Supporting Information for

An Octadentate Bifunctional Chelating Agent for the Development of Stable Zirconium-89 Based Molecular Imaging Probes

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1) Materials

All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or dried over molecular sieves while required. All preparations were carried out using standard Schlenk techniques. Desferrioxamine (DFO) mesylate was purchased from Sigma-Aldrich and synthon **2** was prepared following a literature procedure.[1] Fmoc-amino acids, Rink Amide MBHA LL resin (100–200 mesh), HOBt, HATU, and TBTU were purchased from Merck Biosciences (Nottingham, UK). Bombesin(1–14) trifluoroacetate (BBS(1-14)) was purchased from Bachem (Bubendorf, Switzerland). Deferoxamine-*p*-SCN (DFO-*p*-SCN) was purchased from Macrocyclics (Dallas, Texas, USA). HPLC solvents were purchased from Acros Organics (Geel, Belgium), Merck (Darmstadt, Germany) and Sigma-Aldrich (Buchs, Switzerland). Polypropylene syringes for manual peptide couplings, fitted with polypropylene frits and a polypropylene plunger, were obtained from MultiSyntech (Witten, Germany). Water for injection (WFI) from Bichsel (Unterseen, Switzerland) was used for all radiolabelling reagent setups. $[^{89}Zr(C_2O_4)]^{4-}$ in 1 M oxalic acid was obtained from Perkin Elmer (USA).

All culture reagents, except FBS, were purchased from BioConcept (Allschwil, Switzerland). Heat-inactivated fetal bovine serum was obtained from Oxoid (FBS superior, Pratteln, Switzerland). Human Caucasian prostate adenocarcinoma (PC-3) cells were obtained from HPA Culture Collections (Salisbury, UK).

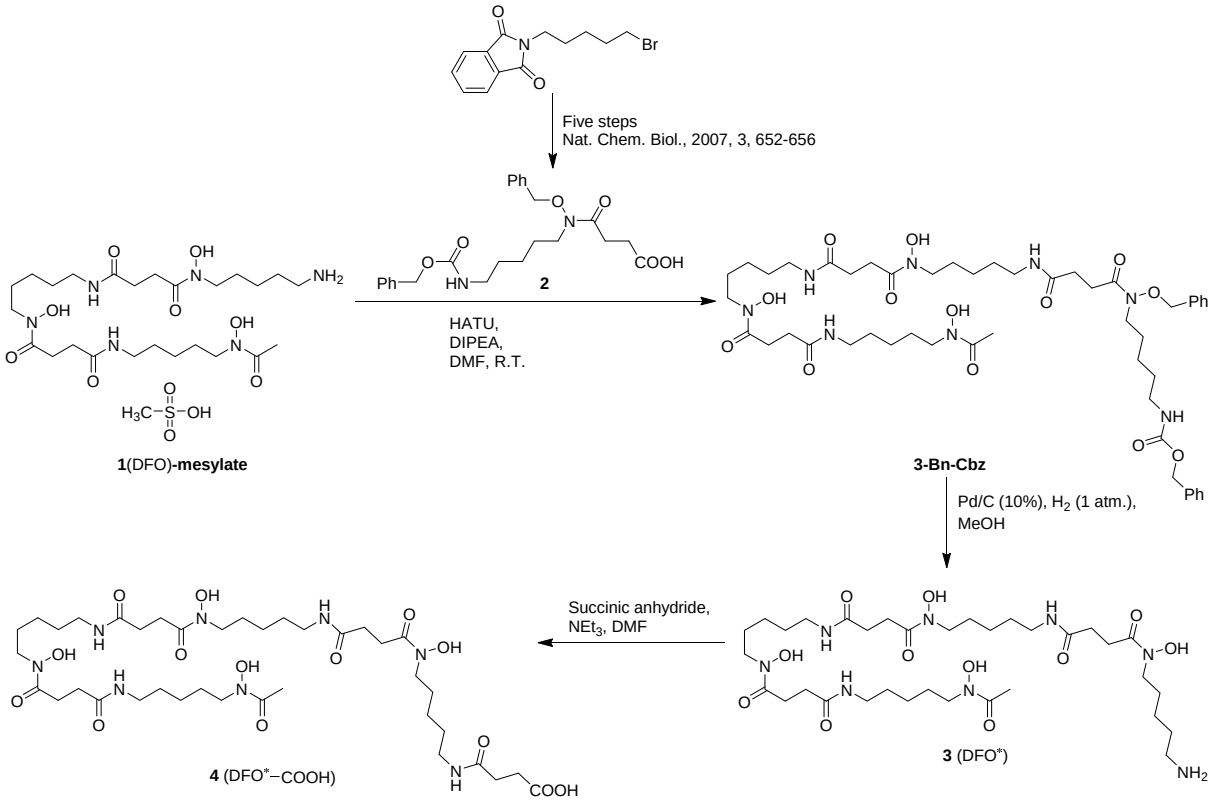
[1] N. Kadi, D. Oves-Costales, F. Barona-Gomez and G. L. Challis, *Nat. Chem. Biol.*, 2007, **3**, 652-656.

2) Instrumentation and methods

^{1}H and *^{13}C NMR spectra* were recorded in deuterated solvents on Bruker 400 or 500 spectrometers at room temperature. The chemical shifts, δ, are reported in ppm (parts per million). The residual solvent peaks have been used as an internal reference. The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad). Signal intensity is abbreviated br (broad), s (strong), m (medium), and w (weak). *ESI mass spectra* were recorded on a Bruker Esquire 6000 and a Bruker Esquire 3000 plus (Bruker Daltonics GmbH, Bremen, Germany). *MALDI-MS spectra* were measured on a 4800 MALDI TOF/TOF analyser (Applied Biosystems). The samples were co-crystallized with a solution of α-cyano-4-hydroxy-cinnamic acid (CHCA) as a matrix.

Automated solid-phase peptide synthesis (SPPS) was performed on a Pioneer synthesizer (Applied Biosystems). Radioactivity measurements were made by using a Veenstra-405 Dose Calibrator (Veenstra Instruments, The Netherlands) with a calibration factor of 780 for Zr-89. Quantitative γ-counting of experimental samples was performed on a COBRA II auto-gamma system (Model 5003; Packard Instruments, Meriden, CT, USA).

3) Synthesis and Characterization of DFO* (3) and DFO*–COOH (4)



Scheme S1. Synthesis of DFO* (**3**) and DFO*-CO_2H (**4**).

3-Bn-Cbz. To a stirred solution of carboxylic acid **2** (1.13 g, 2.6 mmol) and HATU (1.46 g, 3.8 mmol) in DMF (10 mL), was added DIPEA (0.66 g, 5.1 mmol) under N_2 atmosphere. After stirring the mixture for 40 min at room temperature, desferrioxamine mesylate salt (1.68 g, 2.6 mmol) followed by DIPEA (0.66 g, 5.1 mmol) and 4-methyl morpholine (2 mL) were added. The mixture was then stirred for 48 h at room temperature. The solvent was removed using a high vacuum pump to give an oily residue. Addition of 50 mL ice cold acetone followed by sonication resulted in a white solid which was isolated by centrifugation and decantation of the acetone (procedure was repeated twice). Then, a similar washing procedure was done with doubly distilled water instead of acetone (3×30 mL). The wet white solid was lyophilized to give a white powder. Yield, 841 mg, 34%.

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 1.16-1.27 (m, 8H), 1.32-1.43 (m, 8H), 1.41-1.56 (m, 8H), 1.97 (s, 3H), 2.24-2.33 (m, 6H), 2.54-2.65 (m, 6H), 2.94-3.03 (m, 8H), 3.43-3.48 (m, 6H), 3.53-3.58 (m, 2H), 4.87 (s, 2H), 4.99 (s, 2H), 7.17-7.47 (m, 11H), 7.76 (s, br, 3H), 9.56-9.67 (m, 3H). ^{13}C{^{1}H}NMR (100 MHz, DMSO-d_6): δ (ppm) 20.8, 23.8, 23.9, 26.5, 26.6, 27.7, 28.1, 29.3, 29.4, 30.1, 30.4, 38.9, 45.1, 47.3, 47.6, 65.5, 75.9, 128.2, 128.7, 128.9, 129.1, 129.7, 135.4, 137.8, 156.8, 170.6, 171.4, 171.7, 172.4. ESI-MS (positive detection mode): *m*/*z* (%) 1007.3 (100) $[M+Na]^+$. Anal. Calcd for $C_{49}H_{76}N_8O_{13}$: C 59.74, H 7.78, N 11.37. Found: C 59.99, H 7.67, N 11.73.

3 (DFO*). A mixture of **3-Bn-Cbz** (125 mg, 0.13 mmol) and 100 mL of MeOH was sonicated for 10 min in an ultrasonic bath. The resulting suspension was transferred to an autoclave, 10% Pd/C (38 mg) was added and the mixture was stirred for 6 h under in a H_2 atmosphere (1 bar). The reaction mixture was then filtered through a cotton plug and a filter paper to remove the catalyst. The cotton plug and filter paper was washed with 50 mL of methanol. The combined filtrate was evaporated under reduced pressure to give a white solid that was washed with 2×8 mL of acetonitrile and 20 mL of Et_2O and dried. Yield, 65 mg, 67%. The compound was stored at –20 °C.

^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 1.19-1.28 (m, 8H), 1.33-1.39 (m, 7H), 1.46-1.53 (m, 9H), 1.97 (s, 3H), 2.24-2.33 (m, 8H), 2.55-2.60 (m, 6H), 2.73-2,76 (m, 2H), 2.97-3.03 (m, 6H), 3.41-3.49 (m, 8H), 7.77 (s, br, 4H), 9.60 (s, br, 3H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6): δ (ppm) 20.8, 23.3, 23.9, 26.2, 26.5, 27.1, 27.9, 28.1, 29.2, 30.2, 30.3, 38.9, 39.2, 47.2, 47.5, 170.6, 171.7, 172.4, 172.5. ESI-MS (positive detection mode): *m*/*z* (%) 761.5 (100) $[M+H]^+$. Anal. Calcd for $C_{34}H_{64}N_8O_{11}$: C 53.67, H 8.48, N 14.73. Found: C 53.60, H 8.25, N 14.66.

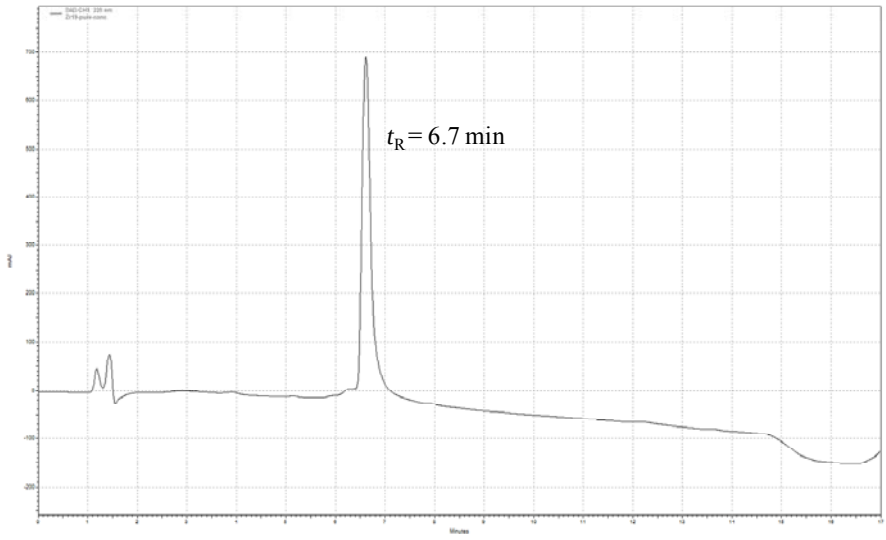
4 (DFO*-COOH). To a stirred mixture of **3** (20 mg, 0.03 mmol) and succinic anhydride (3.9 mg, 0.04 mmol) in 2 mL DMF, was added NEt_3 (7.6 mg, 0.07 mmol) under N_2 atmosphere. After 48 h stirring at room temperature, the solvent was removed in vaccuo and the white solid obtained was washed with small portions acetone and Et_2O. Yield, 18 mg, 80%.

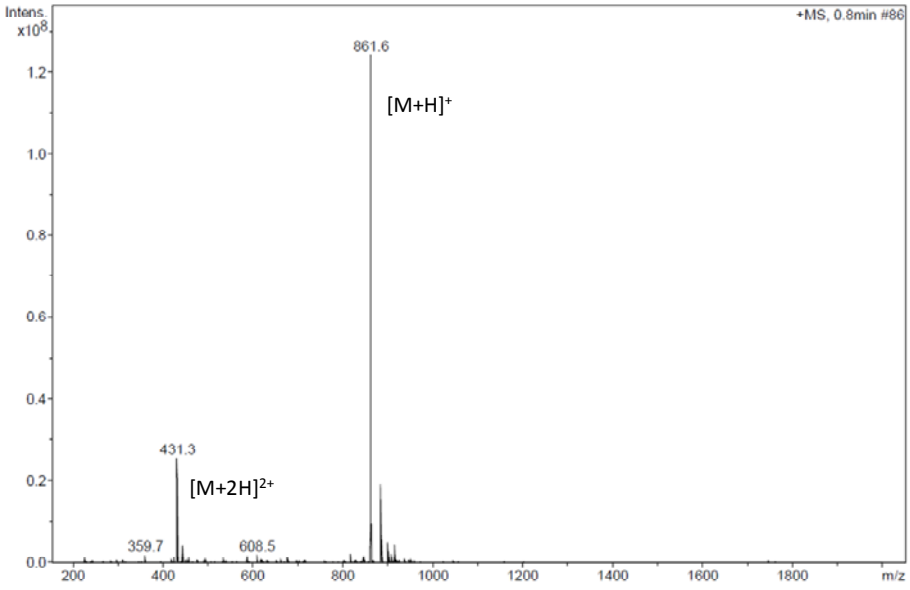
^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) 1.18-1.26 (m, 8H), 1.34-1.42 (m, 8H), 1.46-1.54 (m, 8H), 1.97 (s, 3H), 2.24-2.33 (m, 8H), 2.39-2.43 (m, 2H), 2.56-2.61 (m, 6H), 2.97-3.03 (m, 8H), 3.41-3.49 (m, 8H), 7.77 (s, br, 4H), 9.64 (s, br, 3H), 12.04 (s, br, 1H). ^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6): δ (ppm) 20.8, 23.9, 26.4, 28.1, 29.2, 29.6, 30.3, 30.4, 38.9, 47.2, 47.4, 170.6, 171.2, 171.7, 172.4, 174.3. Anal. Calcd for $C_{38}H_{68}N_8O_{14}$: C 53.01, H 7.96, N 13.01. Found: C 52.89, H 7.73, N 12.88. t_R (LC-MS) 6.7 min. Mass 861.6 $[M+H]^+$, 431.3 $[M+2H]^{2+}$

For bioconjugation purpose, the product was further purified by semi-PREP HPLC (MERCK HITACHI LaChrom equipped with a Zorbax Semi-Preparative column (300SB-C18 9.4 x 250 mm 5 μm)). The purification was performed with a flow rate of 3.5 mL min^{-1} with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade) and B (distilled water containing 0.1% TFA): t = 0-3 min, 10% A; t = 25 min, 50% A; t = 30 min, 100% A; t = 33 min, 100% A; t = 35 min, 10% A.

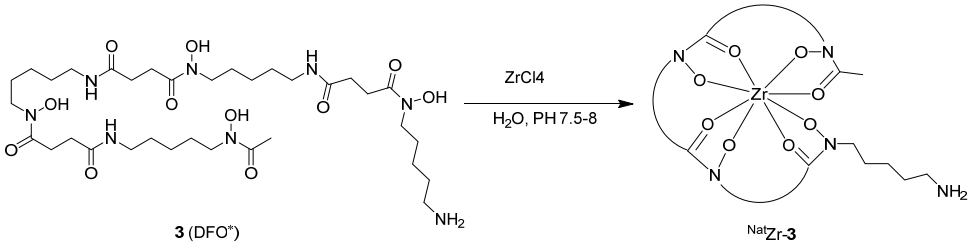
HPLC chromatogram (220 nm) and MS spectra of corresponding peak in the UV trace of HPLC analysis of 4.

Conditions for analytical HPLC analysis. HPLC chromatogram was recorded on a HPLC apparatus (VWR HITACHI CHROMASTER). A Nucleosil 100-5 C18 (250 × 3 mm) reverse phase column was used with a flow rate of 1 mL min^{-1} and UV-absorption was measured at 220 nm. The runs were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade) and B (distilled water containing 0.1% TFA): t = 0-3 min, 5% A; t = 17 min, 100% A; t = 22 min, 100% A; t = 23 min, 5% A; t = 25 min, 5% A.


t_R = 6.7 min


Intens.
x10^8
+MS, 0.8min #86
861.6
[M+H]+
431.3
[M+2H]2+
359.7
608.5
m/z

4) Complexation reaction of DFO* (3) and DFO*–COOH (4) with non-radioactive ^{nat}Zr(IV).

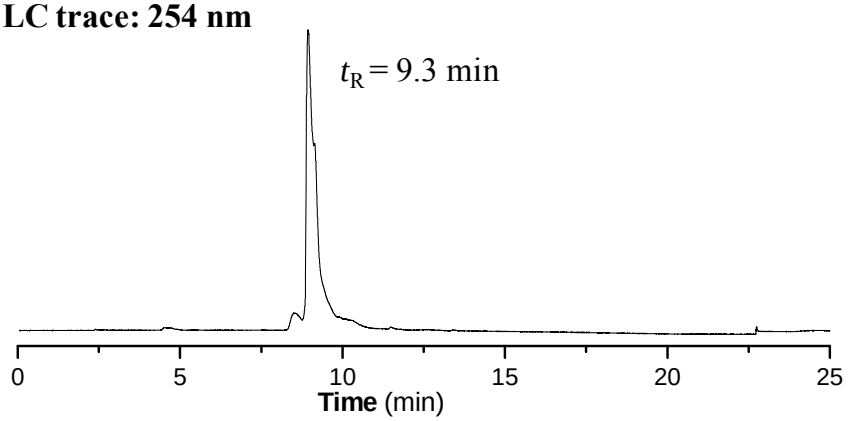


^{Nat}Zr-**3**. 600 uL of a solution of ligand DFO* (**3**; 0.0072 mM) in 0.1 M HCl was added to 200 uL of a solution of $ZrCl_4$ (0.0216 mM) in 0.1 M HCl. The pH of the stirred mixture was then slowly adjusted to *ca.* pH 7.5-8 by slow addition of 0.1 M K_2CO_3 solution and stirred for additional 40 min at room temperature. Then the mixture was lyophilized to give a white solid. Formation of the desired product was confirmed by a single peak in the LC-MS analysis with the expected mass of the protonated adduct of ^{Nat}Zr-**3**. t_R (LC-MS) 9.3 min. Mass 847.3 $[M+H]^+$.

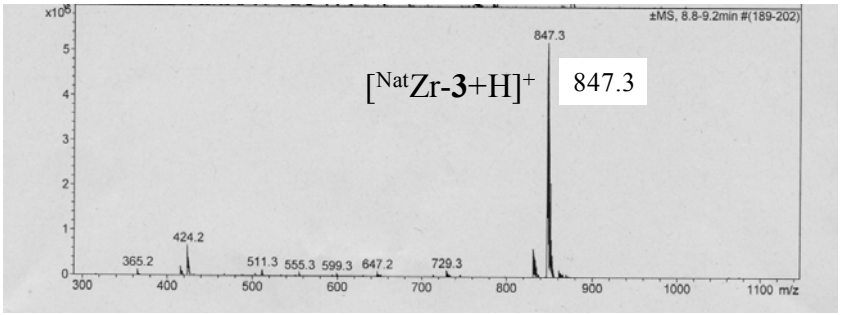
UV trace (254 nm) and MS spectra of corresponding peak in the UV trace of LC-MS analysis of ^{Nat}Zr-3

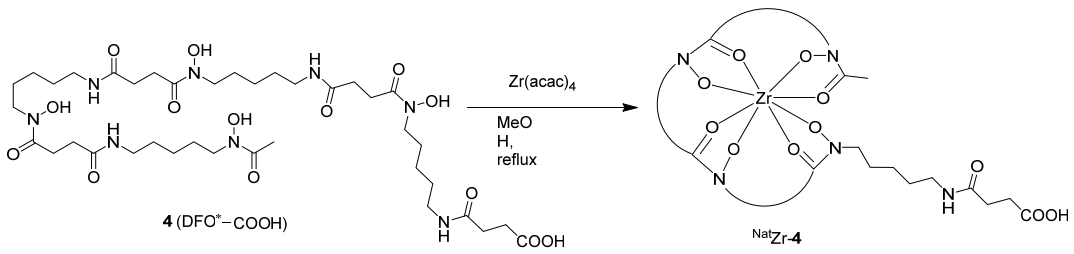
LC-MS condition: LC-MS spectra were recorded on a HPLC apparatus (Acquity Ultra Performance LC, Waters) that was connected to a mass spectrometer (Bruker Esquire 6000) operated in ESI mode. A Nucleosil 100-5 C18 (250 × 3 mm) reverse phase column was used with a flow rate of 0.5 mL min^{-1} and UV-absorption was measured at 254 nm. The runs were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade) and B (distilled water containing 0.02% TFA and 0.05% HCOOH): t = 0-3 min, 5% A; t = 17 min, 100% A; t = 20 min, 100% A; t = 21 min, 5% A; t = 25 min, 5% A.

LC trace: 254 nm



ESI-MS spectra of the peak

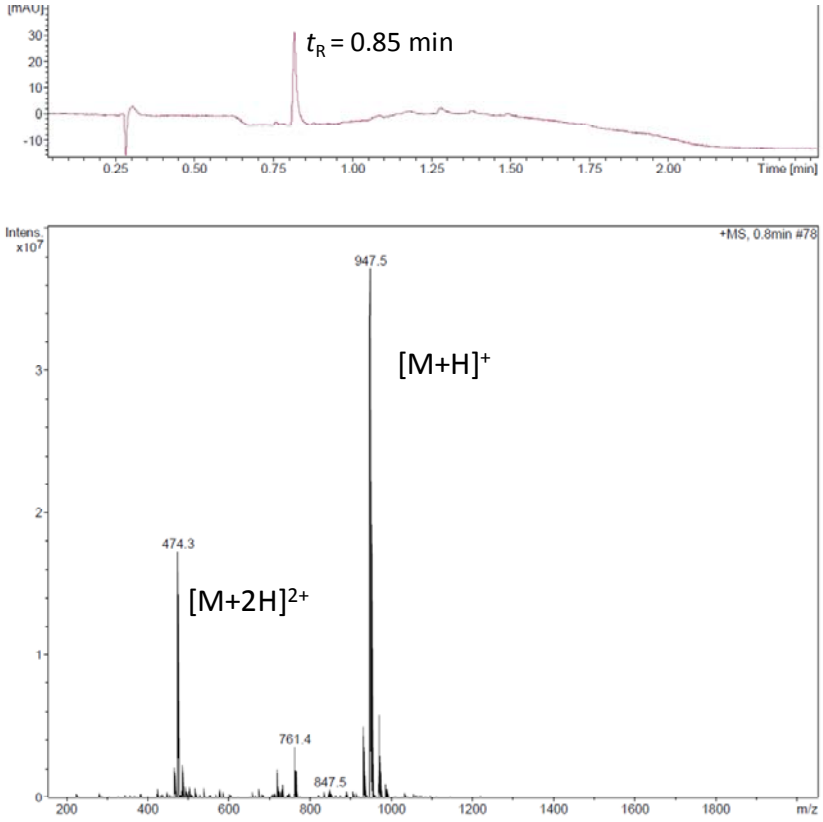




^{Nat}Zr-**4.** Due to the very poor solubility of ligand DFO*–COOH (**4**) in 0.1 M HCl or in H_2O, the complexation was carried out in MeOH using $Zr(acac)_4$. Thus, a mixture of **4** (50 mg, 0.058 mmol) and $Zr(acac)_2$ (28.3 mg, 0.058 mmol) in 15 mL MeOH was stirred at reflux for 15 h under a N_2 atmosphere. The solvent was then removed under reduced pressure and the residue was washed with Et_2O (40 mL) and acetone (40 mL). ^{Nat}Zr-**4** was obtained as an off white solid (12 mg, 22%). The formation of the complex was be confirmed using UPLC-MS. Elemental analysis calculated for $C_{38}H_{64}N_8O_{14}Zr{\cdot}6H_2O.(CH_3)_2CO$, C 44.19, H 7.42, N 10.06. Found C 44.11, H 7.04, N 10.16. t_R (UPLC-MS) 8.7 and 8.8 min. Mass 947.3 $[M+H]^+$

UV trace (254 nm) and MS spectra of corresponding peak in the UV trace of UPLC-MS analysis of ^{Nat}Zr-4

UPLC-MS condition: UPLC-MS spectra were recorded on a HPLC apparatus (Acquity Ultra Performance LC, Waters) that was connected to a mass spectrometer (Bruker Esquire 6000) operated in ESI mode. A UPLC reverse phase column (ACQUITY UPLC BEH C18 1.7 µm, 2.1x50mm) was used with a flow rate of 0.6 mL min^{-1} and UV-absorption was measured at 254 nm. The runs were performed with a linear gradient of A (acetonitrile (Sigma-Aldrich HPLC-grade) and B (distilled water containing 0.1% HCOOH): t = 0-0.25 min, 5% A; t = 1.5 min, 100% A; t = 2.5 min, 100% A.



5) Synthesis of (β-Ala)$_3$[Nle^{14}]BBS(7-14)

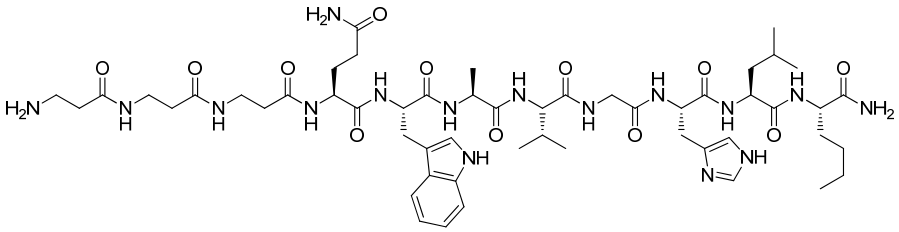


Figure S1: (βAla)$_3$[Nle^{14}]BBS(7-14).

SPPS (scale: 0.1 mmol) of (β-Ala)$_3$[Nle^{14}]BBS(7-14) was done by standard Fmoc chemistry with TBTU–HOBt as coupling reagents and 20% piperidine in DMF as deprotection reagent. The elongation was carried out using a 4-fold excess of protected amino acids and coupling reagents.

HPLC conditions: Analysis was performed with a HPLC system from Bischoff (Leonberg, Germany) equipped with two HPLC pumps 2250, a λ-1010 UV-detector and a reversed phase column from Phenomenex (Jupiter C12, 4µ Proteo 90A, 250 x 4.6 mm). The column was eluted with mixtures of acetonitrile (solvent A) and water with 0.1% trifluoroacetic acid (TFA) (solvent B). Analytical gradient: 0 min 95% B, 12.5 min 50% B at a flow rate of 2 mL/min; ESI-MS: $[M+H]^+$ *m/z* = 1136.3 (calcd. for $C_{53}H_{82}N_{16}O_{12}$: 1135.32)

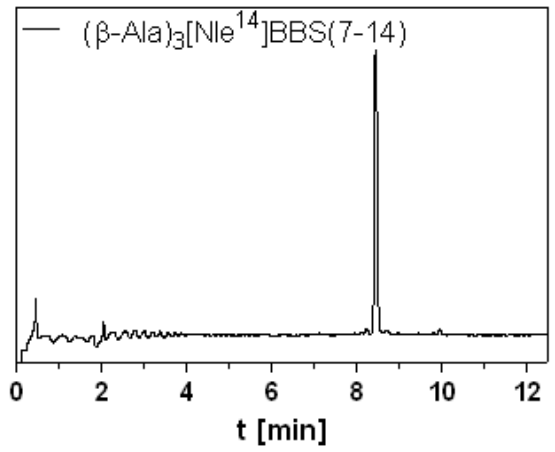


Figure S2: HPLC chromatograph of (βAla)$_3$[Nle^{14}]BBS(7-14) (UV-trace at 214 nm).

6) Synthesis of peptide conjugates 5 and 6

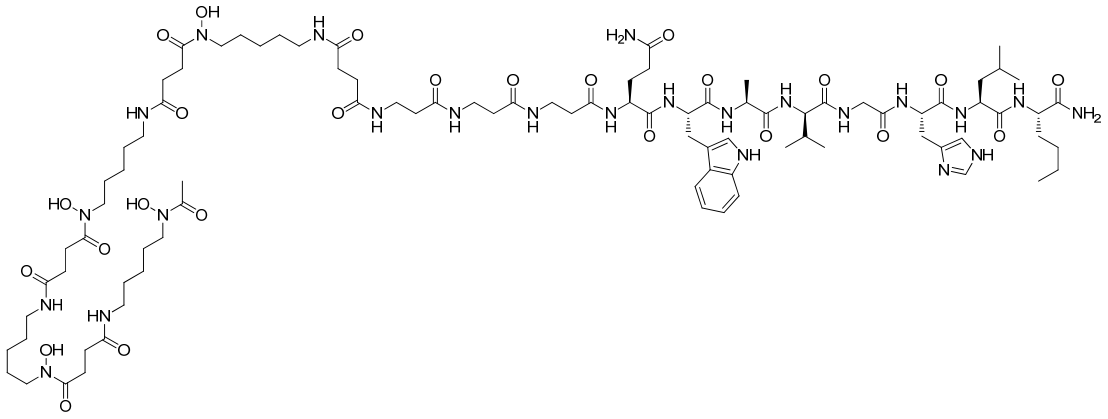


Figure S3: DFO*-(βAla)$_3$[Nle^{14}]BBS(7-14) (**5**).

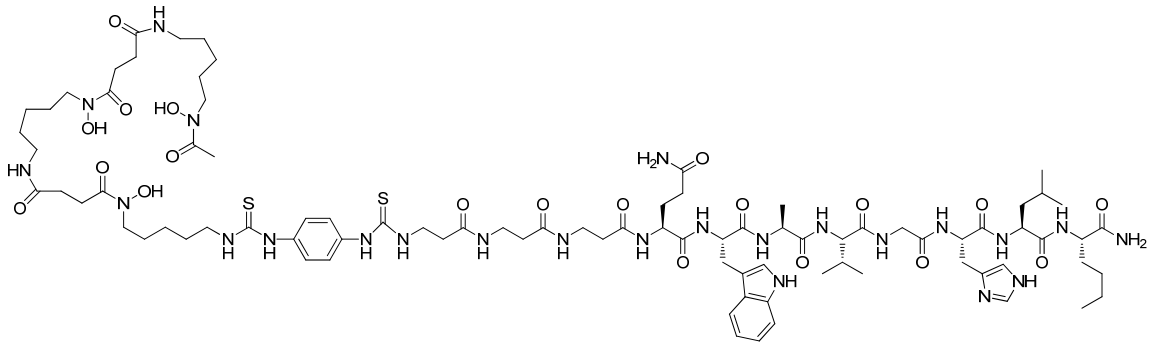


Figure S4: DFO-(βAla)$_3$[Nle^{14}]BBS(7-14) (**6**).

Coupling of DFO* and DFO-*p*-SCN to (βAla)$_3$[Nle^{14}]BBS(7-14) was done by manual synthesis. 50-60 mg (9-12 µmol) of peptide resin was Fmoc-deprotected with 20% piperidine/DMF (5x 2 mL, 3-5 min each). DFO* or DFO-*p*-SCN (2 eq.) was dissolved in 1 mL DMF and shortly warmed up in a heating block at 60-70 °C, then vortexed and sonicated. The solution was transferred to an eppendorf tube containing HATU (2 eq.), and DIPEA (4 eq.) was added. Subsequently, the mixture was transferred to the syringe reactor containing the swollen peptide resin. After 3 h the reaction was stopped and the coupling procedure repeated overnight. Cleavage of the peptide conjugates from the resin and deprotection of the amino acid side chains was done by treatment with ~ 0.6 mL TFA/phenol/TIS/water

(87.5/5/2.5/5) for 3 h and precipitation in ice-cold diethylether. The peptide precipitate was thoroughly washed with diethylether. The remaining crude product was analysed and then purified by semi-preparative RP-HPLC. Combined HPLC fractions were concentrated and lyophilized. The chemical purity of **5** and **6** was determined to be >95% (Figures S9 and S10).

HPLC conditions: Analysis and semi-preparative purifications were performed with a HPLC system from Bischoff (Leonberg, Germany) equipped with two HPLC pumps 2250, a λ-1010 UV-detector and reversed phase columns from Phenomenex (Jupiter C12, 4µ Proteo 90A, 250 x 4.6 mm and Jupiter C18, 4µ Proteo 90A, 250 x 10 mm). Columns were eluted with mixtures of acetonitrile (solvent A) and water with 0.1% trifluoroacetic acid (TFA) (solvent B). Analytical gradient: 0 min 80% B, 12.5 min 60% B at a flow rate of 2 mL/min; Semi-preparative gradient: 0 min 75% B, 20 min 65% B at a flow rate of 3.5 mL/min; MALDI-MS of **5**: $[M+H]^+$ *m/z* = 1978.1 (calcd. for $C_{91}H_{148}N_{24}O_{25}$: 1977.10); MALDI-MS of **6**: $[M+H]^+$ *m/z* = 1887.9 (calcd. for $C_{86}H_{134}N_{24}O_{20}S_2$: 1886.96); The additional signals found in the mass spectra (Figures S5 and S7, S6 and S8) of **5** and **6** can be attributed to the cleavage of hydroxamate-, amide-, and thiourea bonds, which is in agreement with the literature.[2, 3]

[2] G. S. Groenewold, M. J. Van Stipdonk, G. L. Gresham, W. Chien, K. Bulleigh, A. Howard, *J. Mass Spectrom.* **2004**; *39*, 752–761.
[3] S. Saeed, N. Rashid, M. Ali, R. Hussain, P. Jones, *European Journal of Chemistry* **2010**, 1 (3), 221-227.

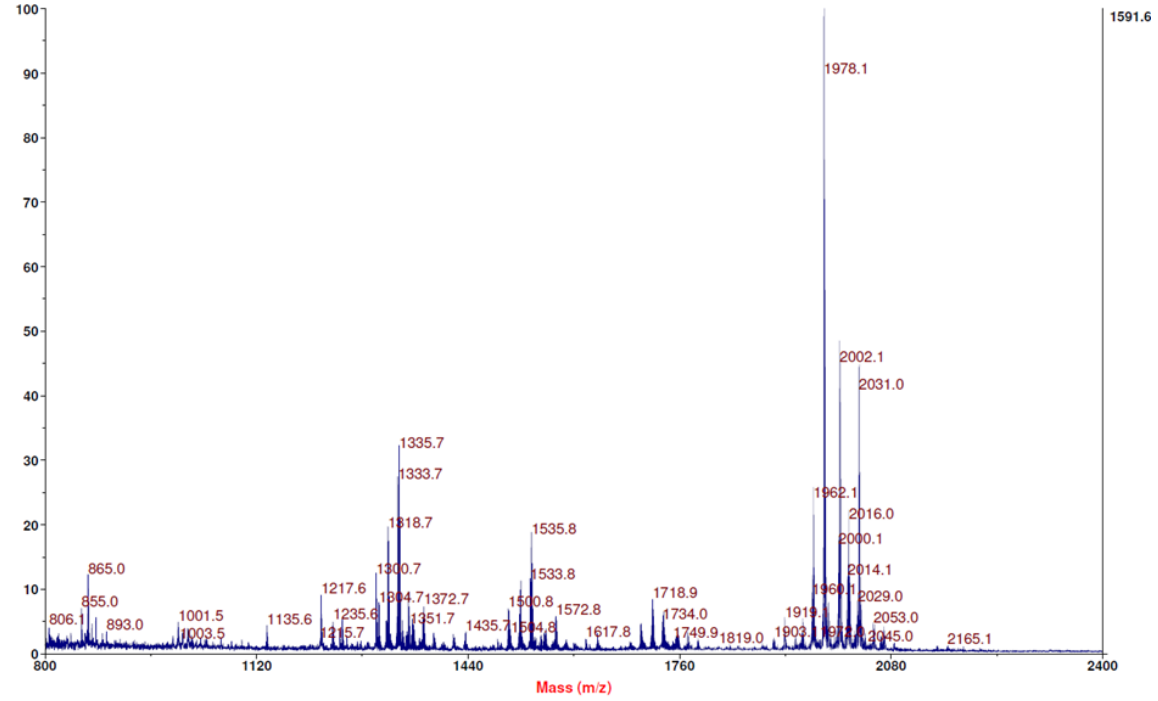


Figure S5: MALDI-MS spectrum of **5**.

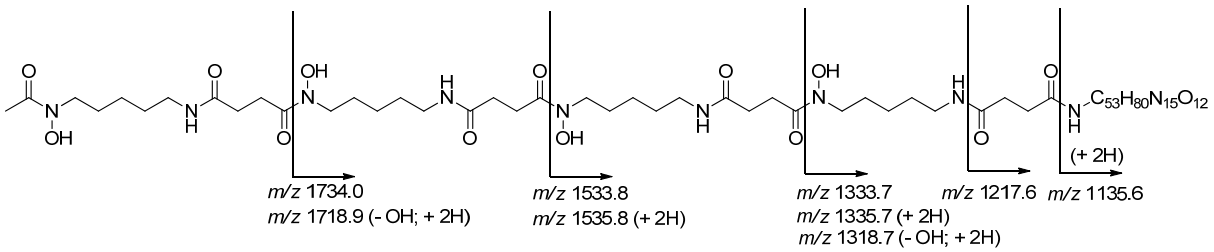


Figure S6: Cleavages accounting for fragment ions observed in the MALDI spectrum of **5**.

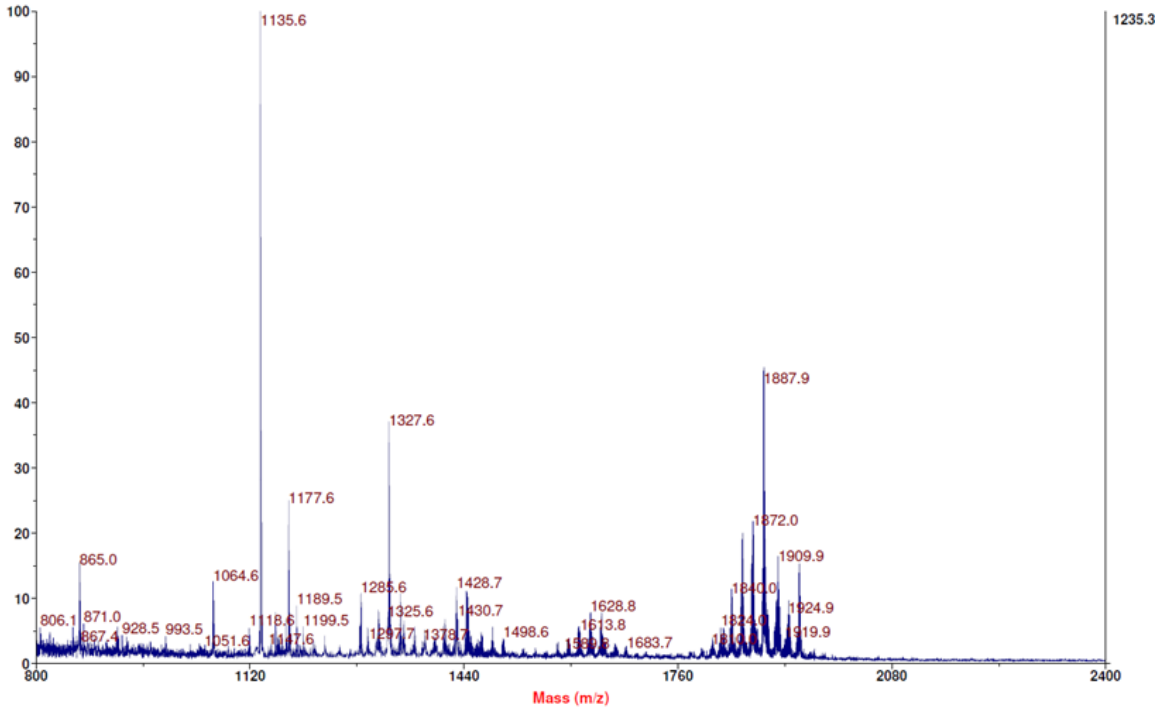


Figure S7: MALDI-MS spectrum of **6**.

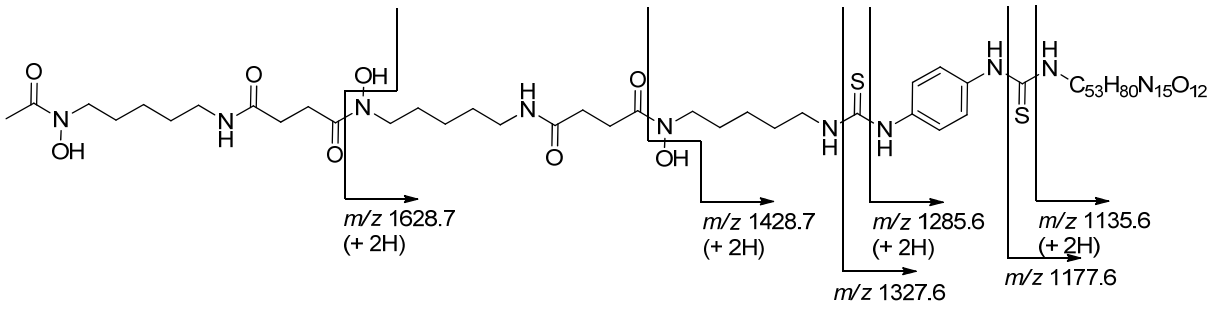


Figure S8: Cleavages accounting for fragment ions observed in the MALDI spectrum of **6**.

7) Labelling of peptide conjugates with ^{89}Zr (IV).

Radiolabelling was performed according to a modified reported procedure.[4] Briefly, lyophilized aliquots of 50 µg of **5** and **6** were prepared, stored at -20 °C and dissolved in 50 µL WFI upon starting the labelling. 10-30 µL Zr-89 (11.2-28.1 MBq) was taken from the stock solution and filled up to 200 µL with 1.0 M oxalic acid. 90 µL of 2.0 M Na_2CO_3 was added and the reaction mixture was incubated for 3 min at RT. 300 µL 0.5 M HEPES (pH 7.3), 710 µL of peptide solution (10 µL (5 nmol, 10 µg) **5** or **6** + 700 µL WFI) and 300 µL 0.5 M HEPES (pH 7.3) were added sequentially. The pH was checked with pH-strips to be between pH 7.0-7.3 and the reaction solution was incubated for 2-16 h at room temperature. Quality control of radiolabelling reactions was performed by means of HPLC and/or ITLC. Radiochemical purity (RCP) and radiochemical yield (RCY) were determined by manual integration of HPLC-, and TLC-chromatograms and ranged between 94-97% (RCP) and >95 % (RCY), respectively. Specific activities A_s of up to 5.6 GBq/µmol were reached.

HPLC conditions: Analyses were performed with a Radio-HPLC system from Bischoff (Leonberg, Germany) equipped with two HPLC pumps 2250, a λ-1010 UV-detector, a Berthold LB509 radioflow detector and a reversed phase column from Phenomenex (Jupiter C12, 4µ Proteo 90A, 250 x 4.6 mm). The column was eluted with mixtures of acetonitrile (solvent A) and water with 0.1% trifluoroacetic acid (TFA) (solvent B). 20 µL of labelling solution were diluted in 50 µL of 0.1 mM Desferrioxamine in 0.5 M HEPES solution (pH 7.3) and 10-20 µL were injected into HPLC. Analytical gradient: 0 min 80% B, 12.5 min 60% B at a flow rate of 2 mL/min

TLC-conditions: 2 µL of radiolabelling solution were spotted directly on Biodex green ITLC-strips and developed in citric acid solution (20 mM, pH 4.8). ITLC strips were analysed with a multisensitive storage film on a Perkin Elmer Cyclone plus phosphor imager. ^{89}Zr-**5** and

[4] R. Cohen, D. J. Vugts, M. Stigter-van Walsum, G. W. M. Visser and G. A. M. S. van Dongen, *Nature Protocols* **2013**, *8*, 1010.

^{89}Zr-**6** remain at the starting line (R_f= 0), while unreacted Zr-89 migrates with the solvent front (R_f= 0.8-0.9).

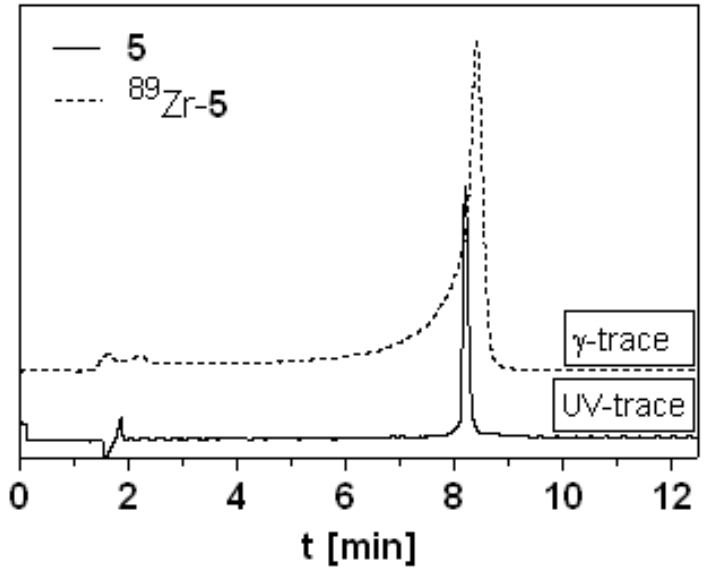


Figure S9: HPLC chromatographs of peptide precursor **5** (UV-trace at 214 nm, straight line) and ^{89}Zr-**5** (γ-trace, dotted line). The small difference of retention times is due to the serial arrangement of the UV- and γ-detectors.

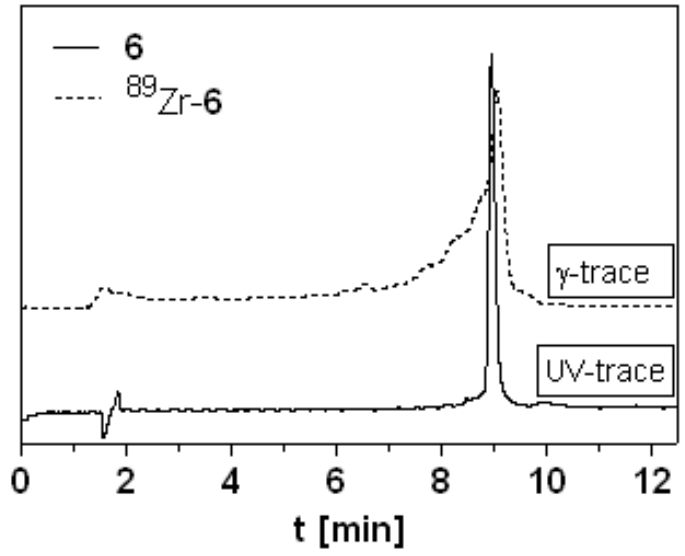


Figure S10: HPLC chromatographs of peptide precursor 6 (UV-trace at 214 nm, straight line) and ^{89}Zr-6 (γ-trace, dotted line). The small difference of retention times is due to the serial arrangement of the UV- and γ-detectors.

At peptide precursor amounts of n= 5 nmol, both compounds **5** and **6** were almost quantitatively radiolabelled after 2 h reaction time, while at n= 2.5 nmol a RCY of 92 ± 4.4% for ^{89}Zr-**5** and 78.5 ± 4.9% for ^{89}Zr-**6** were observed (Figure S11).

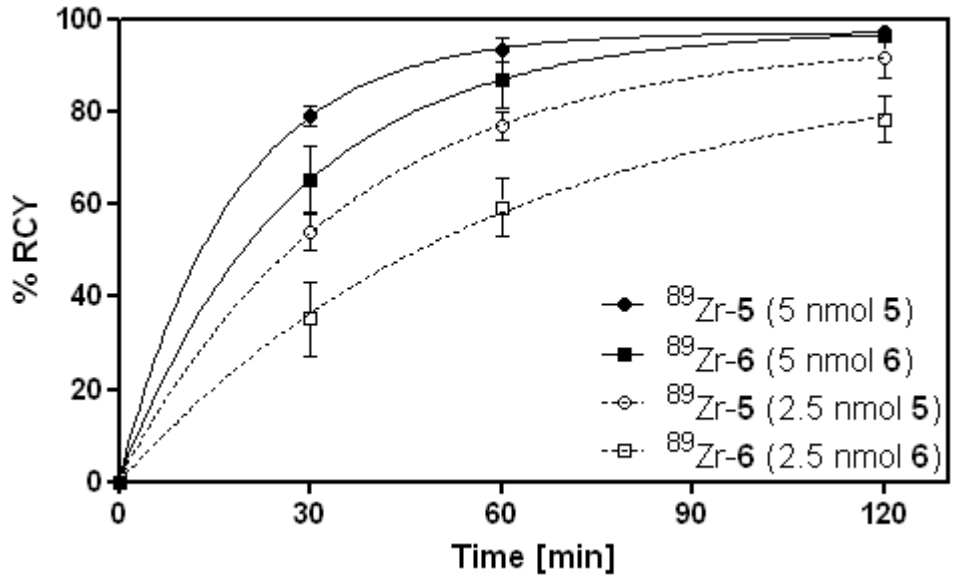
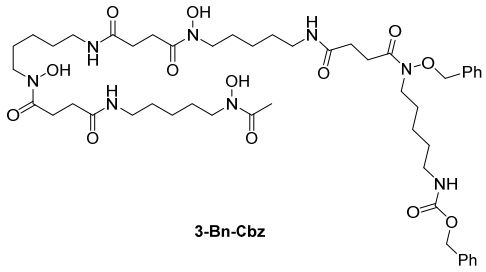


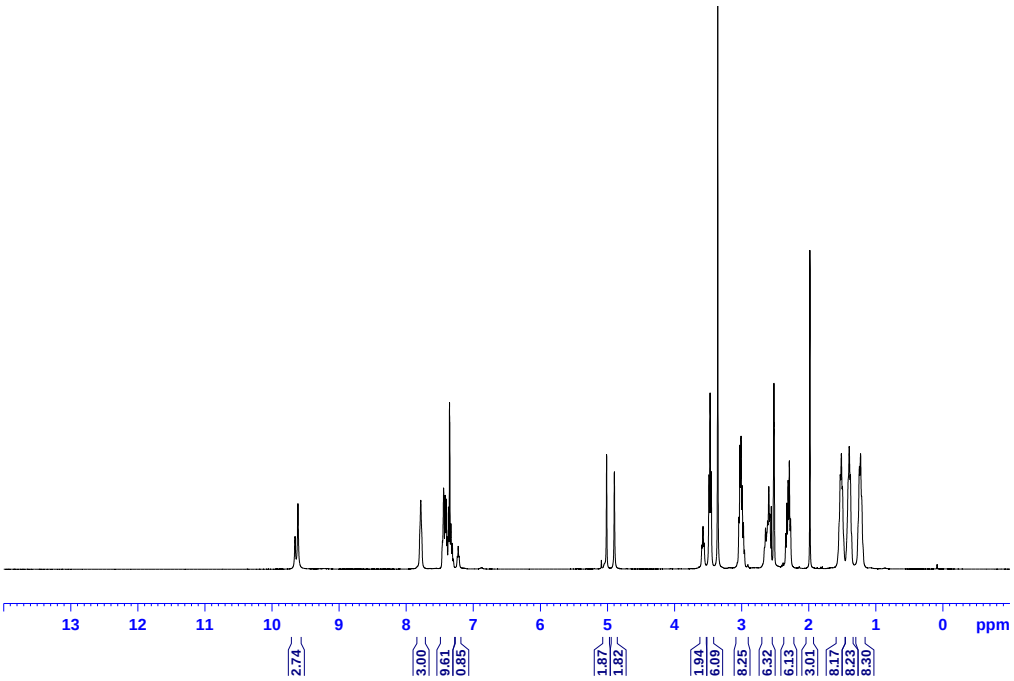
Figure S11: Radiolabelling kinetics of **5** and **6** with Zr-89 (~11 MBq) with varying reaction times (30, 60, 120 min) and amounts of peptide precursor (2.5 nmol vs. 5 nmol).

8) ^{1}H and ^{13}C NMR spectra of compounds.

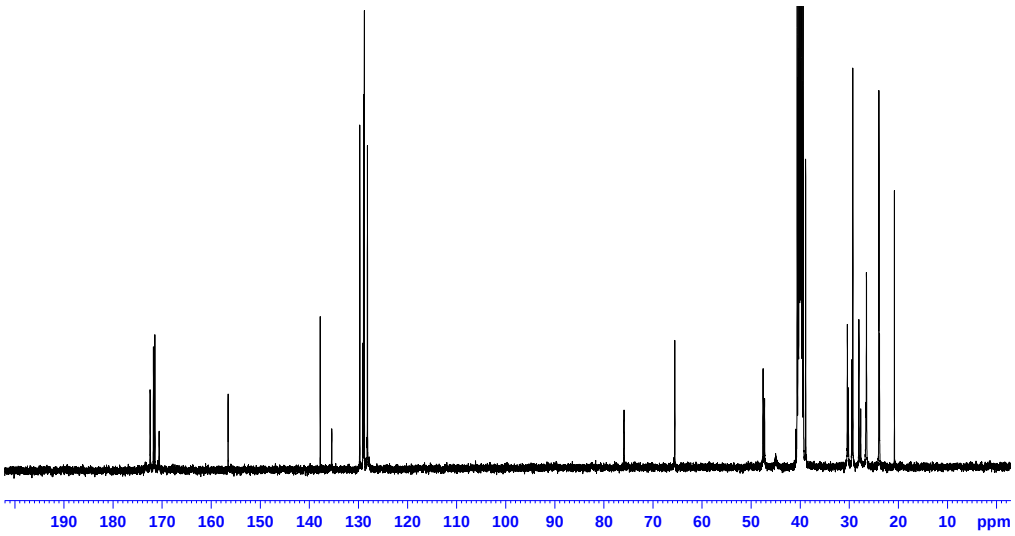
^{1}H NMR (400 MHz, DMSO-d_6).

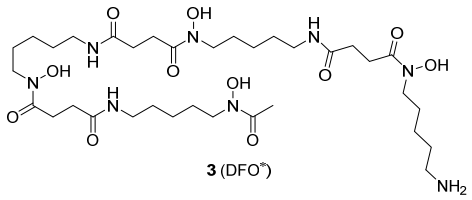


3-Bn-Cbz

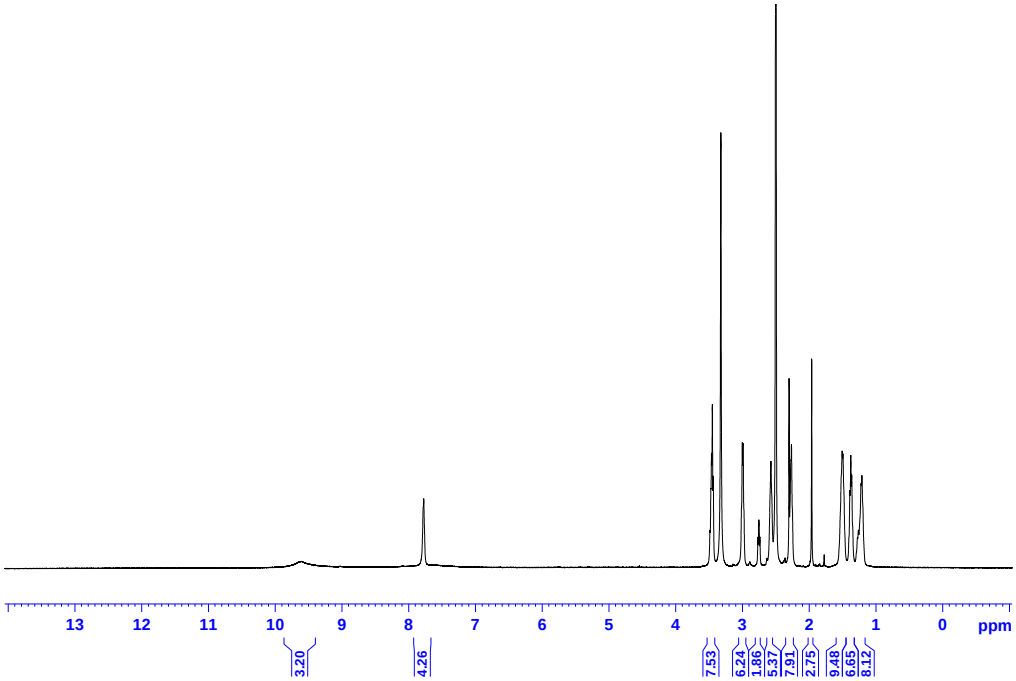


^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6)

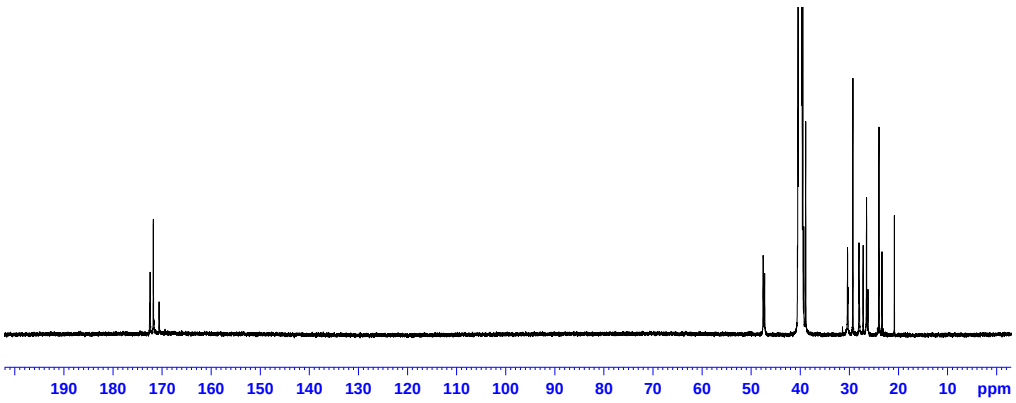


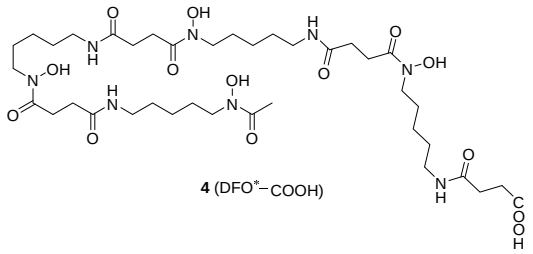


^{1}H NMR (500 MHz, DMSO-d$_6$).

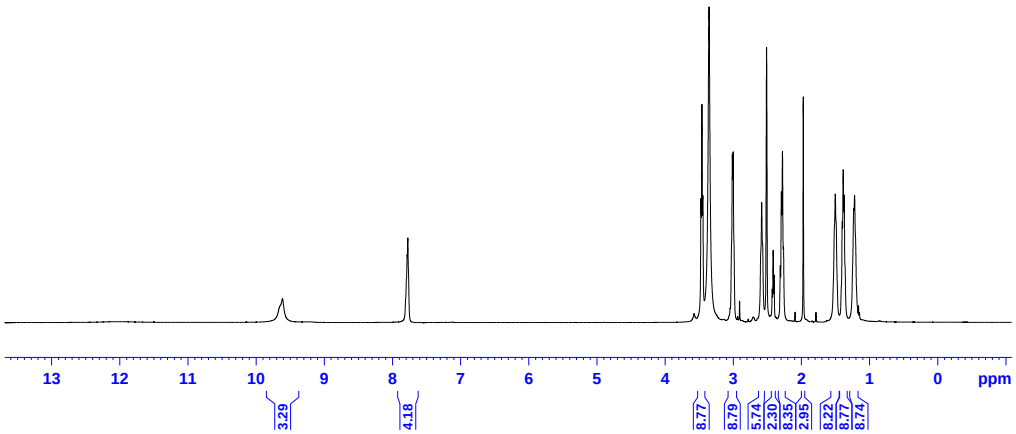


^{13}C{^{1}H} NMR (125 MHz, DMSO-d$_6$)

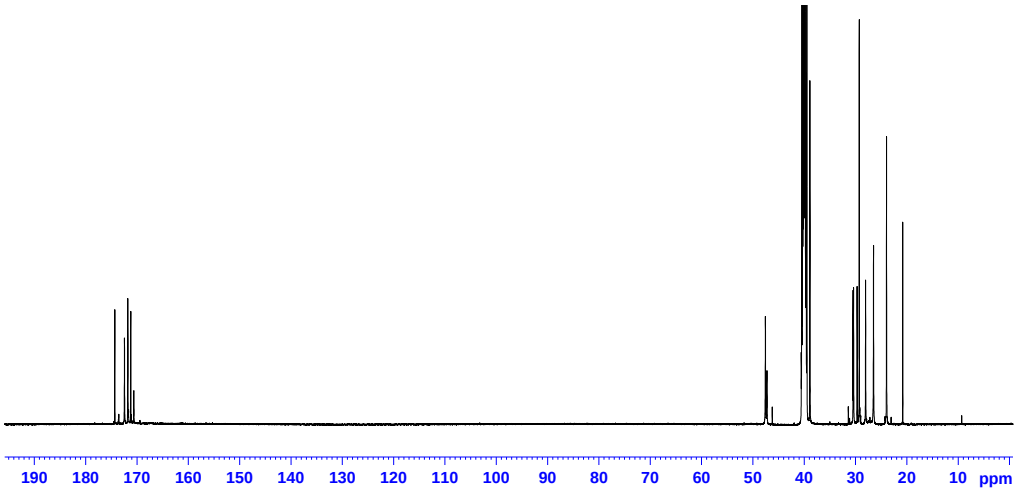




^{1}H NMR (500 MHz, DMSO-d$_6$)



^{13}C{^{1}H} NMR (125 MHz, DMSO-d$_6$):



9) NMR Analysis of Ligand 4 and its Complex ^{nat}Zr-4

All NMR experiments were performed on a Bruker Advance III NMR spectrometer operating at 600.13 MHz proton frequency. The instrument was equipped with a cryogenic 5-mm QCI indirect quadruple resonance probe (H/C/N/F/D) or with a direct observe 5-mm BBFO smart probe, both using shielded z-gradient coils and a GAB gradient amplifier (10 Ampere,

maximum gradient strength 52.5 G/cm). The experiments were performed at 298 K and the temperature was calibrated using a methanol standard showing accuracy within +/- 0.2 K. Standard pulse sequences were used for the cosy, tocsy, hmqc and hmbc two-dimensional NMR experiments.

Upon reaction with Zr^{4+}, significant shift changes for the protons of ligand **4** were found and more complex coupling patterns for the now diastereotopic methylene groups of ^{nat}Zr-**4** can clearly be observed (Fig. S12). This is in perfect agreement with the assumption of a chiral, eight-fold coordinated Zr-complex being formed. A two-dimensional tocsy NMR experiments revealed four C_2H_4– and four C_5H_{10}–spin systems all with different chemical environment as expected. Complete assignment of all types of methylene groups in the complex was achieved by hmqc (Fig. S13) and hmbc experiments. Very sharp resonances were obtained for the terminal $HOOCC_2H_4$–group and the proceeding C_5H_{10}–spin system, indicating a high degree of flexibility in contrast to the other signals, which we interpret as the terminus of the ligand not being attached to the metal as shown in Fig. S13.

The number of CH_2 resonances observed in the hmqc spectrum slightly exceeds the number of carbons that are expected, which clearly indicates the presence of isomers with respect to the wrapping of the ligand around the metal and / or the coordination polyhedron of the Zr atom. This observation is in agreement with the data from HPLC as mentioned in the main body of the text.

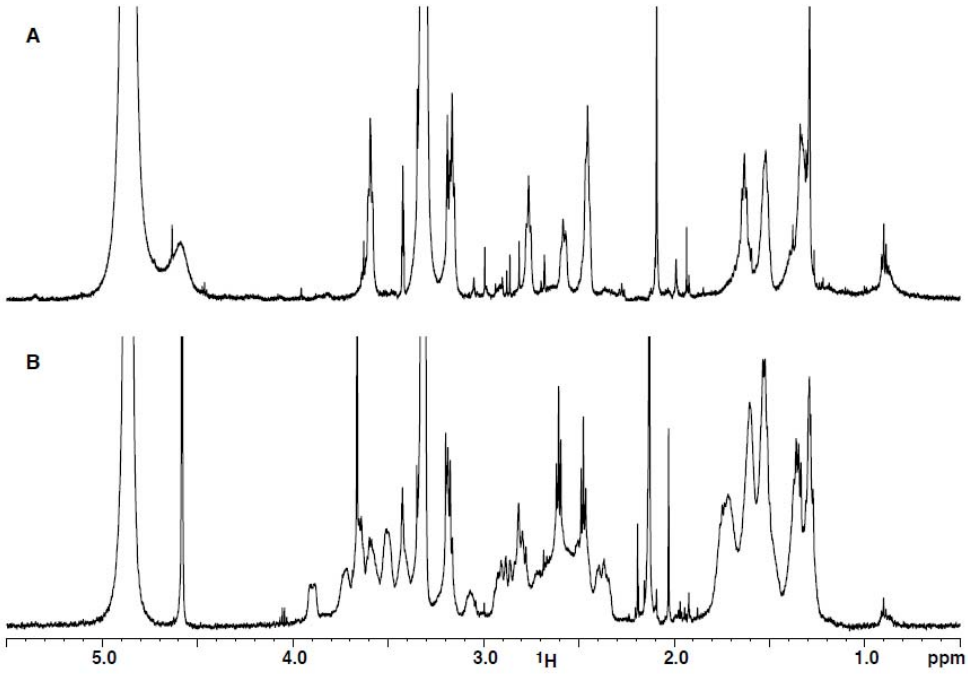


Fig. S12: A) ^{1}H-NMR of the free ligand **4** compared to B) ^{1}H-NMR of the complex ^{nat}Zr-**4**, both in CD_3OD at 298 K

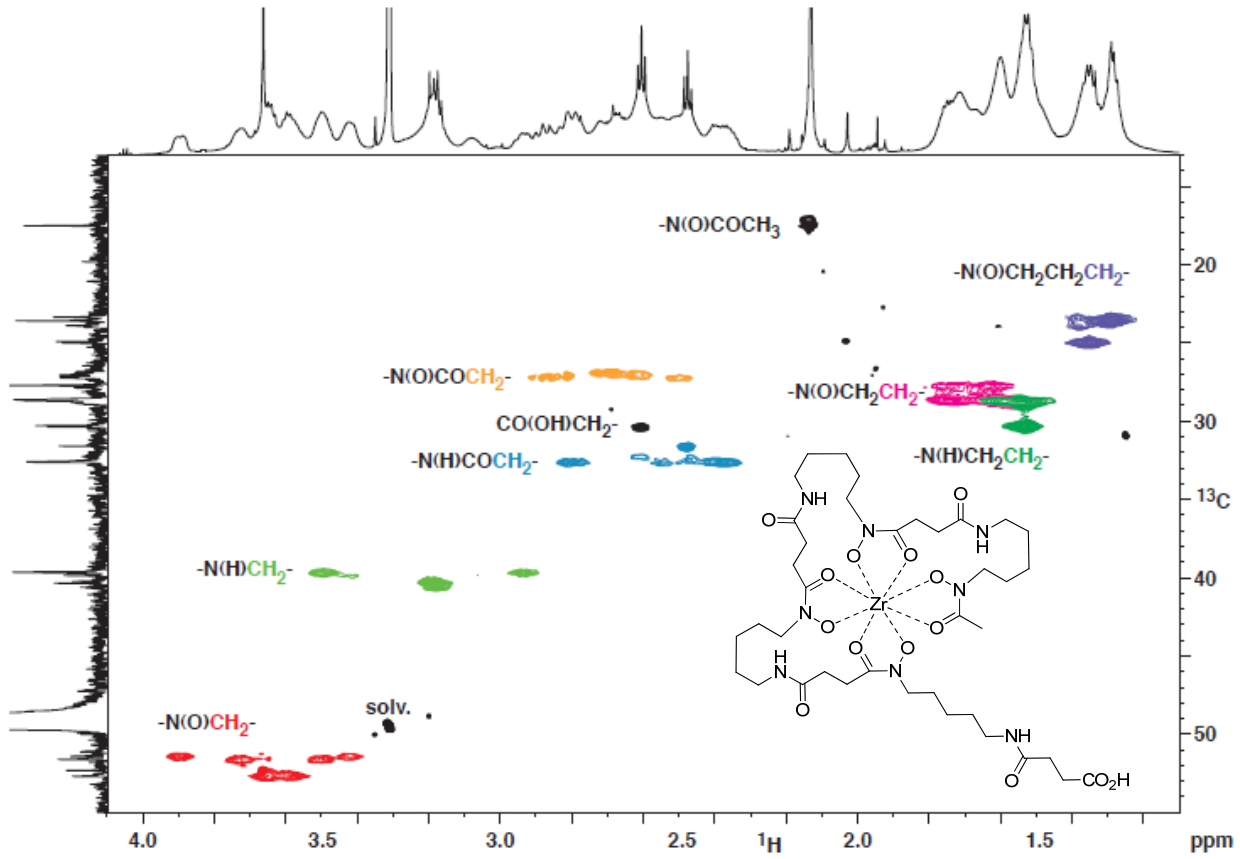


Fig.S13: ^{1}H-^{13}C-HMQC-spectrum of complex ^{nat}Zr-**4** in CD_3OD at 298 K with assignment.

10) Computational Details.

The DFT calculations consisted on the geometry optimization of the Zr-DFO* complex to investigate the coordination around the Zr^{4+} ion, especially in terms of Zr-O bond distances. DFT calculations were performed with the Gaussian03 program package (M. J. Frisch, et al., Gaussian 03, Revision D01, Gaussian, Inc., Wallingford, CT, 2003) using the hybrid functional B3LYP[5] in conjunction with the LanL2DZ basis set.[6,7,8] The nature of the stationary point was checked by computing vibrational frequencies in order to verify the true minimum. Detailed data can be found in the file "DFT_calculation" submitted as an additional ESI document.

11) Stability of ^{89}Zr-complexes in DFO challenging experiments.

A 100 µl (0.31 nmol) aliquot of ^{89}Zr-**5** or ^{89}Zr-**6** was added to 1 mL of 1 mM (~ 3000-fold excess) DFO in 0.5 M HEPES (pH 7.3) or 1 mL of 0.1 mM DFO (~ 300-fold excess) in 0.5 M HEPES (pH 7.3), respectively and incubated at RT. At defined time points (0.5, 1, 2, 4, 24 h) samples of 50 µL were analysed with HPLC using a gradient of 0 min 80% B, 12.5 min 60% B at a flow rate of 2 mL/min.

12) *In vitro*-experiments

Cell culturing: PC-3 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, high glucose) containing 10% (v/v) heat-inactivated FBS, L-glutamine (200 mM), penicillin (100 IU mL^{-1}) and streptomycin (100 µg mL^{-1}). The cells were subcultured weekly after detaching them with a commercial solution of trypsin–EDTA (1 : 250) in PBS. The internalization experiments and saturation binding assays of ^{89}Zr-**5** and ^{89}Zr-**6** were performed as previously described.[9,10]

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[5] A. D. Becke, *J. Chem. Phys.*, 1993, **98**, 5648–5652.
[6] P. J. Hay and W. R. Wadt, *J. Chem. Phys.* , 1985, **82**, 270.
[7] W. R. Wadt and P. J. Hay, *J. Chem. Phys.*, 1985, **82**, 284.
[8] P. J. Hay and W. R. Wadt, *J. Chem. Phys.* , 1985, **82**, 299.
[9] C. A. Kluba, A. Bauman, I. E. Valverde, S. Vomstein, T. L. Mindt, *Org. Biomol. Chem.* **2012**, *10*, 7594-7602.
[10] I. E. Valverde, A. Bauman, C. A. Kluba, S. Vomstein, M. Walter, T. L. Mindt, *Angew. Chem. Int. Ed.* **2013**, *52*, 8957-8960.

Internalisation experiments: Briefly, PC-3 cells were seeded out at a density of 0.8-1.0 million cells per well in six-well plates and kept overnight in cell culture medium (1% FBS in DMEM, high glucose). On the day of the experiment, the medium was replaced and the plates were incubated at 37 °C and 5% CO_2. 0.25 pmol (100 µL) of [^{89}Zr]-**5** or [^{89}Zr]-**6** were added and incubated at 37 °C and 5% CO_2. For determination of non-specific binding the cells were incubated in the presence of 250 pmol BBS(1-14). At preselected time points (0.5, 1, 2, 4 h) the medium was collected and the cells were washed two times with ice-cold PBS. Cells were then treated with glycine buffer (0.05 M, pH 2.8) twice for 5 min at 4 °C to determine the cell surface bound fractions. Finally, the internalized fractions were determined by cell lysis with 1 M NaOH and washed two times (1 mL, 1 M NaOH). The free radioligand, the receptor-bound and the internalized fractions were measured radiometrically in a γ-counter and calculated as percentage of applied dose per 10^6 cells. The kinetics of the internalisation was fitted by non-linear regression using GraphPad Prism 5.0 (Figure S14).

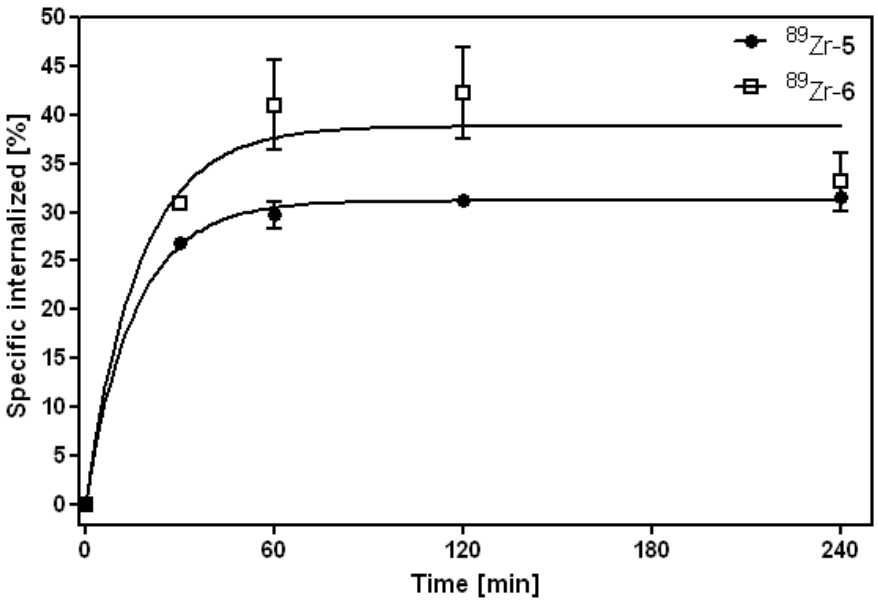

Figure S14: Receptor-specific internalisation of ^{89}Zr-**5** and ^{89}Zr-**6** into GRP-receptor expressing PC-3 cells.

Saturation binding experiment: Briefly, PC-3 cells were seeded out at a density of 0.8-1 million cells per well in six-well plates and kept overnight in culture medium. On the day of the experiment, the medium was replaced and the plates were stored at 37 °C, 5% CO_2. Mounting concentrations of ^{89}Zr-5 or ^{89}Zr-6 (100 µL; c= 1, 5, 10, 50, 100, 250, 500, 750 nM) were added to the medium (final c/well: 0.1, 0.5, 1, 5, 10, 25, 50, 75 nM) and incubated for 2 h at +4 °C. For determination of non-specific binding the cells were incubated in the presence of 250 pmol (c/well= 0.1, 0.5 nM), 2500 pmol (c/well= 1, 5, 10, 25 nM), and 10000 pmol (c/well= 50, 75 nM) of BBS(1-14). After incubation, the supernatant was collected and the cells were washed twice with 1 mL of ice-cold PBS (pH 7.4). To determine the receptor-bound activity, the cells were then lysed by incubation with 1 M NaOH for 10 min at 37 °C (see above). The free radioligand and receptor-bound fractions were measured radiometrically in a γ-counter and calculated as percentage of applied dose per 10^6 cells. The concentration-dependent saturation was fitted by non-linear regression with GraphPad Prism 5.0 (Figure S15). The K_d was determined to be 3.20±0.66 nM (B_{max}= 0.98±0.06) for ^{89}Zr-**5** and 4.17±1.23 nM (B_{max}= 1.22±0.05) for ^{89}Zr-**6**.

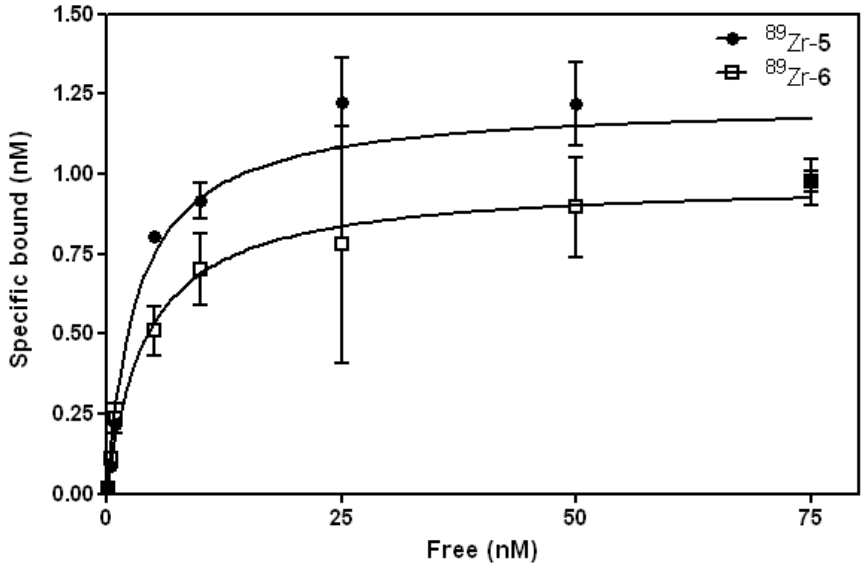

Figure S15: Receptor saturation binding curves of ^{89}Zr-**5** and ^{89}Zr-**6** using GRP-receptor expressing PC-3 cells.

13. Determination of Lipophilicity ($LogD_{7.4}$):

Lipophilicity was assessed by the shake flask method. To a solution containing 500 µL *n*-octanol and 500 µL of PBS (pH 7.4) (obtained from saturated *n*-octanol-PBS solution), 10 µL of a 1 µM solution of ^{89}Zr-**5** or ^{89}Zr-**6** was added. The resulting solutions were vortexed for 1 min at room temperature and centrifuged at 13200 rpm for 10 min. Aliquots of 100 µL were removed from each phase, and the activity was measured in a γ-counter. The lipophilicity was calculated as the average log of the ratio between the radioactivity in the organic and the aqueous fraction from ten samples, resulting in negligible differences for both compounds (Table S1).

Compound	**$LogD_{7.4}$**
^{89}Zr-**5**	-1.61 ± 0.05
^{89}Zr-**6**	-1.52 ± 0.15

Table S1: Partition coefficient in *n*-octanol/PBS (pH 7.4)