIPEA (2.58 mL, 14.8 mmol, 1.5 equiv) and EDC (2.37 g, 12.3 mmol, 1.25 equiv) were added successively to a mixture of Asp(OBn)-D-Phe-O*t*-Bu (approx. 9.87 mmol; calculated as quantitative yield after the first step; Fmoc-deprotection as described above), Fmoc-Gly-OH (3.23 g, 10.9 mmol, 1.1 equiv) and HOBt (2.0 g, 14.8 mmol, 1.5 equiv) in CH_2Cl_2 (500 mL) at 0 °C. The mixture was warmed to r.t. and stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 99:1). Then, the mixture was concentrated under reduced pressure and purified by column chromatography (silica gel, CH_2Cl_2–MeOH, 99:1; R_f = 0.25) to afford **5** (5.5 g, 7.79 mmol; 79%) as colorless crystals; mp 49.5–50 °C.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.42 [s, 9 H, $C(CH_3)_3$], 2.62 (dd, ^3J = 6.5 Hz, ^2J = 17.1 Hz, 1 H, $\beta CH_{2\ Asp}$), 3.01 (m, 1 H, $\beta CH_{2\ Asp}$, 1 H, $\beta CH_{2\ Phe}$), 3.11 (dd, ^3J = 6.1 Hz, ^2J = 14.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.87 (d, ^3J = 5.1 Hz, 2 H, $\alpha CH_{2\ Gly}$), 4.26 (t, ^3J = 6.8 Hz, 1 H, OCH_2CH_{Fmoc}), 4.44 (d, ^3J = 6.5 Hz, 2 H, OCH_2CH_{Fmoc}), 4.70 (m, 1 H, αCH_{Phe}), 4.86 (m, 1 H, αCH_{Asp}), 5.10 (s, 2 H, $COOCH_2Ph$), 5.43 (br s, 1 H, αNH_{Gly}), 7.06 (br d, ^3J = 6.5 Hz, 1 H, αNH_{Phe}), 7.14 (d, ^3J = 6.8 Hz, 2 H, ArH), 7.22 (br d, ^3J = 6.8 Hz, 1 H, αNH_{Asp}), 7.18–7.37 (m, 10 H, ArH, Fmoc), 7.43 (t, ^3J = 7.5 Hz, 2 H, Fmoc), 7.62 (d, ^3J = 7.2 Hz, 2 H, Fmoc), 7.78 (d, ^3J = 7.5 Hz, 2 H, Fmoc).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 27.9 [$C(CH_3)_3$], 35.8 ($\beta CH_{2\ Asp}$), 37.9 ($\beta CH_{2\ Phe}$), 44.6 ($\alpha CH_{2\ Gly}$), 47.1 (OCH_2CH_{Fmoc}), 49.0

(αCH$_{Asp}$), 53.9 (αCH$_{Phe}$), 66.9 (COO*C*H$_2$Ph), 67.4 (O*C*H$_2$CH$_{Fmoc}$), 82.4 [*C*(CH$_3$)$_3$], 120.0 (CH$_{Fmoc}$), 125.1 (CH$_{Fmoc}$), 126.9 (CH$_{Ar}$), 127.1 (CH$_{Ar}$ and CH$_{Fmoc}$), 127.7 (CH$_{Fmoc}$), 128.3 (CH$_{Ar}$), 128.4 (CH$_{Ar}$), 128.6 (CH$_{Ar}$), 129.4 (CH$_{Ar}$), 135.3 (C$_{Ar}$), 136.1 (C$_{Ar}$), 141.3 (C$_{Fmoc}$), 143.7 (C$_{Fmoc}$), 156.6 (NHCOO$_{Fmoc}$), 168.8 (NHCO), 169.3 (NHCO), 170.1 (*C*OO*t*-Bu), 171.5 (*C*OOCH$_2$Ph).

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for C$_{41}$H$_{43}$N$_3$O$_8$Na: 728.2948; found: 728.2968.

Fmoc-Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (6)

Et$_2$NH (22.1 mL, 212.5 mmol, 30 equiv) was added dropwise to a stirred mixture of **5** (5.0 g, 7.08 mmol, 1 equiv) in CH$_2$Cl$_2$ (350 mL) at r.t. and stirred overnight (checked by TLC, CH$_2$Cl$_2$–MeOH, 99:1 and 95:5). Then, the mixture was evaporated and dried overnight under vacuum. The nonpolar fluorenyl side product was removed by flash chromatography (silica gel, CH$_2$Cl$_2$–MeOH, 95:5 to 9:1). Then, the crude D-Phe-Asp(OBn)-Gly was directly used in the second peptide coupling step.

DIPEA (1.85 mL, 10.6 mmol, 1.5 equiv) and EDC (1.76 g, 9.20 mmol, 1.3 equiv) were added successively to a mixture of Gly-Asp(OBn)-D-Phe-O*t*-Bu (approx. 7.08 mmol; calculated as quantitative yield after the first step; Fmoc-deprotection as described above), Fmoc-Arg(Ts)-OH (5.07 g, 9.21 mmol, 1.3 equiv) and HOBt (1.43 g, 10.6 mmol, 1.5 equiv) in CH$_2$Cl$_2$ (500 mL) at 0 °C. The mixture was stirred at 0 °C for 2 h, then warmed to r.t. and stirred overnight (checked by TLC, CH$_2$Cl$_2$–MeOH, 97:3). Then, the mixture was concentrated under reduced pressure and purified by column chromatography (silica gel, CH$_2$Cl$_2$–MeOH, 97:3; R_f = 0.25) to afford **6** (5.05 g, 4.97 mmol; 70%) as yellowish crystals; mp 89–93 °C.

^{1}H NMR (400 MHz, CDCl$_3$): δ = 1.35 [s, 9 H, C(CH$_3$)$_3$], 1.58 (m, 2 H, γCH$_{2\ Arg}$), 1.72 (m, 1 H, βCH$_{2\ Arg}$), 1.88 (m, 1 H, βCH$_{2\ Arg}$), 2.33 (s, 3 H, CH$_{3\ Ts}$), 2.66 (dd, ^3J = 6.2 Hz, ^2J = 17.1 Hz, 1 H, βCH$_{2\ Asp}$), 2.86 (dd, ^3J = 4.8 Hz, ^2J = 17.1 Hz, 1 H, βCH$_{2\ Asp}$), 2.96 (dd, ^3J = 7.2 Hz, ^2J = 14.0 Hz, 1 H, βCH$_{2\ Phe}$), 3.04 (dd, ^3J = 6.2 Hz, ^2J = 14.0 Hz, 1 H, βCH$_{2\ Phe}$), 3.20 (m, 1 H, δCH$_{2\ Arg}$), 3.35 (m, 1 H, δCH$_{2\ Arg}$), 3.83 (dd, ^3J = 4.8 Hz, ^2J = 16.4 Hz, 1 H, αCH$_{2\ Gly}$), 3.96 (dd, ^3J = 4.1 Hz, ^2J = 16.4 Hz, 1 H, αCH$_{2\ Gly}$), 4.15 (t, ^3J = 7.2 Hz, 1 H, OCH$_2$C*H*$_{Fmoc}$), 4.36 (m, 3 H, OC*H*$_2$CH$_{Fmoc}$, αCH$_{Arg}$), 4.61 (m, 1 H, αCH$_{Phe}$), 4.82 (m, 1 H, αCH$_{Asp}$), 5.02 (s, 2 H, COOCH$_2$Ph), 6.13 (d, ^3J = 6.2 Hz, 1 H, αNH$_{Arg}$), 6.46 (br s, 2 H, NH$_{Gdn}$), 7.09–7.39 (m, 18 H, 12 ArH, 4 Fmoc, αNH$_{Phe}$, NH$_{Gdn}$), 7.45 (br d, ^3J = 6.2 Hz, 1 H, αNH$_{Asp}$), 7.57 (t, ^3J = 6.8 Hz, 2 H, Fmoc), 7.75 (m, 5 H, 2 ArH, 2 Fmoc, αNH$_{Gly}$).

^{13}C NMR (100 MHz, CDCl$_3$): δ = 21.4 (CH$_3$PhSO$_2$), 27.8 [C(*C*H$_3$)$_3$], 29.4 (γCH$_{2\ Arg}$), 29.5 (βCH$_{2\ Arg}$), 35.9 (βCH$_{2\ Asp}$), 37.9 (βCH$_{2\ Phe}$), 40.0 (δCH$_{2\ Arg}$), 43.3 (αCH$_{2\ Gly}$), 47.1 (OCH$_2$*C*H$_{Fmoc}$), 49.1 (αCH$_{Asp}$), 54.3 (αCH$_{Phe}$ and αCH$_{Arg}$), 66.8 (COO*C*H$_2$Ph), 67.1 (O*C*H$_2$CH$_{Fmoc}$), 82.3 [*C*(CH$_3$)$_3$], 119.9 (CH$_{Fmoc}$), 125.1 (CH$_{Fmoc}$), 125.9 (CH$_{Ar}$), 126.9 (CH$_{Ar}$), 127.1 (CH$_{Ar}$ and CH$_{Fmoc}$), 127.7 (CH$_{Fmoc}$), 128.2 (CH$_{Ar}$), 128.3 (CH$_{Ar}$), 128.6 (CH$_{Ar}$), 129.2 (CH$_{Ar}$), 129.3 (CH$_{Ar}$), 135.3 (C$_{Ar}$), 136.2 (C$_{Ar}$), 140.5 (C$_{Ar}$), 141.2 (C$_{Fmoc}$), 142.1 (C$_{Ar}$), 143.7 (C$_{Fmoc}$), 156.5 (NHCOO$_{Fmoc}$), 156.6 (C$_{Gdn}$), 169.3 (NHCO), 169.8 (NHCO), 170.5 (*C*OO*t*-Bu), 171.2 (NHCO), 171.3 (*C*OOCH$_2$Ph).

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for C$_{54}$H$_{61}$N$_7$O$_{14}$SNa: 1038.4047; found: 1038.3857; *m*/*z* [M + H]$^+$ calcd for C$_{54}$H$_{62}$N$_7$O$_{14}$S: 1016.4248; found: 1016.4247.

Boc-Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (7)

Et$_2$NH (10.7 mL, 103.2 mmol, 30 equiv) was added dropwise to a stirred mixture of **6** (3.5 g, 3.44 mmol, 1 equiv) in CH$_2$Cl$_2$ (300 mL) at r.t. and stirred overnight (checked by TLC, CH$_2$Cl$_2$–MeOH, 95:5 and 9:1). Then, the mixture was evaporated and dried overnight under vacuum. The nonpolar fluorenyl side product was removed by flash chromatography (silica gel, CH$_2$Cl$_2$–MeOH, 95:5 to 9:1). Then, the crude Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu was directly used in the second peptide coupling step.

DIPEA (0.9 mL, 5.16 mmol, 1.5 equiv) and EDC (0.82 g, 4.3 mmol, 1.25 equiv) were added successively to a mixture of Arg(Ts)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (approx. 3.44 mmol; calculated as quantitative yield after the first step; Fmoc-deprotection as described above), Boc-Lys(*Z*)-OH (1.44 g, 3.78 mmol, 1.1 equiv) and HOBt (0.7 g, 5.16 mmol, 1.5 equiv) in CH$_2$Cl$_2$ (400 mL) at 0 °C. The mixture was warmed to r.t. and stirred overnight (checked by TLC, CH$_2$Cl$_2$–MeOH, 95:5). Then, the mixture was concentrated under reduced pressure and purified by column chromatography (silica gel, CH$_2$Cl$_2$–MeOH, 95:5; R_f = 0.43) to afford **7** (3.19 g, 2.76 mmol; 80%) as yellowish crystals; mp 82–99 °C (dec.).

^{1}H NMR (400 MHz, CDCl$_3$): δ = 1.35 (m, 2 H, γCH$_{2\ Lys}$), 1.38 [s, 9 H, C(CH$_3$)$_3$], 1.41 [s, 9 H, C(CH$_3$)$_3$], 1.48 (m, 4 H, βCH$_{2\ Lys}$, δCH$_{2\ Lys}$), 1.57 (m, 2 H, γCH$_{2\ Arg}$), 1.73 (m, 1 H, βCH$_{2\ Arg}$), 1.88 (m, 1 H, βCH$_{2\ Arg}$), 2.36 (s, 3 H, CH$_{3\ Ts}$), 2.74 (dd, ^3J = 5.8 Hz, ^2J = 17.5 Hz, 1 H, βCH$_{2\ Asp}$), 2.85 (dd, ^3J = 5.5 Hz, ^2J = 17.5 Hz, 1 H, βCH$_{2\ Asp}$), 2.96 (dd, ^3J = 7.2 Hz, ^2J = 13.9 Hz, 1 H, βCH$_{2\ Phe}$), 3.03 (dd, ^3J = 6.4 Hz, ^2J = 13.9 Hz, 1 H, βCH$_{2\ Phe}$), 3.12 (m, 2 H, εCH$_{2\ Lys}$), 3.27 (m, 2 H, δCH$_{2\ Arg}$), 3.81 (m, 1 H, αCH$_{2\ Gly}$), 3.96 (m, 1 H, αCH$_{2\ Gly}$), 4.13 (m, 1 H, αCH$_{Lys}$), 4.47 (m, 1 H, αCH$_{Arg}$), 4.60 (m, 1 H, αCH$_{Phe}$), 4.85 (m, 1 H, αCH$_{Asp}$), 5.04 (s, 2 H, COOCH$_2$Ph), 5.06 (s, 2 H, COOCH$_2$Ph), 5.39 (br s, 1 H, εNH$_{Lys}$), 5.44 (br d, ^3J = 7.2 Hz, 1 H, αNH$_{Lys}$), 6.45 (br s, 2 H, NH$_{Gdn}$), 7.11–7.40 (m, 19 H, 17 ArH, αNH$_{Phe}$, αNH$_{Arg}$, NH$_{Gdn}$), 7.48 (br d, ^3J = 6.2 Hz, 1 H, αNH$_{Asp}$), 7.76 (d, ^3J = 7.1 Hz, 2 H, ArH), 7.84 (br s, 1 H, αNH$_{Gly}$).

^{13}C NMR (100 MHz, CDCl$_3$): δ = 21.4 (CH$_3$PhSO$_2$), 24.3 (γCH$_{2\ Lys}$), 27.8 [C(*C*H$_3$)$_3$], 28.2 [C(*C*H$_3$)$_3$], 29.3 (γCH$_{2\ Arg}$), 29.4 (βCH$_{2\ Arg}$), 30.6 (δCH$_{2\ Lys}$), 32.4 (βCH$_{2\ Lys}$), 36.1 (βCH$_{2\ Asp}$), 37.9 (βCH$_{2\ Phe}$), 40.5 (εCH$_{2\ Lys}$), 41.9 (δCH$_{2\ Arg}$), 43.7 (αCH$_{2\ Gly}$), 49.2 (αCH$_{Asp}$), 53.1 (αCH$_{Arg}$), 54.2 (αCH$_{Phe}$), 54.9 (αCH$_{Lys}$), 66.4 (COO*C*H$_2$Ph), 66.7 (COO*C*H$_2$Ph), 80.2 [*C*(CH$_3$)$_3$], 82.1 [*C*(CH$_3$)$_3$], 125.9 (CH$_{Ar}$), 126.8 (CH$_{Ar}$), 128.0 (CH$_{Ar}$), 128.1 (CH$_{Ar}$), 128.3 (CH$_{Ar}$), 128.4 (CH$_{Ar}$), 128.5 (CH$_{Ar}$), 128.6 (CH$_{Ar}$), 129.1 (CH$_{Ar}$), 129.2 (CH$_{Ar}$), 129.3 (CH$_{Ar}$), 135.4 (C$_{Ar}$), 136.3 (C$_{Ar}$), 136.6 (C$_{Ar}$), 140.5 (C$_{Ar}$), 142.1 (C$_{Ar}$), 156.3 (NHCOO$_{Boc}$), 156.8 (NHCOO$_Z$), 156.9 (C$_{Gdn}$), 169.3 (NHCO), 169.8 (*C*OOCH$_2$Ph), 170.4 (*C*OO*t*-Bu), 170.5 (NHCO), 172.7 (NHCO), 173.6 (NHCO).

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for C$_{58}$H$_{77}$N$_9$O$_{14}$SNa: 1178.5190; found: 1178.5208.

Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-OH·TFA (8)

TFA (10 mL, 131 mmol, 150 equiv) was added dropwise to a stirred mixture of **7** (1 g, 0.87 mmol, 1 equiv) in CH$_2$Cl$_2$ (200 mL) at 0 °C. Then, the mixture was warmed to r.t. and stirred overnight (checked by MS and TLC, CH$_2$Cl$_2$–MeOH, 95:5 and 9:1), evaporated and dried overnight under vacuum. The resulting ammonium salt of linear pentapeptide Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-OH (**8**) was directly used for the second cyclization step.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for C$_{49}$H$_{62}$N$_9$O$_{12}$S: 1000.4239; found: 1000.4243.

***cyclo*[Arg(Ts)-Gly-Asp(OBn)-D-Phe-Lys(*Z*)] (9)**

DIPEA (1.52 mL, 8.7 mmol, 10 equiv) and TBTU (0.56 g, 1.74 mmol, 2 equiv) were added successively to a mixture of Lys(Z)-Arg(Ts)-Gly-Asp(OBn)-D-Phe-OH (**8**, approx. 0.87 mmol; calculated as quantitative yield after the first step: Boc- and *tert*-butyl ester deprotection) and HOBt (2.35 g, 1.74 mmol, 2 equiv) in CH$_2$Cl$_2$ (1 L) at r.t. The mixture was stirred overnight (checked by TLC, CH$_2$Cl$_2$–MeOH, 9:1). Then, the mixture was diluted with H$_2$O (300 mL). The phases were separated and the aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 200 mL). The combined organic layers were dried (MgSO$_4$), filtered, and concentrated under reduced pressure.

The crude product was purified by column chromatography (silica gel, CH_2Cl_2–MeOH, 9:1; R_f = 0.38) to afford **9** (0.67 g, 0.68 mmol; 78%) as yellowish crystals; mp 99–121 °C (dec.).

^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD, 9:1): δ = 1.06 (m, 2 H, $\gamma CH_{2\ Lys}$), 1.28–1.68 (m, 7 H, 2 $\beta CH_{2\ Lys}$, 2 $\delta CH_{2\ Lys}$, 2 $\gamma CH_{2\ Arg}$, 1 $\beta CH_{2\ Arg}$), 1.84 (m, 1 H, $\beta CH_{2\ Arg}$), 2.37 (s, 3 H, $CH_{3\ Ts}$), 2.66 (dd, ^3J = 6.1 Hz, ^2J = 16.4 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.85–3.08 (m, 5 H, 1 $\beta CH_{2\ Asp}$, 2 $\beta CH_{2\ Phe}$, 2 $\varepsilon CH_{2\ Lys}$), 3.15 (m, 2 H, $\delta CH_{2\ Arg}$), 3.32 (m, 2 H, $\alpha CH_{2\ Gly}$), 3.93 (m, 1 H, αCH_{Lys}), 4.21 (m, 1 H, αCH_{Arg}), 4.51 (m, 1 H, αCH_{Phe}), 4.80 (dd, 1 H, ^3J = 6.5 Hz, ^3J = 7.9 Hz, αCH_{Asp}), 5.04 (s, 2 H, $COOCH_2Ph$), 5.06 (s, 2 H, $COOCH_2Ph$), 7.12–7.35 (m, 17 H, ArH), 7.70 (d, ^3J = 8.2 Hz, 2 H, ArH). Signals NH were not detected in the spectrum.

^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD, 9:1): δ = 22.3 (CH_3PhSO_2), 23.9 ($\gamma CH_{2\ Lys}$), 30.1 ($\beta CH_{2\ Arg}$), 30.2 ($\delta CH_{2\ Lys}$), 30.8 ($\beta CH_{2\ Lys}$), 32.4 ($\gamma CH_{2\ Arg}$), 36.2 ($\beta CH_{2\ Asp}$), 38.2 ($\beta CH_{2\ Phe}$), 41.2 ($\varepsilon CH_{2\ Lys}$), 41.4 ($\delta CH_{2\ Arg}$), 44.8 ($\alpha CH_{2\ Gly}$), 50.5 (αCH_{Asp}), 56.1 (αCH_{Arg}), 56.2 (αCH_{Phe}), 56.3 (αCH_{Lys}), 67.7 ($COOCH_2Ph$), 67.9 ($COOCH_2Ph$), 127.0 (CH_{Ar}), 128.1 (CH_{Ar}), 128.9 (CH_{Ar}), 129.2 (CH_{Ar}), 129.3 (CH_{Ar}), 129.5 (CH_{Ar}), 129.6 (CH_{Ar}), 129.7 (CH_{Ar}), 129.8 (CH_{Ar}), 130.2 (CH_{Ar}), 130.4 (CH_{Ar}), 136.6 (C_{Ar}), 137.4 (C_{Ar}), 137.8 (C_{Ar}), 141.7 (C_{Ar}), 143.4 (C_{Ar}), 158.1 ($NHCOO_Z$), 158.5 (C_{Gdn}), 171.7 (NHCO), 171.8 ($COOCH_2Ph$), 172.1 (NHCO), 173.4 (NHCO), 173.6 (NHCO), 174.3 (NHCO).

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{49}H_{59}N_9O_{11}SNa$: 1004.3952; found: 1004.3948.

Fmoc-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (11)

Et_2NH (17.8 mL, 175.5 mmol, 30 equiv) was added dropwise to a stirred mixture of **5** (4.05 g, 5.75 mmol, 1 equiv) in CH_2Cl_2 (350 mL) at r.t. and stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 99:1 and 95:5). Then, the mixture was evaporated and dried overnight under vacuum. The non-polar fluorenyl side product was removed by flash chromatography (silica gel, CH_2Cl_2–MeOH, 95:5 to 9:1). Then, the crude Gly-Asp(OBn)-D-Phe-O*t*-Bu was directly used in the second peptide coupling step.

2,4,6-Collidine (0.76 mL, 5.75 mmol, 1.0 equiv) was added to a mixture of Gly-Asp(OBn)-D-Phe-O*t*-Bu (approx. 5.75 mmol; calculated as quantitative yield after the first step; Fmoc-deprotection as described above), Fmoc-Arg(NO_2)-OH (3.55 g, 8.05 mmol, 1.4 equiv) and PyAOP (4.20 g, 8.05 mmol, 1.4 equiv) in DMF (30 mL) at 0 °C. The mixture was stirred for 5 h at the same temperature and then stored in a refrigerator at 4 °C for 2 d (checked by TLC, CH_2Cl_2–MeOH, 95:5). Then, the mixture was concentrated under reduced pressure and purified by column chromatography (silica gel, CH_2Cl_2–MeOH, 95:5; R_f = 0.22) to afford **11** (3.90 g, 4.30 mmol; 75%) as colorless crystals; mp 110–111 °C.

^{1}H NMR (400 MHz, $CDCl_3$–CD_3OD, 9:1): δ = 1.35 [s, 9 H, $C(CH_3)_3$], 1.61 (m, 2 H, $\gamma CH_{2\ Arg}$), 1.67 (m, 1 H, $\beta CH_{2\ Arg}$), 1.83 (m, 1 H, $\beta CH_{2\ Arg}$), 2.74 (m, 2 H, $\beta CH_{2\ Asp}$), 2.97 (dd, ^3J = 7.5 Hz, ^2J = 14.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.04 (dd, ^3J = 6.5 Hz, ^2J = 14.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.22 (m, 1 H, $\delta CH_{2\ Arg}$), 3.83 (m, 2 H, $\alpha CH_{2\ Gly}$), 4.15 (m, 2 H, OCH_2CH_{Fmoc}, αCH_{Arg}), 4.35 (dd, ^3J = 6.5 Hz, ^2J = 10.6 Hz, 1 H, OCH_2CH_{Fmoc}), 4.42 (dd, ^3J = 6.8 Hz, ^2J = 10.6 Hz, 1 H, OCH_2CH_{Fmoc}), 4.59 (t, ^3J = 6.8 Hz, 1 H, αCH_{Phe}), 4.80 (t, ^3J = 6.5 Hz, 1 H, αCH_{Asp}), 5.04 (s, 2 H, $COOCH_2Ph$), 7.10–7.39 (m, 14 H, 10 ArH, 4 Fmoc), 7.57 (t, ^3J = 6.8 Hz, 2 H, Fmoc), 7.73 (d, ^3J = 7.5 Hz, 2 H, Fmoc). Signals NH were not detected in the spectrum.

^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD, 9:1): δ = 27.8 [$C(CH_3)_3$], 29.0 ($\gamma CH_{2\ Arg}$), 29.1 ($\beta CH_{2\ Arg}$), 36.0 ($\beta CH_{2\ Asp}$), 37.8 ($\beta CH_{2\ Phe}$), 40.6 ($\delta CH_{2\ Arg}$), 42.8 ($\alpha CH_{2\ Gly}$), 47.2 (OCH_2CH_{Fmoc}), 49.3 (αCH_{Asp}), 54.3 (αCH_{Phe} and αCH_{Arg}), 66.9 ($COOCH_2Ph$), 67.0 (OCH_2CH_{Fmoc}), 82.7 [$C(CH_3)_3$], 120.0 (CH_{Fmoc}), 125.0 (CH_{Fmoc}), 125.1 (CH_{Ar}), 126.9 (CH_{Fmoc}), 127.2 (CH_{Fmoc}), 127.8 (CH_{Ar}), 128.3 (CH_{Ar}), 128.4 (CH_{Ar}), 128.6 (CH_{Ar}), 129.4 (CH_{Ar}), 135.5 (C_{Ar}), 136.3 (C_{Ar}), 141.4 (C_{Fmoc}), 143.7 (C_{Fmoc}), 157.0 ($NHCOO_{Fmoc}$), 159.2 (C_{Gdn}), 169.3 (NHCO), 170.1 (*C*O*t*-Bu), 170.8 (NHCO), 171.0 ($COOCH_2Ph$), 173.3 (NHCO).

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{47}H_{54}N_8O_{11}$: 907.3990; found: 907.3962.

Boc-Lys(Z)-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (12)

Et_2NH (11.2 mL, 108.2 mmol, 30 equiv) was added dropwise to a stirred mixture of **11** (3.27 g, 3.61 mmol, 1 equiv) in CH_2Cl_2 (300 mL) at r.t. and stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 95:5 and 9:1). Then, the mixture was evaporated and dried overnight under vacuum. The nonpolar fluorenyl byproduct was removed by flash chromatography (silica gel, CH_2Cl_2–MeOH, 95:5 to 9:1). Then, the crude Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu was directly used in the second peptide coupling step.

DIPEA (0.94 mL, 5.42 mmol, 1.5 equiv) and EDC (0.87 g, 4.51 mmol, 1.25 equiv) were added successively to a mixture of Arg(NO_2)-Gly-Asp(OBn)-D-Phe-O*t*-Bu (approx. 3.61 mmol; calculated as quantitative yield after the first step; Fmoc-deprotection as described above), Boc-Lys(*Z*)-OH (1.51 g, 3.97 mmol, 1.1 equiv) and HOBt (0.73 g, 5.42 mmol, 1.5 equiv) in CH_2Cl_2 (400 mL) at 0 °C. The mixture was warmed to r.t. and stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 95:5). Then, the mixture was concentrated under reduced pressure and purified by column chromatography (silica gel, CH_2Cl_2–MeOH, 95:5; R_f = 0.14) to afford **12** (2.95 g, 2.82 mmol; 78%) as colorless crystals; mp 139–140 °C.

^{1}H NMR (400 MHz, CD_3OD): δ = 1.33 (m, 2 H, $\gamma CH_{2\ Lys}$), 1.39 [s, 9 H, $C(CH_3)_3$], 1.42 [s, 9 H, $C(CH_3)_3$], 1.47 (m, 4 H, $\beta CH_{2\ Lys}$, $\delta CH_{2\ Lys}$), 1.61 (m, 2 H, $\gamma CH_{2\ Arg}$), 1.71 (m, 2 H, $\beta CH_{2\ Arg}$), 2.70 (dd, ^3J = 7.5 Hz, ^2J = 16.4 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.79 (dd, ^3J = 6.1 Hz, ^2J = 16.4 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.98 (dd, ^3J = 8.1 Hz, ^2J = 14.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.07 (dd, ^3J = 6.6 Hz, ^2J = 14.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.09 (m, 2 H, $\varepsilon CH_{2\ Lys}$), 3.25 (m, 2 H, $\delta CH_{2\ Arg}$), 3.84 (m, 2 H, $\alpha CH_{2\ Gly}$), 3.98 (dd, ^3J = 5.6 Hz, ^3J = 8.5 Hz, 1 H, αCH_{Lys}), 4.36 (m, 1 H, αCH_{Arg}), 4.52 (dd, ^3J = 6.6 Hz, ^3J = 8.0 Hz, 1 H, αCH_{Phe}), 4.82 (dd, ^3J = 6.1 Hz, ^3J = 7.5 Hz, 1 H, αCH_{Asp}), 5.05 (s, 2 H, $COOCH_2Ph$), 5.09 (s, 2 H, $COOCH_2Ph$), 7.18–7.33 (m, 15 H, ArH). Signals NH were not detected in the spectrum.

^{13}C NMR (100 MHz, CD_3OD): δ = 24.0 ($\gamma CH_{2\ Lys}$), 28.2 [$C(CH_3)_3$], 28.8 [$C(CH_3)_3$], 29.8 ($\gamma CH_{2\ Arg}$), 29.9 ($\beta CH_{2\ Arg}$), 30.5 ($\delta CH_{2\ Lys}$), 32.5 ($\beta CH_{2\ Lys}$), 37.1 ($\beta CH_{2\ Asp}$), 38.5 ($\beta CH_{2\ Phe}$), 41.4 ($\varepsilon CH_{2\ Lys}$), 41.7 ($\delta CH_{2\ Arg}$), 43.7 ($\alpha CH_{2\ Gly}$), 50.9 (αCH_{Asp}), 54.4 (αCH_{Arg}), 56.0 (αCH_{Phe}), 56.2 (αCH_{Lys}), 67.3 ($COOCH_2Ph$), 67.7 ($COOCH_2Ph$), 80.8 [$C(CH_3)_3$], 83.1 [$C(CH_3)_3$], 127.9 (CH_{Ar}), 128.8 (CH_{Ar}), 128.9 (CH_{Ar}), 129.2 (CH_{Ar}), 129.3 (CH_{Ar}), 129.4 (CH_{Ar}), 129.5 (CH_{Ar}), 129.6 (CH_{Ar}), 130.5 (CH_{Ar}), 137.3 (C_{Ar}), 138.1 (C_{Ar}), 138.4 (C_{Ar}), 158.2 ($NHCOO_{Boc}$), 158.9 ($NHCOO_Z$), 160.9 (C_{Gdn}), 171.2 (NHCO), 171.7 (*C*O*O*CH_2Ph), 171.9 (*C*O*O*t-Bu), 172.1 (NHCO), 174.5 (NHCO), 175.7 (NHCO).

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{51}H_{71}N_{10}O_{14}$: 1047.5151; found: 1047.5133.

Lys(Z)-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-OH·TFA (13)

TFA (10 mL, 131 mmol, 137 equiv) was added dropwise to a stirred mixture of **12** (1 g, 0.96 mmol, 1 equiv) in CH_2Cl_2 (200 mL) at 0 °C. Then, the mixture was warmed to r.t. and stirred overnight (checked by MS and TLC, CH_2Cl_2–MeOH, 95:5 and 9:1), evaporated and dried overnight under vacuum. The resulting ammonium salt of linear pentapeptide Lys(Z)-Arg(NO_2)-Gly-Asp(OBn)-D-Phe-OH (**13**) was directly used in the second cyclization step.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{42}H_{55}N_{10}O_{12}$: 891.4001; found: 891.3987.

***cyclo*[Arg(NO$_2$)-Gly-Asp(OBn)-D-Phe-Lys(*Z*)] (14)**

DPPA (0.79 g, 2.88 mmol, 3 equiv) was added successively to a mixture of Lys(Z)-Arg(NO$_2$)-Gly-Asp(OBn)-D-Phe-OH (**13**, approx. 0.96 mmol; calculated as quantitative yield after the first step: Boc and *tert*-butyl ester deprotection) and $NaHCO_3$ (0.4 g, 4.8 mmol, 5 equiv) in DMF (1 L) at r.t. The mixture was stirred overnight (checked by TLC, CH_2Cl_2–MeOH, 9:1). Then, the solid $NaHCO_3$ was filtered off and DMF evaporated. The residue was dissolved in a mixture of CH_2Cl_2–DMF (95:5, 500 mL) and extracted with H_2O (3 × 300 mL). The organic layer was evaporated and dried overnight under vacuum. The crude product was purified by crystallization (MeOH) to afford **14** (0.71 g, 0.81 mmol; 85%) as colorless crystals; mp 202 °C (dec.); R_f = 0.42 (CH_2Cl_2–MeOH, 9:1).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.05 (m, 2 H, $\gamma CH_{2\ Lys}$), 1.28–1.65 (m, 7 H, 2 $\beta CH_{2\ Lys}$, 2 $\delta CH_{2\ Lys}$, 2 $\gamma CH_{2\ Arg}$, 1 $\beta CH_{2\ Arg}$), 1.74 (m, 1 H, $\beta CH_{2\ Arg}$), 2.59 (dd, ^3J = 6.0 Hz, ^2J = 15.9 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.79–3.00 (m, 5 H, 1 $\beta CH_{2\ Asp}$, 2 $\beta CH_{2\ Phe}$, 2 $\varepsilon CH_{2\ Lys}$), 3.15 (m, 2 H, $\delta CH_{2\ Arg}$), 3.28 (dd, ^2J = 14.9 Hz, ^3J = 4.0 Hz, 1 H, $\alpha CH_{2\ Gly}$), 3.95 (m, 1 H, αCH_{Lys}), 4.08 (dd, ^2J = 14.9 Hz, ^3J = 7.4 Hz, 1 H, $\alpha CH_{2\ Gly}$), 4.21 (m, 1 H, αCH_{Arg}), 4.49 (m, 1 H, αCH_{Phe}), 4.76 (m, 1 H, αCH_{Asp}), 5.05 (s, 2 H, $COOCH_2Ph$), 5.09 (s, 2 H, $COOCH_2Ph$), 7.14–7.46 (m, 16 H, 15 ArH, εNH_{Lys}), 7.57 (d, ^3J = 8.0 Hz, 1 H, αNH_{Arg}), 8.06 (d, ^3J = 7.3 Hz, 1 H, αNH_{Lys}), 8.12 (d, ^3J = 7.3 Hz, 1 H, αNH_{Phe}), 8.17 (d, ^3J = 8.4 Hz, 1 H, αNH_{Asp}), 8.44 (dd, ^3J = 4.1 Hz, ^3J = 7.4 Hz, 1 H, αNH_{Gly}). Signals NH_{Gdn} were not observed in the spectrum.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 23.7 ($\gamma CH_{2\ Lys}$), 29.6 ($\beta CH_{2\ Arg}$), 29.8 ($\delta CH_{2\ Lys}$), 31.6 ($\beta CH_{2\ Lys}$), 31.8 ($\gamma CH_{2\ Arg}$), 36.1 ($\beta CH_{2\ Asp}$), 38.4 ($\beta CH_{2\ Phe}$), 40.9 ($\varepsilon CH_{2\ Lys}$), 41.0 ($\delta CH_{2\ Arg}$), 44.2 ($\alpha CH_{2\ Gly}$), 49.8 (αCH_{Asp}), 52.8 (αCH_{Arg}), 55.3 (αCH_{Phe}), 55.6 (αCH_{Lys}), 66.1 ($COOCH_2Ph$), 67.5 ($COOCH_2Ph$), 128.6 (CH_{Ar}), 128.7 (CH_{Ar}), 128.8 (CH_{Ar}), 128.9 (CH_{Ar}), 129.1 (CH_{Ar}), 129.2 (CH_{Ar}), 129.3 (CH_{Ar}), 129.4 (CH_{Ar}), 130.0 (CH_{Ar}), 137.0 (C_{Ar}), 138.1 (C_{Ar}), 138.2 (C_{Ar}), 157.0 ($NHCOO_Z$), 160.2 (C_{Gdn}), 170.6 (NHCO), 170.7 ($COOCH_2Ph$), 170.9 (NHCO), 171.5 (NHCO), 172.2 (NHCO), 172.9 (NHCO).

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{42}H_{53}N_{10}O_{11}$: 873.3895; found: 873.3854.

***cyclo*[Arg-Gly-Asp-D-Phe-Lys]·AcOH (1) via 'Tosyl' Route**

***cyclo*[Arg(Ts)-Gly-Asp-D-Phe-Lys] (10)**

Pd/C (10 g, 10% weight) was added to a soln of **9** (2.25 g, 2.29 mmol) in a mixture of CH_2Cl_2–MeOH (1:1, 400 mL). The mixture was purged with H_2 three times and stirred under H_2 atmosphere overnight at r.t. The suspension was filtered through a short pad of Celite, washed with MeOH, concentrated and dried overnight under vacuum to afford colorless crystals of the *cyclo*[Arg(Ts)-Gly-Asp-D-Phe-Lys] (**10**, 1.65 g, 2.18 mmol; 95%) which was directly used in the next *N*-tosyl deprotection step.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{34}H_{48}N_9O_9S$: 758.3296; found: 758.3294.

HRMS (ESI–): *m*/*z* [M – H]$^+$ calcd for $C_{34}H_{46}N_9O_9S$: 756.3139; found: 756.3113.

***cyclo*[Arg-Gly-Asp-D-Phe-Lys]·AcOH (1)**

A mixture of *cyclo*[Arg(Ts)-Gly-Asp-D-Phe-Lys] (**10**, 1.53 g, 2.02 mmol, 1 equiv) and anhyd anisole (1 mL) was cooled to –78 °C in a Teflon flask. The anhyd HF (8 mL) was slowly bubbled and condensed from a HF-bomb attached to a Teflon flask via a septum and a Teflon-cannula. The mixture was then warmed to 0 °C and stirred for 2 h. The HF was removed at 0 °C under vacuum (a Teflon-flask with the mixture was connected to vacuum via a safety flask with silica gel). The residue was dried overnight, dissolved in 5% aq AcOH soln and overfilled from a Teflon flask into a glass flask, lyophilized and purified by crystallization (Et_2O–MeOH, 1:1) to afford *cyclo*[Arg-Gly-Asp-D-Phe-Lys]·AcOH (**1**) (1.05 g, 1.74 mmol; 86%; the purity >95% according to NMR) as colorless crystals.

***cyclo*[Arg-Gly-Asp-D-Phe-Lys]·AcOH (1) via 'Nitro' Route**

Pd/C (4.5 g, 10% weight) was added to a soln of **14** (1.5 g, 1.72 mmol) in a mixture of AcOH–MeOH (1:1, 200 mL). The mixture was purged with H_2 three times and stirred under H_2 atmosphere overnight at r.t. The suspension was filtered through a short pad of Celite, washed with a mixture of AcOH–MeOH (1:5), concentrated, and dried overnight under vacuum to afford colorless crystals of *cyclo*[Arg-Gly-Asp-D-Phe-Lys]·AcOH (**1**) (1.03 g, 1.71 mmol; 99%); purity >95% (NMR); mp 191 °C (dec.).

^{1}H NMR (500 MHz, D_2O): δ = 0.70 (m, 2 H, $\gamma CH_{2\ Lys}$), 1.27–1.57 (m, 7 H, 2 $\beta CH_{2\ Lys}$, 2 $\delta CH_{2\ Lys}$, 2 $\gamma CH_{2\ Arg}$, 1 $\beta CH_{2\ Arg}$), 1.72 (m, 1 H, $\beta CH_{2\ Arg}$), 1.76 (s, 3 H, CH_3CO), 2.39 (dd, ^3J = 7.2 Hz, ^2J = 15.4 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.51 (dd, ^3J = 7.0 Hz, ^2J = 15.4 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.71 (t, ^3J = 7.6 Hz, 2 H, $\varepsilon CH_{2\ Lys}$), 2.81 (m, 1 H, $\beta CH_{2\ Phe}$), 2.97 (dd, ^3J = 5.3 Hz, ^2J = 13.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.05 (m, 2 H, $\delta CH_{2\ Arg}$), 3.32 (d, ^2J = 14.6 Hz, 1 H, $\alpha CH_{2\ Gly}$), 3.73 (m, 1 H, αCH_{Lys}), 4.05 (d, ^2J = 14.6 Hz, 1 H, $\alpha CH_{2\ Gly}$), 4.26 (m, 1 H, αCH_{Arg}), 4.42 (dd, ^3J = 5.3 Hz, ^3J = 10.5 Hz, 1 H, αCH_{Phe}), 4.56 (m, 1 H, αCH_{Asp}), 7.11–7.25 (m, 5 H, ArH). Signals NH were not detected in the spectrum.

^{13}C NMR (125 MHz, D_2O): δ = 22.0 ($\gamma CH_{2\ Lys}$), 23.1 (CH_3CO), 24.2 ($\gamma CH_{2\ Arg}$), 25.9 ($\delta CH_{2\ Lys}$), 27.3 ($\beta CH_{2\ Arg}$), 29.5 ($\beta CH_{2\ Lys}$), 36.5 ($\beta CH_{2\ Phe}$), 37.9 ($\beta CH_{2\ Asp}$), 38.9 ($\varepsilon CH_{2\ Lys}$), 40.3 ($\delta CH_{2\ Arg}$), 43.7 ($\alpha CH_{2\ Gly}$), 50.7 (αCH_{Asp}), 52.3 (αCH_{Arg}), 54.7 (αCH_{Phe}), 55.6 (αCH_{Lys}), 127.1 (CH_{Ar}), 128.7 (CH_{Ar}), 129.1 (CH_{Ar}), 135.9 (C_{Ar}), 156.6 (C_{Gdn}), 171.2 ($NHCO_{Gly}$), 172.3 ($NHCO_{Asp}$), 172.5 ($NHCO_{Arg}$), 173.5 ($NHCO_{Phe}$), 174.1 ($NHCO_{Lys}$), 176.6 (CH_3CO), 177.8 ($COOH_{Asp}$).

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{27}H_{42}N_9O_7$: 604.3207; found: 604.3207.

HRMS (ESI–): *m*/*z* [M – H]$^+$ calcd for $C_{27}H_{40}N_9O_7$; 602.3051; found: 602.3057.

The spectroscopic data of the *cyclo*[Arg-Gly-Asp-D-Phe-Lys] (**1**) were in full agreement with those reported in the literature.[4a]

***N*-ε-Azido *cyclo*[Arg-Gly-Asp-D-Phe-Lys] (2)**

Triflyl azide preparation: NaN_3 (0.216 g, 3.32 mmol, 10 equiv) was dissolved in distilled H_2O (8 mL) and CH_2Cl_2 (20 mL) was added. The mixture was cooled in an ice bath and Tf_2O (0.11 mL, 0.664 mmol, 2 equiv) was added dropwise over 5 min. The mixture was stirred for 2 h. The phases were separated and the organic phase was extracted with H_2O (3 × 10 mL). The organic phase was directly used in the next diazo-transfer step.

cyclo[Arg-Gly-Asp-D-Phe-Lys] (**1**, 0.2 g, 0.332 mmol, 1 equiv) was dissolved in a mixture of *t*-BuOH –H_2O (1:1, 20 mL) at r.t. Then, pH was adjusted to 10 by the addition of 1 M NaOH soln. The mixture was treated with $CuSO_4 \cdot 5\ H_2O$ (8 mg, 0.033 mmol, 0.1 equiv), a soln of TfN_3 in CH_2Cl_2 (20 mL, prepared as described above) and stirred overnight at r.t. (checked by MS). Then, the mixture was extracted with CH_2Cl_2 (3 × 10 mL) and the aqueous peptide phase lyophilized. The crude product was purified by Sephadex G-25 gel chromatography (H_2O as solvent) to afford *N*-ε-azido *cyclo*[Arg-Gly-Asp-D-Phe-Lys] (**2**) (0.188 g, 0.299 mmol; 90%; >95% purity according to NMR analysis) as colorless crystals; mp 199 °C (dec.).

IR (film): 2099 cm^{-1}.

^{1}H NMR (500 MHz, D_2O): δ = 0.75 (m, 2 H, $\gamma CH_{2\ Lys}$), 1.26–1.59 (m, 7 H, 2 $\beta CH_{2\ Lys}$, 2 $\delta CH_{2\ Lys}$, 2 $\gamma CH_{2\ Arg}$, 1 $\beta CH_{2\ Arg}$), 1.71 (m, 1 H, $\beta CH_{2\ Arg}$), 2.38 (dd, ^3J = 7.2 Hz, ^2J = 15.7 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.53 (dd, ^3J = 7.0 Hz, ^2J = 15.7 Hz, 1 H, $\beta CH_{2\ Asp}$), 2.80 (m, 1 H, $\beta CH_{2\ Phe}$), 3.00 (dd, ^3J = 5.4 Hz, ^2J = 13.0 Hz, 1 H, $\beta CH_{2\ Phe}$), 3.05

(m, 2 H, $\delta CH_{2\ Arg}$), 3.09 (t, ^3J = 7.0 Hz, 2 H, $\varepsilon CH_{2\ Lys}$), 3.33 (d, ^2J = 15.2 Hz, 1 H, $\alpha CH_{2\ Gly}$), 3.71 (m, 1 H, αCH_{Lys}), 4.06 (d, ^2J = 15.2 Hz, 1 H, $\alpha CH_{2\ Gly}$), 4.27 (m, 1 H, αCH_{Arg}), 4.43 (dd, ^3J = 5.5 Hz, ^3J = 10.8 Hz, 1 H, αCH_{Phe}), 4.56 (m, 1 H, αCH_{Asp}), 7.06–7.28 (m, 5 H, ArH). Signals NH were not detected in the spectrum.

^{13}C NMR (125 MHz, D_2O): δ = 22.4 ($\gamma CH_{2\ Lys}$), 24.1 ($\gamma CH_{2\ Arg}$), 27.1 ($\delta CH_{2\ Lys}$), 27.3 ($\beta CH_{2\ Arg}$), 29.8 ($\beta CH_{2\ Lys}$), 36.6 ($\beta CH_{2\ Phe}$), 37.7 ($\beta CH_{2\ Asp}$), 40.4 ($\delta CH_{2\ Arg}$), 43.7 ($\alpha CH_{2\ Gly}$), 50.5 ($\varepsilon CH_{2\ Lys}$), 50.7 (αCH_{Asp}), 52.2 (αCH_{Arg}), 55.2 (αCH_{Lys}), 55.3 (αCH_{Phe}), 127.2 (CH_{Ar}), 128.8 (CH_{Ar}), 129.1 (CH_{Ar}), 135.8 (C_{Ar}), 156.6 (C_{Gdn}), 171.2 ($NHCO_{Gly}$), 172.3 ($NHCO_{Asp}$), 172.4 ($NHCO_{Arg}$), 173.4 ($NHCO_{Phe}$), 174.4 ($NHCO_{Lys}$), 177.9 ($COOH_{Asp}$).

HRMS (ESI+): *m/z* $[M + H]^+$ calcd for $C_{27}H_{40}N_{11}O_7$: 630.3112; found: 630.3101.

HRMS (ESI–): *m/z* $[M - H]^+$ calcd for $C_{27}H_{38}N_{11}O_7$: 628.2956; found: 628.2944.

The spectroscopic data of the *N*-ε-azido *cyclo*[Arg-Gly-Asp-D-Phe-Lys] (**2**) were in full agreement with those reported in the literature.[4e]

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