Supplementary Material (ESI) for Chemical Communications

A Macrocyclic Coumarin-Containing Tripeptide *via* CuAAC Chemistry

Sander S. van Berkel, Bas van der Lee, Floris L. van Delft and Floris P. J. T. Rutjes*
Institute for Molecules and Materials, Radboud University Nijmegen,
Heyendaalseweg 135, NL-6525 AJ, Nijmegen, The Netherlands.
Fax: (+31) 24 365 2929; Tel: (+31) 24 365 2381
E-mail: F.Rutjes@science.ru.nl;

Supplementary information

Instrumentations

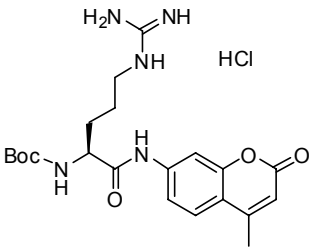
NMR spectra were recorded on *Bruker DPX200* (200 MHz and 50 MHz for ^{1}H and ^{13}C, respectively), *Bruker DMX300* (300 MHz and 75 MHz for ^{1}H and ^{13}C, respectively) and *Varian inova 400* spectrometers. ^{1}H-NMR chemical shifts (δ) are reported in parts per million (ppm) relative to a residual proton peak of the solvent, δ = 3.31 for CD_3OD, δ = 7.26 for $CDCl_3$, and δ = 4.79 for D_2O. Multiplicities are reported as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), dq (double quartet), ddd (double, double doublet), ddt (double, double triplet) or m (multiplet). Broad peaks are indicated by br. Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ^{13}C NMR chemical shifts (δ) are reported in ppm relative to CD_3OD (δ = 49.0) or $CDCl_3$ (δ = 77.0). Electrospray LC/MS analysis was performed using a *Shimadzu LC/MS 2010A system*. Matrix assisted laser desorption/ionisation time-of-flight (MALDI-ToF) spectra were measured on a *Bruker Biflex III* spectrometer and samples were prepared from MeOH solutions using indoleacrylic acid (IAA) (20 mg/mL) as a matrix. LCQ/MS analysis was performed using *Thermo scientific Advantage LCQ* Lineair-Iontrap Electrospray (ESI-MS). Electrospray ionisation time-of-flight (ESI-ToF) spectra were measured with a *JEOL AccuToF*. FT-IR spectra were recorded on an *ATI Matson Genesis Series FTIR* spectrometer with a fitted ATR cell. The vibrations (ν) are given in cm^{-1}. Fluorescence measurents were performed on an Ascent reader, Thermolab systems OY fluorometer equipped with a 390/460 filter set (excitation /emission).

Methods and materials

Unless otherwise stated, all chemicals were obtained from commercial sources and used without further purification. THF was distilled under nitrogen from sodium/benzophenone, CH_2Cl_2, EtOAc and Et_2O were distilled under nitrogen from CaH_2. Analytical thin layer chromatography (TLC) was performed on *Merck* precoated silica gel 60 F-254 plates (layer thickness 0.25 mm) with visualization by ultraviolet (UV) irradiation at λ = 254 nm and/or λ = 366 nm and/or staining with $KMnO_4$. Purifications by silica gel chromatography were performed using *Acros* (0.035 – 0.070 mm, pore diameter ca. 6 nm) silica gel. Preparative thin layer chromatography (Prep-TLC) was performed on *Merck* precoated silica gel 60 F-254 plates (layer thickness 1.00 mm) with concentration zone and visualization by UV irradiation at λ = 254 nm and/or λ = 366 nm. Counter current distribution was carried out using *n*-butanol and water. Water used in the biological procedures was deionised using a *Labconco Water Pro PS* purification system. Bovine serum albumin (BSA) was purchased from *Sigma*, A-7030. Cbz-Gly-Gly-Arg-AMC was purchased from *Bachem*, I-1140. Human thrombin (h-FIIa) and alpha-2-macroglobulin-thrombin complex (α_2M-T) were supplied by *Synapse B.V.*, Maastricht, The Netherlands.

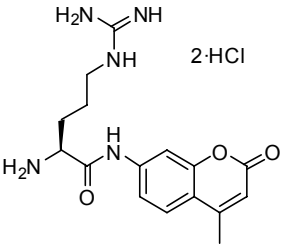
Synthesis

Boc-Arg-AMC·HCl (**I**)



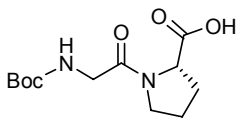
To a cold solution (-15 ºC) of Boc-protected arginine (4.64 g, 5.0 mmol) and 7-amino-4-methyl-coumarin (875 mg, 5.0 mmol) in pyridine (15 mL), phosphoryl chloride (0.51 ml, 5.5 mmol) was added drop wise. A color change going from yellow to orange was observed. The mixture was stirred for 2 hours at -15 ºC and then allowed to warm to room temperature and was stirred for an additional hour. The reaction mixture was quenched with water (15 mL). The solvents were evaporated under reduced pressure. The crude reaction mixture was purification by counter current chromatography using water as stationary phase and *n*-butanol as mobile phase. The product was obtained as a light yellow powder (1.59 g, 68 %). R_F = 0.65 (*n*-BuOH/H_2O/AcOH 4:1:1 v/v). ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 7.70 (d, *J* = 2.13, 1H), 7.60 (d, *J* = 11.6 Hz, 1H), 7.38 (d, *J* = 12 Hz, 1H), 6.13 (d, *J* = 1.6 Hz, 1H), 4.12 (m, 1H), 3.12 (t, *J* = 8.8 Hz, 2H), 2.36 (s, 3H), 1.59 -1.85 (m, 4H), 1.45 (s, 9H). ^{13}C-NMR (50 MHz, CD_3OD) δ (ppm): 173.6, 163.2, 158.6, 158.0, 155.2, 143.3, 126.7, 117.2, 113.6, 108.0, 80.9, 56.4, 42.1, 30.6, 28.8, 26.5, 18.6. HRMS (ESI+) *m/z* calcd for $C_{21}H_{29}N_5O_5$ $[M+H]^+$ 432.2247, found: 432.2247

H-Arg-AMC·2HCl (**II**)



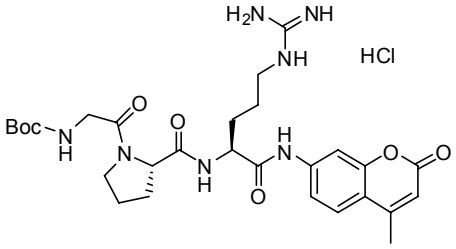
To a suspension of Boc-Arg-AMC·HCl (**I**) (1.18 g, 2.53 mmol) in ether (50 mL), a solution of HCl dissolved in EtOAc (2.6 M, 10 ml) was added. The suspension was stirred overnight at room temperature after which the solvents were removed *in vacuo*. The product was re-suspended in Et_2O (20 mL), stirred for 2 hours and filtered off to yield a white powder (993 mg, 97%). R_F = 0.17 (*n*-BuOH/H_2O/AcOH 4:1:1 v/v). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.91 (s, 1H), 7.76 (d, *J* = 8.8 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 6.27 (s, 1H), 4.14 (m, 1H), 3.27 (t, *J* = 6.8 Hz, 2H), 2.47 (s, 3H), 1.95-2.15 (m, 2H), 1.68-1.82 (m, 2H). ^{13}C-NMR (50 MHz, CD_3OD) δ (ppm): 169.0, 155.4, 155.2, 142.6, 127.0, 117.7, 117.3, 113.9, 108.3, 54.8, 41.8, 29.8, 25.5, 18.7. LRMS (ESI+) *m/z* calcd for $C_{16}H_{21}N_5O_3$ $[M+H]^+$ 332.1, found: 332.2; Anal. $C_{16}H_{21}N_5O_2 \cdot 3.0$ HCl calculated C 45.15% H 5.69% N 16.45%, measured C 45.12% H 5.43% N 16.57%

Boc-Gly-Pro-OH (**III**)



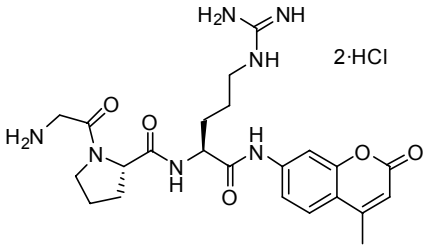
This compound was prepared according to a literature procedure:[S1]
Quantities used; H-Pro-OMe (1.30 g, 7.75 mmol), Boc-Gly-OH (1.26 g, 7.75 mmol), NMM (0.85 mL, 7.75 mmol), HOBt (1.05 g, 7.75 mmol), EtOAc (30 mL) and DCC (1.68 g, 8.14 mmol) The product was purified by column chromatography (CH_2Cl_2/MeOH 9:1). Boc-Gly-Pro-OMe (**III**) was obtained as an off white semisolid (2.15 g (90%)). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 4.46 (dd, *J* = 8.7, 3.4 Hz, 2H), 3.99-3.81 (m, 2H), 3.70 (s, 3H), 3.66-3.52 (m, 2H), 2.28-2.18 (m, 1H), 2.08-1.92 (m, 3H), 1.45 (s, 9H). LRMS (ESI+) *m/z* calcd for $C_{13}H_{22}N_2NaO_5$ $[M+Na]^+$ 309.14, found: 309.1
Subsequent saponification of Boc-Gly-Pro-OMe (2.00 g, 7.50 mmol) in dioxane (13 mL), water (5 mL) using NaOH (2 mL, 2 M). The reaction was stirred for 16 hours at room temperature. After addition of HCl (1.5 mL, 2 M) the product was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried over Na_2SO_4 before the solvents were removed *in vacuo.* The product was obtained as a white powder (1.61 g (79%)). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 4.40 (dd, *J* = 8.7 3.4 Hz, 2H), 3.99-3.81 (m, 2H), 3.66-3.52 (m, 2H), 2.28-2.18 (m, 1H), 2.08-1.92 (m, 3H), 1.45 (s, 9H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 173.7, 168.3, 156.5, 78.7, 58.6, 45.4, 41.5, 28.2, 26.8, 23.8. LRMS (ESI-) *m/z* calcd for $C_{12}H_{19}N_2O_5$ $[M-H]^-$ 271.1, found: 271.1, 542.9 $[2M-H]^-$

Boc-Gly-Pro-Arg-AMC·HCl (**IV**)



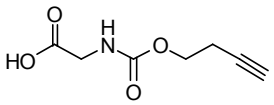
To a cooled solution (0 ºC) of H-Arg-AMC (**II**) (202 mg, 0.5 mmol), Boc-Gly-Pro-OH (**III**) (150 mg, 0.55 mmol), DMAP (122 mg, 1.0 mmol) and HOBt (73.7 mg, 0.55 mmol) in DMF (9 mL), EDC·HCl (116 mg, 0.55 mmol) was added in small portions. The solution was allowed to warm to room temperature and was stirred for 16 hours at room temperature after which the DMF was evaporated *in vacuo*. The crude material was purified using counter current chromatography ($BuOH/H_2O$) to afford an off-white solid (205 mg (70%)) R_F = 0.62 ($BuOH/H_2O$/AcOH 4:1:1). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.87 (d, *J* = 2.0 Hz, 1H), 7.73 (d, *J* = 8.8 Hz, 1H), 7.57 (dd, *J* = 8.8, 2.0 Hz, 1H), 6.25 (d, *J* = 1.2 Hz, 1H), 4.55 (dd, *J* = 4.6, 9.6 Hz, 1H), 4.45 (dd, *J* = 5.0, 8.3 Hz, 1H), 3.93 (q, *J* = 17.0 Hz, 2H), 3.72-3.58 (m, 2H), 3.25 (t, *J* = 7.1 Hz, 2H), 2.34 (d, *J* = 1.2 Hz, 1H), 2.33-2.25 (m, 1H), 2.11-1.94 (m, 4H), 1.87-1.66 (m, 3H), 1.34 (s, 9H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 174.9, 172.9, 172.5, 171.4, 163.2, 158.7, 155.4, 155.2, 143.4, 226.7, 117.4, 117.3, 113.7, 108.2, 80.6, 62.4, 54.9, 48.0, 43.9, 41.9, 30.8, 29.8, 28.7, 26.4, 26.0, 18.6. HRMS (ESI+) *m/z* calcd for $C_{28}H_{40}N_7O_7$ $[M+H]^+$ 586.2989, found: 586.2973.

H-Gly-Pro-Arg-AMC·2HCl (**1**)



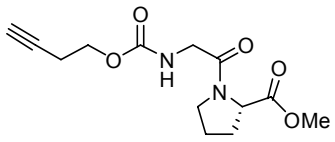
Compound **IV** (200 mg, 0.35 mmol) was suspended in Et_2O (20 mL) and HCl in EtOAc was added (2.6 M, 2 mL). This mixture was stirred for 16 hours and solvents were evaporated *in vacuo*. The crude material was purified by counter current chromatography ($BuOH/H_2O$) and the product was obtained as a white powder (161 mg (95%)). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.87 (d, *J* = 2.0 Hz, 1H), 7.73 (d, *J* = 8.8 Hz, 1H), 7.57 (dd, *J* = 2.0, 8.8 Hz, 1H), 6.25 (d, *J* = 1.2 Hz, 1H), 4.54-4.48 (m, 2H), 3.94 (q, *J* = 12.0 Hz, 2H), 3.77-3.54 (m, 2H), 3.25 (t, *J* = 6.8 Hz, 2H), 2.45 (s, 3H) 2.32-2.25 (m, 1H), 2.08-1.65 (m, 7H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 172.6, 170.7, 165.0, 161.3, 156.7, 153.3, 141.4, 124.8, 115.6, 115.4, 111.8, 106.2, 60.1, 53.6, 40.1, 39.9, 29.1, 28.0, 24.7, 23.8, 16.6. HRMS (ESI+) *m/z* calcd for $C_{23}H_{32}N_7O_5$ $[M+H]^+$ 486.2465, found: 486.2500

N-Butynyloxycarbonyl-Gly-OH (**6a**)



Prepared according to a modified literature procedure: [S2]
To a solution of glycine (150 mg, 2 mmol) in H_2O (15 mL), butynchloroformate (265 mg, 2 mmol) was added drop wise whilst keeping the pH between 9.5 and 10.5 with a 2 M NaOH solution. The reaction was monitored until the pH was stable and then stirred for 72 hours. The solution was transferred to a separation funnel and washed two times with EtOAc (10 mL) after which the water layer was acidified with 2 M HCl (3 mL). The product was extracted from the water layer with EtOAc (2 × 10 mL). The combined organic layers were dried over $MgSO_4$ after which the solvent was removed *in vacuo.* The product was obtained as a yellow oil (332 mg, 97%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.26 (br s, 1H), 4.21 (t, *J* = 6.7 Hz, 2H), 4.04 (d, *J* = 5.7 Hz, 2H), 2.54 (dt, *J* = 6.7, 2.6 Hz, 2H), 2.01 (t, *J* = 2.7 Hz, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.7, 156.1, 77.2, 69.9, 63.2, 42.4, 19.3. HRMS (ESI+) *m/z* calcd. for $C_7H_{10}NO_4$ $[M+H]^+$ 172.0610, found: 172.0605.

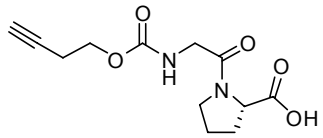
N-Butynyloxycarbonyl-Gly-Pro-OMe (**8**)



To a cooled mixture (0 °C) of *N*-butynyloxycarbonyl-Gly-OH (**6a**) (150 mg, 0.88 mmol), proline methyl ester (113 mg, 0.88 mmol), NMM (89 mg, 0.88 mmol) and HOBt (118 mg, 0.88 mmol) in EtOAc (5 mL), DCC (180 mg, 0.92 mmol) was added in small portions. The mixture was stirred for 30 min at 0 °C and an additional 16 hours at room temperature. DCU was filtered off and the filtrate was transferred to a separation funnel and washed with saturated Na_2CO_3 (2 × 5 mL). The combined aqueous layers were washed with EtOAc (3 × 5 mL) and the combined organic layers were dried over Na_2SO_4 where after

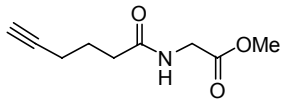
the solvent was removed *in vacuo*. The product was purified by column chromatography (EtOAc/MeOH 20:1) to afford a yellow oil (159 mg, 64%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 4.46 (dd, *J* = 8.7, 4.0 Hz, 1H), 4.12 (dt, *J* = 6.9, 2.3 Hz, 2H), 3.96 (q, *J* = 17.2 Hz, 2H), 3.70 (s, 3H), 3.45-3.65 (m, 2H), 2.51 (dt, *J* = 6.9, 2.7 Hz, 2H), 2.30 (t, *J* = 2.7 Hz, 1H), 2.28-2.18 (m, 1H), 2.11-1.86 (m, 3H). LRMS (ESI+) *m/z* calcd. for $C_{13}H_{18}N_2O_5$ $[M+H]^+$ 283.1, found: 283.1.

N-Butynyloxycarbonyl-Gly-Pro-OH (**5a**)



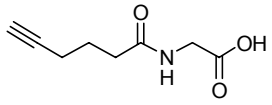
N-Butynyloxycarbonyl-Gly-Pro-OMe (**8**) (158 mg, 0.56 mmol) was dissolved in a mixture of dioxane (13 mL), H_2O (5 mL) and a 2 M NaOH solution (2 mL) was added. The mixture was stirred for 16 hours at room temperature. EtOAc (10 mL) was added to the reaction mixture and it was transferred to a separation funnel. The aqueous layer was acidified with 2 M HCl (2 mL) and washed with EtOAc (3 × 10 mL). The combined organic layers were dried over $MgSO_4$ before the solvent was removed *in vacuo*. The product was purified with gradient column chromatography ($MeOH/CH_2Cl_2$ 1:9 → 1:1) and the product was obtained as yellow oil. (105 mg, 70%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 2.11-1.86 (m, 3H), 2.28-2.18 (m, 1H), 2.30 (t, *J* = 2.7 Hz, 1H), 2.51 (dt, *J* = 6.9, 2.7 Hz, 2H), 3.45-3.65 (m, 2H), 3.96 (q, *J* = 17.2 Hz, 2H), 4.12 (dt, *J* = 6.9, 2.3 Hz, 2H), 4.46 (dd, *J* = 8.7, 4.0 Hz, 1H). LCMS analysis: purity +99%, MS (ESI+) *m/z* calcd. for $C_{12}H_{17}N_2O_5$ $[M+H]^+$ 269.1, found: 269.0.

N-Hex-5-ynoyl-Gly-OMe (**7**)



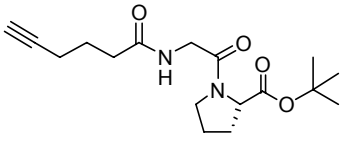
To a cooled solution (0 °C) of H-Gly-OMe·HCl (687.5 mg, 5.5 mmol), 5-hexynoic acid (0.6 mL, 5.5 mmol), and DMAP (1.21 g, 10 mmol) in CH_2Cl_2 (50 mL), EDC·HCl (1.05 g, 5.5 mmol) was slowly added in small portions. The mixture was for 30 min at 0 °C and an additional 16 hours at room temperature. The reaction mixture was poured into 1 M HCl (60 mL) and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layers were washed with sat. $NaHCO_3$ (2 × 50 mL) and dried over Na_2SO_4. The CH_2Cl_2 was removed under reduced pressure to afford the product as a light yellow oil (980 mg, 97%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.00 (br s, 1H, *NH*), 4.05 (d, *J* = 5.2 Hz, 2H), 3.77 (s, 3H), 2.40 (t, *J* = 7.4 Hz, 2H), 2.29 (dt, *J* = 2.7, 6.8 Hz, 2H), 1.98 (t, *J* = 2.7 Hz, 1H), 1.88 (p, *J* = 7.1 Hz, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 172.4, 170.4, 83.4, 69.2, 52.4, 41.2, 34.6, 24.0, 17.8. HRMS (ESI+) *m/z* calcd. for $C_9H_{14}NO_3$ $[M+H]^+$ 184.0974, found: 184.0972.

N-Hex-5-ynoyl-Gly-OH (**6b**)



Compound **7** (550 mg, 3 mmol) was dissolved in THF (30 mL) and cooled to 0 °C. An aqueous solution of NaOH (1 M, 6 mL) was added drop wise. The reaction mixture was stirred for 30 min at 0 °C and an additional 30 min at room temperature. The volume of the reaction was reduced to 50% before aqueous HCl (1 M, 10 mL) and EtOAc (20 mL) were added. The layers were separated after which the water layer was washed with EtOAc (2 × 20 mL). The organic layers were combined and dried over $MgSO_4$. After filtration of $MgSO_4$ the solvents were removed under reduced pressure. The crude product was re-dissolved in EtOAc (30 mL) and an aqueous solution of $NaHCO_3$ (1 M, 20 mL) was added. After extraction and separation, the water layer was acidified with aqueous HCl (2 M, 15 mL) and extracted with EtOAc (2 × 30 mL). The combined organic layers were dried over $MgSO_4$ and after filtration evaporated under reduced pressure. The product was obtained as slightly yellow solid (507 mg, 77%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.19 (br s, 1H, *NH*), 5.36 (br s, 1H, *COOH*), 4.09 (d, *J* = 5.3 Hz, 2H), 2.43 (t, *J* = 7.4 Hz, 2H), 2.29 (dt, *J* = 2.7, 6.8 Hz, 2H), 1.99 (t, *J* = 2.7 Hz, 1H), 1.88 (p, *J* = 6.9 Hz, 2H). ^{13}C-NMR (75 MHz, $CD_3OD/CDCl_3$) δ (ppm): 173.4, 171.6, 83.2, 69.0, 40.9, 34.4, 24.0, 17.6. HRMS (ESI+) *m/z* calcd. for $C_8H_{11}NO_3$ $[M+H]^+$ 170.0817, found: 170.0817.

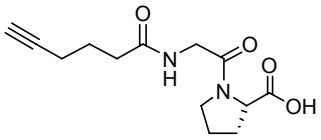
N-Hex-5-ynoyl-Gly-Pro-O^{t}Bu (**9**)



Compound **6b** (169 mg, 1 mmol), H-Pro-O^{t}Bu (180 mg, 1.05 mmol) and DMAP (242 mg, 2 mmol) were dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. To the cooled solution EDC·HCl (210 mg, 1.1 mmol) was added slowly after which the reaction was stirred for 30 min at 0 °C and overnight at room temperature. The reaction mixture was quenched

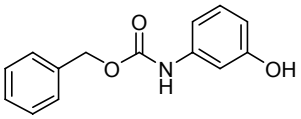
with aqueous HCl (2 M, 20 mL) and the layers were separated. The water layer was extracted with CH_2Cl_2 (2 × 20 mL) and the combined organic layers were dried over Na_2SO_4. After filtration the solvent was removed under reduced pressure. The product was obtained as a yellow oil (315 mg, 97%) ^{1}H-NMR (400 MHz, $CDCl_3$) *S*-cis/trans isomers observed; δ (ppm): 6.51 (br s, 1H), 4.40 (dd, *J* = 8.6, 3.5 Hz) + 4.25 (dd, *J* = 8.2, 2.8 Hz) 2H, AB-system: 4.10 + 3.96 (dd, *J* = 17.7, 4.6 Hz) + AB-system: 4.07 + 3.72 (dd, *J* = 17.2, 3.5 Hz) 2H, 3.67-3.61 (m) + 3.60-3.53 (m) + 3.48- 3.42 (m) 2H, 2.37 (t, *J* = 7.4 Hz) + 2.36 (t, *J* = 7.4 Hz) 2H, 2.27-2.22 (m, 2H), 2.21-1.97 (m, 4H), 1.95 (t, *J* = 2.7 Hz, 1H), 1.89-1.82 (m, 2H), 1.46 (s) + 1.45 (s) 9H. ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 171.7, 170.4, 166.8, 166.3, 82.9, 82.6, 81.2, 68.7, 59.2, 58.8, 46.2, 45.5, 41.6, 41.3, 34.3, 30.9, 28.6, 27.5, 24.0, 23.7, 21.7, 17.5. HRMS (ESI+) *m/z* calcd. for $C_{17}H_{27}N_2O_4$ $[M+H]^+$ 323.1971, found: 323.1966.

N-Hex-5-ynoyl-Gly-Pro-OH (**5b**)



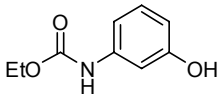
Compound **9** (315 mg, 0.97 mmol) was dissolved in CH_2Cl_2 (15 mL) and TFA (1.5 mL, 20.2 mmol) was added. The reaction was stirred for 16 hours at room temperature. The solvent and excess TFA were removed under reduced pressure after which the crude product was re-dissolved in CH_2Cl_2 (30 mL) and an aqueous solution of $NaHCO_3$ (1 M, 20 mL) was added. After extraction and separation, the water layer was acidified with aqueous HCl (2 M, 15 mL) and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic layers were dried over $MgSO_4$ and after filtration evaporated under reduced pressure. The product was obtained as slightly brown oil (290 mg, 99%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 9.29 (br s, 1H), 7.16 (br s, 1H), 4.53 (dd, *J* = 8.3, 4.0 Hz, 1H), AB-system: 4.19 + 4.04 (dd, *J* = 17.6, 4.6 Hz, 2H), 3.57 (m, 2H), 2.45 (t, *J* = 7.8 Hz, 2H), 2.21 (dt, *J* = 6.8 Hz, 2H), 2.33-2.14 (m, 2H), 2.07 (m, 2H), 1.94 (t, *J* = 2.6 Hz, 1H), 1.82 (m, 2H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 175.7, 175.6, 169.5, 84.3, 70.2, 60.5, 47.4, 42.6, 35.6, 30.2, 25.9, 25.7, 18.7. HRMS (ESI+) *m/z* calcd. for $C_{13}H_{19}N_2O_4$ $[M+H]^+$ 267.1345, found: 267.1349.

N-Carbobenzyloxy-3-aminophenol (**10a**)



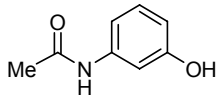
Prepared according to a literature procedure: [S3]
Quantities used; 3-aminophenol (2.5 g, 23 mmol), benzyl chloroformate (2.0 g, 11.5 mmol), Et_2O (175 mL). The product was obtained as a white powder (2.85 g, 51 %). ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 9.62 (s, 1H), 9.33 (s, 1H), 7.41 (m, 5H), 7.02 (t, *J* = 8.1 Hz, 1H), 7.01 (t, *J* = 2.0 Hz, 1H), 6.85 (ddd, *J* = 8.3, 1.9, 0.9 Hz, 1H), 6.38 (ddd, *J* = 8.1, 2.4, 1.0 Hz, 1H), 5.13 (s, 2H). LRMS (ESI+) *m/z* calcd. for $C_{14}H_{14}NO_3$ $[M+H]^+$ 244.1, found: 244.0.

N-Ethoxycarbonyl-3-aminophenol (**10b**)



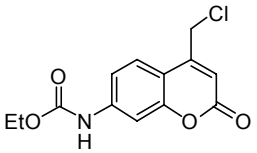
Prepared according to a literature procedure: [S4]
Quantities used; 3-aminophenol (12.8 g, 117 mmol), ethyl chloroformate (25.6 g, 237 mmol), Et_2O (450 mL) and Et_3N (1 mL). The product was obtained as white crystals (11.4 g, 54%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.33 (br, 1H), 7.13 (t, *J* = 8.1 Hz, 1H), 6.66 (dd, *J* = 2.0, 0.8 Hz, 1H), 6.64 (dd, *J* = 2.0, 0.8 Hz, 1H), 6.57 (ddd, *J* = 8.1, 2.4, 0.8 Hz, 1H), 6.19 (br, 1H), 4.23 (q, *J* = 7.1 Hz, 2H), 1.31 (t, *J* = 6.8 Hz, 3H). LRMS (ESI+) *m/z* calcd. for $C_9H_{12}NO_3$ $[M+H]^+$ 182.1, found: 182.1.

N-Acetyl-3-aminophenol (**10c**)



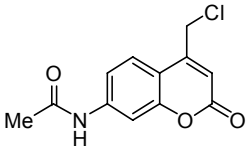
Prepared according to a literature procedure: [S5]
Quantities used; 3-aminophenol (10.9 g, 0.10 mol) acetic anhydride (21.3 mL, 0.22 mol) The product was obtained as a light brown powder (15.0 g, 99%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.15 (t, *J* = 2.1 Hz, 1H), 7.08 (t, *J* = 8.2 Hz, 1H), 6.91 (ddd, *J* = 8.1, 2.0, 1.0 Hz, 1H), 6.52 (ddd, *J* = 8.1, 2.4, 1.0 Hz, 1H), 2.09 (s, 3H). LRMS (ESI+) *m/z* calcd. for $C_8H_{10}NO_2$ $[M+H]^+$ 152.1, found: 152.0.

N-Ethoxycarbonyl-7-amino-4-chloromethylcoumarin (**11b**)



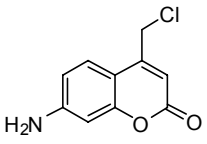
Prepared according to a literature procedure: [S6]
Quantities used; ethyl 4-chloroacetoacetate (2.3 g, 14.4 mmol) *N*-ethoxycarbonyl-3-aminophenol (**10b**) (2.0 g, 12 mmol) and H_2SO_4 (40 mL, 60% in H_2O). The product was obtained as a light pink powder (2.4 g, 70%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 10.19 (br, 1H), 7.75 (d, *J* = 8.8 Hz, 1H), 7.59 (d, *J* = 1.9 Hz, 1H), 7.42 (dd, *J* = 8.8, 2.0 Hz, 1H), 6.51 (s, 1H), 4.97 (s, 2H), 4.17 (d, *J* = 7.1 Hz, 1H),1.26 (t, *J* = 7.1 Hz, 1H). LRMS (ESI+) *m/z* calcd. for $C_{13}H_{13}ClNO_4$ $[M+H]^+$ 282.1, found: 282.1.

N-Acetyl-7-amino-4-chloromethylcoumarin (**11c**)



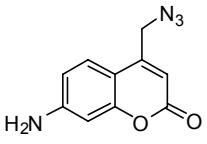
Ethyl 4-chloroacetoacetate (1.8 g, 11.12 mmol) was added to a solution of *N*-acetyl-3-aminophenol (**10c**) (1.4 g, 9.27 mmol) in H_2SO_4 (30 mL, 70% in H_2O). The suspension was stirred for 4 days at 45 °C. The mixture was transferred to a separation funnel and the product was extracted with EtOAc (3 × 35 mL). Solid material was filtered off and the filtrate was dried over anhydrous Na_2SO_4 after which the solvent was removed *in vacuo*. The product was purified two times by column chromatography (MeOH/CH_2Cl_2 1:9) and was obtained as brown powder (465 mg, 20%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 10.42 (s, 1H), 7.81 (d, *J* = 2.0 Hz, 1H), 7.78 (d, *J* = 8.7 Hz, 1H), 7.47 (dd, *J* = 8.8, 2.1 Hz, 1H), 6.54 (s, 1H), 4.98 (d, *J* = 0.7 Hz, 2H), 2.10 (s, 3H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ (ppm): 169.1, 159.8, 154.0, 150.4, 142.8, 125.7, 115.0, 112.9, 112.1, 105.5, 41.1, 24.1. HRMS (ESI+) *m/z* calcd. for $C_{12}H_{11}ClNO_3$ $[M+H]^+$ 252.0428, found: 252.0433.

7-Amino-4-chloromethylcoumarin (**12**)



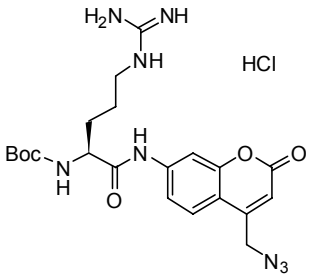
To a solution of concentrated HCl (0.20 mL) in 2-propanol (1 mL) was added *N*-acetyl-7-amino-4-chloromethylcoumarin (**10c**) (200 mg, 1.20 mmol). The solution was heated to reflux and stirred for 16 hours. The mixture was then cooled to room temperature and subsequently transferred to a separation funnel. After addition of H_2O (5 mL), the product was extracted with EtOAc (2 × 15 mL). The combined organic layers were washed with H_2O (10 mL) and dried over Na_2SO_4 after which the solvents were removed *in vacuo.* The product was obtained as a brown solid. (181 mg, 72%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 4.87 (d, *J* = 0.6 Hz, 2H), 6.17 (s, 1H), 6.22 (br s, 2H), 6.44 (d, *J* = 2.2 Hz, 1H), 6.59 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H). ^{13}C-NMR (75 MHz, CD_3OD/DMSO-d_6) δ (ppm): 157.7, 154.9, 153.3, 127.1, 113.0, 109.3, 108.4, 100.6, 42.3. HRMS (ESI+) *m/z* calcd. for $C_{10}H_9ClNO_2$ $[M+H]^+$ 210.3022, found: 210.0322.

7-Amino-4-azidomethylcoumarin (**13**)



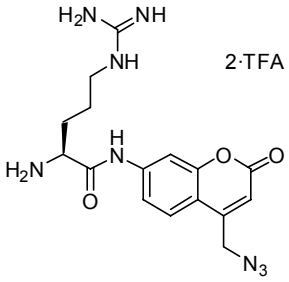
To a suspension of NaN_3 (1.14 g, 17.5 mmol) in acetone and acetonitril (1:1 v/v, 70 mL), 7-Amino-4-chloromethylcoumarin (**12**) (730 mg, 3.5 mmol) was added. The mixture was stirred for 48 hours after which the solvent was evaporated *in vacuo*. The mixture was suspended in EtOAc (35 mL) and the precipitated salts were removed by filtration. The resulting organic layer was dried over Na_2SO_4 and the solvents were removed under reduced pressure. The product was obtained as a brown powder (725 mg, 95%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm): 7.48 (d, *J* = 2.1 Hz, 1H), 6.58 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.44 (d, *J* = 2.1 Hz, 1H), 6.23 (br s, 2H), 6.18 (s, 1H), 4.87 (d, *J* = 0.7 Hz, 2H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 164.2, 157.5, 154.9, 152.7, 126.5, 113.2, 108.6, 107.7, 51.6. HRMS (ESI+) *m/z* calcd. for $C_{10}H_9N_4O_2$ $[M+H]^+$ 217.0726, found: 217.0730. FT-IR ν_{max} film (cm^{-1}): 2569, 2116, 1688, 1601, 1320, 849, 603.

Boc-Arg-7-amino-4-azidomethylcoumarin·HCl (**14**)



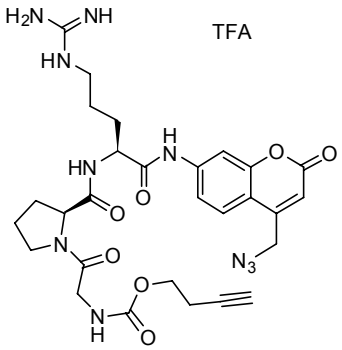
To a cooled solution (-15 °C) of Boc-protected arginine (328 mg, 1.00 mmol) and 7-amino-4-azidomethylcoumarin (**13**) (216 mg, 1.00 mmol) in pyridine (3 mL) was added drop wise phosphoryl chloride (102 mg, 1.1 mmol). The mixture was stirred for 5 min at -15 °C and was allowed to warm to room temperature and stirred for an additional hour. The solvent was evaporated under reduced pressure and purification was performed two times using counter current chromatography (*n*-BuOH/H_2O) to obtain the product as a white powder (208 mg, 41%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.86 (d, *J* = 2.0 Hz, 1H), 7.68 (d, *J* = 8.6 Hz, 1H), 7.50 (d, *J* = 8.6 Hz, 1H), 6.44 (s, 1H), 4.73 (s, 2H), 3.23 (t, *J* = 6.8 Hz, 2H), 1.90 (s, 1H), 1.82-1.62 (m, 3H), 1.46 (s, 9H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 173.6, 162.6, 158.7, 158.0, 155.7, 151.7, 151.6, 143.6, 126.2, 117.3, 144.8, 113.0, 108.3, 81.0, 56.4, 51.4, 42.1, 30.6, 28.7, 26.5. HRMS (ESI+) *m/z* calcd. for $C_{21}H_{29}N_8O_5$ $[M+H]^+$ 473.2261, found: 473.2285.

H-Arg-7-amino-4-azidomethylcoumarin·2TFA (**4**)



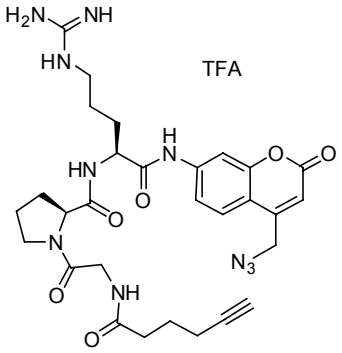
To a suspension of Boc-Arg-7-amino-4-azidomethylcoumarin (**14**) (295 mg, 0.58 mmol) in CH_2Cl_2 (20 mL) was added trifluoroacetic acid (TFA, 0.5 mL, 6.7 mmol). The suspension was stirred for 48 hours after which the solvent and excess TFA were removed *in vacuo* to afford a orange/brown powder (299 mg, 86%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.96 (d, *J* = 2.0 Hz, 1H), 7.72 (d, *J* = 8.7 Hz, 1H), 7.56 (dd, *J* = 8.7, 2.1 Hz, 1H), 6.47 (t, *J* = 1.2 Hz, 1H), 4.74 (d, *J* = 1.3 Hz, 2H), 3.27 (t, *J* = 6.9 Hz, 2H), 2.15-2.02 (m, 2H),1.85-1.68 (m, 2H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 169.0, 162.5, 158.7, 155.6, 151.6, 142.9, 126.3, 117.3, 115.2, 113.2, 108.4, 54.9, 51.4, 41.8, 29.8, 25.6. HRMS (ESI+) *m/z* calcd. for $C_{16}H_{23}Cl_2N_8O_3$ $[M+H]^+$ 445.1270, calcd. for $C_{16}H_{21}N_8O_3$ $[M+H]^+$ 373.1734, found: 373.1747. FT-IR ν_{max} film (cm^{-1}): 3339, 3170, 2142, 1701, 1662, 1610, 1584, 1528, 1416, 1394, 1225, 1001, 858.

N-Butynyloxycarbonyl-Gly-Pro-Arg-7-amino-4-azidomethylcoumarin·TFA (**3a**)



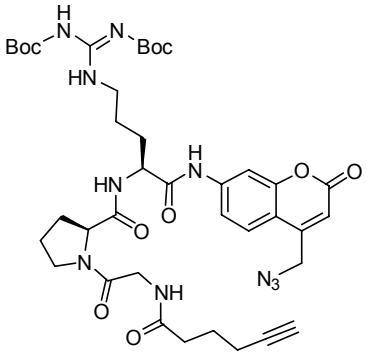
To a cooled solution (0 °C) of *N*-butynyloxycarbonyl-Gly-Pro-OH (**5a**) (113 mg, 0.42 mmol), H-Arg-7-amino-4-azidomethyl-coumarin·2TFA (**4**) (252 mg, 0.42 mmol), HOBt (57 mg, 0.42 mmol) and DMAP (103 mg, 0.42 mmol) in DMF (10 mL), EDC·HCl (89 mg, 0.46 mmol) was slowly added in small portions. The mixture was allowed to warm to room temperature and was stirred for an additional 16 hours. The DMF was removed under reduced pressure and purification was performed three times using counter current chromatography (BuOH/H_2O) to give the product as a yellow powder (66 mg, 24%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.94 (s, 1H), 7.64 (s, 2H), 7.34 (br s, 1H), 7.12 (br s, 1H), 6.42 (s, 1H), 4.71 (s, 2H), 4.51-4.58 (m, 1H), 4.42-4.47 (m, 1H), 3.80-4.05 (m, 4H), 3.55-3.75 (m, 2H), 3.23 (t, *J* = 6.4 Hz, 2H), 2.36 (t, *J* = 6.8 Hz, 2H), 2.26-2.32 (m, 1H), 2.25 (t, *J* = 2.8 Hz, 4H), 1.92-2.10 (m, 4H), 1.63-1.86 (m, 4H), MALDI-TOF (ESI+) *m/z* calcd. for $C_{28}H_{35}N_{10}O_7$ $[M+H]^+$ 623.2690, found: 623.2733, calcd for $C_{28}H_{34}NaN_{10}O_7$ $[M+Na]^+$ 645.2510, found: 645.2515.

N-[(*N*-Hex-5-ynoyl)-Gly-Pro-Arg]-7-amino-4-azidomethylcoumarin·TFA (**3b**)



N-Hex-5-ynoyl-Gly-Pro-OH (**5b**) (133 mg, 0.5 mmol) and H-Arg-7-amino-4-azidomethylcoumarin·2TFA (**4**) (295 mg, 0.5 mmol) were dissolved in DMF (15 mL) and cooled to 0 °C. DMAP (121 mg, 1.0 mmol) and EDC·HCl (105 mg, 0.55 mmol) were added to the cooled solution which was subsequently stirred for 30 min at 0 °C before allowing the mixture to warm to room temperature. The reaction mixture was stirred for an additional 16 hours at room temperature after which the solvent was removed under reduced pressure. The crude mixture was purified by counter current chromatography (*n*-BuOH/H_2O 1:1) to afford a white solid (200 mg, 54%) R_F = 0.36 (BuOH/AcOH/H_2O 4:1:1). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.92 (d, *J* = 1.8 Hz, 1H), 7.70-7.63 (m, 2H), 6.42 (s, 1H), 4.72 (d, *J* = 0.9 Hz, 2H), 4.54 (dd, *J* = 9.7, 4.5 Hz, 1H), 4.47 (dd, *J* = 8.6, 4.8 Hz, 1H), 4.15 + 3.94 (AB-system, *J* = 16.7, 2H), 3.79-3.74 (m, 1H), 3.70-3.64 (m, 1H), 3.25 (dt, *J* = 6.8, 6.8, 6.7 Hz, 2H), 2.42-2.22 (m, 4H), 2.18 (t, *J* = 2.6 Hz, 1H), 2.13 (dt, *J* = 7.0, 2.6 Hz, 2H), 2.09-1.97 (m, 4H), 1.91-1.60 (m, 6H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 176.2, 174.9, 172.5, 171.0, 162.6, 158.7, 155.7, 151.6, 143.6, 126.1, 117.5, 14.9, 113.1, 108.4, 84.1, 70.2, 62.5, 54.90, 54.84, 51.45, 43.2, 41.9, 35.5, 30.8, 29.7, 26.5, 26.0, 25.7, 18.7. HRMS (ESI+) *m/z* calcd. for $C_{29}H_{37}N_{10}O_6$ $[M+H]^+$ 621.2897, found: 621.2882. FT-IR ν_{max} film (cm^{-1}): 3287, 2941, 2111, 1653, 1610, 1528, 1420, 1195, 1135.

N-[(*N*-Hex-5-ynoyl)-Gly-Pro-Arg$^{\omega,\omega'}$(bis-Boc)]-7-amino-4-azidomethylcoumarin (**15**)



Compound **3b** (57 mg, 77.0 µmol) was dissolved in THF (1.5 mL) and DMAP (18.6 mg (0.15 mmol) was added. The reaction mixture was cooled to 0 °C and $(Boc)_2O$ (67.1 mg, 0.31 mmol) was added slowly. The mixture was stirred at 0 °C for 30 min and an additional 72 hours at r.t. The solvent was removed under reduced pressure and the crude mixture was purified by column chromatography (CH_2Cl_2/MeOH 9:1) to afford the $^{\omega,\omega'}$bis-(Boc) product as a white solid (50 mg, 79%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 7.92 (dd, *J* = 6.6 Hz, 1H), 7.74 (ddd, *J* = 8.6, 3.0, 2.0 Hz, 1H), 7.65 (d, *J* = 8.6 Hz, 1H), 6.43 (s, 1H), 4.73 (d, *J* = 0.9 Hz, 2H), 4.47 (m, 2H), 4.12 (d, *J* = 16.4 Hz, 1H), 3.96 (dd, *J* = 16.8, 4.2 Hz, 1H), 3.80-3.73 (m, 1H), 3.68-3.62 (m, 1H), 3.46-3.38 (m, 1H), 2.40-2.20 (m, 4H), 2.16 (t, *J* = 2.6 Hz, 1H), 2.12 (ddt, *J* = 7.0, 4.3, 2.7 Hz, 2H), 2.08-1.98 (m, 6H), 1.88-1.58 (m, 4H), 1.52 (d, *J* = 2.9 Hz, 9H), 1.46 (s, 9H) ^{13}C-NMR (75 MHz,CD_3OD) δ (ppm): 175.9, 174.8, 172.7, 170.9, 162.6, 160.7, 157.6, 155.6, 154.1, 151.6, 143.6, 126.0, 117.4, 114.7, 112.9, 108.3, 85.3, 84.5, 84.2, 80.4, 70.3, 62.6, 55.4, 51.4, 45.5, 43.2, 41.3, 35.4, 30.8, 29.6-29.5 (d), 28.7, 28.3, 27.1-27.0 (d), 26.0, 25.6, 18.7. HRMS (ESI+) *m/z* calcd. for $C_{39}H_{53}N_{10}O_{10}$ $[M+H]^+$ 821.3946, found: 821.3939.

Macrocyclization reactions

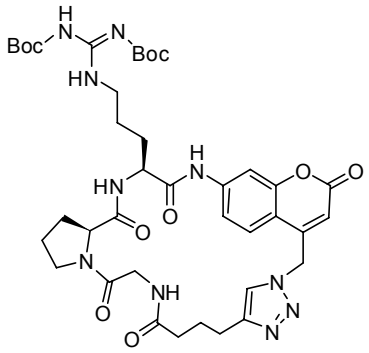
Starting with linear precursor **3a** first the 1,3-dipolar cycloaddition was performed in the presence of copper(I) bromide and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in toluene at 40 °C giving rise to multiple products (Table S1, entry 1). Applying $CuSO_4$ in combination with sodium ascorbate in a solvent mixture of *tert*-butanol/water again produced multiple products, including the desired cyclic product (Table S1, entry 2). Disturbingly, identification of the crude products with ^{1}H-NMR spectroscopy was complicated due to complexation of copper ions to the guanidine moiety giving rise to broadening of the signals. A convenient way to get around this problem involves pressure-promoted cycloaddition in the absence of copper.[S7] Hence, a high pressure experiment was performed (DMF, 50 °C, 5 days), but unfortunately again a variety of products was formed (entry 3). The use of copper-wire activated by Et_3N in a solvent mixture of MeOH/MeCN required prolonged heating (65 °C, 7 days), leading to the formation of both the desired product and by-products (entry 4).
The macrocyclization reactions performed on compound **3b** proceeded more successfully, giving clean conversions to the desired product **2b** according to TLC analysis (Table S1, entries 5 and 6). However, the unprotected side-chain of arginine hindered purification and thus isolation of the product.

Table S1. Copper catalyzed macrocyclization reactions with linear peptides **3a** and **3b**

Entry	Compound	Conditions	Time	Temp	S	P	B
1	**3a**	CuBr, DBU, toluene	48 h	40 °C	✓		✓
2	**3a**	$CuSO_4$, Na-Asc., $^tBuOH/H_2O$	16 h	40 °C	✓	✓	✓
3	**3a**	DMF[a]	5 d	50 °C	✓		✓
4	**3a**	Cu-wire, Et_3N, MeOH/MeCN	7 d	65 °C		✓	✓
5	**3b**	CuI, TBTA[b], Et_3N, DMF	18 h	40 °C		✓	
6	**3b**	Cu-wire, Et_3N, MeCN	18 h	40 °C		✓	

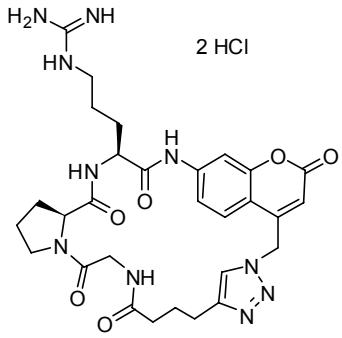
[a] Reaction performed at 15 kPa, no additives, [b] TBTA = *tris*-(benzyltriazolylmethyl)amine, S = starting material, P = product, B = by-product

Cyclo-[-*N*-(*N*-(1,2,3-triazol-4-yl)-butanoyl-glycyl-prolyl-arginyl$^{\omega,\omega'}$(bis-Boc))-7-amino-4-methylenecoumarin-] (**16**)



Compound (**15**) (47 mg, 57.6 µmol) was dissolved in dry and degassed (N_2) THF (25 mL) and kept under a nitrogen atmosphere. A volume of 5 mL of a stock solution containing CuBr (85 mg, 576 µmol) and *N*,*N*,*N*',*N*',*N*''-pentamethyl- diethylenetriamine (PMDETA) (105 mg, 576 µmol) in dry and degassed (N_2) THF (50 mL) was slowly added to the reaction (a colour change of yellow to green is observed). The reaction mixture was warmed to 40 °C and stirred for 7 hours and subsequently quenched with MeOH (10 mL). The solvents were removed under reduced pressure and the crude mixture was purified by preparative TLC (CH_2Cl_2/MeOH 9:1) to afford a light yellow solid (11 mg, 23%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 8.33 (dd, *J* = 4.2, 2.1 Hz, 1H), 7.79 (s, 1H), 7.30 (ddd, *J* = 8.6, 5.7, 2.1 Hz, 1H), 6.77 (d, *J* = 8.76 Hz, 1H), 6.65 (s, 1H), 6.09 + 5.49 (AB-system, *J* = 15.3, 2H), 4.57-4.53 (m, 1H), 4.32 (dd, *J* = 8.8, 5.8 Hz, 1H), 4.24 + 3.86 (AB-system, *J* = 17.2 Hz, 2H), 3.70 (t, *J* = 6.7 Hz, 2H), 3.61-3.57 (m, 1H), 3.40-3.34 (m, 1H), 2.73 (t, *J* = 5.8 Hz, 2H), 2.38-2.11 (m, 3H), 2.06-1.92 (m, 2H), 1.89-1.78 (m, 4H), 1.71-1.56 (m, 4H), 1.51 (d, *J* = 14.5 Hz, 9H), 1.43 (d, *J* = 3.3 Hz, 9H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 175.6, 174.9, 172.4, 171.2, 162.3, 157.7, 156.24, 156.15, 148.9, 148.5, 143.4, 125.3, 125.0, 117.6, 117.4, 114.4, 108.3, 85.4, 84.5, 80.4, 68.9, 63.64, 63.60, 54.8, 54.7, 52.8, 47.8, 45.4, 42.9, 41.3, 35.3, 31.0, 28.7, 28.3, 27.4, 27.1, 26.3, 26.1, 25.1. HRMS (ESI+) *m/z* calcd. for $C_{39}H_{53}N_{10}O_{10}$ $[M+H]^+$ 821.3946, found: 821.3942. FT-IR ν_{max} film (cm^{-1}): 3304, 2976, 2928, 2872, 1722, 1645, 1567, 1143, 1057.

Cyclo-[-*N*-(*N*-(1,2,3-triazol-4-yl)-butanoyl-glycyl-prolyl-arginyl)-7-amino-4-methylenecoumarin-]·2HCl (**2b**)



A solution of compound **16** (10 mg, 12.2 µmol) in Et_2O (2.5 mL) was treated with 0.5 mL 2.6 M HCl in EtOAc. The clear solution became a suspension upon stirring for 72 hours. The solvents were removed by a nitrogen airflow and the remaining solid was dried *in vacuo* to yield a yellow solid (8 mg, 99%). ^{1}H-NMR (400 MHz, CD_3OD) δ (ppm): 9.17 (s, 1H, *NH*), 8.29 (s, 1H), 8.13 (s, 1H), 7.34 (d, *J* = 8.8 Hz, 1H), 6.86 (d, *J* = 8.8 Hz, 1H), 6.69 (s, 1H), 6.15 + 5.60 (AB-system, *J* = 15.1 Hz, 1.3, 2H), 4.53 (dd, *J* = 10.8, 3.7 Hz, 1H), 4.33 (dd, *J* = 8.0, 6.3 Hz, 1H), 4.25 + 3.95 (AB-system, *J* = 17.7 Hz, 2H), 3.73 (m, 1H), 3.59 (m, 1H), 3.21 (t, *J* = 6.7 Hz, 2H), 2.81 (m, 2H), 2.43-2.36 (m, 1H), 2.27-2.13 (m, 1H), 2.09 (dt, *J* = 6.2, 12.4 Hz, 2H), 1.94-178 (m, 4H), 1.75-1.54 (m, 4H). ^{13}C-NMR (75 MHz, CD_3OD) δ (ppm): 175.5, 175.1, 172.3, 171.4, 162.3, 158.7, 156.2, 148.7, 143.4, 125.6, 125.3, 117.7, 117.6, 114.5, 108.4, 63.7, 54.3, 53.0, 47.9, 43.0, 41.9, 35.2, 31.1, 30.8, 28.5, 26.9, 26.13, 26.10, 24.9. HRMS (ESI+) *m/z* calcd. for $C_{29}H_{37}N_{10}O_6$ $[M+H]^+$ 621.2898, found: 621.2851.

Biological evaluation

*Determination kinetic parameters for H-Gly-Pro-Arg-AMC·2HCl (**1**):*

The following solutions were prepared and stored at the indicated temperatures:

Substrate: H-Gly-Pro-Arg-AMC (55.7 mg, 0.1 mmol) was dissolved in 1.0 mL $BSA60^+$ buffer (60 mg/mL, containing NaCl (8.18 mg/mL)) to give a 100 mM stock solution. From this stock solution a series of dilutions was prepared (total volume of 500 µL) to give final substrate concentrations in the well of 0, 200, 400, 600, 800, 1000, 1200, and 1400 µM. The dilutions are kept at 37 °C.
Fluo: Cbz-Gly-Gly-Arg-AMC·□□□ (61.5 mg, 0.1 mmol) was dissolved in 1.0 mL DMSO to give a 100 mM stock solution. 7.5 µL of the 100 mM stock solution and 292.5 µL BSA60 buffer (NOT containing NaCl) were shortly vigorously mixed to give a *Fluo*-solution of 2.5 mM. This solution was kept at room temperature.
h-Thrombin: A solution of 1.2 µM activated humane thrombin (denoted FIIa) was prepared from an 18.8 µM FIIa stock solution via dilution with BSA5 buffer (5 mg/mL). The FIIa solution was kept at 0 °C. Prior to use it was warmed to 37 °C.
Calibrator: Lyophilized α_2-MT complex was reconstituted in 1 mL deionised water to give a final enzyme concentration of 600 nM. The calibrator solution was kept at 0 °C. Prior to use it was warmed to 37 °C.

A set of four wells (quadruple measurement) of an Immulon 2HB, round-bottom 96-well plate, was filled with 100 µL of each appropriate substrate concentration (total 4 × 8 wells). A set of four wells in a 96 wells plate was filled with 80 µL BSA5 buffer followed by 20 µL of *Fluo*-solution. The 96 wells plate was placed in a Ascent reader, Thermolab systems OY fluorometer equipped with a 390/460 filter set (excitation /emission) and allowed to warm to 37 °C (approximately 5 minutes). To all the wells containing substrate, 20 µL of 0.6 µM FIIa solution was added to each well (4 × 8 wells) to initiate hydrolysis (200 nM end concentration of FIIa in wells). To the wells containing the *Fluo*-solution, 20 µL of calibrator was added to initiate hydrolysis. In a typical experiment fluorescence was measured continuously during 40 minutes at 37 °C. The obtained data was organized in Microsoft Excel and the kinetic parameters were obtained by fitting the data to the Michaels-Menten equation. For the determination of K_M and k_{cat} the initial reaction velocity of the first 10 minutes was used. The amidolytic activity was calculated by comparing the arbitrary fluorescence values to those of an AMC-calibration curve. Results of different experiments can be compared by correcting the amidolytic activity of each experiment with the amidolytic activity found for the calibrator which was measured in the same 96 wells plate.

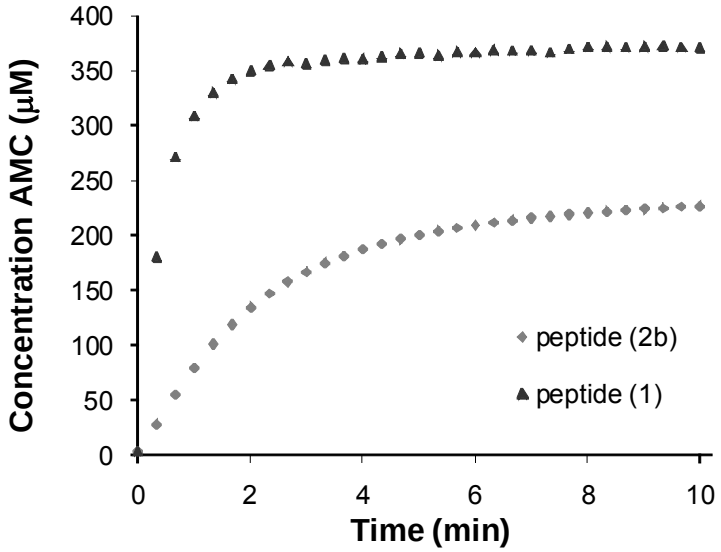
*Determination kinetic parameters for cyclo-[-N-(N-(1,2,3-triazol-4-yl)-butanoyl-glycyl-prolyl-arginyl)-7-amino-4-methylenecoumarin-]·2HCl (**2b**):*

Using the experimental procedure as described *vide supra* utilizing substrate **2b**.

The following solution was prepared and stored at the indicated temperature:

Substrate: *c*(-Gly-Pro-Arg-AMC-[triazole]-spacer-) (**2b**, 1.4 mg, 0.025 mmol) was dissolved in 0.25 mL $BSA60^+$ buffer (60 mg/mL containing NaCl (8.18 mg/mL)) to give a 100 mM stock solution. From this stock solution a series of dilutions was prepared (total volume of 500 µL) to give final substrate concentrations in the well of 0, 50, 100, 200, 400, 600, 800 and 1000 µM. The dilutions are kept at 37 °C.

In brief, kinetic parameters were determined by monitoring the fluorescence increase in time as a result of the hydrolysis of the arginine-coumarin bond. The fluorescent counts were subsequently converted to AMC concentrations using a calibration curve. Plotting of the calculated AMC concentration versus time gave curves as depicted in Graph S1. While complete substrate consumption was observed after approximately 3 minutes for the linear Gly-Pro-Arg peptide, complete substrate consumption for the cyclic Gly-Pro-Arg peptide required more than 10 minutes. For both the linear and cyclic peptide the curves obtained at different substrate concentrations did not allow linear regression processing so that the initial rate constants were determined *via* non-linear regression by fitting of the data to a 6^{th} order polynomial. From these polynomials the initial rate constants were determined.



Graph S1 Normalized plots of the hydrolysis of the linear and cyclic peptide at a substrate concentration of 600 µM where (▲) = linear peptide (**1**), conc. FIIa = 1.11 µM and (♦) = cyclic peptide (**2b**), conc. FIIa = 0.45 µM

Subsequently applying the Michaelis-Menten equation on the obtained rate constants for linear peptide **1**, we found a poor overlap of the fitted data with the experimental data which hampered accurate determination of the kinetic parameters. To assess the kinetic constants more accurately, a Lineweaver-Burk plot was constructed by plotting the reciprocal substrate concentrations against the reciprocal rate constants. The slope of the obtained linear line equals k_{cat} and from the x-intercept, the K_M could be determined ($x = -1/K_M$). Statistical analysis showed a tolerable R^2 of 0.97. From calculations a K_M value of 746.9 µM and a k_{cat} of 29.3 s^{-1} were obtained for the linear peptide **1** (Table S2, entry 1). Utilizing the Michaelis-Menten equation on the initial rate constants obtained for compound **2b**, gave a good overlap of the fitted and experimental data, resulting in a k_{cat} = 23.3 s^{-1}, and a K_M = 3693.9 µM (Table S2, entry 2).

Table S2. Kinetic parameters for the linear peptide **1** and cyclic peptide **2b**

Entry	Substrate	E (µM)	K_M (µM)	k_{cat} (s^{-1})	v_{max} $(M \cdot s)^{-1}$
1	[b] H-Gly-Pro-Arg-AMC (**1**)	1.11	746.9	29.3	39228
2	[a] *c*[Pro-Arg-AMC-ψ(triazole)-Gly] (**2b**)	0.45	3693.9	23.3	6308

[a] Calculated via Michaelis-Menten method, [b] Calculated via Lineweaver-Burk method, E = FIIa, $v_{max} = k_{cat} / K_M$

References

S1 B. Poojary, K. H. Kumar, S. L. Belagali, *Z. Naturforsch. B*, **2004**, *59*, 817-820.
S2 D. J. Maly, F. Leonetti, B. J. Backes, D. S. Dauber, J. L. Harris, C. S. Craik, A. Ellman, *J. Org. Chem.* **2002**, *67*, 910-915.
S3 E. R. Bissell, *Synthesis* **1982**, 846-848.
S4 B. Cürten, P. H. M. Kullmann, M. E. Bier, K. Kandler, B. F. Schmidt, *Photochem. Photobiol.* **2005**, *81*, 641-648.
S5 A. Kakefuda, T. Suzuki, T. Tobe, J. Tsukada, A. Tahara, S. Sakamoto, S. Tsukamoto, *J. Med. Chem.* **2002**, 45, 2589-2598.
S6 R. G. Bhat, S. Sinha, S. Chandrasekaran, *Chem. Commun.* **2002**, 812-813.
S7 V. Melai, A. Brillante, P. Zanirato, *J. Chem. Soc*., *Perkin Trans. 2* **1998**, 2447-2449