

Supporting Information

Exploring Structural Parameters for Pretargeting Radioligand Optimization

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1 | General information

1.1 | Reagent and instrument specifications

NOTA-Bn-p-NCS and NODA-Bn-p-NCS were purchased from Macrocyclics. All other starting materials were purchased from Sigma-Aldrich (synthetic-grade). All starting materials were used without further purification. All solvents used for HPLC analysis and purification were purchased from Fisher Scientific (HPLC grade). Metal-free DMSO ($\geq 99.9995\%$) and MeCN ($\geq 99.999\%$) were purchased from Sigma-Aldrich. Water ($>18.2\text{ M}\Omega\text{ cm}^{-1}$ at 25 °C) was obtained from an Alpha-Q Ultrapure water system from Millipore (Bedford, MA). Proton (^1H) NMR spectra were measured on a BrukerAvance Ultra Shield (500 MHz) spectrometer at ambient temperature. Data were recorded as follows: chemical shift in ppm from internal reference tetramethylsilane on the scale, multiplicity (s = singlet; d = doublet; t = triplet; m = multiplet), coupling constant (Hz), integration, and assignment. Due to the low material amounts (microgram scale) utilized for precursor synthesis, no carbon (^{13}C) spectra were recorded for the compounds presented within this study. Non-carrier-added (n.c.a.) [^{18}F]fluoride was obtained via the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ nuclear reaction of 11-MeV protons in an EBCO TR-19/9 cyclotron using enriched ^{18}O -water. QMA light ion-exchange cartridges and C18 light Sep-Pak® cartridges were obtained from Waters (Milford, MA). $^{68}\text{Ga}^{3+}$ was received from received from an Eckert & Ziegler ^{68}Ga -generator (Model IGG 100). C18 cartridges were equilibrated using absolute ethanol (10 mL) followed by deionized water (5 mL). QMA cartridges used a Chromafix 30-PS-HCO₃-resin for ion-exchange and were equilibrated using KHCO₃-solution (0.4 M, 5 mL) followed by deionized water (10 mL). High performance liquid chromatography (HPLC) purification and analysis was performed on a Shimadzu UFLC HPLC system equipped with a DGU-20A degasser, a SPD- M20A UV detector, a RF-20Axs fluorescence detector, a LC-20AB pump system, and a CBM-20A communication BUS module. A LabLogic Scan-RAM radio-TLC/HPLC-detector was used for purifications while a PosiRAM Model 4 was used for analysis. HPLC solvents (Buffer A: 0.1% TFA in water, Buffer B: 0.1% TFA in MeCN) were filtered before use. HPLC analysis of radioactive and non-radioactive compounds was performed on a reversed phase Atlantis T3 column (C18, 5 μm , 4.6 mm \times 250 mm). Preparative HPLC purification was carried out on a reversed phase Waters XTerra Prep C18 OBD (C18, 10 μm , 19 mm \times 250 mm). For radioactive thin-layer chromatography (TLC) analysis throughout this work, Merck precoated TLC plates (C18, reversed-phase) were used. Radio-TLC was performed using a Bioscan AR-2000 (Eckert & Ziegler, Berlin, Germany) instrument. Radioactivity was determined using a calibrated ion chamber (Capintec CRC-15R). Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded with a Shimadzu LC-2020 with electrospray ionization SQ detector. High-resolution mass spectrometry (ESI-HRMS) was carried out on a Micromass LCT Premier XE using a reversed phase Waters XBridge column (C18, 5 μm , 4.6 mm \times 50 mm). All PET imaging experiments were conducted on a Focus 120 MicroPET camera (Siemens, Knoxville, TN).

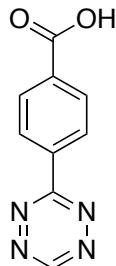
1.2 | Animal protocol, cell culture and xenograft procedures

All animal experiments within this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of MSKCC and followed National Institutes of Health guidelines for animal welfare. Female athymic nude CrTac:NCr-Foxn1^{nu} mice at age 6-8 weeks were purchased from Charles River Laboratories.

BxPC3 cells were purchased from ATCC (Manassas, VA) and grown in RPMI modified to contain 4.5g/L glucose and 1.5g/L sodium bicarbonate and supplemented with 10% (v/v) fetal calf serum, 10mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 10cc/L non-essential amino acids, 100IU penicillin and 100ug streptomycin. The human colorectal cancer cell line SW1222 was obtained from Sigma Aldrich and maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% heat-inactivated fetal bovine serum, 2.0 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin in a 37°C environment containing 5% CO₂. Cell lines were harvested and passaged weekly using a formulation of 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium. For subcutaneous injections, mice were anesthetized with 2% isoflurane (Baxter Healthcare) (2 L/min medical air) before BxPC3 cells were implanted subcutaneously (5×10^6 cells in 150 μL 1:1 growth media/Matrigel® (BD Biosciences, San Jose, CA) in the right shoulder and allowed to grow for approximately 3-4 weeks until the tumors reached 5-10 mm in size. For all intravenous injections, mice were gently warmed with a heat lamp and placed on a restrainer. The tails were sterilized with alcohol pads, and injection took place via the lateral tail vein. Healthy mice were given a two-week recovery period before reuse in another experiment.

2 | Synthetic chemistry data

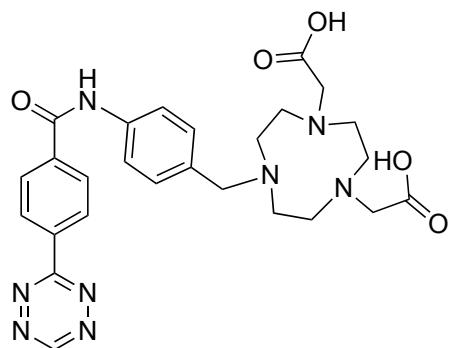
4-(1,2,4,5-tetrazin-3-yl)benzoic acid:



1, MW = 202.17 g/mol, C₉H₆N₄O₂

The title compound was synthesized according to the previously published procedure from Karver, *et al.*¹ Obtained analytical data were consistent with the reported data. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.66 (s, 1 H), 8.62 (d, *J* = 8.3 Hz, 2 H), 8.22 (d, *J* = 8.3 Hz, 2 H); MS (ESI) *m/z* 239.2 [M+Cl]⁺.

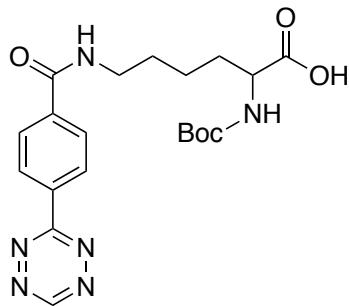
2,2'-(7-(4-(4-(1,2,4,5-tetrazin-3-yl)benzamido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (Tz-1-NODA):



15, MW = 534.58 g/mol, C₂₆H₃₀N₈O₅

4-(1,2,4,5-tetrazin-3-yl)benzoic acid (5 mg, 26.7 μmol) was dissolved in DMSO (0.5 mL) before p-Bn-NODA-NCS (11.1 mg, 26.7 μmol) and triethylamine (5 μL) were added. The pink reaction mixture was stirred at room temperature for 1 h (general procedure for the isothiocyanate-amine addition reaction). After completion of the reaction (monitored by LC-MS), precursor **15** was purified (R_t = 12.4 min). The collected fraction was concentrated under reduced pressure and dried over night under high vacuum. Precursor **15** (purity >97%) was obtained as pink oil (8.9 mg, 58%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.32 (s, 1 H), 8.41 (d, *J* = 8.2 Hz, 2 H), 8.29 (d, *J* = 8.3 Hz, 2 H), 7.62 (d, *J* = 7.8 Hz, 2 H), 7.37 (d, *J* = 7.5 Hz, 2 H), 4.16 (s, 2 H), 3.12 – 3.04 (m, 12 H), 2.42 – 2.11 (m, 5 H); MS (ESI) *m/z* 535.4 [M+H]⁺, HRMS (ESI) calcd. for C₂₆H₃₀N₈NaO₅ [= M + Na]⁺ *m/z* 557.3256 found 557.3348.

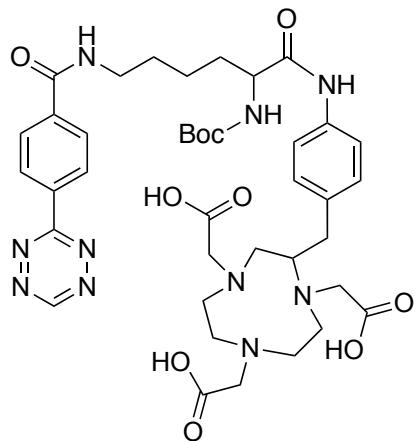
***N*⁶-(4-(1,2,4,5-tetrazin-3-yl)benzoyl)-*N*²-(*tert*-butoxycarbonyl)lysine (Tz-1-Lysine-Boc):**



MW = 430.47 g/mol, C₂₀H₂₆N₆O₅

Tz 1 (5 mg, 26.7 μ mol) was dissolved in dry DMF (0.5 mL) under nitrogen atmosphere before Boc-Lysine (5.8 mg, 29 μ mol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (10.7 mg, 60 μ mol), and hydroxybenzotriazole (6.8 mg, 60 μ mol) were added to the pink solution. The reaction mixture was stirred at room temperature for 4-6 h until all starting material was converted according to LC-MS (general amide coupling conditions). The mixture was purified (R_t = 14.1 min) and the product solution was subsequently concentrated and dried to yield the above shown compound as pink oil (6.4 mg, 58%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.41 (s, 1 H), 8.52 (t, *J* = 7.9 Hz, 1 H), 8.40 (d, *J* = 7.9 Hz, 2 H), 7.76 (s, 1 H), 7.54 (d, *J* = 8.0 Hz, 2 H), 6.99 (d, *J* = 7.7 Hz, 1 H), 3.00 (s, 2 H), 2.84 – 2.73 (m, 3 H), 1.70 – 1.49 (m, 4 H), 1.42 (s, 9 H). (ESI) *m/z* 431.5 [M+H]⁺.

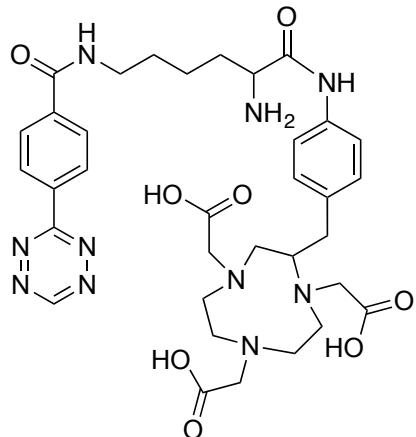
2,2',2''-(2-(4-(4-(1,2,4,5-tetrazin-3-yl)benzamido)-2-((*tert*-butoxycarbonyl)amino)-hexanamido)-benzyl)-1,4,7-triazonane-1,4,7-triyltriacetic acid (Tz-1-Lysine-Boc-NOTA):



MW = 820.91 g/mol, C₃₉H₅₂N₁₀O₁₀

The title compound was obtained using Tz-1-Lysine-Boc (5 mg, 12.1 μ mol) and an equimolar amount of p-Bn-NOTA-NCS, as well as the general conditions for the isothiocyanate-amine addition reaction. The product was purified (R_t = 16.6 min) and dried under reduced pressure and furnished as pink oil (8.2 mg, 78%). ¹H NMR (500 MHz, Chloroform-*d*) δ 10.20 (s, 1 H), 8.53 (d, *J* = 8.0 Hz, 2 H), 7.73 (d, *J* = 7.9 Hz, 2 H), 7.56 (d, *J* = 8.2 Hz, 2 H), 7.47 (d, *J* = 7.9 Hz, 2 H), 7.37 (t, *J* = 7.9 Hz, 3 H), 7.31 – 7.25 (m, 7 H), 6.72 (s, 1 H), 5.52 (s, 1 H), 4.49 (dd, *J* = 9.8, 6.8 Hz, 7 H), 4.16 (d, *J* = 21.1 Hz, 2 H), 3.12 – 3.09 (m, 4 H), 2.02 – 1.71 (m, 9 H), 1.41 (s, 9 H). (ESI) *m/z* 821.7 [M+H]⁺.

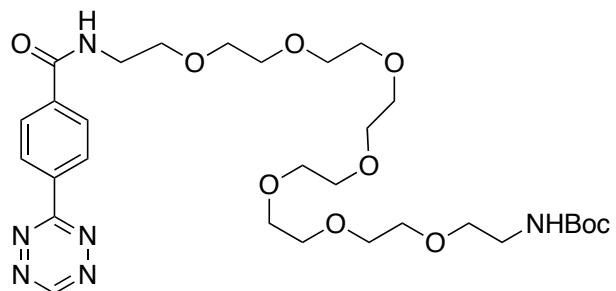
2,2',2''-(2-(4-(4-(1,2,4,5-tetrazin-3-yl)benzamido)-2-aminohexanamido)benzyl)-1,4,7-triazonane-1,4,7-triyltriacetic acid:



16, MW = 720.78 g/mol, C₃₄H₄₄N₁₀O₈

Tz-1-Lysine-Boc-NOTA (6 mg, 7 μ mol) was dissolved in DCM (0.5 mL) before TFA (0.3 mL) was added dropwise to the solution under vigorous stirring. The mixture was stirred at room temperature for 45 min before DCM was removed under reduced pressure (General TFA-deprotection procedure). The remaining crude product was purified (R_t = 12.3 min, >96%), yielding precursor **14** as pink oil (4.8 mg, 90%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.10 (s, 1 H), 8.42 (d, *J* = 8.1 Hz, 2 H), 7.78 (d, *J* = 8.1 Hz, 2 H), 7.43 (d, *J* = 9.1 Hz, 2 H), 7.34 (d, *J* = 8.9 Hz, 2 H), 6.72 (s, 1 H), 5.52 (s, 1 H), 4.49 (m, 1 H), 4.16 (m, 2 H), 3.82 – 3.69 (m, 6 H), 3.12 – 3.09 (m, 9 H), 2.02 – 1.71 (m, 15 H); MS (ESI) *m/z* 721.4 [M+H]⁺, HRMS (ESI) calcd. for C₃₄H₄₄N₁₄NaO₈ [= M + Na]⁺ *m/z* 743.3381 found 743.3373.

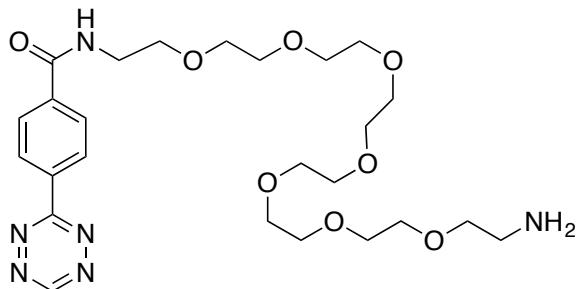
tert-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-heptaoxa-2-azapentacosan-25-yl)carbamate (Tz-1-PEG₇-NH₂Boc):



MW = 652.75 g/mol, C₃₀H₄₈N₆O₁₀

Tz-1 (10 mg, 0.05 mmol) was dissolved in anhydrous dimethylsulfoxide (DMSO, 0.5 mL) before *O*-(2-aminoethyl)-*O'*-[2-(boc-amino)ethyl]hexaethylene glycol (18.4 mg, 0.0375 mmol) and TEA (0.0057 mL, 0.0375 mmol) were added. The reaction mixture was stirred at room temperature for 1 h (general NHS ester coupling procedure). After completion of the reaction (monitored by LC-MS) the product was isolated and purified using preparative HPLC (R_t = 15.3 min). ¹H NMR (500 MHz, Chloroform-*d*) δ 10.21 (s, 1 H), 8.58 (d, *J* = 8.2 Hz, 2 H), 7.54 (d, *J* = 8.2 Hz, 2 H), 6.89 (m, 2 H), 6.45 (m, 3 H), 5.08 – 4.88 (m, 4 H), 4.56 (d, *J* = 8.0 Hz, 2 H), 3.63 (d, *J* = 8.0 Hz, 2 H), 3.54 (dt, *J* = 10.4, 4.9 Hz, 6 H), 3.42 (q, *J* = 5.2 Hz, 3 H), 3.33 – 3.27 (m, 5 H), 2.37 (t, *J* = 10.2 Hz, 2 H), 2.29 (d, *J* = 6.9 Hz, 2 H), 2.01 (q, *J* = 7.1 Hz, 3 H), 1.44 (s, 9 H); MS (ESI) *m/z* 653.6 [M+H]⁺.

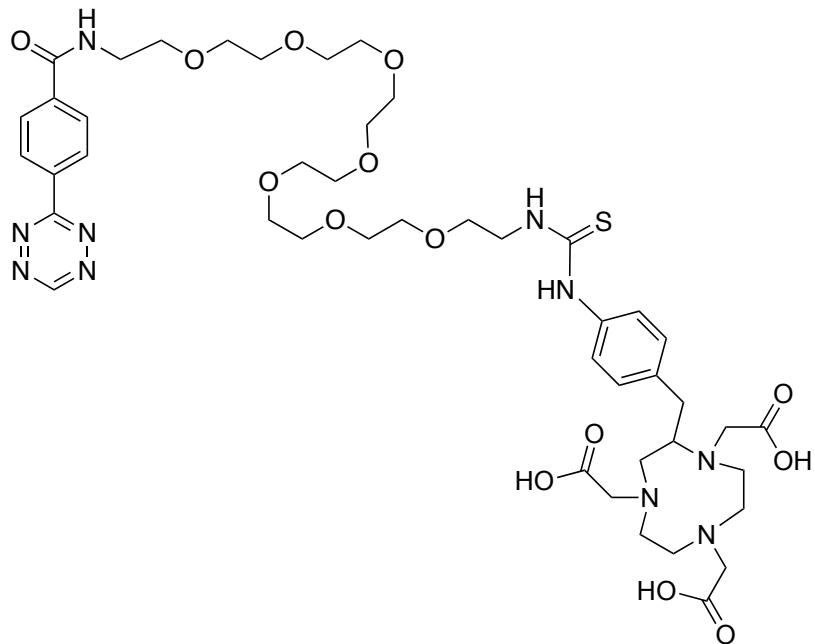
***N*-(23-amino-3,6,9,12,15,18,21-heptaoxatricosyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (Tz-1-PEG₇-NH₂):**



MW = 552.62 g/mol, C₂₅H₄₀N₆O₈

The title compound was obtained from the Boc-protected starting material Tz-1-PEG₇-NH₂Boc (5 mg, 6.7 μ mol) using the general TFA deprotection procedure. After purification (R_t = 10.7 min) and lyophilization the product was furnished as a pink oil (3.9 mg, 89%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.17 (s, 1 H), 8.47 (d, *J* = 8.1 Hz, 2 H), 7.52 (d, *J* = 8.2 Hz, 2 H), 6.58 (m, 2 H), 6.32 – 6.12 (m, 3 H), 5.11 – 5.02 (m, 4 H), 4.46 (d, *J* = 6.0 Hz, 2 H), 3.76 (d, *J* = 6.0 Hz, 2 H), 3.42 (dt, *J* = 10.4, 4.9 Hz, 6 H), 3.21 (q, *J* = 5.2 Hz, 3 H), 3.33 – 3.27 (m, 6 H), 2.37 (t, *J* = 10.2 Hz, 2 H), 2.29 (d, *J* = 6.9 Hz, 2 H), 2.01 (q, *J* = 7.1 Hz, 3 H). MS (ESI) *m/z* 553.8 [M+H]⁺.

2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-heptaoxa-2-azapenta-cosan-25-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:

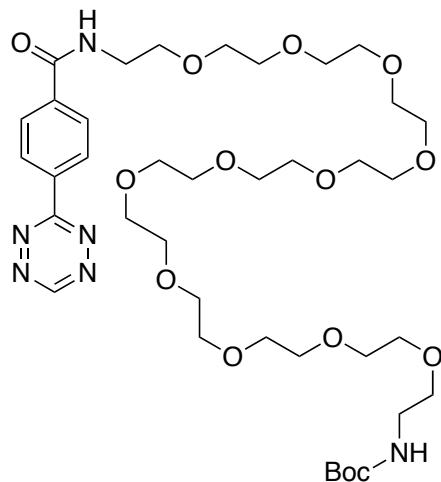


17, MW = 1003.14, C₄₅H₆₆N₁₀O₁₄S

Precursor **17** was obtained using the starting material Tz-1-PEG₇-NH₂ (3.5 mg, 5.4 μ mol) and p-Bn-NOTA-NCS (2.7 mg, 6 μ mol) using the general isothiocyanate-amide addition reaction conditions as described above (see precursor **15**). HPLC purification (>96%) and subsequent lyophilization furnished precursor **15** as a pink oil in high purities and good yield (5.1 mg, 83%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.51 (s, 1 H), 8.39 (d, *J* = 7.7 Hz, 2 H), 7.46 (d, *J* = 8.0 Hz, 2 H), 7.35 (d, *J* = 8.1 Hz, 2 H), 7.12 (d, *J* = 8.1 Hz, 2 H), 4.32 (d, *J* = 5.9 Hz, 3 H), 3.92 (d, *J* = 17.9 Hz, 3 H), 3.74 (d, *J* = 18.0 Hz, 3 H), 3.56 (d, *J* = 5.8 Hz, 2 H), 3.48 (d, *J* = 5.5 Hz, 2 H), 3.33 (t, *J* = 8.2 Hz, 3 H), 3.21 – 3.08 (m, 16 H), 3.01 – 2.90 (m, 18 H), 2.11 (t, *J* = 7.9 Hz, 2 H), 2.03 (t, *J* = 5.4 Hz, 2 H), 1.69 (p, *J* = 7.2

Hz, 3 H). MS (ESI) m/z 1004.1 $[M+H]^+$, HRMS (ESI) calcd. for $C_{45}H_{66}N_{10}NaO_{14}S$ [= M + Na] $^+$ m/z 1026.5165 found 1026.5156.

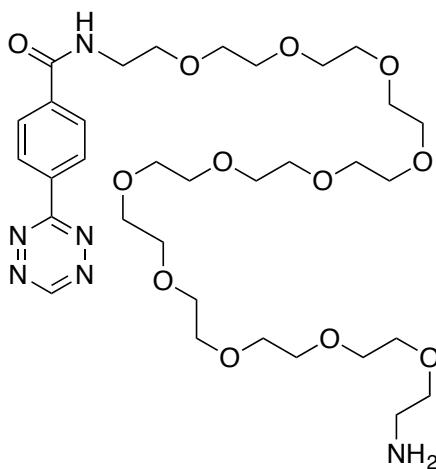
tert-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23,26,29,32,35-undecaoxa-2-azaheptatriacontan-37-yl)carbamate (Tz-1-PEG₁₁-NHBoc):



MW = 828.96 g/mol; $C_{38}H_{64}N_6O_{14}$

The title compound was prepared according to the preparation of Tz-1-PEG₇-NHBoc using *O*-(2-Aminoethyl)-*O'*-[2-(Boc-amino)ethyl]decaethylene glycol (24.2 mg, 0.0375 mmol) instead. After completion of the reaction (monitored by HPLC, 5% MeCN/H to 95% MeCN over 20 min, R_t = 14.2 min, 1 mL/min) the product was purified using preparative HPLC (5% MeCN/H to 95% MeCN over 20 min, R_t = 14.5 min, 8 mL/min) with purity >95%. The product was furnished as a pink solid (18.2 mg, 96%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.65 (s, 1 H), 8.77 (t, J = 6.4 Hz, 1 H), 8.65 – 8.57 (m, 2 H), 8.16 – 8.12 (m, 2 H), 6.77 – 6.73 (m, 7 H), 3.62 – 3.42 (m, 37 H), 3.38 (t, J = 6.3 Hz, 3 H), 3.07 (q, J = 5.8 Hz, 2 H), 1.38 (s, 9 H); MS (ESI) m/z 829.8 $[M+H]^+$.

N-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)-4-(1,2,4,5-tetrazin-3-yl)-benzamide (Tz-1-PEG₁₁-NH₂):

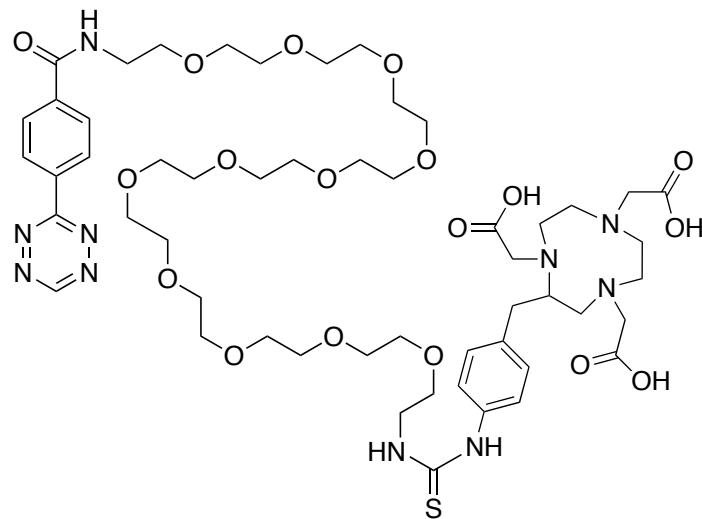


MW = 728.84 g/mol; $C_{33}H_{56}N_6O_{12}$

The title compound was furnished using Tz-1-PEG₁₁-NHBoc (16.3 mg, 0.0216 mmol) and the standard TFA reaction conditions (see Tz-1-PEG₇-NH₂). The solvent was removed under reduced pressure before the deprotected product was purified via preparative HPLC (5% MeCN/H to 95% MeCN over 20 min, R_t = 11.6 min, 8 mL/min)

with purity >97%. The product was furnished as a pink solid (14.4 mg, 92%). ^1H NMR (500 MHz, DMSO- d_6) δ 10.59 (s, 1 H), 8.74 (t, J = 5.2 Hz, 1 H), 8.61-8.55 (m, 2 H), 8.12-8.09 (m, 2 H), 6.79 – 6.74 (m, 1 H), 3.68 – 3.36 (m, 42 H), 3.34 (t, J = 6.1 Hz, 4 H), 3.10 (q, J = 6.0 Hz, 2 H); MS (ESI) m/z 829.7 [M+H] $^+$; HRMS (ESI) calcd for $\text{C}_{33}\text{H}_{57}\text{N}_6\text{O}_{12}$ [= M + H] $^+$ m/z 729.7719 found 729.7742.

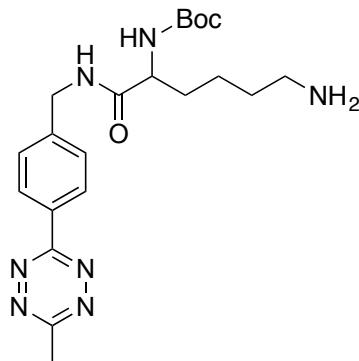
2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23,26,29,32,35-undecaoxa-2-azaheptatriacontan-37-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (Tz-1-PEG₁₁-NOTA):



18, MW = 1179.35 g/mol; $\text{C}_{53}\text{H}_{82}\text{N}_{10}\text{O}_{18}\text{S}$

Precursor **18** was obtained using Tz-1-PEG₁₁-NH₂ (13.5 mg, 0.0216 mmol) and NOTA-Bn-NCS (20.2 mg, 0.036 mmol) under the general conditions for isothiocyanate-amine addition reaction. After completion of the reaction (monitored by HPLC, 5% MeCN/H₂O to 95% MeCN over 20 min, R_t = 13.5 min, 1 mL/min) the product was purified using preparative HPLC (5% MeCN/H₂O to 95% MeCN over 30 min, R_t = 13.6 min, 8 mL/min) with purity >97%. The product was furnished as a pink solid (20.2 mg, 73%). ^1H NMR (500 MHz, DMSO- d_6) δ 10.59 (s, 1 H), 8.47 (d, J = 7.3 Hz, 2 H), 7.87 (t, J = 7.2 Hz, 3 H), 7.55 (d, J = 7.6 Hz, 3 H), 7.43 (d, J = 7.5 Hz, 3 H), 7.20 (d, J = 7.7 Hz, 2 H), 4.41 (d, J = 5.8 Hz, 3 H), 4.00 (d, J = 17.5 Hz, 2 H), 3.82 (d, J = 17.9 Hz, 4 H), 3.51 (s, 49 H), 2.20 (t, J = 7.4 Hz, 3 H), 2.12 (t, J = 5.5 Hz, 3 H), 1.78 – 1.68 (m, 4 H); MS (ESI) m/z 1180.9 [M+H] $^+$; HRMS (ESI) calcd for $\text{C}_{53}\text{H}_{81}\text{N}_{10}\text{O}_{18}\text{S}$ [= M - H] $^+$ m/z 1178.2335 found 1178.2345.

tert-butyl-(6-amino-1-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-1-oxohexan-2-yl)carbamate (Tz-2-Lysine-Boc):

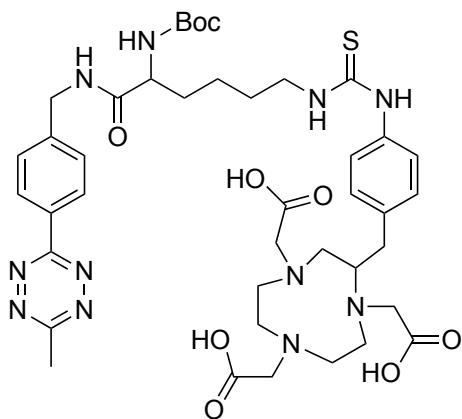


MW = 429.53 g/mol, $\text{C}_{21}\text{H}_{31}\text{N}_7\text{O}_3$

The title compound was synthesized following the general amide coupling procedure as described for compound Tz-1-Lysine-NHBoc and identical amounts of material. After full conversion of the starting material (monitored by LC-

MS), the product was purified using preparative HPLC ($R_t = 14.4$ min) and subsequently dried over night on the lyophilizer. The title compound was furnished as pink oil (6.8 mg, 59%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.52 (t, $J = 5.9$ Hz, 1 H), 8.40 (d, $J = 5.9$ Hz, 2 H), 7.76 (s, 2 H), 7.54 (d, $J = 8.0$ Hz, 2 H), 6.99 (d, $J = 7.9$ Hz, 1 H), 3.00 (s, 3 H), 2.84 – 2.73 (m, 2 H), 1.70 – 1.49 (m, 3 H), 1.42 (s, 6 H), 1.35 (s, 9 H); MS (ESI) m/z 430.5 [$\text{M}+\text{H}]^+$.

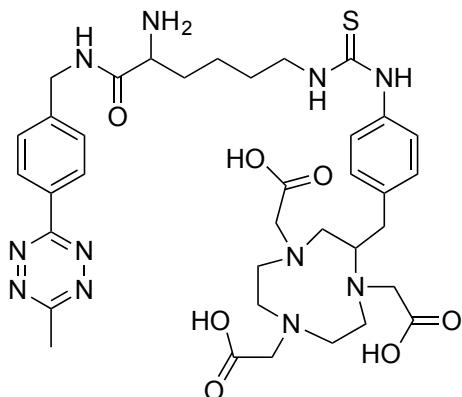
2,2',2''-(2-(4-(3-(5-((tert-butoxycarbonyl)amino)-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (Tz-2-Lysine-Boc):



MW = 880.04 g/mol, $\text{C}_{41}\text{H}_{57}\text{N}_{11}\text{O}_{9}\text{S}$

The title compound was synthesized according to the general isothiocyanate-amine addition procedure using Tz-2-Lysine-Boc (5 mg, 11.7 μmol) and p-Bn-NOTA-NCS (6.6 mg, 12 μmol). The target compound was purified ($R_t = 15.1$ min) and concentrated to yield the product as pink solid (8.8 mg, 86%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.50 (t, $J = 6.1$ Hz, 1 H), 8.41 (d, $J = 6.2$ Hz, 1 H), 7.98 – 7.88 (m, 2 H), 7.56 (dd, $J = 12.6, 8.2$ Hz, 2 H), 7.45 (d, $J = 8.3$ Hz, 2 H), 6.98 (d, $J = 12.8$ Hz, 1 H), 4.42 (d, $J = 5.9$ Hz, 1 H), 4.32 (s, 1 H), 3.96 (d, $J = 5.9$ Hz, 2 H), 3.50 (s, 2 H), 3.47 (s, 2 H), 3.34 (s, 2 H), 3.31 (s, 1 H), 3.22 – 3.11 (m, 1 H), 3.05 (dd, $J = 13.5, 5.9$ Hz, 1 H), 2.99 (s, 1 H), 2.82 (d, $J = 8.2$ Hz, 2 H), 2.74 – 2.60 (m, 7 H), 1.71 – 1.64 (m, 9 H), 1.62 – 1.51 (m, 8 H), 1.41 (s, 1 H), 1.35 (s, 9 H). MS (ESI) m/z 881.1 [$\text{M}+\text{H}]^+$.

2,2',2''-(2-(4-(3-(5-amino-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)thioureido)-benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid

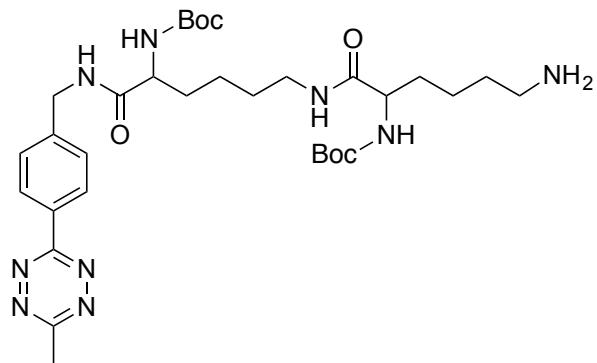


19, MW = 779.92 g/mol, $\text{C}_{36}\text{H}_{49}\text{N}_{11}\text{O}_{7}\text{S}$

Deprotection of MeTz-Lysine-Boc-NOTA was performed under standard TFA deprotection conditions to yield precursor **19** as pink oil in quantitative yield (>98%) and high purity (>99%). compound was purified ($R_t = 15.1$ min) and concentrated to yield the product as pink solid (8.8 mg, 86%). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.45 (t, $J = 6.0$ Hz, 1 H), 8.40 (d, $J = 6.1$ Hz, 1 H), 7.98 – 7.82 (m, 2 H), 7.56 (dd, $J = 12.5, 7.4$ Hz, 2 H), 7.41 (d, $J = 12.2$ Hz, 2 H), 6.98 (d, $J = 7.4$ Hz, 1 H), 4.42 (d, $J = 5.9$ Hz, 1 H), 4.32 (s, 1 H), 3.96 (d, $J = 5.9$ Hz, 2 H), 3.70 (s, 2 H), 3.57

(s, 2 H), 3.44 (s, 2 H), 3.36 (s, 1 H), 3.32 – 3.21 (m, 1H), 3.12 (dd, $J = 13.5, 5.9$ Hz, 1 H), 2.99 (s, 1 H), 2.92 (d, $J = 8.1$ Hz, 2 H), 2.70 – 2.51 (m, 7 H), 2.21 – 1.94 (m, 9 H), 1.92 – 1.81 (m, 10 H); MS (ESI) m/z 780.9 [M+H]⁺; HRMS (ESI) calcd for C₃₆H₄₉N₁₁NaO₇S [= M + Na]⁺ m/z 802.8435 found 802.8431.

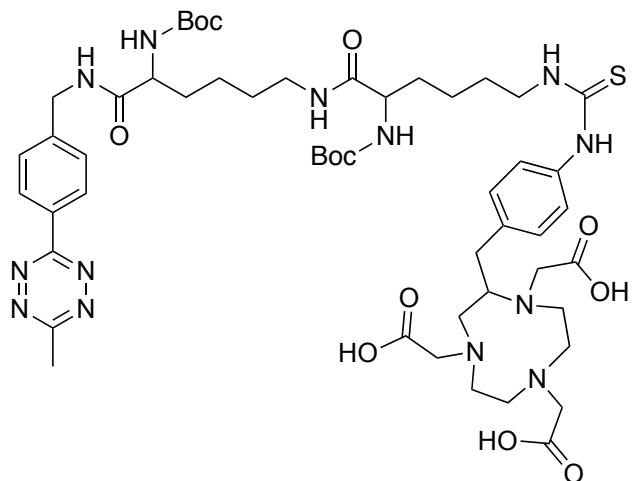
tert-butyl-(11-(4-aminobutyl)-15,15-dimethyl-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,13-trioxo-14-oxa-2,9,12-triazahexadecan-4-yl)carbamate [Tz-2-(Lysine-Boc)₂]:



MW = 657.82 g/mol, C₃₂H₅₁N₉O₆

The title compound was synthesized using the general amide coupling procedure as presented for Tz-2-Lysine-Boc. In order to attach two lysine moieties instead of one, the molar ratios of all the reagents except the tetrazine were doubled as follows: (4-(1,2,4,5-Tetrazin-3-yl)phenyl)methanamine (5.2 mg, 30 μ mol), Boc-Lysine (11.8 mg, 60 μ mol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (21.4 mg, 120 μ mol) and hydroxybenzotriazole (13.6 mg, 120 μ mol). After full conversion of the tetrazine starting material, the desired product was isolated and concentrated to yield the title compound as pink oil (7.4 mg, 38%). Note: *the relatively low yield can be explained by the simultaneous formation of the mono- and tri-lysine compounds MeTz-Lysine-Boc-NH₂ (3.1 mg, 34%) and MeTz-(Lysine-Boc)₃-NH₂ (4.2 mg, 28%), respectively.* ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.47 (t, $J = 6.0$ Hz, 1 H), 8.40 (d, $J = 8.2$ Hz, 1 H), 7.78 (t, $J = 6.1$ Hz, 1 H), 7.66 (s, 1 H), 7.53 (d, $J = 8.2$ Hz, 1 H), 6.94 (d, $J = 8.0$ Hz, 1 H), 6.76 (d, $J = 8.0$ Hz, 1 H), 4.41 (s, 1 H), 3.97 – 3.89 (m, 10 H), 3.87 – 3.80 (m, 4 H), 3.11 (s, 3 H), 3.00 (s, 1 H), 2.76 (q, $J = 7.7, 6.4$ Hz, 1 H), 1.64 – 1.47 (m, 2 H), 1.41 (s, 3 H), 1.38 (s, 4 H), 1.32 – 1.21 (m, 15 H). MS (ESI) m/z 658.8 [M+H]⁺.

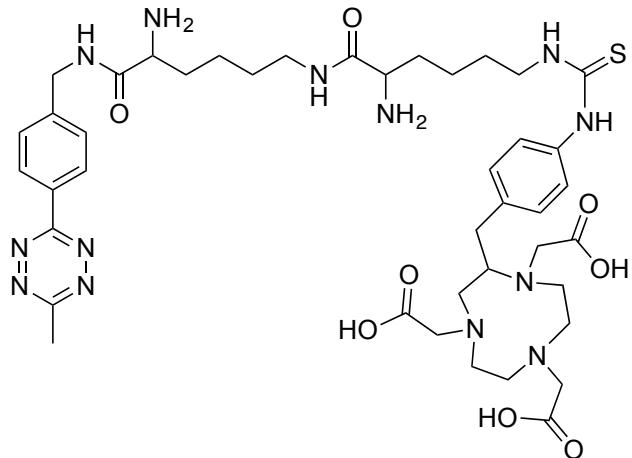
2,2',2''-(2-(4-(3-((tert-butoxycarbonyl)amino)-6-((5-((tert-butoxycarbonyl)amino)-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyltriacetic acid [Tz-2-(Lysine-Boc)₂-NOTA]:



MW = 1108.33 g/mol, C₅₂H₇₇N₁₃O₁₂S

The title compound was obtained using Tz-2-(Lysine-Boc)₂ (5 mg, 7.6 μ mol) and the general isothiocyanate-amine addition procedure as described above. Upon purification, the title compound was obtained as pink solid (6.4 mg, 76%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.42 (t, *J* = 6.1 Hz, 1 H), 8.40 (d, *J* = 6.2 Hz, 1 H), 7.88 – 7.72 (m, 2 H), 7.56 (dd, *J* = 12.5, 7.4 Hz, 2 H), 7.41 (d, *J* = 7.2 Hz, 2 H), 6.98 (d, *J* = 12.6 Hz, 1 H), 4.42 (d, *J* = 5.7 Hz, 1 H), 4.32 (s, 1 H), 3.96 (d, *J* = 5.7 Hz, 2 H), 3.70 (s, 2 H), 3.57 (s, 2 H), 3.44 (s, 2 H), 3.36 (s, 1 H), 3.32 – 3.21 (m, 1 H), 3.12 (dd, *J* = 13.5, 5.9 Hz, 1 H), 2.99 (d, *J* = 13.4 Hz, 1 H), 2.91 (d, *J* = 5.9 Hz, 1 H), 2.60 – 2.48 (m, 8 H), 2.34 – 2.03 (m, 10 H), 1.82 – 1.71 (m, 15 H), 1.53 (s, 9 H), 1.47 (s, 10 H); MS (ESI) *m/z* 1109.4 [M+H]⁺.

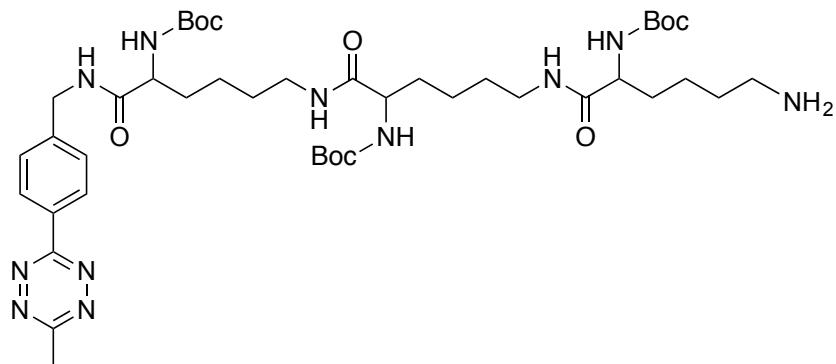
2,2',2''-(2-(4-(3-(5-amino-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)-amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyltriacetic acid:



20, MW = 908.09 g/mol, C₄₂H₆₁N₁₃O₈S

Starting from MeTz-(Lysine-Boc)₂-NOTA (6.3 mg, 5.6 μ mol), precursor **20** was obtained as a pink oil (3.9 mg, 77%, purity >98%) by applying the general TFA-based deprotection procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.11 (t, *J* = 5.9 Hz, 1 H), 8.47 (d, *J* = 5.9 Hz, 1 H), 8.23 – 8.18 (m, 2 H), 8.16 – 8.07 (m, 2 H), 7.74 (s, 1 H), 7.59 (s, 1 H), 4.62 – 4.41 (m, 1 H), 3.90 – 3.79 (m, 8 H), 3.74 – 3.64 (m, 5 H), 3.10 (q, *J* = 6.6 Hz, 1 H), 3.01 (s, 1 H), 2.81 – 2.69 (m, 2 H), 1.83 – 1.73 (m, 2 H), 1.72 – 1.65 (m, 10 H), 1.57 – 1.49 (m, 12 H), 1.48 – 1.42 (m, 4 H), 1.39 – 1.21 (m, 8 H); MS (ESI) *m/z* 909.4 [M+H]⁺; HRMS (ESI) calcd for C₄₂H₆₁N₁₃NaO₈S [= M + Na]⁺ *m/z* 931.4384 found 931.4379.

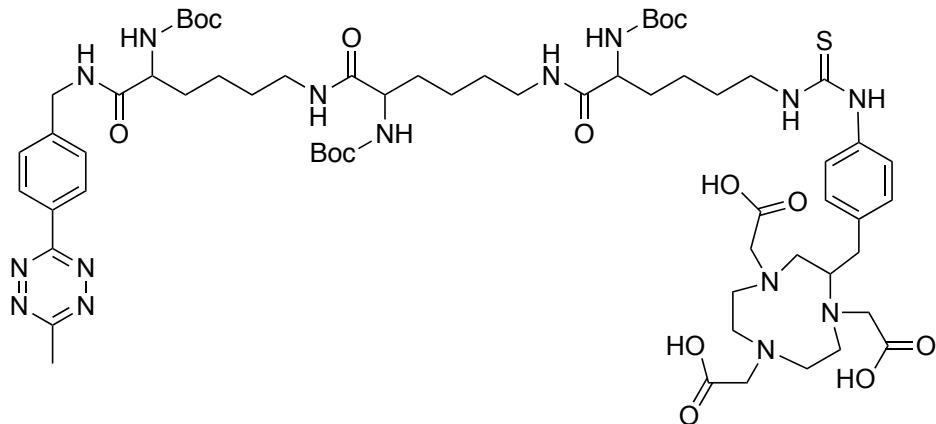
tert-butyl-(18-(4-aminobutyl)-11-((tert-butoxycarbonyl)amino)-22,22-dimethyl-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,17,20-tetraoxo-21-oxa-2,9,16,19-tetraazatricosan-4-yl)carbamate [Tz-2-(Lysine-Boc)₃]:



MW = 886.11 g/mol, C₄₃H₇₁N₁₁O₉

The title compound was obtained in the process of synthesizing Tz-2-(Lysine-Boc)₂ as described above and was furnished as pink oil (4.2 mg, 28%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.47 (t, *J* = 6.0 Hz, 1 H), 8.40 (d, *J* = 8.0 Hz, 2 H), 7.76 (d, *J* = 6.1 Hz, 2 H), 7.63 (s, 3 H), 7.53 (d, *J* = 8.0 Hz, 2 H), 6.93 (d, *J* = 7.9 Hz, 1 H), 6.74 (d, *J* = 8.0, 2 H), 4.41 – 4.28 (m, 2 H), 3.92 (d, *J* = 7.6 Hz, 1 H), 3.82 (d, *J* = 7.7 Hz, 2 H), 3.00 (s, 7 H), 2.83 – 2.70 (m, 6 H), 1.67 – 1.44 (m, 13 H), 1.53 – 1.40 (m, 27 H); MS (ESI) *m/z* 887.2 [M+H]⁺.

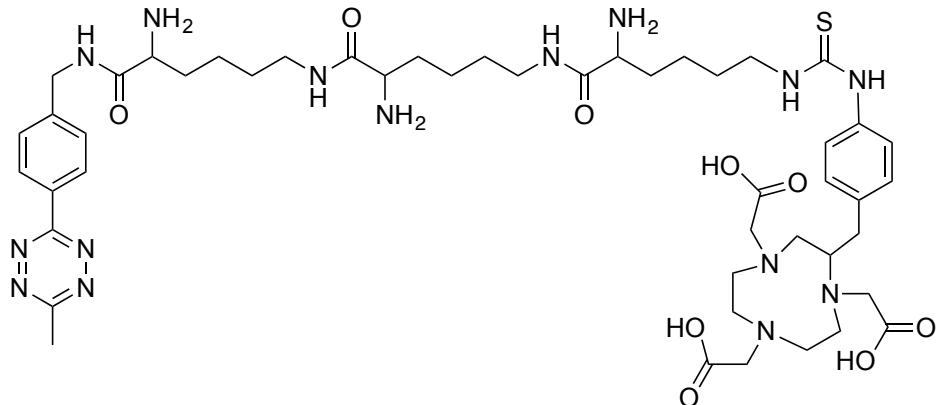
2,2',2''-(2-(4-(3-(13,20-bis((tert-butoxycarbonyl)amino)-2,2-dimethyl-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamoyl)-4,12,19-trioxo-3-oxa-5,11,18-triazatetraacosan-24-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl triacetic acid [Tz-2-(Lysine-Boc)₃-NOTA]:



MW = 1336.62 g/mol, C₆₃H₉₇N₁₅O₁₅S

The title compound was obtained using MeTz-(Lysine-Boc)₃-NH₂ (4.1 mg, 4.7 μmol) and the general isothiocyanate-amine addition procedure as described above. Upon purification, the title compound was obtained as pink oil (3.7 mg, 60%); ¹H NMR (500 MHz, Chloroform-*d*) δ 8.47 (t, *J* = 6.0 Hz, 1 H), 8.40 (d, *J* = 8.0 Hz, 2 H), 7.76 (d, *J* = 6.1 Hz, 2 H), 7.63 (s, 3 H), 7.53 (d, *J* = 8.0 Hz, 2 H), 6.93 (d, *J* = 7.9 Hz, 1 H), 6.74 (dd, *J* = 19.2, 8.0 Hz, 2 H), 4.66 (s, 2 H), 4.41 (d, *J* = 5.9 Hz, 2 H), 3.92 (d, *J* = 7.6 Hz, 1 H), 3.82 (d, *J* = 7.7 Hz, 2 H), 3.00 (s, 7 H), 3.56 – 3.49 (m, 10 H), 2.83 – 2.70 (m, 6 H), 2.53 – 2.41 (m, 13 H), 1.67 – 1.44 (m, 13 H), 1.53 – 1.40 (m, 27 H); MS (ESI) *m/z* 1337.8 [M+H]⁺.

2,2',2''-(2-(4-(3-(5-amino-6-((5-amino-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl triacetic acid:

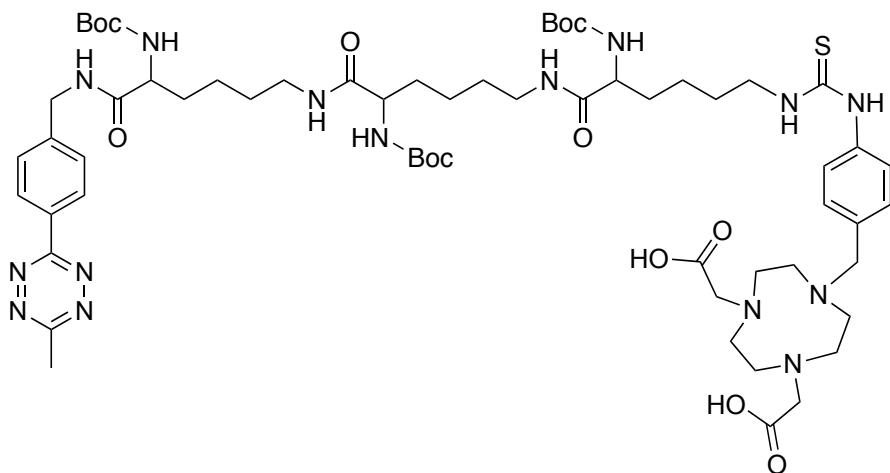


21, MW = 1036.27 g/mol, C₄₈H₇₃N₁₅O₉S

The title compound was obtained using MeTz-(Lysine-Boc)₃-NOTA (3.7 mg, 2.8 μmol) and the general isothiocyanate-amine addition procedure as described above. Upon purification, precursor **19** was obtained as pink oil (2.5 mg, 86%, purity >97%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.61 (s, *J* = 6.5 Hz, 1 H), 8.32 (d, *J* = 8.0 Hz, 2

H), 7.76 (d, $J = 6.4$ Hz, 2 H), 7.53 – 7.49 (m, 2 H), 7.47 (d, $J = 8.0$ Hz, 2 H), 6.81 (d, $J = 7.9$ Hz, 1 H), 6.74 (dd, $J = 19.2, 8.0$ Hz, 2 H), 4.66 (s, 2 H), 4.32 (m, 2 H), 3.92 (d, $J = 7.1$ Hz, 1 H), 3.82 (d, $J = 7.1$ Hz, 2 H), 3.51 – 3.40 (m, 7 H), 3.32 – 3.29 (m, 18 H) 2.73 – 2.60 (m, 5 H), 2.63 – 2.44 (m, 12 H), 1.61 – 1.42 (m, 11 H); MS (ESI) m/z 1037.3 [M+H]⁺; HRMS (ESI) calcd for C₄₈H₇₃N₁₅NaO₉S [= M + Na]⁺ m/z 1058.5334 found 1058.5330.

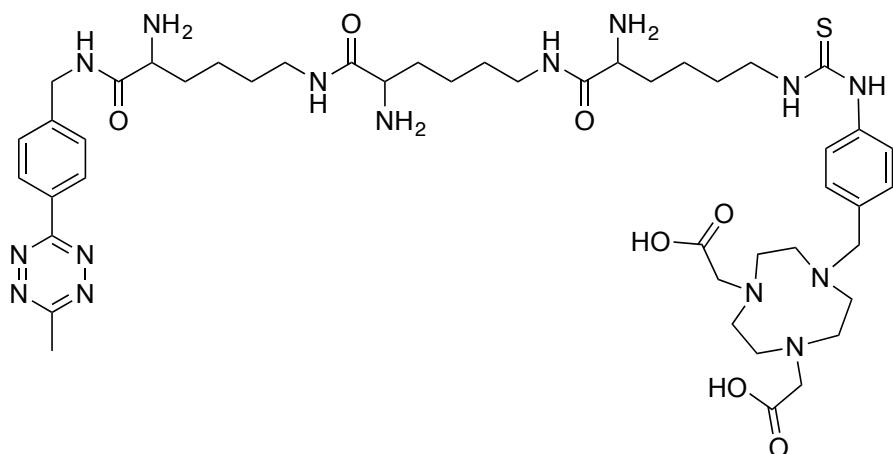
2'-(7-(4-(3-(13,20-bis((tert-butoxycarbonyl)amino)-2,2-dimethyl-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamoyl)-4,12,19-trioxo-3-oxa-5,11,18-triazatetracosan-24-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyli diacetic acid [Tz-2-(Lysine-Boc)₃-NODA]:



MW = 1278.58 g/mol, C₆₁H₉₅N₁₅O₁₃S

The title compound was obtained using MeTz-(Lysine-Boc)₃-NH₂ (3.5 mg, 2.9 μ mol) and the general isothiocyanate-amine addition procedure using p-Bn-NODA-NCS as described above. Upon purification, the title compound was obtained as pink oil (3.6 mg, 68%). ¹H NMR (500 MHz, Chloroform-*d*) δ 8.47 (t, $J = 6.0$ Hz, 1 H), 8.40 (d, $J = 8.0$ Hz, 2 H), 7.76 (d, $J = 6.1$ Hz, 2 H), 7.63 (s, 3 H), 7.53 (d, $J = 8.0$ Hz, 2 H), 6.93 (d, $J = 7.9$ Hz, 1 H), 6.74 (dd, $J = 19.2, 8.0$ Hz, 2 H), 4.66 (s, 2 H), 4.41 (d, $J = 5.9$ Hz, 2 H), 3.92 (d, $J = 5.9$ Hz, 1 H), 3.82 (d, $J = 7.7$ Hz, 2 H), 3.00 (s, 7 H), 3.56 – 3.49 (m, 9 H) 2.83 – 2.70 (m, 10 H), 2.53 – 2.41 (m, 6 H), 1.77 – 1.71 (m, 11 H), 1.67 – 1.32 (m, 29 H); MS (ESI) m/z 1279.8 [M+H]⁺.

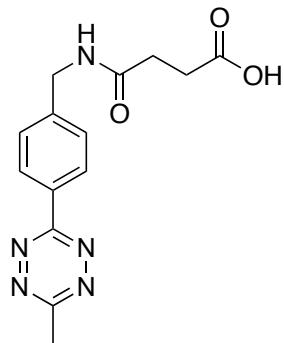
2,2'-(7-(4-(3-(5-amino-6-((5-amino-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyli diacetic acid:



22, MW = 987.23 g/mol, C₄₆H₇₁N₁₅O₇S

The title compound was obtained using MeTz-(Lysine-Boc)₃-NODA (3.5 mg, 2.7 μ mol) and the general isothiocyanate-amine addition procedure as described above. Upon purification, precursor **20** was obtained as pink oil (2.5 mg, 94%, purity >97%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.79 (t, *J* = 5.4 Hz, 2 H), 8.60 (d, *J* = 8.4 Hz, 2 H), 8.13 (d, *J* = 8.4 Hz, 2 H), 7.43 (d, *J* = 5.3 Hz, 2 H), 7.20 (d, *J* = 8.3 Hz, 1 H), 7.11 (d, *J* = 8.2 Hz, 1 H), 4.00 (d, *J* = 17.6 Hz, 2 H), 3.81 (d, *J* = 17.4 Hz, 3 H), 3.64 (s, 3 H), 3.57 – 3.45 (m, 39 H), 3.31 – 3.20 (m, 1 H), 3.05 – 2.94 (m, 5 H), 2.86 – 2.74 (m, 8 H); MS (ESI) *m/z* 988.4 [M+H]⁺.

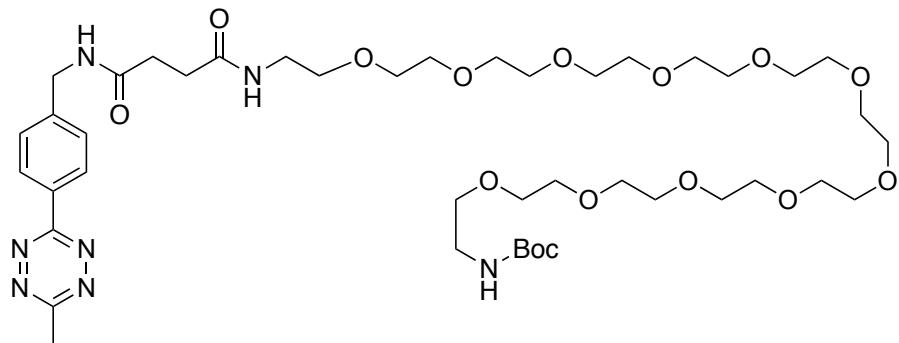
4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-4-oxobutanoic acid (Tz-2-succinate):



MW = 301.31 g/mol, C₁₄H₁₅N₅O₃

The title compound was obtained using Tz-2 amine (10 mg, 49 μ mol), succinic anhydride (5 mg, 50 μ mol), and TEA (5 μ L) in dry DMF. The mixture was stirred at room temperature for 4 h and monitored by LC-MS. The product was then purified as pink solid (11.6 mg, 79%) using preparative HPLC and subsequent drying; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.60 (d, *J* = 8.4 Hz, 2 H), 8.13 (d, *J* = 8.4 Hz, 2 H), 7.73 (s, 1 H, NH), 4.21 (s, 2 H), 3.81 – 3.77 (m, 2 H), 3.57 – 3.49 (m, 2 H), 2.74 (s, 3 H); MS (ESI) *m/z* 302.4 [M+H]⁺.

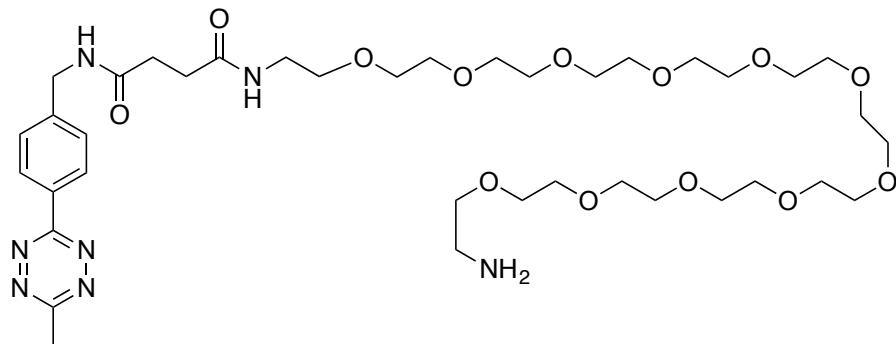
***tert*-butyl-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,6-dioxo-10,13,16,19,22,25,28,31,34,37,40-undeca-oxa-2,7-diazadotetracontan-42-yl)carbamate (Tz-2-PEG₁₁-NH₂Boc):**



MW = 928.09 g/mol, C₄₃H₇₃N₇O₁₅

The title compound was obtained from Tz-2-succinate (10 mg, 30.1 μ mol) and NH₂-PEG₁₁-NH₂Boc (1.2 eq.) using the amide coupling conditions requiring *in situ* acid activation. Upon purification and lyophilization, the title compound was obtained as pink solid (19 mg, 68%); ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.79 (t, *J* = 5.4 Hz, 2 H), 8.60 (d, *J* = 5.4 Hz, 2 H), 4.21 (s, 2 H), 4.08 – 4.02 (m, 5 H), 3.81 – 3.77 (m, 2 H), 3.57 – 3.49 (m, 2 H), 3.47 – 3.33 (m, 24 H) 2.74 (s, 3 H), 2.67 – 2.46 (m, 24 H), 1.45 (s, 7 H); MS (ESI) *m/z* 929.2 [M+H]⁺.

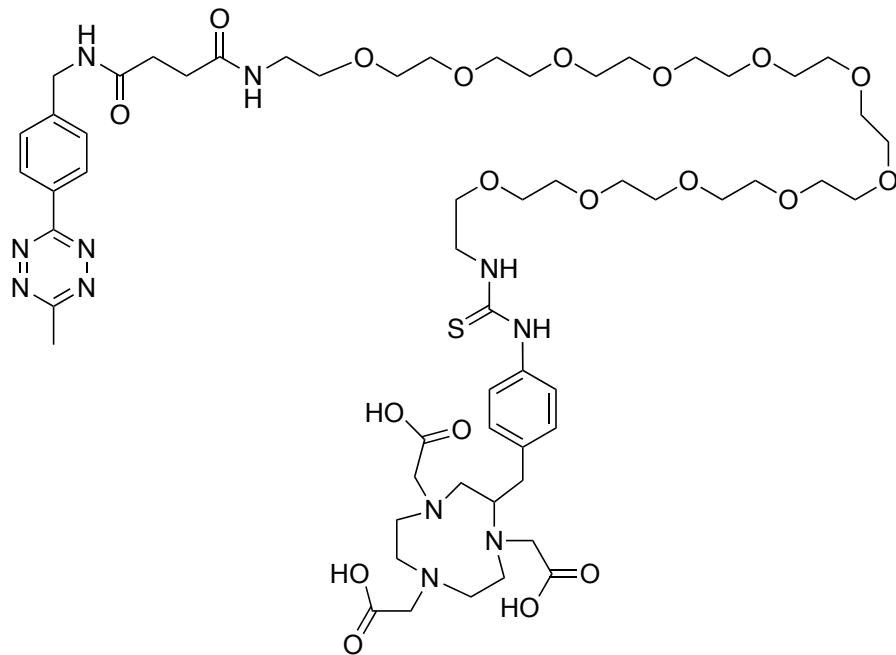
*N*¹-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)-*N*⁴-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)succinamide (Tz-2-PEG₁₁-NH₂):



MW = 827.97 g/mol, C₃₈H₆₅N₇O₁₃

The title compound was obtained using Tz-2-PEG₁₁-NHBoc (17 mg, 18.3 μ mol) as starting material and the standard TFA-mediated deprotection conditions, furnishing the title compounds as pink oil (11.5 mg, 76%); ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.58 (d, *J* = 5.9 Hz, 2 H), 8.67 (d, *J* = 5.9 Hz, 2 H), 4.21 (s, 2 H), 4.18 – 4.12 (m, 4 H), 3.77 – 3.69 (m, 9 H), 3.52 – 3.32 (m, 8 H), 3.27 – 3.13 (m, 21 H) 2.71 (s, 3 H), 2.44 – 2.26 (m, 14 H); MS (ESI) *m/z* 828.9 [M+H]⁺.

2,2',2''-(2-(4-(3-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,6-dioxo-10,13,16,19,22,25,28,31-34,37,40-undecaoxa-2,7-diazadotetracontan-42-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:

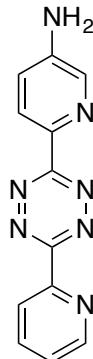


23, MW = 1278.48 g/mol, C₅₈H₉₁N₁₁O₁₉S

The title compound was obtained using Methyltetrazine-PEG₁₂-NHS ester (6.8 mg, 7.5 μ mol) and p-Bn-NOTA-NH₂ (5.6 mg, 10 μ mol) and the general NHS ester coupling procedure as described for the synthesis of Tz-PEG₇-NHBoc. Precursor **23** was furnished as pink oil (7.1 mg, 89%, purity >98%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.43 (d, *J* = 5.7 Hz, 2 H), 7.54 (d, *J* = 5.7 Hz, 2 H), 7.23 (d, *J* = 8.6 Hz, 2 H), 7.17 (d, *J* = 8.5 Hz, 2 H), 7.11 (s, 1 H), 6.56 (s, 2 H), 4.27 – 4.23 (m, 7 H), 3.83 – 3.79 (m, 8 H), 3.70 (t, *J* = 6.3 Hz, 7 H), 3.65 – 3.61 (m, 4 H), 3.59 – 3.49 (m, 49 H),

2.97 – 2.76 (m, 6 H); MS (ESI) m/z 1279.5 [M+H]⁺; HRMS (ESI) calcd for C₅₈H₉₁N₁₁NaO₁₉S [= M + Na]⁺ m/z 1301.5836 found 1301.5831.

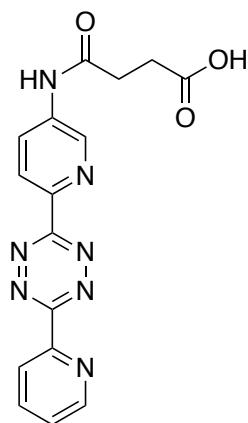
6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine:



3, MW = 251.09 g/mol, C₁₂H₉N₇

The title compound was synthesized according to the previously published procedure from Devaraj, *et al.*¹ Obtained yield and analytical data were consistent with the reported data. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.89 (s, 1 H), 8.53 (s, 1 H), 8.58-8.51 (m, 2 H), 7.99-7.92 (m, 2 H), 7.61 (d, *J* = 8.5 Hz, 1 H), 7.48 (m, 1 H), 7.10-6.99 (dd, *J* = 8.6 Hz, 2.7 Hz, 1 H), 5.78 (s, 2 H).

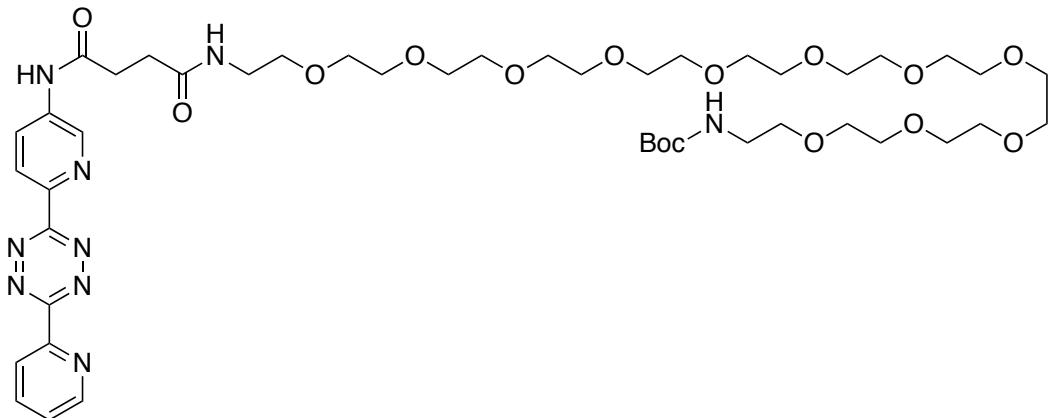
4-oxo-4-((6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)butanoic acid (Tz-3-succinate):



MW = 351.33 g/mol, C₁₆H₁₃N₇O₃

The title compound was obtained using the same procedure as stated for Tz-2-succinate (same molarities). Tz-2-succinate was furnished as pink solid (9.6 mg, 55%); ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.95 (s, 1 H), 8.79 (t, *J* = 5.4 Hz, 1 H), 8.60 (t, *J* = 8.4 Hz, 1 H), 8.33 (d, *J* = 8.4 Hz, 1 H), 8.29 (dd, *J* = 5.4 Hz, 1 H), 8.10 (dd, *J* = 8.6 Hz, 1 H), 7.52 – 7.44 (m, 2 H), 7.13 – 7.10 (m, 1 H), 2.72 – 2.63 (m, 4 H); MS (ESI) m/z 352.4 [M+H]⁺.

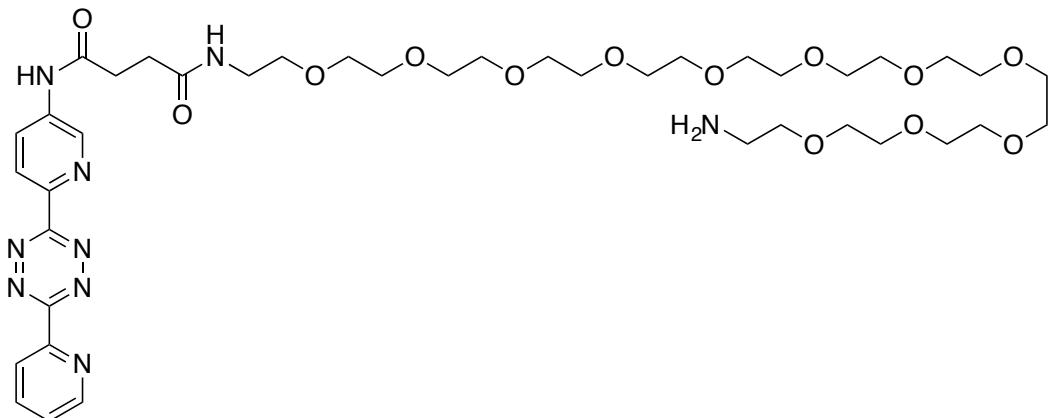
tert-butyl-(37,40-dioxo-40-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)-3,6,9,12,15,18,-21,24,27,30,33-undecaoxa-36-azatetracontyl)carbamate (Tz-3-PEG₁₁-NHBoc):



MW = 978.11 g/mol, C₄₅H₇₁N₉O₁₅

The title compound was obtained using the general amide bond formation procedure. The title compound was then obtained as pink oil (4.5 mg, 4.6 μ mol). ¹H NMR (500 MHz, Chloroform-*d*) δ 10.16 (s, 1 H), 9.09 – 9.03 (m, 2 H), 8.98 (d, *J* = 5.6 Hz, 1 H), 8.72 (dd, *J* = 11.1, 8.3 Hz, 2 H), 8.55 (dd, *J* = 8.4, 2.4 Hz, 1 H), 8.01 (dd, *J* = 8.3, 2.2 Hz, 1 H), 7.60 – 7.56 (m, 2 H), 3.68 – 3.56 (m, 37 H), 3.55 – 3.51 (m, 4 H), 3.48 (t, *J* = 7.7 Hz, 3 H), 3.30 (s, 4 H), 2.84 (t, *J* = 7.6 Hz, 3 H), 2.71 – 2.67 (m, 1 H), 1.44 (s, 9 H). MS (ESI) *m/z* 979.3 [M+H]⁺.

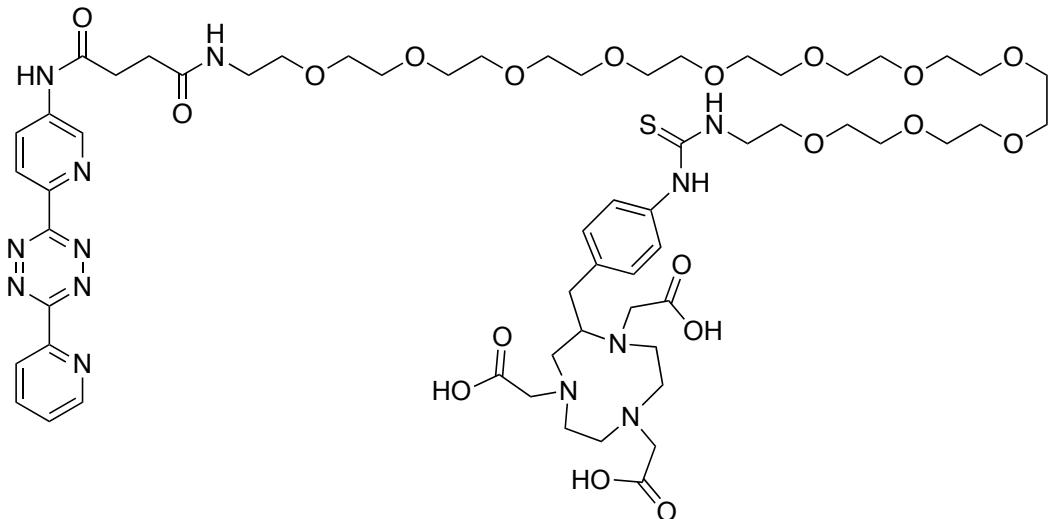
N¹-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)-N⁴-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)succinamide (Tz-3-PEG₁₁-NH₂):



MW = 877.99 g/mol, C₄₀H₆₃N₉O₁₃

The title compound was obtained from Tz-3-PEG₁₁-NHBoc (4.3 mg, 4.4 μ mol) using the standard TFA deprotection protocol. The title compound was obtained as pink oil (3.8 mg, 98%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.62 (dd, *J* = 15.9, 8.2 Hz, 1 H), 8.42 (dd, *J* = 8.2, 2.3 Hz, 1 H), 8.19 – 8.14 (m, 4 H), 7.99 (t, *J* = 7.4 Hz, 1 H), 7.74 (dd, *J* = 7.5, 2.7 Hz, 2 H), 3.62 – 3.37 (m, 49 H), 3.22 (q, *J* = 5.6 Hz, 2 H), 2.99 (t, *J* = 5.5 Hz, 2 H), 2.68 (t, *J* = 5.8 Hz, 1 H). MS (ESI) *m/z* 878.9 [M+H]⁺.

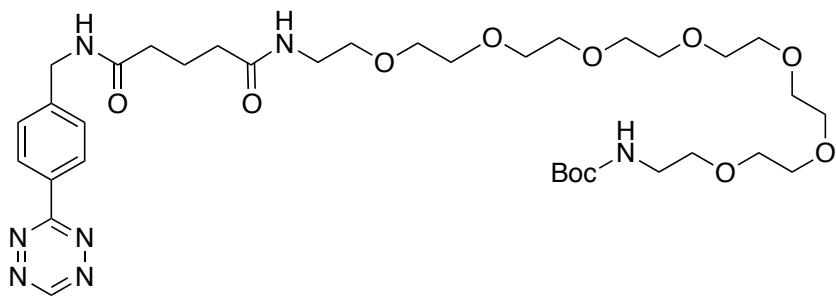
2,2',2''-(2-(4-(37,40-dioxo-40-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)-3,6,9,12,15,-18,21,24,27,30,33-undecaoxa-36-azatetracontyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



24, MW = 1328.50 g/mol, C₆₀H₈₉N₁₃O₁₉S

The title compound was obtained from Tz-3-PEG₁₁-NH₂ (3.6 mg, 4.1 μ mol) and p-Bn-NOTA-NCS using the standard isothiocyanate-amine addition procedure. Precursor **24** was obtained as pink oil (4.4 mg, 81%, purity >96%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.64 (s, 1 H), 9.07 (s, 1 H), 8.94 (d, *J* = 4.8 Hz, 2 H), 8.61 (dd, *J* = 15.5, 8.9 Hz, 1 H), 8.42 (dd, *J* = 9.0, 4.8 Hz, 1 H), 8.16 (t, *J* = 8.0 Hz, 1 H), 7.99 (t, *J* = 6.0 Hz, 1 H), 7.74 (dd, *J* = 7.9, 5.9 Hz, 2 H), 7.43 (d, *J* = 8.3 Hz, 1 H), 7.20 (d, *J* = 8.4 Hz, 1 H), 4.34 – 4.21 (m, 16 H), 4.00 (d, *J* = 18.2 Hz, 1 H), 3.81 (d, *J* = 18.3 Hz, 1 H), 3.58 – 3.47 (m, 45 H), 3.42 – 3.32 (m, 5 H), 3.23 – 3.28 (m, 6 H), 2.67 – 2.54 (m, 3 H); MS (ESI) *m/z* 1329.7 [M+H]⁺; HRMS (ESI) calcd for C₆₀H₈₉N₁₃NaO₁₉S [= M + Na]⁺ *m/z* 1351.6016 found 1351.6012.

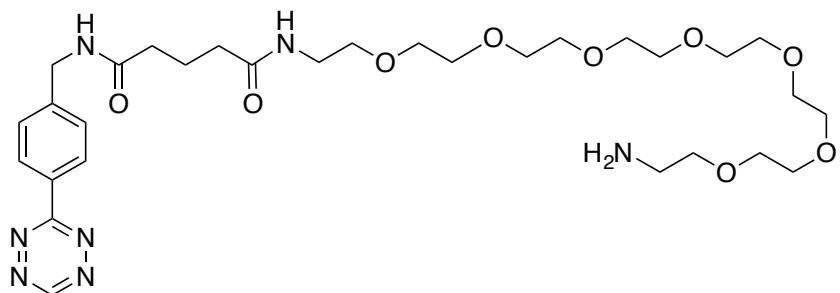
tert-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-heptaoxa-2-azapentacosan-25-yl)carbamate (Tz-4-PEG₇-NHBOC):



MW = 751.88 g/mol, C₃₅H₅₇N₇O₁₁

The title compound was obtained using the NHS-activated Tz 4 (6.1 mg, 30 μ mol), NH₂-PEG₇-NHBOC (12.4 mg, 26.4 μ mol), and TEA (4.5 μ L, 3 eq.) in anhydrous DMSO (400 μ L). The resulting pink mixture was stirred at room temperature for 1 h. The desired compound was furnished as pink oil (14.3 mg, 85%), and all spectral data were in line with previously published reports³. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.21 (s, 1 H), 8.43 – 8.31 (m, 2 H), 7.75 – 7.71 (m, 1 H), 7.54 (s, 2 H), 6.69 – 6.54 (m, 1 H), 4.33 – 4.28 (m, 3 H), 3.48 – 3.39 (m, 22 H), 3.33 – 3.28 (m, 2 H), 3.39 – 3.19 (m, 14 H), 1.29 (s, 9H).

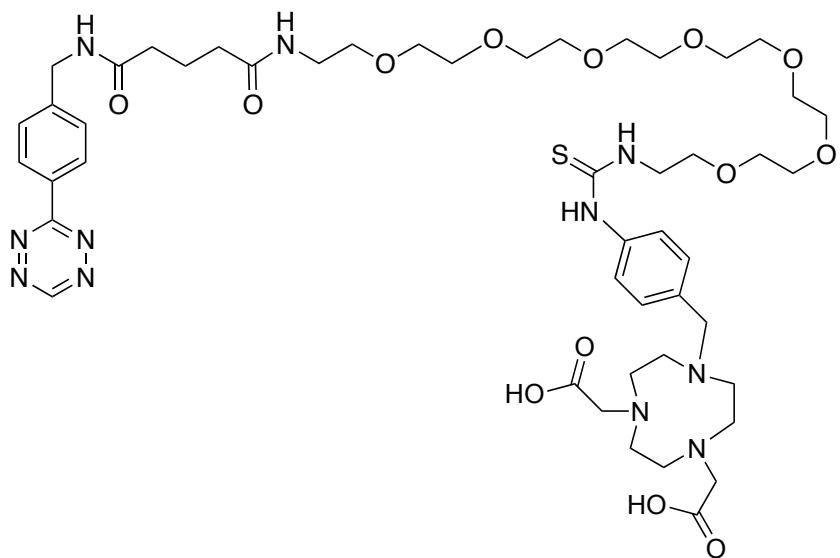
*N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(23-amino-3,6,9,12,15,18,21-heptaoxatricosyl)glutaramide:



MW = 651.76 g/mol, C₃₀H₄₉N₇O₉

The title compound (9.6 mg, 79%) was obtained using the standard TFA deprotection conditions and in accordance to previously published reports. All spectral data were in line with previously published results². ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.21 (s, 1 H), 8.76 – 8.72 (m, 1 H), 7.72 – 7.69 (m, 2 H), 7.51 (s, 2 H), 6.62 – 6.52 (m, 2 H), 4.30 – 4.24 (m, 4 H), 3.58 – 3.51 (m, 18 H), 3.48 – 3.42 (m, 3 H), 3.31 – 3.22 (m, 16 H).

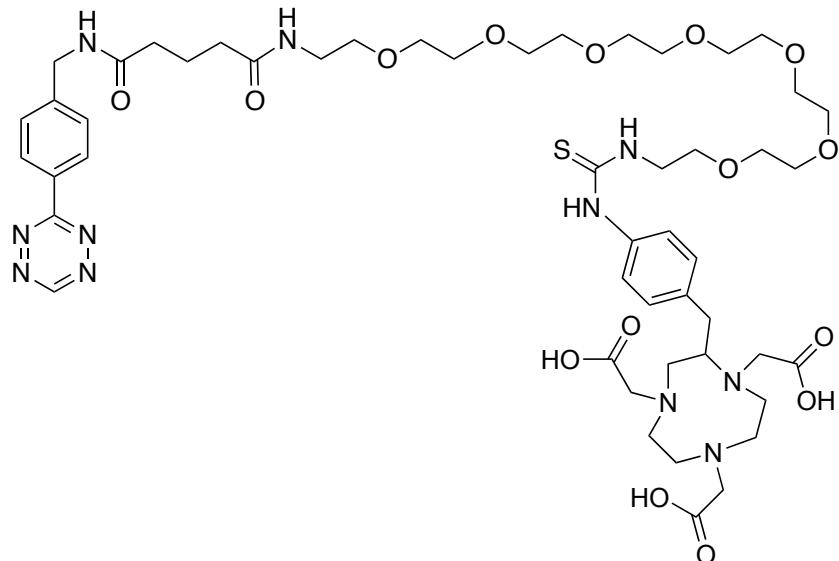
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-heptaoxa-2-azapentacosan-25-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyli)diacetic acid (Tz-4-PEG₇-NODA):



25, MW = 1044.24 g/mol, C₄₈H₇₃N₁₁O₁₃S

Precursor **25** (6.6 mg, 77%, purity >95%) was obtained as pink solid using Tz-4-PEG₇-NH₂ (5 mg, 9.1 μ mol) and p-Bn-NODA-NCS as well as the standard isothiocyanate-amine addition conditions. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.91 (s, 1 H), 8.50 (d, *J* = 5.9 Hz, 1 H), 8.42 (d, *J* = 5.9 Hz, 1 H), 7.74 – 7.69 (m, 2 H), 7.54 (d, *J* = 8.0 Hz, 2 H), 7.18 (d, *J* = 8.1 Hz, 2 H), 4.41 (d, *J* = 5.9 Hz, 2 H), 3.99 (d, *J* = 5.8 Hz, 2 H), 3.83 (d, *J* = 5.0 Hz, 1 H), 3.79 (s, 1 H), 3.68 (dd, *J* = 11.0, 4.9 Hz, 2 H), 3.55 – 3.44 (m, 31 H), 3.43 – 3.24 (m, 5 H), 3.18 (s, 15 H), 2.41 – 2.32 (m, 5 H). MS (ESI) *m/z* 1045.4 [M+H]⁺; HRMS (ESI) calcd for C₄₈H₇₃N₁₁NaO₁₃S [=M+Na]⁺ *m/z* 1067.4324 found 1067.4318.

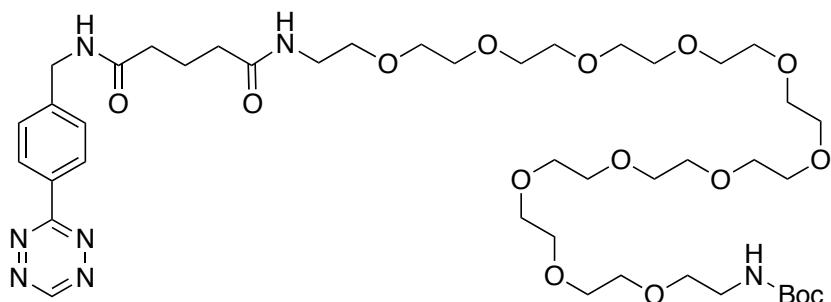
2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29-heptaoxa-2,8-diaza-hentriacontan-31-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl triacetic acid:



26, MW = 1102.27 g/mol, C₅₀H₇₅N₁₁O₁₅S

Precursor **26** (4.1 mg, 45%, purity >98%) was obtained as pink solid using Tz-4-PEG₇-NH₂ (5 mg, 9.1 μ mol) and p-Bn-NOTA-NCS as well as the standard isothiocyanate-amine addition conditions. All obtained spectral data was in line with previously published reports³. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.51 (s, 1 H), 9.50 (s, 1 H), 8.40 – 8.27 (m, 3 H), 7.79 – 7.68 (m, 1 H), 7.62 – 7.59 (m, 1 H), 7.47 (d, *J* = 5.9 Hz, 2 H), 7.35 (d, *J* = 5.8 Hz, 2 H), 7.03 – 6.92 (m, 2 H), 4.43 (d, *J* = 7.4 Hz, 2 H), 4.00 – 3.20 (m, 48 H), 3.12 – 3.02 (m, 6 H), 2.11 – 2.01 (m, 6 H).

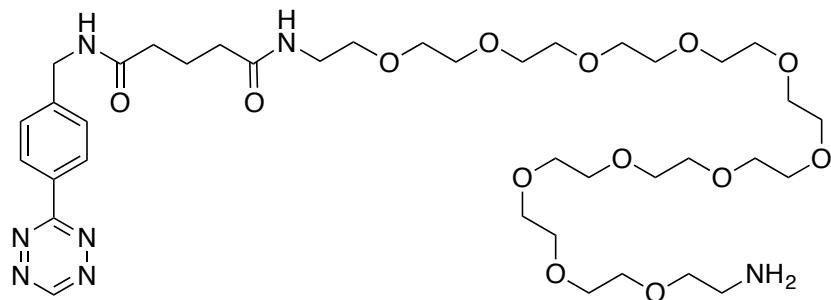
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8-diazatritetracontan-43-yl)carbamate (Tz-4-PEG₁₁-NH_{Boc}):**



MW = 928.09 g/mol, C₄₃H₇₃N₇O₁₅

The title compound was synthesized according to the PEG₇ derivative (identical stoichiometry) and obtained as pink oil (15.7 mg, 63%). All spectral data were in line with previously published reports⁴. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.65 (s, 1 H), 8.77 (t, *J* = 5.4 Hz, 1 H), 8.65-8.57 (m, 2 H), 8.16-8.12 (m, 2 H), 6.77-6.73 (m, 1 H), 3.62-3.42 (m, 46 H), 3.38 (t, *J* = 6.1 Hz, 3 H), 3.07 (q, *J* = 5.8 Hz, 2 H), 1.38 (s, 9 H).

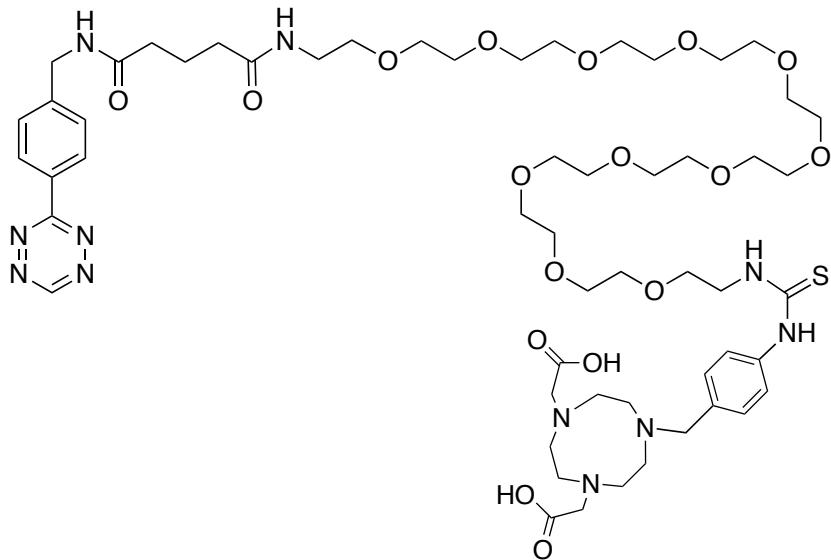
*N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxapenta-triacontyl)-glutaramide (Tz-4-PEG₁₁-NH₂):



MW = 827.97 g/mol, C₃₈H₆₅N₇O₁₃

The title compound was synthesized according to the PEG₇ derivative using the TFA deprotection conditions and was obtained as pink oil (10.9 mg, 89%). All spectral data were in line with previously published reports⁴. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.59 (s, 1 H), 8.74 (t, *J* = 5.2 Hz, 1 H), 8.61-8.55 (m, 2 H), 8.12-8.09 (m, 2 H), 6.79-6.74 (m, 1 H), 3.68-3.36 (m, 46 H), 3.34 (t, *J* = 6.1 Hz, 3 H), 3.10 (q, *J* = 5.8 Hz, 2 H).

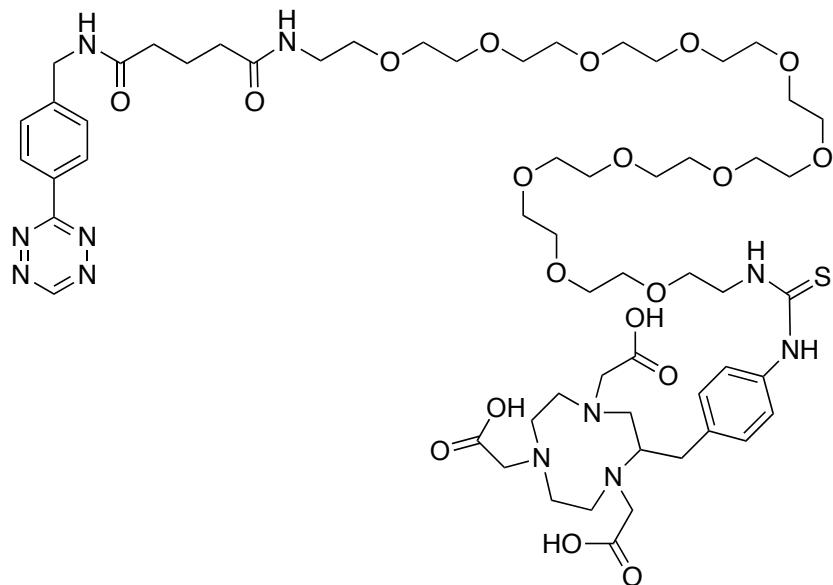
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8-diazatritetracontan-43-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyi)diacetic acid:



27, MW = 1206.34 g/mol, C₅₆H₈₉N₁₀O₁₇S

Precursor **27** was synthesized according to the PEG₇ derivative using the standard isothiocyanate-amine addition conditions and isolated as pink solid (12.2 mg, 73%, purity >98%); ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.66 (s, 1 H), 8.78 (t, *J* = 8.2 Hz, 1 H), 8.60 (d, *J* = 8.2 Hz, 1 H), 8.14 (d, *J* = 8.2 Hz, 2 H), 7.67 – 7.61 (m, 3 H), 7.43 (d, *J* = 8.1 Hz, 2 H), 7.20 (d, *J* = 8.4 Hz, 2 H), 4.00 (d, *J* = 18.2 Hz, 1 H), 3.82 (d, *J* = 18.1 Hz, 2 H), 3.61 – 3.43 (m, 63 H), 3.36 – 3.21 (m, 1 H), 3.13 – 2.99 (m, 6 H), 2.87 – 2.72 (m, 2 H), 2.64 (d, *J* = 12.4 Hz, 2 H); MS (ESI) *m/z* 1207.5 [M+H]⁺; HRMS (ESI) calcd for C₅₆H₈₉N₁₀O₁₇S [= M + Na]⁺ *m/z* 1229.5341 found 1229.5333.

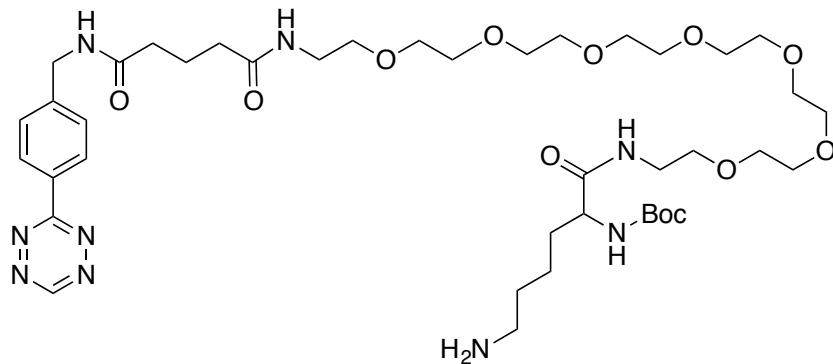
2,2',2''-(2-(4-(3-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8-diazatritetracontan-43-ylthioureido)benzyl)-1,4,7-triazonane-1,4,7-triyltriacetic acid:



28, MW = 1278.48 g/mol, C₅₈H₉₁N₁₁O₁₉S

Precursor **28** was synthesized as recently reported³. Analytical data matched previously established protocols. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.59 (s, 1H), 8.47 (d, *J* = 7.3 Hz, 3H), 7.87 (t, *J* = 5.2 Hz, 3H), 7.55 (d, *J* = 7.6 Hz, 3H), 7.43 (d, *J* = 8.1 Hz, 3H), 7.20 (d, *J* = 7.7 Hz, 2H), 4.41 (d, *J* = 5.8 Hz, 3H), 4.00 (d, *J* = 17.5 Hz, 2H), 3.82 (d, *J* = 17.9 Hz, 4H), 3.51 (s, 53H), 2.20 (t, *J* = 7.4 Hz, 3H), 2.12 (t, *J* = 7.5 Hz, 5H), 1.78 (dt, *J* = 14.4, 7.2 Hz, 4H).

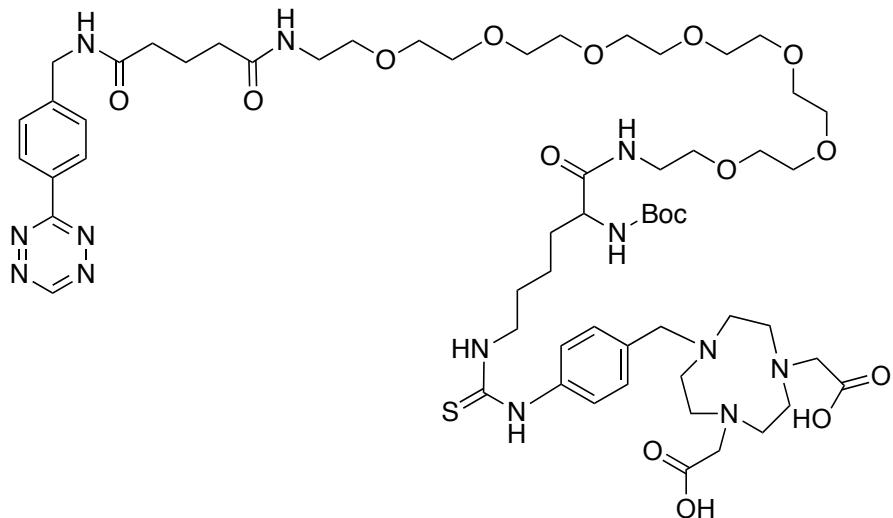
tert-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-38-amino-3,7,33-trioxo-11,14,17,20,23,26,29-heptaoxa-2,8,32-triazaoctatriacontan-34-yl)carbamate (Tz-4-PEG₇-Lysine-Boc):



MW = 880.05 g/mol, C₄₁H₆₉N₉O₁₂

The title compound was obtained using Tz-4-PEG₇-NH₂ (8.6 mg, 15.6 μmol) and the standard amide coupling conditions using Boc-lysine (5.0 mg, 20 μmol). The title compound was furnished as pink oil (9.2 mg, 76%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.91 (s, 1H), 8.50 (d, *J* = 5.9 Hz, 1H), 8.42 (d, *J* = 5.9 Hz, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.18 (d, *J* = 8.0 Hz, 1H), 4.41 (d, *J* = 5.9 Hz, 2H), 3.99 – 3.77 (m, 6H), 3.83 (d, *J* = 5.9 Hz, 2H), 3.79 (s, 1H), 3.68 (dd, *J* = 11.0, 4.7 Hz, 2H), 3.55 – 3.44 (m, 34H), 3.43 – 3.24 (m, 5H), 3.18 (s, 1H), 2.44 (t, *J* = 4.6 Hz, 2H), 1.67 (s, 9H); MS (ESI) *m/z* 881.1 [M+H]⁺.

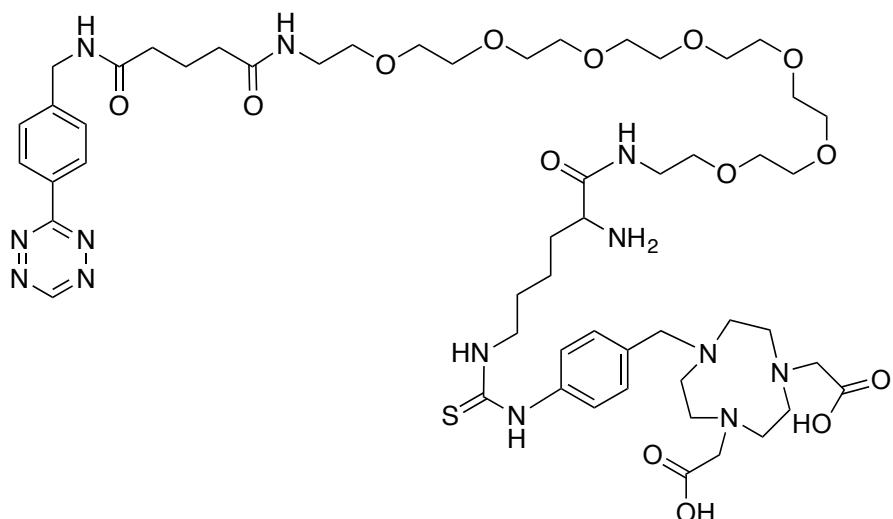
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-34-((tert-butoxycarbonyl)amino)-3,7,33-trioxo-11,14,17,20,23,26,29-heptaoxa-2,8,32-triazaoctatriacontan-38-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyli)diacetic acid (Tz-4-PEG₇-Lysine-Boc-NODA):



MW = 1272.53 g/mol, C₅₉H₉₃N₁₃O₁₆S

The title compound (1.9 mg, 72%) was obtained as pink solid using Tz-4-PEG₇-Lysine-Boc-NH₂ (1.5 mg, 2 μ mol), p-Bn-NODA-NCS (1.3 mg, 3 μ mol), as well as the general isothiocyanate-amine addition conditions. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.82 (s, 1 H), 8.43 (d, *J* = 5.6 Hz, 2 H), 8.41 (d, *J* = 5.6 Hz, 2 H), 7.24 (d, *J* = 7.9 Hz, 1 H), 7.16 (d, *J* = 8.0 Hz, 2 H), 6.9 (d, *J* = 8.4 Hz, 2 H), 4.55 – 4.51 (m, 1 H), 3.92 (d, *J* = 17.8 Hz, 2 H), 3.83 (d, *J* = 5.0 Hz, 2 H), 3.79 – 3.70 (m, 15 H), 3.68 (dd, *J* = 17.6, 4.9 Hz, 2 H), 3.55 – 3.44 (m, 47 H), 3.41 – 3.34 (m, 3 H), 2.44 – 2.38 (m, 2 H), 1.67 (s, 9 H); MS (ESI) *m/z* 1273.6 [M+H]⁺.

2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-34-amino-3,7,33-trioxo-11,14,17,20,23,26,29-heptaoxa-2,8,32-triazaoctatriacontan-38-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyli)diacetic acid:

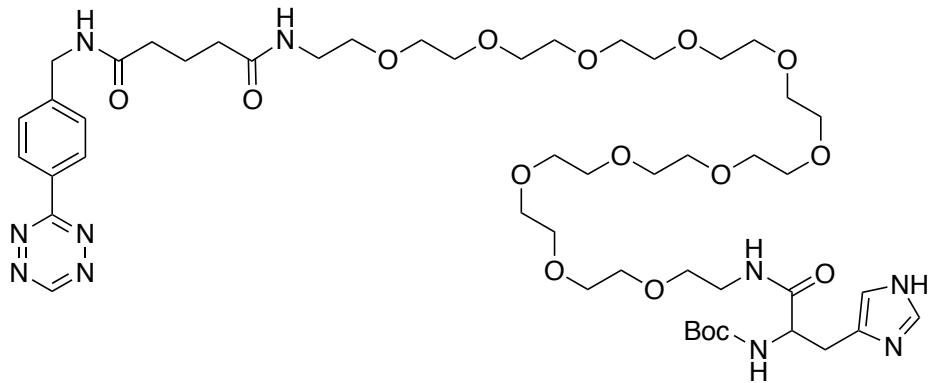


29, MW = 1172.41 g/mol, C₅₄H₈₅N₁₃O₁₄S

Precursor **29** (1.3 mg, 81%, purity >97%) was obtained as pink solid from the previous title compound (1.9 mg, 1.5 μ mol) using standard TFA deprotection conditions. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.91 (s, 1 H), 8.50 (d, *J* = 5.9

Hz, 1 H), 8.42 – 8.35 (m, 2 H), 7.54 (d, J = 5.9 Hz, 1 H), 7.18 (d, J = 8.1 Hz, 2 H), 6.89 (d, J = 8.1 Hz, 2 H), 3.99 (d, J = 17.8 Hz, 2 H), 3.83 (d, J = 4.4 Hz, 2 H), 3.79 – 3.70 (m, 15 H), 3.68 (dd, J = 17.5, 4.2 Hz, 2 H), 3.55 – 3.44 (m, 43 H), 3.43 – 3.24 (m, 4 H), 2.44 – 2.33 (m, 8 H); MS (ESI) m/z 1273.6 [M+H]⁺; MS (ESI) m/z 1173.5 [M+H]⁺; HRMS (ESI) calcd for C₅₄H₈₅N₁₃NaO₁₄S [= M + Na]⁺ m/z 1195.5273 found 1195.5263.

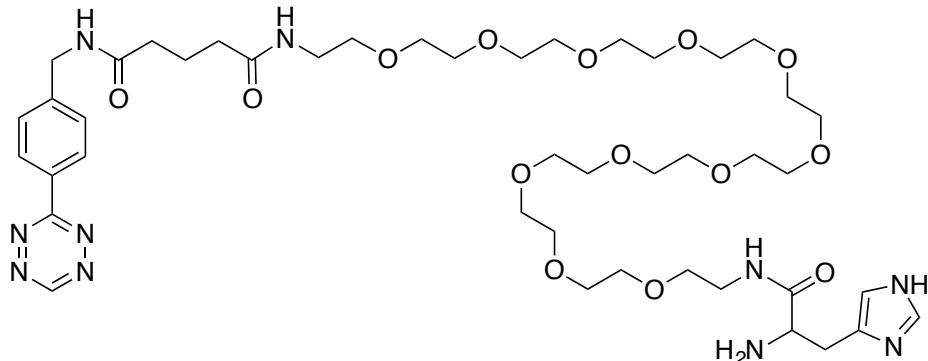
tert-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-47-(1H-imidazol-4-yl)-3,7,45-trioxo-11,14,17,20,23,26-29,32,35,38,41-undecaoxa-2,8,44-triazaheptatetracontan-46-yl)carbamate (Tz-4-PEG₁₁-His-Boc):



MW = 1065.23 g/mol, C₄₉H₈₀N₁₀O₁₆

The title compound (12.7 mg, 72%) was obtained as pink solid using Tz-4-PEG₁₁-NH₂ (10 mg, 18 μ mol), Boc-Histidine (5.9 mg, 25 μ mol), and the general reaction conditions for amide bond formations. ¹H NMR (500 MHz, Chloroform-*d*) δ 12.53 (s, 1 H), 9.66 (s, 1 H), 8.50 (d, J = 5.9 Hz, 1 H), 8.42 (d, J = 5.8 Hz, 1 H), 8.35 (s, 1 H), 7.54 (d, J = 7.9 Hz, 1 H), 7.41 (s, 1 H), 7.18 (d, J = 8.0 Hz, 1 H), 7.09 – 7.01 (m, 2 H), 4.23 (dd, J = 17.8, 6.5 Hz, 1 H), 4.08 (dd, J = 17.5, 6.2 Hz, 1 H), 3.99 (d, J = 17.8 Hz, 2 H), 3.92 (dd, J = 16.3, 6.1 Hz, 1 H), 3.83 (d, J = 5.0 Hz, 2 H), 3.79 – 3.70 (m, 15 H), 3.68 (dd, J = 16.1, 4.9 Hz, 2 H), 3.51 – 3.35 (m, 27 H), 3.33 – 3.21 (m, 4 H), 2.42 – 2.23 (m, 6 H), 1.82 (s, 9 H); MS (ESI) m/z 1066.4 [M+H]⁺.

***N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(38-amino-39-(1H-imidazol-4-yl)-37-oxo-3,6,9,12,15,18,21,24-27,30,33-undecaoxa-36-azanonatriacontyl)glutaramide (Tz-4-PEG₁₁-His):**

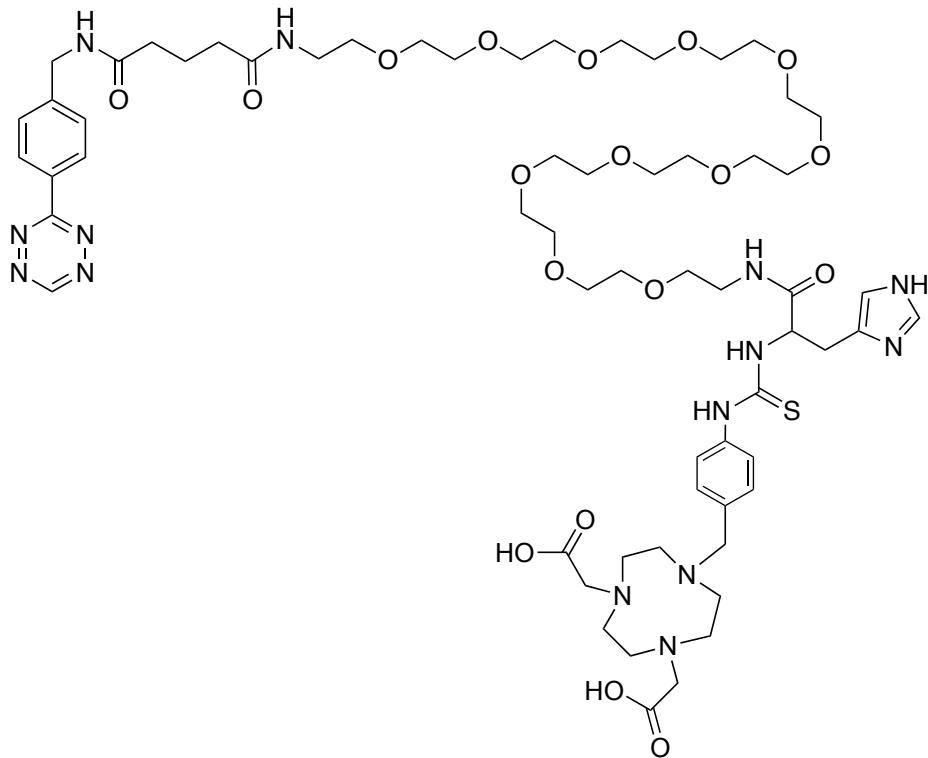


MW = 965.12 g/mol, C₄₄H₇₂N₁₀O₁₄

The title compound (8.8 mg, 87%) was obtained as pink solid using Tz-4-PEG₁₁-Histidine-Boc-NH₂ (12.7 mg, 13 μ mol) and the general TFA deprotection procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.71 (s, 1 H), 9.34 (s, 1 H), 8.53 (d, J = 8.0 Hz, 1 H), 8.33 (d, J = 8.0 Hz, 1 H), 8.18 (s, 1 H), 7.55 (d, J = 7.9 Hz, 1 H), 7.45 (s, 1 H), 7.21 (d, J = 7.9 Hz, 1 H), 7.11 – 7.02 (m, 3 H), 4.56 (dd, J = 17.8, 6.5 Hz, 1 H), 4.22 – 4.13 (m, 2 H), 4.01 (dd, J = 17.6, 6.3 Hz, 1 H), 3.89 (d, J = 17.8 Hz, 2 H), 3.84 (dd, J = 17.6, 6.3 Hz, 1 H), 3.78 (d, J = 6.2 Hz, 2 H), 3.71 – 3.60 (m, 13 H),

3.52 (dd, $J = 17.4, 6.2$ Hz, 2 H), 3.41 – 3.35 (m, 26 H), 3.29 – 3.21 (m, 5 H), 2.42 – 2.23 (m, 6 H); MS (ESI) m/z 966.2 [M+H]⁺.

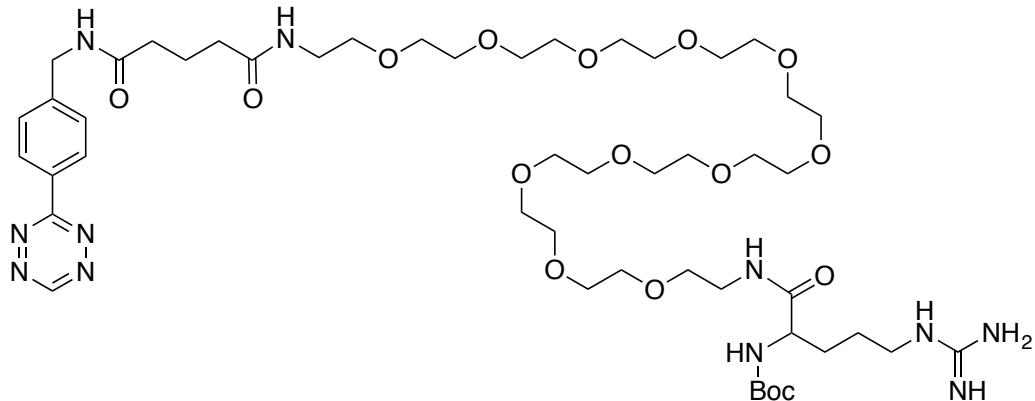
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-41-(1H-imidazol-4-yl)-1,39-dioxo-5,8,11,14,17-20,23,26-,29,32,35-undecaoxa-2,38-diazahentetracontan-40-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (Tz-4-PEG₁₁-His-NODA):



30, MW = 1357.59 g/mol, C₆₂H₉₆N₁₄O₁₈S

Precursor **30** (6.8 mg, 91%, purity >98%) was furnished as a pink oil using Tz-4-PEG₁₁-Histidine-NH₂ (5 mg, 5.8 μ mol), p-Bn-NODA-NCS, and the established isothiocyanate-amine addition procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.61 (s, 1 H), 9.71 (s, 1 H), 8.74 (d, $J = 5.3$ Hz, 1 H), 8.51 (d, $J = 5.2$ Hz, 1 H), 8.01 (s, 1 H), 7.49 (d, $J = 7.5$ Hz, 1 H), 7.41 (s, 1 H), 7.33 (d, $J = 7.6$ Hz, 2 H), 7.20 (d, $J = 7.9$ Hz, 1 H), 7.14 (m, 4 H), 6.89 (d, $J = 7.7$ Hz, 2 H), 4.78 (dd, $J = 15.3, 6.2$ Hz, 1 H), 4.22 – 4.04 (m, 8 H), 4.01 (dd, $J = 15.1, 6.2$ Hz, 1 H), 3.89 (d, $J = 17.8$ Hz, 2 H), 3.84 (dd, $J = 17.5, 5.1$ Hz, 1 H), 3.78 (d, $J = 5.0$ Hz, 2 H), 3.71 – 3.60 (m, 13 H), 3.58 (dd, $J = 10.0, 4.2$ Hz, 2 H), 3.52 – 3.44 (m, 11 H), 3.41 – 3.35 (m, 25 H), 3.29 – 3.21 (m, 4 H), 2.12 – 2.02 (m, 10 H); MS (ESI) m/z 1358.7 [M+H]⁺; HRMS (ESI) calcd for C₆₂H₉₆N₁₄NaO₁₈S [= M + Na]⁺ m/z 1380.7961 found 1380.7955.

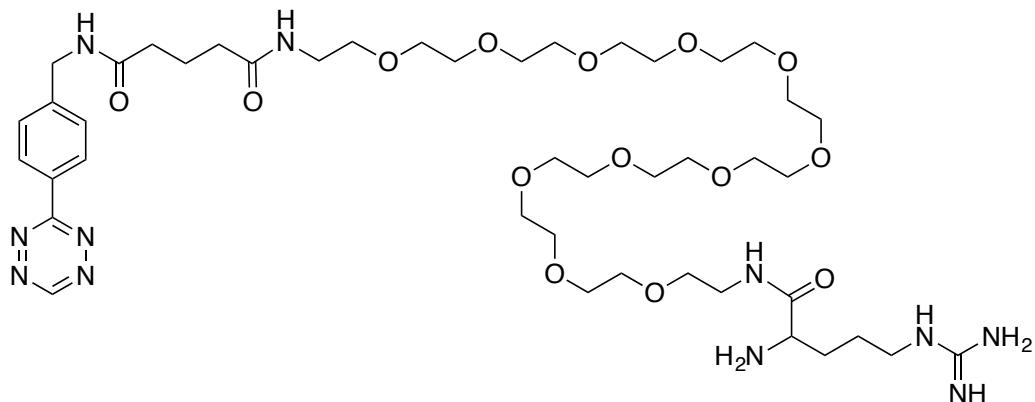
tert-butyl-(51-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-amino-1-imino-7,45,49-trioxo-11,14,17,20,23,26-29,32,35,38,41-undecaoxa-2,8,44,50-tetraazahenpentacantan-6-yl)carbamate (Tz-4-PEG₁₁-Arg-Boc):



MW = 1084.28 g/mol, C₄₉H₈₅N₁₁O₁₆

The title compound (6.8 mg, 80%) was obtained as pink solid using Tz-4-PEG₁₁-NH₂ (5 mg, 9 μ mol), Boc-Arginine (5 mg, 13 μ mol), and the general reaction conditions for amide bond formations. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.88 (s, 1 H), 8.45 (d, *J* = 6.1 Hz, 1 H), 8.34 (d, *J* = 6.2 Hz, 1 H), 8.24 (s, 1 H), 7.54 (d, *J* = 7.9 Hz, 1 H), 7.19 (d, *J* = 7.9 Hz, 1 H), 7.12 (s, 1 H), 4.19 (d, *J* = 5.9 Hz, 2 H), 3.91 (d, *J* = 17.8 Hz, 2 H), 3.83 (d, *J* = 5.8 Hz, 2 H), 3.79 (s, 1 H), 3.68 (dd, *J* = 11.0, 4.7 Hz, 2 H), 3.55 – 3.44 (m, 38 H), 3.41 – 3.23 (m, 19 H), 3.17 (s, 1 H), 2.33 – 2.28 (m, 2 H), 1.52 (s, 9 H); MS (ESI) *m/z* 1085.4 [M+H]⁺.

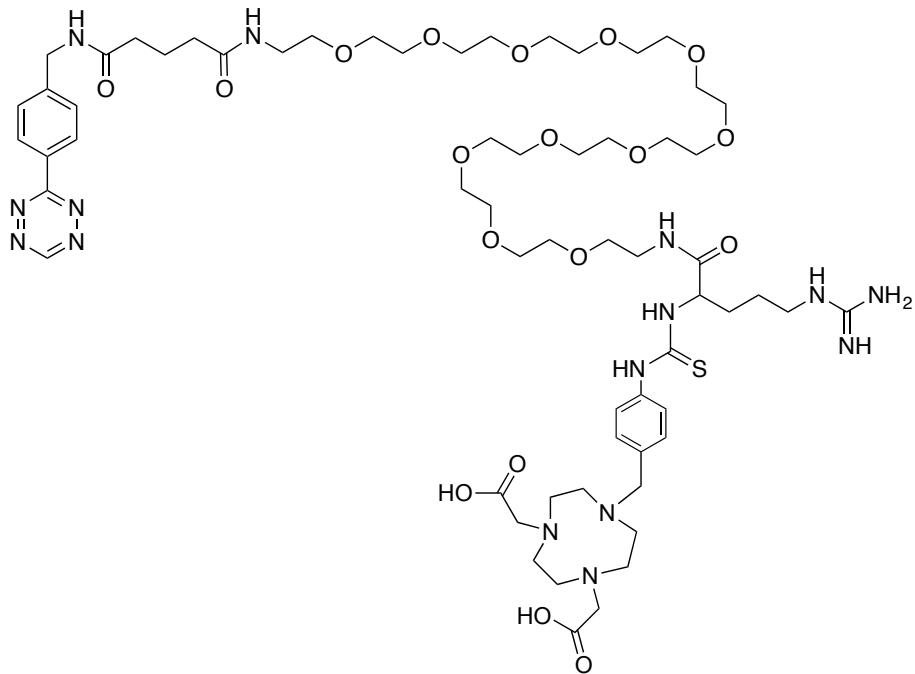
*N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(1,6-diamino-1-imino-7-oxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8-diazatritetracontan-43-yl)glutaramide (Tz-4-PEG₁₁-Arg):



MW = 984.16 g/mol, C₄₄H₇₇N₁₁O₁₄

The title compound (4.7 mg, 84%) was obtained as pink solid using Tz-4-PEG₁₁-Arginine-Boc-NH₂ (6.6 mg, 6.3 μ mol) and the general TFA deprotection procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.21 (s, 1 H), 8.69 (d, *J* = 6.7 Hz, 1 H), 8.52 (d, *J* = 6.8 Hz, 1 H), 8.42 (s, 1 H), 7.83 (d, *J* = 7.2 Hz, 1 H), 7.52 (d, *J* = 7.3 Hz, 1 H), 7.11 (s, 1 H), 7.03 (s, 1 H), 4.19 (d, *J* = 5.9 Hz, 2 H), 4.08 – 3.98 (m, 4 H), 3.91 (d, *J* = 13.2 Hz, 2 H), 3.83 (d, *J* = 5.8 Hz, 2 H), 3.79 (s, 1 H), 3.68 (dd, *J* = 13.2, 3.9 Hz, 2 H), 3.55 – 3.44 (m, 35 H), 3.41 – 3.23 (m, 11 H), 3.17 (s, 1 H), 2.33 – 2.24 (m, 8 H); MS (ESI) *m/z* 985.2 [M+H]⁺.

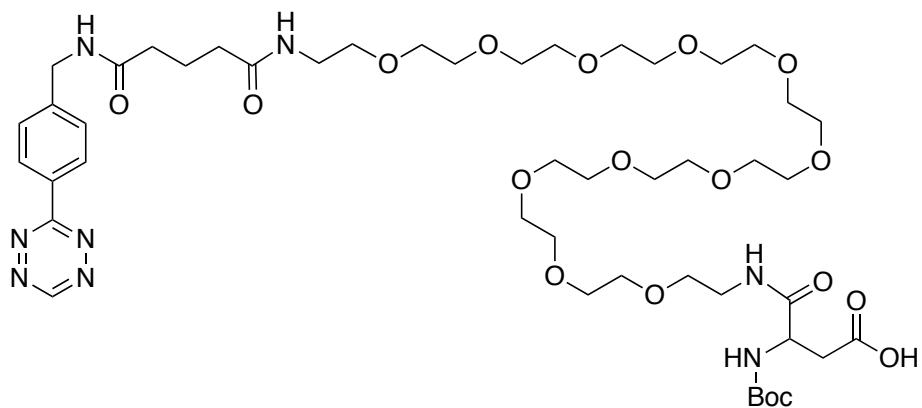
2,2'-(7-(4-(3-(51-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-amino-1-imino-7,45,49-trioxo-11,14,17,20,23-26,29,32,35,38,41-undecaoxa-2,8,44,50-tetraazahenpentacontan-6-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyldiacetic acid:



31, MW = 1376.64 g/mol, C₆₂H₁₀₁N₁₅O₁₈S

Precursor **28** (5.1 mg, 75%, purity >95%) was furnished as a pink oil using Tz-4-PEG₁₁-Arginine-NH₂ (4.7 mg, 5.3 μ mol), p-Bn-NODA-NCS, and the established isothiocyanate-amine addition procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.61 (s, 1 H), 9.71 (s, 1 H), 8.74 (d, *J* = 8.2 Hz, 1 H), 8.66 (s, 1 H), 8.51 (d, *J* = 8.2 Hz, 1 H), 8.01 (s, 1 H), 7.49 (d, *J* = 7.5 Hz, 1 H), 7.41 (s, 1 H), 7.33 (d, *J* = 7.5 Hz, 2 H), 7.20 (d, *J* = 7.9 Hz, 1 H), 7.14 – 7.04 (m, 4 H), 6.89 (d, *J* = 6.7 Hz, 2 H), 4.74 (dd, *J* = 6.6, 13.3 Hz, 1 H), 4.22 – 4.14 (m, 9 H), 4.09 (dd, *J* = 12.8, 6.3 Hz, 1 H), 3.92 (d, *J* = 12.9 Hz, 2 H), 3.84 (dd, *J* = 6.2, 16.1 Hz, 1 H), 3.76 (d, *J* = 6.2 Hz, 2 H), 3.73 – 3.64 (m, 11 H), 3.62 (dd, *J* = 9.6, 6.1 Hz, 2 H), 3.58 – 3.54 (m, 5 H) 3.51 – 3.45 (m, 17 H), 3.39 – 3.31 (m, 9 H), 2.31 – 2.02 (m, 24 H); MS (ESI) *m/z* 1377.7 [M+H]⁺; HRMS (ESI) calcd for C₆₂H₁₀₁N₁₅NaO₁₈S [= M + Na]⁺ *m/z* 1399.6383 found 1399.6371.

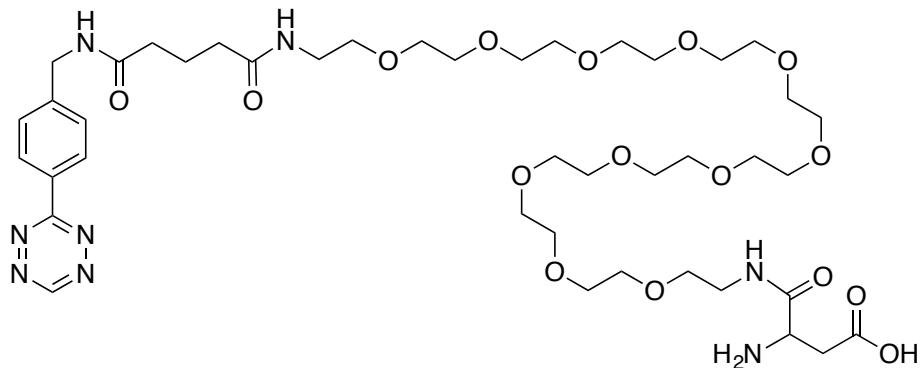
1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-46-((*tert*-butoxycarbonyl)amino)-3,7,45-trioxo-11,14,17,20,23-26,29,32,35,38,41-undecaoxa-2,8,44-triazaoctatetracontan-48-oic acid (Tz-4-PEG₁₁-Asp-Boc):



MW = 1043.18 g/mol, C₄₇H₇₈N₈O₁₈

The title compound (6.2 mg, 73%) was obtained as pink solid using Tz-4-PEG₁₁-NH₂ (5 mg, 9 μ mol), Boc-Aspartate (2.5 mg, 10 μ mol), and the general reaction conditions for amide bond formations. ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.21 (s, 1 H), 8.61 (d, *J* = 5.7 Hz, 1 H), 8.47 (d, *J* = 8.3 Hz, 1 H), 7.81 (d, *J* = 5.6 Hz, 1 H), 7.42 (d, *J* = 8.4 Hz, 1 H), 4.41 (d, *J* = 5.9 Hz, 2 H), 3.99 (d, *J* = 5.8 Hz, 2 H), 3.83 – 3.74 (m, 7 H), 3.79 (s, 1 H), 3.68 (dd, *J* = 11.0, 4.7 Hz, 2 H), 3.55 – 3.44 (m, 39 H), 3.43 – 3.24 (m, 5 H), 3.18 (s, 1 H), 2.44 (t, *J* = 6.3 Hz, 5 H), 1.56 (s, 9 H); MS (ESI) *m/z* 1044.3 [M+H]⁺.

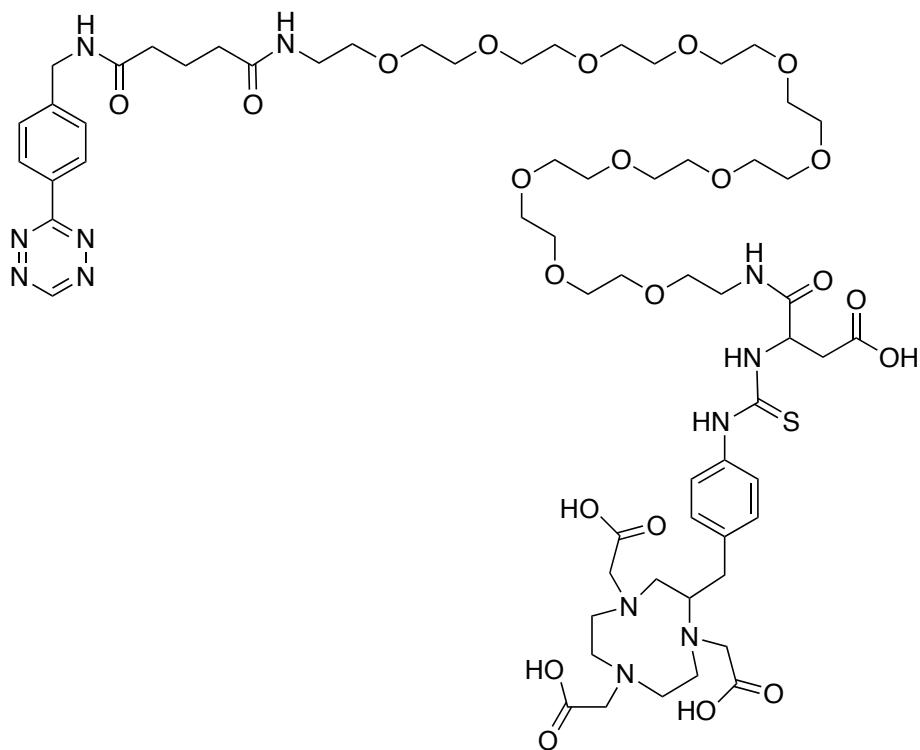
1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-46-amino-3,7,45-trioxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8,44-triazaoctatetracontan-48-oic acid (Tz-4-PEG₁₁-Asp):



MW = 943.06 g/mol, C₄₂H₇₀N₈O₁₆

The title compound (5.1 mg, 92%) was obtained as pink solid using Tz-4-PEG₁₁-Aspartate-Boc-NH₂ (6.2 mg, 6.6 μ mol) and the general TFA deprotection procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.04 (s, 1 H), 8.73 (d, *J* = 5.8 Hz, 1 H), 8.44 (d, *J* = 5.7 Hz, 1 H), 7.88 (d, *J* = 7.2 Hz, 1 H), 7.51 (d, *J* = 7.1 Hz, 1 H), 4.41 (d, *J* = 7.2 Hz, 2 H), 4.28 (d, *J* = 14.1 Hz, 2 H), 3.98 – 3.91 (m, 5 H), 3.87 (s, 1 H), 3.78 (dd, *J* = 11.0, 4.7 Hz, 2 H), 3.71 – 3.54 (m, 39 H), 3.43 – 3.24 (m, 8 H), 2.32 – 2.21 (m, 5 H); MS (ESI) *m/z* 944.1 [M+H]⁺.

2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-47-carboxy-3,7,45-trioxo-11,14,17,20,23,26-29,32,35,38,41-undecaoxa-2,8,44-triazaheptatetracontan-46-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



32, MW =1393.57 g/mol, C₆₂H₉₆N₁₂O₂₂S

Precursor **32** (5.8 mg, 74%, purity >97%) was furnished as a pink oil using Tz-4-PEG₁₁-Aspartate-NH₂ (5.1 mg, 6.0 μ mol), p-Bn-NOTA-NCS, and the established isothiocyanate-amine addition procedure. ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.22 (s, 1 H), 9.38 (s, 1 H), 9.12 (s, 1 H), 8.81 (d, *J* = 5.9 Hz, 1 H), 8.42 (d, *J* = 5.8 Hz, 1 H), 7.92 (d, *J* = 7.5 Hz, 1 H), 7.72 (d, *J* = 7.4 Hz, 1 H), 7.56 (s, 1 H), 6.88 (s, 1 H), 4.62 – 4.52 (m, 13 H), 4.38 (d, *J* = 15.5 Hz, 2 H), 4.29 (d, *J* = 15.3 Hz, 2 H), 4.18 – 3.97 (m, 10 H), 3.82 (s, 1 H), 3.78 (dd, *J* = 11.0, 4.7 Hz, 2 H), 3.63 – 3.49 (m, 43 H), 3.41 – 3.22 (m, 8 H), 2.12 (t, *J* = 6.1 Hz, 5 H); MS (ESI) *m/z* 1394.6 [M+H]⁺; HRMS (ESI) calcd for C₆₂H₉₆N₁₂NaO₂₂S [= M + Na]⁺ *m/z* 1416.5696 found 1416.5687.

3 | Radiochemistry

General ¹⁸F-labeling procedure: The [¹⁸F]fluoride (non-carrier added) received from the cyclotron was trapped on a pre-conditioned anion-exchange (QMA, Waters) cartridge. The cartridge was subsequently washed with metal-free water (10-15 mL, pH 6) before the [¹⁸F]fluoride (30-110 mCi, 1.1-4.1 GBq) was eluted using 0.4 M KHCO₃-solution (0.2 mL) into an Eppendorf tube. The pH of the solution was adjusted to ~pH 3.5-4 using metal-free glacial acetic acid (15-20 μ L), followed by the addition of 2 mM metal-free AlCl₃-solution (25 μ L, 50 nmol). The resulting solution was incubated at 40 °C for 20 min with agitation (700 rpm) to form the Al-¹⁸F complex. In the meantime, the thawed precursor solution (50 nmol in 50 μ L of DMSO) was diluted with metal-free MeCN (700 μ L). The aqueous Al-¹⁸F-solution was combined with the organic precursor solution and the resulting mixture was stirred on a hot plate for 12 min at 90 °C. After the given period of time the reaction vial was cooled using dry ice before the reaction mixture was diluted with 18mΩ H₂O (20 mL). The obtained aqueous solution containing the labeled product was then flushed through a pre-conditioned C18 cartridge. An additional 10 mL of 18mΩ H₂O water was used to remove unreacted ¹⁸F from the cartridge. The product was subsequently eluted with ethanol (0.2-0.35 mL) and analyzed for purity using radio-HPLC (5% MeCN/H₂O to 95% MeCN over 20 min, R_t = 10.7-12.2 min, 1 mL/min). Final tracers using the Al-¹⁸F-methodology were isolated in 34-75% RCY (d.c.) with SAs ranging from 0.52-1.1 mCi/nmol (19.3-40.7 MBq/nmol). For further animal studies, 0.9% sterile saline was added to reduce ethanol content <10%.

General ^{68}Ga -labeling procedure: $^{68}\text{Ga}^{3+}$ (9.5-15.8 mCi, 0.35-0.59 GBq) was received from the $^{68}\text{Ge}/^{68}\text{Ga}$ -Generator as an aqueous solution (500 μL , 0.5 M NaOH, pH = 10-11). The solution was acidified (pH = 4.5) using metal-free glacial acetic acid (18.5 μL). Subsequently, the tetrazine precursor (5-15 nmol, 4.8-19.5 μg) dissolved in metal-free DMSO (5-25 μL) was added to the aqueous $^{68}\text{Ga}^{3+}$ -solution. The mixture was incubated at 40 °C for 10 min with agitation (700 rpm) using a thermomixer. The reaction mixture was diluted to a total volume of 1-1.5 mL using 18mΩ H_2O , and slowly flushed through a pre-equilibrated C18 light cartridge (Waters). The cartridge, containing the labeled product, was washed with water (10-15 mL) to elute residual $^{68}\text{Ga}^{3+}$ off the cartridge before the final tracer was eluted using ethanol (molecular biology grade, 100-300 μL). The identity and purity was determined using radio-HPLC (5% MeCN/ H_2O to 95% MeCN over 20 min, R_t = 11.1-12.3 min, 1 mL/min). ^{68}Ga -labeled tracers were obtained in 66-95% decay-corrected (d.c.) isolated radiochemical yield (RCY) with specific activities (SAs) ranging from 0.73-1.3 mCi/nmol (27.1-48.2 MBq/nmol). Finally, 0.9% sterile saline was added to reduce ethanol content of the final tracer solution to <10% for injections.

All tracers were evaluated for chemical and radiochemical purity using analytical radio-HPLC (5% MeCN/ H_2O to 95% MeCN over 20 min, R_t = 10.7-12.2 min, 1 mL/min). Further, the pH of the final tracer solution was confirmed to be in the range of 6.8-7.2 prior to injection.

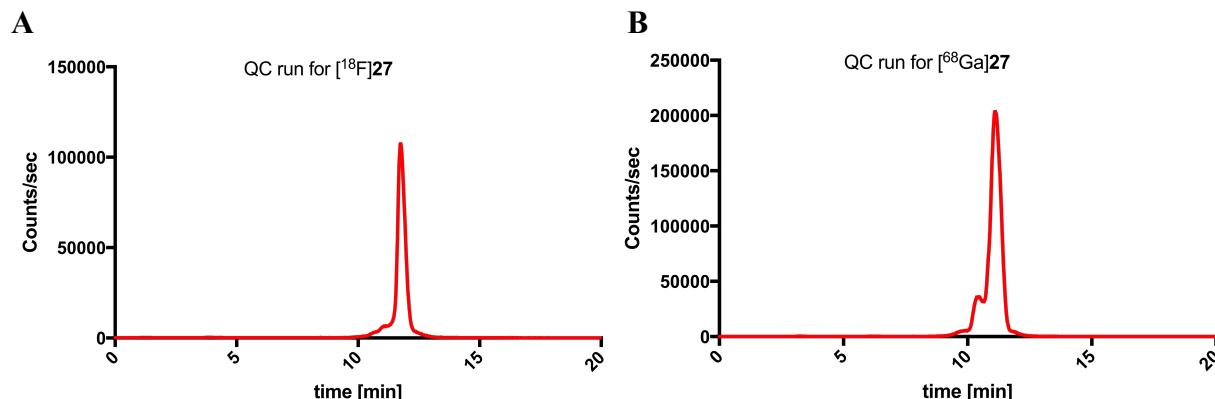


Figure S1. Representative radio-HPLC traces of the quality control runs for both lead compounds $[^{18}\text{F}]27$ and $[^{18}\text{F}]68$. Lead compounds $[^{18}\text{F}]27$ and $[^{18}\text{F}]68$ were furnished in high (>97%) radiochemical purities with retention times of 11.7 and 11.1 minutes, respectively.

4 | In vitro stability, plasma protein binding, and distribution coefficients

4.1 | In vitro stability and plasma protein binding

Radioligands (2-3 MBq) were incubated with agitation (600 rpm) at 37 °C in 500 μL of human serum. After 4 h, the solution was transferred into a 1.7 mL centrifuge tube. 300 μL of MeCN was added to the solution and the resulting mixture was vortexed and centrifuged (13,000 rpm) for 5 min. The clear supernatant was removed, moved to a new centrifuge tube followed by additional centrifugation at 13,000 rpm for 5 min. The resulting clear supernatant was used for HPLC analysis, and the residual protein was checked for residual radioactivity. The fraction of intact radioligand was calculated by dividing the peak area corresponding to the tracer by the integral of the entire HPLC run. The observed decomposition of the radioligand in human serum is likely related to the presence of nucleophilic sulfhydryl and amino groups in the serum that could, even at neutral pH, have a measurable negative effect on the stability of the tetrazine moiety.

4.2 | Distribution coefficients

The logD values of all tracers were determined using the shake-flask method. Tracer (500 μCi , 1-2 nmol) was dissolved in a 1:1 mixture of PBS (pH 6.8) and 1-octanol with a total volume of 500 μL (n = 3). The mixture was incubated at 37 °C for 30 min with agitation (450 rpm). The final logD values were calculated using the activity concentrations of both layers obtained from radioactivity counting in pre-weighed gamma-counter tubes using the following formula:

$$\log D_{oct/wat} = \log \left(\frac{[solute]_{octanol}^{ionized} + [solute]_{octanol}^{un-ionized}}{[solute]_{water}^{ionized} + [solute]_{water}^{un-ionized}} \right)$$

Tracer	Net charge	Serum stability [%-intact]	Protein binding [%]	LogD
[¹⁸ F]15	0	82.7 ± 6.21	<2	-1.79 ± 0.19
[¹⁸ F]16	0	84.0 ± 4.75	<2	-1.42 ± 0.08
[¹⁸ F]17	-1	85.2 ± 8.17	<2	-1.23 ± 0.13
[¹⁸ F]18	-1	72.67 ± 6.57	<2	-1.44 ± 0.15
[¹⁸ F]19	0	73.13 ± 4.34	<2	-1.30 ± 0.19
[⁶⁸ Ga]19	1	70.4 ± 4.2	<2	-1.84 ± 0.17
[¹⁸ F]20	2	84.87 ± 4.25	12.8 ± 4.32	-1.26 ± 0.14
[⁶⁸ Ga]20	1	65.7 ± 3.18	<2	-2.26 ± 0.04
[¹⁸ F]21	2	78.87 ± 5.65	8.1 ± 2.6	-1.12 ± 0.23
[⁶⁸ Ga]21	3	67.9 ± 7.04	10.3 ± 4.2	-1.13 ± 0.18
[⁶⁸ Ga]22	4	71.67 ± 6.17	6.2 ± 3.9	-1.28 ± 0.1
[¹⁸ F]23	-1	91.9 ± 3.5	<2	-1.5 ± 0.23
[¹⁸ F]24	-1	66.9 ± 7.85	<2	-1.01 ± 0.12
[⁶⁸ Ga]24	0	57.63 ± 8.92	<2	-1.11 ± 0.12
[¹⁸ F]25	0	85.2 ± 8.17	<2	-2.35 ± 0.04
[⁶⁸ Ga]26	0	83.4 ± 6.08	<2	-2.09 ± 0.06
[¹⁸ F]27	0	88.53 ± 5.49	<2	-1.34 ± 0.18
[⁶⁸ Ga]27	1	73.53 ± 4.34	<2	-1.45 ± 0.12
[¹⁸ F]28	-1	75.1 ± 7.32	<2	-1.49 ± 0.1
[⁶⁸ Ga]28	0	72.43 ± 2.25	<2	-1.65 ± 0.12
[¹⁸ F]29	1	79.7 ± 7.45	<5.3 ± 2.23	-2.39 ± 0.1
[¹⁸ F]30	2	73.67 ± 5.71	<2	-2.33 ± 0.12
[⁶⁸ Ga]30	1	87.33 ± 4.7	<2	-1.74 ± 0.13
[¹⁸ F]31	1	83.27 ± 4.32	<2	-2.75 ± 0.08
[¹⁸ F]32	-2	77.6 ± 3.56	<2	-2.94 ± 0.18

Table S1 | Summary of molecular net charges, serum stabilities, protein binding, and logD values for all 25 radioligands.

5 | Plasma half-life and *in vivo* stability

5.1 | Plasma half-life determination

Radioligands (4-6 MBq) were injected into healthy athymic nude mice (n = 3) via the lateral tail vein. Blood (25-50 µL) was collected at 2, 5, 15, 30, 45, 60, 90, and 120 minutes p.i. either via tail vein or saphenous vein. Both procedures furnished consistent and reproducible results. The collection tubes were weighed before and after blood collection and measured for radioactivity in order to calculate the %ID/g values for each time point and sample. The calculated %ID/g values were subsequently plotted as a function of time. A bi-exponential decay curve was used as fit function, accounting for a short blood half-life (a-phase) during the initial phase directly after injection, and a longer blood half-life during terminal phase (b-phase). The data analysis program Prism 7™ was used to determine both half-lives, $t_{1/2}$ -a and $t_{1/2}$ -b, as well as their relative contribution (in %) to the overall half-life ($t_{1/2}$). Subsequently, both half-lives were weighed according to their relative contribution to the overall blood half-life $t_{1/2}$ using the following formula:

$$t_{1/2} = \frac{(t_a \times \%_{fast}) + t_b \times (100 - \%_{fast})}{100}$$

At the time of tracer injection (t = 0), it was assumed that %ID/g = 50. The injected dose at t = 0 equals 100%, divided by the total mass of blood (2 g in case of 6-8 weeks old mice) gives %ID/g_{t=0} = 50.

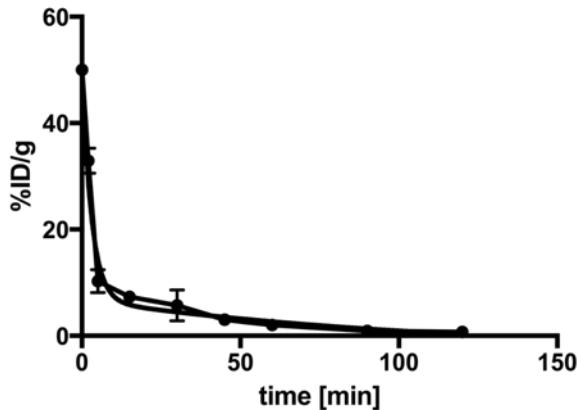


Figure S2. Example blood time-activity curve for $[^{68}\text{Ga}]27$, including bi-exponential curve fit. Based on the assumption that total blood volume is approximately 2 mL (with a total mass of 2 g), the $\%ID/g_{t=0} = 50$ at $t = 0$. With $t_1 = 2.05$ min, $t_2 = 74.5$ min and %-fast = 81.9%, the blood half-life of compound $[^{68}\text{Ga}]27$ was calculated to 15.11 min.

5.2 | In vivo stability

In vivo stabilities were determined for all 15 radioligands evaluated in pretargeting experiments. Depending on plasma half-life, *in vivo* stability (given as %-intact tracer at 3 h p.i.) was determined by either blood draw via the tail vein or terminal blood draw via cardiac puncture. In general, for tracers with $t_{1/2} > 7-8$ min, the blood volume obtained via the tail or saphenous vein was sufficient signal detection during radio-HPLC. In contrast, for tracers with $t_{1/2} < 7$ min terminal blood draw was necessary in order to obtain a sufficient amount of radioactivity signal during radio-HPLC.

Tracer #	t (slow) [min]	t (fast [min]	%-fast	Plasma half-life [min]	Plasma stability [%-intact]
$[^{18}\text{F}]15$	18	1.1	57.1	8.35	68.1 ± 8.4
$[^{18}\text{F}]16$	23.25	2.56	85.32	5.60	-
$[^{18}\text{F}]17$	25.39	1.268	34.63	17.04	62.6 ± 12.3
$[^{18}\text{F}]18$	24.03	1.607	52.67	12.22	71.2 ± 8.5
$[^{18}\text{F}]19$	21.32	0.8043	86.37	3.60	55.6
$[^{68}\text{Ga}]19$	30.26	1.013	80.97	6.58	-
$[^{18}\text{F}]20$	11.92	0.897	79.21	3.19	58.3 ± 8.8
$[^{68}\text{Ga}]20$	20.18	1.021	89.47	3.04	-
$[^{18}\text{F}]21$	15.99	1.409	85.77	3.48	51.2 ± 7.9
$[^{68}\text{Ga}]21$	16.43	0.82	92.37	2.01	-
$[^{68}\text{Ga}]22$	13.23	0.9	91.55	1.94	-
$[^{18}\text{F}]23$	26.23	1.414	59.97	11.35	55.9 ± 8.9
$[^{18}\text{F}]24$	31.55	5.089	60.56	15.53	31.6 ± 6.3
$[^{68}\text{Ga}]24$	18.98	0.1	23.74	14.50	-
$[^{18}\text{F}]25$	25.39	1.268	54.63	12.21	66.3 ± 8.2
$[^{68}\text{Ga}]26$	39.58	1.569	58.58	17.31	n/a
$[^{18}\text{F}]27$	34.6	2.02	53.8	17.07	73.1 ± 9.3
$[^{68}\text{Ga}]27$	26.71	3.563	50.1	15.11	69.2 ± 4.2
$[^{18}\text{F}]28$	20.23	2.301	39.44	13.16	66.7 ± 6.5
$[^{68}\text{Ga}]28$	23.77	1.3	60.67	10.14	78.2 ± 9.1
$[^{18}\text{F}]29$	18.85	1.295	77.71	5.21	63.8 ± 11.9
$[^{18}\text{F}]30$	28.61	1.766	76.181	8.16	-
$[^{68}\text{Ga}]30$	23.35	2.086	79.11	6.53	56.3 ± 7.9
$[^{18}\text{F}]31$	16.27	1.11	54.69	7.98	-
$[^{18}\text{F}]32$	17.2	1.469	56.65	8.29	-

Table S2 | Plasma half-lives were calculated using Prism 7TM software and a two-phase decay model. In vivo stabilities were determined for compounds that were employed in pretargeting experiments. Regarding our previous report³, this table includes the corrected plasma half-life for radioligand $[^{18}\text{F}]28$. Instead of 71.2 min, as stated in

our previous study, we found a plasma half-life of 13.2 min for this compound. The observed difference is due to the different approaches used to calculate the plasma half-life. In the study at hand, we considered a bi-exponential clearance pattern as the more accurate way to calculate plasma half-lives. Our values are in line with experimental in vivo data⁴.

6 | Preparation of antibody-TCO conjugates

The anti-CA19.9 mAb 5B1 was modified using TCO-NHS as previously described³.

The amine-reactive ester TCO-NHS (*N*-succinimidyl 5-((4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-5-oxopentanoate) was obtained from Sigma Aldrich. A solution of huA33 (1.79 mg) was prepared in 1 mL of phosphate buffered saline (PBS, pH 7.4). The pH of the resultant solution was adjusted to 8.8-8.9 with a 0.1 M aqueous solution of NaHCO₃. The appropriate amount of TCO-NHS (25 mg/mL in DMF) was then added such that the ratio of TCO:mAb was 25:1, and the reaction mixture was incubated for 1 h at RT with agitation (550 rpm). The consequent huA33-TCO was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 2*1.0 mL fractions of PBS, pH 7.4) and concentrated, if necessary, via centrifugal filtration.

Determination of number of TCO groups/mAb: A solution of mAb-TCO (75 µg) was prepared in 300 µL of PBS (pH 7.4). The appropriate amount of Tz-PEG₇-AF488 (200 µM in DMSO) was then added such that the ratio of Tz:mAb was 20:1, and the reaction mixture was incubated for 1 hour at RT with agitation (550 rpm). The consequent mAb-AF488 was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 1.0 mL fractions of PBS, pH 7.4) and concentrated, if necessary, via centrifugal filtration. The number of active TCO moieties was determined as follows:

$$A_{mAb} = A_{280} - (A_{495} * CF)$$

$$DOL = [A_{max} * MW_{mAb}] / [[mAb] * \epsilon_{AF488}]$$

in which the correction factor for AF488 was given as 0.11 by the supplier, MW_{mAb} = 150,000, ε_{495,AF488} = 71,000, and ε_{280,mAb} = 210,000. The degree of labeling of AF488 was taken as the degree of labeling of TCO due to the rapid and quantitative nature of the Tz/TCO reaction.

7 | Ex vivo biodistribution and PET imaging experiments

Ex vivo biodistribution: In healthy mice and pretargeted biodistribution experiments, the radioligands were injected into either healthy or BxPC3-xenograft (72 h after the injection of 5B1-TCO) bearing athymic nude mice (n = 4) via the tail vein, respectively. The organs were harvested at the appropriate time points after the animals were euthanized by CO₂ asphyxiation. The collected organs were weighed and counted in a WIZARD² automatic γ-counter (PerkinElmer, Boston, MA). The tissue mass and the decay-corrected injected radioactivity dose per animal was used to calculate the %ID/g values for each organ of interest.

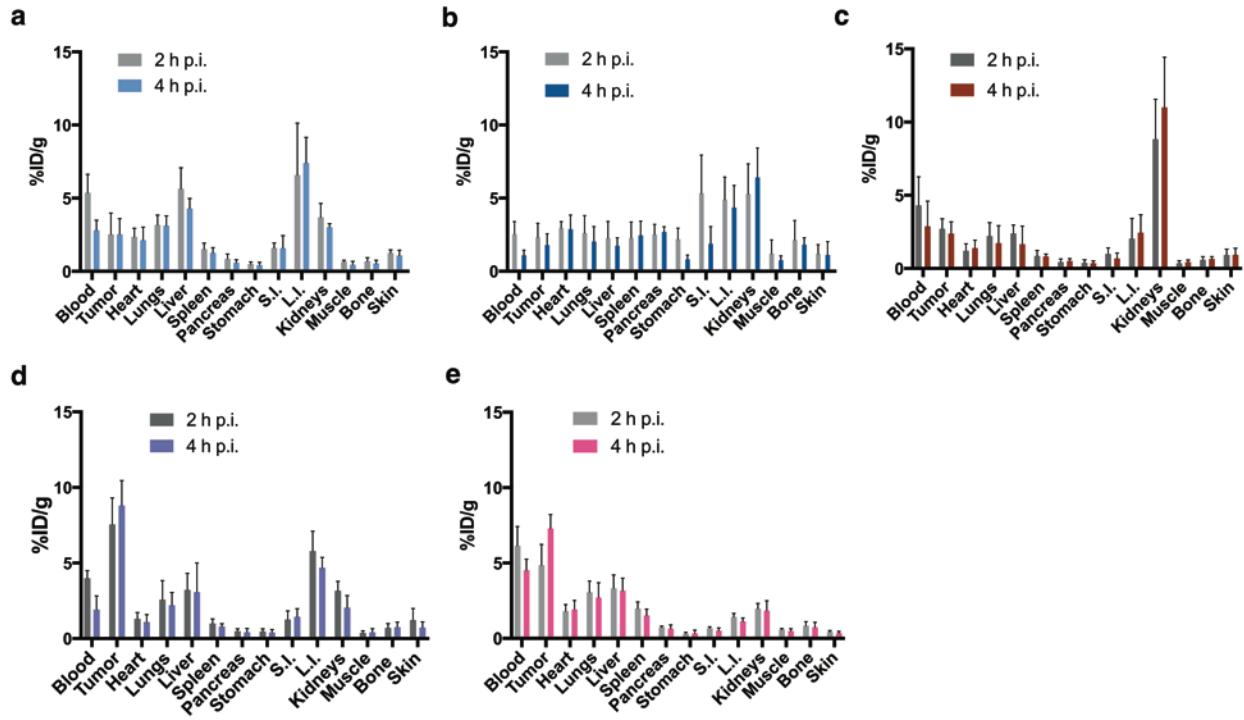


Figure S3 | Ex vivo biodistribution data obtained for radioligands $[^{18}\text{F}]15$, $[^{18}\text{F}]19$, and $[^{18}\text{F}]29$ (a-c), as well as for lead compounds $[^{18}\text{F}]27$ (d) and $[^{68}\text{Ga}]27$ (e). Organ uptake values were determined at 2 h and 4 h p.i.

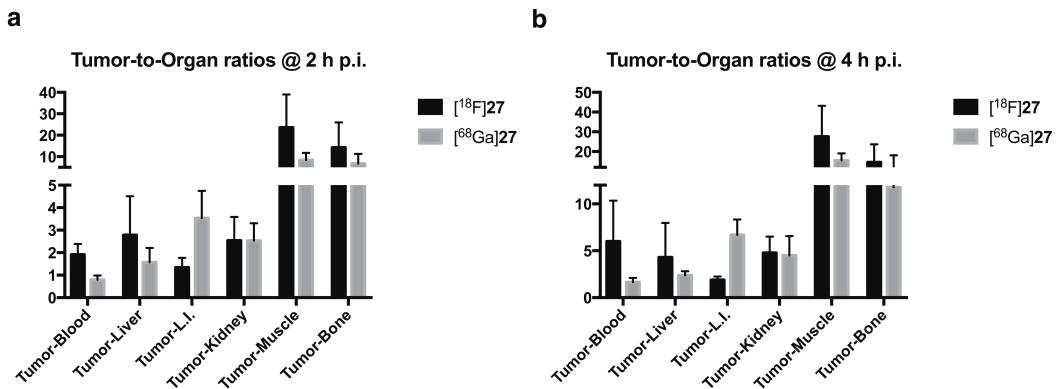
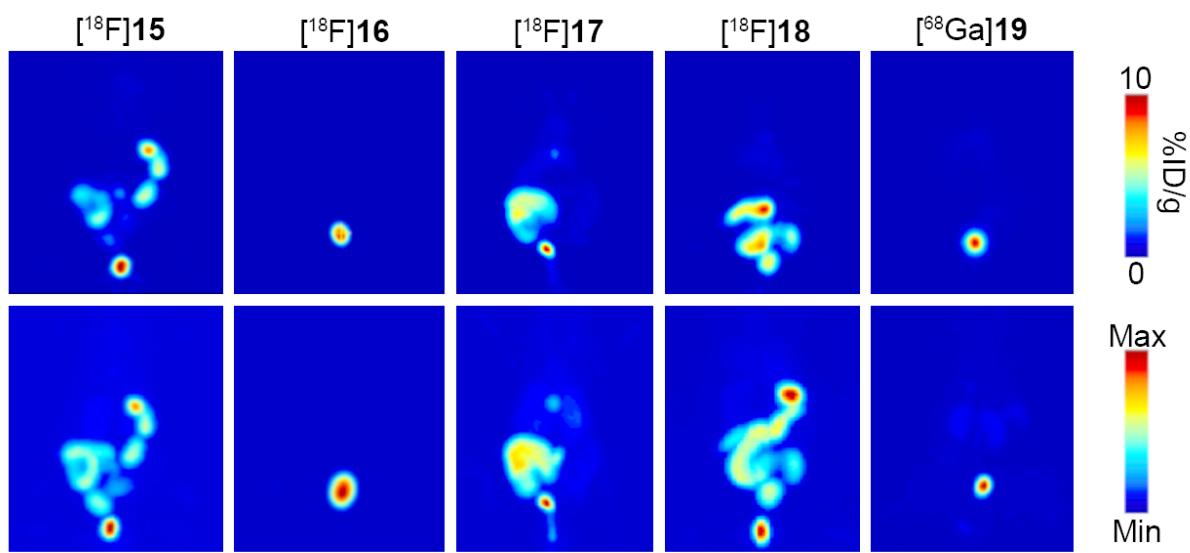
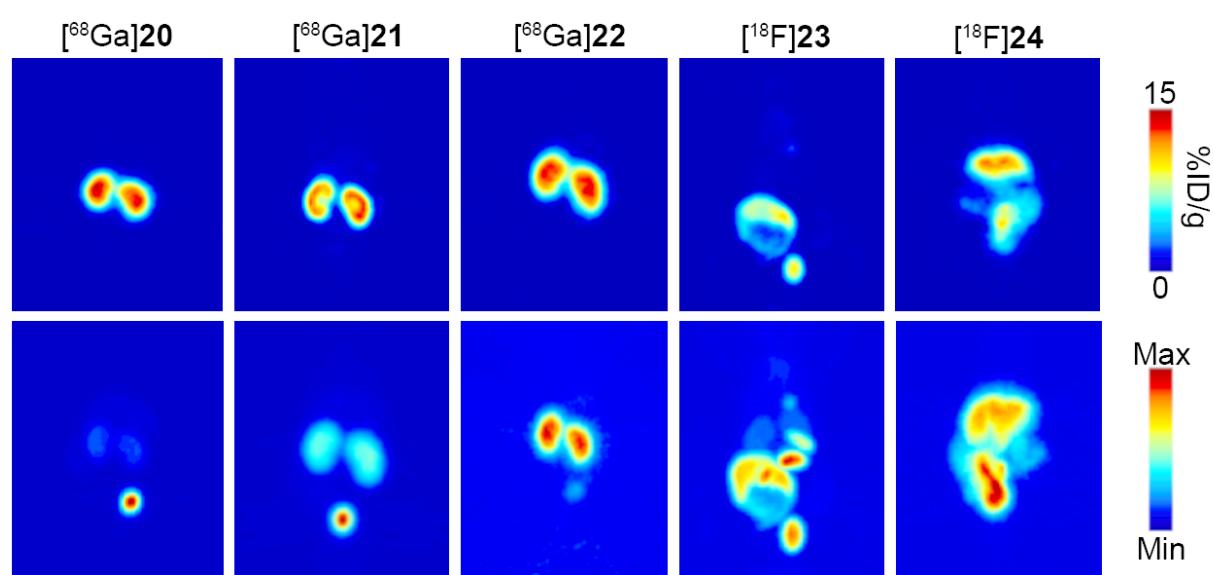


Figure S4 | Calculated tumor-to-organ ratios at 2 h (a) and 4 h (b) p.i. for both lead compounds.

PET imaging: PET imaging experiments were carried out on a microPET Focus 120 (Siemens). After the administration of the radiolabeled tracer (18.5 – 24.1 MBq/mouse [500-650 μCi]) in 150-200 μL sterile saline) via intravenous tail vein injection ($t = 0$), anesthesia was maintained using 1.5% isoflurane/oxygen gas mixture and static scans were recorded at various time points with a minimum of 12 million coincident events (8-25 m total scan time). An energy window of 350-700 keV and a coincidence time window of 6 ns were used. Data were sorted into 2-dimensional histograms by Fourier re-binning, and transverse images were reconstructed by filtered back-projection into a $128 \times 128 \times 63$ ($0.72 \times 0.72 \times 1.3$ mm) matrix. The image data were normalized to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection but no attenuation, scatter, or partial-volume averaging correction was applied. Activity concentrations (as %ID/g) were determined by conversion of the counting rates from the reconstructed (filtered back-projection) images. Maximum intensity projection (MIP) images were generated from 3-dimensional ordered subset expectation maximization reconstruction (3DOSEM). All of the resulting images were analyzed using ASIPro VM™ software. For the determination of specific organ radioactivity uptake (as %ID/g), decay-corrected PET image slices were used to establish region of interests (ROI analysis). ASIPro VM™ software then calculates the mean %ID/g for the given ROI.

a**b**

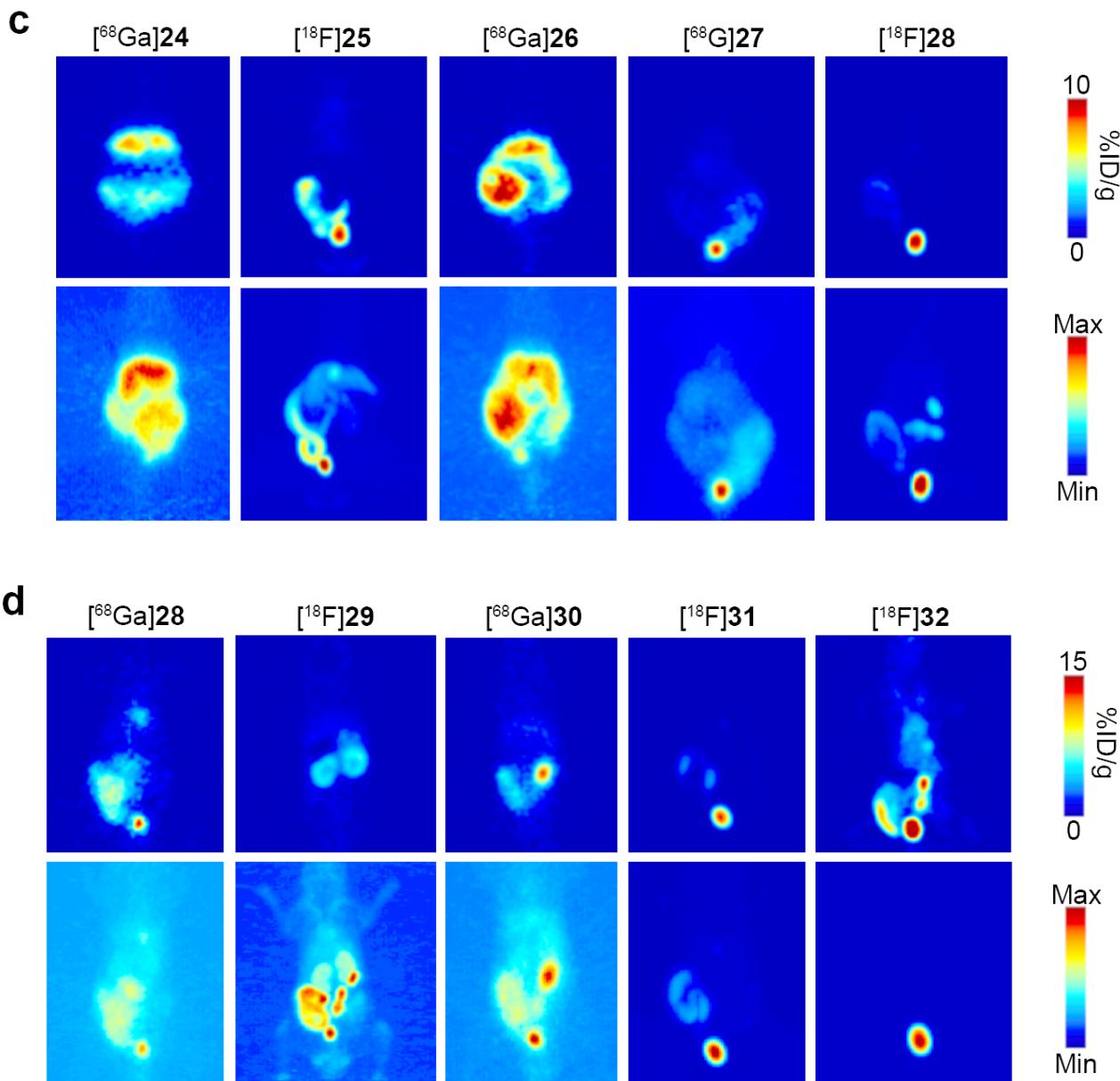
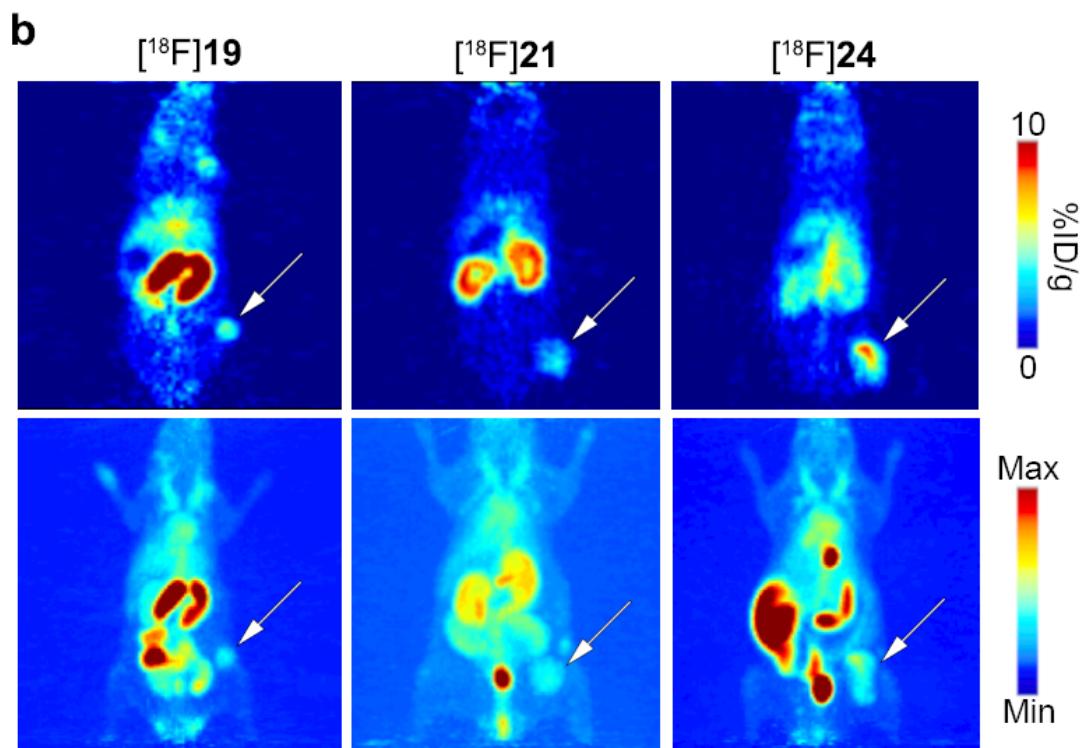
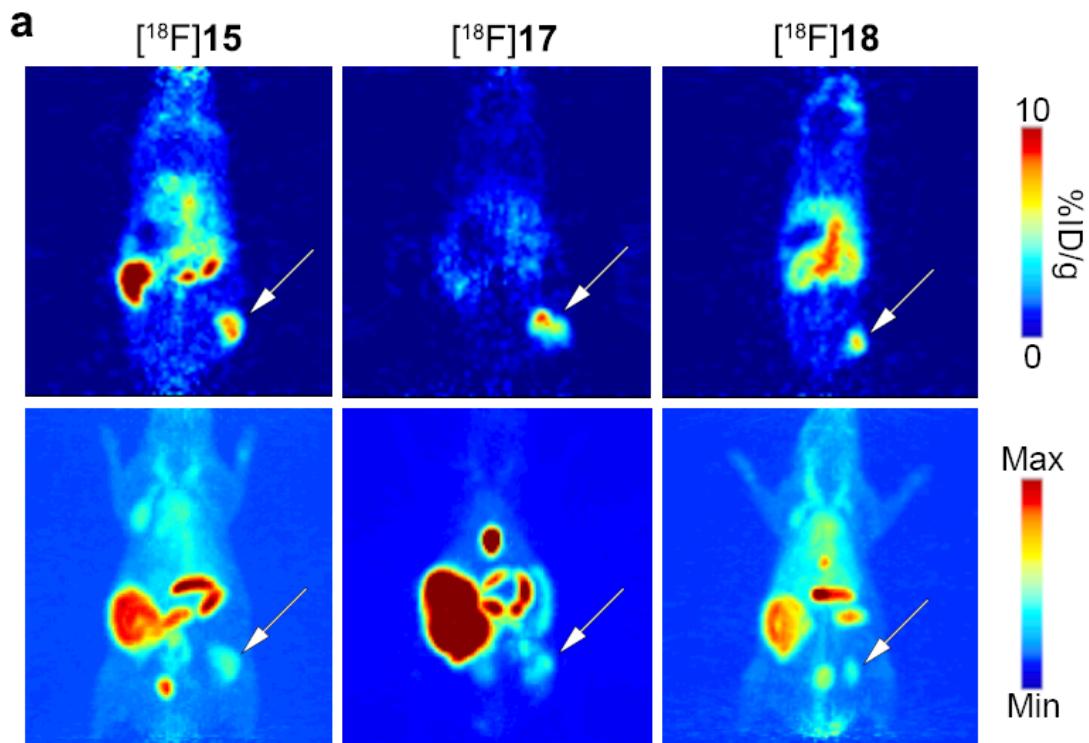


Figure S5 | PET images acquired 2 h p.i. of the radioligand. Images were acquired using healthy athymic nude for exploration of biodistribution. The upper panel displays transverse slices and the bottom rows show the corresponding MIPs.



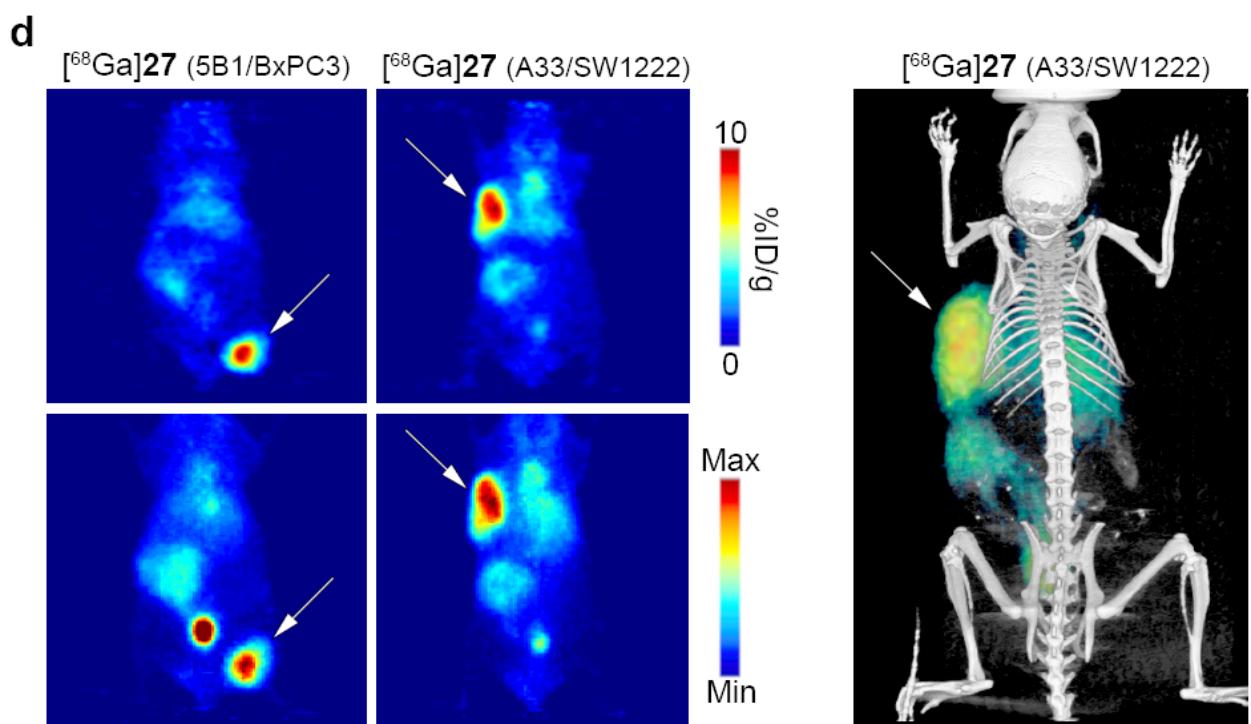
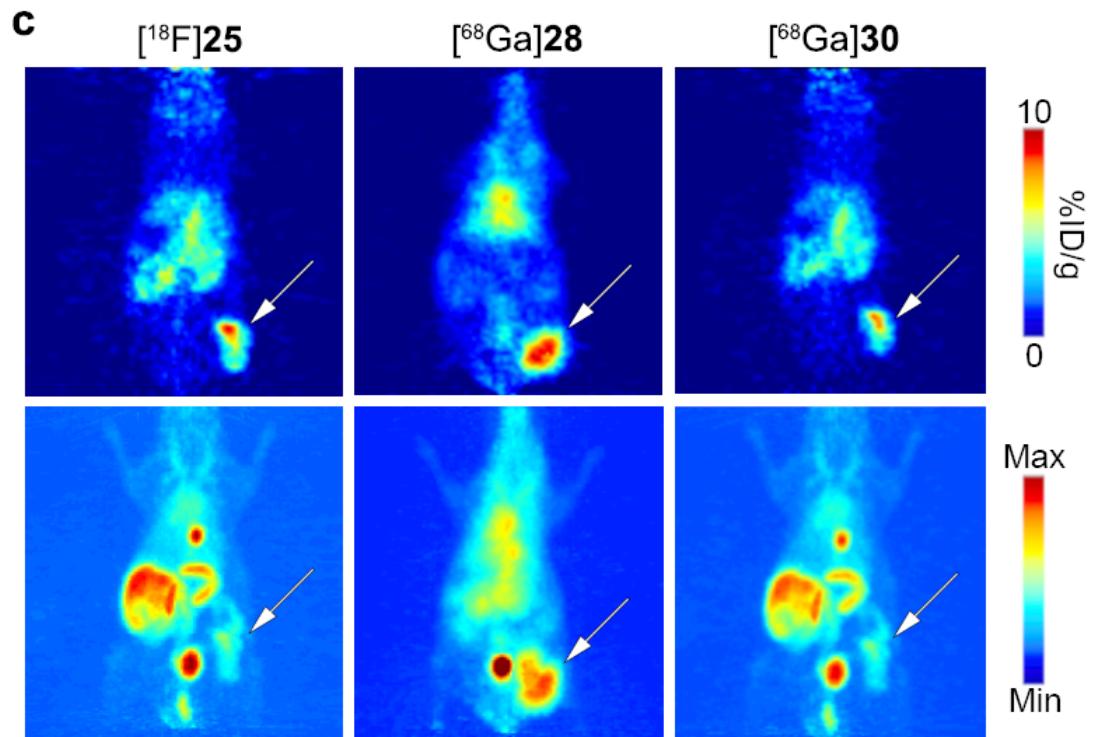


Figure S6 | Pretargeted PET images acquired 2 h p.i. of all remaining radioligands that were not shown in the manuscript. The upper panel displays transverse slices and the bottom rows show the corresponding MIPs. Location of tumor tissue is indicated by white arrows.

8 | Dosimetry

The pretargeted biodistribution data obtained from the utilized mouse model were first expressed as normal-organ mean standard uptake values (SUVs) versus time post-injection. It was assumed that SUVs are, in first order, independent of body mass and hence the same among species. The mean SUVs for the appropriate mouse organs were then used to calculate the mean SUVs of the same organs in a human using the organ and total-body masses of the 70kg Standard Man anatomic model. These data were then corrected for radioactive decay to the time of injection and subsequently fitted to a mono-exponential or bi-exponential time-activity function, depending on the organ. This information was subsequently used to determine the organ residence times which were then entered into the OLINDA computer program to yield the mean organ absorbed doses and effective dose in mSv/MBq⁵. The data obtained from the herein presented ¹⁸F/⁶⁸Ga-based pretargeting approach were compared to the CA19.9 targeting and directly radiolabeled immunoconjugate ⁸⁹Zr-DFO-5B1 by Viola-Villegas et al.⁶.

Organ	Absorbed doses [mSv/MBq]		
	[¹⁸ F]27	[⁶⁸ Ga]27	⁸⁹ Zr-DFO-5B1
Adrenals	0.0089	0.0126	2.22
Brain	0.0078	0.0120	1.7
Breasts	0.0065	0.0109	1.36
Gallbladder Wall	0.0096	0.0132	2.13
LLI Wall	0.0162	0.0129	2.22
Small Intestine	0.0114	0.0138	2.1
Stomach Wall	0.0111	0.0132	2.2
ULI Wall	0.0140	0.0135	1.98
Heart Wall	0.0085	0.0143	2.15
Kidneys	0.0316	0.0371	2.86
Liver	0.0100	0.0141	2.52
Lungs	0.0060	0.0084	2.52
Muscle	0.0042	0.0037	1.59
Ovaries	0.0093	0.0131	1.98
Pancreas	0.0097	0.0137	2.26
Red Marrow	0.0070	0.0098	4.01
Osteogenic Cells	0.0114	0.0168	5.08
Skin	0.0055	0.0100	1.17
Spleen	0.0207	0.0611	3.7
Testes	0.0071	0.0114	1.51
Thymus	0.0071	0.0114	1.71
Thyroid	0.0071	0.0114	1.69
Urinary Bladder Wall	0.0087	0.0127	1.86
Uterus	0.0094	0.0134	1.99
Total Body	0.0072	0.0118	1.86
Effective Dose (mSv/MBq)	0.0093	0.0125	2.02

Table S3 | Mean organ and effective whole body doses (given in mSv/MBq) for ¹⁸F- and ⁶⁸Ga-labeled lead compounds [¹⁸F]27 and [⁶⁸Ga]27, respectively, as well as for the ⁸⁹Zr-labeled radioimmunoconjugate 5B1-DFO for comparison. The pretargeting approach offers significant dosimetric advantages over directly labeled radioimmunoconjugates.

9 | Statistics

Tumor %ID/g vs. plasma half-life: To evaluate the effect of plasma half-life within each drug, the tumor percent uptake was assessed using a random effect model. In this model it is assumed that the tumor uptake profile of each mouse (within a given drug) deviates in a random fashion from an average profile. The model for drug *i* in mouse *j*,

in which μ is the average intercept, α_j is a random intercept (accounting for random effect) for mouse j , β is the slope, and ϵ_{ij} is a random error term, is:

$$Tumor\%_{ij} = \mu + \alpha_j + \beta * plasma\ half-life + \epsilon_{ij}$$

Using a Wald Test, we observe a significant increase in tumor percent uptake as plasma half-life increases (p-value < 0.0001; test-statistic = 6.5). All models were fit using the lme4 package in R version 3.1.1. The estimate of the slope and the standard error used to calculate the test-statistic are 0.26 (std. error = 0.04), meaning that for every incremental increase in the plasma-half life of a drug, on average we can expect the tumor %ID/g to increase by 0.24.

Molecular charge vs. logD: To evaluate the effect of molecular charge within each drug, the logD was assessed using a random effect model. In this model it is assumed that the logD profile of each well *in vitro* experiment (within a given drug) deviates in a random fashion from an average profile. The model for drug i in well experiment j , in which μ is the average intercept, α_j is a random intercept (accounting for random effect) for experiment j , β is the slope, and ϵ_{ij} is a random error term, is:

$$logD_{ij} = \mu + \alpha_j + \beta * molecular\ charge + \epsilon_{ij}$$

Using a Wald Test, we observe a significant decrease in logD as molecular charge increases (p-value < 0.0001; test-statistic = -8.2). All models were fit using the lme4 package in R version 3.1.1. The estimate of the slope (and the standard error) used to calculate the test-statistic are -0.28 (std. error = 0.03), meaning that for every unit increase in the molecular charge, on average we can expect logD to decrease by -0.28.

We note a limitation of the two above shown analyses. There is some selection bias in the compounds considered. That is we evaluate more compounds within a given class that correspond to a longer plasma-half life and zero molecular charge. Consequently, we have limited data for compounds that exhibit shorter plasma-half lives and extreme molecular charges, which might have adversely affected our ability to accurately estimate and predict at the boundaries; however, our goal in these analyses is to identify general trends of association.

Plasma half-life vs. LogD: We can use simple linear regression to associate logD and plasma half-life and a Wald Test to test the significance of this association. Here, we observe a significant increase in logD as we increase plasma half-life (p-value < 0.0001, test-statistic = 6.7).

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