

Exploring Structural Parameters for Pretargeting Radioligand Optimization

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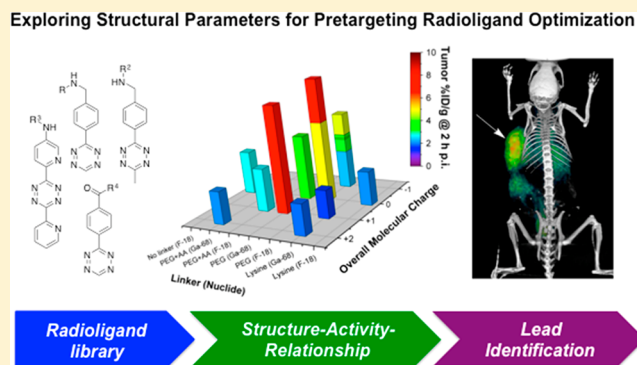
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Supporting Information

ABSTRACT: Pretargeting offers a way to enhance target specificity while reducing off-target radiation dose to healthy tissue during payload delivery. We recently reported the development of an ¹⁸F-based pretargeting strategy predicated on the inverse electron demand Diels–Alder reaction as well as the use of this approach to visualize pancreatic tumor tissue in vivo as early as 1 h postinjection. Herein, we report a comprehensive structure: pharmacokinetic relationship study of a library of 25 novel radioligands that aims to identify radiotracers with optimal pharmacokinetic and dosimetric properties. This investigation revealed key relationships between molecular structure and in vivo behavior and produced two lead candidates exhibiting rapid tumor targeting with high target-to-background activity concentration ratios at early time points. We believe this knowledge to be of high value for the design and clinical translation of next-generation pretargeting agents for the diagnosis and treatment of disease.



INTRODUCTION

In vivo pretargeting for diagnostic^{1–6} and therapeutic⁷ applications has emerged over the last three decades as a powerful technology to enhance target specificity and reduce off-target effects.^{2,8} Generally speaking, pretargeting strategies strive to combine the inherent advantages of macromolecular targeting vectors and small molecules, specifically high target specificity and short organ and tissue residence times, respectively.^{1,4} To achieve this, the targeting vector is administered first and allowed to accumulate at the target site and clear from off-target organs prior to the injection of a small effector molecule carrying the payload of interest (e.g., radionuclide; Figure 1).^{4,5,9} To enable their in vivo recombination, both entities are equipped with complementary functionalities that enable an in vivo ligation reaction.^{10,11} Appropriate pairs of reaction partners that have been employed in in vivo pretargeting approaches include streptavidin–biotin,¹² complementary oligonucleotide strands,¹³ and click chemistry-based reaction pairs.^{10,14,15} While strategies based on streptavidin–biotin have shown somewhat deflating outcomes in the clinic,¹⁶ the use of a bispecific, CEA-targeting antibody in combination with a radiolabeled hapten peptide has shown very promising clinical results.¹⁷

One of the newer members of the click chemistry toolbox, the inverse electron-demand Diels–Alder (IEDDA) reaction between *trans*-cyclooctene (TCO) and tetrazine (Tz) has proven particularly well suited for in vivo pretargeting.^{2–5,7,10,11} The IEDDA ligation is selective and bioorthogonal, but its principal advantage for pretargeting compared to other click reactions, such as the Staudinger ligation¹⁸ or the strain-promoted alkyne–azide cycloaddition¹⁴ (SPAAC), lies in its speed. To wit, the first-order rate constants of the Tz/TCO ligation lie in the realm of 10^4 – 10^5 M^{−1} s^{−1}, orders of magnitude faster than the rates of the Staudinger and SPAAC reactions (0.002 M^{−1} s^{−1} and 0.07 M^{−1} s^{−1}, respectively).¹¹ The in vivo feasibility of the IEDDA reaction between a TCO-modified monoclonal antibody (mAb) and a radiolabeled Tz has been demonstrated by various groups using a wide range of antigen-targeting immunoconjugates and tetrazines labeled with an array of radionuclides for imaging (including ¹¹¹In, ⁶⁴Cu, ^{99m}Tc, ¹⁸F, ⁶⁸Ga, and ¹¹C)^{1,4,5,19–21} and therapy (¹⁷⁷Lu).⁷

Received: August 1, 2017

Published: August 31, 2017

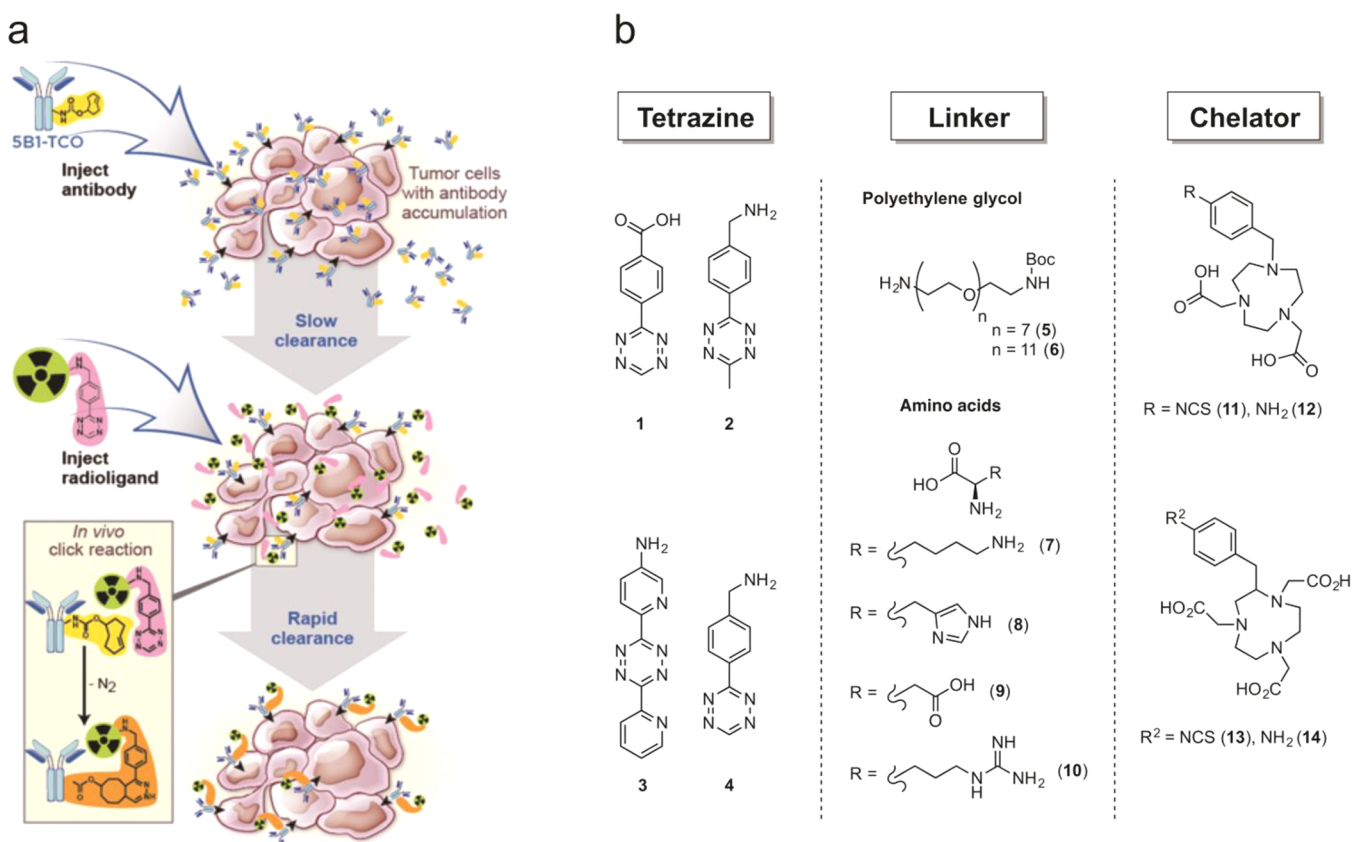


Figure 1. (a) Schematic illustration of the pretargeting approach: a macromolecular targeting vector (in our case an antibody–TCO conjugate) is injected first and allowed to reach the target site while clearing slowly from systemic circulation. After a specific accumulation time, the small molecule effector probe (in this case a radiolabeled tetrazine probe) is administered systemically and undergoes bioorthogonal click reaction with the TCO groups of the immunoconjugate at the target site. (b) Modular chemistry approach for radioligand design: radioligands consisted of a Tz moiety for in vivo click chemistry, a linker for altering the biodistribution, and a chelator for the attachment of the positron-emitting metal ions $^{68}\text{Ga}^{3+}$ and ^{18}F -AlF $^{2+}$.

In terms of pretargeted positron emission tomography (PET) imaging, Zeglis et al. presented promising results in 2013 in pretargeting experiments using the gpA33-targeting mAb huA33-TCO and a ^{64}Cu -labeled tetrazine radioligand.⁴ Shortly thereafter, our laboratories developed a second-generation Tz for ^{64}Cu -based pretargeted PET imaging applications by integrating the sarcophagine chelator system into the radioligand structure.³ At the same time, our laboratories demonstrated that an ^{18}F -labeled Tz-based radioligand in combination with the carbohydrate antigen 19.9 (CA19.9)-targeting fully human mAb 5B1-TCO²² allowed for the successful PET imaging of subcutaneous (sc) pancreatic cancer xenografts as early as 1 h postinjection (p.i.).¹ Critically, this new pretargeting approach utilized the short-lived radionuclide ^{18}F ($t_{1/2} = 109$ min), resulting in only a fraction of off-target radiation doses to healthy tissues compared to directly labeled immunoconjugates with long-lived isotopes (^{124}I or ^{89}Zr , $t_{1/2} > 3$ days). Despite clear delineation of tumor tissue at early imaging time points demonstrating the general feasibility of this approach, the relatively low tumor-to-background activity concentration ratios at even 4 h p.i., such as tumor-to-intestines (1.6 ± 0.1) and tumor-to-kidney (1.8 ± 0.4) ratios, inspired us to undertake a thorough investigation into the fundamental relationships between molecular structure, pharmacokinetics, and pretargeting performance.

This first-of-its-kind structure–pharmacokinetics relationship (SPR) study was further fueled by the increased popularity of IEDDA pretargeting strategy. However, current approaches lack fundamental insight into the relationship between physicochemical

properties and pretargeting performance. To address those issues, the study at hand was designed with two main objectives: (1) to identify a radiopharmaceutical lead candidate suitable for clinical development and (2) to generate experimental evidence for a rational understanding of how molecular parameters such as overall molecular net charge, distribution coefficient, plasma half-life (PHL), and stability influence the in vivo performance of small-molecule radioligands in pretargeting systems.

Tracer Library and SPR Study Design. The synthesis of the radioligands library as the first step of this study was based on previously reported protocols.^{1,2,4} New reaction routes and radiolabeling procedures developed within this study are described in the Supporting Information (sections 2 and 3). Overall, the radioligands were designed to display structural variation in order to cover a broad spectrum of physicochemical properties, thereby enabling the study of the relationship between structure and in vivo behavior (Table 1). Each radioligand is composed of three different structural building blocks (Table 1; Figure 2a). First, a Tz component (1–4) (Scheme 1) was selected for in vivo click chemistry. Second, a linker moiety consisting of polyethylene glycol [PEG₇ (5) or PEG₁₁ (6)], amino acids (AA) [AA = lysine (K, 7), histidine (H, 8), aspartate (R, 9), and arginine (D, 10)], or a combination of both is attached. Finally, a bifunctional chelator, either 1,4,7-triazacyclononane-1,4-diacetic acid (NODA, 11,12) or 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA, 13,14), was introduced, allowing for the installation of ^{18}F and ^{68}Ga radionuclides. Tz moieties 1–4 were selected based on their previously reported

Table 1. All 25 Radioligands Were Employed in the First Characterization Process^a

Tz	Linker		Chelator	Precursor	Tracer
	PEG _x (x = 7,11)	AA (D, H, K, R)			
1	-	-	NODA	15	[¹⁸ F]15
1	-	K	NOTA	16	[¹⁸ F]16
1	7	-	NOTA	17	[¹⁸ F]17
1	11	-	NOTA	18	[¹⁸ F]18
2	-	K	NOTA	19	[¹⁸ F]19
					[⁶⁸ Ga]19
2	-	K-K	NOTA	20	[¹⁸ F]20
					[⁶⁸ Ga]20
2	-	K-K-K	NOTA	21	[¹⁸ F]21
					[⁶⁸ Ga]21
2	-	K-K-K	NODA	22	[⁶⁸ Ga]22
2	11	-	NOTA	23	[¹⁸ F]23
3	11	-	NOTA	24	[¹⁸ F]24
					[⁶⁸ Ga]24
4	7	-	NODA	25	[¹⁸ F]25
4	7	-	NOTA	26	[⁶⁸ Ga]26
4	11	-	NODA	27	[¹⁸ F]27
					[⁶⁸ Ga]27
4	11	-	NOTA	28	[¹⁸ F]28
					[⁶⁸ Ga]28
4	7	K	NODA	29	[¹⁸ F]29
4	11	H	NODA	30	[¹⁸ F]30
					[⁶⁸ Ga]30
4	11	R	NODA	31	[¹⁸ F]31
4	11	D	NOTA	32	[¹⁸ F]32

^aOn the basis of those results, 15 radioligands were selected for the next step of testing to investigate their performance in pretargeting experiments (blue). Finally, radioligands [¹⁸F]27 and [⁶⁸Ga]27 were identified as lead compounds based on their overall tumoral uptake and tumor-to-NT activity concentration ratios (red).

stability and reaction kinetics with TCO to ensure a wide range of properties.^{4,5,21,23} The use of PEG linkers to modulate in vivo PK of small molecules has previously been reported, including accelerated nontarget organ clearance as well as increased renal clearance.^{1,2,24} We included them into our study in order to investigate their impact on radiotracer PK alone or in combination with AA linkers (which, to the best of our knowledge, have not yet been systematically reported in any SPR study). The amino acids lysine, arginine, histidine, and aspartate were regarded as useful structural components to significantly influence in vivo behavior and PK parameters. It was reasoned that their charged side chains should have a measurable effect on tracer PK and would further allow us to establish a correlation between molecular net charge and PK parameters. Both bifunctional chelator moieties NODA and NOTA currently find broad application in preclinical^{25,26} and clinical²⁷ research for the radiolabeling of biological macromolecules and small molecule targeting probes. Precursors 15–32 were synthesized in good overall chemical yields (18–37%) via 3–8-step syntheses, depending on the starting materials (Figure 2b).

All ¹⁸F- and ⁶⁸Ga-labeled [*t*_{1/2} (⁶⁸Ga) = 68 min] Tz-derived radioligands were furnished in high radiochemical yields [RCYs,

>55% (¹⁸F); >83% (⁶⁸Ga)], with specific activities (SAs) of >19 MBq/nmol and high radiochemical and radionuclidic purity (Supporting Information, section 3). For in vitro analysis, purified tracers were incubated in human serum at 37 °C to analyze their stability under physiological conditions. Further, the distribution coefficient (log *D*, *n* = 3) of all radioligands in a 1:1 mixture of PBS:1-octanol was determined using the shake-flask method. Subsequently, the tracers (4–8 MBq, 0.5–1 nmol) were injected into healthy athymic nude mice via the lateral tail vein. At various time points (between 2–120 min p.i.), blood was drawn via the lateral tail vein or saphenous vein (*n* = 4) to calculate PHLs (all radioligands, *n* = 1) and plasma stabilities (*n* = 3). Tracers (11–14 MBq, 0.8–1.3 nmol) were injected into healthy athymic nude mice, and general biodistribution experiments for all tracers were performed using serial PET imaging hourly between 1 and 4 h p.i., unless stated otherwise (*n* = 4). Decay-corrected PET imaging data and reconstructed 3D images of the tracer distribution were then used to determine radioactivity concentrations (given as percent injected dose per gram, %ID/g) in the kidney and large intestine (quantitative ROI analysis). Additional ex vivo organ uptake values were determined for selected compounds and were found to be in line with

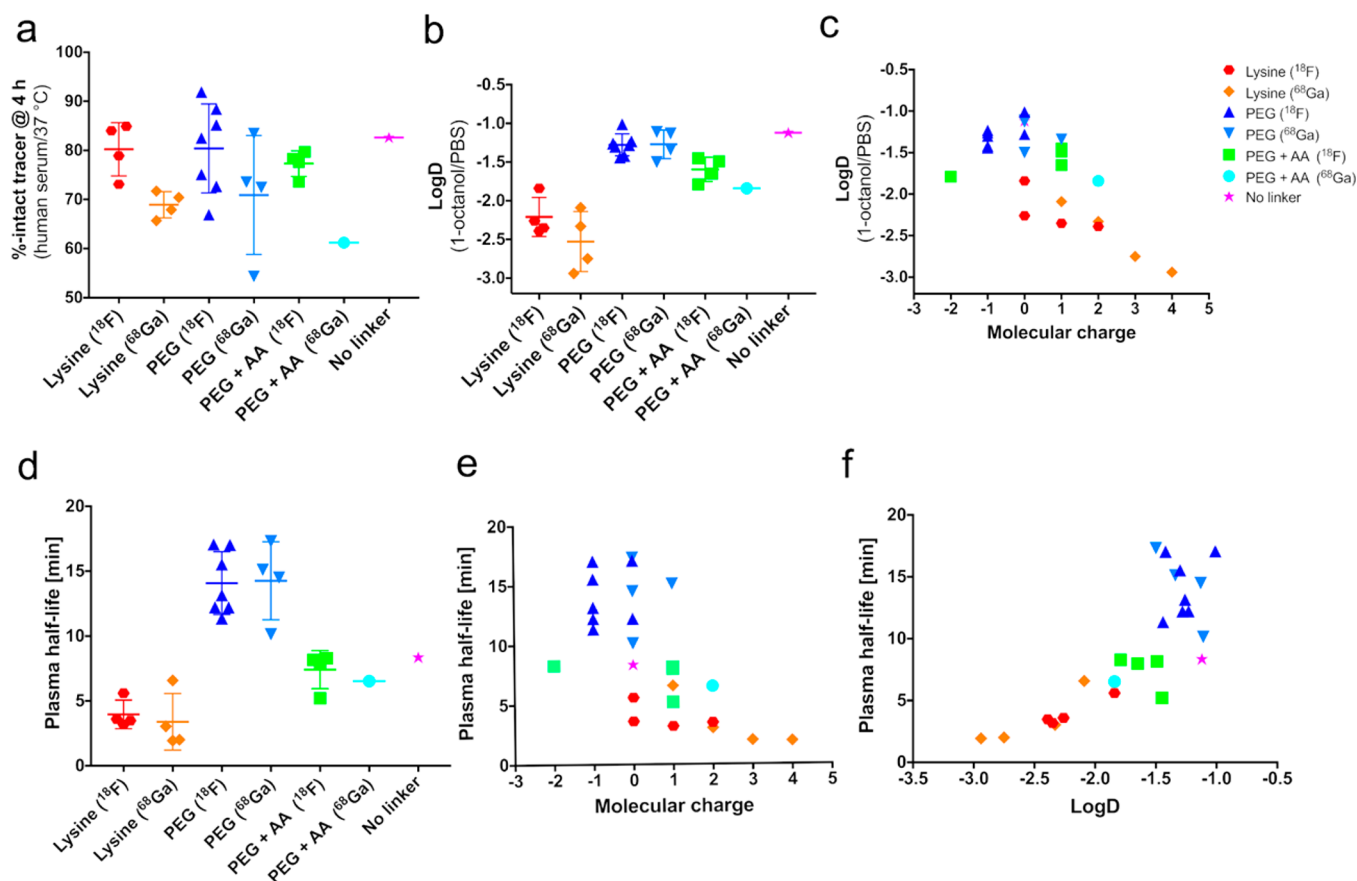


Figure 2. Correlation diagrams showing how structural and physicochemical parameters (e.g., stability, linker, molecular charge, log *D*, PHL) influence each other. (a) Tracer stabilities after a 3 h incubation time in human serum are shown after tracers were divided into groups with the same linker and radionuclide. Serum stabilities were generally >60%, except for ⁶⁸Ga-labeled compound 46. (b) Log *D* values (median shown, *n* = 3) summarized as a group diagram. (c) Distribution coefficients in relation to molecular charge (under physiological conditions) showed a significant correlation. (d–f) in vivo PHLs plotted as a group diagram (d), in dependence of molecular net charge (e), as well as plotted against log *D* (f). Lysine-containing compounds showed overall fast clearance from circulation, with PEGylated compounds having three times longer PHLs. Interestingly, a positive correlation between a tracer's PHL and its log *D* value was found.

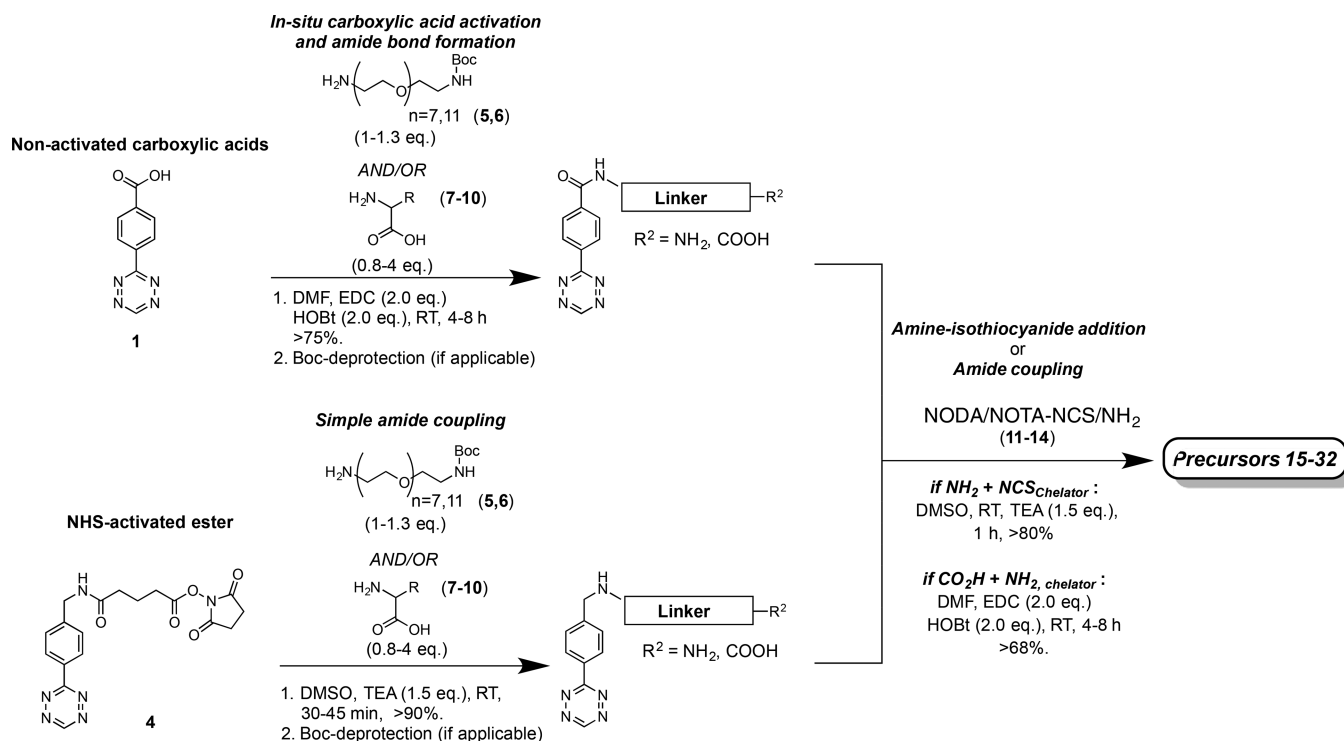
image-derived uptake data, justifying the use of image-derived organ uptake data for the majority of compounds in order to reduce the number of animals euthanized. Data analysis led to the selection of 15 radioligands (Table 1, highlighted in blue) that were further tested in in vivo pretargeting experiments using athymic nude mice bearing subcutaneous pancreatic ductal adenocarcinoma (PDAC) xenografts (*n* = 4). A TCO-modified immunoconjugate of the CA19.9-targeting antibody 5B1 (5B1–TCO; 1.3 nmol, 200 μg per mouse) was injected 72 h prior to the injection of the small molecule radioligands (1.3–1.6 nmol, 0.6–1.4 μg, 1–1.2 equiv). PET images were acquired hourly between 1–4 h p.i. unless stated otherwise. In addition, ex vivo biodistribution experiments were conducted for selected tracers in order to obtain quantitative information on tracer distribution in up to 14 organs. Ultimately, this investigation identified two radioligands ([¹⁸F]27 and [⁶⁸Ga]27) as the most promising lead compounds (Table 1, highlighted in red).

Physicochemical and Pharmacokinetic Properties of Tz-Derived PET Tracer. Structural, in vitro, and pharmacokinetic (PK) data obtained for all 25 tracers were used to investigate potential correlations between physicochemical parameters. Tracer stabilities (given as % intact, *n* = 3) in human serum (incubation for 4 h at 37 °C, Figure 3c) ranged from >90% ([¹⁸F]23) to 54 ± 7% ([⁶⁸Ga]24) (Figure 3a, Supporting Information, section 4). Considering all of the structural

elements of the radioligands, we reasoned that both the tetrazine moiety and the metal complex would have an impact on in vitro (and presumably in vivo) stability. However, we found that instability was due primarily to the decomposition of the tetrazine moiety and that the radioactive metal complexes were stable over the course of our experiments. The majority of the radioligands did not show any elevated protein binding (<2% of total radioactivity, Supporting Information, section 4), with the exception of [¹⁸F]20, [¹⁸F]21, [⁶⁸Ga]21, [⁶⁸Ga]22, and [¹⁸F]29, each of which exhibited up to 16% protein-bound radioactivity. This result may be explained by the high lysine content residues and positive charge of these radioligands, prompting electrostatic interactions between the tracers and plasma proteins.²⁴

Not surprisingly, a significant negative correlation between the log *D* value of a tracer and its overall molecular charge under physiological conditions was found (*p* value <0.0001, Supporting Information, sections 4 and 9). Generally speaking, the closer the net charge of a tracer is to 0, the higher its partition coefficient. However, it is not quite that simple. For instance, radioligands possessing the same overall molecular charge but a different number of formally charged functional groups could still show significant differences in their distribution coefficients. One explanation could be enhanced solvation by water molecules through charge–dipole interactions: if the molecule exhibits a greater number of formal charges, it would lead to a higher

Scheme 1. Reaction Scheme Exemplifying the Synthesis of Precursor Molecules Using Tz 1 and Tz 4 via Two Different Synthetic Routes, Both of Which Furnish Final Compounds in Good Overall Yields and High Chemical Purities^a



^aFor Tz starting materials such as Tz 1, in situ activation of the carboxylic acid group under mild conditions was performed using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT), enabling subsequent amide coupling with the terminal amine of an appropriate linker moiety. NHS-activated, commercially available starting materials such as Tz 4 could be coupled to the linker moiety under weakly basic conditions with high conversion rates and without the need for further activation reagents. After TFA-mediated deprotection of the terminal Boc-group (if applicable), terminal amines (or carboxylic acids) were reacted with a bifunctional NODA or NOTA chelator construct that possessed a complementary functional group for chemical ligation.

degree of solvation.²⁸ For example, radioligands with a net charge of 0 containing a PEG linker (or no linker) exhibited log *D* values between −1.0 and −1.5, while radioligands with the same overall charge but with lysine residues in the linker displayed log *D* values between −1.8 and −3.1. PHLs for the radioligands correlated (*p* value <0.0001, Supporting Information, sections 5 and 9) with their net charge. Tracers with lower (more negative) log *D* values and higher net charges possessed shorter PHLs (Figure 3d,e). The replacement of a PEG linker by AAs or the incorporation of AA(s) to an already existing PEG linker resulted in reduced PHLs. PEG-containing tracers [¹⁸F]21–[⁶⁸Ga]28, even though ranging from −1 to +1 in terms of net charge, possessed calculated PHLs of >10 min. For instance, lead compounds [¹⁸F]27 and [⁶⁸Ga]27 exhibited PHL of 17.1 and 15.1 min, respectively. In contrast, AA-containing radioligands exhibited PHLs < 10 min. Tracers that solely contained lysine moieties as linkers displayed the shortest PHLs, values that decreased further as the number of lysine residues increased. For example, [⁶⁸Ga]22 (with a charge of +4 and 3 lysine residues) possessed a PHL of 1.9 min, the shortest of all tracers. Longitudinal PET imaging experiments conducted in healthy animals revealed a significant difference in biodistribution patterns and clearance pathways between tracers (Supporting Information, section 7). In general, lysine-containing radioligands (regardless of the presence of a PEG group) showed fast clearance from circulation and relatively high kidney radioactivity concentration of >5%ID/g as early as 1 h p.i. Radioactivity uptake and retention increased in a nearly linear fashion with the number of lysine residues per radioligand

(Figure 4a–c). High retention of the tracers containing lysine residues was likely due to reabsorption of those tracers in the proximal tubules of the kidney. In fact, recent studies have shown that peptides high in lysine residues are powerful kidney targeting agents, facilitating the uptake and retention of those constructs in the renal clearing organs.^{29,30} All other radioligands that did not contain lysine residues exhibited significantly lower kidney activity concentrations (<3.5%ID/g, Figure 4). Compound [⁶⁸Ga]30, for instance, exhibited the lowest uptake values of 1.2 ± 0.3%ID/g at 2 h p.i., although it also displayed higher liver (>1.8%ID/g) and intestinal uptake (>3%ID/g). Generally, tracers with overall net charges were cleared faster from circulation through globular filtration, whereas compounds with low or no net charge exhibited elongated circulation times and were predominantly cleared hepatically and excreted via the intestines.

Evaluation of Pretargeting Performance in a PDAC Xenograft Model. On the basis of this in vitro and PK data, 15 radioligands were selected for in vivo pretargeting experiments to probe correlations between PK parameters and pretargeting performance. Compounds were selected in order to cover a broad range of structural and physicochemical diversity. Generally, we reasoned that PHL and the primary clearance pathway of a tracer should have significant impact on its pretargeting performance. PHL would determine how much tracer molecules reach the target site before clearance, and the clearance pathway should influence the target-to-background ratio, as renal clearance should reduce background noise more quickly than hepatic clearance.

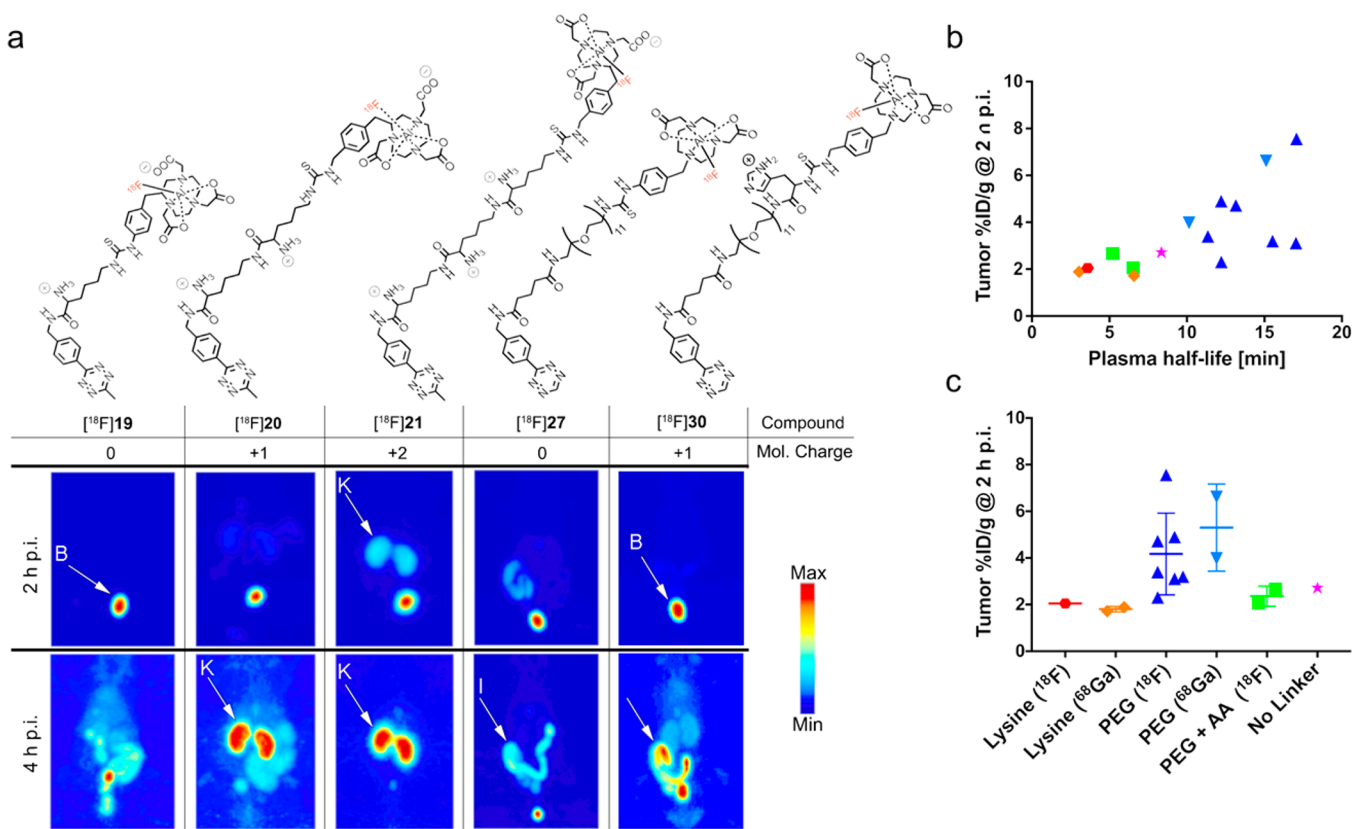


Figure 3. (a) Chemical structures and maximum intensity projections (MIPs) acquired 2 and 4 h p.i. for five selected radioligands ([¹⁸F]19, [¹⁸F]20, [¹⁸F]21, [¹⁸F]27, and [¹⁸F]30) in healthy athymic nude mice (B = bladder, K = kidney, I = intestine). Tracers (11–14 MBq, 0.8–1.3 nmol) were administered via the lateral tail vein ($t = 0$). Notably, lysine-containing radioligands [¹⁸F]19, [¹⁸F]20, and [¹⁸F]21 showed fast renal clearance, whereas [¹⁸F]30 showed more predominantly uptake in the intestines. Lead compound [¹⁸F]27 showed clearance via both excretion routes. The increasing uptake and retention in the kidneys facilitated by an increasing number of lysine residues in the radioligand structure is well visible for [¹⁸F]19–21. (b) Kidney uptake of radioligands at 2 h p.i. is shown as a function of molecular net charge. (c) Kidney uptake values at 2 h p.i. dependent on the linker and radionuclide. Solely lysine containing radioligands exhibited elevated kidney uptake values of up to 16.0 ± 5.8 (2 h p.i.) and 21.1 ± 5.6 ID/g (4 h p.i.) for [⁶⁸Ga]22. Even one lysine residue had a dramatic effect on kidney uptake as shown for [¹⁸F]29, where a lysine residue was integrated between the PEG₇ linker and the chelator moiety, increasing kidney uptake from 2.6 ± 0.3 ([¹⁸F]25, Tz-PEG₇-NODA) to 8.3 ± 1.5 ID/g ([¹⁸F]29, Tz-PEG₇-Lysine-NODA). Substitution of lysine by other amino acids with either positively or negatively charged side chains reduced kidney uptake to below 4%ID/g.

All initial pretargeting experiments were carried out in mice bearing sc BxPC3 xenografts. Approximately 3–4 weeks after inoculation (2.5×10^6 cells), TCO-modified anti-CA19.9 mAb SB1–TCO (1.33 nmol, 200 μ g in 150 μ L of 0.9% saline) was injected via the tail vein. Then 72 h later, Tz-derived radioligands (1.3–1.6 nmol, 1–1.2 equiv, in 150 μ L of 0.9% saline, containing <5% v/v ethanol) were injected into the opposite lateral tail vein ($t = 0$).^{1,2}

PET imaging and ex vivo biodistribution experiments were conducted at 2 and 4 h p.i. Tumor uptake values of all the radioligands were determined via PET image analysis (ROI analysis) as well as via ex vivo biodistribution analysis for selected compounds (Supporting Information, sections 6 and 7). The median tumoral activity concentration values at 2 h p.i. ranged from 7.6 ± 1.8 ID/g for ¹⁸F-labeled lead compound [¹⁸F]27, to 1.7 ± 0.8 ID/g for the fast-clearing tracer [¹⁸F]20 (Figures 5 and 6). Lysine-containing radioligands generally possessed tumor uptake values of <3%ID/g at all time points, with tumor uptake values of <2%ID/g for compounds solely containing a lysine linker. The highest tumor uptake values were observed for radioligands containing PEG linkers only. The introduction of AA linker led to accelerated plasma clearance and thus reduced the tumoral uptake due to fewer tracer molecules capable of reaching the

tumor site. As expected, the PHL of a radiotracer correlated (p -value <0.0001, Supporting Information, section 9) with the tracer's overall tumoral uptake, as illustrated for the 2 and 4 h time points (Figure 5c,d). Additionally, tracer stabilities in vivo were determined to be >50% at 3 h p.i. and did not correlate with tumoral uptake (Figure 5b).

In case of tracer [⁶⁸Ga]24, possessing low stabilities in both human serum ($66.9 \pm 7.9\%$) and in vivo ($31 \pm 6.3\%$), stability may indeed explain the relatively poor tumoral uptake of 3.1 ± 0.7 ID/g at 2 h p.i., despite exhibiting a PHL (15.5 min) that would allow for higher accumulation at the target site over time.

Identification of Lead Candidates. Taking all of the data into account, our study identified two lead candidates: [¹⁸F]27 and [⁶⁸Ga]27. Both tracers possessed good stabilities in vitro as well as in vivo and exhibited PHLs (17.1 and 15.1 min, respectively) that allowed for a fast enough clearance from circulation without impairing tumor accumulation. [¹⁸F]27 showed tumor uptake values of 7.6 ± 1.8 ID/g at 2 h p.i. and 8.8 ± 1.7 ID/g after 4 h, as well as promising tumor-to-NT (nontarget) activity concentration ratios, although it did display some uptake in the intestines (Figure 6a,b). [⁶⁸Ga]27 exhibited tumoral uptake values of 6.8 ± 1.4 ID/g at 2 h p.i. and 7.1 ± 1.8 ID/g at 4 h p.i. and boasted superior tumor-to-NT activity concentration

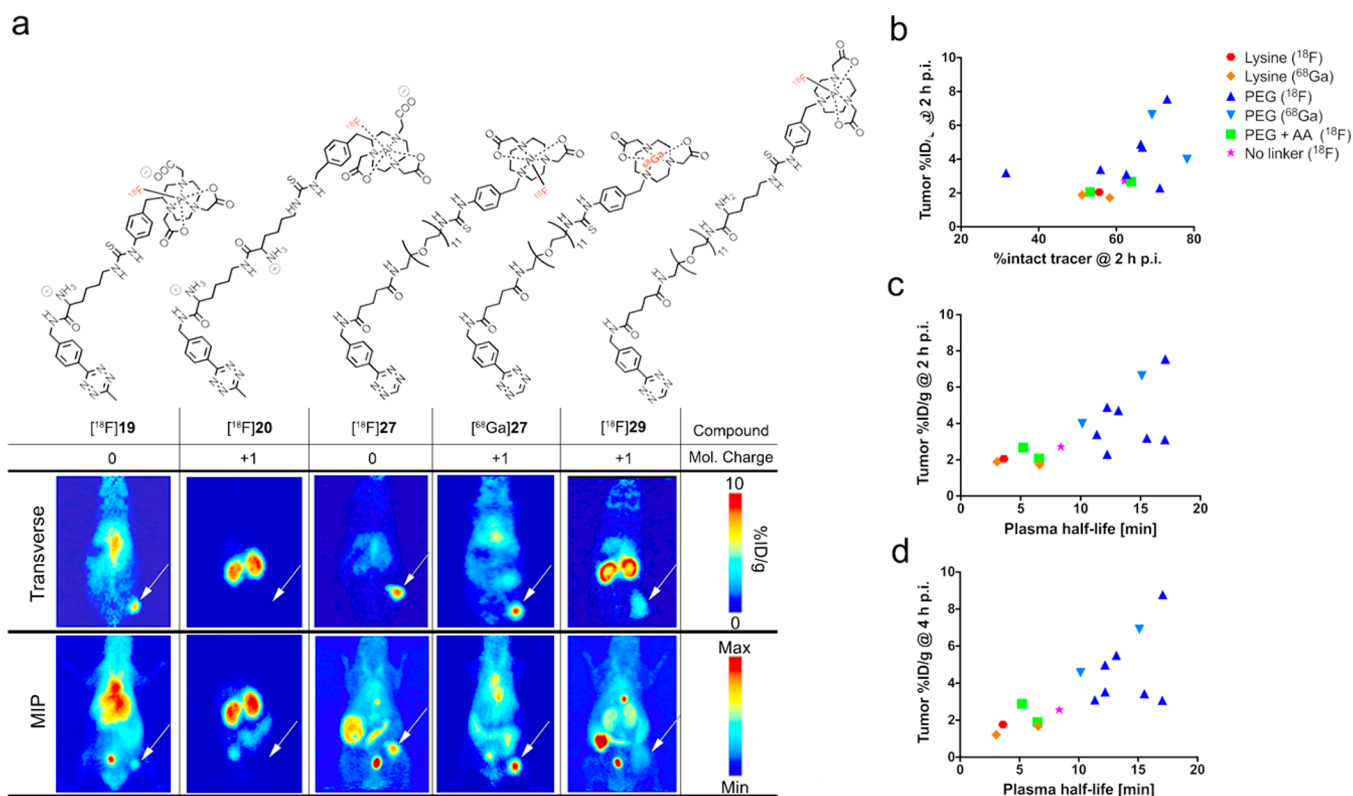


Figure 4. (a) MIPs and transverse image slices acquired in pretargeted PET imaging experiments 2 h after radioligand administration. White arrows indicate tumor tissue. To this end, SB1-TCO (200 μg , 1.33 nmol, in 150 μL of 0.9% saline) was injected into the lateral tail vein 72 h prior to the radioligand (1.3–1.6 nmol in 150 μL of 0.9% saline, containing <5% v/v ethanol). $[^{18}\text{F}]19$ exhibited slightly higher tumor uptake ($2.2 \pm 0.2\%$ ID/g, one lysine residue) than $[^{18}\text{F}]20$ ($1.7 \pm 0.2\%$ ID/g, two lysine residues), where the tumor was not visible due to the high background of kidney. However, those uptake values were significantly lower compared to the PEGylated lead compounds $[^{18}\text{F}]27$ ($7.6 \pm 1.8\%$ ID/g) and $[^{68}\text{Ga}]27$ ($6.6 \pm 1.4\%$ ID/g). Incorporation of a lysine residue as for $[^{18}\text{F}]29$ (PEG + Lysine) increased kidney uptake, while decreasing PHL and tumoral uptake, indicating a positive correlation. (b–d) Plots showing tumoral uptake in dependence of in vivo stability (2 h p.i.) and PHL (2, 4 h p.i.). In vivo stability (shown as % intact tracer and determined at 2 h p.i.) of all radioligands employed in pretargeting experiments was >50%, except for $[^{18}\text{F}]24$ with a measured stability of $31.6 \pm 6.3\%$. In vivo stability had no measurable effect on the pretargeting performance, most likely because the majority of intact tracer had undergone click reaction at the target site within the first 2 h of the experiment. Tumor uptake of radioligands did however correlate with their PHLs: the longer the PHL the higher the overall tumoral uptake.

ratios compared to its ^{18}F -labeled counterpart, especially with regard to the large intestines (>3 at 2 h p.i. compared to ~ 1.2 for $[^{18}\text{F}]27$; Supporting Information, section 6).

However, $[^{68}\text{Ga}]27$ showed elevated activity concentrations in the blood pool in both tumor models with tumor-to-blood ratios of 0.8 ± 0.2 and 1.7 ± 0.4 at 2 and 4 h p.i., respectively, in the PDAC model, suggesting that a significant amount of SB1-TCO was still present in circulation when the tracer was administered. High tumor uptake as well as similar residual blood radioactivity concentrations were observed when $[^{68}\text{Ga}]27$ was tested in a sc colorectal cancer (CRC, SW1222 cell line, Supporting Information, section 7) model, performed to demonstrate the versatility and modularity of this approach. To this end, the anti-A33 mAb huA33-TCO was injected 48 h prior to the radioligand.^{31,32} Despite a relatively low tumor-to-blood activity concentration ratio due to residual circulating mAb, $[^{68}\text{Ga}]27$ showed excellent tumor uptake as early as 1 h p.i., with maximum uptake values of $7.7 \pm 1.0\%$ ID/g at 4 h p.i. (Figure 6a,b). In accordance to our data showing the influence of molecular net charge, $[^{68}\text{Ga}]27$ (+1) showed significantly reduced uptake in the intestines in both in vivo models compared to $[^{18}\text{F}]27$ (0). Instead, enhanced renal clearance was observed that led to an increased tumor-to-background signal. Furthermore, dosimetric calculations based on ex vivo biodistribution data gave whole-body effective doses

of 0.009 mSv/MBq ($[^{18}\text{F}]27$) and 0.013 mSv/MBq ($[^{68}\text{Ga}]27$) for both lead compounds. Compared to approaches using longer-lived radionuclides (^{89}Zr), this means a >70-fold reduction in radiation doses to healthy tissue (Supporting Information, section 8). Ultimately, $[^{18}\text{F}]27$ and $[^{68}\text{Ga}]27$ showed the most favorable pretargeting PK with high tumor accumulation with minimal off-target radiation. As a result, both compounds are currently being evaluated to enter the clinical development phase.

DISCUSSION AND CONCLUSION

Since the advent of in vivo pretargeting as a diagnostic and therapeutic tool, clinical success so far has been limited. Despite the ability to successfully target tumor tissue, peripheral issues (such as the immunogenicity of streptavidin) have impaired clinical development, although recent results obtained with bispecific antibodies are extremely promising. The question whether click chemistry-based approaches, most notably those based on the IEDDA reaction, will have more success in the clinic has yet to be answered.

In recent years, the IEDDA reaction utilizing full-sized, TCO-modified mAbs in combination with small, Tz-derived imaging probes has attracted by far the most attention in the scientific community. Because of its nearly ideal properties such

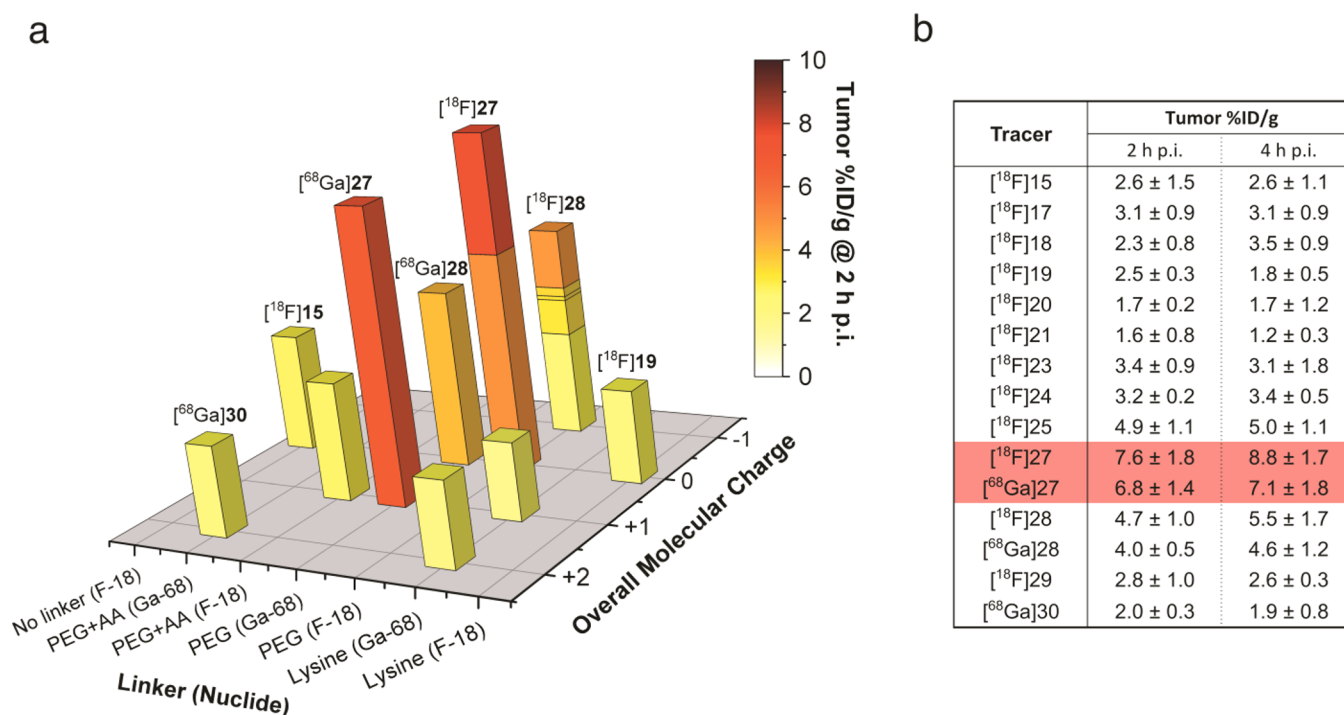


Figure 5. (a) 3D bar diagram summarizing tumoral uptake values (Z-axis) for all evaluated radioligands at 2 h p.i. (compound numbers are given for selected compounds only). Uptake values are presented in relation to the employed linker moiety (X-axis) and molecular net charge (Y-axis). In opposition to kidney uptake data, tumoral uptake values of lysine containing and other, fast-clearing radioligands were generally low (<3.5%ID/g for all time points). Tracer with PHLs of >10 min generally exhibited higher tumoral uptake, and the highest values were measured for PEGylated compounds based on Tz 4, including lead compounds [¹⁸F]27 and [⁶⁸Ga]27. (b) Summary of tumor uptake values of all 15 radioligands tested in pretargeting experiments using the PDAC model.

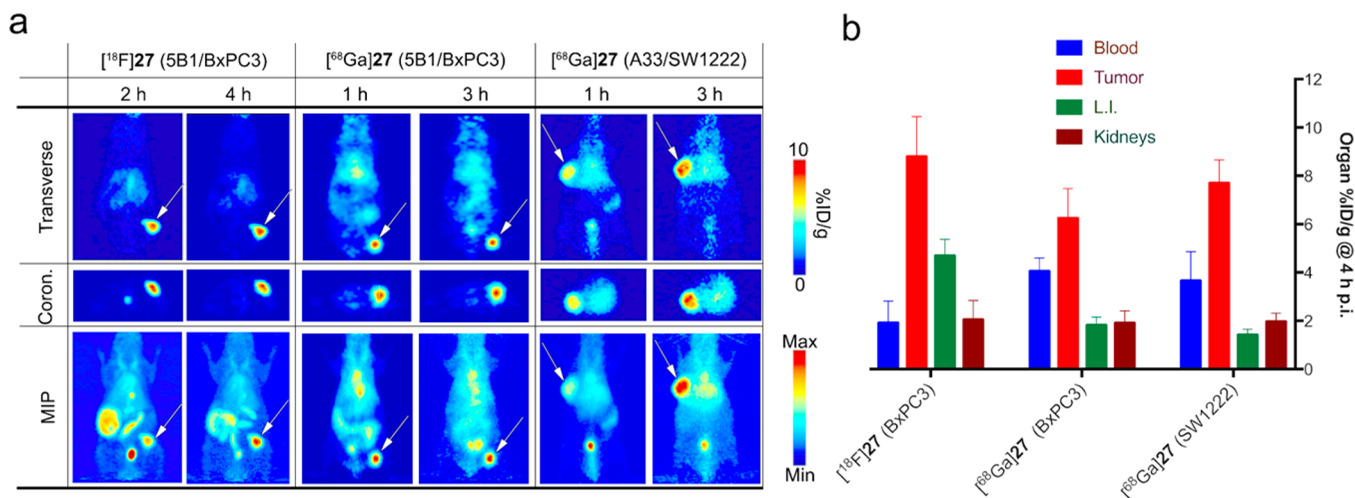


Figure 6. (a) Transverse (top) and coronal (middle) slices as well as MIPs (bottom) of pretargeted PET imaging experiments using lead compounds [¹⁸F]27 (left) and [⁶⁸Ga]27 (center) in the BxPC3-based PDAC model. PET images show high tumor uptake of >6%ID/g 2 h p.i. In addition, the ⁶⁸Ga-labeled tracer [⁶⁸Ga]27 was tested in a pretargeting study using a subcutaneous CRC model (SW1222, right). The gpA33-targeting immunoconjugate huA33-TCO (0.95 nmol) was injected 48 h prior to tracer [⁶⁸Ga]27. Good delineation of tumor tissue was achieved as early as 1 h p.i., with the tumor showing highest uptake at 3 h p.i. These data confirming results obtained from the PDAC model and supported the overall promising performance of both radioligands. (b) Quantitative comparison of uptake values calculated for tumor, blood, large intestines, and kidneys. [¹⁸F]27 showed overall the highest tumor uptake (8.8 ± 1.7%ID/g) but also the highest residual radioactivity in the intestines. [⁶⁸Ga]27 showed highest tumor-to-clearance organ ratios in both models but exhibited relatively high residual blood radioactivity concentrations, most likely due to residual antibody in the bloodstream at the time of tracer injection.

as speed, selectivity, and bioorthogonality, the IEDDA reaction is currently regarded as an approach with high clinical potential. Despite its popularity, limited efforts to investigate the influence of small molecule PK have been reported. Thus, a comprehensive investigation of how small-molecule in vivo behavior would

significantly improve our current understanding of how molecular parameters influence pretargeting performance and increase the potential for designing and identifying clinical candidates. The feasibility of combining the pretargeting approach with short-lived ($t_{1/2} < 2$ h) radionuclides was only recently demonstrated.^{1,21}

Improving the small molecule's PK in order to achieve high target-to-NT activity concentration ratios as early as possible after tracer administration would be highly valuable from a clinical perspective. To achieve this goal, effector molecules with the ability to accumulate quickly at the target site while simultaneously exhibiting short off-target residence times are needed. Taken together, these issues motivated us to pursue a comprehensive SPR study. The study at hand includes the *in vitro* and *in vivo* evaluation of 25 novel ^{18}F - and ^{68}Ga -labeled Tz-derived radioligands capable of *in vivo* click reaction with clinically validated mAbs as pretargeting constructs.

Molecular and structural parameters were correlated with *in vitro* and *in vivo* results in order to investigate relationships between structure and *in vivo* behavior. Overall, four major trends were observed. First, the overall molecular charge of the radioligand had a significant impact on *in vivo* behavior, as high net charge reduced lipophilicity and shortened the PHL. Second, the introduction of amino acid residues into the radioligand structure did not only lead to reduced circulation times but also influenced whether the tracer was eliminated renally or hepatically. Charged molecules were predominantly cleared through glomerular filtration in the kidneys, whereas compounds without an overall net charge displayed higher uptake in the liver and intestines. Importantly, the introduction of lysine residues into the radioligand structure dramatically decreased the tracer's PHL (<6 min), while it was quickly and significantly taken up and retained in the proximal tubules of the kidneys. Kidney uptake values of these radioligands were up to 20%ID/g at 3 h p.i. (^{68}Ga]**22**), while their tumoral uptake values were dramatically reduced to <2%ID/g. Those results appeared to be in line with recently published data demonstrating that peptide sequences high in lysine residues are powerful delivery agents to specifically target the proximal tubules of the kidneys.^{29,30} Third, the substitution of lysine by other amino acids (such as arginine) resulted in a significant reduction of kidney targeting ability. Those results are consistent with data presented in this study, showing that kidney uptake was decreased >4-fold when lysine was substituted by an arginine or histidine. Most notably, the incorporation of a lysine residue into the structure of ^{18}F]**25** from radioligand ^{18}F]**29** dramatically changed *in vivo* behavior. Compared to ^{18}F]**25**, ^{18}F]**29** exhibited a significantly reduced PHL and a lower tumor uptake value of $2.9 \pm 0.3\%$ ID/g at 2 h p.i., with the majority of radioactive tracer being accumulated in the kidneys ($8.3 \pm 2.1\%$ ID/g, Figure 6a, Supporting Information, section 7), and finally, tumor uptake was mainly determined by the PHL of the radioligand. We found a significant and positive correlation between a radioligand's tumor uptake and PHL (p -value <0.0001), explaining the observations made in our *in vivo* studies. The PHL of a radioligand had to be sufficiently long, approximately >10 min, to enable effective tumor accumulation.

Overall, we observed that the incorporation of amino acid residues, and with that the alteration of molecular net charge, had a more profound effect on tracer PK than the introduction of PEG-linkers alone. Although PEG-linker increased the kidney-to-intestines uptake ratios as expected, no change in PHL could be observed. Instead, our results suggest that the molecular net charge had the most significant impact on radioligand PK.

Lead compounds ^{18}F]**27** and ^{68}Ga]**27** contained PEG₁₁ linkers, exhibited PHLs of approximately 15 min, and possessed net molecular charges of 0 and +1, respectively. Even though the ^{18}F -labeled tracer ^{18}F]**27** was predominantly excreted via the intestines, it showed the highest overall tumoral uptake of all

radioligands investigated, with $8.8 \pm 1.7\%$ ID/g at 4 h p.i. The ^{68}Ga -labeled lead compound ^{68}Ga]**27** was tested in two tumor models with different targeting vectors and showed high tumor uptake values in both models at 4 h p.i.: $6.9 \pm 1.8\%$ ID/g (PDAC, BxPC3) and $7.7 \pm 1\%$ ID/g (CRC, SW1222). However, as for all other radioligands that were employed in pretargeting experiments, both lead compounds showed significant residual blood radioactivity concentrations that cannot be explained by the clearance of the tracer alone (Supporting Information, section 7). Instead, we believe that residual mAb circulating in the blood at the time of tracer injection and subsequent click chemistry in circulation led to elevated blood radioactivity concentrations. We applied the established accumulation intervals between the mAb-TCO and tracer injections of several days (2 and 3 days for huA33 and 5B1, respectively) to allow mAb accumulation at the target site and clear from circulation. Because of the inherent instability of the TCO group (formation of the more stable *cis*-isomer over time)^{3,31} and internalization of some antibodies (e.g., 5B1) the accumulation interval cannot be extended infinitely. Instead, a mAb-dependent time interval between the injections of mAb-TCO and the Tz probe has to be determined.³ Indeed, pretargeting studies conducted by our groups that used longer accumulation intervals for 5B1-TCO did not lead to dramatically improved tumor-to-blood activity concentration ratios.² Although a decreased antibody concentration in the blood pool can be achieved, the increased decomposition of TCO at the tumor site led to overall reduced tumoral uptake and TTB values.² In case of noninternalizing huA33-TCO, however, longer intervals of up to 120 h yielded slightly higher TTB ratios.³ New technologies such as innovative clearing agents³³ or the use of smaller antibody fragments have the potential to further improve this strategy by reducing residual activity concentrations in the blood.

We initially speculated that the pretargeting performance of a radioligand could be improved by solely increasing the renal-to-gut excretion ratio. However, our data suggest that it is not that simple. To facilitate sufficient delivery of radioligand to the tumor site a compromise, or "sweet spot", between the excretion pathways, PHL, and tumor signal needs to be identified, and that achieving high on one of those parameters will most likely have a negative impact on the others. As an example, short PHL and fast organ clearance had a negative effect on tumor accumulation. On the other hand, molecules with no net charge or low overall net charge exhibited longer PHLs and higher tumoral uptake values but also higher background noise levels due to a slower excretion predominantly via the intestines.

At this point of the discussion, it is important to highlight the focus of this study to develop radioligands specifically for pretargeting approaches utilizing short-lived radionuclides with physical half-lives <2 h. Short physical half-lives, however, limit the time window for high-quality PET image acquisition to a maximum of 6–8 h after tracer injection (preclinically). Thus, radioligands have to possess PK properties that allow for successful tumor visualization with high tumor-to-background ratios at early time points, preferably between 1 and 4 h p.i. Conversely, approaches that apply longer-lived radionuclides such as ^{64}Cu ($t_{1/2} = 12.7$ h) allow image acquisition at later time points (up to 48 h p.i.) and are thus less sensitive to early radioligand PK. At the same time, it is important to note that time restraints are easily justified and out-weighed by the dosimetric advantages that the use of short-lived radionuclides entails. In addition, the ability of virtually any major hospital and cancer center to produce ^{68}Ga - and ^{18}F -labeled clinical-grade

Table 2. Summary of Purities and Chemical Yields (Last Step of Synthesis) Obtained for All Precursor Molecules 15–32^a

precursor	purity [%]	yield [%]	tracer	RC purity [%]	dc RC yield [%]	SA [MBq/nmol]
15	>97	58	[¹⁸ F]15	>98	44 ± 8	23 ± 3
16	>96	90	[¹⁸ F]16	>98	49 ± 5	28 ± 2
17	>96	83	[¹⁸ F]17	>99	64 ± 7	38 ± 4
18	>97	73	[¹⁸ F]18	>98	56 ± 4	31 ± 7
19	>99	98	[¹⁸ F]19	>97	38 ± 5	29 ± 3
			[⁶⁸ Ga]19	>98	85 ± 7	24 ± 5
20	>98	77	[¹⁸ F]20	>98	51 ± 3	33 ± 5
			[⁶⁸ Ga]20	>98	91 ± 4	26 ± 4
21	>97	86	[¹⁸ F]21	>97	62 ± 8	37 ± 5
			[⁶⁸ Ga]21	>98	78 ± 12	27 ± 3
22	>97	94	[⁶⁸ Ga]22	>98	88 ± 6	22 ± 2
23	>98	89	[¹⁸ F]23	>97	38 ± 4	29 ± 5
24	>96	81	[¹⁸ F]24	>96	60 ± 6	44 ± 7
			[⁶⁸ Ga]24	>99	87 ± 5	29 ± 5
25	>95	77	[¹⁸ F]25	>97	44 ± 9	39 ± 4
26	>98	45	[⁶⁸ Ga]26	>98	78 ± 4	36 ± 5
27	>98	73	[¹⁸ F]27	>98	66 ± 9	46 ± 4
			[⁶⁸ Ga]27	>98	86 ± 3	36 ± 5
28	>97	73	[¹⁸ F]28	>97	49 ± 11	48 ± 3
			[⁶⁸ Ga]28	>98	77 ± 3	38 ± 3
29	>97	81	[¹⁸ F]29	>97	62 ± 9	46 ± 6
30	>98	91	[¹⁸ F]30	>98	51 ± 3	41 ± 4
			[⁶⁸ Ga]30	>99	90 ± 5	37 ± 2
31	>95	75	[¹⁸ F]31	>95	61 ± 9	39 ± 6
32	>97	74	[¹⁸ F]32	>97	42 ± 8	40 ± 4

^aOn the right hand site are the radiochemical (RC) purity and the decay-corrected (dc) RC yields that were observed for all tracers that were evaluated in this study (all *n* = 3).

radiopharmaceuticals on site and on a daily basis is a tremendous logistical advantage and thus justifies the development of these tracers.

Ultimately, this SPR study revealed fundamental relationships between the structure and in vivo behavior of tetrazine-based radioligands, allowing for a more rational approach to design next-generation radiopharmaceuticals for in vivo pretargeting. As discussed earlier, the second goal of this study was to generate candidates worthy of clinical translation. We believe that with lead compounds [¹⁸F]27 and [⁶⁸Ga]27, we have identified two promising candidates that show high tumor targeting abilities and favorable dosimetries, justifying an IND application in the near future.

EXPERIMENTAL SECTION

General Information. All reactions involving tetrazine moieties were carried out using aluminum foil covered reaction vials due to light sensitivity of the tetrazine structure. Further, all reaction mixtures were purified using preparative HPLC with the following gradient: 5–95% of MeCN in H₂O over 20 min, 8 mL/min. The final purity of all molecules was determined using analytical HPLC (see Table 2 for precursors 15–32; gradient 5–95% of MeCN in H₂O over 20 min, 1 mL/min) to achieve purities of >95%. Retention times (*R_t*) were assigned to all compounds. Upon purification and freeze-drying, all final precursor compounds 15–32 were aliquoted into 25 μL (50 nmol) fractions using metal-free, anhydrous DMSO. It is worth noting that reaction conditions, in particular for the formation of amide bonds, had to be designed with respect to the well-known sensitivity of tetrazines to harsh synthetic conditions, such as high pH and elevated temperatures.^{23,34} For instance, the presence of bases such as pyridine, triethylamine, and reagents such as *N,N'*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide used for in situ carboxylic acid activation, led to decomposition of the tetrazine moiety, which was easily visible by eye due to loss of purple color and the absence of absorption at 525 nm. To overcome this

limitation, we found that amide bonds could be formed in high yields and without impairing the tetrazine moiety by using 2 equiv of both 1-ethyl-3-(3-demethylamunopropyl)carbodiimide and hydroxybenzotriazole in dry DMF or DMSO. The procedures and analytical data for the synthesis of all precursor molecules can be found in the Supporting Information (sections 2 and 3). Summarized below (Table 2) are the yield and purity data obtained for all precursor and tracer molecules. Further, the general ¹⁸F and ⁶⁸Ga-labeling procedures that were developed for this study are presented.

Synthesis of Precursor Molecules 15–32. 4-(1,2,4,5-Tetrazin-3-yl)benzoic Acid. The title compound was synthesized according to the previously published procedure from Karver et al.²³ Obtained analytical data were consistent with the reported data. ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.66 (s, 1H), 8.62 (d, *J* = 8.3 Hz, 2H), 8.22 (d, *J* = 8.3 Hz, 2H). MS (ESI) *m/z* [*M* + *Cl*][−]: 239.2.

2,2'-(7-(4-(1,2,4,5-Tetrazin-3-yl)benzamido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic Acid (Tz-1-NODA, 15). 4-(1,2,4,5-Tetrazin-3-yl)benzoic acid (26.7 μmol) was dissolved in DMSO (0.5 mL) before p-Bn-NODA-NCS (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 overnight under high vacuum. Precursor 15 (purity >97%) was obtained as pink oil (8.9 mg, 58%). ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.32 (s, 1H), 8.41 (d, *J* = 8.2 Hz, 2H), 8.29 (d, *J* = 8.3 Hz, 2H), 7.62 (d, *J* = 7.8 Hz, 2H), 7.37 (d, *J* = 7.5 Hz, 2H), 4.16 (s, 2H), 3.12–3.04 (m, 12H), 2.42–2.11 (m, 5H). MS (ESI) *m/z* [*M* + *H*]⁺: 535.4. HRMS (ESI) *m/z* calcd for C₂₆H₃₀N₈NaO₅ [*M* + *Na*]⁺ 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). Tz 1 (5 mg, 26.7 μmol) was dissolved in dry DMF (0.5 mL) under nitrogen atmosphere before Boc-lysine (5.8 mg, 29 μmol), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (10.7 mg, 60 μmol), and hydroxybenzotriazole (6.8 mg, 60 μmol) 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 (ppm): 10.41 (s, 1H), 8.52 (t, J = 7.9 Hz, 1H), 8.40 (d, J = 7.9 Hz, 2H), 7.76 (s, 1H), 7.54 (d, J = 8.0 Hz, 2H), 6.99 (d, J = 7.7 Hz, 1H), 3.00 (s, 2H), 2.84–2.73 (m, 3H), 1.70–1.49 (m, 4H), 1.42 (s, 9H). (ESI) m/z $[\text{M} + \text{H}]^+$: 431.5.

2,2',2''-(2-(4-(6-(4-(1,2,4,5-Tetrazin-3-yl)benzamido)-2-((tert-butoxycarbonyl)amino)-hexanamido)-benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid (Tz-1-lysine-Boc-NOTA). The title compound was obtained using Tz-1-Lysine-Boc (5 mg, 12.1 μmol) 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 (ppm): 10.20 (s, 1H), 8.53 (d, J = 8.0 Hz, 2H), 7.73 (d, J = 7.9 Hz, 2H), 7.56 (d, J = 8.2 Hz, 2H), 7.47 (d, J = 7.9 Hz, 2H), 7.37 (t, J = 7.9 Hz, 3H), 7.31–7.25 (m, 7H), 6.72 (s, 1H), 5.52 (s, 1H), 4.49 (dd, J = 9.8, 6.8 Hz, 7H), 4.16 (d, J = 21.1 Hz, 2H), 3.12–3.09 (m, 4H), 2.02–1.71 (m, 9H), 1.41 (s, 9H). (ESI) m/z $[\text{M} + \text{H}]^+$: 821.7.

2,2',2''-(2-(4-(6-(4-(1,2,4,5-Tetrazin-3-yl)benzamido)-2-aminohexanamido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid (16). Tz-1-Lysine-Boc-NOTA (7 μmol) 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 (ppm): 10.10 (s, 1H), 8.42 (d, J = 8.1 Hz, 2H), 7.78 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 9.1 Hz, 2H), 7.34 (d, J = 8.9 Hz, 2H), 6.72 (s, 1H), 5.52 (s, 1H), 4.49 (m, 1H), 4.16 (m, 2H), 3.82–3.69 (m, 6H), 3.12–3.09 (m, 9H), 2.02–1.71 (m, 15H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 721.4. HRMS (ESI) calcd for $\text{C}_{34}\text{H}_{44}\text{N}_{14}\text{NaO}_8$ $[\text{M} + \text{Na}]^+$ 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-heptaosa-2-azapentacosan-25-yl)carbamate (Tz-1-PEG₇-NH₂Boc). Tz-1 (10 mg, 0.05 mmol) was dissolved in anhydrous dimethyl sulfoxide (DMSO, 0.5 mL) before O-(2-aminoethyl)-O-[2-(boc-amino)ethyl]hexaethylene glycol (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 (ppm): 10.21 (s, 1H), 8.58 (d, J = 8.2 Hz, 2H), 7.54 (d, J = 8.2 Hz, 2H), 6.89 (m, 2H), 6.45 (m, 3H), 5.08–4.88 (m, 4H), 4.56 (d, J = 8.0 Hz, 2H), 3.63 (d, J = 8.0 Hz, 2H), 3.54 (dt, J = 10.4, 4.9 Hz, 6H), 3.42 (q, J = 5.2 Hz, 3H), 3.33–3.27 (m, 5H), 2.37 (t, J = 10.2 Hz, 2H), 2.29 (d, J = 6.9 Hz, 2H), 2.01 (q, J = 7.1 Hz, 3H), 1.44 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 653.6.

N-(23-Amino-3,6,9,12,15,18,21-heptaotricosyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (Tz-1-PEG₇-NH₂). The title compound was obtained from the Boc-protected starting material Tz-1-PEG₇-NH₂Boc (5 mg, 6.7 μmol) 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 (ppm): 10.17 (s, 1H), 8.47 (d, J = 8.1 Hz, 2H), 7.52 (d, J = 8.2 Hz, 2H), 6.58 (m, 2H), 6.32–6.12 (m, 3H), 5.11–5.02 (m, 4H), 4.46 (d, J = 6.0 Hz, 2H), 3.76 (d, J = 6.0 Hz, 2H), 3.42 (dt, J = 10.4, 4.9 Hz, 6H), 3.21 (q, J = 5.2 Hz, 3H), 3.33–3.27 (m, 6H), 2.37 (t, J = 10.2 Hz, 2H), 2.29 (d, J = 6.9 Hz, 2H), 2.01 (q, J = 7.1 Hz, 3H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 553.8.

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-heptaosa-2-azapenta-cosan-25-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid (17). Precursor **17** was obtained using the starting material Tz-1-PEG₇-NH₂ (3.5 mg, 5.4 μmol) and p-Bn-NOTA-NCS (2.7 mg, 6 μmol) 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 (ppm): 10.51

(s, 1H), 8.39 (d, J = 7.7 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 7.12 (d, J = 8.1 Hz, 2H), 4.32 (d, J = 5.9 Hz, 3H), 3.92 (d, J = 17.9 Hz, 3H), 3.74 (d, J = 18.0 Hz, 3H), 3.56 (d, J = 5.8 Hz, 2H), 3.48 (d, J = 5.5 Hz, 2H), 3.33 (t, J = 8.2 Hz, 3H), 3.21–3.08 (m, 16H), 3.01–2.90 (m, 18H), 2.11 (t, J = 7.9 Hz, 2H), 2.03 (t, J = 5.4 Hz, 2H), 1.69 (p, J = 7.2 Hz, 3H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1004.1. HRMS (ESI) m/z calcd for $\text{C}_{45}\text{H}_{66}\text{N}_{10}\text{NaO}_{14}\text{S}$ $[\text{M} + \text{Na}]^+$ 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-undecaosa-2-azaheptatriacontan-37-yl)carbamate (Tz-1-PEG₁₁-NH₂Boc). The title compound was prepared according to the preparation of Tz-1-PEG₇-NH₂Boc 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 (ppm): 10.65 (s, 1H), 8.77 (t, J = 6.4 Hz, 1H), 8.65–8.57 (m, 2H), 8.16–8.12 (m, 2H), 6.77–6.73 (m, 7H), 3.62–3.42 (m, 37H), 3.38 (t, J = 6.3 Hz, 3H), 3.07 (q, J = 5.8 Hz, 2H), 1.38 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 829.8.

N-(35-Amino-3,6,9,12,15,18,21,24,27,30,33-undecaaxapentatriacontyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (Tz-1-PEG₁₁-NH₂). The title compound was furnished using Tz-1-PEG₁₁-NH₂Boc (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 (ppm): 10.59 (s, 1H), 8.74 (t, J = 5.2 Hz, 1H), 8.61–8.55 (m, 2H), 8.12–8.09 (m, 2H), 6.79–6.74 (m, 1H), 3.68–3.36 (m, 42H), 3.34 (t, J = 6.1 Hz, 4H), 3.10 (q, J = 6.0 Hz, 2H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 829.7. HRMS (ESI) m/z calcd for $\text{C}_{33}\text{H}_{57}\text{N}_{10}\text{O}_{12}$ $[\text{M} + \text{H}]^+$ 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-undecaosa-2-azaheptatriacontan-37-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid (Tz-1-PEG₁₁-NOTA, 18). 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/H 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 (ppm): 10.59 (s, 1H), 8.47 (d, J = 7.3 Hz, 2H), 7.87 (t, J = 7.2 Hz, 3H), 7.55 (d, J = 7.6 Hz, 3H), 7.43 (d, J = 7.5 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, 49H), 2.20 (t, J = 7.4 Hz, 3H), 2.12 (t, J = 5.5 Hz, 3H), 1.78–1.68 (m, 4H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1180.9. HRMS (ESI) m/z calcd for $\text{C}_{53}\text{H}_{81}\text{N}_{10}\text{O}_{18}\text{S}$ $[\text{M} - \text{H}]^-$ 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). The title compound was synthesized following the general amide coupling procedure as described for compound Tz-1-Lysine-NH₂Boc 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 overnight on the lyophilizer. The title compound was furnished as pink oil (6.8 mg, 59%). ^1H NMR (500 MHz, DMSO- d_6) δ (ppm): 8.52 (t, J = 5.9 Hz, 1H), 8.40 (d, J = 5.9 Hz, 2H), 7.76 (s, 2H), 7.54 (d, J = 8.0 Hz, 2H), 6.99 (d, J = 7.9 Hz, 1H), 3.00 (s, 3H), 2.84–2.73 (m, 2H), 1.70–1.49 (m, 3H), 1.42 (s, 6H), 1.35 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 430.5.

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). 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 (ppm): 8.50 (t, $J = 6.1$ Hz, 1H), 8.41 (d, $J = 6.2$ Hz, 1H), 7.98–7.88 (m, 2H), 7.56 (dd, $J = 12.6, 8.2$ Hz, 2H), 7.45 (d, $J = 8.3$ Hz, 2H), 6.98 (d, $J = 12.8$ Hz, 1H), 4.42 (d, $J = 5.9$ Hz, 1H), 4.32 (s, 1H), 3.96 (d, $J = 5.9$ Hz, 2H), 3.50 (s, 2H), 3.47 (s, 2H), 3.34 (s, 2H), 3.31 (s, 1H), 3.22–3.11 (m, 1H), 3.05 (dd, $J = 13.5, 5.9$ Hz, 1H), 2.99 (s, 1H), 2.82 (d, $J = 8.2$ Hz, 2H), 2.7–2.60 (m, 7H), 1.71–1.64 (m, 9H), 1.62–1.51 (m, 8H), 1.41 (s, 1H), 1.35 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 881.1.

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). 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 (ppm): 8.45 (t, $J = 6.0$ Hz, 1H), 8.40 (d, $J = 6.1$ Hz, 1H), 7.98–7.82 (m, 2H), 7.56 (dd, $J = 12.5, 7.4$ Hz, 2H), 7.41 (d, $J = 12.2$ Hz, 2H), 6.98 (d, $J = 7.4$ Hz, 1H), 4.42 (d, $J = 5.9$ Hz, 1H), 4.32 (s, 1H), 3.96 (d, $J = 5.9$ Hz, 2H), 3.70 (s, 2H), 3.57 (s, 2H), 3.44 (s, 2H), 3.36 (s, 1H), 3.32–3.21 (m, 1H), 3.12 (dd, $J = 13.5, 5.9$ Hz, 1H), 2.99 (s, 1H), 2.92 (d, $J = 8.1$ Hz, 2H), 2.70–2.51 (m, 7H), 2.21–1.94 (m, 9H), 1.92–1.81 (m, 10H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 780.9. HRMS (ESI) m/z calcd for $\text{C}_{36}\text{H}_{49}\text{N}_{11}\text{NaO}_7\text{S}$ $[\text{M} + \text{Na}]^+$ 802.8435, 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)]. The title compound was synthesized using the general amide coupling procedure as presented for Tz-2-Lysine-Boc. 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 μmol), Boc-lysine (11.8 mg, 60 μmol), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (21.4 mg, 120 μmol), and hydroxybenzotriazole (13.6 mg, 120 μmol). 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 trilycine compounds MeTz-lysine-Boc-NH₂ (3.1 mg, 34%) and MeTz-(Lysine-Boc)₃-NH₂ (4.2 mg, 28%), respectively. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 8.47 (t, $J = 6.0$ Hz, 1H), 8.40 (d, $J = 8.2$ Hz, 1H), 7.78 (t, $J = 6.1$ Hz, 1H), 7.66 (s, 1H), 7.53 (d, $J = 8.2$ Hz, 1H), 6.94 (d, $J = 8.0$ Hz, 1H), 6.76 (d, $J = 8.0$ Hz, 1H), 4.41 (s, 1H), 3.97–3.89 (m, 10H), 3.87–3.80 (m, 4H), 3.11 (s, 3H), 3.00 (s, 1H), 2.76 (q, $J = 7.7, 6.4$ Hz, 1H), 1.64–1.47 (m, 2H), 1.41 (s, 3H), 1.38 (s, 4H), 1.32–1.21 (m, 15H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 658.8.

2,2',2''-(2-(4-(3-(5-(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-triyl)triacetic Acid [Tz-2-(lysine-Boc)₂-NOTA]. The title compound was obtained using Tz-2-(Lysine-Boc)₂ (5 mg, 7.6 μmol) 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, $\text{DMSO}-d_6$) δ (ppm): 8.42 (t, $J = 6.1$ Hz, 1H), 8.40 (d, $J = 6.2$ Hz, 1H), 7.88–7.72 (m, 2H), 7.56 (dd, $J = 12.5, 7.4$ Hz, 2H), 7.41 (d, $J = 7.2$ Hz, 2H), 6.98 (d, $J = 12.6$ Hz, 1H), 4.42 (d, $J = 5.7$ Hz, 1H), 4.32 (s, 1H), 3.96 (d, $J = 5.7$ Hz, 2H), 3.70 (s, 2H), 3.57 (s, 2H), 3.44 (s, 2H), 3.36 (s, 1H), 3.32–3.21 (m, 1H), 3.12 (dd, $J = 13.5, 5.9$ Hz, 1H), 2.99 (d, $J = 13.4$ Hz, 1H), 2.91 (d, $J = 5.9$ Hz, 1H), 2.60–2.48 (m, 8H), 2.34–2.03 (m, 10H), 1.82–1.71 (m, 15H), 1.53 (s, 9H), 1.47 (s, 10H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1109.4.

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 (20). Starting from MeTz-(lysine-Boc)₂-NOTA (6.3 mg, 5.6 μmol), 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, $\text{DMSO}-d_6$) δ (ppm): 9.11 (t, $J = 5.9$ Hz, 1H), 8.47 (d, $J = 5.9$ Hz, 1H), 8.23–8.18 (m, 2H), 8.16–8.07 (m, 2H), 7.74 (s, 1H), 7.59 (s, 1H), 4.62–4.41 (m, 1H), 3.90–3.79 (m, 8H), 3.74–3.64 (m, 5H), 3.10 (q, $J = 6.6$ Hz, 1H), 3.01 (s, 1H), 2.81–2.69 (m, 2H), 1.83–1.73 (m, 2H), 1.72–1.65 (m, 10H), 1.57–1.49 (m, 12H), 1.48–1.42

(m, 4H), 1.39–1.21 (m, 8H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 909.4. HRMS (ESI) m/z calcd for $\text{C}_{42}\text{H}_{61}\text{N}_{13}\text{NaO}_8\text{S}$ $[\text{M} + \text{Na}]^+$ 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]. 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, $\text{DMSO}-d_6$) δ (ppm): 8.47 (t, $J = 6.0$ Hz, 1H), 8.40 (d, $J = 8.0$ Hz, 2H), 7.76 (d, $J = 6.1$ Hz, 2H), 7.63 (s, 3H), 7.53 (d, $J = 8.0$ Hz, 2H), 6.93 (d, $J = 7.9$ Hz, 1H), 6.74 (d, $J = 8.0$ Hz, 2H), 4.41–4.28 (m, 2H), 3.92 (d, $J = 7.6$ Hz, 1H), 3.82 (d, $J = 7.7$ Hz, 2H), 3.00 (s, 7H), 2.83–2.70 (m, 6H), 1.67–1.44 (m, 13H), 1.53–1.40 (m, 27H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 887.2.

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-triazatetracosan-24-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid [Tz-2-(lysine-Boc)₃-NOTA]. 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 (ppm): 8.47 (t, $J = 6.0$ Hz, 1H), 8.40 (d, $J = 8.0$ Hz, 2H), 7.76 (d, $J = 6.1$ Hz, 2H), 7.63 (s, 3H), 7.53 (d, $J = 8.0$ Hz, 2H), 6.93 (d, $J = 7.9$ Hz, 1H), 6.74 (dd, $J = 19.2, 8.0$ Hz, 2H), 4.66 (s, 2H), 4.41 (d, $J = 5.9$ Hz, 2H), 3.92 (d, $J = 7.6$ Hz, 1H), 3.82 (d, $J = 7.7$ Hz, 2H), 3.00 (s, 7H), 3.56–3.49 (m, 10H), 2.83–2.70 (m, 6H), 2.53–2.41 (m, 13H), 1.67–1.44 (m, 13H), 1.53–1.40 (m, 27H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1337.8.

2,2',2''-(2-(4-(3-(5-Amino-6-((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). 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, $\text{DMSO}-d_6$) δ (ppm): 8.61 (s, $J = 6.5$ Hz, 1H), 8.32 (d, $J = 8.0$ Hz, 2H), 7.76 (d, $J = 6.4$ Hz, 2H), 7.53–7.49 (m, 2H), 7.47 (d, $J = 8.0$ Hz, 2H), 6.81 (d, $J = 7.9$ Hz, 1H), 6.74 (dd, $J = 19.2, 8.0$ Hz, 2H), 4.66 (s, 2H), 4.32 (m, 2H), 3.92 (d, $J = 7.1$ Hz, 1H), 3.82 (d, $J = 7.1$ Hz, 2H), 3.51–3.40 (m, 7H), 3.32–3.29 (m, 18H), 2.72–2.60 (m, 5H), 2.63–2.44 (m, 12H), 1.61–1.42 (m, 11H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1037.3. HRMS (ESI) m/z calcd for $\text{C}_{48}\text{H}_{73}\text{N}_{15}\text{NaO}_9\text{S}$ $[\text{M} + \text{Na}]^+$ 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-diyl)diacetic Acid [Tz-2-(lysine-Boc)₃-NODA]. The title compound was obtained using MeTz-(Lysine-Boc)₃-NH₂ (3.5 mg, 2.9 μmol) 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 (ppm): 8.47 (t, $J = 6.0$ Hz, 1H), 8.40 (d, $J = 8.0$ Hz, 2H), 7.76 (d, $J = 6.1$ Hz, 2H), 7.63 (s, 3H), 7.53 (d, $J = 8.0$ Hz, 2H), 6.93 (d, $J = 7.9$ Hz, 1H), 6.74 (dd, $J = 19.2, 8.0$ Hz, 2H), 4.66 (s, 2H), 4.41 (d, $J = 5.9$ Hz, 2H), 3.92 (d, $J = 5.9$ Hz, 1H), 3.82 (d, $J = 7.7$ Hz, 2H), 3.00 (s, 7H), 3.56–3.49 (m, 9H), 2.89–2.70 (m, 10H), 2.53–2.41 (m, 6H), 1.77–1.71 (m, 11H), 1.67–1.32 (m, 29H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1279.8.

2,2'-(7-(4-(3-(5-Amino-6-((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-diyl)diacetic Acid (22). The title compound was obtained using MeTz-(lysine-Boc)₃-NODA (3.5 mg, 2.7 μmol) 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, $\text{DMSO}-d_6$) δ (ppm): 8.79 (t, $J = 5.4$ Hz, 2H), 8.60 (d, $J = 8.4$ Hz, 2H), 8.13 (d, $J = 8.4$ Hz, 2H), 7.43 (d, $J = 5.3$ Hz, 2H), 7.20 (d, $J = 8.3$ Hz, 1H), 7.11 (d, $J = 8.2$ Hz, 1H), 4.00 (d, $J = 17.6$ Hz, 2H), 3.81 (d, $J = 17.4$ Hz, 3H), 3.64 (s, 3H), 3.57–3.45 (m, 39H), 3.31–3.20 (m, 1H), 3.05–2.94 (m, 5H), 2.86–2.74 (m, 8H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 988.4.

4-((4-(6-Methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-4-oxobutanoic Acid (Tz-2-succinate). 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 (ppm): 8.60 (d, J = 8.4 Hz, 2H), 8.13 (d, J = 8.4 Hz, 2H), 7.73 (s, 1H, NH), 4.21 (s, 2H), 3.81–3.77 (m, 2H), 3.57–3.49 (m, 2H), 2.74 (s, 3H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 302.4.

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-NHBoc). The title compound was obtained from Tz-2-succinate (10 mg, 30.1 μ mol) and NH₂-PEG₁₁-NHBoc (1.2 equiv) 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 (ppm): 8.79 (t, J = 5.4 Hz, 2H), 8.60 (d, J = 5.4 Hz, 2H), 4.21 (s, 2H), 4.08–4.02 (m, 5H), 3.81–3.77 (m, 2H), 3.57–3.49 (m, 2H), 3.47–3.33 (m, 24H), 2.74 (s, 3H), 2.67–2.46 (m, 24H), 1.45 (s, 7H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 929.2.

*N*¹-(35-Amino-3,6,9,12,15,18,21,24,27,30,33-undeca-oxapentatriacontyl)-*N*⁴-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)succinamide (Tz-2-PEG₁₁-NH₂). 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 (ppm): 8.58 (d, J = 5.9 Hz, 2H), 8.67 (d, J = 5.9 Hz, 2H), 4.21 (s, 2H), 4.18–4.12 (m, 4H), 3.77–3.69 (m, 9H), 3.52–3.32 (m, 8H), 3.27–3.13 (m, 21H), 2.71 (s, 3H), 2.44–2.26 (m, 14H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 828.9.

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-undeca-oxa-2,7-diazadotetracontan-42-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)-triacetic Acid (23). The title compound was obtained using methyl-tetrazine-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 (ppm): 8.43 (d, J = 5.7 Hz, 2H), 7.54 (d, J = 5.7 Hz, 2H), 7.23 (d, J = 8.6 Hz, 2H), 7.17 (d, J = 8.5 Hz, 2H), 7.11 (s, 1H), 6.56 (s, 2H), 4.27–4.23 (m, 7H), 3.83–3.79 (m, 8H), 3.70 (t, J = 6.3 Hz, 7H), 3.65–3.61 (m, 4H), 3.59–3.49 (m, 49H), 2.97–2.76 (m, 6H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1279.5. HRMS (ESI) m/z calcd for C₅₈H₉₁N₁₁NaO₁₉S $[\text{M} + \text{Na}]^+$ 1301.5836, found 1301.5831.

6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (3). 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 (ppm): 8.89 (s, 1H), 8.53 (s, 1H), 8.58–8.51 (m, 2H), 7.99–7.92 (m, 2H), 7.61 (d, J = 8.5 Hz, 1H), 7.48 (m, 1H), 7.10–6.99 (dd, J = 8.6 Hz, 2.7 Hz, 1H), 5.78 (s, 2H).

4-Oxo-4-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)butanoic Acid (Tz-3-succinate). 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 (ppm): 8.95 (s, 1H), 8.79 (t, J = 5.4 Hz, 1H), 8.60 (t, J = 8.4 Hz, 1H), 8.33 (d, J = 8.4 Hz, 1H), 8.29 (dd, J = 5.4 Hz, 1H), 8.10 (dd, J = 8.6 Hz, 1H), 7.52–7.44 (m, 2H), 7.13–7.10 (m, 1H), 2.72–2.63 (m, 4H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 352.4.

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-undeca-oxa-36-azatetracontyl)carbamate (Tz-3-PEG₁₁-NHBoc). 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 (ppm): 10.16 (s, 1H), 9.09–9.03 (m, 2H), 8.98 (d, J = 5.6 Hz, 1H), 8.72 (dd, J = 11.1, 8.3 Hz, 2H), 8.55 (dd, J = 8.4, 2.4 Hz, 1H), 8.01 (dd, J = 8.3, 2.2 Hz, 1H), 7.60–7.56 (m, 2H), 3.68–3.56 (m, 37H), 3.55–3.51 (m, 4H), 3.48 (t, J = 7.7 Hz, 3H), 3.30 (s, 4H), 2.84 (t, J = 7.6 Hz, 3H), 2.71–2.67 (m, 1H), 1.44 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 979.3.

*N*¹-(35-Amino-3,6,9,12,15,18,21,24,27,30,33-undeca-oxapentatriacontyl)-*N*⁴-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)-

succinamide (Tz-3-PEG₁₁-NH₂). 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 (ppm): 8.62 (dd, J = 15.9, 8.2 Hz, 1H), 8.42 (dd, J = 8.2, 2.3 Hz, 1H), 8.19–8.14 (m, 4H), 7.99 (t, J = 7.4 Hz, 1H), 7.74 (dd, J = 7.5, 2.7 Hz, 2H), 3.62–3.37 (m, 49H), 3.22 (q, J = 5.6 Hz, 2H), 2.99 (t, J = 5.5 Hz, 2H), 2.68 (t, J = 5.8 Hz, 1H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 878.9.

2,2',2''-(2-(4-(3-(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-undeca-oxa-36-azatetracontyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)-triacetic Acid (24). 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 (ppm): 10.64 (s, 1H), 9.07 (s, 1H), 8.94 (d, J = 4.8 Hz, 2H), 8.61 (dd, J = 15.5, 8.9 Hz, 1H), 8.42 (dd, J = 9.0, 4.8 Hz, 1H), 8.16 (t, J = 8.0 Hz, 1H), 7.99 (t, J = 6.0 Hz, 1H), 7.74 (dd, J = 7.9, 5.9 Hz, 2H), 7.43 (d, J = 8.3 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 4.34–4.21 (m, 16H), 4.00 (d, J = 18.2 Hz, 1H), 3.81 (d, J = 18.3 Hz, 1H), 3.58–3.47 (m, 45H), 3.42–3.32 (m, 5H), 3.23–3.28 (m, 6H), 2.67–2.54 (m, 3H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1329.7. HRMS (ESI) m/z calcd for C₆₀H₈₉N₁₃NaO₁₉S $[\text{M} + \text{Na}]^+$ 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-hepta-oxa-2-azapentacosan-25-yl)carbamate (Tz-4-PEG₇-NHBoc). 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 equiv) 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 reports.² ^1H NMR (500 MHz, DMSO- d_6) δ (ppm): 10.21 (s, 1H), 8.43–8.31 (m, 2H), 7.75–7.71 (m, 1H), 7.54 (s, 2H), 6.69–6.54 (m, 1H), 4.33–4.28 (m, 3H), 3.48–3.39 (m, 22H), 3.33–3.28 (m, 2H), 3.39–3.19 (m, 14H), 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-hepta-oxatricosyl)-glutaramide. 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 results.² ^1H NMR (500 MHz, DMSO- d_6) δ (ppm): 10.21 (s, 1H), 8.76–8.72 (m, 1H), 7.72–7.69 (m, 2H), 7.51 (s, 2H), 6.62–6.52 (m, 2H), 4.30–4.24 (m, 4H), 3.58–3.51 (m, 18H), 3.48–3.42 (m, 3H), 3.31–3.22 (m, 16H).

2,2',2''-(7-(4-(3-(1-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-hepta-oxa-2-azapentacosan-25-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic Acid (Tz-4-PEG₇-NODA, 25). 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 (ppm): 9.91 (s, 1H), 8.50 (d, J = 5.9 Hz, 1H), 8.42 (d, J = 5.9 Hz, 1H), 7.74–7.69 (m, 2H), 7.54 (d, J = 8.0 Hz, 2H), 7.18 (d, J = 8.1 Hz, 2H), 4.41 (d, J = 5.9 Hz, 2H), 3.99 (d, J = 5.8 Hz, 2H), 3.83 (d, J = 5.0 Hz, 1H), 3.79 (s, 1H), 3.68 (dd, J = 11.0, 4.9 Hz, 2H), 3.55–3.44 (m, 31H), 3.43–3.24 (m, 5H), 3.18 (s, 15H), 2.41–2.32 (m, 5H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 1045.4. HRMS (ESI) m/z calcd for C₄₈H₇₃N₁₁NaO₁₃S $[\text{M} + \text{Na}]^+$ 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-hepta-oxa-2,8-diaza-hentriacontan-31-yl)-thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)-triacetic Acid (26). 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 reports.² ^1H NMR (500 MHz, DMSO- d_6) δ (ppm): 10.51 (s, 1H), 9.50 (s, 1H), 8.40–8.27 (m, 3H), 7.79–7.68 (m, 1H), 7.62–7.59 (m, 1H), 7.47 (d, J = 5.9 Hz, 2H), 7.35 (d, J = 5.8 Hz, 2H), 7.03–6.92 (m, 2H), 4.43 (d, J = 7.4 Hz, 2H), 4.00–3.20 (m, 48H), 3.12–3.02 (m, 6H), 2.11–2.01 (m, 6.4H).

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-undeca-oxa-2,8-diazatritetracontan-43-yl)carbamate (Tz-4-PEG₁₁-NHBoc). 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 reports.¹ ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.65 (s, 1H), 8.77 (t, *J* = 5.4 Hz, 1H), 8.65–8.57 (m, 2H), 8.16–8.12 (m, 2H), 6.77–6.73 (m, 1H), 3.62–3.42 (m, 46H), 3.38 (t, *J* = 6.1 Hz, 3H), 3.07 (q, *J* = 5.8 Hz, 2H), 1.38 (s, 9H).

N¹-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)-N⁵-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undecaaxapenta-triacontyl)-glutaramide (Tz-4-PEG₁₁-NH₂). 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 reports.¹ ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 10.59 (s, 1H), 8.74 (t, *J* = 5.2 Hz, 1H), 8.61–8.55 (m, 2H), 8.12–8.09 (m, 2H), 6.79–6.74 (m, 1H), 3.68–3.36 (m, 46H), 3.34 (t, *J* = 6.1 Hz, 3H), 3.10 (q, *J* = 5.8 Hz, 2H).

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-undecaaxa-2,8-diazatritetracontan-43-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic Acid (27). 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 (ppm): 10.66 (s, 1H), 8.78 (t, *J* = 8.2 Hz, 1H), 8.60 (d, *J* = 8.2 Hz, 1H), 8.14 (d, *J* = 8.2 Hz, 2H), 7.67–7.61 (m, 3H), 7.43 (d, *J* = 8.1 Hz, 2H), 7.20 (d, *J* = 8.4 Hz, 2H), 4.00 (d, *J* = 18.2 Hz, 1H), 3.82 (d, *J* = 18.1 Hz, 2H), 3.61–3.43 (m, 63H), 3.36–3.21 (m, 1H), 3.13–2.99 (m, 6H), 2.87–2.72 (m, 2H), 2.64 (d, *J* = 12.4 Hz, 2H). MS (ESI) *m/z* [M + H]⁺: 1207.5. HRMS (ESI) *m/z* calcd for C₅₆H₈₉N₁₀NaO₁₇S [M + Na]⁺ 1229.5341, found 1229.5333.

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-undecaaxa-2,8-diazatritetracontan-43-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid (28). Precursor 28 was synthesized as recently reported.⁴ Analytical data matched previously established protocols. ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 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-trioxa-11,14,17,20,23,26,29-heptaaxa-2,8,32-triazaoctatriacontan-34-yl)carbamate (Tz-4-PEG₇-lysine-Boc). The title compound was obtained using Tz-4-PEG₇-NH₂ (8.6 mg, 15.6 μ mol) and the standard amide coupling conditions using Boc-lysine (5.0 mg, 20 μ mol). The title compound was furnished as pink oil (9.2 mg, 76%). ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 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* [M + H]⁺: 881.1.

2,2'-(7-(4-(3-(1-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-34-(tert-butoxycarbonyl)amino)-3,7,33-trioxa-11,14,17,20–23,26,29-heptaaxa-2,8,32-triazaoctatriacontan-38-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic Acid (Tz-4-PEG₇-Lysine-Boc-NODA). 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 (ppm): 9.82 (s, 1H), 8.43 (d, *J* = 5.6 Hz, 2H), 8.41 (d, *J* = 5.6 Hz, 2H), 7.24 (d, *J* = 7.9 Hz, 1H), 7.16 (d, *J* = 8.0 Hz, 2H), 6.9 (d, *J* = 8.4 Hz, 2H), 4.55–4.51 (m, 1H), 3.92 (d, *J* = 17.8 Hz, 2H), 3.83 (d, *J* = 5.0 Hz, 2H), 3.79–3.70 (m, 15H), 3.68 (dd, *J* = 17.6, 4.9 Hz, 2H), 3.55–3.44 (m, 47H), 3.41–3.34 (m, 3H), 2.44–2.38 (m, 2H), 1.67 (s, 9H). MS (ESI) *m/z* [M + H]⁺: 1273.6.

2,2'-(7-(4-(3-(1-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-34-amino-3,7,33-trioxa-11,14,17,20,23–26,29-heptaaxa-2,8,32-triazaoctatriacontan-38-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic Acid (29). 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 (ppm): 9.91 (s, 1H), 8.50 (d, *J* = 5.9 Hz, 1H), 8.42–8.35 (m, 2H), 7.54 (d, *J* = 5.9 Hz, 1H), 7.18 (d, *J* = 8.1 Hz, 2H), 6.89 (d, *J* = 8.1 Hz, 2H), 3.99 (d, *J* = 17.8 Hz, 2H), 3.83 (d, *J* = 4.4 Hz, 2H), 3.79–3.70

(m, 15H), 3.68 (dd, *J* = 17.5, 4.2 Hz, 2H), 3.55–3.44 (m, 43H), 3.43–3.24 (m, 4H), 2.44–2.33 (m, 8H). MS (ESI) *m/z* [M + H]⁺: 1273.6. HRMS (ESI) *m/z* calcd for C₅₄H₈₅N₁₃NaO₁₄S [M + Na]⁺ 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-trioxa-11,14,17,20,23,26–29,32,35,38,41-undecaaxa-2,8,44-triazaheptatetracontan-46-yl)carbamate (Tz-4-PEG₁₁-His-Boc). 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 (ppm): 12.53 (s, 1H), 9.66 (s, 1H), 8.50 (d, *J* = 5.9 Hz, 1H), 8.42 (d, *J* = 5.8 Hz, 1H), 8.35 (s, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.41 (s, 1H), 7.18 (d, *J* = 8.0 Hz, 1H), 7.09–7.01 (m, 2H), 4.23 (dd, *J* = 17.8, 6.5 Hz, 1H), 4.08 (dd, *J* = 17.5, 6.2 Hz, 1H), 3.99 (d, *J* = 17.8 Hz, 2H), 3.92 (dd, *J* = 16.3, 6.1 Hz, 1H), 3.83 (d, *J* = 5.0 Hz, 2H), 3.79–3.70 (m, 15H), 3.68 (dd, *J* = 16.1, 4.9 Hz, 2H), 3.51–3.35 (m, 27H), 3.33–3.21 (m, 4H), 2.42–2.23 (m, 6H), 1.82 (s, 9H). MS (ESI) *m/z* [M + H]⁺: 1066.4.

N¹-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)-N⁵-(38-amino-39-(1H-imidazol-4-yl)-37-oxo-3,6,9,12,15,18,21,24–27,30,33-undecaaxa-36-azanonatriacontyl)glutaramide (Tz-4-PEG₁₁-His). 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 (ppm): 12.71 (s, 1H), 9.34 (s, 1H), 8.53 (d, *J* = 8.0 Hz, 1H), 8.33 (d, *J* = 8.0 Hz, 1H), 8.18 (s, 1H), 7.55 (d, *J* = 7.9 Hz, 1H), 7.45 (s, 1H), 7.21 (d, *J* = 7.9 Hz, 1H), 7.11–7.02 (m, 3H), 4.56 (dd, *J* = 17.8, 6.5 Hz, 1H), 4.22–4.13 (m, 2H), 4.01 (dd, *J* = 17.6, 6.3 Hz, 1H), 3.89 (d, *J* = 17.8 Hz, 2H), 3.84 (dd, *J* = 17.6, 6.3 Hz, 1H), 3.78 (d, *J* = 6.2 Hz, 2H), 3.71–3.60 (m, 13H), 3.52 (dd, *J* = 17.4, 6.2 Hz, 2H), 3.41–3.35 (m, 26H), 3.29–3.21 (m, 5H), 2.42–2.23 (m, 6H). MS (ESI) *m/z* [M + H]⁺: 966.2.

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-undecaaxa-2,38-diazaheptatetracontan-40-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic Acid (Tz-4-PEG₁₁-His-NODA, 30). 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 (ppm): 12.61 (s, 1H), 9.71 (s, 1H), 8.74 (d, *J* = 5.3 Hz, 1H), 8.51 (d, *J* = 5.2 Hz, 1H), 8.01 (s, 1H), 7.49 (d, *J* = 7.5 Hz, 1H), 7.41 (s, 1H), 7.33 (d, *J* = 7.6 Hz, 2H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.14 (m, 4H), 6.89 (d, *J* = 7.7 Hz, 2H), 4.78 (dd, *J* = 15.3, 6.2 Hz, 1H), 4.22–4.04 (m, 8H), 4.01 (dd, *J* = 15.1, 6.2 Hz, 1H), 3.89 (d, *J* = 17.8 Hz, 2H), 3.84 (dd, *J* = 17.5, 5.1 Hz, 1H), 3.78 (d, *J* = 5.0 Hz, 2H), 3.71–3.60 (m, 13H), 3.58 (dd, *J* = 10.0, 4.2 Hz, 2H), 3.52–3.44 (m, 11H), 3.41–3.35 (m, 25H), 3.29–3.21 (m, 4H), 2.12–2.02 (m, 10H). MS (ESI) *m/z* [M + H]⁺: 1358.7. HRMS (ESI) *m/z* calcd for C₆₂H₉₆N₁₄NaO₁₈S [M + Na]⁺ 1380.7961, found 1380.7955.

tert-Butyl-(51-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-amino-1-imino-7,45,49-trioxa-11,14,17,20–23,26–29,32,35,38,41-undecaaxa-2,8,44,50-tetraazahenpentacontan-6-yl)carbamate (Tz-4-PEG₁₁-Arg-Boc). 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 (ppm): 9.88 (s, 1H), 8.45 (d, *J* = 6.1 Hz, 1H), 8.34 (d, *J* = 6.2 Hz, 1H), 8.24 (s, 1H), 7.54 (d, *J* = 7.9 Hz, 1H), 7.19 (d, *J* = 7.9 Hz, 1H), 7.12 (s, 1H), 4.19 (d, *J* = 5.9 Hz, 2H), 3.91 (d, *J* = 17.8 Hz, 2H), 3.83 (d, *J* = 5.8 Hz, 2H), 3.79 (s, 1H), 3.68 (dd, *J* = 11.0, 4.7 Hz, 2H), 3.55–3.44 (m, 38H), 3.41–3.23 (m, 19H), 3.17 (s, 1H), 2.33–2.28 (m, 2H), 1.52 (s, 9H). MS (ESI) *m/z* [M + H]⁺: 1085.4.

N¹-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)-N⁵-(1,6-diamino-1-imino-7-oxo-11,14,17,20,23,26,29–32,35,38,41-undecaaxa-2,8-diazatritetracontan-43-yl)glutaramide (Tz-4-PEG₁₁-Arg). 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 (ppm): 10.21 (s, 1H), 8.69 (d, *J* = 6.7 Hz, 1H), 8.52 (d, *J* = 6.8 Hz, 1H), 8.42 (s, 1H), 7.83 (d, *J* = 7.2 Hz, 1H), 7.52 (d, *J* = 7.3 Hz, 1H), 7.11 (s, 1H), 7.03 (s, 1H), 4.19 (d, *J* = 5.9 Hz, 2H), 4.08–3.98 (m, 4H), 3.91 (d, *J* = 13.2 Hz, 2H), 3.83 (d, *J* = 5.8 Hz, 2H), 3.79 (s, 1H), 3.68 (dd, *J* = 13.2, 3.9 Hz, 2H), 3.55–3.44

(m, 35H), 3.41–3.23 (m, 11H), 3.17 (s, 1H), 2.33–2.24 (m, 8H). MS (ESI) m/z 985.2 $[M + H]^+$: 985.2.

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-undeca-oxa-2,8,44,50-tetraazahenpentacontan-6-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (31). Precursor 31 (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 (ppm): 12.61 (s, 1H), 9.71 (s, 1H), 8.74 (d, *J* = 8.2 Hz, 1H), 8.66 (s, 1H), 8.51 (d, *J* = 8.2 Hz, 1H), 8.01 (s, 1H), 7.49 (d, *J* = 7.5 Hz, 1H), 7.41 (s, 1H), 7.33 (d, *J* = 7.5 Hz, 2H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.14–7.04 (m, 4H), 6.89 (d, *J* = 6.7 Hz, 2H), 4.74 (dd, *J* = 6.6, 13.3 Hz, 1H), 4.22–4.14 (m, 9H), 4.09 (dd, *J* = 12.8, 6.3 Hz, 1H), 3.92 (d, *J* = 12.9 Hz, 2H), 3.84 (dd, *J* = 6.2, 16.1 Hz, 1H), 3.76 (d, *J* = 6.2 Hz, 2H), 3.73–3.64 (m, 11H), 3.62 (dd, *J* = 9.6, 6.1 Hz, 2H), 3.58–3.54 (m, 5H), 3.51–3.45 (m, 17H), 3.39–3.31 (m, 9H), 2.31–2.02 (m, 24H). MS (ESI) m/z $[M + H]^+$: 1377.7. HRMS (ESI) m/z calcd for C₆₂H₁₀₁N₁₅NaO₁₈S $[M + Na]^+$ 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-undeca-oxa-2,8,44-triazaoctatetracontan-48-oic Acid (Tz-4-PEG₁₁-Asp-Boc). 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 (ppm): 10.21 (s, 1H), 8.61 (d, *J* = 5.7 Hz, 1H), 8.47 (d, *J* = 8.3 Hz, 1H), 7.81 (d, *J* = 5.6 Hz, 1H), 7.42 (d, *J* = 8.4 Hz, 1H), 4.41 (d, *J* = 5.9 Hz, 2H), 3.99 (d, *J* = 5.8 Hz, 2H), 3.83–3.74 (m, 7H), 3.79 (s, 1H), 3.68 (dd, *J* = 11.0, 4.7 Hz, 2H), 3.55–3.44 (m, 39H), 3.43–3.24 (m, 5H), 3.18 (s, 1H), 2.44 (t, *J* = 6.3 Hz, 5H), 1.56 (s, 9H). 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-undeca-oxa-2,8,44-triazaoctatetracontan-48-oic Acid (Tz-4-PEG₁₁-Asp). 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 (ppm): 11.04 (s, 1H), 8.73 (d, *J* = 5.8 Hz, 1H), 8.44 (d, *J* = 5.7 Hz, 1H), 7.88 (d, *J* = 7.2 Hz, 1H), 7.51 (d, *J* = 7.1 Hz, 1H), 4.41 (d, *J* = 7.2 Hz, 2H), 4.28 (d, *J* = 14.1 Hz, 2H), 3.98–3.91 (m, 5H), 3.87 (s, 1H), 3.78 (dd, *J* = 11.0, 4.7 Hz, 2H), 3.71–3.54 (m, 39H), 3.43–3.24 (m, 8H), 2.32–2.21 (m, 5H). MS (ESI) m/z 944.1 $[M + H]^+$: 944.1.

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-undeca-oxa-2,8,44-triazaheptatetracontan-46-yl)thioureido)-benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic Acid (32). 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 (ppm): 11.22 (s, 1H), 9.38 (s, 1H), 9.12 (s, 1H), 8.81 (d, *J* = 5.9 Hz, 1H), 8.42 (d, *J* = 5.8 Hz, 1H), 7.92 (d, *J* = 7.5 Hz, 1H), 7.72 (d, *J* = 7.4 Hz, 1H), 7.56 (s, 1H), 6.88 (s, 1H), 4.62–4.52 (m, 13H), 4.38 (d, *J* = 15.5 Hz, 2H), 4.29 (d, *J* = 15.3 Hz, 2H), 4.18–3.97 (m, 10H), 3.82 (s, 1H), 3.78 (dd, *J* = 11.0, 4.7 Hz, 2H), 3.63–3.49 (m, 43H), 3.41–3.22 (m, 8H), 2.12 (t, *J* = 6.1 Hz, 5H). MS (ESI) m/z $[M + H]^+$: 1394.6. HRMS (ESI) m/z calcd for C₆₂H₉₆N₁₂NaO₂₂S $[M + Na]^+$ 1416.5696, found 1416.5687.

General ¹⁸F-Labeling Procedure. The [¹⁸F]fluoride (noncarrier added) received from the cyclotron was trapped on a preconditioned 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 18 m Ω H₂O (20 mL). The obtained aqueous solution containing the labeled product was then flushed through a preconditioned C18 cartridge. An additional 10 mL of 18 m Ω 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 Al¹⁸F methodology were isolated in 34–75% RCY (dc), SAs ranging from 0.52 to 1.1 mCi/nmol (19.3–40.7 MBq/nmol), and radiochemical purities >96% (see Table 2). For further animal studies, 0.9% sterile saline was added to reduce ethanol content <10%.

General ⁶⁸Ga-Labeling Procedure. ⁶⁸Ga³⁺ (9.5–15.8 mCi, 0.35–0.59 GBq) was received from the ⁶⁸Ge/⁶⁸Ga generator as an aqueous solution (500 μ L, 0.5 M NaOH, pH = 10–11). The solution was acidified (pH = 4.5) using metal-free glacial acetic acid (18.5 μ L). Subsequently, the tetrazine precursor (5–15 nmol, 4.8–19.5 μ g) dissolved in metal-free DMSO (5–25 μ L) was added to the aqueous ⁶⁸Ga³⁺ 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 18 m Ω H₂O 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 ⁶⁸Ga³⁺ off the cartridge before the final tracer was eluted using ethanol (molecular biology grade, 100–300 μ L). The identity and purity was determined using radio-HPLC (5% MeCN/H₂O to 95% MeCN over 20 min, *R*_t = 11.1–12.3 min, 1 mL/min). ⁶⁸Ga-labeled tracers were obtained in 66–95% decay-corrected (dc) isolated radiochemical yield (RCY), specific activities (SAs) ranging from 0.73 to 1.3 mCi/nmol (27.1–48.2 MBq/nmol), and radiochemical purities >97% (see Table 2). Finally, 0.9% sterile saline was added to reduce ethanol content of the final tracer solution to <10% for injections.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01108.

Further general experimental and equipment information, chemical structures and additional data of all precursor molecules, representative radio-HPLC traces, physicochemical data obtained for all radioligands and detailed procedures for their determination, Ex vivo biodistribution data and additional PET imaging data, details on statistical analyses performed (PDF)

Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge the MSKCC Small Animal Imaging Core Facility as well as the Radiochemistry and Molecular Imaging Probe core, which were supported in part by NIH grant P30 CA08748. The authors also would like to thank the NIH (F32 CA210396, J.P.M.; R00 CA1440138, B.M.Z.). We also thank Wolfgang W. Scholz and MabVax Therapeutics (San Diego, CA)

for providing the fully human monoclonal antibody SB1. We gratefully acknowledge William H. and Alice Goodwin and the Commonwealth Foundation for Cancer Research and The Center for Experimental Therapeutics of Memorial Sloan Kettering Cancer Center.

■ ABBREVIATIONS USED

IEDDA, inverse electron-demand Diels–Alder; Tz, tetrazine; TCO, *trans*-cyclooctene; PET, positron emission tomography; SPR, structure-pretargeting relationship; %ID/g, percent injected dose per gram; SA, specific activity; PDAC, pancreatic ductal adenocarcinoma; CA19.9, carbohydrate antigen 19.9; PHL, plasma half-life; TTB, tumor-to-background; PK, pharmacokinetics

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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 ($\geq 99.99995\%$) 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 $^{18}\text{O}(\text{p,n})^{18}\text{F}$ 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 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- HCO_3 -resin for ion-exchange and were equilibrated using KHCO_3 -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, $5 \mu\text{m}$, $4.6 \text{ mm} \times 250 \text{ mm}$). Preparative HPLC purification was carried out on a reversed phase Waters XTerra Prep C18 OBD (C18, $10 \mu\text{m}$, $19 \text{ mm} \times 250 \text{ mm}$). 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, $5 \mu\text{m}$, $4.6 \text{ mm} \times 50 \text{ mm}$). All PET imaging experiments were conducted on a Focus 120 MicroPET camera (Siemens, Knoxville, TN).

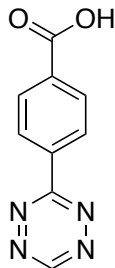
1.2 | Animal protocol, cell culture and xenograft procedures

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% CO_2 . 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 $150 \mu\text{L}$ 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

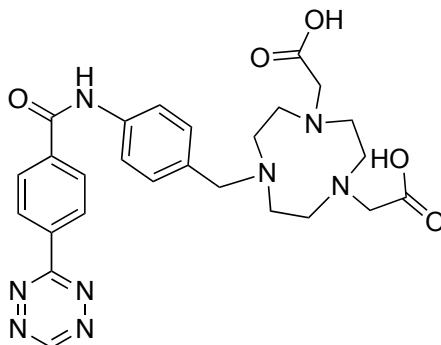
4-(1,2,4,5-tetrazin-3-yl)benzoic acid:



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]⁻.

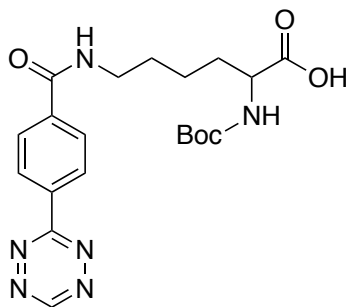
2,2'-(7-(4-(4-(1,2,4,5-tetrazin-3-yl)benzamido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (Tz-1-NODA):



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.

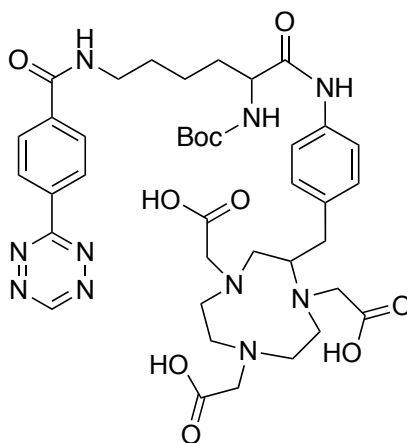
***N*⁶-(4-(1,2,4,5-tetrazin-3-yl)benzoyl)-*N*²-(*tert*-butoxycarbonyl)lysine (Tz-1-Lysine-Boc):**



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]⁺.

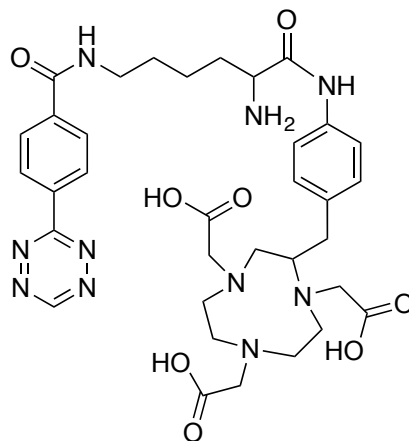
2,2',2''-(2-(4-(6-(4-(1,2,4,5-tetrazin-3-yl)benzamido)-2-((*tert*-butoxycarbonyl)amino)-hexanamido)-benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (Tz-1-Lysine-Boc-NOTA):



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]⁺.

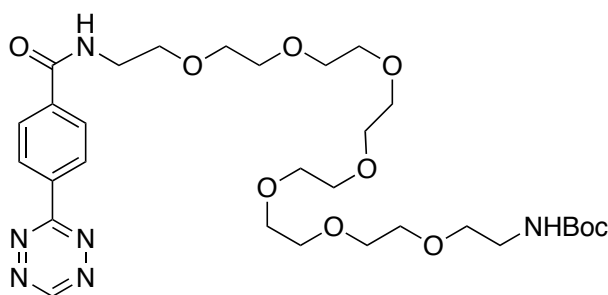
2,2',2''-(2-(4-(6-(4-(1,2,4,5-tetrazin-3-yl)benzamido)-2-aminohexanamido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



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.

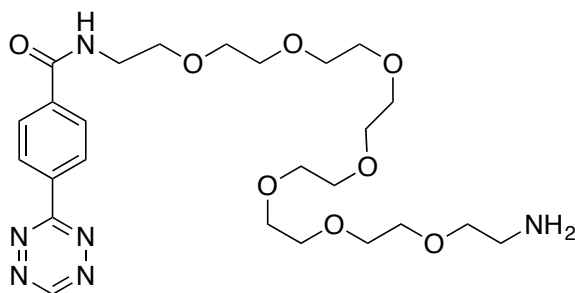
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-hepta-oxa-2-azapentacosan-25-yl)carbamate (Tz-1-PEG₇-NH₂Boc):**



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]⁺.

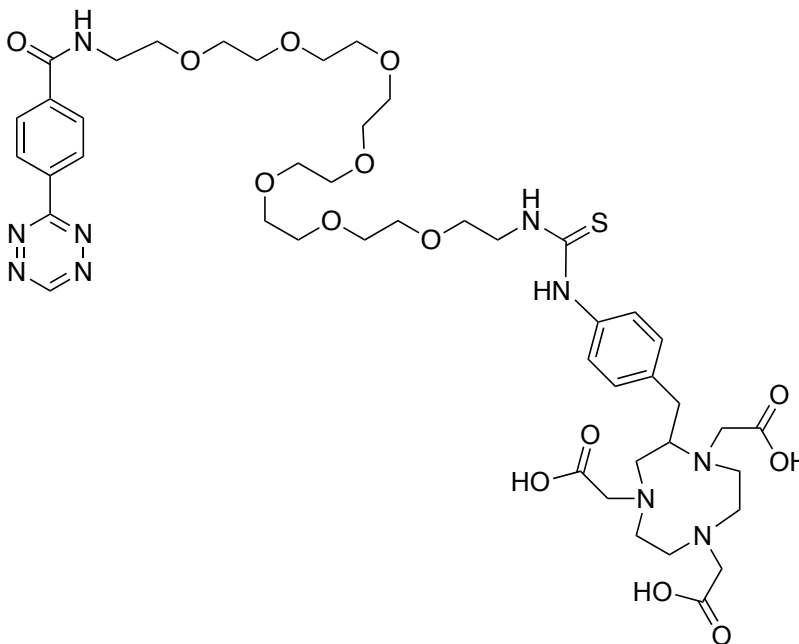
***N*-(23-amino-3,6,9,12,15,18,21-heptaotricosyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (Tz-1-PEG₇-NH₂):**



MW = 552.62 g/mol, C₂₅H₄₀N₆O₈

The title compound was obtained from the Boc-protected starting material Tz-1-PEG₇-NHBoc (5 mg, 6.7 μmol) 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-heptaosa-2-azapenta-cosan-25-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:

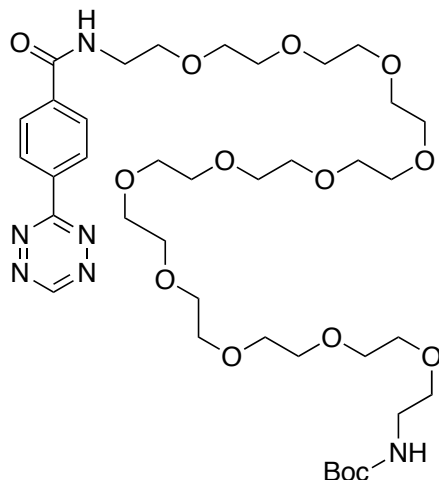


17, MW = 1003.14, C₄₅H₆₆N₁₀O₁₄S

Precursor **17** was obtained using the starting material Tz-1-PEG₇-NH₂ (3.5 mg, 5.4 μmol) and p-Bn-NOTA-NCS (2.7 mg, 6 μmol) 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.

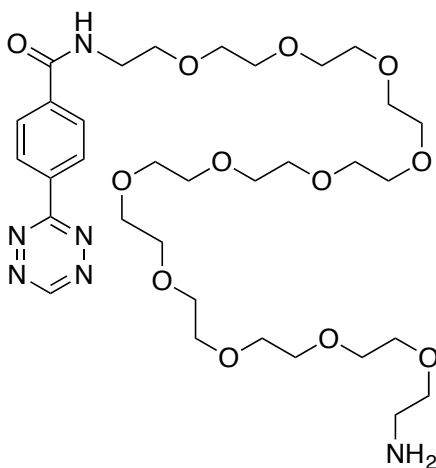
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23,26,29,32,35-undecaoxa-2-azaheptatriacontan-37-yl)carbamate (Tz-1-PEG₁₁-NHBoc):**



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):

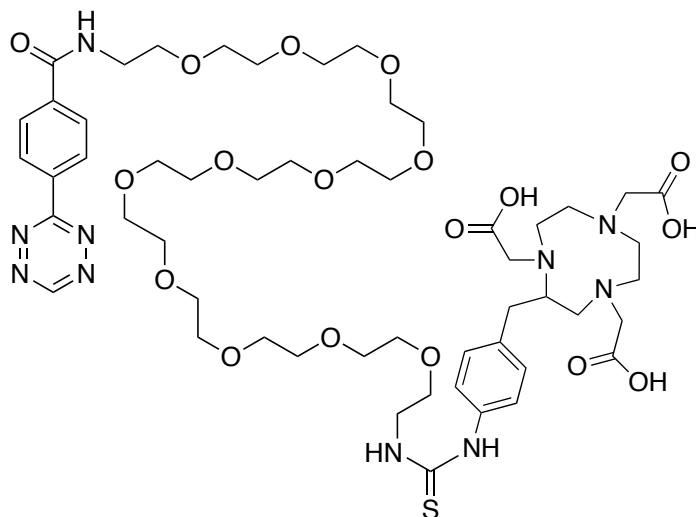


MW = 728.84 g/mol; $C_{33}H_{56}N_6O_{12}$

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, $\text{DMSO-}d_6$) 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 $[\text{M}+\text{H}]^+$, HRMS (ESI) calcd for $\text{C}_{33}\text{H}_{57}\text{N}_6\text{O}_{12}$ $[\text{M} + \text{H}]^+$ m/z 729.7719 found 729.7742.

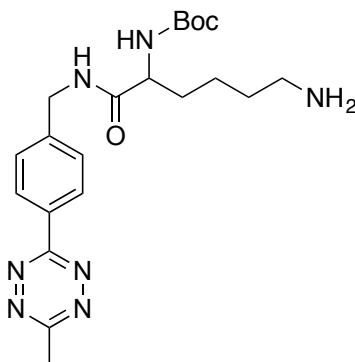
2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23,26,29,32,35-undecaoxa-2-azaheptatriacontan-37-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (Tz-1-PEG₁₁-NOTA):



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, $\text{DMSO-}d_6$) 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 $[\text{M}+\text{H}]^+$; HRMS (ESI) calcd for $\text{C}_{53}\text{H}_{81}\text{N}_{10}\text{O}_{18}\text{S}$ $[\text{M} - \text{H}]^-$ 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):

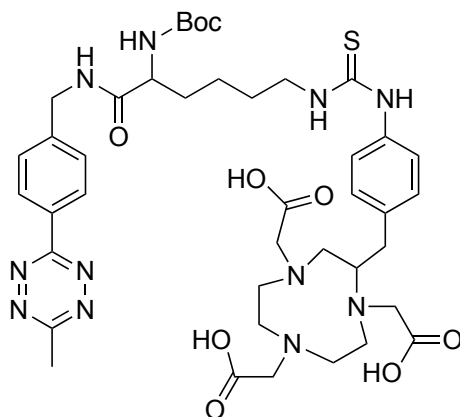


MW = 429.53 g/mol, $\text{C}_{21}\text{H}_{31}\text{N}_7\text{O}_3$

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 $[\text{M}+\text{H}]^+$.

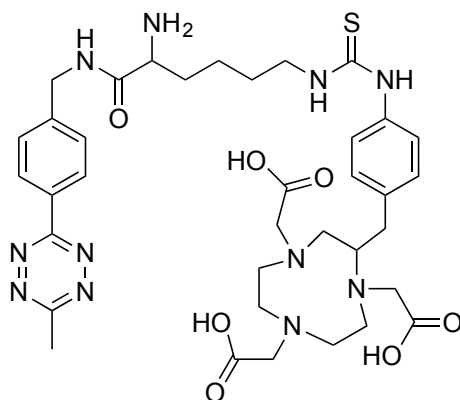
2,2',2''-(2-(4-(3-(5-((tert-butoxycarbonyl)amino)-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (Tz-2-Lysine-Boc):



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

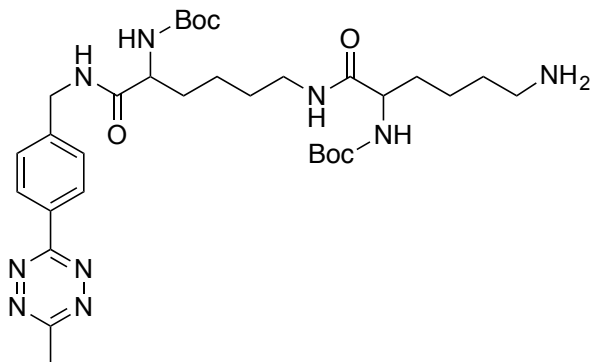


19, MW = 779.92 g/mol, $\text{C}_{36}\text{H}_{49}\text{N}_{11}\text{O}_7\text{S}$

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 $C_{36}H_{49}N_{11}NaO_7S$ $[M + Na]^+$ m/z 802.8435 found 802.8431.

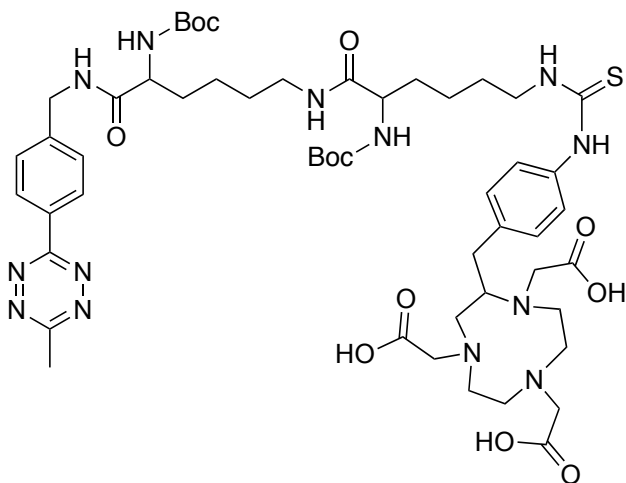
tert-butyl-(11-(4-aminobutyl)-15,15-dimethyl-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,13-trioxo-14-oxa-2,9,12-triazahehexadecan-4-yl)carbamate [Tz-2-(Lysine-Boc)]:



MW = 657.82 g/mol, $C_{32}H_{51}N_9O_6$

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 $[M+H]^+$.

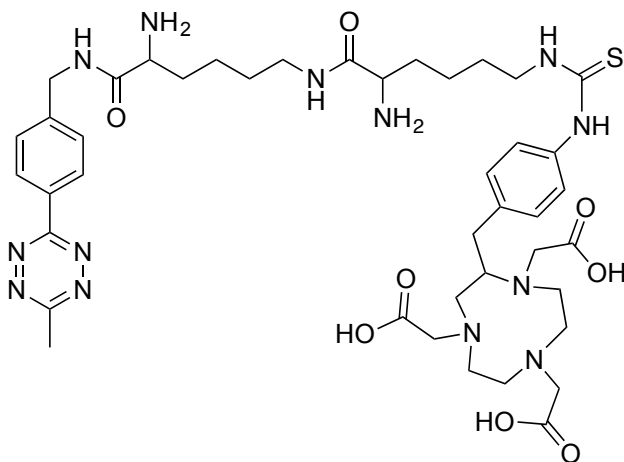
2,2',2''-(2-(4-(3-(5-((tert-butoxycarbonyl)amino)-6-((5-((tert-butoxycarbonyl)amino)-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid [Tz-2-(Lysine-Boc)₂-NOTA]:



MW = 1108.33 g/mol, $C_{52}H_{77}N_{13}O_{12}S$

The title compound was obtained using Tz-2-(Lysine-Boc)₂ (5 mg, 7.6 μmol) 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]⁺.

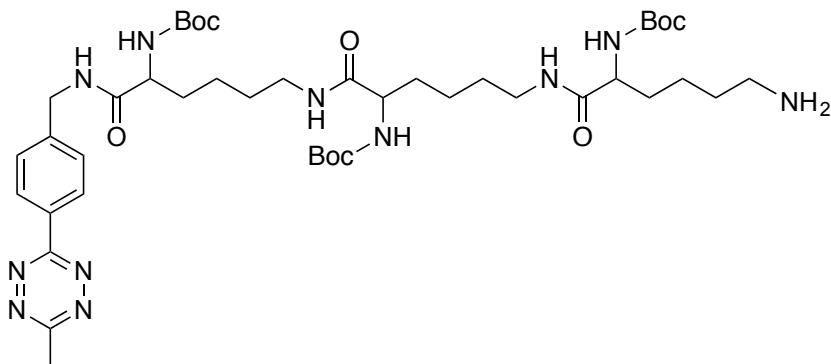
2,2',2''-(2-(4-(3-(5-amino-6-((5-amino-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)-amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



20, MW = 908.09 g/mol, C₄₂H₆₁N₁₃O₈S

Starting from MeTz-(Lysine-Boc)₂-NOTA (6.3 mg, 5.6 μmol), 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.

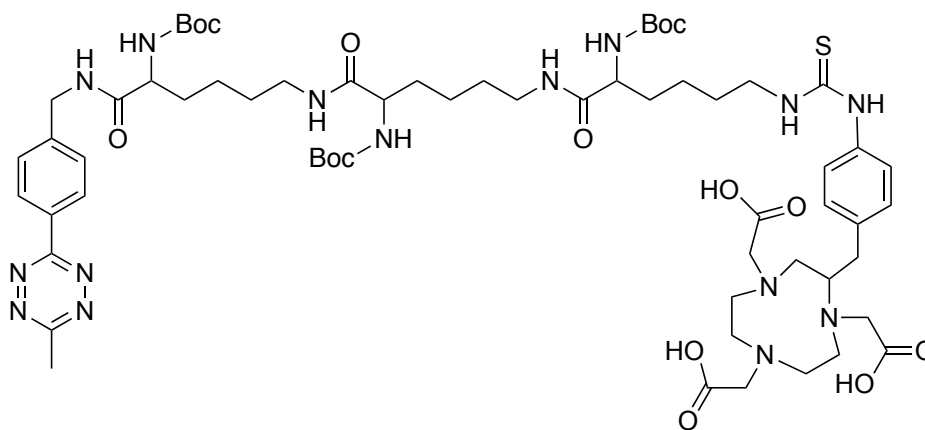
tert-butyl-(18-(4-aminobutyl)-11-((tert-butoxycarbonyl)amino)-22,22-dimethyl-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,17,20-tetraoxo-21-oxa-2,9,16,19-tetraazatricosan-4-yl)carbamate [Tz-2-(Lysine-Boc)₃]:



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]⁺.

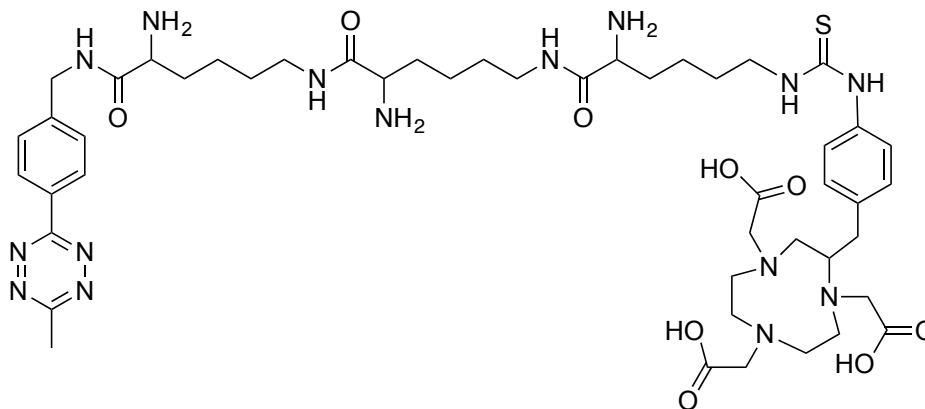
2,2',2''-(2-(4-(3-(13,20-bis((tert-butoxycarbonyl)amino)-2,2-dimethyl-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamoyl)-4,12,19-trioxo-3-oxa-5,11,18-triazatetracosan-24-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid [Tz-2-(Lysine-Boc)₃-NOTA]:



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-((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:

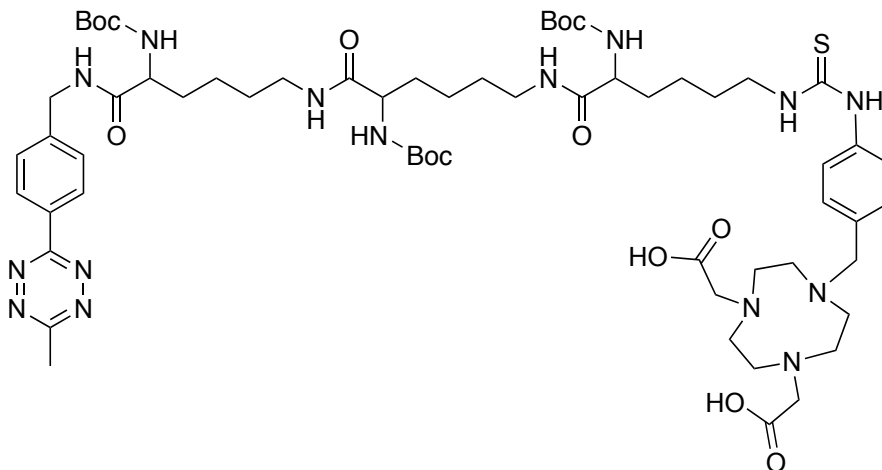


21, MW = 1036.27 g/mol, C₄₈H₇₃N₁₅O₉S

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 $C_{48}H_{73}N_{15}NaO_9S$ $[= M + Na]^+$ m/z 1058.5334 found 1058.5330.

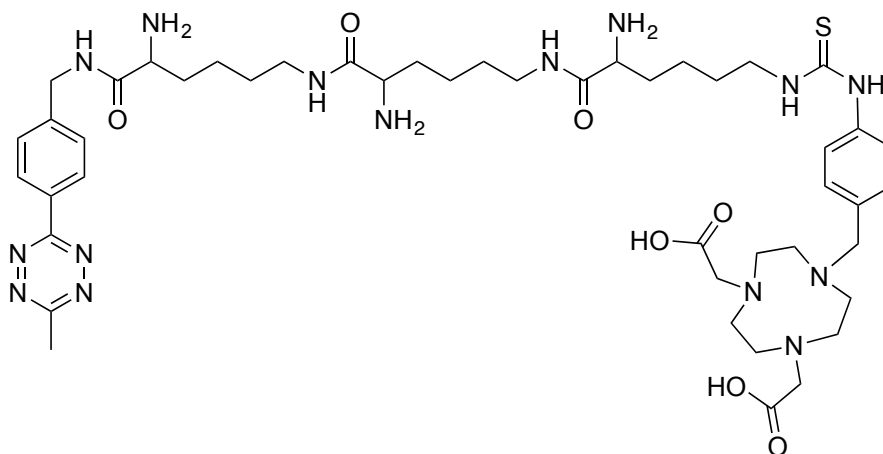
2'-(7-(4-(3-(13,20-bis((*tert*-butoxycarbonyl)amino)-2,2-dimethyl-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamoyl)-4,12,19-trioxo-3-oxa-5,11,18-triazatetracosan-24-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid [Tz-2-(Lysine-Boc)₃-NODA]:



MW = 1278.58 g/mol, $C_{61}H_{95}N_{15}O_{13}S$

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 $[M+H]^+$.

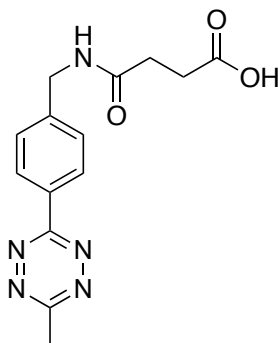
2,2'-(7-(4-(3-(5-amino-6-((5-amino-6-((5-amino-6-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-6-oxohexyl)amino)-6-oxohexyl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid:



22, MW = 987.23 g/mol, $C_{46}H_{71}N_{15}O_7S$

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]⁺.

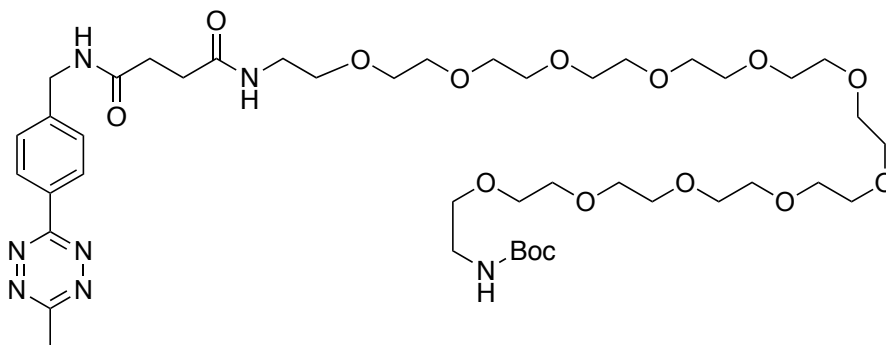
4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)amino)-4-oxobutanoic acid (Tz-2-succinate):



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]⁺.

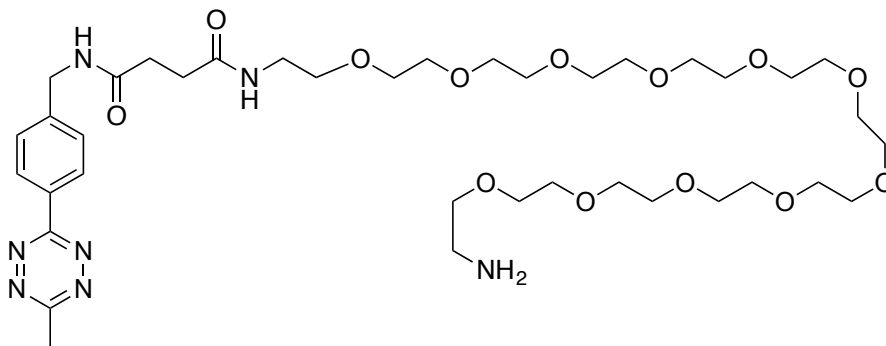
***tert*-butyl-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,6-dioxo-10,13,16,19,22,25,28,31,34,37,40-undeca-oxa-2,7-diazadotetracontan-42-yl)carbamate (Tz-2-PEG₁₁-NHBoc):**



MW = 928.09 g/mol, C₄₃H₇₃N₇O₁₅

The title compound was obtained from Tz-2-succinate (10 mg, 30.1 μ mol) and NH₂-PEG₁₁-NHBoc (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]⁺.

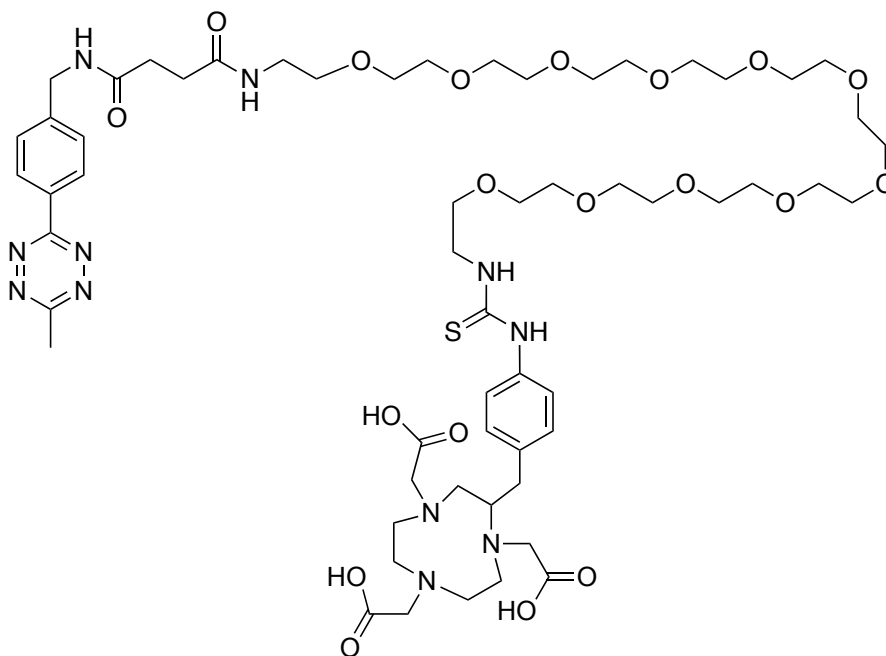
***N*¹-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undeca-oxapentatriacontyl)-*N*⁴-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)succinamide (Tz-2-PEG₁₁-NH₂):**



MW = 827.97 g/mol, C₃₈H₆₅N₇O₁₃

The title compound was obtained using Tz-2-PEG₁₁-NHBoc (17 mg, 18.3 μmol) 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-undeca-oxa-2,7-diazadotetracontan-42-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:

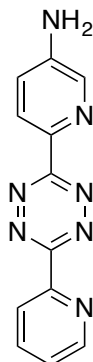


23, MW = 1278.48 g/mol, C₅₈H₉₁N₁₁O₁₉S

The title compound was obtained using Methyltetrazine-PEG₁₂-NHS ester (6.8 mg, 7.5 μmol) and p-Bn-NOTA-NH₂ (5.6 mg, 10 μmol) 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 $C_{58}H_{91}N_{11}NaO_{19}S$ $[= M + Na]^+$ m/z 1301.5836 found 1301.5831.

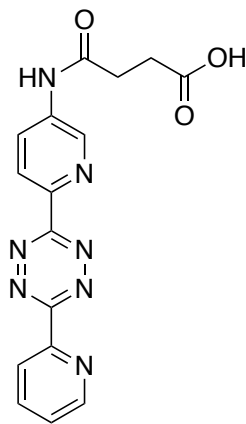
6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine:



3, MW = 251.09 g/mol, $C_{12}H_9N_7$

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).

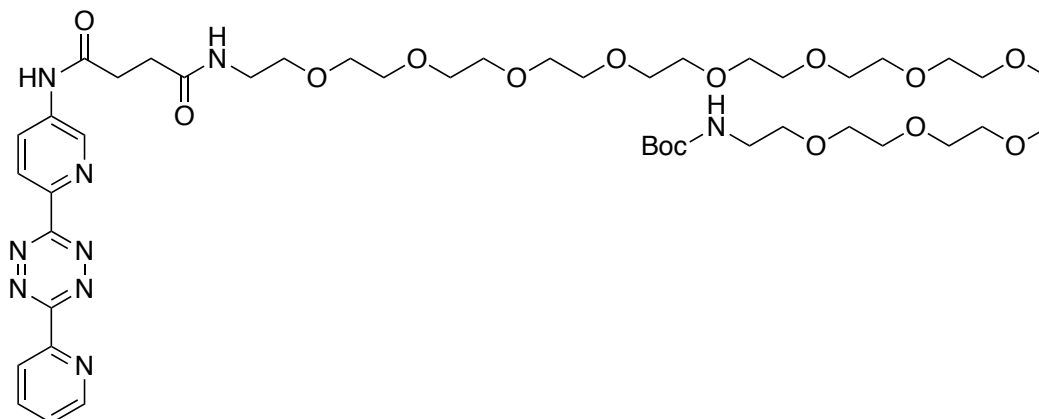
4-oxo-4-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)butanoic acid (Tz-3-succinate):



MW = 351.33 g/mol, $C_{16}H_{13}N_7O_3$

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 $[M+H]^+$.

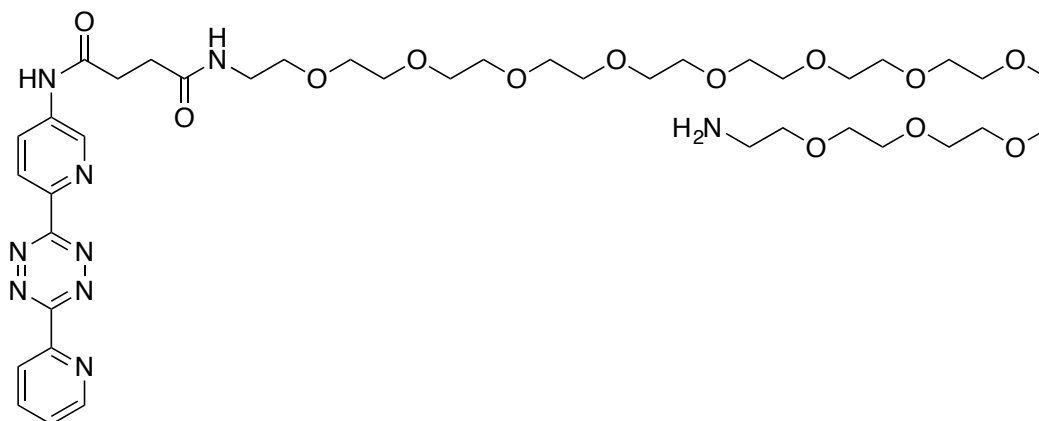
tert-butyl-(37,40-dioxo-40-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)-3,6,9,12,15,18,-21,24,27,30,33-undeca-oxa-36-azatetracontyl)carbamate (Tz-3-PEG₁₁-NHBoc):



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 μmol). 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]⁺.

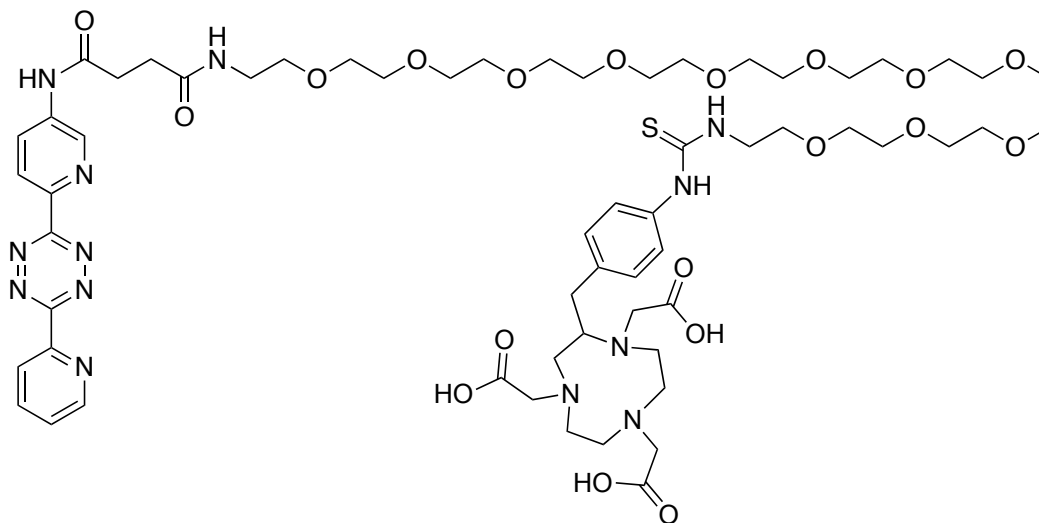
N¹-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undeca-oxapentatriacontyl)-N⁴-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)succinamide (Tz-3-PEG₁₁-NH₂):



MW = 877.99 g/mol, C₄₀H₆₃N₉O₁₃

The title compound was obtained from Tz-3-PEG₁₁-NHBoc (4.3 mg, 4.4 μmol) 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]⁺.

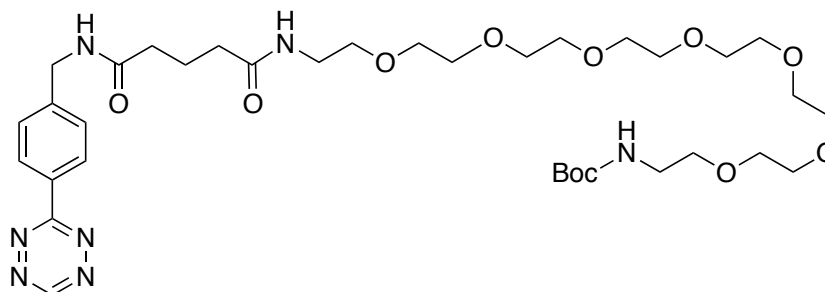
2,2',2''-(2-(4-(3-(37,40-dioxo-40-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)-3,6,9,12,15,-18,21,24,27,30,33-undecaoxa-36-azatetracontyl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



24, MW = 1328.50 g/mol, C₆₀H₈₉N₁₃O₁₉S

The title compound was obtained from Tz-3-PEG₁₁-NH₂ (3.6 mg, 4.1 μmol) 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.

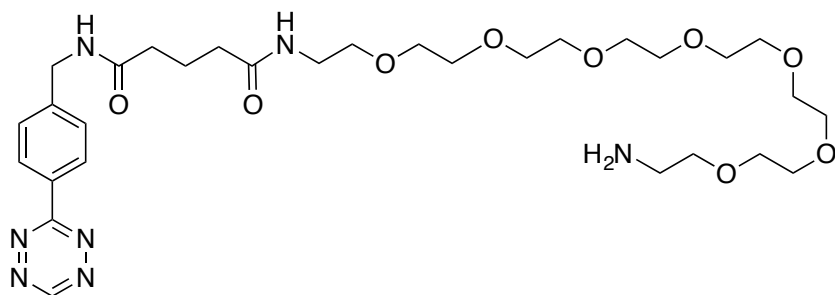
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-hepta-oxa-2-azapentacosan-25-yl)carbamate (Tz-4-PEG₇-NHBoc):**



MW = 751.88 g/mol, C₃₅H₅₇N₇O₁₁

The title compound was obtained using the NHS-activated Tz 4 (6.1 mg, 30 μmol), NH₂-PEG₇-NHBoc (12.4 mg, 26.4 μmol), and TEA (4.5 μL, 3 eq.) in anhydrous DMSO (400 μL). 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).

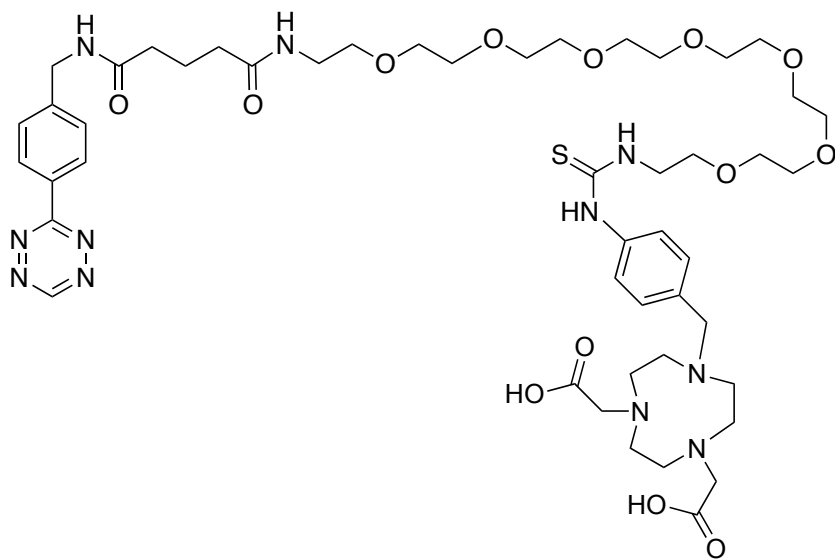
***N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(23-amino-3,6,9,12,15,18,21-heptaotricosyl)glutaramide:**



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).

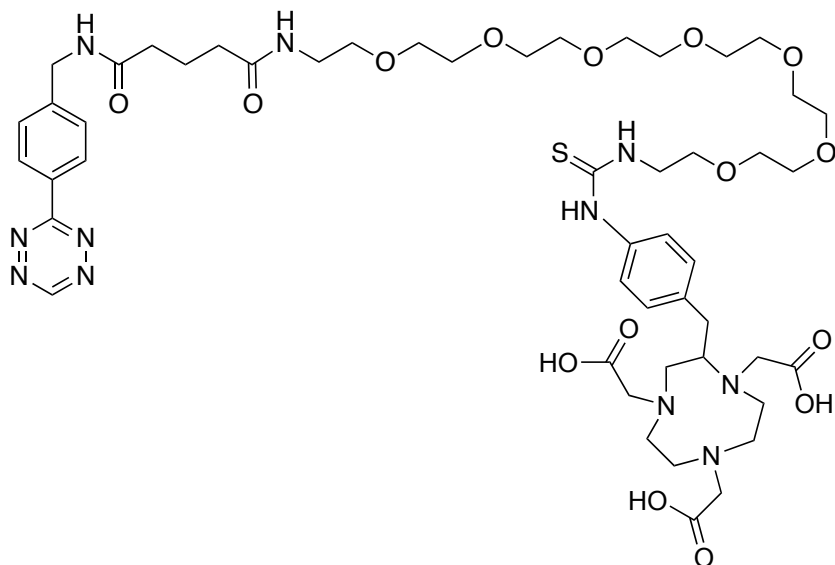
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-oxo-5,8,11,14,17,20,23-heptaoxa-2-azapentacosan-25-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (Tz-4-PEG₇-NODA):



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 μmol) 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.

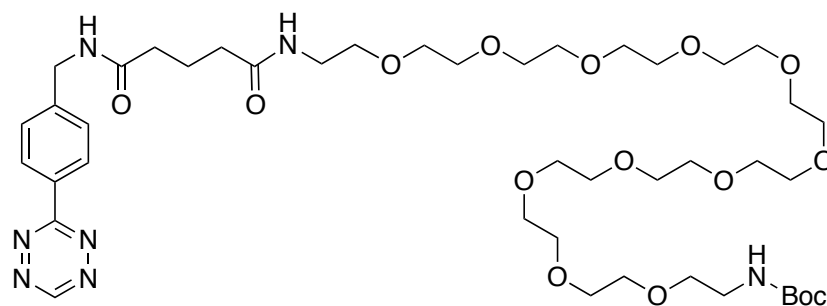
2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29-hepta-oxa-2,8-diazahentriacontan-31-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



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 μmol) 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).

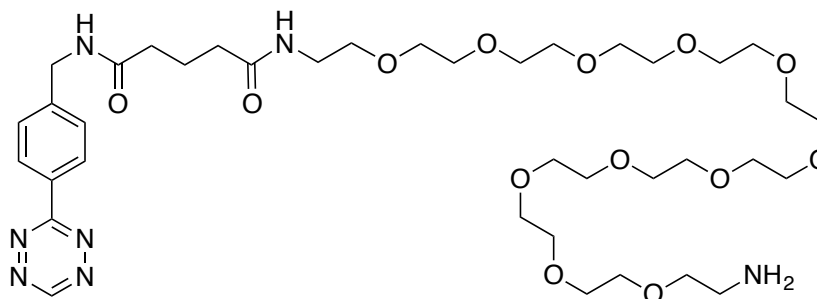
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29,32,35,38,41-undeca-oxa-2,8-diazatritetracontan-43-yl)carbamate (Tz-4-PEG₁₁-NH-Boc):**



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).

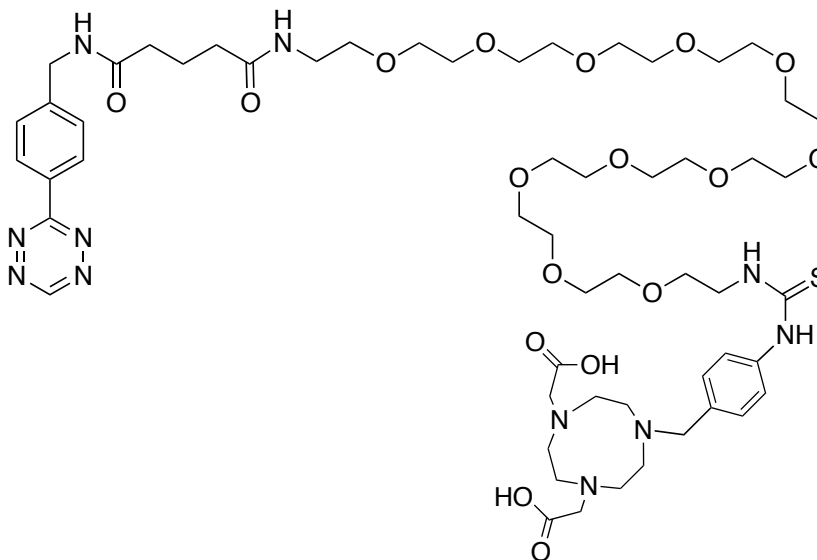
***N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(35-amino-3,6,9,12,15,18,21,24,27,30,33-undeca-oxapenta-triacontyl)-glutaramide (Tz-4-PEG₁₁-NH₂):**



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).

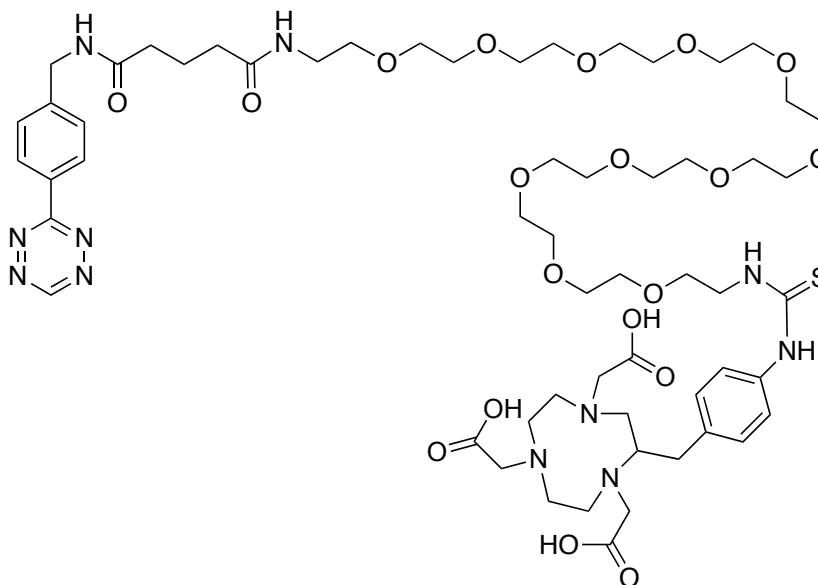
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29,32,35,38,41-undeca-oxa-2,8-diazatritetracontan-43-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid:



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 C₅₆H₈₉N₁₀NaO₁₇S [= M + Na]⁺ *m/z* 1229.5341 found 1229.5333.

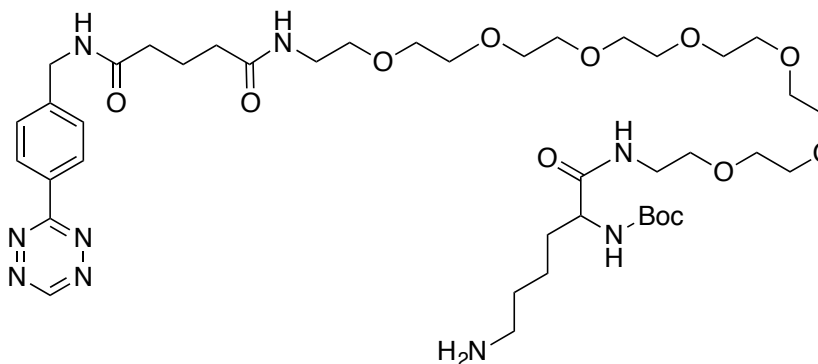
2,2',2''-(2-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8-diazatritetracontan-43-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid:



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).

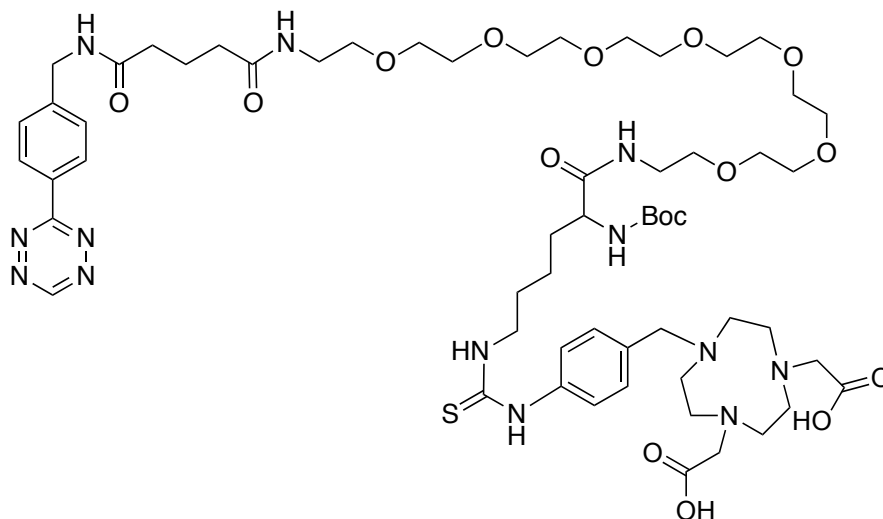
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-38-amino-3,7,33-trioxa-11,14,17,20,23,26,29-hepta-2,8,32-triazaoctatriacontan-34-yl)carbamate (Tz-4-PEG₇-Lysine-Boc):**



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, 1 H), 8.50 (d, *J* = 5.9 Hz, 1 H), 8.42 (d, *J* = 5.9 Hz, 1 H), 7.54 (d, *J* = 7.9 Hz, 1 H), 7.18 (d, *J* = 8.0 Hz, 1 H), 4.41 (d, *J* = 5.9 Hz, 2 H), 3.99 – 3.77 (m, 6 H), 3.83 (d, *J* = 5.9 Hz, 2 H), 3.79 (s, 1 H), 3.68 (dd, *J* = 11.0, 4.7 Hz, 2 H), 3.55 – 3.44 (m, 34 H), 3.43 – 3.24 (m, 5 H), 3.18 (s, 1 H), 2.44 (t, *J* = 4.6 Hz, 2 H), 1.67 (s, 9 H); MS (ESI) *m/z* 881.1 [M+H]⁺.

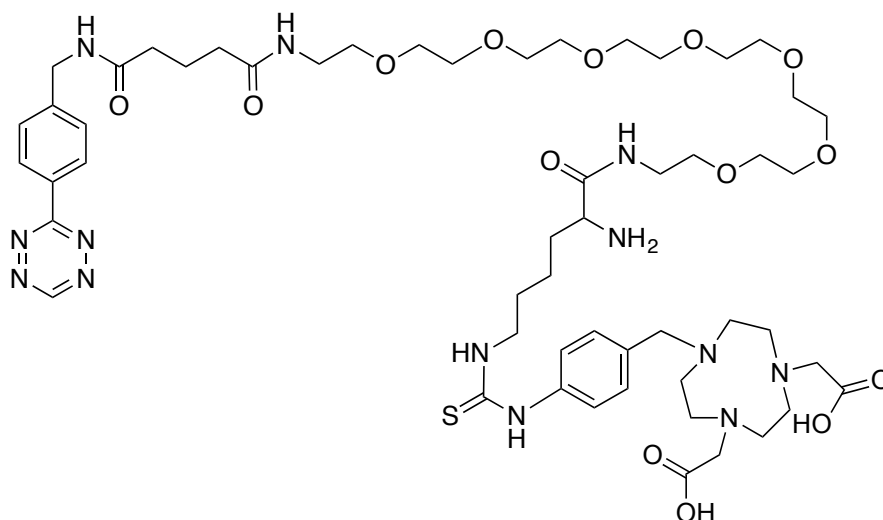
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-34-((*tert*-butoxycarbonyl)amino)-3,7,33-trioxo-11,14,17,20,23,26,29-hepta-2,8,32-triazaoctatriacontan-38-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (Tz-4-PEG₇-Lysine-Boc-NODA):



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 μmol), p-Bn-NODA-NCS (1.3 mg, 3 μmol), 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-hepta-2,8,32-triazaoctatriacontan-38-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid:



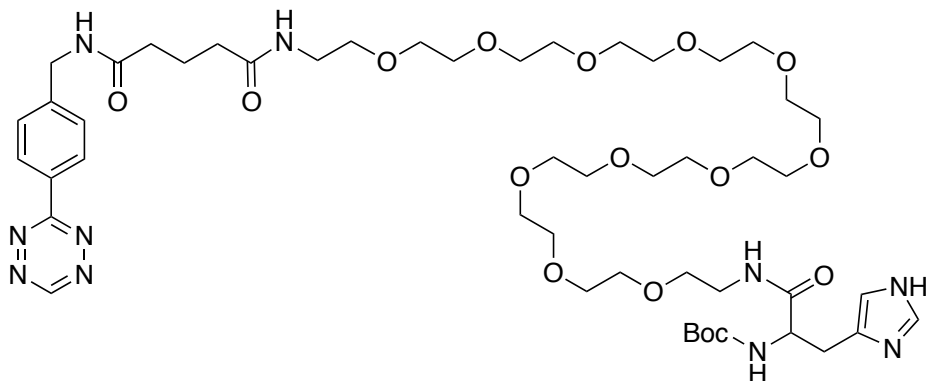
29, MW = 1172.41 g/mol, C₅₄H₈₅N₁₃O₁₄S

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

S23

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 $C_{54}H_{85}N_{13}NaO_{14}S$ $[M + Na]^+$ m/z 1195.5273 found 1195.5263.

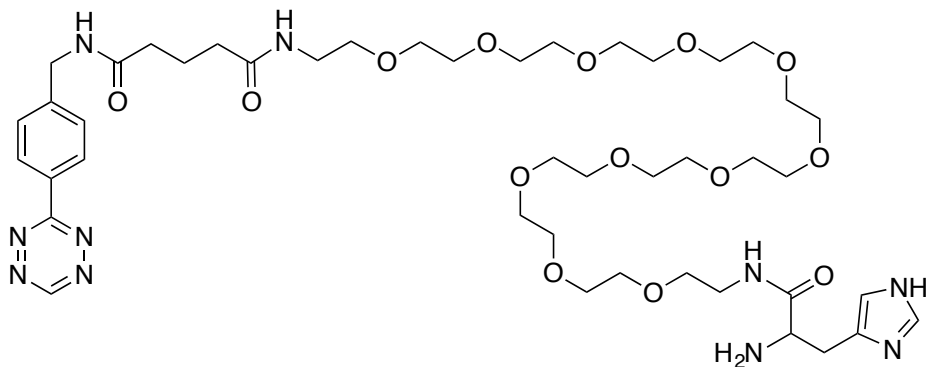
***tert*-butyl-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-47-(1*H*-imidazol-4-yl)-3,7,45-trioxo-11,14,17,20,23,26-29,32,35,38,41-undecaoxa-2,8,44-triazaheptatetracontan-46-yl)carbamate (Tz-4-PEG₁₁-His-Boc):**



MW = 1065.23 g/mol, $C_{49}H_{80}N_{10}O_{16}$

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-(1*H*-imidazol-4-yl)-37-oxo-3,6,9,12,15,18,21,24-27,30,33-undecaoxa-36-azanonatricontyl)glutaramide (Tz-4-PEG₁₁-His):**

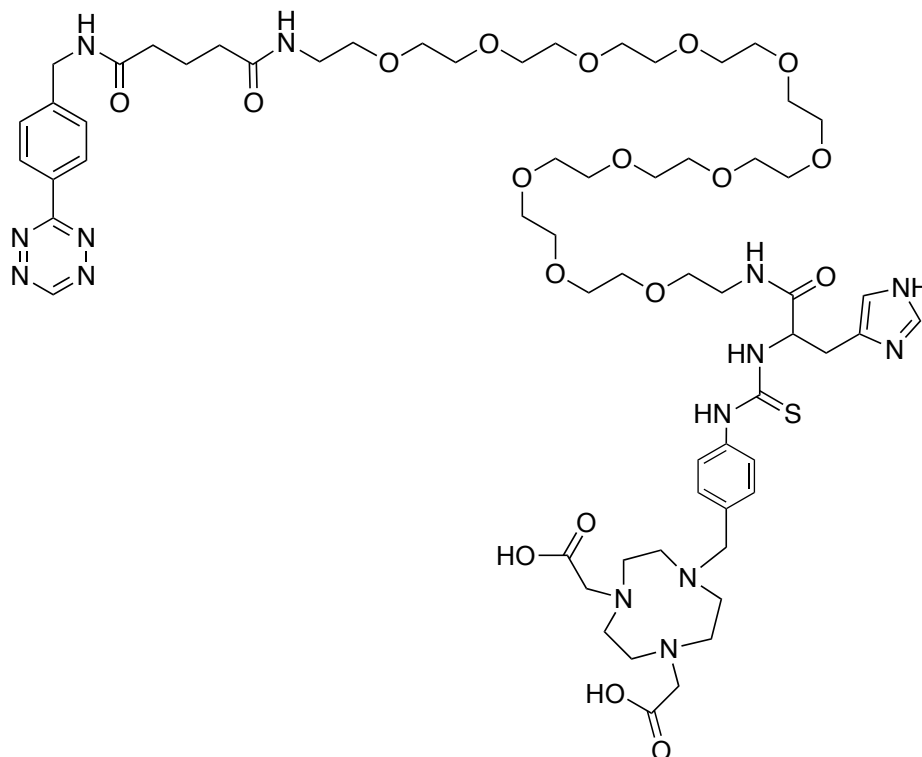


MW = 965.12 g/mol, $C_{44}H_{72}N_{10}O_{14}$

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 $[M+H]^+$.

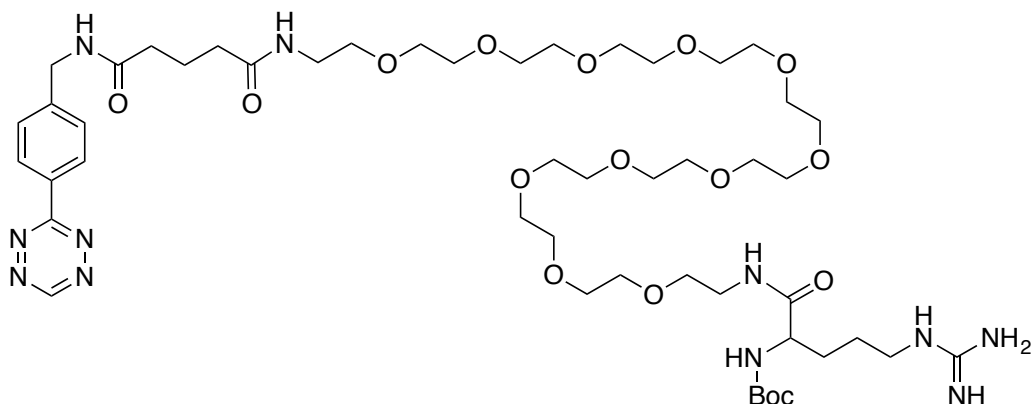
2,2'-(7-(4-(3-(1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-41-(1*H*-imidazol-4-yl)-1,39-dioxo-5,8,11,14,17-20,23,26-,29,32,35-undeca-2,38-diazahentetracontan-40-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid (Tz-4-PEG₁₁-His-NODA):



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 μmol), 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.

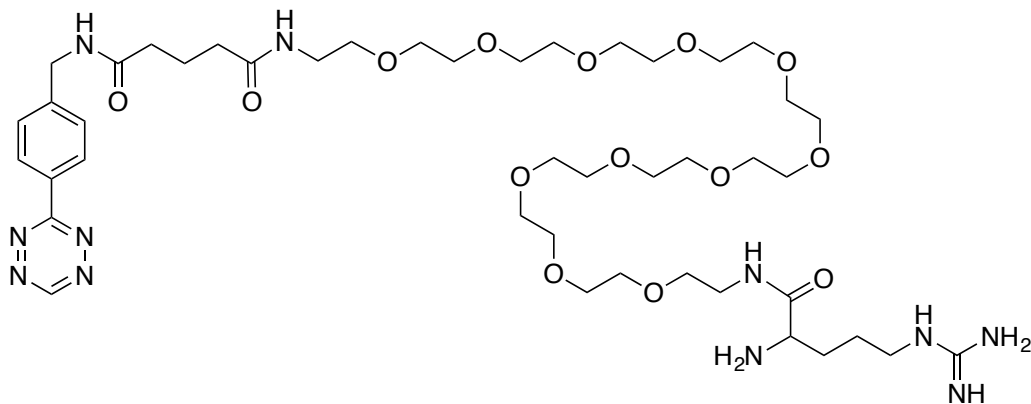
***tert*-butyl-(51-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-amino-1-imino-7,45,49-trioxo-11,14,17,20,23,26-29,32,35,38,41-undecaoxa-2,8,44,50-tetraazahenpentacontan-6-yl)carbamate (Tz-4-PEG₁₁-Arg-Boc):**



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 μmol), Boc-Arginine (5 mg, 13 μmol), 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]⁺.

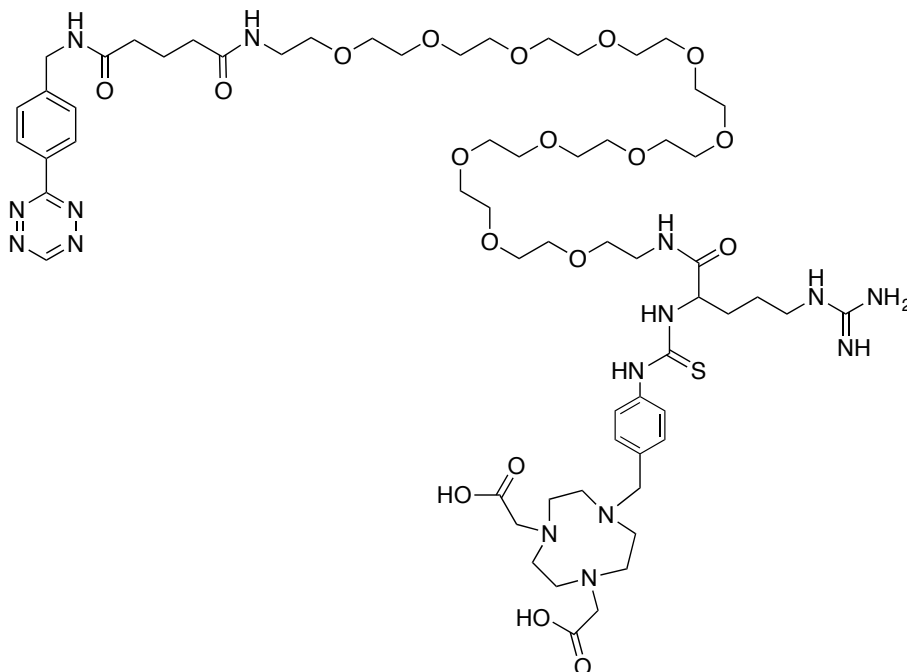
***N*¹-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-*N*⁵-(1,6-diamino-1-imino-7-oxo-11,14,17,20,23,26,29,32,35,38,41-undecaoxa-2,8-diazatritetracontan-43-yl)glutaramide (Tz-4-PEG₁₁-Arg):**



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 μmol) 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]⁺.

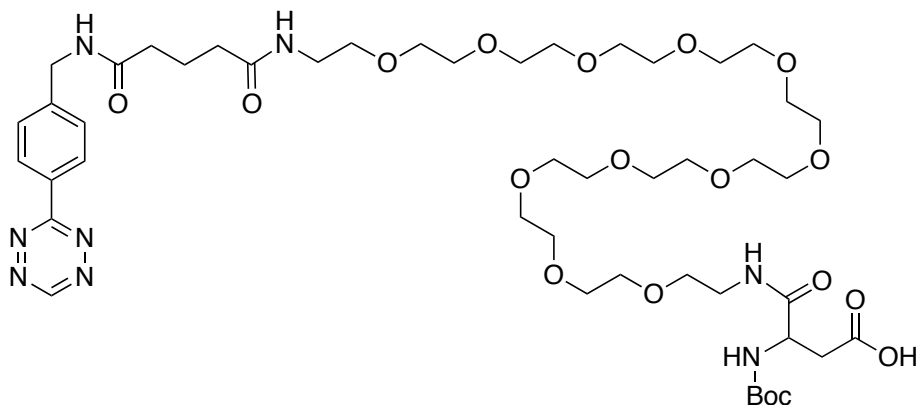
2,2'-(7-(4-(3-(51-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-1-amino-1-imino-7,45,49-trioxo-11,14,17,20,23-26,29,32,35,38,41-undecaoxa-2,8,44,50-tetraazahenpentacontan-6-yl)thioureido)benzyl)-1,4,7-triazonane-1,4-diyl)diacetic acid:



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 μmol), 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.

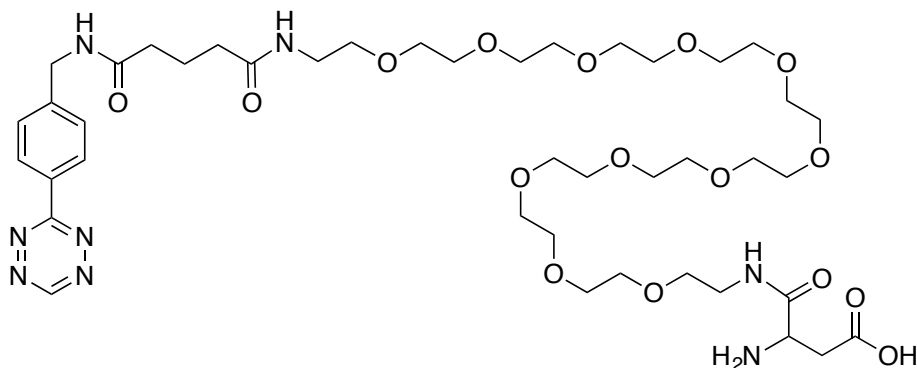
1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-46-((*tert*-butoxycarbonyl)amino)-3,7,45-trioxo-11,14,17,20,23-26,29,32,35,38,41-undeca-oxa-2,8,44-triazaoctatetracontan-48-oic acid (Tz-4-PEG₁₁-Asp-Boc):



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 μmol), Boc-Aspartate (2.5 mg, 10 μmol), 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]⁺.

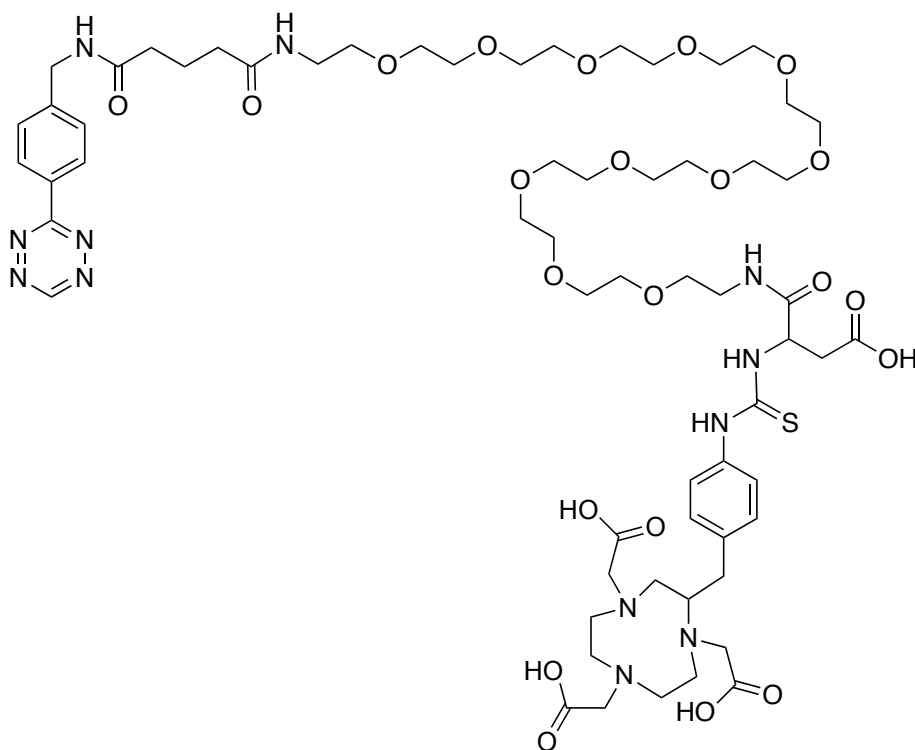
1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-46-amino-3,7,45-trioxo-11,14,17,20,23,26,29,32,35,38,41-undeca-oxa-2,8,44-triazaoctatetracontan-48-oic acid (Tz-4-PEG₁₁-Asp):



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 μmol) 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 μmol), 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 μL), followed by the addition of 2 mM metal-free AlCl₃-solution (25 μL, 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 μL of DMSO) was diluted with metal-free MeCN (700 μL). 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 Al¹⁸F-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 $^{\circ}\text{C}$ 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 18m Ω 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.

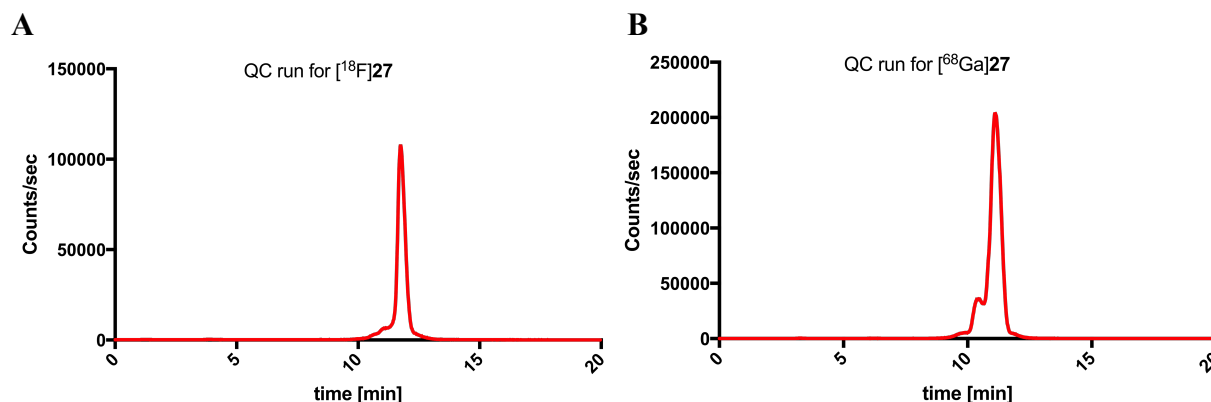


Figure S1. Representative radio-HPLC traces of the quality control runs for both lead compounds [^{18}F]27 and [^{18}F]68. Lead compounds [^{18}F]27 and [^{18}F]68 were furnished in high (>97%) radiochemical purities with retention times of 11.7 and 11.1 minutes, respectively.

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 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ 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.

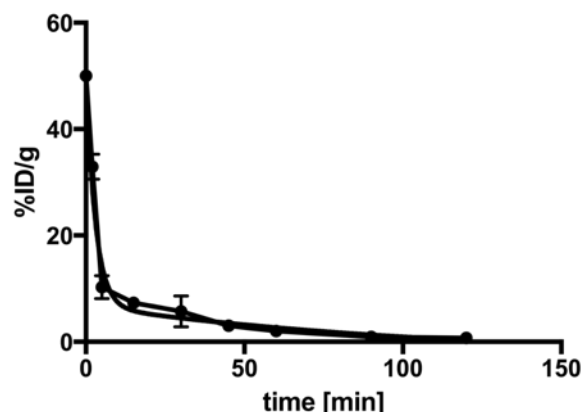


Figure S2. Example blood time-activity curve for [^{68}Ga]27, including bi-exponential curve fit. Based on the assumption that total blood volume is approximately 2 mL (with a total mass of 2 g), the $\%ID/g_{t=0} = 50$ at $t = 0$. With $t_1 = 2.05$ min, $t_2 = 74.5$ min and $\% \text{-fast} = 81.9\%$, the blood half-life of compound [^{68}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 $\% \text{-intact}$ 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 $t_{1/2} > 7\text{-}8$ 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}F]15	18	1.1	57.1	8.35	68.1 ± 8.4
[^{18}F]16	23.25	2.56	85.32	5.60	-
[^{18}F]17	25.39	1.268	34.63	17.04	62.6 ± 12.3
[^{18}F]18	24.03	1.607	52.67	12.22	71.2 ± 8.5
[^{18}F]19	21.32	0.8043	86.37	3.60	55.6
[^{68}Ga]19	30.26	1.013	80.97	6.58	-
[^{18}F]20	11.92	0.897	79.21	3.19	58.3 ± 8.8
[^{68}Ga]20	20.18	1.021	89.47	3.04	-
[^{18}F]21	15.99	1.409	85.77	3.48	51.2 ± 7.9
[^{68}Ga]21	16.43	0.82	92.37	2.01	-
[^{68}Ga]22	13.23	0.9	91.55	1.94	-
[^{18}F]23	26.23	1.414	59.97	11.35	55.9 ± 8.9
[^{18}F]24	31.55	5.089	60.56	15.53	31.6 ± 6.3
[^{68}Ga]24	18.98	0.1	23.74	14.50	-
[^{18}F]25	25.39	1.268	54.63	12.21	66.3 ± 8.2
[^{68}Ga]26	39.58	1.569	58.58	17.31	n/a
[^{18}F]27	34.6	2.02	53.8	17.07	73.1 ± 9.3
[^{68}Ga]27	26.71	3.563	50.1	15.11	69.2 ± 4.2
[^{18}F]28	20.23	2.301	39.44	13.16	66.7 ± 6.5
[^{68}Ga]28	23.77	1.3	60.67	10.14	78.2 ± 9.1
[^{18}F]29	18.85	1.295	77.71	5.21	63.8 ± 11.9
[^{18}F]30	28.61	1.766	76.181	8.16	-
[^{68}Ga]30	23.35	2.086	79.11	6.53	56.3 ± 7.9
[^{18}F]31	16.27	1.11	54.69	7.98	-
[^{18}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}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:

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

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

in which the correction factor for AF488 was given as 0.11 by the supplier, $MW_{\text{mAb}} = 150,000$, $\epsilon_{495, \text{AF488}} = 71,000$, and $\epsilon_{280, \text{mAb}} = 210,000$. 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.

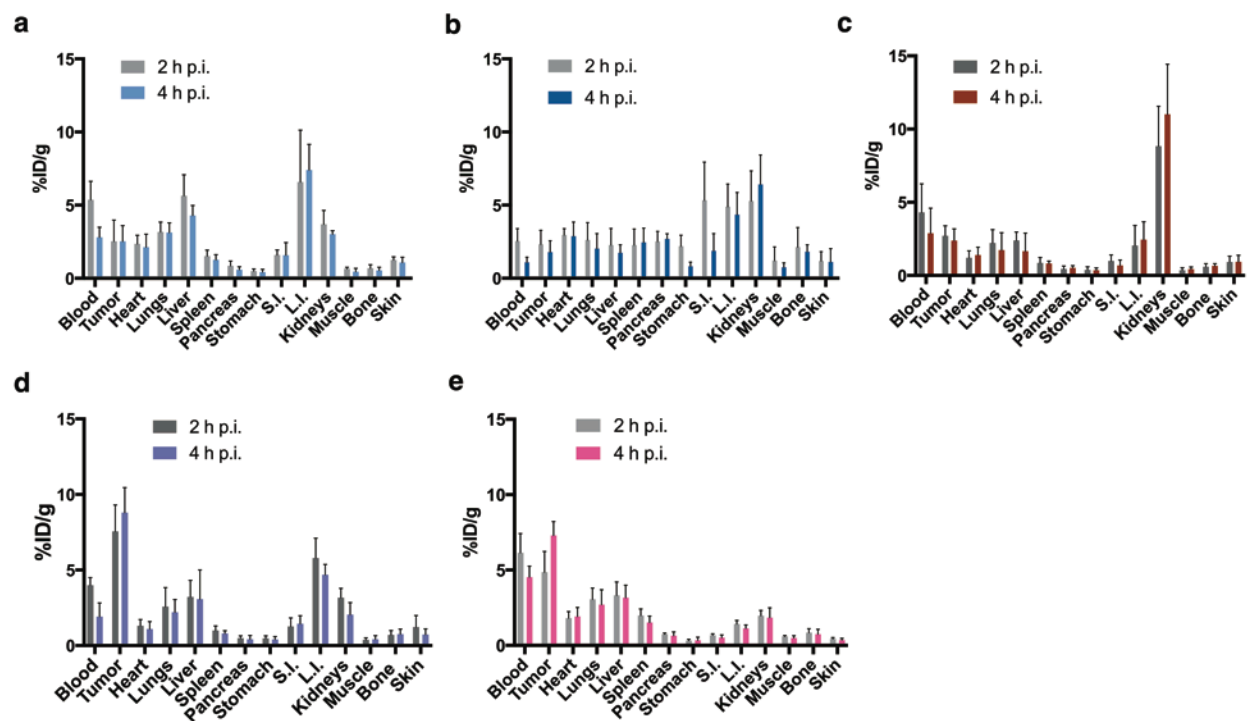


Figure S3 | Ex vivo biodistribution data obtained for radioligands [^{18}F]15, [^{18}F]19, and [^{18}F]29 (a-c), as well as for lead compounds [^{18}F]27 (d) and [^{68}Ga]27 (e). Organ uptake values were determined at 2 h and 4 h p.i.

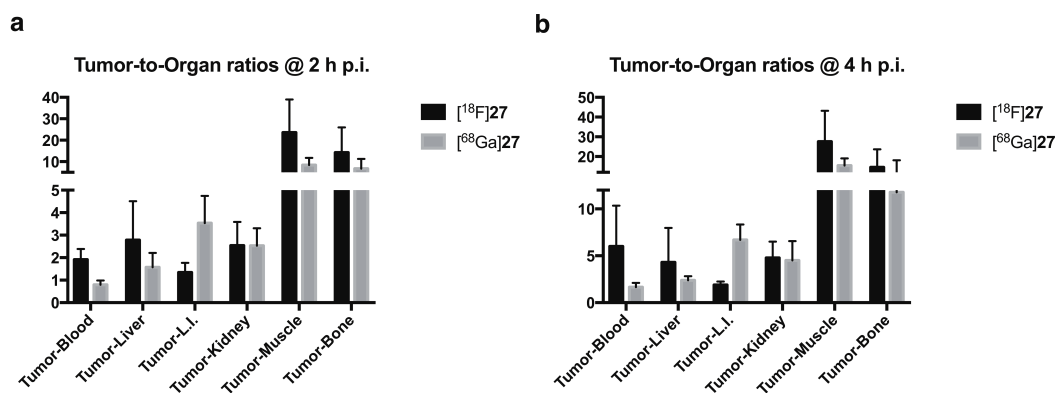
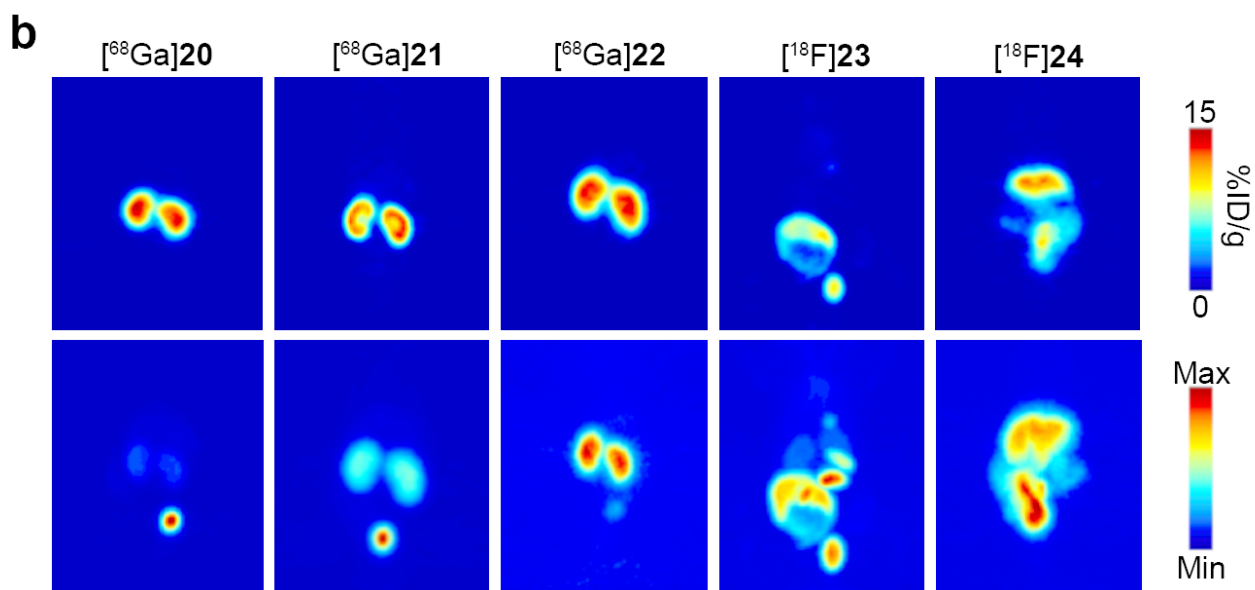
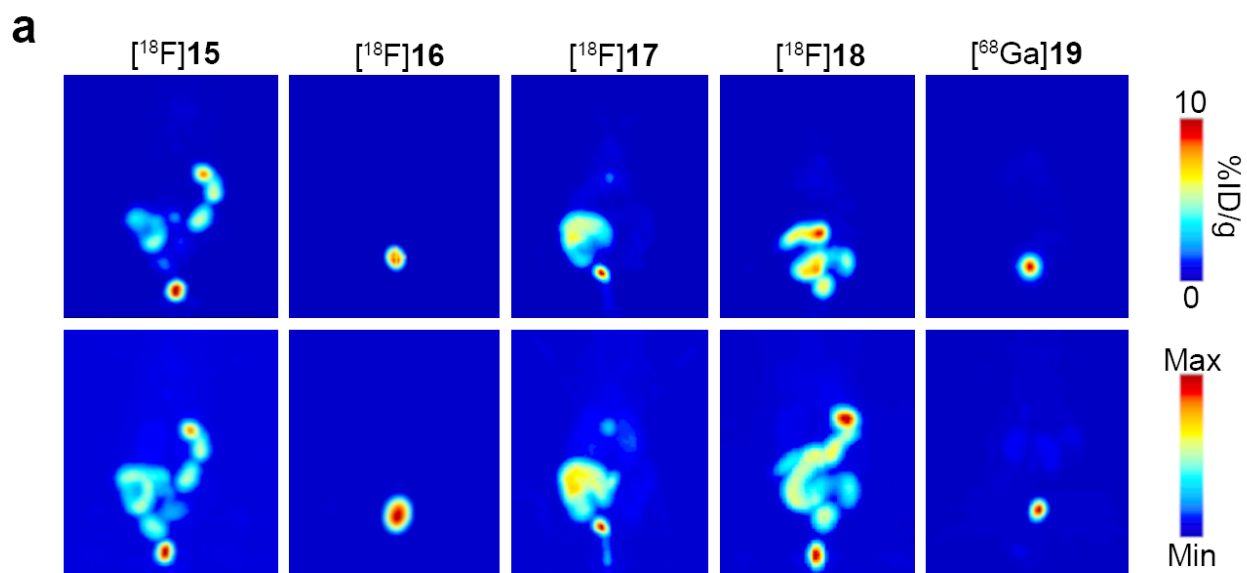


Figure S4 | Calculated tumor-to-organ ratios at 2 h (a) and 4 h (b) p.i. for both lead compounds.

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 VMTM 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 VMTM software then calculates the mean %ID/g for the given ROI.



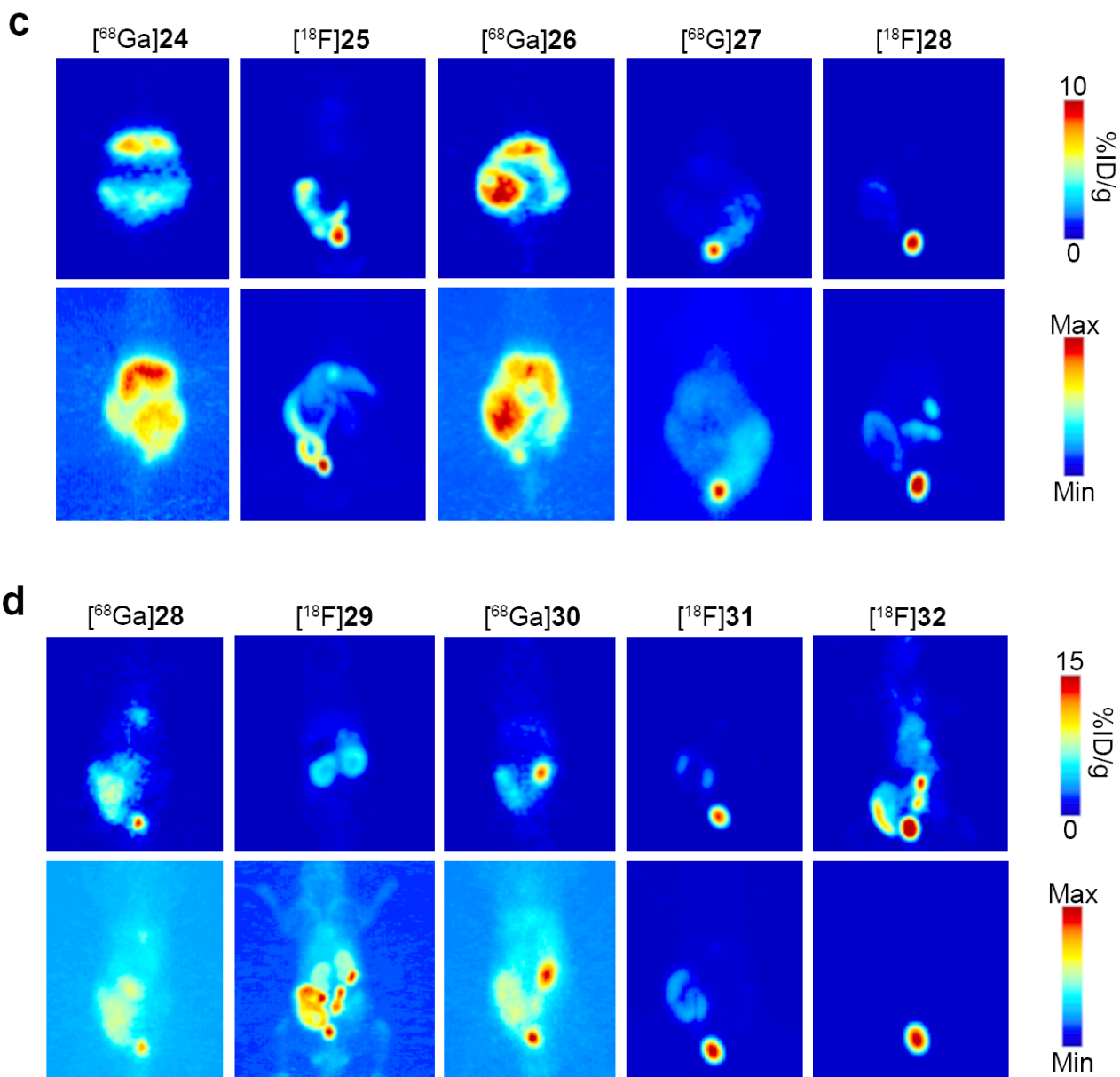
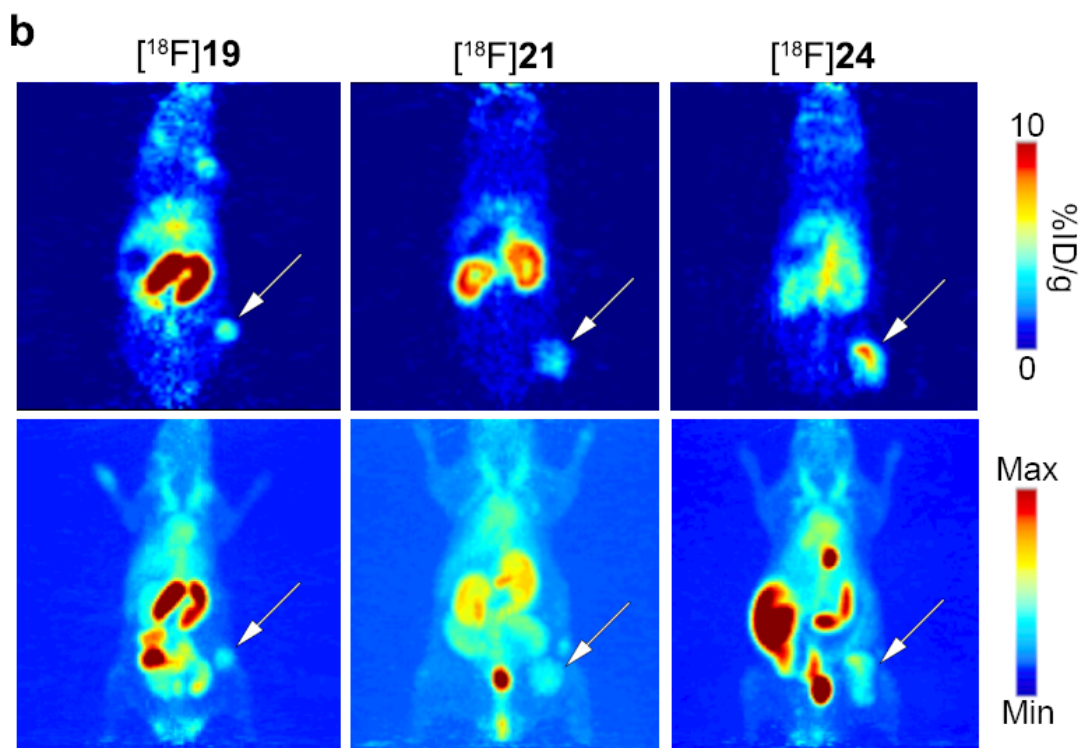
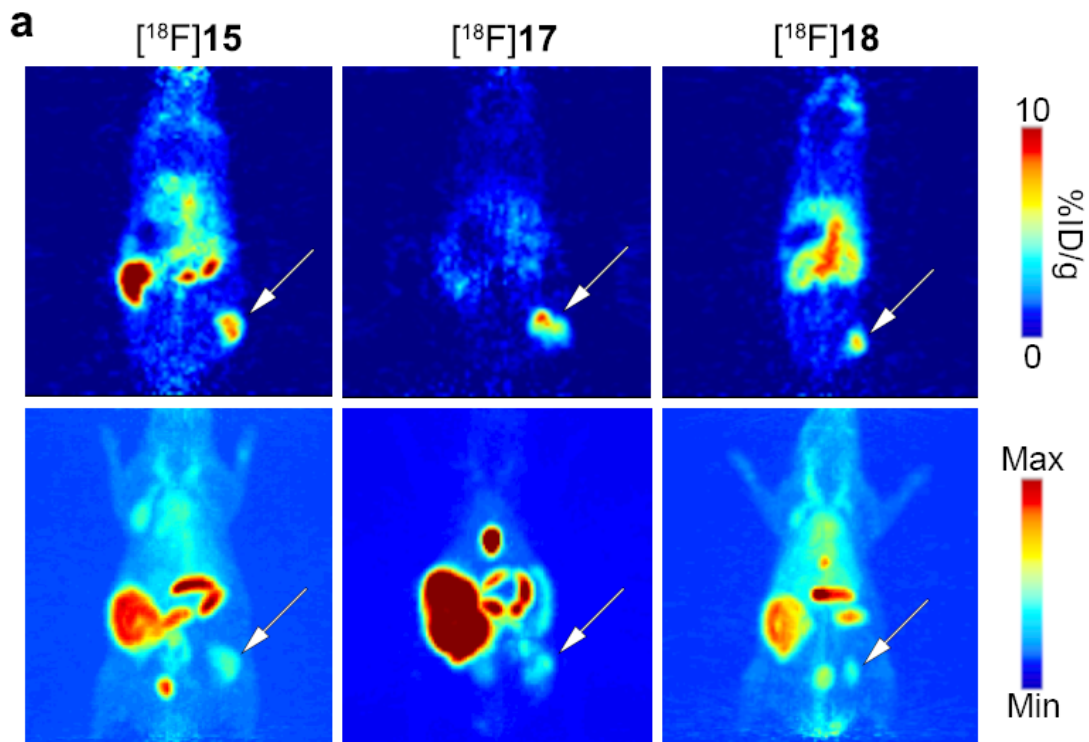


Figure S5 | PET images acquired 2 h p.i. of the radioligand. Images were acquired using healthy athymic nude for exploration of biodistribution. The upper panel displays transverse slices and the bottom rows show the corresponding MIPs.



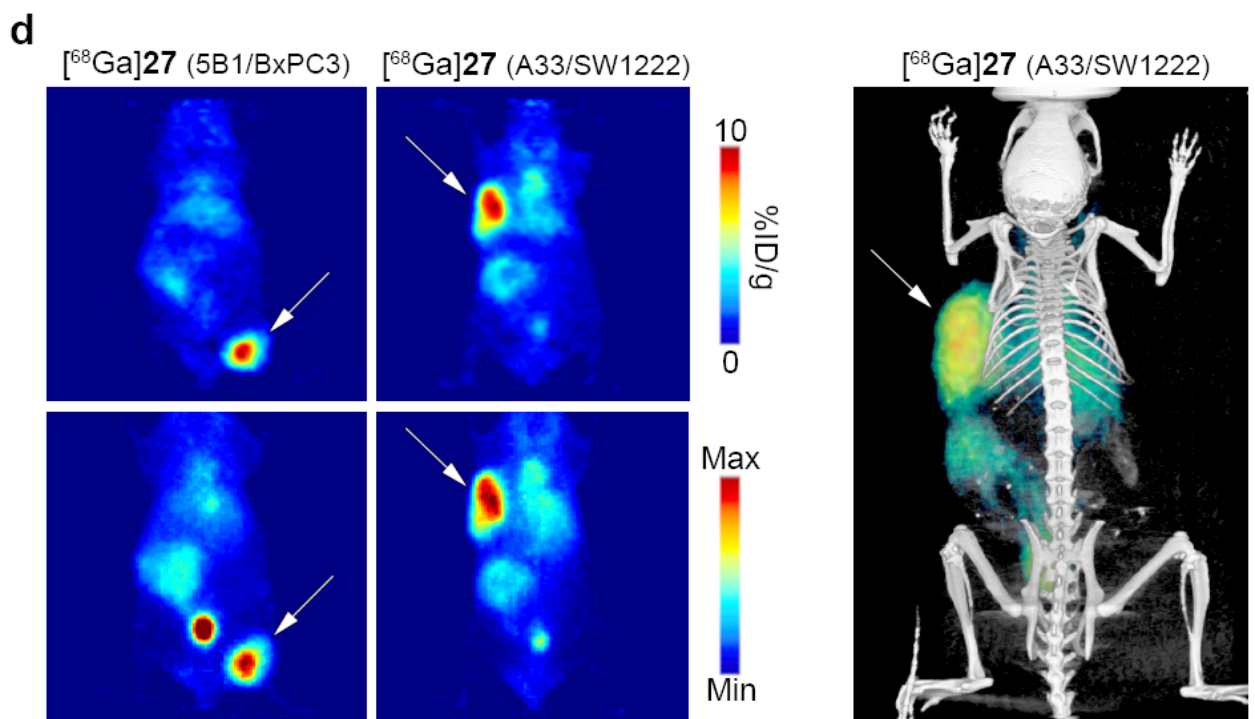
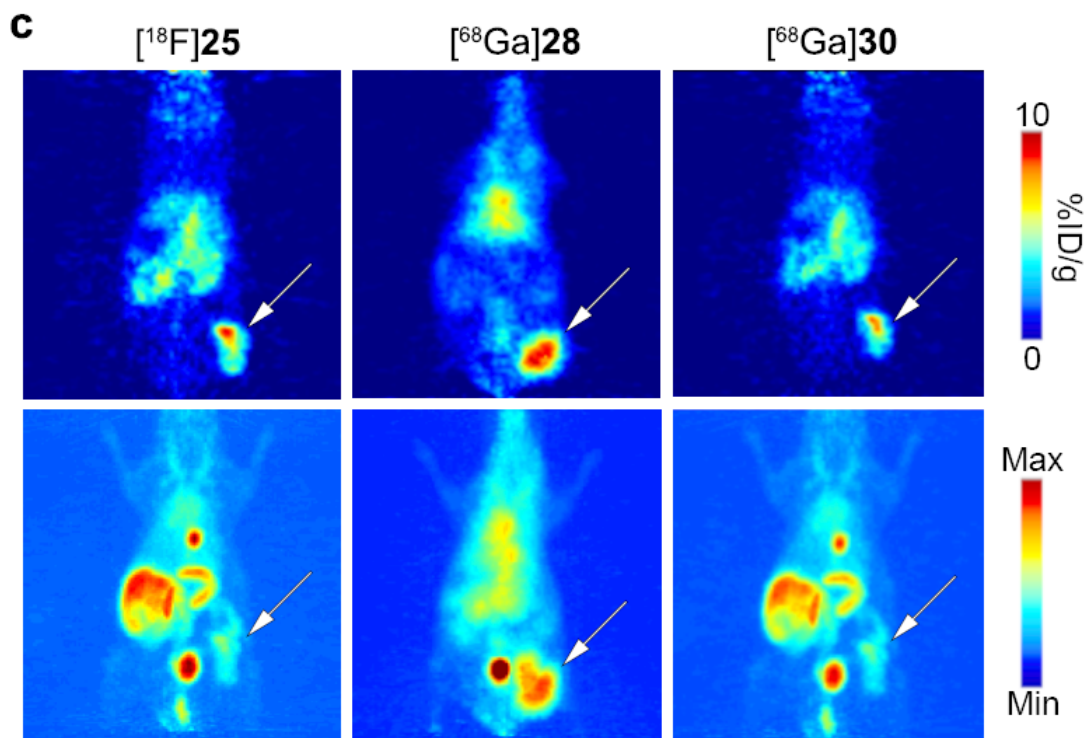


Figure S6 | Pretargeted PET images acquired 2 h p.i. of all remaining radioligands that were not shown in the manuscript. The upper panel displays transverse slices and the bottom rows show the corresponding MIPs. Location of tumor tissue is indicated by white arrows.

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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Compound	SMILES	Overall net charge	LogD [1-Octanol/water]	Plasma Half-life in mice [min]
[18F]19	<chem>O=C(C(CCCCNC(NC1=CC=C(CC2C[N@@]3(CC([O-])=O)CC[N@@]4(CC(O5)=O)CC[N@]2(CC(O6)=O)[Al]4365[18F])C=C1)=S)[NH3+])NCC(C=C7)=CC=C7C8=NN=C(C)N=N8</chem>		0 -1.3 ± 0.19	3.6
[18F]20	<chem>O=C(C(CCCCNC(C(CCCCNC(NCC1=CC=C(CC2C[N@@]3(CC([O-])=O)CC[N@@]4(CC(O5)=O)CC[N@]2(CC(O6)=O)[Al]4365[18F])C=C1)=S)[NH3+])=O)[NH3+])NCC(C=C7)=CC=C7C8=NN=C(C)N=N8</chem>		1 -1.26 ± 0.14	3.19
[18F]21	<chem>O=C(C(CCCCNC(C(CCCCNC(C([NH3+])CCCCNC(NCC1=CC=C(CC2C[N@]3(CC([O-])=O)CC[N@]4(CC(O5)=O)CC[N@@]2(CC(O6)=O)[Al]4365[18F])C=C1)=S)=O)[NH3+])=O)[NH3+])NCC(C=C7)=CC=C7C8=NN=C(C)N=N8</chem>		2 -1.12 ± 0.23	3.48
[18F]27	<chem>O=C(CCCC(NCCOCCNC(NC1=CC=C(C[N]2([Al]3456[18F])CC[N@]4(CC(O5)=O)CC[N]3(CC(O6)=O)CC2)C=C1)=S)=O)NCC(C=C7)=CC=C7C8=NN=CN=N8</chem>		0 -1.34 ± 0.18	17.07
[68Ga]27	<chem>O=C(CCCC(NCCOCCNC(NC1=CC=C(C[N]2([Ga68]3450C6=O)CC[N]4(C6)CC[N]3(CC(O5)=O)CC2)C=C1)=S)=O)NCC(C=C7)=CC=C7C8=NN=CN=N8</chem>		1 -1.45 ± 0.12	15.11
[18F]29	<chem>O=C(CCCC(NCCOCCNC(C([NH3+])CCCCNC(NC(C=C1)=CC=C1C[N]2(CC3)[Al]45(O6)([18F])OC(C[N]4(CC2)CC[N@]53CC6=O)=O)=S)=O)NCC(C=C7)=CC=C7C8=NN=CN=N8</chem>		1 -2.39 ± 0.1	5.21
[18F]30	<chem>O=C(NCCOCCNC(CCCC(NCC(C=C1)=CC=C1C2=NN=CN=N2)=O)=O)C(CC3=CN=C[NH2+]3)NC(NCC4=CC=C(C[N]5([Al]6789[18F])CC[N@@]7(CC(O8)=O)C[N]6(CC(O9)=O)CC5)C=C4)=S</chem>		2 -2.33 ± 0.12	8.16