Angewandte Chemie

Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Functionalizing αvβ3- or α5β1-Selective Integrin Antagonists for Surface Coating: A Method to Discriminate Integrin Subtypes In Vitro**

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Methods

Nanopatterned gold surfaces

In a typical synthesis, 7 mg/mL of polystyrene(154)-block-poly(2-vinylpyridine)(33) (PS154-b-P2VP33, Polymer Source) was dissolved at room temperature in p-xylene (Sigma-Aldrich, St. Louis, USA) and stirred for 2 days. The quantity of inorganic precursor was calculated relative to the number of P2VP units (NP2VP) and defined as the loading parameter (L). L was kept constant in all experiments, equal to 0.5 (i.e., 1 molecule of $HAuCl_4$ per 2 P2VP units). Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$; Sigma-Aldrich) was added to the block copolymer solution and stirred for 2 days in a sealed glass vessel.

Glass coverslips (Carl Roth, Karlsruhe, Germany) were cleaned in a piranha solution for at least 5 h and extensively rinsed with MilliQ water and dried under a stream of nitrogen. Micellar monolayers were prepared by dip-coating a glass coverslip into the previously prepared solutions with a constant velocity equal to 24 mm/min. Finally, to remove the organic template and form inorganic nanoparticles, the dip-coated glass slides were exposed to oxygen plasma (150 W, 0.15 mbar, 45 min, PVA TEPLA 100 Plasma System).

To prevent nonspecific protein adsorption or cell binding, the substrate area surrounding or in between the gold nanoparticles was covalently modified with polyethylene glycol (PEG; molecular weight 2000).[1] The gold nanoparticles were then functionalized with peptidomimetics by incubating the PEG-functionalized substrates in 100 µl of a 100 µM ethanolic solution. The substrates were then thoroughly rinsed with ethanol and water, incubated overnight in water, and finally dried with nitrogen.

Cell experiments

Mouse fibroblast cell lines expressing either αvβ3 or α5β1 were generated from fibroblasts derived from the kidney of αvflox/flox, β1flox/flox, β2-/- , β7-/- mice (manuscript in preparation). Cells were immortalized by retroviral transduction of the SV40 large T and cloned. Subsequently the cells were retrovirally transduced

with mouse αv or β1 integrin expression constructs. Endogenous β1 and αv integrin loci were deleted by adenoviral cre-recombinase transduction. Selective expression of αvβ3 or α5β1 was verified by flow cytometry and immunoblotting.
Single integrin-expressing fibroblasts were cultivated by using DMEM supplemented with 10% FBS (both from Gibco, Invitrogen, Darmstadt, Germany), 1% penicillin / streptomycin (PAA, Cölbe, Germany) in a culture incubator at 37 °C in an atmosphere containing 5 % CO_2. After reaching 80% confluence, cells were first rinsed with sterile PBS and then released with a 1% trypsin-EDTA solution (Gibco, Invitrogen, Darmstadt, Germany) for about 3-5 minutes. Trypsinisation was stopped by adding 10% FBS supplemented DMEM to the released cells, which were centrifuged at 900 rpm for 3 minutes. The resulting cell pellet was resuspended in pre-warmed growth medium and the cells were seeded again in cell culture dishes.
To test the selectivity of compounds **3** and **4**, both cells lines were serum-starved overnight, trypsinised, resuspended in DMEM containing 0.5 % FBS and seeded (approximately $2 \cdot 10^3$ cells) on functionalized gold nanoarrays. Live cell imaging was performed at 37°C and 5% CO_2 on a Zeiss Axiovert 200M microscope with a 10×1.6 objective; the microscope was equipped with a motorized stage (Märzhäuser, Wetzlar, Germany), an environment chamber (EMBL Precision Engineering, Heidelberg, Germany) and a cooled CCD camera (Roper Scientific, Princeton, NJ). Time-lapse movies (30 frame per second) were made by capturing images every 5 minutes for 6 hours. Spreading area was manually quantified with ImageJ (version 1.43u)[2] on a population of 30 cells. Error bars correspond to the standard deviation.

Competitive solid-phase integrin binding assay

The *in vitro* inhibition of integrin – extracellular matrix protein binding was measured using a solid-phase binding assay with soluble integrins and coated extracellular matrix ligands. Human integrin αvβ3 was purchased from Millipore, α5β1 from R&D Systems and αIIbβ3 from Enzyme Research Laboratories. Vitronectin was purchased from Millipore, fibronectin from Sigma and fibrinogen

from Calbiochem. The integrin antibodies were purchased from Pharmingen, BD Bioscience Europe (mouse anti-human CD49e, mouse anti-human CD51/CD61 and mouse anti-human CD41b) and Sigma (anti-mouse IgG-peroxidase). The detection of HRP was performed using HRP substrate solution 3.3.5.5'-tetramethylethylenediamine (TMB, Seramun, Germany) and 3 M H_2SO_4 for stopping the reaction. The absorbance (450 nm) was recorded with a POLARstar Galaxy plate reader (BMG Labtechnologies). Every concentration was analyzed in duplicates and the resulting inhibition curves were analyzed using OriginPro 7.5G software, the turning point describes the IC_{50} value. Each plate contained Cilengitide[3] or Tirofiban[4] as internal control. Blocking and binding steps were always performed with TS buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) containing 1% BSA (TSB-buffer). Washings after each incubation step were done with PBST buffer (10 mM Na_2HPO_4 pH 7.5, 150 mM NaCl, and 0.01% Tween 20).

Fibronectin-α5β1 assay. Flat-bottom 96-well ELISA plates were coated overnight at 4 °C with 100 µL/well of 0.50 µg/mL of fibronectin in 15 mM of Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 (carbonate buffer). Plates were subsequently blocked for 1 h at room temperature. Next, 1.0 µg/mL of soluble integrin α5β1 and a serial dilution of integrin inhibitors and the control Cilengitide were incubated in the coated wells for 1 h at room temperature. After washing three times, the plate was treated with 100 µL/well of primary antibody (CD49e) at 1.0 µg/mL (1:500 dilution) and secondary antibody (anti-mouse IgG-peroxidase) at 2.0 µg/mL (1:385 dilution) for 1 h each at room temperature. After this treatment the plate was washed three times and the binding visualized with TMB as previously described.

Vitronectin-αvβ3 assay. For the αvβ3 assay, plates were coated with vitronectin (1.0 µg/mL) in carbonate buffer and blocked as described for α5β1. Soluble αvβ3 (1.0 µg/mL) was incubated with a serial dilution of integrin inhibitors and the control Cilengitide for 1 h at room temperature. After washing three times, primary (CD51/CD61, 2.0 µg/mL, 1:250 dilution) and secondary antibody (anti-

mouse-HRP conjugate, 1.0 µg/mL, 1:770 dilution) were applied for 1 h each at room temperature and the binding visualized as explained for α5β1.

Fibrinogen-αIIbβ3 assay. Flat-bottom 96-well ELISA plates were coated overnight at 4 °C with 100 µL/well of 10.0 µg/mL of fibrinogen in carbonate buffer. After blocking the plates, 2.5 µg/mL of soluble integrin αIIbβ3 and a serial dilution of integrin inhibitors and the control molecule Tirofiban were incubated in the coated wells for 1 h at room temperature. The plate was then washed three times and subsequently treated with 100 µL/well of primary antibody (CD41b) at 2.0 µg/mL (1:250 dilution) and secondary antibody (anti-mouse IgG-peroxidase) at 1.0 µg/mL (1:770 dilution) for 1 h each at room temperature. The binding was visualized as explained above.

Supplementary Figures

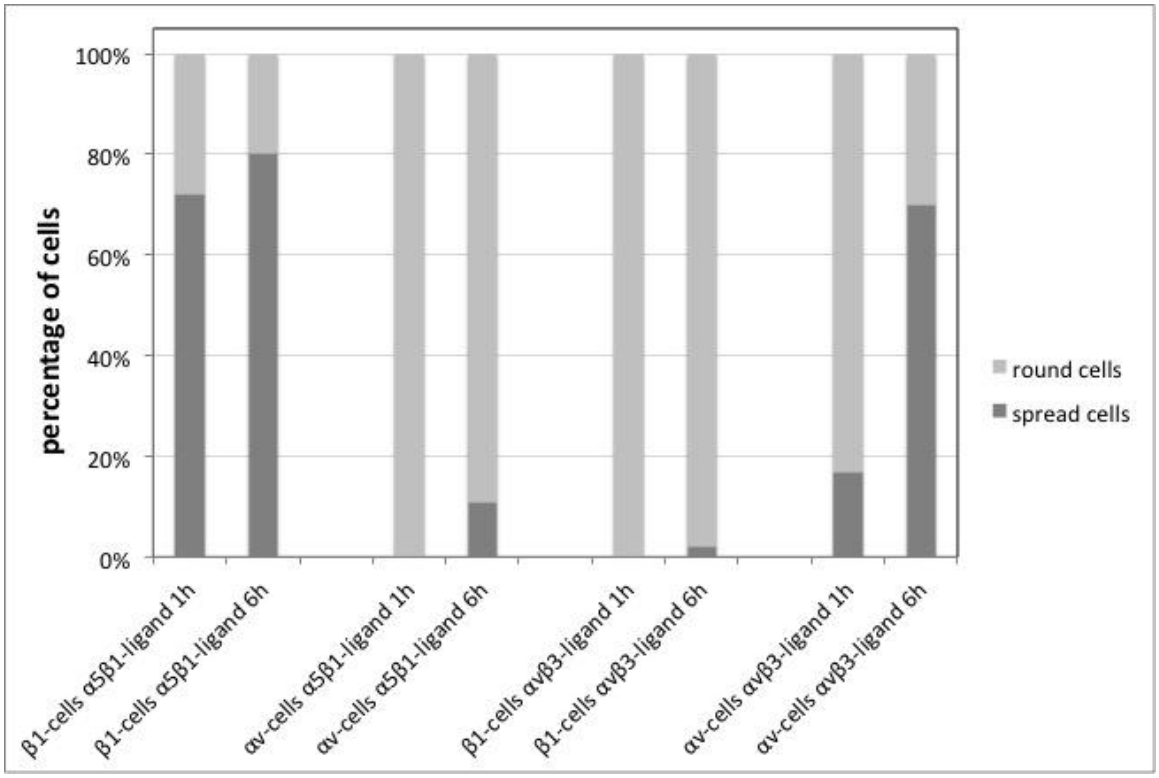


Figure S1: Diagram displaying the percentage of spread and round cells expressing α5β1 or αvβ3 integrin on gold nanoarrays functionalized with α5β1- or αvβ3-ligands in culture for 1h and 6h. A population of about 100 cells has been considered.

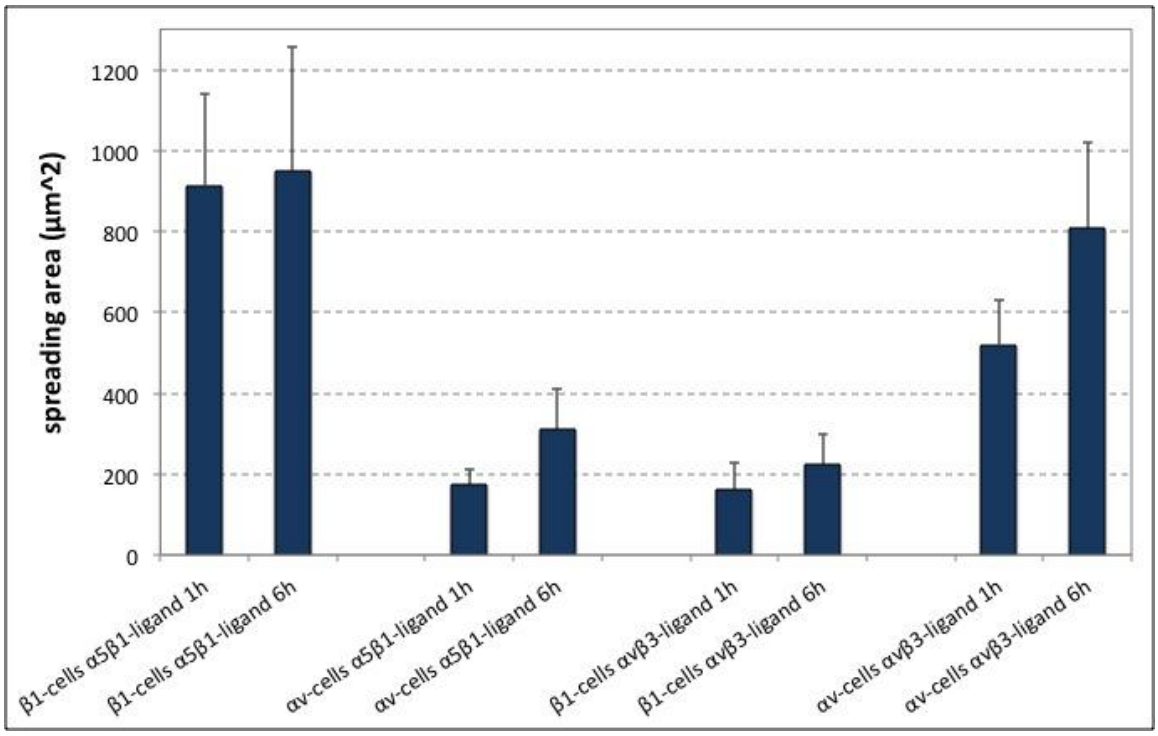


Figure S2: Diagram displaying the spreading area of α5β1- and αvβ3-expressing cells at two different time points on gold nanoarrays functionalized with α5β1- and αvβ3-selective compounds. The number of analyzed cells for each population is n = 30.

General Procedures and Synthesis

Solvents and Reagents. Solvents were purchased from *Aldrich*, *Fluka, Merck and Prolabo*. Reactions sensible to oxygen or water were performed in flame-dried reaction vessels under an argon atmosphere (99.996%). Protected Fmoc-amino acids and coupling reagents were purchased from *Novabiochem* (Schwalbach, Germany), *Iris Biotech GmbH* (Marktredwitz, Germany) and *Medalchemy* (Alicante, Spain). All other chemicals and organic solvents were purchased from commercial suppliers at the highest purity available and used without further purification. Flash chromatographic purification was performed using silica gel 60 (40-63 μm) from *Merck* at 1-1.5 atm pressure.

Analysis. Analytical high-performance liquid chromatography (HPLC) was performed using an *Amersham Pharmacia Biotech* Äkta Basic 10F equipment, with a P-900 pump system, a reversed-phase YMC-ODS-A C18 column (4.6 × 250 mm, 5 μm), and UV detection (UV-900, 220 and 254 nm). The system was run at a flow rate of 1.0 ml/min over 30 min using H_2O (0.1% (v/v) trifluoroacetic acid, [TFA]) and MeCN (0.1% (v/v) TFA) as solvents. Semi-preparative HPLC was carried out on a *Beckmann* instrument (system gold, solvent delivery module 126, UV detector 166) using an YMC ODS-A column (20 × 250 mm, 5 μm), at a flow rate of 8 ml/min. Linear gradients using H_2O (0.1% (v/v) TFA) and MeCN (0.1% (v/v) TFA) were run over varying periods of time. HPLC-mass spectrometry (MS) analyses were performed on a *Hewlett Packard Series* HP 1100 with a *Finnigan LCQ* mass spectrometer using a YMC Hydrosphere C18 column (2.1 ×125 mm, 3 μm). The system uses H_2O (0.1% (v/v) formic acid) and MeCN (0.1% (v/v) formic acid) as eluents. High resolution mass spectrometry (HR-MS) was recorded on a *Thermo Finnigan* LTQ-FT (ESI-ICR) spectrometer. ^{1}H-NMR and ^{13}C NMR spectra were recorded at 295 K on a 500 MHz *Bruker* DMX, 360 MHz *Bruker* AV or a 250 MHz *Bruker* AV spectrometer (*Bruker*, Karlsruhe, Germany). Chemical shifts (δ) are given in *parts per million* (ppm). Following solvent peaks were used as internal standards: dimethyl sulfoxide (DMSO)-d_5: 2.50 ppm (^{1}H-NMR) and 39.52 ppm (^{13}C-NMR); $CHCl_3$: 7.26 ppm (^{1}H-NMR) and 77.16 ppm (^{13}C-NMR).[5]

GP1 Boc deprotection in solution. For Boc deprotection the compound was stirred for 30 min in TFA/DCM (1:1; v/v). The solvent was removed under reduced pressure to obtain the deprotected compound.

GP2 Coupling with HATU/HOAt in solution. A solution of the acid (1 eq.), the amine (1 eq.), HATU (1.3 eq.), HOAt (1.3 eq.) and DIEA (5 eq.) in DMF was stirred over night. After removing the DMF under reduced pressure, the residue was redissolved in EtOAc, washed consecutively with saturated NH_4Cl, saturated $NaHCO_3$ and *brine* and dried over Na_2SO_4. After evaporation of the solvent the crude product was purified by reverse phase HPLC to afford the final compound.

GP3 Methyl deprotection. The compound was dissolved in MeOH/H_2O (3:1; v/v) and LiOH (5 eq.) was added. The reaction was stirred for 4 h to 12 h at ambient temperature while the course of reaction was followed by analytical HPLC.

GP4 Loading of tritylchloride (TCP) resin. Solid phase synthesis was carried out using TCP resin (0.94 mmol/g) following standard Fmoc strategy. Fmoc-Aax-OH (1.2 eq.) was attached to the TCP resin with *N,N*-diisopropylethylamine (DIEA, 2.5 eq.) in anhydrous DCM (10 ml/g resin) at room temperature for 1 h. The remaining trityl chloride groups were capped by addition of a solution of MeOH/DIEA (4/1, v/v, 1 ml/g resin) for 15 min. The resin was filtered and washed thoroughly with DCM (2x), *N*-methyl-2-pyrrolidone (NMP, 3x) and MeOH (5x). The loading capacity was determined by weight after drying the resin under vacuum.

GP5 Solid phase *N*-Fmoc deprotection. The resin-bound Fmoc peptide was treated with 20% piperidine in NMP (v/v) for 5 min and a second time for 10 min. The resin was washed with NMP (5x).

GP6 Solid phase coupling with HATU/HOAt. A solution of the acid (2 eq.), HATU (2 eq.), HOAt (2 eq.), DIEA (5 eq.) in NMP was added to the resin-bound free amine peptide and shaken for 1.5 h at room temperature. The resin was washed with NMP (5x).

GP7 Solid phase *N*-Alloc deprotection. The resin was washed with DCM (3x) and then treated with a solution of *tetrakis*-triphenylphosphinepalladium (0.25

eq.) and phenylsilane (10 eq.) in DCM at ambient temperature. Care had to be taken due to gas evolution and the pressure had to be released from the reaction vessel from time to time. After 30 min of shaking, the mixture was filtered and the resin washed twice with a 0.5% solution of DDTC (sodium *N*,*N*-diethyldithiocarbamate) in DMF and a 0.5% solution of DIEA in DMF. The washing procedure was repeated (3x) and the resin washed with NMP (5x).

GP8 Guanidinylation. The amino-functionalized resin was added to a solution of 10 eq. *N*,*N*'-bis-Boc-guanidinylpyrazole in absolute DCM (10 ml/g resin) in a closed reaction vessel. The mixture was shaken over night at room temperature. The resin was filtered and washed five times with DCM. The unconsumed guanidinylpyrazole could be recycled by concentration of the filtrate and recrystallization from hexane/ethyl acetate.

GP9 Cleavage from TCP resin. The resin-bound compound was treated three times with a 20% solution of HFIP (hexafluoroisopropanol) in DCM (v/v) for 10 min. The collected solutions were concentrated *in vacuo*.

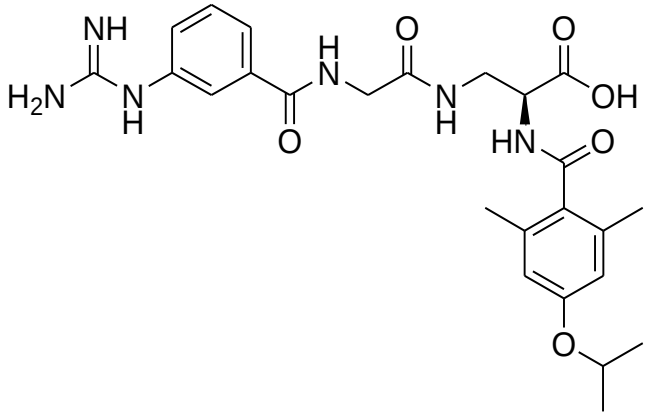
GP10 Boc, *t*Bu and trityl deprotection in solution. Deprotection was achieved by treatment with TFA/TIS/H_2O (95/2.5/2.5, v/v/v) in solution for 1 h. The mixture was coevaporated with toluene (3x).

GP11 Reductive cleavage of benzyl and carboxybenzyl protecting groups. The starting material (1 eq.) was dissolved in MeOH. After addition of the catalyst (5% Pd/C, 15 mg/mmol starting material) the mixture was hydrogenated (1 atm, H_2) at room temperature. The progress of the reaction was monitored by analytical HPLC until all starting material was consumed. The catalyst was removed by filtration over Celite® and the solvent removed under reduced pressure.

GP12 Synthesis of ethers by *Mitsunobu* reaction. The aromatic alcohol (1 eq.), the aminoalcohol (1.2 eq) and tributylphosphine (1.2 eq.) were dissolved in dry THF and stirred at 0 °C under argon atmosphere. Azodicarboxydipiperidide (ADDP, 1.2 eq.) was dissolved in absolute THF and added to the reaction mixture over a period of 4 h. The resulting suspension was allowed to warm to

room temperature over night. The solvent was removed and the residue purified by flash chromatography on silica gel.

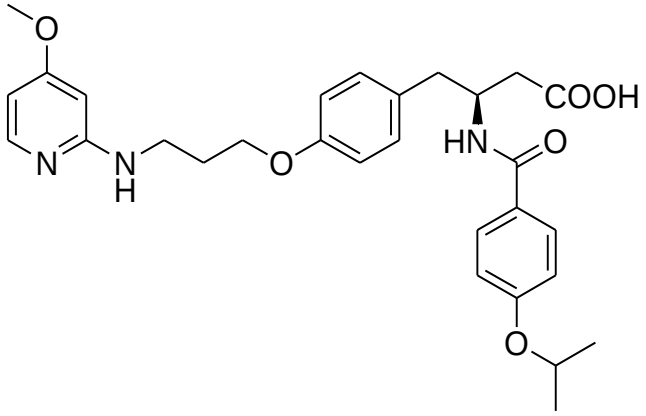
(S)-3-(2-(3-guanidinobenzamido)acetamido)-2-(4-isopropoxy-2,6-dimethylbenzamido)propanoic acid) (1)



$C_{25}H_{32}N_6O_6$
Exact Mass: 512,24
Mol. Wt.: 512,56

Preparation of compound **1** according to literature[6] gave 15.13 mg (26 µmol, 22%) as colorless solid. **^{1}H-NMR** (500 MHz, DMSO): δ = 12.71 (bs, 1H), 9.95 (bs, 1H), 8.79 (t, ^3J = 5.8 Hz, 1H), 8.27 (d, ^3J = 6.6 Hz, 1H), 8.02 (t, ^3J = 5.7 Hz, 1H), 7.77 (d, ^3J = 7.9 Hz, 1H), 7.73 (s, 1H), 7.55-7.42 (m, 4H), 7.39 (d, ^3J = 9.1 Hz, 1H), 6.57 (s, 2H), 4.62-4.56 (m, 1H), 4.50-4.46 (m, 1H), 3.98-3.87 (m, 3H), 3.91 (dd, ^2J = 16.3 Hz, ^3J = 5.9 Hz, 1H), 3.83 (dd, ^2J = 16.3 Hz, ^3J = 5.8 Hz, 1H), 3.56-3.51 (m, 1H), 3.44-3.39 (m, 1H), 2.20 (s, 6H), 1.24 (d, ^3J = 6.0 Hz, 6H). **^{13}C NMR** (126 MHz, DMSO-d6) δ = 171.9, 169.2, 165.7, 156.9, 155.8, 135.7, 135.6, 135.4, 130.6, 129.7, 127.2, 125.2, 123.4, 114.0, 68.8, 52.0, 42.5, 21.8, 19.1. **RP-HPLC** (10-90%, 30 min) R_t = 13.50 min. **MS** (ESI): m/z = 513.2 $[m+H]^+$, 535.2 $[m+Na]^+$. **HR-MS** (ESI) calc. for $C_{25}H_{33}N_6O_6^+$: 513.24561; found: 513.24585.

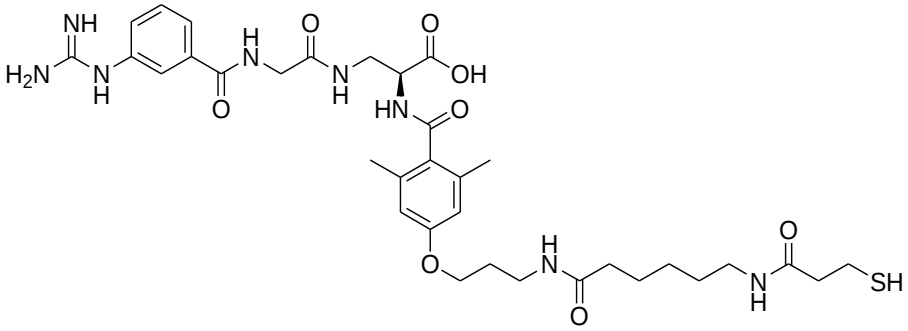
(S)-3-(4-isopropoxybenzamido)-4-(4-(3-((4-methoxypyridin-2-yl)amino)propoxy)phenyl)butanoic acid (2)



$C_{29}H_{35}N_3O_6$
Exact Mass: 521,25
Mol. Wt.: 521,6

The deprotection of the ligand precursor **15** (336 mg, 0.71 mmol, 1.0 eq.) and coupling with 4-isopropoxybenzoic acid (166 mg, 0.92 mmol, 1.3 eq.) followed GP1 and GP2. The crude product was deprotected according GP3. Semipreparative HPLC purification yielded the title compound **2** (32.7 mg, 62.7 µmol, 9%). **^{1}H-NMR** (500 MHz, DMSO): δ [ppm] = 12.87 (bs, 1H), 12.17 (bs, 1H), 8.28 (bs, 1H), 8.13 (d, ^3J = 8.3 Hz, 1H), 7.80 (d, ^3J = 7.1 Hz, 1H), 7.72 (d, ^3J = 8.8 Hz, 2H), 7.13 (d, ^3J = 8.5 Hz, 2H), 6.94 (d, ^3J = 8.8 Hz, 2H), 6.83 (d, ^3J = 8.6 Hz, 2H), 6.46 (dd, ^3J = 7.0 Hz, ^4J = 2.1 Hz, 1H), 6.33 (d, ^4J = 1.9 Hz, 1H), 4.72-4.65 (m, 1H), 4.44-4.37 (m, 1H), 4.01 (t, ^3J = 6.0 Hz, 2H), 3.86 (s, 3H), 3.46-3.42 (m, 2H), 2.80 (dd, ^2J = 13.6 Hz, ^3J = 8.0 Hz, 1H), 2.73 (dd, ^2J = 13.6 Hz, ^3J = 5.8 Hz, 1H), 2.52-2.48 (m, 1H), 2.41 (dd, ^2J = 15.4 Hz, ^3J = 6.2 Hz, 1H), 2.02-1.97 (m, 2H), 1.27 (d, ^3J = 6.0 Hz, 6H). **^{13}C-NMR** (125 MHz, DMSO): δ [ppm] = 172.5, 165.1, 159.7, 156.8, 130.9, 130.1, 129.0, 126.5, 114.7, 114.1, 103.9, 99.4, 69.3, 64.6, 56.4, 48.3, 40.1, 39.8, 38.7, 27.9, 21.7. **RP-HPLC** (10-90%): t_R = 17.63 min. **MS** (ESI): *m/z* (%) = 522.4 $[m+H]^+$. **HR-MS** (ESI) calc. for $C_{29}H_{36}N_3O_6^+$: 522.25986; found: 522.25795.

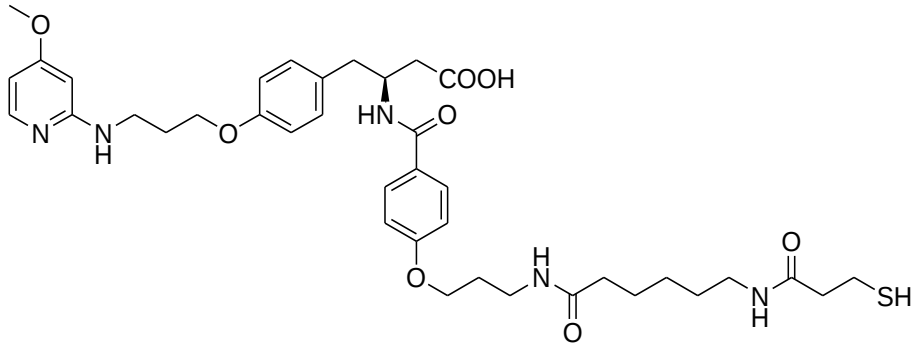
(S)-3-(2-(3-guanidinobenzamido)acetamido)-2-(4-(3-(6-(3-mercaptopropan-amido)hexanamido)propoxy)-2,6-dimethylbenzamido)propanoic acid (3)



$C_{34}H_{48}N_8O_8S$
Exact Mass: 728,33
Mol. Wt.: 728,86

Prepared from *N*-α-Fmoc-*N*-β-Alloc-l-diaminopropionic acid according the following reaction sequence: Loading of 1 g resin (real loading: ~693 µmol, GP4), Fmoc deprotection (GP5), coupling of **12** (GP6), Fmoc deprotection (GP5), coupling of 6-(*tert*-butoxycarbonyl)aminohexanoic acid (GP6), Fmoc deprotection (GP5), coupling of 3-(trityl)mercaptopropionic acid (GP6), Alloc deprotection (GP7), coupling of Fmoc-Gly-OH (GP6), Fmoc deprotection (GP5), coupling of 3-Fmoc-aminobenzoic acid (GP6), Fmoc deprotection (GP5), guanidinylation (GP8), cleavage from the resin (GP9), Trityl and Boc deprotection (GP10). After semipreparative HPLC purification compound **3** (15.5 mg, 21.3 µmol, 3%) was obtained as colorless solid. **^{1}H-NMR** (500 MHz, DMSO): δ = 12.46 (bs, 1H), 9.95 (s, 1H), 8.80 (t, ^3J = 5.8 Hz, 1H), 8.31 (d, ^3J = 7.0 Hz, 1H), 8.04 (t, ^3J = 5.6 Hz, 1H), 7.84 (t, ^3J = 5.3 Hz, 2H), 7.76 (d, ^3J = 7.8 Hz, 1H), 7.72 (s, 1H), 7.58-7.45 (m, 4H), 7.39 (d, ^3J = 8.9 Hz, 1H), 6.58 (s, 2H), 4.52-4.45 (m, 1H), 3.98-3.87 (m, 3H), 3.82 (dd, ^2J = 16.4 Hz, ^3J = 5.7 Hz, 1H), 3.58-3.50 (m, 1H), 3.45-3.38 (m, 1H), 3.18-3.15 (m, 2H), 3.03-3.00 (m, 2H), 2.66-2.61 (m, 2H), 2.35 (t, ^3J = 6.9 Hz, 2H), 2.24 (t, ^3J = 8.0 Hz, 1H), 2.21 (s, 6H), 2.04 (t, ^3J = 7.4 Hz, 2H), 1.83-1.78 (m, 2H), 1.51-1.45 (m, 2H), 1.40-1.34 (m, 2H), 1.25-1.19 (m, 2H). **^{13}C NMR** (126 MHz, DMSO-d6) δ = 172.0, 171.9, 169.7, 169.2, 169.1, 165.7, 158.0, 155.8, 135.7, 135.6, 135.4, 130.7, 129.7, 127.2, 125.1, 123.4, 112.9, 65.1, 52.1, 42.5, 38.4, 35.4, 35.3, 35.0, 34.0, 28.9, 28.9, 26.1, 25.0, 19.1. **RP-HPLC** (10-90%, 30 min) R_t = 11.48 min. **MS** (ESI): m/z = 729.3 $[m+H]^+$, 751.3 $[m+Na]^+$. **HR-MS** (ESI) calc. for $C_{34}H_{49}N_8O_8S^+$: 729.33941; found: 729.33903.

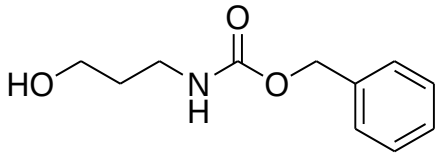
(S)-3-(4-(3-(6-(3-mercaptopropanamido)hexanamido)propoxy)benzamido)-4-(4-(3-((4-methoxypyridin-2-yl)amino)propoxy)phenyl)butanoic acid (4)



$C_{38}H_{51}N_5O_8S$
Exact Mass: 737,35
Mol. Wt.: 737,91

After Boc deprotection of ligand precursor **15** (32 mg, 67.6 µmol, 1.0 eq.) according to GP1 the solvent was evaporated *in vacuo*. The deprotected compound was coupled with **17** (52 mg, 81.1 µmol, 1.2 eq.) (GP2) and deprotected according to GP3 and GP1. Semipreparative HPLC purification afforded the title compound **4** (4.05 mg, 5.49 µmol, 8%) as colorless solid. **^{1}H-NMR** (500 MHz, DMSO): δ = 12.69 (bs, 1H), 12.17 (bs, 1H), 8.40 (bs, 1H), 8.15 (d, ^3J = 8.4 Hz, 1H), 7.87-7.82 (m, 2H), 7.79 (t, ^3J = 6.4 Hz, 1H), 7.74 (d, ^3J = 8.8 Hz, 2H), 7.13 (d, ^3J = 8.6 Hz, 2H), 6.96 (d, ^3J = 8.8 Hz, 2H), 6.83 (d, ^3J = 8.6 Hz, 2H), 6.51 (dd, ^3J = 7.2 Hz, ^4J = 2.4 Hz, 1H), 6.36 (d, ^4J = 2.3 Hz, 1H), 4.44-4.37 (m, 1H), 4.04-3.99 (m, 4H), 3.88 (s, 3H), 3.46-3.42 (m, 2H), 3.20-3.16 (m, 2H), 3.04-3.00 (m, 2H), 2.80 (dd, ^2J = 13.6 Hz, ^3J = 8.0 Hz, 1H), 2.73 (dd, ^2J = 13.6 Hz, ^3J = 5.9 Hz, 1H), 2.66-2.61 (m, 2H), 2.53-2.48 (m, 1H), 2.41 (dd, ^2J = 15.4 Hz, ^3J = 6.2 Hz, 1H), 2.35 (t, ^3J = 6.9 Hz, 2H), 2.24 (t, ^3J = 8.0 Hz, 1H), 2.07-1.98 (m, 4H), 1.87-1.81 (m, 2H), 1.51-1.45 (m, 2H), 1.40-1.34 (m, 2H), 1.25-1.19 (m, 2H). **^{13}C-NMR** (125 MHz, DMSO): δ = 172.6, 172.1, 170.0, 169.3, 165.1, 160.8, 157.8, 156.8, 154.6, 131.0, 130.1, 129.0, 126.8, 114.2, 113.8, 104.4, 91.7, 65.5, 64.5, 56.6, 48.4, 38.8, 38.4, 35.4, 28.9, 27.8, 26.1, 25.0, 20.0. **HPLC** (10-90%, 30 min) t_R = 15.43 min. **MS** (ESI): 738.5 $[m+H]^+$, 369.9 $[m/2+H]^+$. **HR-MS** (ESI): calc. for $C_{38}H_{52}N_5O_8S^+$: 738.35311; found: 738.35471.

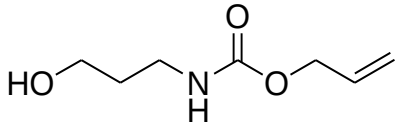
3-(Benzyloxycarbonylamino)-propan-1-ol (5)



$C_{11}H_{15}NO_3$
Exact Mass: 209,11
Mol. Wt.: 209,24

3-Aminopropanol (3.0 ml, 39.2 mmol, 1 eq.) and $NaHCO_3$ (8.23 g, 98.0 mmol, 2.5 eq.) were dissolved in 80 ml THF/H_2O (1:1; v/v). After addition of benzylchloroformiate (5.60 ml, 39.2 mmol, 1 eq.) over a period of 30 min and stirring for 12 h the THF was removed. The aqueous phase was extracted with EtOAc, the organic phase washed with *brine*, dried over Na_2SO_4 and the solvent removed under reduced pressure. Purification via flash chromatography (hexane/EtOAc 2:3) gave **5** (6.31 g, 30.2 mmol, 77%) as colorless solid. **^{1}H-NMR** (250 MHz, DMSO): δ = 7.29-7.39 (m, 5H, Ar-*H*), 7.12 (t, ^3J = 6.5 Hz, 1H, -N*H*-), 5.00 (s,2H, -C*H*$_2$-Ar), 4.40 (bs, 1H, -O*H*), 3.37-3.43 (m, 2H, -C*H*$_2$-OH), 3.04 (q, 2H, ^3J = 6.5 Hz, -C*H*$_2$-NH-),1.50-1.61 (m, 2H, -CH_2-C*H*$_2$-CH_2-). **^{13}C-NMR** (90 MHz, DMSO): δ = 156.3, 137.4, 128.5, 127.9, 127.8, 65.2, 58.5, 37.7, 32.7. **RP-HPLC** (50-100%, 30 min) R_t = 13.42 min. **MS** (ESI): m/z = 210.1 $[m+H]^+$, 232.3 $[m+Na]^+$.

Allyl 3-hydroxypropylcarbamate (6)

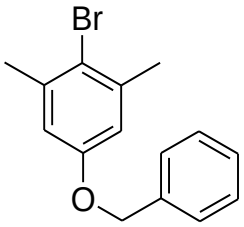


$C_7H_{13}NO_3$
Exact Mass: 159,09
Mol. Wt.: 159,18

3-Aminopropanol (3.00 g, 39.9 mmol, 1.0 eq.) and Na_2CO_3 (4.44 g, 41.9 mmol, 1.05 eq.) were dissolved in 60 ml H_2O/acetonitrile (2:1) and cooled to 0 °C. Allyl chloroformate (5.29 g, 43.9 mmol, 1.1 eq.) was added over 30 min and the mixture was allowed to warm to room temperature over night. After evaporation of acetonitrile, conc. HCl was added to the residue (pH 2) and taken up in EtOAc. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated to give 5.77 g (36.2 mmol, 91%) of a colorless solid. **^{1}H-NMR** (360 MHz, $CDCl_3$): δ = 5.94 - 5.83 (m, 1H), 5.27 (dd, ^3J = 17.2 Hz, ^2J = 1.4 Hz, 1H), 5.18 (dd, ^3J = 10.4 Hz, ^2J = 1.2 Hz, 1H), 5.09 (bs, 1H), 4.53 (d, ^3J = 5.4 Hz, 2H),

3.65 (t, ^{3}J = 5.7 Hz, 2H), 3.32-3.30 (m, 2H), 2.44 (bs, 1H), 1.81-1.65 (m, 2H). **^{13}C-NMR** (90 MHz, $CDCl_3$): δ = 157.4, 133.0, 117.9, 65.9, 59.8, 37.9, 32.7. **MS** (ESI): 160.0 $[m+H]^+$.

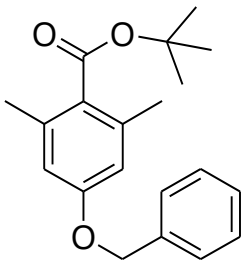
1-((4-bromo-3,5-dimethylphenoxy)methyl)benzene (7)



$C_{15}H_{15}BrO$
Exact Mass: 290,03
Mol. Wt.: 291,18

4-Bromo-3,5-dimethylphenole (5.00 g, 24.9 mmol, 1 eq.) was dissolved in 75 ml dry DMF and cooled to 0 °C. NaH (1.30 g, 29.9 mmol, 1.3 eq., 60% in paraffin oil) was added and the solution allowed to stir for 30 min. After addition of benzylbromide (2.95 ml, 24.9 mmol, 1 eq.) the solution was stirred for another 2 h at room temperature. After addition of 50 ml saturated aqueous NH_4Cl, the solution was extracted with diethylether and the organic layer washed with *brine* and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude product purified via flash chromatography (hexane/EtOAc 9:1) to give **7** (6.47 g, 22.2 mmol, 89%) as colorless crystals. **^{1}H-NMR** (250 MHz, DMSO): δ = 7.45-7.28 (m, 5H), 6.87 (s, 2H), 5.06 (s, 2H), 2.31 (s, 6H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 157.3, 139.1, 136.9, 128.6, 128.0, 127.4, 118.6, 114.7, 70.1, 24.1. **RP-HPLC** (50-100%, 30 min) R_t = 22.66 min.

***tert*-Butyl 4-(benzyloxy)-2,6-dimethylbenzoate (8)**

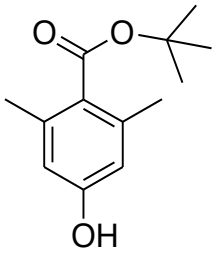


$C_{20}H_{24}O_3$
Exact Mass: 312,17
Mol. Wt.: 312,4

7 (5.50 g, 18.9 mmol, 1 eq.) was dissolved in 100 ml dry THF and cooled to -78 °C. Then, *n*-Butyllithium (2.5 m in hexane, 9.00 ml, 22.5 mmol, 1.2 eq.) was added and the solution allowed to stir for 30 min. After addition of Boc_2O (4.15 g,

19 mmol, 1 eq.) the solution was again stirred for 30 min before it was allowed to come to room temperature within 1.5 h. The mixture was acidified with 1 n HCl, the THF evaporated and the aqueous phase extracted with EtOAc. The organic layer was washed with *brine*, dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified via flash chromatography (hexane/EtOAc 26:1) to give **8** (5.58 g, 17.9 mmol, 95%) as a colorless solid. **^{1}H-NMR** (250 MHz, DMSO): δ = 7.45-7.30 (m, 5H), 6.63 (s, 2H), 5.04 (s, 2H), 2.22 (s, 6H), 1.40 (s, 9H). **^{13}C-NMR** (90 MHz, DMSO): δ = 168.4, 158.5, 138.7, 137.4, 128.4, 127.8, 127.6, 122.4, 112.5, 80.7, 69.0, 27.6, 21.1. **RP-HPLC** (50-100%, 30 min) R_t = 20.51 min.

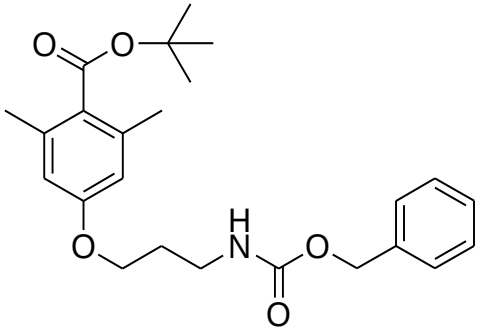
***tert*-Butyl 4-hydroxy-2,6-dimethylbenzoate (9)**



$C_{13}H_{18}O_3$
Exact Mass: 222,13
Mol. Wt.: 222,28

Prepared from **8** (5.58 g, 17.9 mmol) according to GP11 gives **9** (3.96 mg, 17.8 mmol, 100%) as a pale yellow liquid. **^{1}H-NMR** (250 MHz, DMSO): δ = 9.52 (s, 1H), 6.43 (s, 2H), 2.17 (s, 6H), 1.51 (s, 9H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 169.2, 155.7, 137.1, 128.1, 114.4, 81.5, 28.3, 19.8. **RP-HPLC** (50-100%, 30 min) R_t = 8.80 min.

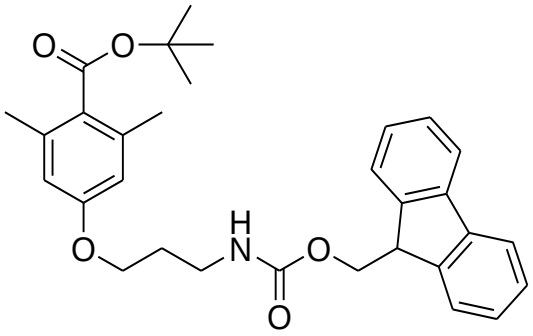
Benzyl 4-(4-(*tert*-butoxycarbonyl)-3,5-dimethylphenoxy)butylcarbamate (10)



$C_{24}H_{31}NO_5$
Exact Mass: 413,22
Mol. Wt.: 413,51

Prepared from **9** (1.77 g, 7.96 mmol, 1 eq.) and **5** (1.83 g, 8.76 mmol, 1.1 eq.) according to GP12. Purification via flash chromatography (hexane/EtOAc 3:1) of the crude product gives **10** (3.16 g, 7.64 mmol, 96%) as a colorless oil. **^{1}H-NMR** (250 MHz, DMSO): δ = 7.38-7.29 (m, 5H), 6.61 (s, 2H), 5.01 (s, 2H), 3.97 (t, ^3J = 6.4 Hz, 2H), 3.15 (q, ^3J = 6.4 Hz, 2H), 2.22 (s, 6H), 1.89-1.79 (m, 2H), 1.52 (s, 9H). **^{13}C-NMR** (90 MHz, DMSO): δ = 168.4, 158.5, 156.2, 137.3, 135.9, 128.4, 127.8, 127.8, 127.7, 113.4, 65.2, 65.1, 37.4, 29.1, 27.8, 19.5. **RP-HPLC** (50-100%, 30 min) R_t = 17.54 min. **MS** (ESI): m/z = 436.3 $[m+Na]^+$.

***tert*-Butyl 4-(4-(9H-fluoren-9-yl)methyl)-carbaminobutoxy)-2,6-dimethylbenzoate (11)**

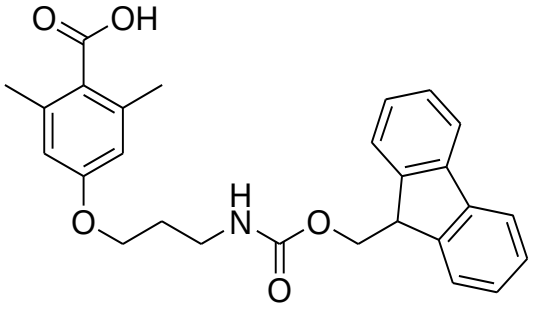


$C_{31}H_{35}NO_5$
Exact Mass: 501,25
Mol. Wt.: 501,61

After deprotection of **10** (2.97 g, 7.19 mmol) according to GP11 the obtained oil was dissolved in 120 ml THF/H_2O (1:1) and $NaHCO_3$ (1.21 g, 14.4 mmol, 2 eq.) was added. After addition of *N*-(9-Fluorenylmethoxycarbonyloxy) succinimide (2.76 g, 8.19 mmol, 1.1 eq.) over a period of 30 min and stirring for 12 h the THF was removed. The aqueous phase was extracted with EtOAc, the organic phase washed with *brine*, dried over Na_2SO_4 and the solvent removed under reduced

pressure. Purification via flash chromatography (hexane/EtOAc 3:1) yields **11** (3.41 g, 6.80 mmol, 95%) as a colorless solid. **^{1}H-NMR** (250 MHz, DMSO): δ = 7.88 (d, ^{3}J = 7.2 Hz, 1H), 7.68 (d, ^{3}J = 7.2 Hz, 1H), 7.45-7.27 (m, 5H), 6.61 (s, 2H), 4.31 (d, ^{3}J = 6.8 Hz, 2H), 4.21 (t, ^{3}J = 6.8 Hz, 1H), 3.96 (t, ^{3}J = 6.4 Hz, 2H), 3.14 (q, ^{3}J = 6.4 Hz, 2H), 2.22 (s, 6H), 1.88-1.77 (m, 2H), 1.52 (s, 9H). **^{13}C-NMR** (90 MHz, DMSO): δ = 168.4, 161.2, 157.5, 143.9, 135.9, 136.0, 135.4, 133.3, 129.5, 124.0, 121.2, 114.2, 113.4, 81.0, 80.7, 65.1, 53.9, 28.8, 27.9, 19.5. **RP-HPLC** (50-100%, 30 min) R_t = 22.46 min. **MS** (ESI): m/z = 524.4 $[m+Na]^+$, 468.6 $[m-t\text{Bu}+Na]^+$.

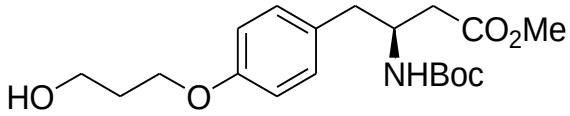
4-(4-(9H-fluoren-9-yl)methyl)-carbaminobutoxy)-2,6-dimethylbenzoic acid (12)



$C_{27}H_{27}NO_5$
Exact Mass: 445,19
Mol. Wt.: 445,51

Prepared from **11** (3.41 g, 6.80 mmol, 1 eq.) according to GP10. Purification via flash chromatography (hexane/EtOAc 1:2) yields 2.18 g (4.89 mmol, 72%) **12** as colorless solid. **^{1}H-NMR** (250 MHz, DMSO): δ = 12.68 (s, 1H), 7.88 (d, ^{3}J = 7.2 Hz, 1H), 7.68 (d, ^{3}J = 7.2 Hz, 1H), 7.45-7.27 (m, 5H), 6.62 (s, 2H), 4.31 (d, ^{3}J = 6.8 Hz, 2H), 4.21 (t, ^{3}J = 6.8 Hz, 1H), 3.96 (t, ^{3}J = 6.1 Hz, 2H), 3.13 (q, ^{3}J = 6.1 Hz, 2H), 2.25 (s, 6H), 1.88-1.78 (m, 2H). **^{13}C-NMR** (90 MHz, DMSO): δ = 170.6, 158.5, 156.2, 144.0, 140.8, 136.2, 127.7, 127.7, 127.1, 125.2, 120.2, 113.43 65.3, 65.1, 46.9, 37.3, 29.1, 19.9. **RP-HPLC** (10-90%, 30 min) R_t = 25.32 min. **MS** (ESI): m/z = 929.4 $[2m+K]^+$.

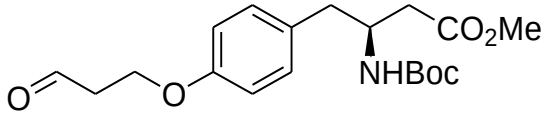
(*S*)-Methyl-3-(*tert*-butoxycarbonylamino)-4-(4-(3-hydroxypropoxy)phenyl) butanoat (13)



$C_{19}H_{29}NO_6$
Exact Mass: 367,2
Mol. Wt.: 367,44

A solution of *N*-Boc-β-tyrosine methyl ester[6] (520 mg, 1.68 mmol, 1.0 eq.) and potassium carbonate (581 mg, 4.20 mmol, 2.5 eq.) in absolute DMF (15 ml) were stirred for 30 min at 0 °C. 3-Brom-1-propanol (161 µL, 1.85 mmol, 1.1 eq.) was added and the mixture was stirred over night while warming to room temperature. The solvent was evaporated *in vacuo*, the residue was taken up with 1 N HCl and extracted with EtOAc. The combined organic layers were washed with *brine* and dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by flash chromatography (DCM/EtOAc 3:1) to give 562 mg (1.53 mmol, 91%) of a colorless oil. **^{1}H-NMR** (500 MHz, DMSO): δ = 7.06 (d, ^3J = 8.5 Hz, 2H), 6.82 (d, ^3J = 8.3 Hz, 2H), 6.79 (d, ^3J = 8.6 Hz, 1H), 4.51 (bs, 1H), 3.98 (t, ^3J = 6.4 Hz, 2H), 3.93-3.85 (m, 1H), 3.54 (s, 3H), 3.33-3.29 (m, 2H), 2.63-2.59 (m, 2H), 2.38 (d, ^3J = 6.9 Hz, 2H), 1.86-1.80 (m, 2H), 1.32 (s, 9H). **^{13}C-NMR** (125 MHz, DMSO): δ = 171.3, 157.1, 154.8, 130.2, 130.1, 114.1, 77.6, 64.4, 57.3, 51.3, 49.4, 38.8, 32.1, 28.2. **RP-HPLC** (10-100%): t_R = 18.34 min. **MS** (ESI): *m/z* (%) = 757.1 $[2m+Na]^+$, 390.2 $[m+Na]^+$, 268.3 $[m-Boc+H]^+$.

(*S*)-Methyl-3-(*tert*-butoxycarbonylamino)-4-(4-(3-oxopropoxy)phenyl) butanoat (14)

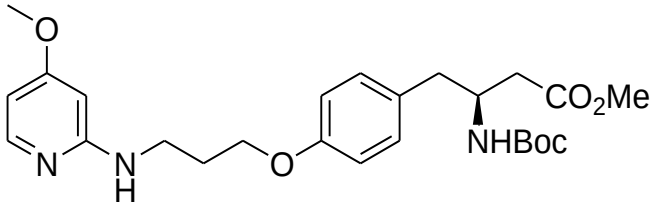


$C_{19}H_{27}NO_6$
Exact Mass: 365,18
Mol. Wt.: 365,42

Compound **13** (562 mg, 1.53 mmol, 1.0 eq.) was dissolved in DMSO (15 ml) and IBX[7] (890 mg, 3.18 mmol, 2.0 eq.) was added. The suspension was stirred for additional 6 h at ambient temperature. DCM was added and the mixture was extracted with saturated Na_2CO_3. The organic layers were combined, washed with *brine* and dried over Na_2SO_4. The solvent was evaporated *in vacuo* and compound **14** was obtained as brown oil (584 mg). The compound was used

directly in the next step without further purification. **^{1}H-NMR** (360 MHz, DMSO): δ = 9.72 (t, ^{3}J = 1.7 Hz, 1H), 7.08 (d, ^{3}J = 8.6 Hz, 2H), 6.84 (d, ^{3}J = 8.5 Hz, 2H), 6.80 (d, ^{3}J = 8.9 Hz, 1H), 4.24 (t, ^{3}J = 5.9 Hz, 2H), 3.94-3.82 (m, 1H), 3.54 (s, 3H), 2.87-2.83 (m, 2H), 2.64-2.35 (m, 2H), 2.44-2.33 (m, 2H), 1.32 (s, 9H). **^{13}C-NMR** (90 MHz, DMSO): δ = 201.8, 171.3, 156.7, 154.8, 130.7, 130.1, 114.2, 77.6, 61.5, 51.3, 49.3, 42.7, 28.2. **RP-HPLC** (10-90%): t_R = 21.91 min. **MS** (ESI): *m/z* (%) = 388.1 $[m+Na]^+$, 332.3 $[m-{}^{t}Bu+Na]^+$, 266.1 $[m-Boc+H]^+$.

(*S*)-Methyl-3-(*tert*-butoxycarbonylamino)-4-(4-(3-(4-methoxypyridin-2-ylamino)-propoxy)phenyl)butanoat (15)

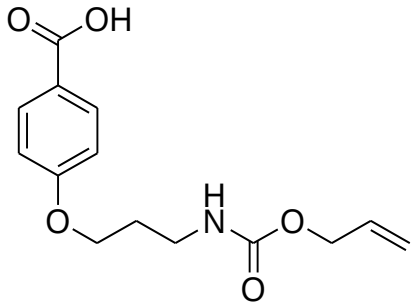


$C_{25}H_{35}N_3O_6$
Exact Mass: 473,25
Mol. Wt.: 473,56

Compound **14** (584 mg crude product, 1.60 mmol, 1 eq.) was dissolved in absolute DCM (50 ml). 2-Amino-4-methoxypyridine (238 mg, 1.92 mmol, 1.2 eq.) and 868 mg magnesium sulfate monohydrate (0.96 g, 7.99 mmol, 5 eq.) were added. After stirring the suspension for 1 h at room temperature sodium triacetoxyborohydride (1.90 g, 8.95 mmol, 5.6 eq.) was added and stirring was continued over night. After addition of 50 ml saturated $NaHCO_3$ solution and further 30 min of stirring the organic layer was extracted twice with DCM (50 ml) and dried over Na_2SO_4. After filtration the solvent was concentrated to dryness and the crude product was purified by flash chromatography (DCM/EtOAc 1:1) to give 220 mg of a colorless oil (0.46 mmol, 29% over two steps). **^{1}H-NMR** (500 MHz, DMSO): δ [ppm] = 7.77 (d, ^{3}J = 5.8 Hz, 1H), 7.07 (d, ^{3}J = 8.5 Hz, 2H), 6.86-6.84 (m, 3H), 6.49 (t, ^{3}J = 5.5 Hz, 1H), 6.11 (dd, ^{3}J = 5.8 Hz, ^{4}J = 2.2 Hz, 1H), 5.94 (d, ^{4}J = 2.2 Hz, 1H), 3.99 (t, ^{3}J = 6.3 Hz, 2H), 3.92-3.85 (m, 1H), 3.69 (s, 3H), 3.54 (s, 3H), 3.36-3.32 (m, 2H), 2.65-2.56 (m, 2H), 2.37 (d, ^{3}J = 6.9 Hz, 2H), 1.96-1.90 (m, 2H), 1.32 (s, 9H). **^{13}C-NMR** (125 MHz, DMSO): δ [ppm] = 171.4, 166.0, 160.7, 157.1, 154.9, 148.7, 130.4, 130.2, 114.2, 100.9, 90.7, 77.7, 65.3,

54.6, 51.4, 49.5, 37.8, 28.9, 28.3. **RP-HPLC** (10-90%): t_R = 19.15 min. **MS** (ESI): *m/z* (%) = 474.2 $[m+H]^+$, 418.3 $[m-{}^tBu+H]^+$, 374.3 $[m-Boc+H]^+$.

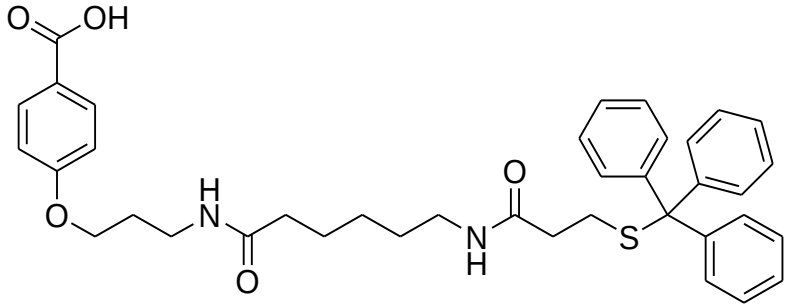
4-[3-(((Allyloxy)carbonyl)amino)propoxy]benzoic acid (16)



$C_{14}H_{17}NO_5$
Exact Mass: 279,11
Mol. Wt.: 279,29

Compound **16** was prepared from methyl 4-hydroxy-benzoate (1.00 g, 6.57 mmol, 1.0 eq.) and allyl-3-hydroxypropylcarbamate **6** (1.15 g, 7.23 mmol, 1.2 eq.) according to GP12 (hexane/EtOAc 2:1). The compound was deprotected according to GP3 to give a colorless solid (1.77 g, 6.35 mmol, 97%). **^{1}H-NMR** (360 MHz, DMSO): δ = 12.59 (bs, 1H), 7.89-7.86 (d, ^3J = 10.8 Hz, 2H), 7.29 (t, ^3J = 5.3 Hz, 1H), 7.01-6.98 (d, ^3J = 10.8 Hz, 2H), 5.90 (m, 1H), 5.21 (m, 2H), 4.46 (d, ^3J = 5.3 Hz, 2H), 4.06 (t, ^3J = 6.2 Hz, 2H), 3.15 (m, 2H), 1.87 (m, 2H). **^{13}C-NMR** (90 MHz, DMSO): δ = 166.9, 162.2, 155.9, 133.8, 131.3, 122.9, 116.9, 114.2, 65.4, 64.2, 37.2, 29.0. **RP-HPLC** (10-90%, 30 min) t_R = 16.97 min. **MS** (ESI): 876.0 $[3m+K]^+$,860.1 $[3m+Na]^+$, 597.2 $[2m+K]^+$, 581.2 $[2m+Na]^+$, 280.1 $[m+H]^+$.

4-(3-(6-(3-(Tritylthio)propanamido)hexanamido)propoxy)benzoic acid (17)



$C_{38}H_{42}N_2O_5S$
Exact Mass: 638,28
Mol. Wt.: 638,82

Prepared from **16** (77 mg, 0.28 mmol) according to the following reaction sequence: Loading of 0.25 g resin (real loading: ~186 µmol, GP4), Alloc

deprotection (GP7), coupling of Fmoc-6-aminohexanoic acid (GP6), Fmoc deprotection (GP5), coupling of 3-(Trityl)mercaptopropionic acid (GP6), cleavage from the resin (GP9). The title compound **16** (95 mg, 149 µmol, 80%) was obtained as colorless solid. **^{1}H-NMR** (500 MHz, DMSO): δ = 12.66 (bs, 1H), 7.90-7.87 (m, 3H), 7.81 (t, ^{3}J = 5.5 Hz, 1H), 7.34-7.22 (m, 15H), 6.99 (d, ^{3}J = 8.6 Hz, 2H), 4.03 (t, ^{3}J = 6.2 Hz, 2H), 3.20-3.16 (m, 2H), 2.98-3.94 (m, 2H), 2.21 (t, ^{3}J = 7.3 Hz, 2H), 2.12 (t, ^{3}J = 7.2 Hz, 2H), 2.02 (t, ^{3}J = 7.5 Hz, 2H), 1.86-1.81 (m, 2H), 1.48-1.42 (m, 2H), 1.36-1.30 (m, 2H), 1.21-1.15 (m, 2H). **^{13}C-NMR** (125 MHz, DMSO): δ = 172.1, 169.8, 167.1, 162.2, 144.5, 131.4, 129.1, 128.1, 126.8, 123.1, 114.2, 66.0, 65.5, 38.4, 35.4, 35.3, 34.0, 28.9, 28.8, 27.6, 26.1, 25.1. **RP-HPLC** (10-90%, 30 min) t_R = 26.17 min. **MS** (ESI): 1315.5 $[2m+K]^+$,1299.4 $[2m+Na]^+$, 1277.3 $[2m+H]^+$, 661.3 $[m+Na]^+$, 638.9 $[m+H]^+$.

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