Electronic Supplementary Information (ESI)

Protease responsive nanoprobes with tethered fluorogenic peptidyl 3-arylcoumarin substrates

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General chemistry

Chemicals and solvents were purchased from standard suppliers and used without further purification. Subtilisin A (EC 3.4.21.62) was purchased from Calbiochem and chymotrypsin (from bovine pancreas) (EC 3.4.31.1) from Fluka. Silica Gel 60 Å, 40–63 μm, for flash chromatography was supplied by Fluorochem and deuterated solvents were purchased from Goss or Sigma Aldrich. Anhydrous solvents were purchased from Fluka or Acros except toluene and THF which were distilled over metallic sodium and benzophenone. All other solvents were used as supplied (Analytical or HPLC grade), without prior purification. Milli-Q water was used for chemical reactions and enzymatic cleavage reactions. All reactions using anhydrous conditions were performed using flame- or oven dried apparatus under an atmosphere of nitrogen. Reactions were monitored by analytical thin-layer chromatography on commercially available precoated aluminium packed plates (Merck Kieselgel 60 F_{254}). Visualization of the silica plates was achieved using a UV lamp (λ_{max} = 254 nm) and potassium permanganate staining. Organic solvents were evaporated under reduced pressure at ≤ 35 °C (water bath temperature).

Melting points were recorded on a Stuart Scientific melting point apparatus (SMP3). Infrared spectra were measured on an Avatar 360 Nicolet FT-IR spectrophotometer in the range of 4000–500 cm^{-1} using KBr discs. Absorption maxima (υ_{max}) are reported in wavenumbers (cm^{-1}) and classified as strong (s), medium (m) or broad (br). Only signals representing functional groups are reported. Absorptions from the fingerprint region are not listed.

Mass spectra (TOF-ES) were recorded on a Waters 2795 separation module/micromass LCT platform. Values of hydrodynamic diameter were obtained by dynamic light scattering (DLS) using a Viscotec Model 802 instrument equipped with an internal laser (825-832 nm) with a maximum radiation power of 60 mW. Data processing was performed with the software program OmniSize2.

Proton nuclear magnetic resonance (δ^H) and carbon nuclear magnetic resonance (δ^C) spectra were recorded at 20 °C on a Bruker AV400 operating at 400.13 MHz and 101.62 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm), referenced to either $CDCl_3$ (^{1}H, 7.26 ppm; ^{13}C, 77.16 ppm), [D_6]-DMSO (^{1}H, 2.50 ppm; ^{13}C, 39.51 ppm) or CD_3OD (^{1}H, 3.31 ppm; ^{13}C, 77.23 ppm). Coupling constants (J) are recorded in Hz and significant multiplicities described by singlet (s), doublet (d), triplet (t), quadruplet (q), broad

(br), multiplet (m) or doublet of doublets (dd). Spectra were assigned using appropriate COSY, DEPT, HSQC and HMBC sequences. Fluorescence spectra were recorded on a Cary Eclipse fluorimeter. UV-Vis-spectra were recorded on a Cary 50 spectrophotometer. All solvents used for steady state and life time measurements were of the highest commercially available grade and used without further purification.

Analytical high performance liquid chromatography (HPLC) was performed using either system 1 or system 2 to confirm purity. All retention times are quoted in minutes.

System 1: Hypersil^{TM} Pep 100-C18 (4.6 x 150 mm), flow rate 1.20 mL min^{-1} and UV detection at 220 nm. Linear gradient: 20 - 100% solvent B in 30 minutes. Solvent A: 0.06% TFA in water; solvent B: 0.06% TFA in CH_3CN/water (90/10).
System 2: Hypersil^{TM} Pep 100-C18 (4.6 x 150 mm), flow rate of 1.20 mL min^{-1} and UV detection at 220 nm. Linear gradient: 20 - 60% solvent B in 25 minutes. Solvent A: 0.06% TFA in water; solvent B: 0.06% TFA in CH_3CN/water (90/10).

Preparative HPLC was performed using either system 3 or 4.
System 3: Phenomenex Kromasil (5 µ, 100 A) reverse phase C_{18} column (250 x 21.2 mm), a flow rate of 21.20 mL min^{-1} and UV detection at 220 nm.
System 3a: Linear gradient: 40 – 75% solvent B in 10 minutes.
System 3b: Linear gradient: 35 – 60% solvent B in 10 minutes.
System 3c: Linear gradient: 35 – 75% solvent B in 15 minutes.
Solvent A: 0.06% TFA in water; solvent B: 0.06% TFA in CH_3CN.

System 4: Phenomenex Onyx Monolithic reverse phase C_{18} column (100 x 10 mm), a flow rate of 14.10 mL/min and UV detection at 220 nm.
System 4a: Linear gradient: 10 – 40% B in 8 min
System 4b: Linear gradient: 2 - 35% B in 10 min
System 4c: Linear gradient: 15 - 40% B in 12 min
System 4d: Linear gradient: 20 - 50% B in 19 min
Solvent A: 0.06% TFA in water; solvent B: 0.06% TFA in CH_3CN.

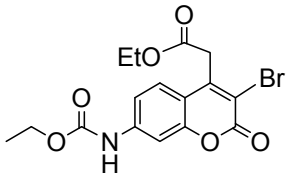
Experimental procedures

Ethyl 7-*N*-(carbethoxy)aminocoumarin-4-acetate 2

To a 250 mL round bottom flask was added 7-*N*-(carbethoxy)aminocoumarin-4-acetic acid[1] **1** (2.86 g, 9.84 mmol) and *p*-toluenesulfonic acid mono-hydrate (0.183 mg, 0.096 mmol) suspended in EtOH (170 mL). The white suspension was then heated to reflux, whereupon a clear solution was obtained. The reaction was carried out in the dark for 18 hours and monitored by TLC. The EtOH was subsequently removed by rotary evaporation yielding a white solid, which was further washed with a saturated $NaHCO_3$ solution (20 mL), water (20 mL) and hexane (30 mL). After drying *in vacuo* a white solid was obtained (2.90 g, 9.08 mmol, 92%). R_f = 0.3 (50:50 = Hex:EtOAc). mp: 173–174 °C; υ_{max} (KBr): 3286 (m, NH) 2984 (br, aliphatic C-H) 1704 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, [D_6]-DMSO, 400 MHz): 10.17 (s, 1H, C-N*H*-C), 7.60 (d, J = 8.8 Hz, 1H, C_5-*H* (coumarin)), 7.57 (d, J = 2.0 Hz, 1H, C_8-*H* (coumarin)), 7.39 (dd, J = 8.8/2.0 Hz, 1H, C_6-*H* (coumarin)), 4.17 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.11 (q, J = 7.1 Hz, 2H, CH_2CH_3), 3.96 (s, 2H, CH_2CO_2Et), 1.26 (t, J = 7.1 Hz, 3H, CH_2CH_3), 1.18 (t, J= 7.1 Hz, 3H, CH_2CH_3); ^{13}C-NMR (δ, [D_6]-DMSO, 100 MHz): 169.1 (C), 159.9 (C), 154.1 (C), 153.3 (C), 149.3 (C), 143.0 (C), 126.1 (CH), 114.3 (CH), 113.8 (CH), 113.5 (C), 104.5 (CH), 60.9 (CH_2), 60.8 (CH_2), 36.7 (CH_2), 14.4 (CH_3), 14.0 (CH_3); m/z 320.1151 (MH^+), calc. 320.1134.

[1] D. J . Maley, F. Leonetti, J. Backes, D. S. Dauber, J. L. Dauber, J. L. Harris, C. S. Craik, J. A. Ellman, *J. Org. Chem.* **2002**, *67*, 910-915.

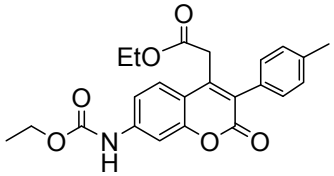
Ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate 3



To a dry 250 mL round bottom flask was added ethyl 7-*N*-(carbethoxy)aminocoumarin-4-acetate **2** (5.27 g, 16.50 mmol) and dry THF (130 mL). *N*-bromosuccinimide (5.84 g, 32.80 mmol, 2.0 eq.) dissolved in dry THF (25 mL) was then added dropwise to the white suspension. The reaction mixture was stirred under a nitrogen atmosphere in the dark at ambient temperature for 18 hours. The THF was then removed by rotary evaporation yielding a yellow solid. The crude product was dissolved in DCM (200 mL) and washed with water (3 x 200 mL). After drying the organic phase over $MgSO_4$, it was concentrated under reduced pressure to yield a pale yellow solid which was further washed with CH_3CN (10 mL). The obtained white solid was then dried *in vacuo* (5.06 mg, 12.71 mmol, 77%). R_f = 0.3 (Hex:EtOAc = 50:50). mp: 200–201 °C; υ_{max} (KBr): 3321 (m, NH) 2983 (br, aliphatic) 1712 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 7.51 – 7.49 (m, 2H, C_5-*H* (coumarin)), C_8-*H* (coumarin)), 7.34 (dd, J = 8.8/1.8 Hz, 1H, C_6-*H* (coumarin)), 6.90 (s, 1H, C-N*H*-C), 4.26 (q, J = 7.1 Hz, 2H, CH_2CH$_3$), 4.19 (q, J = 7.1 Hz, 2H, CH_2CH$_3$), 4.08 (s, 2H, CH_2CO$_2$Et), 1.33 (t, J = 7.1 Hz, 3H, CH$_2$CH_3), 1.25 (t, J = 7.1 Hz, 3H, CH$_2$CH_3); ^{13}C-NMR (δ, [D$_6$]-DMSO, 100 MHz): 169.2 (C), 156.5 (C), 153.3 (C), 152.5 (C), 148.7 (C), 143.3 (C), 126.9 (C), 114.9 (CH), 113.8 (CH), 111.2 (C), 104.2 (CH), 60.9 (2 x CH_2), 38.3 (CH_2), 14.4 (2 x (CH_3). *m*/*z* 398.0283, 400.0278 (MH^+), calc. 398.0239, 400.0219.

General procedure for the Suzuki coupling of ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate with aromatic boronic acids will be illustrated with a specific example

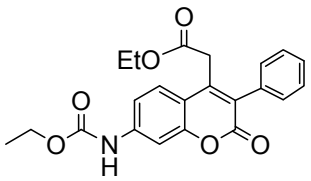
Ethyl 7-*N*-(carbethoxy)amino-3-(*p*-tolyl)-coumarin-4-acetate 4b



To a three neck round bottom flask equipped with an upright condenser and N_2 inlet was added ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate **3** (0.298 g, 0.75 mmol) suspended in dry toluene (37 mL). 2 M aqueous K_2CO_3 (0.754 mL, 1.51 mmol, 2.0 eq.), p-tolylboronic acid (0.154 g, 1.13 mmol, 1.5 eq.) and the catalyst $Pd(PPh_3)_4$ (48.9 mg, 5.7 mol%) were subsequently added. The yellow clear reaction mixture was stirred at 90 °C for 150 min under N_2 and monitored by TLC. Upon allowing the reaction mixture to cool to room temperature, a white precipitate formed which was collected by filtration and washed with cold toluene (4 mL), distilled water (6 mL) and hexane (7 mL). The white solid was then dried *in vacuo*. (0.235 g, 0.57 mmol, 76%). R_f = 0.4 (Hex:EtOAc = 50:50). mp: 186–187 °C; υ_{max} (KBr): 3320 (m, NH) 2983 (br, aliphatic C-H) 1696 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, [D$_6$]-DMSO, 400 MHz): 10.18 (bs, 1H, C-N*H*-C), 7.65 (d, J = 8.8 Hz, 1H, C_5-*H* (coumarin)), 7.62

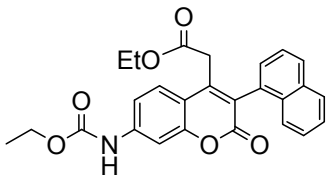
(d, *J* = 2.0 Hz, 1H, C_8-*H* (coumarin)), 7.40 (dd, *J* = 8.8/2.0 Hz, 1H, C_6-*H* (coumarin)), 7.26 (d, *J* = 8.0 Hz, 2H, $C_{2'}$-*H* (p-tolyl), $C_{6'}$-*H* (p-tolyl)), 7.12 (d, *J* = 8.0 Hz, 2H, $C_{3'}$-*H* (p-tolyl), $C_{5'}$-*H* (p-tolyl)), 4.18 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 4.06 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 3.70 (s, 2H, C*H*$_2$CO$_2$Et), 2.36 (s, 3H, Phe-C*H*$_3$), 1.27 (t, *J* = 7.1 Hz , 3H, CH$_2$C*H*$_3$), 1.13 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR (δ, [D$_6$]-DMSO, 100 MHz): 169.2 (C), 160.2 (C), 153.4 (C), 153.1 (C), 144.0 (C), 142.7 (C), 137.6 (C), 131.1 (CH), 129.6 (2 x CH), 128.9 (2 x CH), 126.6 (CH), 125.8 (C), 114.5 (CH), 114.0 (C), 104.2 (CH), 61.0 (CH$_2$), 60.8 (CH$_2$), 35.3 (CH$_2$), 20.9 (CH$_3$), 14.43 (CH$_3$), 13.93 (CH$_3$); *m/z* 410.1740 (MH$^+$), calc. 410.1604.

Ethyl 7-*N*-(carbethoxy)amino-3-phenyl-coumarin-4-acetate 4a



According to the general procedure, ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate **3** (0.497 g, 1.25 mmol) was treated with 2 M aqueous K_2CO_3 (1.26 mL, 2.52 mmol, 2.0 eq.), phenylboronic acid (0.230 g, 1.89 mmol, 1.5 eq.) and the catalyst Pd(PPh$_3$)$_4$ (77.0 mg, 4.1 mol%) in dry toluene (50 mL) for 150 min at 90 °C. Further work up and purification as described in the general procedure provided the product as a white solid (0.445 g, 1.13 mmol, 90%). R_f = 0.4 (Hex:EtOAc = 50:50). mp: 226–228 °C; υ_{max} (KBr): 3319 (m, NH) 2986 (br, aliphatic C-H) 1689 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, [D$_6$]-DMSO, 400 MHz): 10.21 (s, 1H, C-N*H*-C), 7.68 (d, *J* = 8.8 Hz, 1H, C_5-*H* (coumarin)), 7.62 (d, *J* = 1.8 Hz, 1H, C_8-*H* (coumarin)), 7.48 – 7.40 (m, 4H, 4 x Phe-*H*), 7.26 - 7.24 (m, 2H, C_6-*H* (coumarin), Phe-*H*), 4.18 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 4.06 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 3.70 (s, 2H, C*H*$_2$CO$_2$Et), 1.27 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$), 1.12 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR (δ, [D$_6$]-DMSO, 100 MHz): 169.6 (C), 161.3 (C), 153.9 (C), 153.1 (C), 143.5 (C), 141.5 (C), 134.0 (C), 130.0 (2 x CH), 128.7 (3 x CH), 127.6 (CH), 125.80 (C), 115.1 (CH), 114.8 (C), 105.9 (CH), 61.9 (CH$_2$), 61.8 (CH$_2$), 36.2 (CH$_2$), 14.6 (CH$_3$), 14.2 (CH$_3$); *m/z* 396.1518 (MH$^+$), calc. 396.1447.

Ethyl 7-*N*-(carbethoxy)amino-3-(1-napthyl)-coumarin-4-acetate 4c.

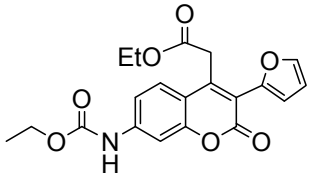


According to the general procedure, ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate **3** (0.081 g, 0.202 mmol) was treated with 2 M aqueous K_2CO_3 (0.20 mL, 0.400 mmol, 2.0 eq.), 1-napthylboronic acid (0.0524 g, 0.305 mmol, 1.5 eq.) and the catalyst Pd(PPh$_3$)$_4$ (12.4 mg, 5.3 mol%) in dry toluene (10 mL) for 150 min at 90 °C. Further work up and purification as described in the general procedure provided the product as a white solid. (0.076 g, 0.170 mmol, 84%). R_f = 0.4 (Hex:EtOAc = 50:50). mp: 219–220 °C; υ_{max} (KBr): 3326 (m, NH) 2981 (br, aliphatic C-H) 1693 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, [D$_6$]-DMSO, 400 MHz): 10.23 (s, CON*H*C), 8.02 (dd, *J* = 8.2/3.2 Hz, 2H, 2 x napthyl-*H*), 7.76 (d, *J* = 8.8 Hz,

1H, C_5-*H* (coumarin)), 7.70 (d, *J* = 2.0 Hz, 1H, C_8-*H* (coumarin)), 7.61 – 7.54 (m, 3H, 3 x napthyl-*H*), 7.48 – 7.44 (m, 2H, 2 x napthyl-*H*), 7.36 (m, 1H, C_6-*H* (coumarin)), 4.20 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 3.88 (q, *J* = 7.1, 2H, C*H*$_2$CH$_3$), 3.72 (d, *J* = 16.4 Hz, 1H, C*H*$_2$CO$_2$Et), 3.45 (d, *J* = 16.4 Hz, 1H, C*H*$_2$CO$_2$Et), 1.29 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$), 0.96 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR (δ, [D$_6$]-DMSO, 100 MHz): 168.7 (C), 159.9 (C), 153.6 (C), 153.4 (C) 146.3 (C), 142.9 (C), 133.2 (C), 131.7 (C), 128.8 (CH), 128.4 (CH), 127.7 (C), 126.9 (C), 126.5 (C), 126.2 (C), 125.6 (C), 125.0 (C), 124.3 (C), 114.5 (CH), 114.0 (C), 104.4 (CH), 60.8 (CH$_2$), 60.8 (CH$_2$), 35.2 (CH$_2$), 14.4 (CH$_3$), 13.7 (CH$_3$); *m*/*z* 446.1759 (MH$^+$), calc. 446.1604.

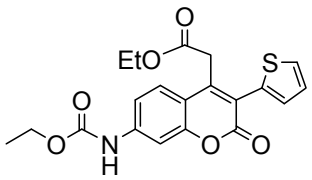
General procedure for the Suzuki coupling of ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate with heteroaromatic boronic acids will be illustrated with a specific example

Ethyl 7-*N*-(carbethoxy)amino-3-(2-furyl)-coumarin-4-acetate 4e



To a three neck round bottom flask equipped with an upright condenser and N_2-inlet was added ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate **3** (1.01 g, 2.53 mmol) and 2-furanboronic acid (0.562 g, 5.02 mmol, 2.0 eq.) suspended in 1-propanol (140 mL). Pd(OAc)$_2$ (38.9 mg, 0.058 mmol, 0.02 eq.), triphenylphosphine (48.7 mg, 0.185 mmol, 0.07 eq.) and 2 M Na_2CO_3 (1.51 mL, 3.01 mmol, 1.2 eq.) were then added and the reaction mixture was heated at 100 °C for 4 h under a N_2 atmosphere. The hot reaction mixture was then filtered under gravity. Upon allowing the filtrate to cool to ambient temperature, a yellow precipitate formed, which was collected by filtration and washed with cold 1-propanol (10 mL) and hexane (10 mL). The yellow solid was then dried *in vacuo* (0.730 g, 1.90 mmol, 75%). R_f = 0.3 (Hex:EtOAc = 50:50). mp: 199-201 °C; υ_{max} (KBr): 3298 (m, NH) 2976 (br, aliphatic C-H) 1697 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 7.55 – 7.53 (m, 2H, C_5-*H* (coumarin), furyl-*H*), 7.48 (d, *J* = 1.8 Hz, 1H, C_8-*H* (coumarin)), 7.35 (m, 1H, C_6-*H* (coumarin)), 7.11 (m, 1H, fury-*H*), 6.87 (bs, 1H, CON*H*C), 6.55 – 6.54 (m, 1H, furyl-*H*), 4.27 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 4.21 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 4.06 (s, 2H, C*H*$_2$CO$_2$Et), 1.33 (t, 7.1 Hz, 3H, CH$_2$C*H*$_3$), 1.25 (t, 7.1 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR (δ, [D$_6$]-DMSO, 100 MHz): 169.2 (C), 158.4 (C), 153.4 (C), 152.7 (C), 146.8 (C), 143.9 (CH), 143.8 (C), 143.1 (C), 127.0 (CH), 114.8 (C), 114.7 (CH), 114.2 (C), 113.6 (CH), 111.6 (CH), 104.1 (CH), 60.8 (2 x CH$_2$), 35.4 (CH$_2$), 14.4 (CH$_3$), 14.1 (CH$_3$); *m*/*z* 386.1262 (MH$^+$), calc. 386.1240.

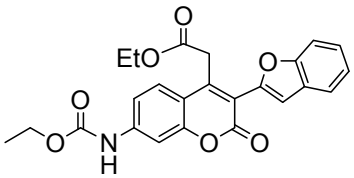
Ethyl 7-*N*-(carbethoxy)amino-3-(2-thienyl)-coumarin-4-acetate 4d.



According to the general procedure, ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate **3** (0.516 g, 1.30 mmol) was treated with 2-thiophene boronic acid (0.426 g, 3.33

mmol, 2.6 eq.), $Pd(OAc)_2$ (22.5 mg, 0.033 mmol, 0.03 eq.), triphenylphosphine (27.4 mg, 0.105 mmol, 0.08 eq.), 2 M Na_2CO_3 (0.777 mL, 1.55 mmol, 1.2 eq.) in 1-propanol (72 mL) at 100 °C for 5 h. The reaction mixture was then cooled to room temperature and filtered through a short pad of Celite. Further purification of the crude product by flash chromatography (Hex:EtOAc = 70:30) provided the final compound as a yellow solid (0.473 mg, 1.18 mmol, 91%). R_f = 0.3 (Hex:EtOAc = 50:50). Decomposed over 168 °C; υ_{max} (KBr): 3352 (m, NH) 2977 (br, aliphatic C-H) 1731 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 7.51 – 4.48 (m, 3H, C_5-*H* (coumarin), C_8-*H* (coumarin), thienyl-*H*), 7.36 (dd, *J* = 8.6/1.8 Hz, 1H, C_6-*H* (coumarin)), 7.18 (dd, *J* = 3.6/1.2 Hz, 1H, thienyl-*H*), 7.12 - 7.10 (m, 1H, thienyl-*H*), 6.85 (bs, 1H, CON*H*C), 4.28 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 4.22 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 3.85 (s, 2H, C*H*$_2$CO$_2$Et), 1.33 (t, 7.1 Hz, 3H, CH$_2$C*H*$_3$), 1.26 (t, 7.1 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR (δ, [D_6]-DMSO, 100 MHz): 169.5 (C), 160.8 (C), 153.8 (C), 153.1 (C), 145.4 (C), 141.9 (C), 134.0 (C), 129.8 (CH), 128.2 (CH), 127.0 (CH), 126.1 (CH), 120.0 (C), 115.05 (C), 114.9 (CH), 106.0 (CH), 62.1 (2 x CH_2), 36.7 (CH_2), 14.7 (CH_3), 14.3 (CH_3); *m*/*z* 402.0670 (MH^+), calc. 402.1011.

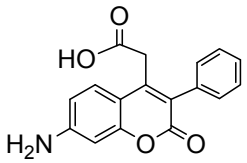
Ethyl 7-*N*-(carbethoxy)amino-3-(benzo[*b*]furyl)-coumarin-4-acetate 4f
(KAWE-142, KAWE-216)



According to the general procedure, ethyl 3-bromo-7-*N*-(carbethoxy)aminocoumarin-4-acetate **3** (1.01 g, 2.52 mmol) was treated with benzo[*b*]furanboronic acid (0.816 g, 5.04 mmol, 2.0 eq.), $Pd(OAc)_2$ (38.0 mg, 0.056 mmol, 0.02 eq.), triphenylphosphine (56.2 mg, 0.214 mmol, 0.08 eq.), 2 M Na_2CO_3 (1.51 mL, 3.01 mmol, 1.2 eq.) in 1-propanol (140 mL) at 100 °C for 125 minutes. Further workup and purification as described in the general procedure provided the product as a yellow solid (0.776 g, 1.78 mmol, 71%). R_f = 0.3 (Hex:EtOAc = 60:40). mp: 218-220 °C; υ_{max} (KBr): 3290 (m, NH) 2980 (br, aliphatic C-H) 1694 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, [D_6]-DMSO, 400 MHz): 10.30 (bs, 1H, CON*H*C), 7.89 (d, *J* = 8.9 Hz, 1H, C_5-*H* (coumarin)), 7.74 (d, *J* = 7.6 Hz, 1H, benzo-*H*), 7.64 (d, *J* = 1.9 Hz, 1H, C_8-*H* (coumarin)), 7.52 (d, *J* = 8.2 Hz, 1H, benzo-*H*), 7.44 (dd, *J* = 8.8/1.9 Hz, 1H, C_6-*H* (coumarin)), 7.40 – 7.36 (m, 2H, 2 x benzo-*H*), 7.31 – 7.27 (m, 1H, benzo-*H*), 4.19 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 4.15 (s, 2H, C*H*$_2$CO$_2$Et), 4.14 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 1.28 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$), 1.18 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$); ^{13}C-NMR (δ, [D_6]-DMSO, 100 MHz): 169.1 (C), 158.4 (C), 154.0 (C), 153.3 (C), 153.1 (C), 149.10 (C), 146.4 (C), 143.6 (C), 127.80 (C), 127.4 (CH), 125.3 (CH), 123.3 (CH), 121.7 (CH), 114.8 (CH), 114.4 (C), 114.1 (C), 110.9 (CH), 109.8 (CH), 104.1 (CH), 61.0 (CH_2), 60.9 (CH_2), 35.6 (CH_2), 14.4 (CH_3), 14.1 (CH_3); *m*/*z* 436.1507 (MH^+), calc. 436.1396.

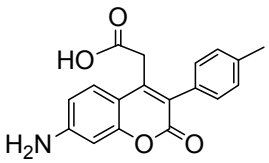
General procedure for the saponification reaction will be illustrated for a specific example

7-Amino-3-phenylcoumarin-4-acetic acid 5a



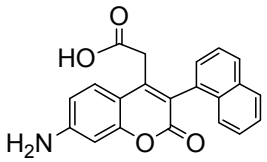
To a 25 mL two neck round bottom flask equipped with a condenser was added ethyl-7-*N*-(carbethoxy)amino-3-phenyl-4-coumarin-4-acetate **4a** (0.397 g, 1.00 mmol) and NaOH (0.408 g, 10.2 mmol, 10 eq.). After adding distilled water (12.6 mL) and EtOH (1.4 mL), the white suspension was heated to reflux, whereupon a clear yellow solution was obtained. The reaction was carried out in the dark for 3 h and monitored by TLC and ES-MS. After cooling to room temperature, the pH of the reaction mixture was adjusted to 2 by the dropwise addition of concentrated H_2SO_4. The resulting yellow precipitate was collected by filtration, washed with distilled water (2 mL) and hexane (2 mL) and dried *in vacuo* overnight (0.272 mg, 0.921 mmol, 91%). Analytical HPLC analysis (system 1): t_R = 12.80 min, 96% purity. According to ^{1}H-NMR the purity of the crude product was over 95% purity. The crude product was further purified by preparative HPLC (system 3a) (33 mg of crude product yielded 14.9 mg of pure product in form of a yellow solid). Decomposed over 260 °C; υ_{max} (KBr): 3500 – 2500 (br, COOH), 3473 (m, NH_2), 3360 (m, NH_2), 1676 (s, C=O), 1619 8 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, CD_3OD, 400 MHz): 7.46 - 7.34 (m, 4H, $C_{2'}$-*H* (Phe), $C_{6'}$-*H* (Phe), $C_{4'}$-*H* (Phe), C_5-*H* (coumarin)), 7.32 – 7.29 (m, 2H, $C_{3'}$-*H* (Phe), $C_{5'}$-*H* (Phe)), 6.66 (dd, J = 8.7/2.2 Hz, 1H, C_6-*H* (coumarin)), 6.57 (d, J = 2.2 Hz, 1H, C_8-*H* (coumarin)), 3.53 (s, 2H, CH_2CO_2H); ^{13}C-NMR (δ, CD_3OD/[D_6]-DMSO (91/9): 173.2 (C), 163.8 (C), 156.5 (C), 154.3 (C), 147.6 (C), 136.3 (C), 131.4 (2 x CH), 129.5 (2 x CH), 129.1 (CH), 127.8 (CH), 123.4 (C), 113.2 (C), 111.0 (CH), 100.4 (CH), 36.8 (CH_2); m/z 296.0908 (MH^+), calc. 296.0923.

7-Amino-3-(*p*-tolyl)coumarin-4-acetic acid 5b



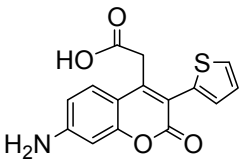
Crude Yield: 27.7 mg (58%). Analytical HPLC analysis (system 1): t_R = 10.83 min, over 95% purity. According to ^{1}H-NMR the purity of the crude product was over 92%. The crude product was further purified by preparative HPLC (system 3a) (45.3 mg of crude product yielded 35.5 mg of pure product in form of a yellow solid). Decomposed over 282 °C; υ_{max} (KBr): 3500 – 2500 (br, COOH), 3453 (m, NH_2), 3359 (m, NH_2), 1678 (s, C=O), 1622 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, CD_3OD, 400 MHz): 7.41 (d, J = 8.8 Hz, 1H, C_5-*H*(coumarin)), 7.24 (d, J = 8.0 Hz, 2H, $C_{2'}$-*H* (p-tolyl), $C_{6'}$-*H* (p-tolyl)), 7.19 (d, J = 8.0 Hz, 2H, $C_{3'}$-*H* (p-tolyl), $C_{5'}$-*H* (p-tolyl)), 6.66 (dd, J = 8.8/2.4 Hz, 1H, C_6-*H*(coumarin)), 6.56 (d, J = 2.4 Hz, 1H, C_8-*H*(coumarin)), 3.62 (s, 2H, CH_2CO_2H), 2.39 (s, 3H, CH_3(p-tolyl)); ^{13}C-NMR (δ, CD_3OD/[D_6]-DMSO (83/17), 100 MHz): 173.1 (C), 163.5 (C), 156.4 (C), 154.1 (C), 147.2 (C), 139.0 (C), 133.2 (C), 131.2 (2 x CH), 130.1 (2 x CH), 127.8 (CH), 123.4 (C), 113.1 (CH), 110.9 (C), 100.3 (CH), 37.0 (CH_2), 21.4 (CH_3); m/z 310.1124 (MH^+), calc. 310.1079.

7-Amino-3-(1-naphtyl)coumarin-4-acetic acid 5c



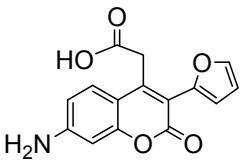
Crude Yield: 49.0 mg (61%). Analytical HPLC analysis (system 1): t_R = 13.38 min, 56% purity. The crude product was further purified by preparative HPLC (system 3a) (53.3 mg of crude product yielded 14.7 mg of pure product in form of a yellow fluffy solid). Decomposed over 230 °C. υ_{max} (KBr): 3500 – 2500 (br, COOH), 3443 (m, NH_2), 3362 (m, NH_2), 1684 (C=O), 1616 (C=O) cm^{-1}; ^{1}H-NMR (δ, $CDCl_3$, 400 MHz): 8.00 - 7.95 (m, 2H, C_5'-H (napthyl), C_8'-H (napthyl)), 7.64 – 7.61 (m, 1H, $C_{4'}$-*H* (napthyl), 7.56 – 7.40 (m, 5H, C_5-*H*(coumarin), $C_{2'}$-*H* (napthyl), $C_{3'}$-*H* (napthyl), $C_{6'}$-*H* (napthyl), $C_{7'}$-*H* (napthyl)), 6.71 (dd, *J* = 8.8/2.4 Hz, 1H, C_6-*H*(coumarin)), 6.64 (d, *J* = 2.4 Hz, 1H, C_8-*H*(coumarin)), 3.64 (d, *J* = 16.4 Hz, 1H, C*H*$_2$CO$_2$H), 3.33 (d, *J* = 16.4 Hz, 1H, C*H*$_2$CO$_2$H); ^{13}C-NMR (δ, CD_3OD, 100 MHz): 173.2 (C), 164.0 (C), 156.9 (C), 154.5 (C), 149.7 (C), 135.3 (C), 133.6 (C), 133.6 (C), 129.8 (CH), 129.5 (CH), 129.3 (CH), 127.9 (CH), 127.4 (CH), 127.1 (CH), 126.5 (CH), 126.2 (CH), 121.6 (C), 113.4 (CH), 111.0 (C), 100.5 (CH), 36.6 (CH_2); *m*/*z* 346.1181 (MH^+), calc. 346.1079.

7-Amino-3-(2-thienyl)coumarin-4-acetic acid 5d



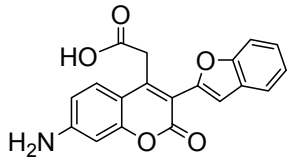
Yield: 224.0 mg (0.74 mmol, 81%). Analytical HPLC analysis (system 1): t_R = 13.50 min, 98% purity. According to ^{1}H-NMR the purity of the crude product was over 94%. The crude product was further purified by preparative HPLC (system 3a) (58.8 mg of crude product yielded 33.0 mg of pure product in form of a yellow fluffy solid). Decomposed over 256 °C; υ_{max} (KBr): 3500 – 2500 (br, COOH), 3473 (m, NH_2), 3359 (m, NH_2), 1684 (s, C=O), 1622 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, CD_3OD/[D_6]-DMSO (83/17), 400 MHz): 7.59 (dd, *J* = 4.8/1.6 Hz, 1H, thienyl-*H*), 7.46 (d, *J* = 8.4 Hz, 1H, C_5-*H* (coumarin)), 7.15 – 7.12 (m, 2H, thienyl-*H*), 6.68 (dd, *J* = 8.8/2.0 Hz, 1H, C_6-*H* (coumarin)), 6.56 (d, *J* = 2.4 Hz, 1H, C_8-*H* (coumarin)), 3.79 (s, 2H, C*H*$_2$CO$_2$H); ^{13}C-NMR (δ, CD_3OD/[D_6]-DMSO (75/25), 100 MHz): 172.7 (C), 162.7 (C), 156.5 (C), 154.7 (C), 149.2 (C), 136.6 (C), 130.3 (CH), 128.6 (CH), 128.3 (CH), 127.8 (CH), 116.0 (C), 113.3 (CH), 110.6 (C), 100.1 (CH), 37.0 (CH_2); *m*/*z* 302.0518 (MH^+), calc. 302.0487.

7-Amino-3-(2-furyl)coumarin-4-acetic acid 5e



Yield: 228.2 mg (51%). Analytical HPLC analysis (system 2): t_R = 11.04 min, over 96% purity. According to ^{1}H-NMR the purity of the crude product was over 92%. The crude product was further purified by preparative HPLC (system 3b) (67.0 mg of crude product yielded 28.4 mg of pure product in form of a yellow solid). Decomposed over 130 °C; υ_{max} (KBr): 3500 – 2500 (br, COOH), 3452 (m, NH_2), 3361 (m, NH_2), 1671 (s, C=O), 1620 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, CD_3OD, 400 MHz): 7.60 – 7.59 (m, 1H, C*H* (furyl)), 7.48 (d, J = 8.8 Hz, 1H, C_5-*H* (coumarin)), 6.79 (dd, J = 3.3/0.6 Hz, 1H, C*H* (furyl)), 6.67 (dd, J = 8.8/2.3 Hz, 1H, C_6-*H* (coumarin)), 6.55 – 6.54 (m, 1H, C*H* (furyl)), 6.53 (d, J = 2.3 Hz, 1H, C_8-*H* (coumarin)), 3.94 (s, 2H, CH_2CO_2H); ^{13}C-NMR (δ, CD_3OD, 100 MHz): 173.5 (C), 162.5 (C), 156.4 (C), 154.8 (C), 149.1 (C), 148.1 (C), 143.8 (CH), 128.0 (CH), 113.6 (CH), 113.5 (CH), 112.8 (C), 112.1 (CH), 111.1(C), 100.2 (CH), 36.7 (CH_2); *m*/*z* 286.0727 (MH^+), calc. 286.0715.

7-Amino-3-(benzo[*b*]furyl)coumarin-4-acetic acid 5f

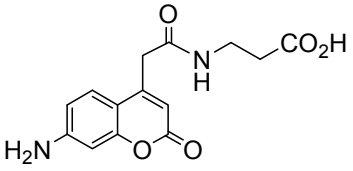


Crude Yield: 17.5 mg (34%). Analytical HPLC analysis (system 2): t_R = 18.72 min, over 56% purity. The crude product was further purified by preparative HPLC (system 3c) (77.5 mg of crude product yielded 23.2 mg of pure product in form of a yellow solid). Decomposed over 278 °C; υ_{max} (KBr): 3500 – 2500 (br, COOH), 3464 (m, NH_2), 3355 (m, NH_2), 1674 (s, C=O), 1620 (s, C=O) cm^{-1}; ^{1}H-NMR (δ, CD_3OD, 400 MHz): 7.64 – 7.62 (m, 1H, C-*H* (benzo)), 7.57 (d, J = 8.8 Hz, 1H, C_5-*H* (coumarin)), 7.49 (m, 0.5H, C-*H* (benzo)), 7.47 (m, 0.5H, C-*H* (benzo)), 7.32 – 7.20 (m, 3H, C-*H* (benzo)), 6.71 (dd, J = 8.8/2.0 Hz, 1H, C_6-*H* (coumarin)), 6.55 (d, J = 2.0 Hz, 1H, C_8-*H* (coumarin)), 4.02 (s, 2H, CH_2CO_2H); ^{13}C-NMR (δ, CD_3OD/[D_6]-DMSO (65/35), 100 MHz):172.5 (C), 161.2 (C), 156.7 (C), 155.7 (C), 155.2 (C), 151.7 (C), 149.5 (C), 129.7 (C), 128.8 (CH), 125.9 (CH), 124.3 (CH), 122.5 (CH), 113.5 (CH), 112.1 (CH), 111.8 (C), 110.6 (C), 109.0 (CH), 99.8 (CH), 36.9 (CH_2); *m*/*z* 336.0891 (MH^+), calc. 336.0872.

Note: An almost immediate decarboxylation of the compounds **5a** – **5f** was observed in [D_6]-DMSO. The products were stable in CD_3OD and stable for 24 hours in CD_3OD /[D_6]-DMSO (65:35 v/v).

General procedure for the preparation of ACA(R)-β-Ala-OH will be illustrated with a specific example

ACA-β-Ala-OH 8



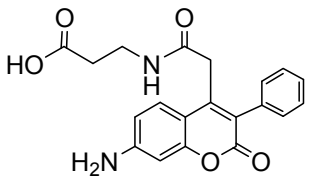
Loading of ACA to the Fmoc-β-Ala-Wang resin

Fmoc-*β*-Ala-Wang resin (100 mg, 0.066 mmol, theoretical loading 0.66 mmol g^{-1}) was added to a peptide column and left to swell in DMF (4 mL) for 9 hours. The resin was then washed with DMF (5 min, 2.5 mL min^{-1}), treated with 10% v/v piperidine in DMF and subsequently washed with DMF (10 min, 2.5 mL min^{-1}) on the peptide synthesizer. To the resin was then added the coupling solution consisting of ACA (58.6 mg, 0.27 mmol, 4.1 eq.), HOAt (35.2 mg, 0.26 mmol, 4.0 eq.) and DIC (46.4 µL, 0.30 mmol, 4.5 eq.) dissolved in DMF (2 mL). The reaction mixture was stirred on intermittent stir in the dark for 36 hours. After washing the yellow resin on the peptide synthesizer with DMF; it was collected by filtration, followed by successively washing with DMF, DCM and hexane. The yellow resin was then dried *in vacuo* overnight (70.4 mg).

Cleavage of ACA-β-Ala-OH from the resin

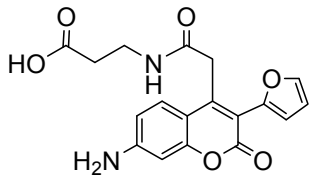
The dry resin was placed in a 50 mL round bottom flask, to which triisopropylsilane (1 mL), distilled water (1 mL) and TFA (18 mL) were added. The suspension was left to stir for 2 hours in the dark, after which time the resin was filtered. The TFA and the scavenger mixture were then evaporated yielding a yellow glassy film, which was gently washed with Et_2O (3 x 20 mL). After lyophilization the peptide was obtained as a yellow fluffy solid (18.5 mg, 0.064 mmol). Analytical HPLC (system 2): t_R = 4.94 min, over 77% purity. The crude product was further purified via preparative RP chromatography (system 4b) (0.8 mg, 0.003 mmol, 4%); *m*/*z* 291.1051 (MH^+), calc. 291.0981, 313.0898 (MNa^+), calc. 313.2611.

ACA(phenyl)-β-Ala-OH 8a



After lyophilization, the crude product was obtained as a yellow solid (30.7 mg). Analytical HPLC (system 2): t_R = 14.06 min, 40% purity. The crude product was further purified by preparative RP chromatography (system 4c) (28.7 mg of crude product yielded 3.9 mg of pure product); ^{1}H-NMR (δ, [D_6]-DMSO, 400 MHz): 11.85 (bs, 1H, COO*H*), 8.12 (t, *J* = 5.6 Hz, 1H, CO-N*H*-CH_2), 7.41 – 7.23 (m, 6H, 5 x Phe-H, C_5-*H* (coumarin)), 6.54 (dd, *J* = 8.8/2.2 Hz, 1H, C_6-*H* (coumarin)), 6.45 (d, *J* = 2.4 Hz, 1H, C_8-*H* (coumarin)), 6.11 (bs, 2H, Ar-NH_2), 3.40 (s, 2H, CCH_2CO), 3.23 (q, *J* = 6.4 Hz, 2H, CH_2(β-Ala)), 2.35 (t, *J* = 6.8 Hz, 2H; CH_2(β-Ala)); *m*/*z* 367.1323 (MH^+), calc. 367.1294.

ACA(furyl)-β-Ala-OH 8e



After lyophilization, the crude product was obtained as a yellow solid (12.7 mg). Analytical HPLC (system 2): t_R = 10.86 min, over 77% purity. The crude product was further purified by preparative RP chromatography (system 4a) (9.9 mg of crude product yielded 7.3 mg of pure product). ^{1}H-NMR (δ, [D$_6$]-DMSO, 400 MHz): 12.22 (bs, 1H, COO*H*), 8.16 (t, *J* = 5.6 Hz, 1H, CO-N*H*-CH$_2$), 7.72 (dd, *J* = 2.0/0.8 Hz, 1H, furyl-*H*), 7.40 (d, *J* = 8.8 Hz, C$_5$-*H* (coumarin)), 6.67 (dd, *J* = 3.3/0.7 Hz, furyl-*H*), 6.57 - 6.55 (m, 2H, C$_6$-*H* (coumarin), furyl-*H*), 6.43 (d, *J* = 2.2 Hz, 1H, C$_8$-*H* (coumarin)), 6.24 (bs, 2H, Ar-N*H*$_2$), 3.68 (s, 2H, CC*H*$_2$CO), 3.25 (q, *J* = 6.4 Hz, 2H, C*H*$_2$(β-Ala)), 2.38 (t, *J* = 6.8 Hz, 2H; C*H*$_2$(β-Ala)); *m*/*z* 357.1217 (MH$^+$), calc. 357.1987.

General procedure for the preparation of Z-Gly-Gly-Leu-ACA(R)-β-Ala-OH

Loading of ACA(R) to the Fmoc-β-Ala-Wang resin

Fmoc-*β*-Ala-Wang resin (100 mg, 0.066 mmol, theoretical loading 0.66 mmol g^{-1}) was placed in a peptide column and left to swell in DMF (4 mL) overnight. The column was then washed with DMF, 10% v/v piperidine/DMF and DMF on the peptide synthesizer. To the column was then added the coupling solution consisting of the fluorophores (0.264 mmol, 4.0 eq.), HOAt (35.9 mg, 0.264 mmol. 4.0 eq.) and DIC (46.5 µL, 0.30 mmol, 4.5 eq.) dissolved in DMF (1mL). The reaction mixture was left to stir on intermittent stir for 36 hours in the dark,

Coupling of amino acids (Fmoc-Leu-OH, Fmoc-Gly-OH and Z-Gly-OH) to the ACA(R)-β-Ala-OH-Wang resin

After washing the resin with DMF on the peptide synthesizer, the excess of DMF was removed and Fmoc-Leu-OH (116.6 mg, 0.33 mmol, 5 eq.), HATU (128.5 mg, 0.33 mmol, 5 eq.) and 2,4,6-collidine (43.6 µL, 0.33 mmol, 5 eq.) dissolved in DMF (1 mL) were added. The reaction mixture was then gently stirred in the dark for 8 hours, after which time the resin was washed with DMF. Subsequently, a second coupling of Fmoc-Leu-OH was carried out using the same amount of amino acid and coupling reagents (Fmoc-substitution: 0.35 - 0.45 mmol g^{-1}, 68 - 79 % efficiency). Before the coupling of Fmoc-Gly-OH, the resin was washed with DMF, treated with 10% v/v piperidine in DMF and washed with DMF (3 x 10 min, 2.5 mL min^{-1}) on the peptide synthesizer. The excess of DMF was then removed and Fmoc-Gly-OH (78.9 mg, 0.264 mmol, 4.0 eq.), HATU (100.4 mg, 0.264 mmol, 4.0 eq.) and DIPEA (92 µL, 0.53 mmol, 4.5 eq.) dissolved in DMF (1 mL) were added. The reaction mixture was stirred on intermittent stir for 8 hours, after which time the peptide column was washed with DMF, treated with 10% v/v piperidine in DMF, and washed with DMF (3 x 10 min, 2.5 mL min^{-1}) on the peptide synthesizer. Z-Gly-OH (55.2 mg, 0.264 mmol, 4.0 eq.), HATU (100.4 mg, 0.264 mmol, 4.0 eq.) and DIPEA (92 µL, 0.53 mmol, 4.5 eq.) dissolved in DMF (1 mL) were then added and the reaction mixture left to stir overnight in the dark. The resin was then

washed with DMF (10 min, 2.5 mL min^{-1}) and collected by filtration followed by successively washing with DMF, DCM and hexane. The resin was then dried *in vacuo*.

Z-Gly-Gly-Leu-ACA-β-Ala-OH 6

The dry resin was transferred to a 50 mL round bottom flask, to which triisopropylsilane (1 mL) distilled water (1 mL) and TFA (18 mL) were added. After gently stirring the reaction mixture in the dark for 2 hours, the resin was collected by filtration. The TFA and scavenger mixture was evaporated yielding a glassy film which was washed with Et_2O (3 x 20 mL). The crude peptide was then lyophilized for 2 days.

After lyophilization, the crude product was obtained as a fluffy white solid. (22.4 mg). Analytical HPLC (system 2): t_R = 17.50 min, 47% purity. The crude product was further purified by preparative RP chromatography (system 4d) (5.4 mg, 14%). ^{1}H-NMR (δ,[D_6]-DMSO, 400 MHz): 12.28 (bs, 1H, COO*H*), 10.40 (bs, 1H, Ar-N*H*), 8.35 (t, *J* = 5.6 Hz, N*H* (β-Ala)), 8.22 – 8.16 (m, 2H, N*H* (Leu), N*H* (Gly)), 7.81 (d, *J* = 2.0 Hz, 1H, C_8-*H* (coumarin)) , 7.69 (d, *J* = 8.8 Hz, 1H, C_5-*H* (coumarin)), 7.57 (t, *J* = 5.9 Hz, 1H, N*H* (Gly)), 7.52 (dd, *J* = 8.8/1.7 Hz, 1H, C_6-*H* (coumarin)), 7.37 – 7.29 (m, 5 H, Cbz-*H*), 6.29 (s, 1H, C_3-*H* (coumarin)), 5.02 (s, 2H, CH_2 (Cbz)), 4.48 (m, 1H, *CH*-NH (Leu)), 3.77 (d, *J* = 5.6 Hz, CH_2(Gly)), 3.67 – 3.64 (m, 4H, CH_2(ACA)), CH_2(Gly)), 3.26 (q, *J* = 6.4 Hz, 2H, CH_2(β-Ala)), 2.39 (t, *J* = 6.8 Hz, 2H, CH_2(β-Ala)), 1.58 (m, 3H, C*H*(Leu), CH_2(Leu)), 0.92 – 0.87 (m, 6H, 2 x CH_3(Leu)); *m*/*z* 652.2579 (MH^+), calc. 652.2579; 674.2197 (MNa^+), calc. 674.2438; 608.2564 (MH^+-CO_2), calc. 608.2720.

Z-Gly-Gly-Leu-ACA(2-furyl)-β-Ala-OH 6e

After lyophilization, the crude product was obtained as a fluffy yellow solid. (30.0 mg). Analytical HPLC (system 1): t_R = 20.6 min, 47% purity. The crude product was further purified by preparative RP chromatography (system 4d) (8.6 mg, 18%).

^{1}H-NMR (δ, [D_6]-DMSO, 400 MHz): 12.29 (bs, 1H, COO*H*), 10.46 (bs, 1H, Ar-N*H*), 8.24 - 8.17 (m, 3H, N*H* (Leu), N*H* (β-Ala), N*H* (Gly)), 7.85 (d, *J* = 2.0 Hz, 1H, C_8-*H* (coumarin)*)*, 7.80 – 7.79 (m, 1H, furyl-*H*), 7.72 (d, *J* = 8.8 Hz, 1H, C_5-*H* (coumarin)*)*, 7.59 - 7.52 (m, 2H, N*H*(Gly), C_6-*H* (coumarin)*)*, 7.37 – 7.29 (m, 5 H, Cbz-H), 6.85 (m, 1H, furyl-*H*), 6.62 (m, 1H, furyl-*H*), 5.02 (s, 2H, CH_2 (Cbz)), 4.49 (m, 1H, *CH*-NH (Leu)), 3.83 (s, 2H, CH_2 (ACA)), 3.79 (d, *J* = 5.6 Hz, 2H, CH_2(Gly)), 3.65 (d, *J* = 6.0 Hz, 2H, CH_2(Gly)), 3.26 (q, *J* = 6.6 Hz, 2H, CH_2(β-Ala)), 2.39 (t, *J* = 6.6 Hz, CH_2(β-Ala)), 1.59 (m, 3H, C*H*(Leu), CH_2(Leu)), 0.92 (m, 6H, 2 x CH_3(Leu); *m*/*z* 718.2672 (MH^+), calc. 718.2724; 740.2392 (MNa^+), calc. 740.2544; 674.2704 (MH^+-CO_2), calc. 674.2826.

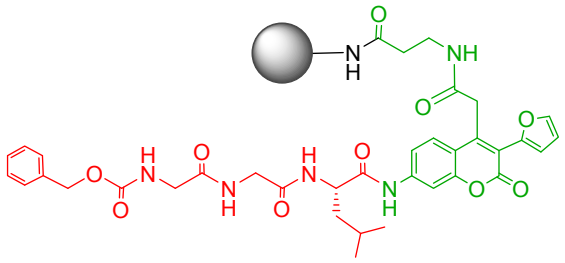
Z-Gly-Gly-Leu-ACA(phenyl)-β-Ala-OH 6a

After lyophilization, the crude product was obtained as a fluffy white solid. (21.3 mg). Analytical HPLC (system 1): t_R = 21.3 min, 41% purity. The crude product was further purified by preparative RP chromatography (system 4d) (4.1 mg, 4%). ^{1}H-NMR (δ, [D_6]-DMSO, 400 MHz): 12.19 (bs, 1H, COO*H*), 10.40 (bs, 1H, Ar-N*H*), 8.21 – 8.15 (m, 3H, N*H* (Leu), N*H* (β-Ala), N*H* (Gly)), 7.85 (d, *J* = 2.0 Hz, 1H, C_8-*H* (coumarin)), 7.62 (d, *J* = 8.9 Hz, 1H, C_5-*H* (coumarin)), 7.55 (m, 2H, C_6-*H* (coumarin), N*H* (Gly)), 7.46 – 7.31 (m, 10H, 5 x Phe-*H*, 5 x Cbz-*H*), 5.03 (s, 2H, CH_2 (Cbz)), 4.50 (m, 1H, *CH*-NH (Leu)), 3.79 (d, *J* = 5.6 Hz, CH_2(Gly)), 3.66 (d, *J* = 6.0 Hz, CH_2(Gly)), 3.50 (s, 2H, CH_2 (ACA)), 3.23 (q, *J* = 6.6 Hz, 2H, CH_2 (β-Ala)), 2.36 (t, *J* = 6.6 Hz, CH_2(β-Ala)), 1.61 (m, 3H, C*H*(Leu), CH_2(Leu)), 0.93 – 0.87 (m, 6H, 2 x CH_3(Leu)); *m*/*z* 728.2821 (MH^+), calc. 728.2932; 750.2652 (MNa^+), calc. 750.2757; 684.3329 (MH^+-CO_2), calc. 684.3000.

Generic preparation of amine-functionalized nanoparticles

A water-in-oil microemulsion was established by dissolving the surfactants Brij 30 (3.08 g) and AOT (1.59 g) in deoxygenated hexane (42 mL). To the mixture was then added the aqueous phase (2 mL), which consisted of the monomers acrylamide (529 mg, 7.44 mmol) and *N*-(3-aminopropyl)methacrylamide (27.2 mg, 0.15 mmol) and the crosslinker *N,N'*-methylene bisacrylamide (160 mg, 1.04 mmol). The polymerization was initiated by the addition of ammonium persulphate (30 µL, 10% w/v) and *N,N,N',N'*-tetramethylenediamine (15 µL). The reaction was allowed to proceed for 2 hours while being stirred under an argon atmosphere. After this time, the hexane was removed by rotary evaporation yielding an opaque, viscous residue. To remove surfactants and unreacted monomers, the particles were washed 10 times with absolute ethanol. The particles were then collected by vacuum filtration using a Millipore filtration system with a 0.02 µm Anodisc filter. After drying under vacuum the particles were obtained as a white powder. (491 mg, 69%).

Coupling of fluorogenic peptides to amine-functionalized nanoparticles will be illustrated with a specific example.



To a dry vial was added Z-Gly-Gly-Leu-ACA(2-furyl)-β-Ala-OH **6e** (4.0 mg, 0.006 mmol) and HOAt (2.2 mg, 0.016 mmol, 4.0 eq.), which were subsequently dissolved in DMF (1mL). DIC (2 µL, 0.013 mmol, 3.1 eq.) was then added. After 1 minute, the coupling solution was transferred to a 10 mL round bottom flask containing NH_2-functionalized nanoparticles (37.6 mg, assuming loading of 0.109 mmol g^{-1}) suspended in DMF (1.5 mL). The reaction mixture was stirred under N_2 at room temperature overnight. Thereafter, the nanoparticles were washed with DMF (10 mL) and ethanol (10 mL) until no fluorescence was detected in the washing solution. The particles were then collected by filtration using a Millipore filtration system (Anodisc filter 0.02 µm) and dried under vacuum (pale yellow powder, 31.4 mg).

Determination of extinction coefficient

The molar absorption coefficient (L mol^{-1} cm^{-1}) was determined from the Lambert Beer's – Law Plot. The molar absorption coefficient corresponds to the slope obtained when different concentrations of the fluorophore were plotted against the absorbance (Figure S1). Stock solutions of the fluorophores were prepared in DMSO. The stock solutions were further diluted with EtOH to obtain concentrations ranging from 6 to 60 µM. The final concentration of DMSO was less than 5%.

Electronic Supplementary Information (ESI)

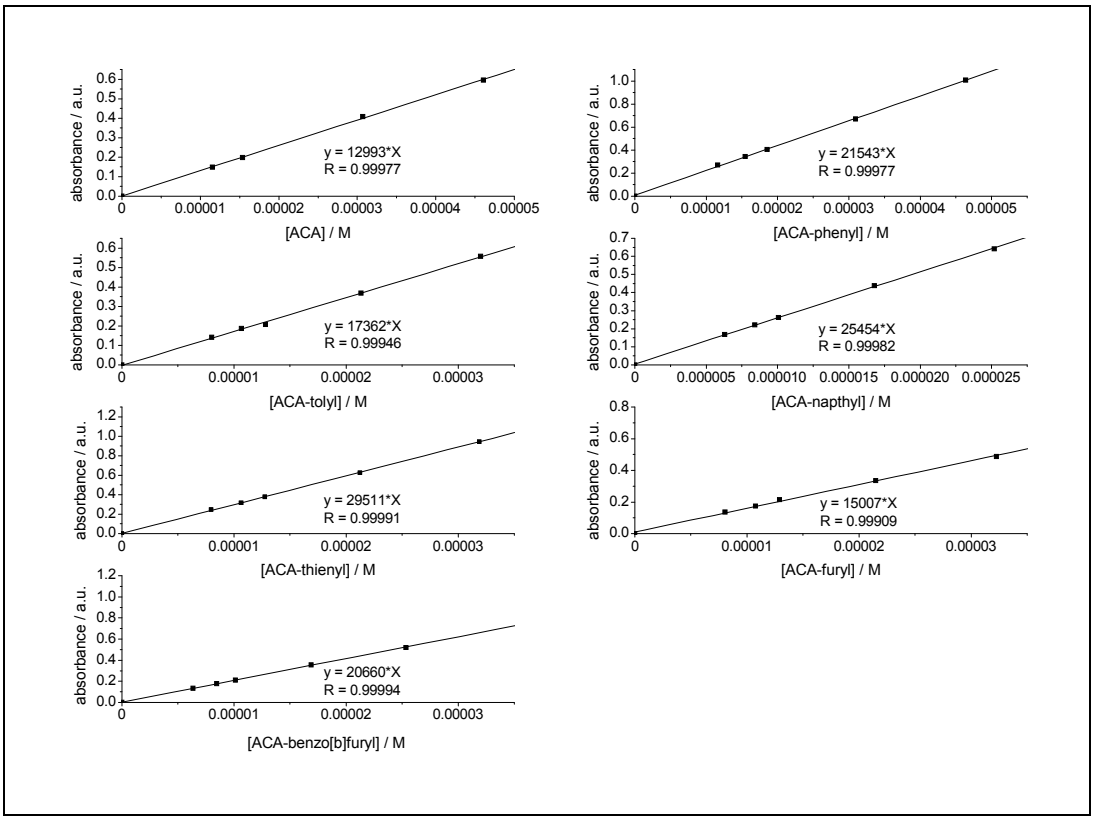


Figure S1: Lambert Beer's plot of ACA and newly synthesized derivatives

Quantum yield and life time measurements.

Sample preparation

Sample solutions of the fluorophores were prepared in PBS/10% DMSO buffer solutions (n_D = 1.3800). In order to minimise re-absorption effects, the absorbance was kept below 0.1.[2] Prior to the measurement, the freshly prepared sample solutions were degassed by purging with argon for 15 minutes.

Determination of quantum yield

Absorption spectra were recorded on a Cary 50 spectrophotometer, while fluorescence spectra were measured on a Cary Eclipse fluorimeter (2.5 nm slit) in a 1 cm quartz cell. Emission spectra were recorded upon excitation at 350 nm and corrected for the wavelength-dependent sensitivity of the detector. Quantum yield measurements were performed against two quantum counters, 9,10-diphenylanthracene in cyclohexane (ϕ_f, = 0.90[3]) and 7-amino-4-methylcoumarin in water (ϕ_f, = 0.94[4]). Relative errors in the quantum yield values are estimated to ± 10%.

[2] S. Dhami , A. J. de Mello, G. Rumbles, S. M. Bishop, D. Philipps, A .Beeby, *Photochem. Photobiol*., **1995**, *61*, 341-346.
[3] S. Hamai; F. Hirayama , *J. Phys. Chem.,* **1983**, *87*, 83-89.
[4] T. L. Arbeloa., F. L. Arbeloa, M. J. Tapia, I. Arbeloa, *J. Phys. Chem*., **1993**, *97*, 4704-4707.

Time resolved fluorescence measurements

Excited-state lifetime measurements in the nanosecond time scale were carried out with the time-correlated single photon counting (TCSPC) technique. Excitation was performed at a repetition rate of 20 MHz with about 65 ps pulses generated by a laser diode at 395 nm (Picoquant model LDH-P-C-400B). The average power was about 0.5 mW at 20 MHz. The scattering reference was a toluene/water emulsion. Fluorescence was collected at 90°C at magic angle with respect to the polarization of the pump pulses. A 420 or 540 nm cut-off filter placed in front of the photomultiplier tube (Hamamatsu, H5783-P-01) ensured that no scattering excitation light could reach the detector, whose output was connected to the input of a TCSPC computer board module (Becker and Hickl, SP-300-12). The full width at the half maximum (fwhm) of the instrument response function (IRF) was around 200 ps. All measurements were performed in a 1 cm quartz cell. The accuracy of the lifetime by this method is estimated approximately 0.3 ns. Time-resolved fluorescence data were analyzed by iterative reconvolution of the instrument response function with trial functions using a nonlinear least-squares fitting procedures (Igor Pro, Wavemetrics Inc.).

Optimized conditions for cleavage reaction

In order to determine the optimum excitation and emission maximum for the enzymatic cleavage reaction, 10 μM solutions of the β-Ala-modified fluorophores (**8**, **8a**, **8e**) and peptide substrates (**6**, **6a**, **6e**) in Tris–HCl buffer (pH = 8.20, 8% DMSO) were excited at different wavelengths. The excitation wavelength at which the peptides only showed a minimal absorbance and the modified fluorophores retained between 25% - 50% of their maximal fluorescence was then chosen in order to calculate the ratio of the fluorescence (F_{rel}) signal between the fluorophore and the corresponding peptide (see Figures S2 and S3). Results are summarized in Table S1. The information is used as a guide for the proteolysis of peptide substrate-bound nanoparticles.

Table S1: Optimized conditions for the cleavage reaction.

Derivatives	opt. λ_{ex} / nm	opt. λ_{em} / nm	F_{rel} / a.u.
-ACA-	380	460	450
-ACA(Phe)-	390	470	300
-ACA(2-furyl)-	430	510	40

Electronic Supplementary Information (ESI)

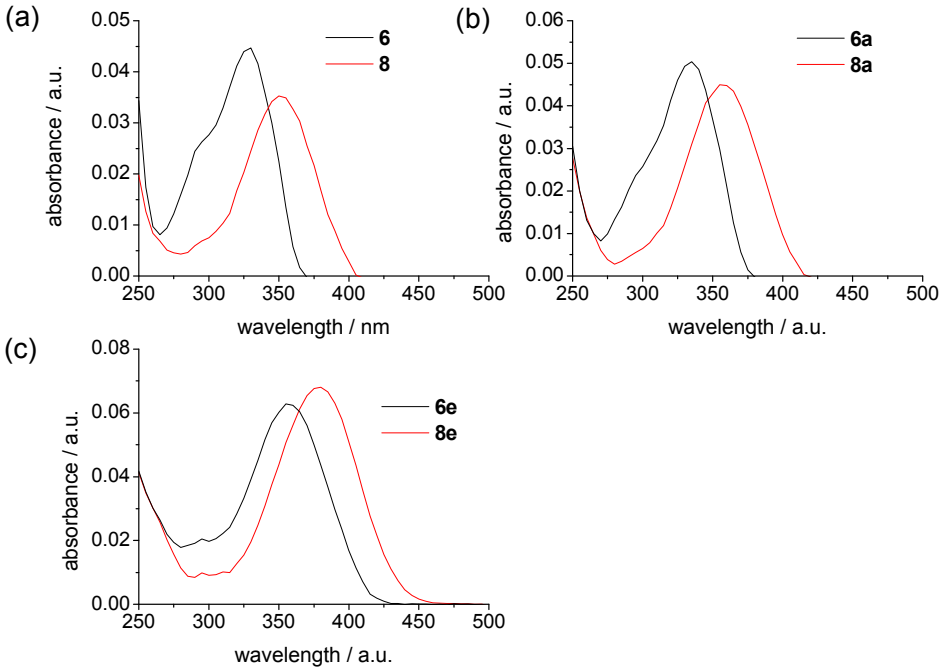


Figure S2: Absorption spectra of 10 µM solutions of β-Ala modified fluorophores (**8**, **8a**, **8e**) and peptide substrates (**6**, **6a**, **6e**) in Tris–HCl buffer.

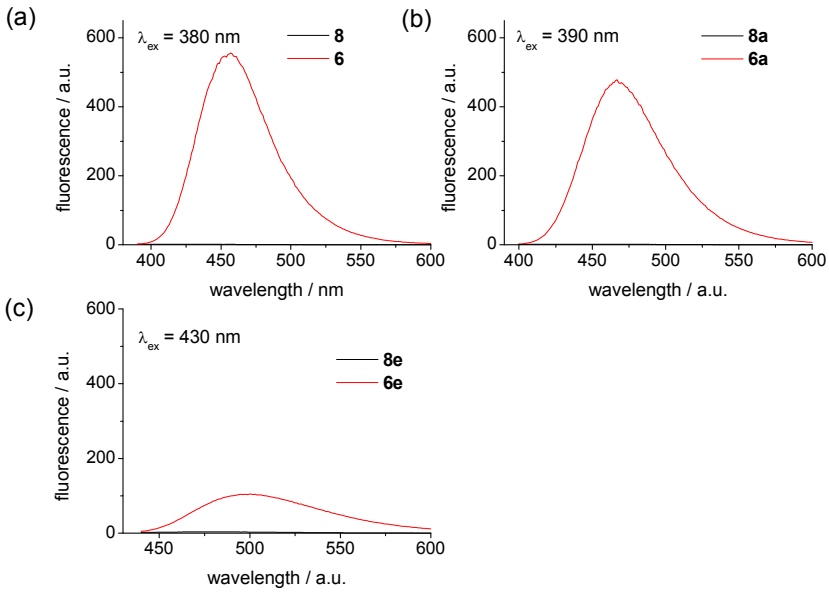
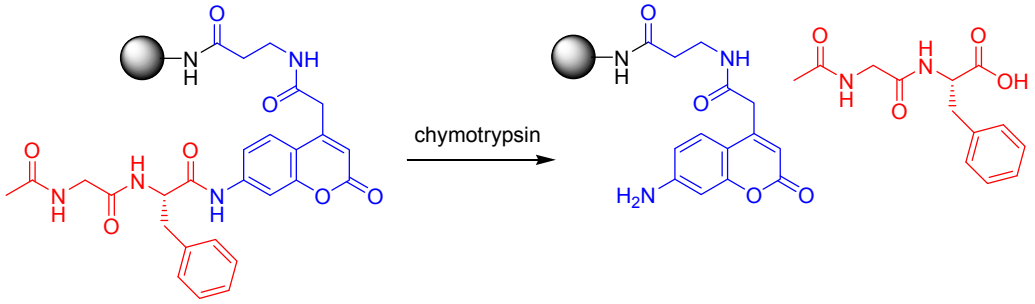


Figure S3: Emission spectra of 10 µM solutions of β-Ala modified fluorophores (**8**, **8a**, **8e**) and peptide substrates (**6**, **6a**, **6e**) in Tris–HCl buffer. For **6** and **6a**, the emission spectra were barely visible as a result of the selected $\lambda_{ex/opt}$.

Experimental setup for subtilisin mediated cleavage reaction.
Cleavage reactions of peptide substrates bound nanoparticle (1mg mL^{-1}) were performed at 37°C in fresh Tris–HCl buffer solutions (50 mM, pH = 8.20) using a Cary Eclipse fluorescence spectrophotometer. After pipetting the substrate (990 μL) into a quartz cuvette and placing the latter into the fluorimeter, the sample solution was incubated at 37°C for 3 minutes. The cleavage reaction was initiated by the addition of a subtilisin solution (0.033 mM, 10 μL) in Tris–HCl buffer. The change in the fluorescence was monitored over a time period of 30 min (λ_{ex} (**6**) = 380 nm, λ_{ex} (**6e**) = 430 nm).

Kinetic analysis
The method of initial rates was used in order to characterize enzyme-substrate interactions. Enzyme activity was monitored at 37°C in Tris–HCl buffer (50 mM, pH = 8.20). Stock solutions of the peptides **6**, **6a** and **6e** were prepared in DMSO and further diluted with Tris–HCl buffer containing 8% DMSO. Initial rates were determined for substrate concentrations ranging from 0.5 to 0.05 mM. In each instance these initial rates, expressed in AU's/min were transformed into the appropriate mM/min units. The corresponding calibrations curves were obtained from solutions of the β-Ala modified fluorophores **8**, **8a** and **8e**. Subtilisin was present in a concentration of 0.33 μM. Hydrolysis of the ACA peptides was monitored fluorimetrically using the optimized conditions for the cleavage reaction. Values of kinetic constants were obtained by fitting the data directly to the Michaelis-Menten equation using nonlinear regression (PRISM).

Example of a chymotrypsin mediated cleavage reaction of polymeric nanoprobes with tethered Ac-Gly-Phe-ACA-β-Ala-OH



Cleavage reaction of nanoparticle bound Ac-Gly-Phe-ACA-β-Ala (1.8 mg mL^{-1}) was performed at 37 °C using a fresh phosphate buffer solution (0.2 M, pH = 7.80). After pipetting the substrate (3 mL) into a cuvette and placing the latter into a fluorimeter, the sample solution was incubated at 37 °C for 3 minutes. The proteolysis was initiated by adding a chymotrypsin solution (200 µL, 0.16 mM) in phosphate buffer. The change in the fluorescence was monitored over a time period of 1 hour, whereupon an emission spectrum was taken every minute (λ_{ex} = 345 nm; not optimised) (Figure S4).

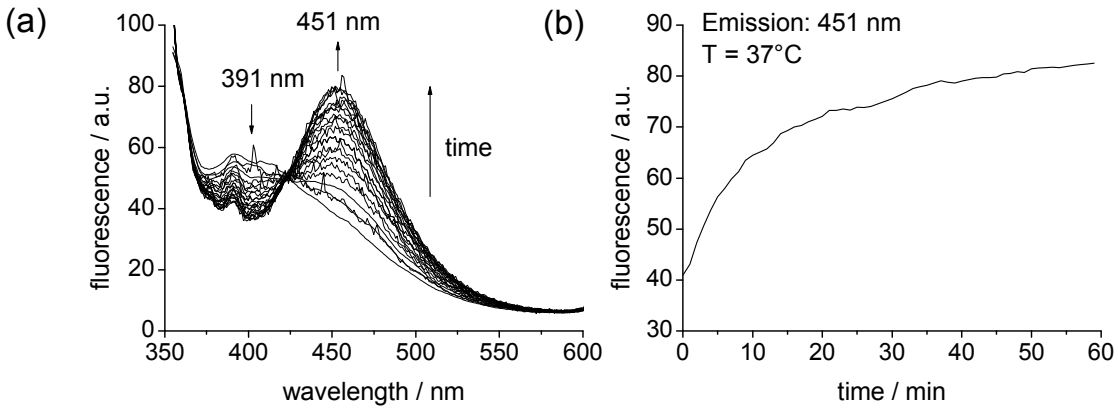


Figure S4: (a) Cleavage of nanoparticle bound Ac-Gly-Phe-ACA-β-Ala by chymotrypsin (λ_{ex} = 345 nm). (b) Rate of the appearance of the tethered [NH_2]-ACA- β-Ala.