

# Hybrid Bombesin Analogues – Combining an Agonist and an Antagonist in Defined Distances for Optimized Tumor Targeting

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## 1. General Aspects and Materials

Materials and reagents were of the highest commercially available grade and used without further purification. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 plates. Compounds were visualized by UV and ninhydrin. Flash chromatography was performed using Fluka silica gel 60, particle size 40 - 63  $\mu\text{m}$ .  $^1\text{H-NMR}$  spectra were recorded on a Bruker DPX 400 spectrometer. Chemical shifts are reported in ppm using TMS or the solvent as a reference.

A Bruker Esquire 3000plus instrument was used for electrospray ionization (ESI) mass spectrometry measurements. LC-MS analyzes were performed in combination with an Agilent 1100series HPLC using Reprisil Gold 120 C18 3 $\mu\text{m}$  (125x3 mm) and Reprisil 100 C18 5 $\mu\text{m}$  (150x4 mm) columns from Dr Maisch (Ammerbuch-Entringen, Germany). Preparative HPLC was carried out on a Reprisil Gold 120 C18 5  $\mu\text{m}$  (150 x 16 mm). Acetonitril (solvent A) and 0.1% TFA and 1% Acetonitril in water (solvent B) served as the mobile phase for both compound analyses and purifications.

High-resolution mass spectrometry measurements were performed on a Bruker maXis ESI-QTOF with an accuracy of 5 ppm and are reported deconvoluted.

For automated peptide synthesis, a Syro I Peptide Synthesizer (MultiSynTech GmbH, Witten, Germany) was employed. Reactions under microwave irradiation were carried out using an Initiator from Biotage (Uppsala, Schweden). UV-Vis absorptions for determining the concentration were measured on a CARY 300 Bio from Varian.

Quality controls of the radiolabeled compounds were carried out on a HPLC coupled to a radioactivity detector from Raytest (Straubenhardt, Germany). The radioactive samples from the *in vitro* and *in vivo* experiments were quantified on a Cobra II Model 5003 Gamma-counter from Packard. Lutetium-177 was obtained from itm (isotopen technologie münchen AG), Gallium-68 was eluted from a  $^{68}\text{Ge}/^{68}\text{Ga}$  generator (Eckert & Ziegler, Berlin), an automatic synthesis module, also from Eckert & Ziegler, was applied for the  $^{68}\text{Ga}$  labelings.

Cell culture: Human prostate cancer cells (PC-3) were obtained from American Type Culture Collection; cultured at 37°C and 5%  $\text{CO}_2$  in DMEM containing 2 mmol/L L-glutamine and supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin. All culture reagents were from Invitrogen or BioConcept.

The PET and PET/CT studies were performed using a small animal microPET (Focus 120, Concorde Microsystems, Knoxville, Tennessee) and a small animal CT (CT Imaging GmbH, Erlangen, Germany).

## 2. Synthesis and Analytical Details of the Oligoprolin-Bombesin Conjugates 1 – 8

### 2.1. Synthesis of sidechain-protected, alkynylated Bombesin derivatives

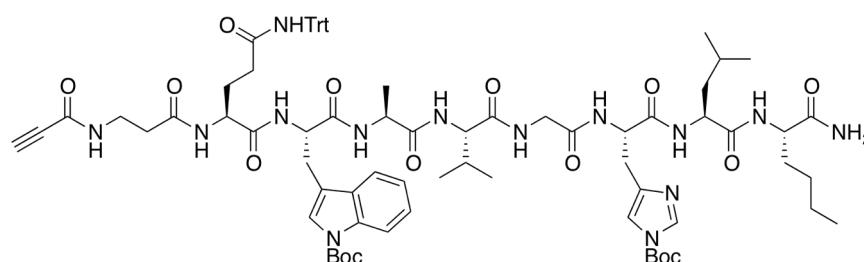
Sieber amide resin (1 eq, 0.69 mmol/g) was placed in a syringe equipped with a teflon frit, washed with  $\text{CH}_2\text{Cl}_2$  and swollen in dimethylformamide (DMF). The Fmoc protecting group was removed with 20 % piperidine in DMF (2 min than 15 min), the resin was thoroughly washed (5x) with DMF afterwards. 5 eq of the respective amino acid and 4.9 eq of HCTU were dissolved in DMF and 10 eq of  $^i\text{Pr}_2\text{NEt}$ . After 2 min the solution was poured on the resin and the mixture was agitated for 60 min. The resin was thoroughly washed with DMF (5x) afterwards; success of the coupling was controlled with the Kaiser Test. The Fmoc protecting group was removed with 20 % piperidine in DMF (2 min than 15 min), the resin was thoroughly washed with DMF (5x) afterwards.

The subsequent amino acids were coupled using the same protocol.

The N-terminal propiolic acid was coupled with EEDQ on solid phase to the peptide.<sup>[1]</sup> Before the coupling, the resin was washed with anhydrous  $\text{CH}_2\text{Cl}_2$ . Propiolic acid (3 eq) and EEDQ (3.1 eq) were dissolved in anhydrous  $\text{CH}_2\text{Cl}_2$ , added to the resin and the reaction was shaken for 3 hours. Afterwards the resin was thoroughly washed with  $\text{CH}_2\text{Cl}_2$  (5x).

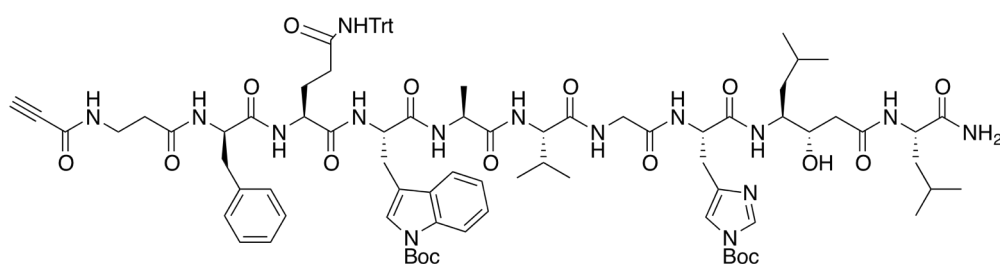
The peptide was cleaved stepwise from the sieber amide resin using 1% TFA in  $\text{CH}_2\text{Cl}_2$  (10 x 2 min). The fractions were collected and immediately neutralized with 10 % pyridine in methanol. The fractions were combined, concentrated *in vacuo* and the peptide was precipitated in ice cold water. After centrifugation and washing with water (3x) the peptide was suspended in a mixture of water and acetonitril (1:4) and lyophilized.

### Prs- $\beta$ Ala-QWAVGHLZ (Z=Nle, Agonist) 9



LC-ESI-MS: 90-0 % B in 16 min, 0.5 ml/min, 50°C;  $R_t = 13.16$  min,  $m/z = 1146.3$  [(M-Boc-Trt)+ $\text{H}^+$ ]<sup>+</sup>, 1388.4 [(M-Boc)+ $\text{H}^+$ ]<sup>+</sup> (1487.74 g/mol calculated for  $\text{C}_{79}\text{H}_{102}\text{N}_{14}\text{O}_{15}$ ) The intactness of the protecting groups was confirmed by direct injection ESI-MS (no TFA present, lower injection temperature):  $m/z = 1388.4$  [(M-Boc)+ $\text{H}^+$ ]<sup>+</sup>, 1488.4 [M+ $\text{H}^+$ ]<sup>+</sup>, 1510.3 [M+ $\text{Na}^+$ ]<sup>+</sup>

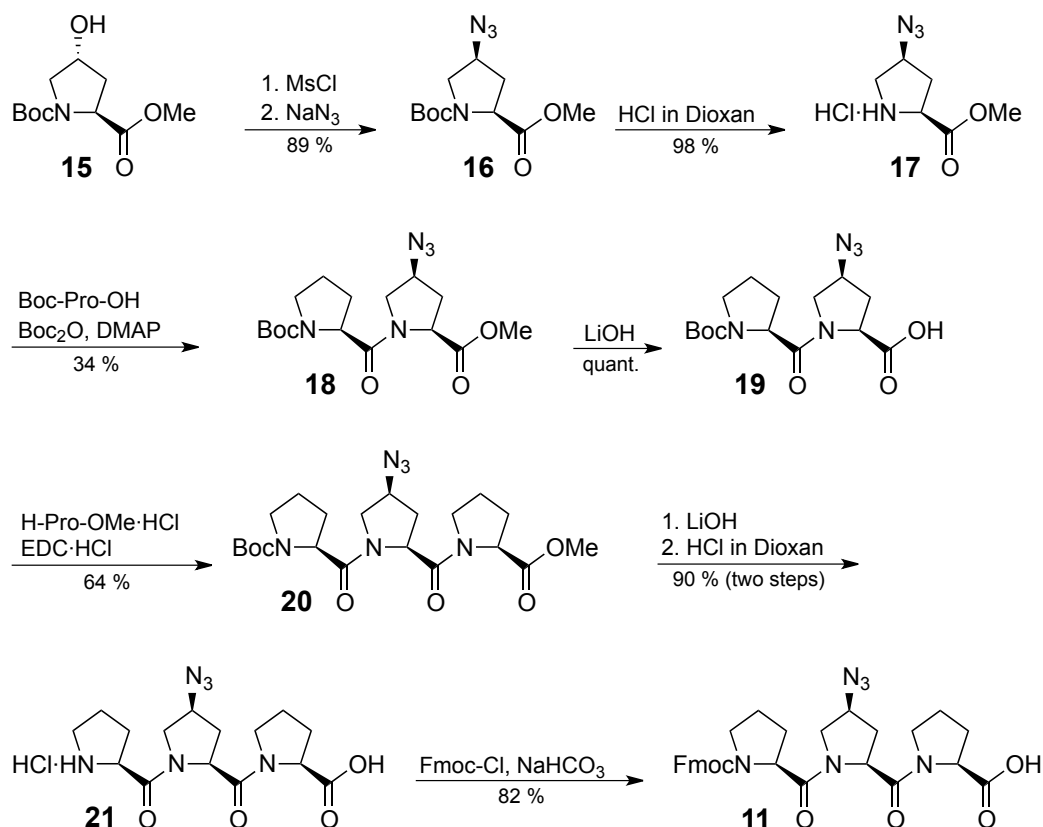
The alkynylated peptide was used without further purification.



LC-ESI-MS: 90-0 % B in 16 min, 0.5 ml/min, 50°C;  $R_t = 13.55$  min;  $m/z = 1579.8 [(M-Boc)+H]^+$   
(1678.97 g/mol calculated for  $C_{90}H_{115}N_{15}O_{17}$ )

The alkylnated peptide was used without further purification.

## 2.2. Peptide Syntheses in Solution



**Scheme 1.** Synthesis of azido-functionalized building block **11**

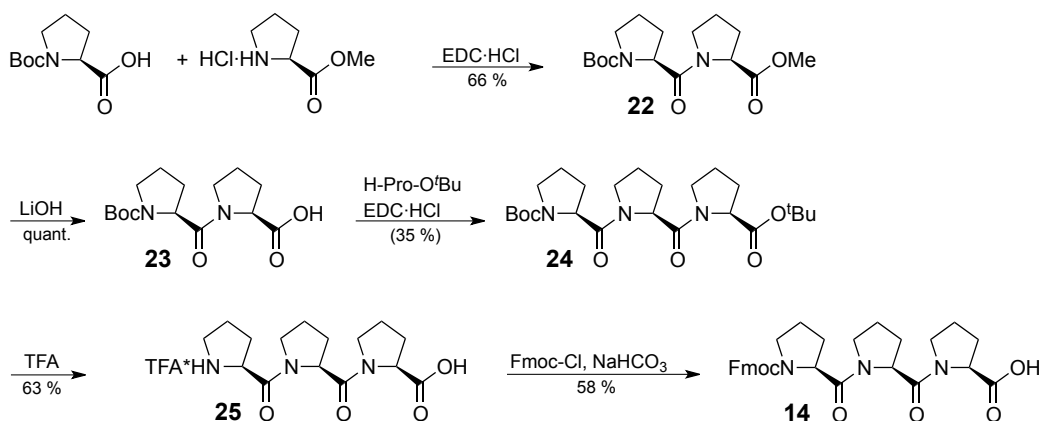
The synthesis of the azido-functionalized building block **11** (Scheme 1) is described in detail in the supporting information of a previous publication.<sup>[21]</sup> The analytical data of **11** are in agreement with those described previously.

**Fmoc-Pro-(4S)Azp-Pro-OH 11**NMR spectra show a double set of peaks ( $\approx 2.9 : 1$ )

due to the *cis* and *trans* conformers around the tertiary carbamate. Major conformer  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ,  $25^\circ\text{C}$ ):  $\delta = 7.78$  (m, 2H, Fmoc), 7.64-7.56 (m, 2H, Fmoc), 7.46-7.32 (m, 2H, Fmoc), 4.75 (t,  $J = 8.1$  Hz), 1H, Azp- $\text{H}^\alpha$ ), 4.63 (dd,  $J = 8.4$  Hz, 3.4 Hz, 1H, Pro $^{\text{C}}$ - $\text{H}^\alpha$ ), 4.57 (m, 1H, Pro $^{\text{N}}$ - $\text{H}^\alpha$ ), 4.38-4.31 (m, 2H, Fmoc  $\text{CH}_2$ ), 4.28-4.23 (m, 3H, Fmoc  $\text{CH}$ , Azp- $\text{H}^\gamma$ , Azp- $\text{H}^\beta$ ), 3.79-3.70 (m, 2H, Pro $^{\text{N}}$ - $\text{H}^\delta$ , Pro $^{\text{C}}$ - $\text{H}^\delta$ ), 3.60-3.42 (m, 3H, Pro $^{\text{N}}$ - $\text{H}^\delta$ , Pro $^{\text{C}}$ - $\text{H}^\delta$ , Azp- $\text{H}^\delta$ ), 2.61 (m, 1H, Azp- $\text{H}^\beta$ ), 2.27-1.90 (m, 9H, Azp- $\text{H}^\beta$ , all Pro- $\text{H}^\beta$ , all Pro- $\text{H}^\gamma$ )

Isolated signals of the minor conformer:  $\delta = 4.15$  (t,  $J = 5$  Hz, 1H, Fmoc  $\text{CH}$ ), 4.02 (dd,  $J = 8.4$  Hz, 3.3 Hz, 1H, Pro $^{\text{N}}$ - $\text{H}^\alpha$ ), 3.93 (q, 1H, Azp- $\text{H}^\gamma$ ), 3.23 (dd,  $J = 9.8$  Hz, 8.0 Hz, 1H, Azp- $\text{H}^\delta$ ), 2.47 (m, 2H, Azp- $\text{H}^\beta$ ) 2.46 (m, 1H, Azp- $\text{H}^\beta$ )

LC-ESI-MS: 95-40% B in 15 min, 0.5 ml/min,  $50^\circ\text{C}$ ;  $R_t = 15.83$  min;  $m/z = 573.2$   $[\text{M}+\text{H}^+]^+$  (572.61 g/mol calculated for  $\text{C}_{30}\text{H}_{32}\text{N}_6\text{O}_6$ )

**Scheme 2.** Synthesis of building block **14**

The building block **14** was obtained following the same route as previously described for building block **11**.

**Fmoc-Pro-Pro-Pro-OH 14**NMR spectrum shows a double set of peaks ( $\approx 1.6 : 1$ ) due to

the *cis* and *trans* conformers around the tertiary carbamate. Major conformer  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ,  $25^\circ\text{C}$ ):  $\delta = 7.74$  (m, 2H, Fmoc); 7.60 (m, 2H, Fmoc); 7.39 (m, 2H, Fmoc); 7.29 (m, 2H, Fmoc); 4.73 (dd,  $J = 7.6$  Hz, 4.4 Hz, 1H, Pro- $\text{H}^\alpha$ ); 4.58-4.12 (m, 5H, 2x Pro- $\text{H}^\alpha$ , Fmoc- $\text{CH}_2$  /  $\text{CH}$ ); 3.81 - 3.40 (m, 6H, Pro- $\text{H}^\delta$ ); 2.22 - 1.72 (m, 12H, Pro- $\text{H}^{\beta/\gamma}$ );

Isolated signals of the minor conformer:  $\delta = 4.44$  (dd,  $J = 7.4$  Hz, 4.4 Hz, 1H Pro- $\text{H}^\alpha$ )

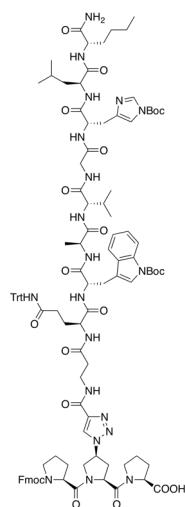
LC-ESI-MS: 90-5 % B in 20 min, 0.7 ml/min,  $50^\circ\text{C}$ ;  $R_t = 12.13$  min; Purity 95 %;  $m/z = 532.2$   $[\text{M}+\text{H}^+]^+$  (531.60 g/mol calculated for  $\text{C}_{30}\text{H}_{33}\text{N}_3\text{O}_6$ )

### 2.3. Cu(I)-catalyzed azide alkyne Cycloaddition (“Click-reaction“)

For the ligation of the azido functionalized prolin-trimer **11** with the alkynylated bombesin derivatives **9** and **10** the following procedure was used:

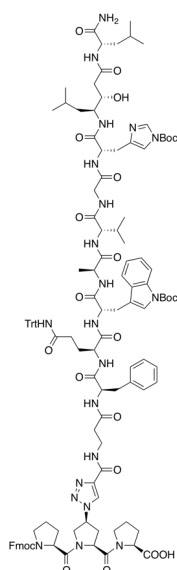
The alkynylated peptide (1.1 eq) was dissolved in a minimal amount of a degassed mixture of DMF and NMP (2:1). The azide **11** (1 eq) and  $[\text{Cu}(\text{CH}_3\text{CN})_4]\text{PF}_6$  (0.3 eq) were added and the reaction was heated in a closed vessel (80°C, Microwave) for 2 h. The reaction mixture was stirred with polymer-bound EDTA to remove the copper catalyst and was used without further purification for solid phase peptide synthesis.

#### Fmoc-Pro-(4S)Azp(Triazolyl-βAla-[Nle<sup>14</sup>]Bombesin(7-14))-Pro-OH **12**



LC-ESI-MS: 90-0 % B in 16 min 0.5 ml/min, 50°C;  $R_t = 14.08$  min;  $m/z = 1961.5$   $[\text{M-Boc}+\text{H}^+]^+$   
(2060.35 g/mol calculated for  $\text{C}_{109}\text{H}_{134}\text{N}_{20}\text{O}_{21}$ )

#### Fmoc-Pro-(4S)Azp(Triazol-βAla-JMV594)-Pro-OH **13**



LC-ESI-MS: 90-0 % B in 16 min, 0.5 ml/min, 50°C  $R_t = 14.39$  min,  $m/z = 2152.9$   $[\text{M-Boc}+\text{H}^+]^+$   
(2251.58 g/mol calculated for  $\text{C}_{120}\text{H}_{147}\text{N}_{21}\text{O}_{23}$ )

## 2.4. Solid Phase Peptide Synthesis of Oligoprolin-Bombesin Conjugates 1-8

ChemMatrix Rink Amide Resin (0.47 mmol/g, 1 eq) was washed with  $\text{CH}_2\text{Cl}_2$  and swollen in DMF. The building block (**12**, **13** or **14**, 3 eq) was preactivated with HATU (2.95 eq) and  $i\text{Pr}_2\text{NEt}$  (6 eq) and added to the resin. After 2 h of agitation the resin was washed with DMF, remaining copper traces were washed away with diethyldithiocarbamate (0.5 % w/v) and  $i\text{Pr}_2\text{NEt}$  (0.5 % v/v) in DMF.

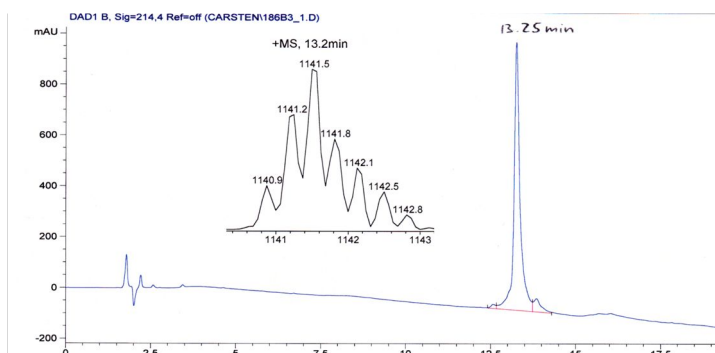
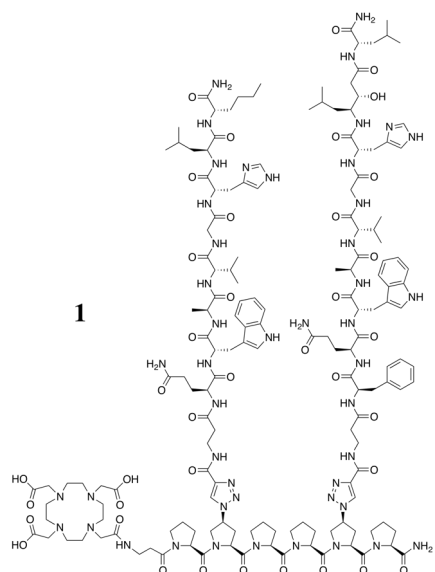
The Fmoc-protecting group was removed by treating the immobilized peptide with 20% piperidine in DMF. The resin was thoroughly washed (5x) with DMF afterwards.

The building blocks (**12**, **13** or **14**), Fmoc- $\beta$ -Alanine and DOTA(tBu)<sub>3</sub> were coupled using the same reagents and conditions.

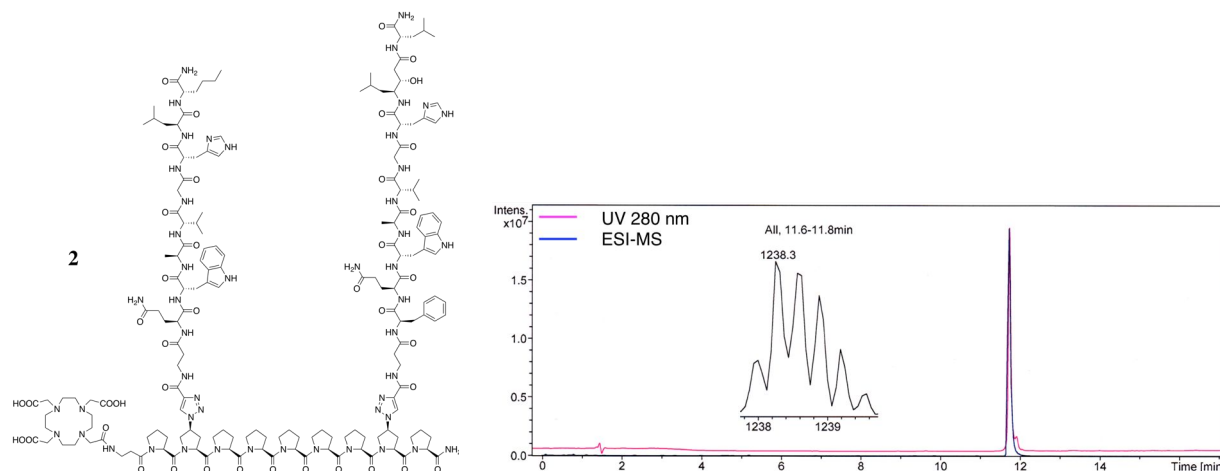
Before cleaving the peptide from the resin, the resin was washed with  $\text{CH}_2\text{Cl}_2$ . Then a cleavage cocktail consisting of TFA (95 %), triisopropylsilane (2.5 %) and water (2.5 %) was poured on the resin and the suspension was shaken for 90 min. The solution was collected after filtration from the resin and a second portion of the cleavage cocktail was added. After 60 min, the second portion was combined with the first fraction, the volume was reduced to one third *in vacuo* and the peptide was precipitated in cold diethyl ether. The oligoprolin-bombesin conjugates were purified by preparative HPLC.

Hybrid ligand 1 HR-ESI-MS: 3482.6931 (calculated for  $\text{C}_{160}\text{H}_{231}\text{CuN}_{47}\text{O}_{38}$ \*: 3482.6918,  $\Delta = 0.4$  ppm) \*In low resolution LC-MS the free ligand without copper is observed. Also the subsequent, successful radiolabeling with Lutetium-177 proves, that only traces of copper were present.

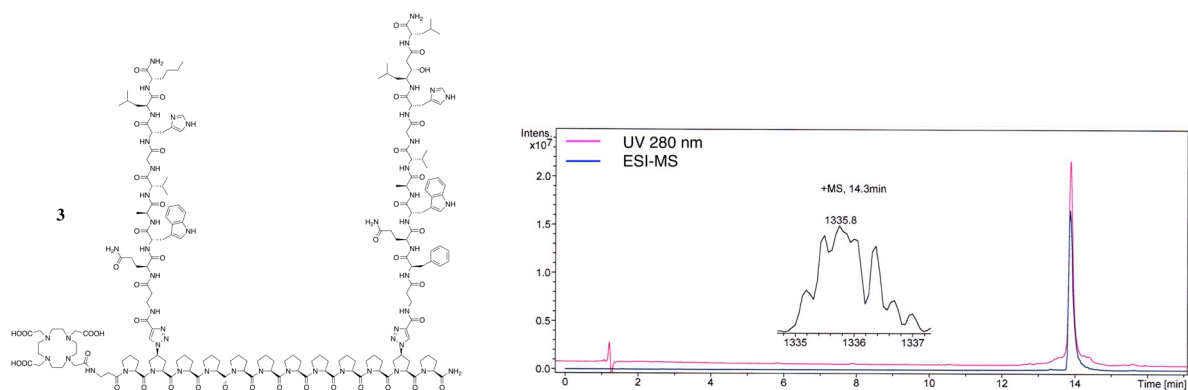
LC-ESI-MS: 95-50 % B in 14 min, 0.5 ml/min, 50°C;  $R_t = 13.25$  min;  $m/z = 1141.5$   $[\text{M}+3\text{H}^+]^{3+}$ , 1711.5  $[\text{M}+2\text{H}^+]^{2+}$  (3420.83 calculated for  $\text{C}_{160}\text{H}_{231}\text{N}_{47}\text{O}_{38}$ )



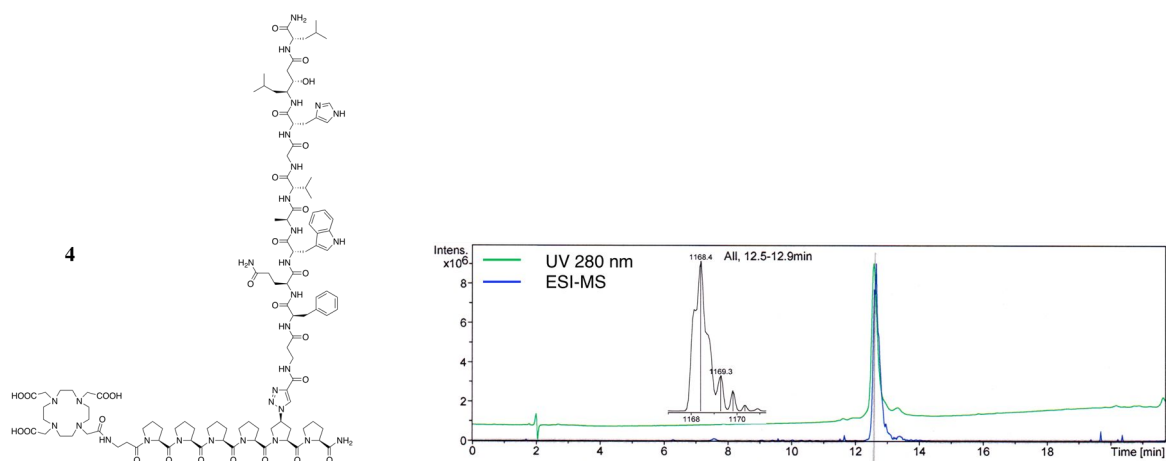
**Hybrid ligand 2** HR-ESI-MS: 3711.9130 (3711.9238 calculated for  $C_{175}H_{252}N_{50}O_{41}$ ,  $\Delta = 2.9$  ppm); LC-ESI-MS: 95-50 % B in 14 min, 0.5 ml/min, 50°C;  $R_t = 12.81$  min;  $m/z = 1238.3$   $[M+3H^+]^{3+}$



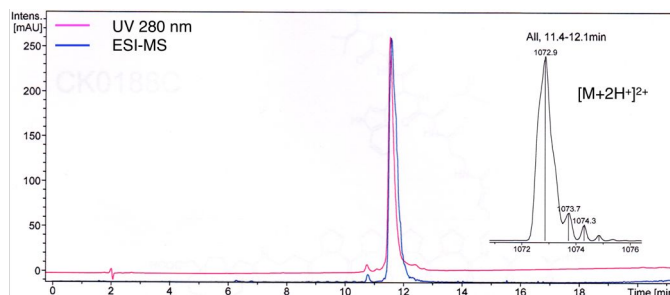
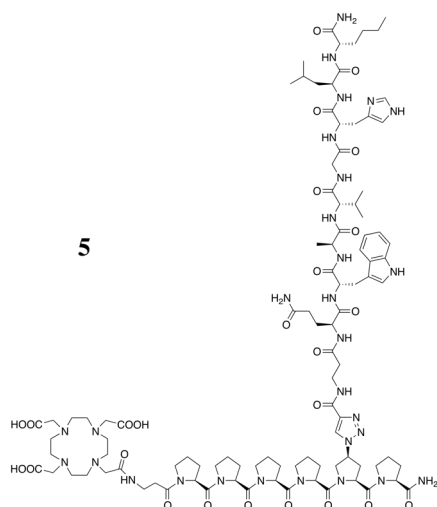
**Hybridligand 3** HR-ESI-MS: 4003.0726 (4003.0821 calculated for  $C_{190}H_{273}N_{53}O_{44}$ ,  $\Delta = 2.4$  ppm); LC-ESI-MS: 95-50 % B in 15 min, 0.5 ml/min, 50°C;  $R_t = 14.15$  min;  $m/z = 1335.8$   $[M+3H^+]^{3+}$



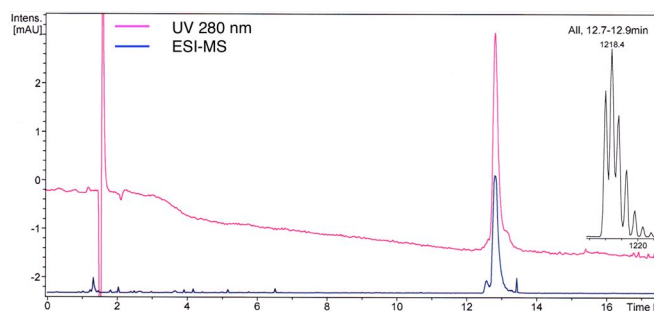
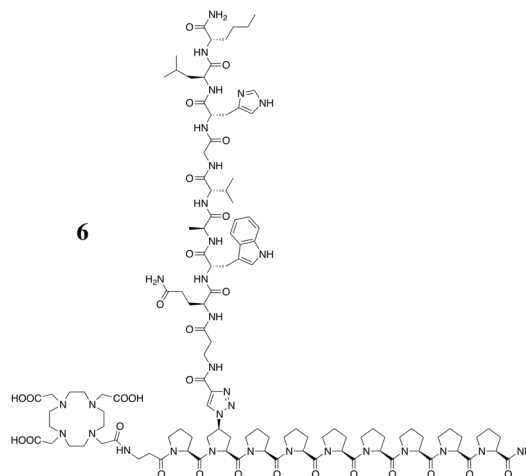
**Monovalent Antagonist 4** HR-ESI-MS: 2334.2246 (2334.2103 calculated for  $C_{110}H_{160}N_{30}O_{27}$ ,  $\Delta = 6.1$  ppm); LC-ESI-MS: 90-35 % B in 19 min, 0.7 ml/min, 50°C,  $R_t = 12.76$  min;  $m/z = 1168.4$   $[M+2H^+]^{2+}$



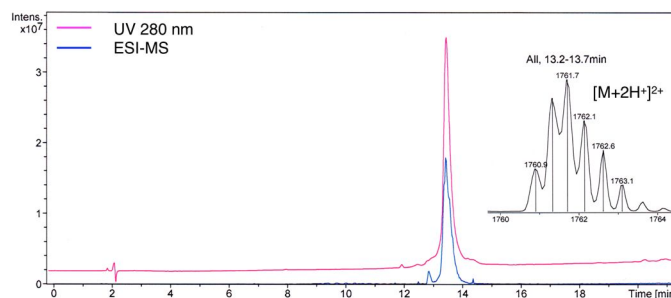
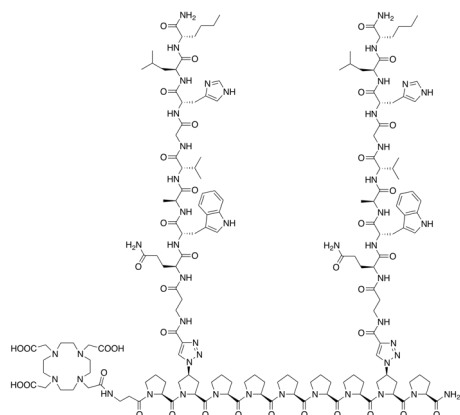
**Monovalent Agonist 5** HR-ESI-MS: 2143.1263 (2143.1156 calculated for  $C_{99}H_{147}N_{29}O_2$ ,  $\Delta = 5.0$  ppm); LC-ESI-MS: 90-35 % B in 19 min, 0.7 ml/min, 50°C;  $R_t = 11.72$  min;  $m/z = 1072.9 [M+2H^+]^{2+}$



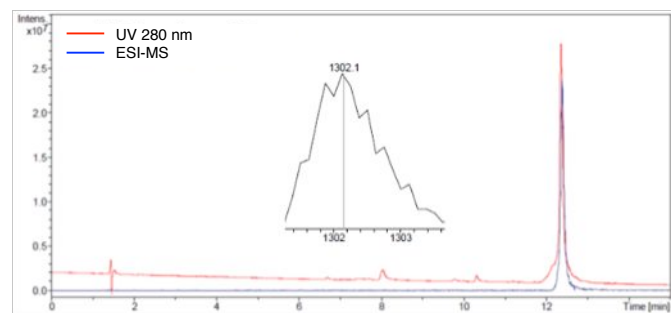
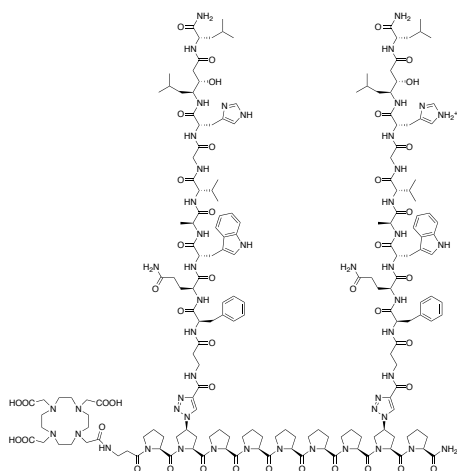
**Monovalent Agonist 6** HR-ESI-MS: 2471.2293 (2471.2270 calculated for potassium adduct  $C_{114}H_{167}KN_{32}O_{28}$ ,  $\Delta = 0.9$  ppm); LC-ESI-MS: 90-60 % B in 15 min 0.5 ml/min, 50°C;  $R_t = 12.81$  min;  $m/z = 1218.4 [M+2H^+]^{2+}$ ;  $812.7 [M+3H^+]^{3+}$



**Bivalent Agonist 7** HR-ESI-MS: 3518.8354 (3518.8225 calculated for  $C_{164}H_{239}N_{49}O_{39}$ ,  $\Delta = 3.7$  ppm); LC-ESI-MS: 90-35 % B in 19 min 0.7 ml/min, 50°C;  $R_t = 13.55$  min;  $m/z = 1174.9$   $[M+3H]^+{}^{3+}$ ; 1761.7  $[M+2H]^+{}^{2+}$

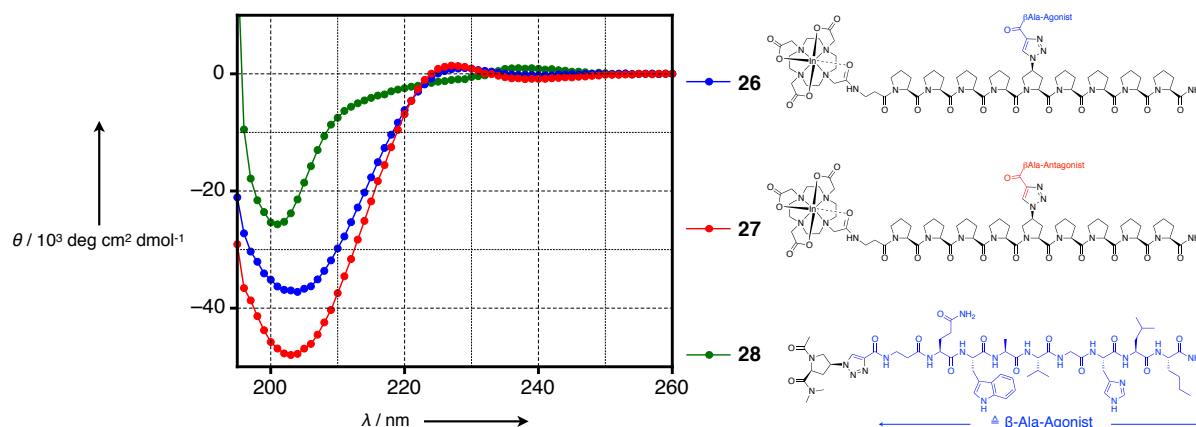


**Bivalent Antagonist 8** HR-ESI-MS: 3902.0074 (3902.0151 calculated for  $C_{186}H_{265}N_{51}O_{43}$ ,  $\Delta = 2.0$  ppm); LC-ESI-MS: 90-50 % B in 15 min 0.5 ml/min, 50°C;  $R_t = 12.35$  min;  $m/z = 1302.1$   $[M+3H]^+{}^{3+}$ ; 1952.6  $[M+2H]^+{}^{2+}$



### 3. Conformational analysis using circular dichroism (CD) spectroscopy

CD spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics), using a spectral bandwidth of 1 nm, at 25 °C with a time constant of 5 s and a step resolution of 1 nm. CD data are given as molar ellipticities ( $\Theta$  in  $\text{deg cm}^2 \text{dmol}^{-1}$ ). A quartz cell with a path length of 2 mm was used with solutions containing approximately 35  $\mu\text{M}$  peptide solutions. All samples were in PBS at pH 7.4 and were equilibrated for at least 12 h before measurement.



**Figure 2.** CD Spectrum of model peptides **26** – **28** and the corresponding structures

The model compounds **26** and **27** consist of the same moieties like the employed ligands: An oligoproline, N-terminally coupled to a  $\beta$ -alanin and a DOTA-chelator and C-terminally carrying a carboxamide. In previous studies, oligoproline-carboxamides were shown to exhibit high conformational stabilities and being locked in the PPII confirmation.<sup>[3]</sup> Attached on one proline *via* a triazol are the two recognition sequences, [Nle<sup>14</sup>]BBN(7-14) and JMV594 respectively. The DOTA chelator holds an Indium(III).

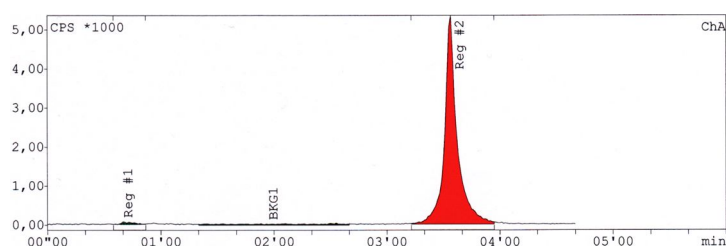
The CD-spectra of **26** and **27** in phosphate buffer are as expected typical for a PPII helix<sup>[4]</sup> (maximum at 227 nm, minimum at 205 nm). The contribution of the bombesin peptide sequences attached to the helix is not obvious, since bombesin peptides mainly adapt a random coil confirmation in aqueous solvents, which gives a similar minimum as the PPII helix.<sup>[5]</sup> The signal of the corresponding agonist **28**, N-terminally coupled to a prolin *via* a triazol accordingly only exhibits a minimum at 201 nm, characteristic for a random coil.

It is almost certain that the presented ligands exhibit the defined PPII conformation also in the applied assays and *in vivo*. CD studies in high concentrations of unpolar solvent (n-PrOH) or in the presence of lipid vesicles at 37°C showed no signs of diminished PPII conformation (data not shown).

#### 4. *in vitro* Experiments with cultivated prostate tumor cells

For the cell assays established protocols which were described before in detail were used.<sup>[6-8]</sup>

**Radiolabeling:** The [<sup>177</sup>Lu]-DOTA-peptide conjugates were prepared by dissolving the peptide (5 nmol) in sodium acetate buffer (250  $\mu$ L, 0.4 mol/L, pH 5.0), followed by incubation with <sup>177</sup>LuCl<sub>3</sub> (40-50 MBq) for 30 min at 95°C. The purity and the labeling yield was controlled by HPLC equipped with a radiodetector. One equivalent of <sup>nat</sup>LuCl<sub>3</sub> was added and the solution was incubated again at 95°C for 20 min to obtain only complexed ligands. The radiotracers were used without further purification. Specific activities were around 9 GBq/ $\mu$ mol.



**Figure 3.** Radio chromatogram, red: [<sup>177</sup>Lu]-1 (Peak 2, 99%); green free Lutetium-177 (Peak 1, <1%)

**Internalization studies:** PC-3 cells were seeded into six-well plates overnight (0.8-1.0 $\cdot$ 10<sup>6</sup> cells per well). On the day of the experiment, the medium was removed, the cells were washed with fresh medium (DMEM, 1% fetal bovine serum, pH 7.4) and incubated in medium (1.3 ml) for 1 h at 37°C. Approximately 20 kBq of [<sup>177</sup>/<sup>nat</sup>Lu]-labeled peptide (2.5 pmol in 100  $\mu$ l 0.9 % NaCl) was added to the medium and the cells were incubated (in triplicates) for 0.5, 1, 2, 4 and 6 h at 37°C, 5% CO<sub>2</sub> with and without an excess of each blocking agent (agonist [Tyr<sup>4</sup>]-Bombesin; antagonist BIM26226<sup>[9]</sup>) to determine nonspecific internalization. The final volume was 1.5 ml. At each time point, the cells were treated in the following way: The internalization was stopped by removal of the medium followed by washing of the cells with ice-cold PBS. Cells were then treated for 5 min (twice) with glycine buffer (0.05 mol/L glycine solution, pH adjusted to 2.8 with 1 mol/L HCl) to distinguish between cell surface-bound (acid releasable) and internalized (acid resistant) radioligand. Finally, cells were detached from the plates by incubation with NaOH solution (1M) for 10 min at 37°C, and the radioactivity was measured in a  $\gamma$ -counter. Internalization data was corrected for unspecific binding.

#### 5. *in vivo* Experiments with PC-3 tumor-bearing mice

All animal experiments were done in compliance with the German regulations. Female nude mice were implanted subcutaneously with 5 million PC-3 tumor cells, which were freshly expanded in sterilized PBS (pH 7.4) and Matrigel. Fourteen days after inoculation, the tumors grew to a size of 5  $\pm$  2 mm.

**PET and PET/CT Imaging of [<sup>68</sup>Ga]-2** [<sup>68</sup>Ga] was eluted and purified from an <sup>68</sup>Ge/<sup>68</sup>Ga-generator.<sup>[10]</sup> Purified <sup>68</sup>Ga<sup>3+</sup> was eluted from a 50W-X8 cation exchanger chromatographic column (Bio-Rad; <400 mesh) with a 97.6% acetone/0.05 M HCl solution (400 μL). This fraction (ca. 400 MBq) was used directly for the labeling of hybrid ligand **2** (7 nmol) in a HEPES solution (0.25 M, 400 μL) at pH 3.6 to 3.9 using an automated synthesis module. Specific activity was 57 MBq/nmol. The purity and the labeling yield were controlled a HPLC equipped with a radiodetector.

Approximately 50 min after intravenous injection of about 2-3 MBq <sup>68</sup>Ga-**2** (100 pmol) into the tail vein, PC-3 tumor-bearing mice were anaesthetized with 4% isoflurane/oxygen and maintained with 2% isoflurane/oxygen. At a constant low breathing frequency (about 60 min<sup>-1</sup>) animals were transferred to the camera bed and fixed for small animal CT, followed by static PET imaging with a duration of 30 min. Breathing frequency and body temperature of the animals were continuously monitored. Images were reconstructed by 2D-OSEM and fused and analyzed using AMIDE (Version 0.92). For blocking the GRP-receptor, 5 minutes prior to the injection of the radiolabeled hybrid ligand, a 1000-fold excess of unlabeled antagonist **4** or unlabeled agonist **5** was injected.

**Biodistribution of [<sup>177</sup>Lu]-2** Six mice were injected into the tail vein with 100 pmol of <sup>177</sup>Lu-radiolabeled peptides (about 1 MBq in 100 μL). Mice were sacrificed at 4 (3 animals) and 21 h (3 animals), and the organs of interest were collected, rinsed of excess blood, weighed, and the radioactivity was counted in a γ-counter. The percentage of injected activity per gram (% IA/g) was calculated for each tissue.

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