General information

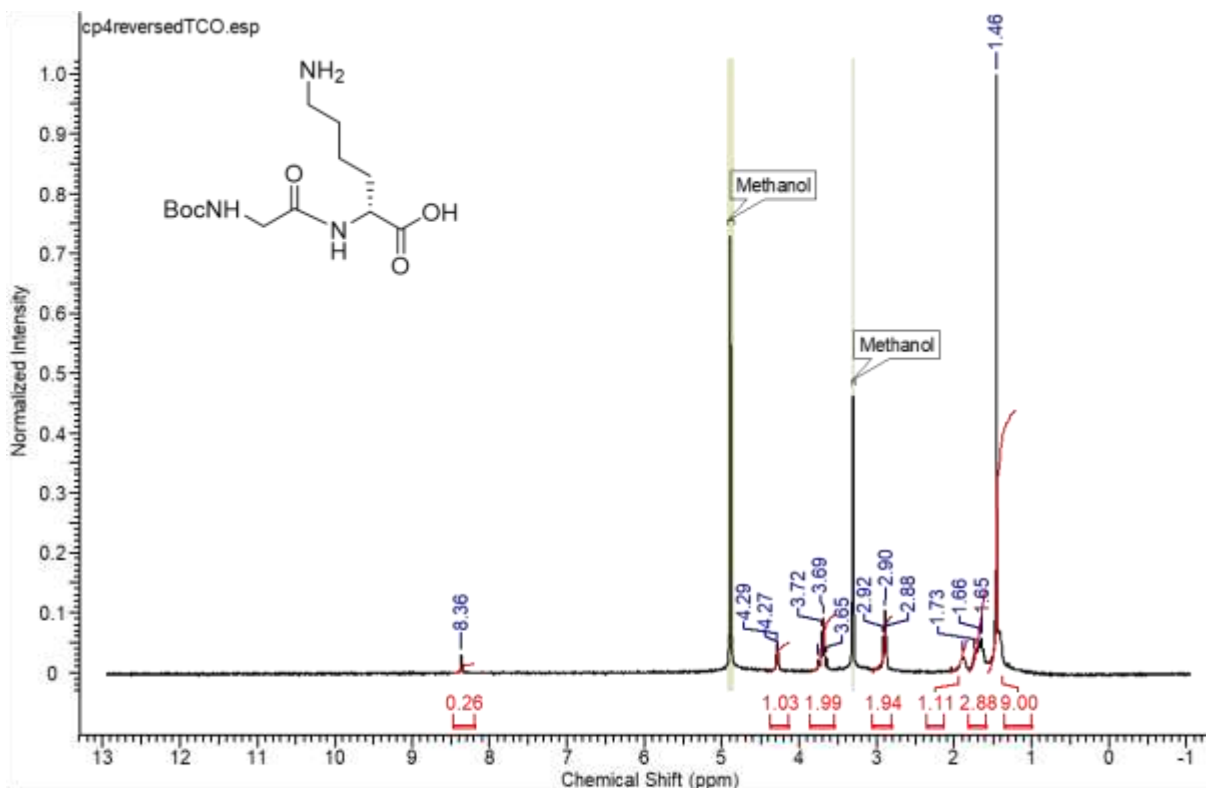
All reagents were purchased from Sigma-Aldrich (St. Louis, MO) except where noted. Sodium ^{125}I-iodide [81.4 TBq (2200 Ci/mmol] in 0.1 N NaOH was obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA). *N*-succinimidyl 3-((1, 2-bis(*tert*-butoxy-carbonyl)guanidino)methyl)-5-iodobenzoate (Boc$_2$-*iso*-SGMIB) and its tin precursor *N*-succinimidyl 3-((1, 2-bis(*tert*-butoxycarbonyl)guanidino)methyl)-5-(tri-n-butylstannyl)benzoate (Boc$_2$-*iso*-SGMTB) were synthesized following reported methods (*1*). *N*^{6}-(((9H-fluoren-9-yl)methoxy)carbonyl)-*N*^{2}-((*tert*-butoxycarbonyl)glycyl)-L-lysine, **1** (*2*) and *N*,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)-carbonyl)pyridin-2-aminium trifluoromethanesulfonate (*3*) were synthesized as previously reported. Methyltetrazine-PEG$_4$-maleimide and TCO-NHS ester were obtained from Click Chemistry Tools (Scottsdale, AZ), Fmoc-PEG$_4$-NHS ester from BroadPharm (San Diego, CA) and immobilized tris-[2-carboxyethyl] phosphine hydrochloride gel from Thermo Fisher Scientific (Waltham, MA). HPLC was performed using the following systems: 1) an Agilent 1260 Infinity System (Santa Clara, CA) equipped with a 1260 Infinity Multiple Wavelength Detector, and an Advion Expression^L Compact Mass Spectrometer (Ithaca, NY) in series; 2) an Agilent 1260 Infinity system equipped with a 1260 Infinity multiple wavelength detector and a LabLogic Dual Scan-RAM (Tampa, FL) flow radioactivity detector/TLC scanner. 3) a system similar to that in the second system but with a LabLogic Flow-RAM flow radioactivity detector. The first system was controlled by Advion Mass Express software and the latter two by LabLogic Laura® software. For both radiolabeled and unlabeled compounds, HPLC was performed using an Agilent Poroshell EC-120 (9.4 mm I.D. × 250 mm, 2.7 µm) reversed-phase semi-preparative column. Vivaspin® 500 centrifugal concentrators used for desalting or buffer exchange of the sdAbs were purchased from Sigma-Aldrich (St. Louis, MO). Empore™ SPE C18 cartridges, used for concentrating HPLC samples, were purchased from 3M (Maplewood, MN). Disposable PD-

10 columns for gel filtration were purchased from GE Healthcare (Piscataway, NJ). Activity levels in various samples were assessed using an automated gamma counter — either an LKB 1282 (Wallac, Finland) or a Perkin Elmer Wizard II (Shelton, CT). Proton NMR spectra of samples were obtained on a 400 MHz spectrometer (Varian/Agilent; Inova) and chemical shifts are reported in δ units using the residual solvent peaks as a reference. Mass spectra were recorded using an Advion Expression[L] Compact Mass Spectrometer for electrospray ionization (ESI) LC/MS (see above) and/or an Agilent LCMS-TOF (ESI); the latter is a high-resolution mass spectrometer. Molecular weights of derivatized sdAbs were determined using the Advion system.

Chemical synthesis

Tert-butoxycarbonyl)glycyl-L-lysine (2)

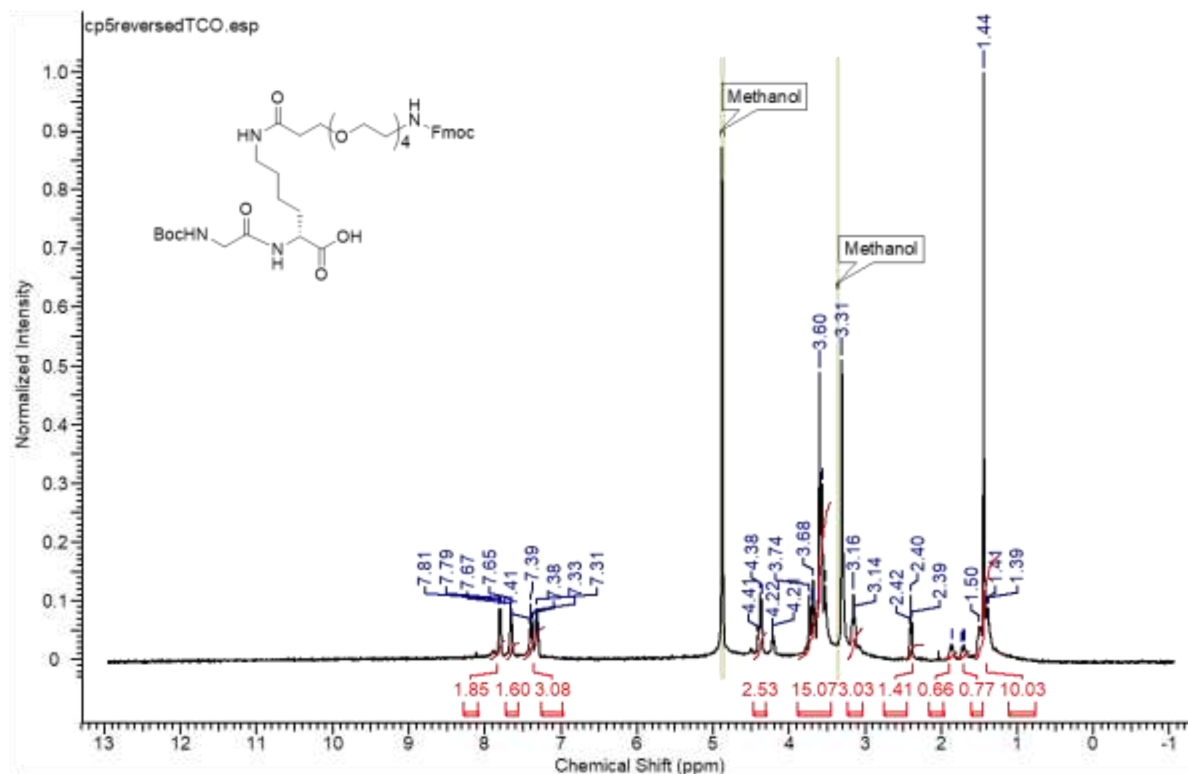
Compound **1** (300 mg, 0.57 mmol) was dissolved in 2 ml of 20% piperidine in DMF and the mixture stirred at 25°C for 30 min. The solvent was removed under vacuum and the residue was washed with ethyl acetate (3 × 20 ml) to obtain 147.3 mg (0.49 mmol, 85.3%) of compound **2** (*4*) as a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ_H = 1.3−1.5 (s, 9H), 1.6−1.8 (m, 3H), 2.8−3.0 (t, 3H), 3.6−3.8 (m, 2H), 4.2−4.3 (t, 1H), 8.3−8.4 (s, 1H). LRMS (LCMS-ESI) *m*/*z*: 304.2 $(M+H)^+$. HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{26}N_3O_5$ $(M+H)^+$: 304.1872; found: 304.1861 ± 0.0002 (n = 4).



(S)-25-(2-((tert-butoxycarbonyl)amino)acetamido)-1-(9H-fluoren-9-yl)-3,19-dioxo-2,7,10,13,16-pentaoxa-4,20-diazahexacosan-26-oic acid (3)

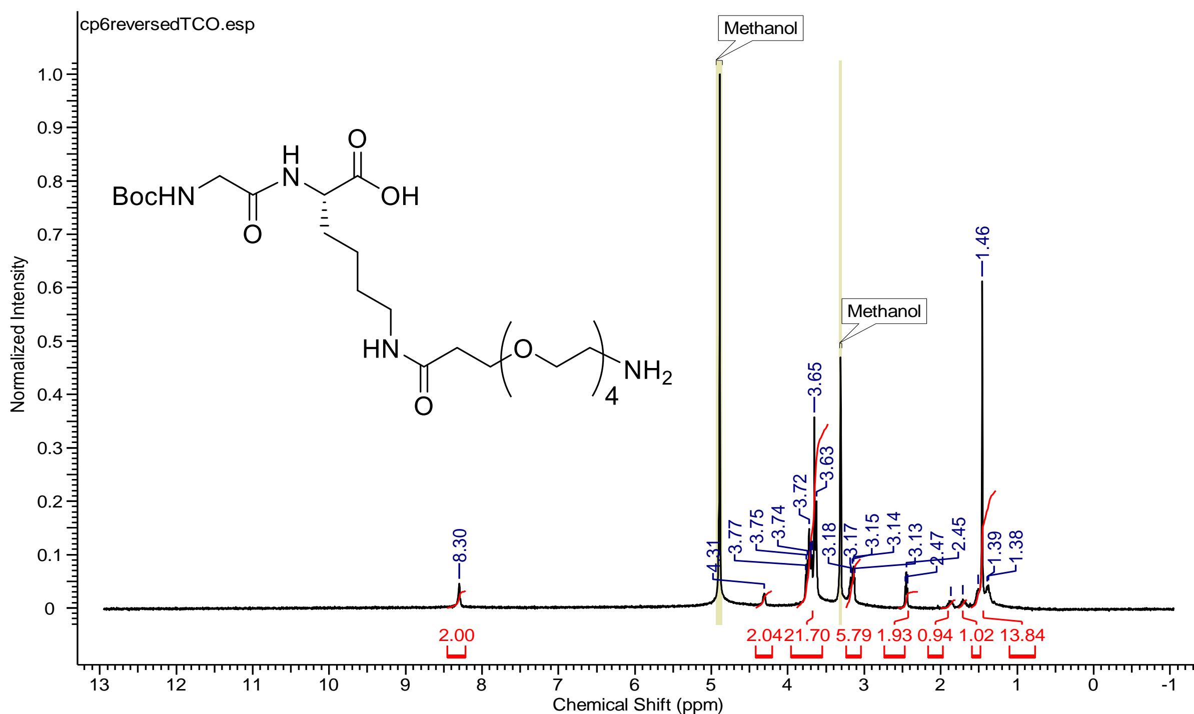
A mixture of **2** (100 mg, 0.33 mmol), Fmoc-PEG$_4$-NHS ester (193 mg, 0.33 mmol) and *N*,*N*-diisopropylethylamine (41 mg, 0.33 mmol) in 5 ml DMF was stirred at 25°C for 2 h. The crude mixture was purified by semipreparative HPLC. For this, the Agilent Poroshell EC-120 (9.4 × 250 mm, 2.7 μm) reversed-phase semipreparative column was eluted at a flow rate of 4 ml/min with a gradient consisting of 0.1% formic acid both in water (solvent A) and acetonitrile (solvent B); the proportion of B was increased linearly from 30% to 70% over 15 min. Pooled HPLC fractions containing **3** (t_R = 10.0 min) were lyophilized to give 184.2 mg (0.24 mmol, 72.3%) of a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ_H = 1.3−1.5 (m, 10H), 1.6−1.8 (m, 1H), 1.8−2.0 (m,1H), 2.3−2.5 (t, 2H), 3.1−3.2 (t, 3H), 3.4−3.8 (m, 15H), 4.3−4.5 (m, 3H), 7.2−7.5 (m, 3H), 7.6−7.7 (d, 2H), 7.7−7.9

(d, 2H). LRMS (LCMS-ESI) *m/z*: 773.0 $(M+H)^+$. HRMS (ESI, *m/z*) calcd for $C_{39}H_{57}N_4O_{12}$ $(M+H)^+$: 773.3973; found: 773.3978± 0.0001 (n = 4).



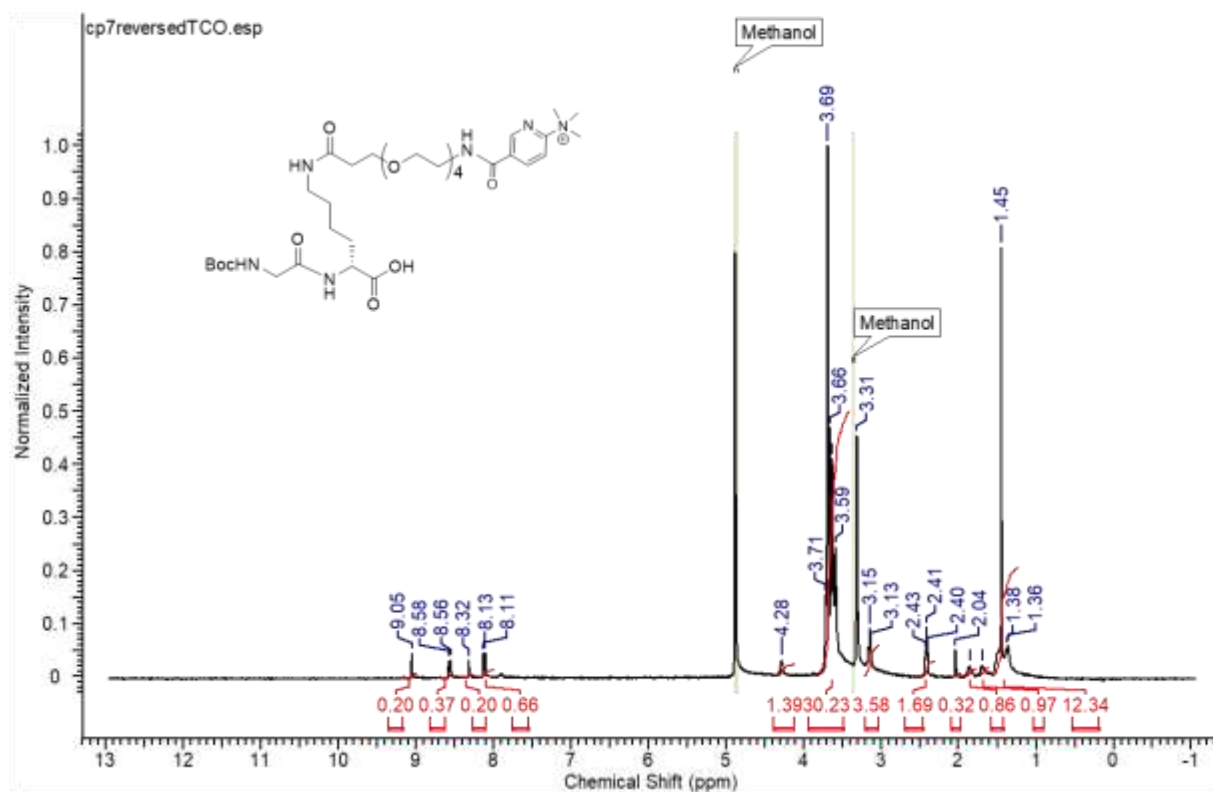
(S)-1-amino-21-(2-((tert-butoxycarbonyl)amino)acetamido)-15-oxo-3,6,9,12-tetraoxa-16-azadocosan-22-oic acid (4)

Compound **3** (100 mg, 0.13 mmol) was dissolved in 2 ml of 20% piperidine in DMF and the mixture stirred at 25°C for 30 min. The solvent was removed under vacuum and the residue washed with ethyl acetate (3 × 20 ml) to obtain 58.3 mg (0.11 mmol, 81.5%) of a colorless oil: ^1H NMR (CD_3OD, 400 MHz) δ_H = 1.3−1.6 (m, 13H), 1.6−1.8 (m,1H), 1.8−2.0 (m, 1H), 2.4−2.5 (t, 2H), 3.0−3.2 (m, 6H), 3.6−3.8 (m, 22H), 4.2−4.4 (t, 2H), 8.2−8.3 (s, 2H). LRMS (LCMS-ESI) *m/z*: 551.1 $(M+H)^+$. HRMS (ESI, *m/z*): calcd for $C_{24}H_{47}N_4O_{10}$ $(M+H)^+$: 551.3292; found: 551.3288 ± 0.0003 (n = 4).



(S)-5-((9-carboxy-2,2-dimethyl-4,7,15-trioxo-3,18,21,24,27-pentaoxa-5,8,14-triazanonacosan-29-yl)carbamoyl)-N,N,N-trimethylpyridin-2-aminium triflate (5)

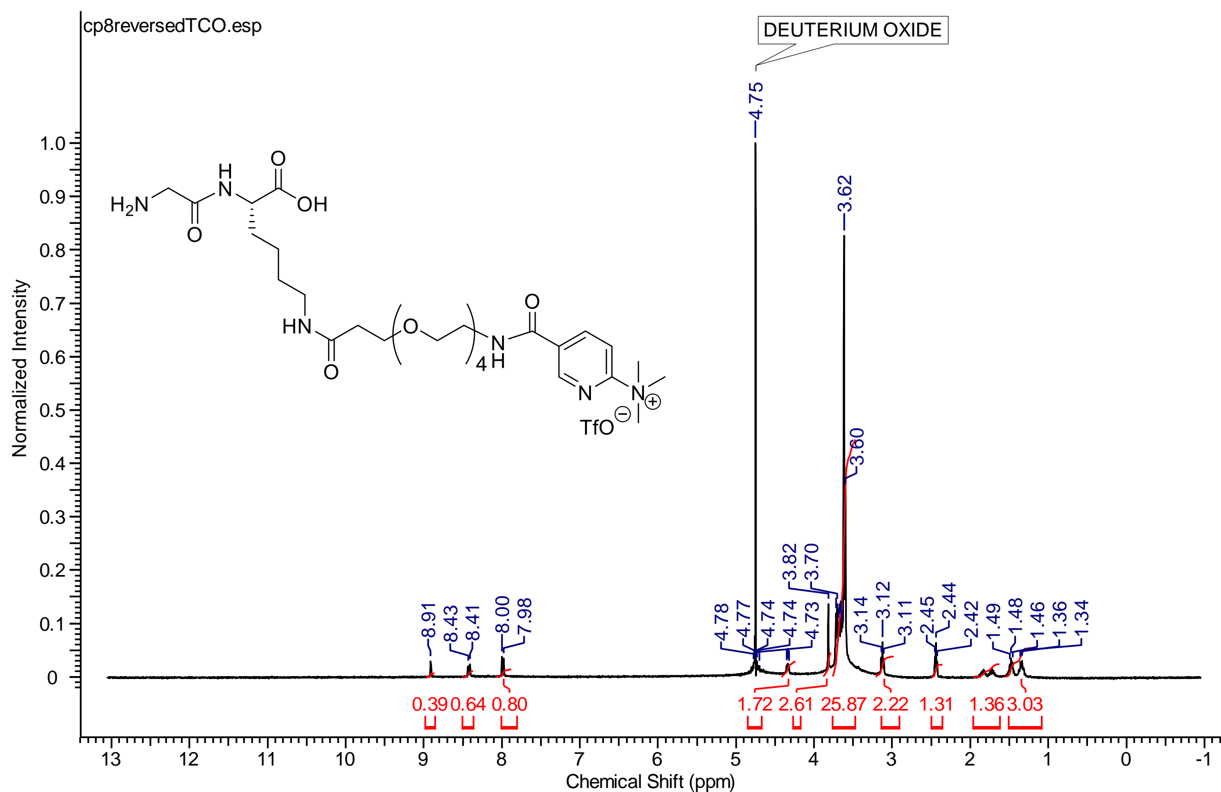
A mixture of **4** (58.3 mg, 0.11 mmol), *N*,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)-carbonyl)pyridin-2-aminium trifluoromethanesulfonate (46.6 mg, 0.10 mmol) and *N*,*N*-diisopropylethylamine (24.8 mg, 0.2 mmol) in 1 ml DMF was stirred at 25°C for 2 h. The product was isolated from this mixture by semi-preparative HPLC as above but using a gradient, wherein the proportion of solvent B was increased linearly from 10% to 30% over 15 min. Lyophilization of pooled HPLC fractions containing **5** (t_R = 12.3 min) delivered 43.5 mg (51.2 μmol, 51.2%) of a white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ_H = 1.2−1.6 (m, 13H), 1.6−1.8 (m, 1H), 1.8−2.0 (m, 1H), 2.3−2.5 (t, 2H), 3.0−3.2 (t, 4H), 3.5−3.8 (m, 30H), 4.2−4.3 (t, 1H), 8.0−8.2 (d, 1H), 8.3-8.4 (s, 1H),



8.5−8.6 (d, 1H), 9.0−9.1 (s, 1H). LRMS (LCMS-ESI) *m/z*: 713.5 M^+. HRMS (ESI, *m/z*) calcd for $C_{33}H_{57}N_6O_{11}^+$ (M^+): 713.4080; found: 713.4074 ± 0.0001 (n = 4).

(S)-5-((24-amino-21-carboxy-15,23-dioxo-3,6,9,12-tetraoxa-16,22-diazatetracosyl) carbamoyl)-N,N,N-trimethylpyridin-2-aminium triflate (6)

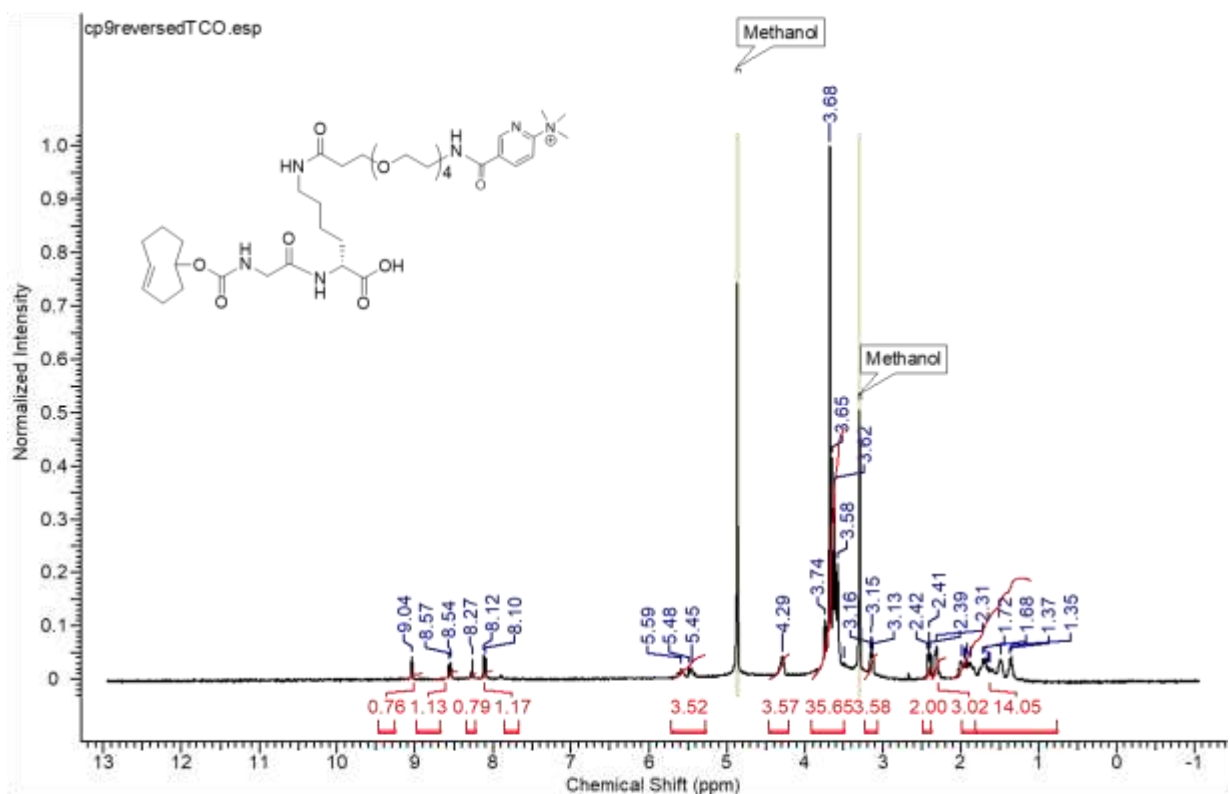
A 95:2.5:2.5 (v/v/v) mixture of TFA:water:triisopropyl silane (0.5 ml) was added to compound **5** (43.5 mg; 51.2 µmol) and the mixture stirred at 25°C for 30 min. Solvents were evaporated to yield 41.0 mg (92.7%, based on trifluoroacetate salt) of compound **6** as a white solid. Because of its extreme polarity, purification by reversed-phase HPLC was not feasible and thus it was carried over as such to the next step. ^{1}H NMR (D_2O, 400 MHz) δ_H =1.3-1.5 (m, 3H), 2.4-2.5 (t, 1H), 3.1-3.2 (t, 3H), 3.5-3.8 (m, 26H), 3.8-3.9 (s, 2H), 4.3-4.4 (t, 2H), 7.9−8.1 (d, 1H), 8.4-8.5 (d,



1H), 8.9-9.0(s,1H). LRMS (LCMS-ESI) *m/z*: 613.5 M$^+$. HRMS (ESI, *m/z*): calcd for $C_{28}H_{49}N_6O_9$ (M$^+$): 613.3556; found: 613.3552 ± 0.0001 (n = 4).

N-(5-(((6S)-6-carboxy-1-(((E)-cyclooct-4-en-1-yl)oxy)-1,4,12-trioxo-15,18,21,24-tetraoxa-2,5,11-triazahexacosan-26-yl)carbamoyl)pyridin-2-yl)-N,N-dimethylmethanideaminium triflate (7)

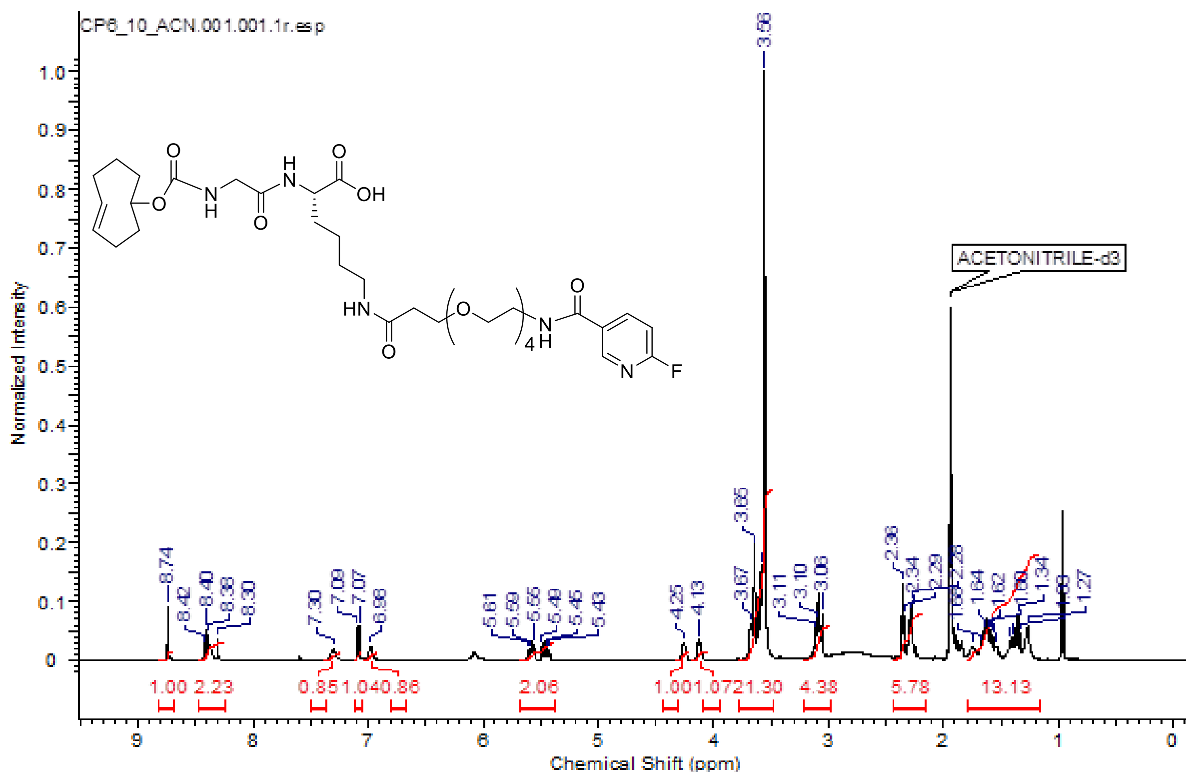
Compound **6** (41 mg, 53.7 μmol), TCO-NHS ester (19 mg, 71.3 μmol) and *N*,*N*-diisopropylethylamine (11.8 mg, 95 μmol) were taken in 1 ml DMF and the mixture stirred at 25°C for 2 h. The mixture was subjected to semipreparative HPLC as above but using a gradient wherein the proportion of solvent B was increased linearly from 5% to 30% over 15 min. Lyophilization of pooled HPLC fractions containing **7** (t_R = 14.1 min) rendered 19.5 mg (21.7 μmol,



45.7%) of a colorless oil: ^{1}H NMR (CD_3OD, 400 MHz) δ_H = 1.0−2.0 (m, 14H), 2.25−2.35 (m, 3H), 2.35−2.45 (t, 2H), 3.1−3.2 (t, 4H), 3.5−3.8 (m, 36H), 4.2−4.4 (m, 4H), 5.3−5.7 (m, 4H), 8.0−8.2 (d, 1H), 8.2−8.3 (s, 1H), 8.5−8.6 (d, 1H), 9.0−9.1 (s, 1H). LRMS (LCMS-ESI) *m/z*: 765.4 (M^+). HRMS (ESI, *m/z*) calcd for $C_{37}H_{61}N_6O_{11}^+$ (M^+): 765.4393; found: 765.4396 ± 0.00004 (n = 4).

(23S)-23-(2-(((((E)-cyclooct-4-en-1-yl)oxy)carbonyl)amino)acetamido)-1-(6-fluoropyridin-3-yl)-1,17-dioxo-5,8,11,14-tetraoxa-2,18-diazatetracosan-24-oic acid, FN-GK-TCO (8)

A 1M solution (3 µl) of tetra-n-butylammonium fluoride in THF (0.8 mg; 3.0 µmol) was added to a solution of **7** (1 mg, 1.1 µmol) in anhydrous acetonitrile (200 µl), and the mixture stirred at 40°C for 30 min. The resultant product was isolated by semi-preparative HPLC as above but using a gradient wherein the proportion of solvent B was increased linearly from 30% to 70% over 15 min. Solvents from the pooled HPLC fractions (t_R = 9.0 min) were removed by lyophilization to



obtain 0.6 mg (0.83 μmol, 75.4%) of **8** as a colorless oil: ^{1}H NMR (CD_3CN, 500 MHz) δH = 1.2-1.8 (m, 13H), 2.2-2.4 (m, 6H), 3.0-3.2 (m, 4H), 3.5-3.8 (m, 21H), 4.1-4.2 (m, 1H), 4.2-4.3(m, 1H), 5.4-5.7 (m, 2), 6.9-7.0 (s, 1H), 7.0-7.1 (m,1H), 7.2-7.4 (s,1H), 8.2-8.5 (m, 2H), 8.7-8.8 (s,1H). LRMS (LCMS-ESI) *m/z*: 726.3 $(M+H)^+$. HRMS (ESI, *m/z*): calcd for $C_{34}H_{53}FN_5O_{11}$ $(M+H)^+$: 726.3726; found: 726.3725 ± 0.0001 (n = 4).

Determination of unlabeled sdAb binding kinetics using surface plasmon resonance

Surface plasmon resonance measurements were performed on a Biacore T200 instrument (Cytiva, Marlborough, MA) at the Duke University Human Vaccine Institute Shared Resource. HER2-Fc protein was immobilized on a CM5 chip via NHS/EDC-mediated amine coupling to 7024 response units (RU). A binding screen of 5F7 and Tz-5F7GGC (50 nM) was performed using the following parameters: 1) flow cells 3 (blank) and 4 (Her2-Fc) (Reference

subtraction, 4-3); 2) association and dissociation for each performed for 120 s at 30 µl/min; and 3) regeneration with 10 mM glycine-HCl, pH 2.0 performed for 45 s at 30 µl/min. For the determination of binding kinetics, immobilized Her2-Fc at a lower density (872 RU) was used. Sensorgrams were generated using a 2-fold titration series of each sdAb (0.39–25 nM) with the following parameters: 1) flow cells 1 (blank) and 2 (Her2-Fc) (Reference subtraction, 2-1); 2) 180-s association and 600-s dissociation at 50 µl/min; and 3) regeneration with 10 mM glycine-HCl, pH 2.0 for 45 s at 30 µl/min. Curves were fit to a 1:1 antigen:analyte binding model using Biacore T200 Evaluation Software to retrieve association rate constants (k_a), dissociation rate constants (k_d), and equilibrium dissociation constants (K_d, a measurement of affinity).

Radiochemistry

Synthesis of ^{18}F-FN-PEG$_4$-GK-TCO (^{18}F-8)

Fluorine-18 activity trapped on a QMA cartridge (Waters Corp, Milford, MA) was obtained from PET-NET Solutions (Durham, NC). The cartridge was eluted with 1 ml of tetraethylammonium bicarbonate in 80% acetonitrile (3 mg/ml). The solvents from the eluate were evaporated at 100°C and the residual water removed by azeotroping with acetonitrile (3 × 0.5 ml) at the same temperature. A solution of **7** (1 mg, 1.1 µmol) in 0.25 ml anhydrous acetonitrile was added to the above dried ^{18}F activity (0.74 – 4.5 GBq), and the mixture heated at 40°C for 15 min. The resultant solution containing ^{18}F-**8** was purified by semi-preparative RP-HPLC as described above for the unlabeled compound using a gradient with the proportion of solvent B increased linearly from 30% to 70% over 15 min. Solvents from the pooled HPLC fractions (t_R = 9.0 min) were concentrated by solid-phase extraction using an Empore™ SPE C18 cartridge eluted with acetonitrile (3 × 150 µl), which was then was evaporated to dryness.

Preparation of iso-^{125}I-SGMIB-5F7

This was performed essentially as described previously (*1,5*). Briefly, Boc_2-*iso*-^{125}I-SGMIB (*1,5*) was first deprotected by treatment with TFA and the resultant *iso*-^{125}I-SGMIB was incubated with a solution of 5F7 sdAb in borate buffer, pH 8.5 (2 mg/ml, 50 µl) and the *iso*-^{125}I-SGMIB-5F7 conjugate was isolated by passage through a PD10 column as above.

Evaluation of radiochemical purity, HER2-binding affinity, and immunoreactivity of ^{18}F-5F7GGC

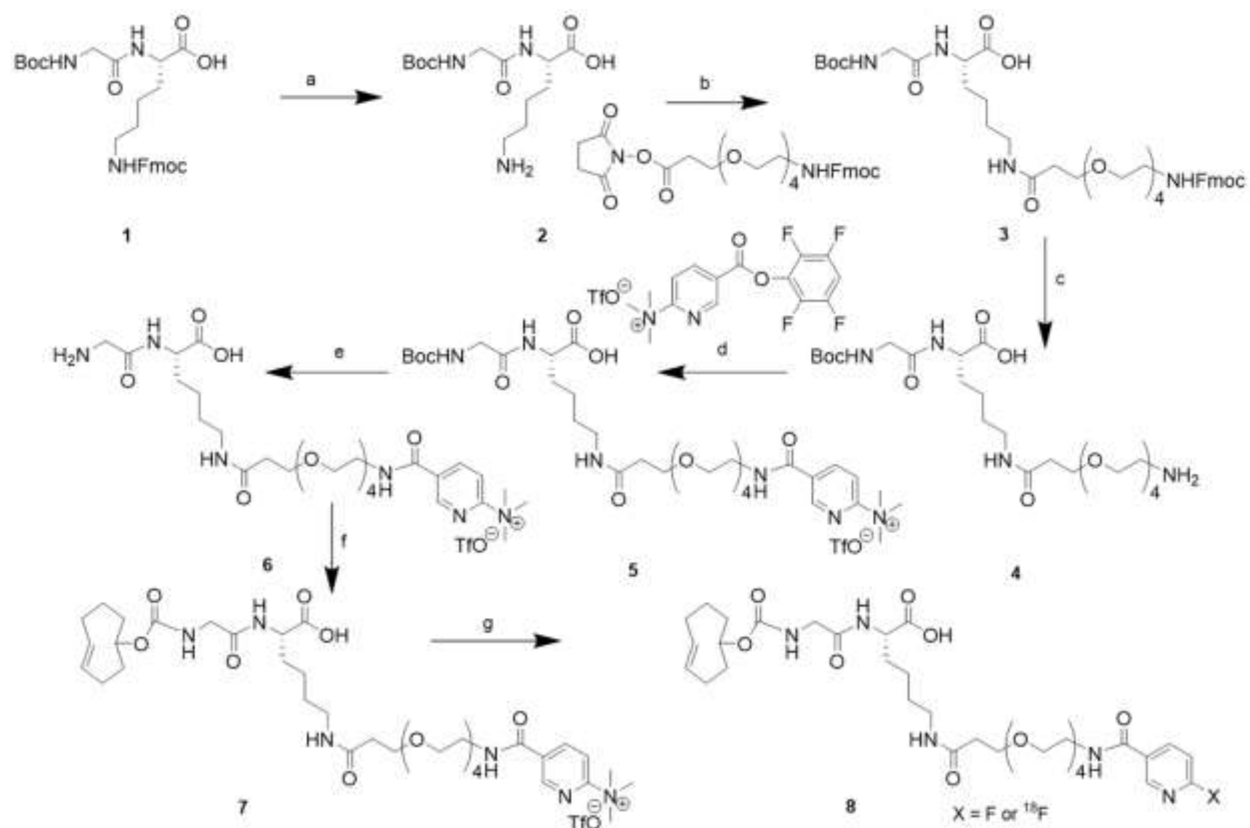
The radiochemical purity of ^{18}F-5F7GGC was determined as described (*2,5*) by SDS-PAGE and size-exclusion HPLC (SE-HPLC). SDS-PAGE was run under non-reducing conditions followed by phosphor imaging using the Storage Phosphor System Cyclone Plus phosphor imager (Perkin-Elmer Life and Analytical Sciences, Downers Grove, IL). SE-HPLC was performed on system 3 using a TSKgel SuperSW2000 (4.6 mm I.D. × 30 cm, 4µm) column (Tosoh Bioscience; Montgomeryville, PA) isocratically eluted with PBS, pH 7.0, at a flow rate of 0.3 ml/min. The binding affinity of ^{18}F-5F7GGC to HER2 on cells was determined by a saturation binding assay using the BT474M line following a reported procedure (*2,5*). Briefly, cells seeded in 24-well plates at a density of 8×10^4 cells/well in 0.5 ml of medium were incubated at 37°C overnight. Cells were then incubated at 4°C for 1 h with fresh cold medium containing increasing concentrations (0.1 to 100 nM; 0.6 ml total volume) of ^{18}F-5F7GGC. The cells were washed twice with cold medium, lysed with 0.1% SDS, and the activity in the cell lysates was counted using an automated gamma counter. Non-specific binding was determined in parallel assays performed by co-incubating the cells with a 100-fold excess of unlabeled trastuzumab. The data were fit using GraphPad Prism software to determine K_d values. The immunoreactive fraction of ^{18}F-5F7GGC was determined using magnetic beads coated with HER2 extracellular domain or human serum albumin (HSA) as a negative control as described (*2,5*). The percentage of specific binding (binding to HER2-immobilized minus that to HSA-immobilized beads) was calculated at each

bead concentration. The reciprocals of the percentage of specific binding were plotted against the reciprocals of bead concentration, and the data were fit to a straight line by linear regression. The immunoreactive fraction was calculated as the reciprocal of the y-intercept value (infinite antigen concentration).

Paired-label uptake of ^{18}F-5F7GGC and iso-^{125}I-SGMIB-5F7 by BT474M1 cells

HER2-expressing BT474M1 cells (8×10^5 cells per well in 2 ml medium) were seeded in 6-well plates and incubated overnight at 37ºC. On the next day, the medium was replaced with 2 ml of medium containing 5 nM each of ^{18}F-5F7GGC and *iso*-^{125}I-SGMIB-5F7, and the cells in triplicate wells were incubated at 37°C. At 1, 2 and 4 h, the cell culture supernatants were collected, the surface-bound activity was stripped by treating the cells twice with 1 ml of 50 mM glycine, pH 2.8, and finally, the cells were lysed with 0.1% SDS. The activity in the cell culture supernatants, membrane-bound fraction and cell lysates was counted in an automated gamma counter. Cell-associated activity was calculated from these as the percentage of input radioactivity dose associated with the cell lysates. To determine nonspecific uptake, a parallel experiment at the 2-h time point was performed as above but with the addition of a 100-fold molar excess of unlabeled trastuzumab to the incubation medium. The entire experiment was repeated twice.

Supplemental Figure 1. Scheme for the synthesis of quaternary salt precursor, FN-PEG$_4$-GK-TCO, and ^{18}F-PEG$_4$-GK-TCO



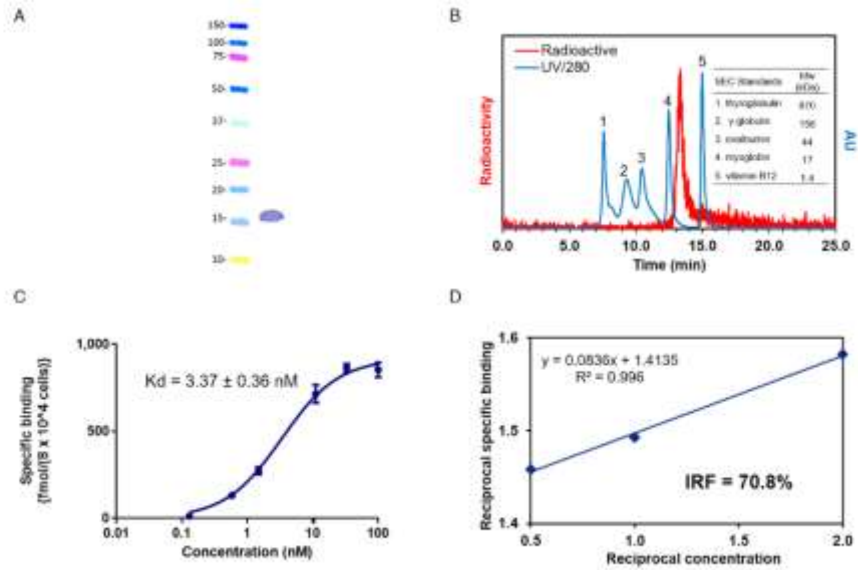
a) 20% piperidine in DMF b) DIEA, DMF c) 20% piperidine in DMF d) DIEA, DMF e) 95:2.5:2.5 TFA:H_2O, Tri-isopropyl silane f) TCO-NHS, DIEA, DMFg) DIEA, DMF h) TBAF or ^{18}F-TEAF, CH_3CN

Supplemental Figure 2: LCMS of Tz- 5F7GGC

Supplemental Figure 3. Characterization of ^{18}F-5F7GGC conjugate

(A) SDS-PAGE/phosphor imaging of ^{18}F-5F7GGC (right lane) and molecular weight markers (left lane). (B) Size-exclusion HPLC: Radioactivity profile in red and UV profile of molecular weight

standards in blue. (C) Saturation binding affinity assay using HER2-positive BT474 cells. (D) Lindmo immunoreactivity assay performed using HER2 ECD- and bovine serum albumin (negative control)-immobilized magnetic beads.



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