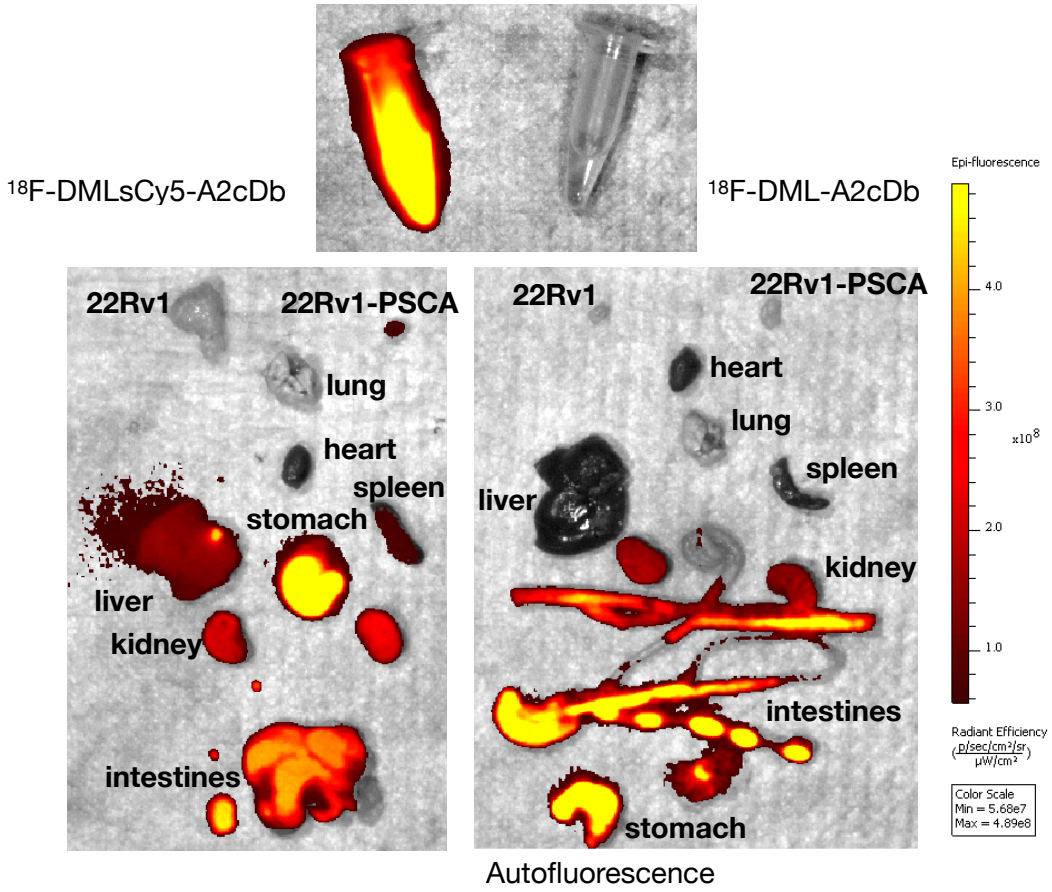


Supplemental Figure 1. Ex vivo optical imaging of tumors and organs from mice injected with the dual-modality ^{18}F-DMLsCy5-A2cDb (left panel) or the single-modality ^{18}F-DML-A2cDb (right panel).

Radiosynthesis of 2-(*E*)-5-(2-[^{18}F]fluoroethoxy)cyclooct-1-ene (^{18}F-TCO)

Reagents and analytical methods

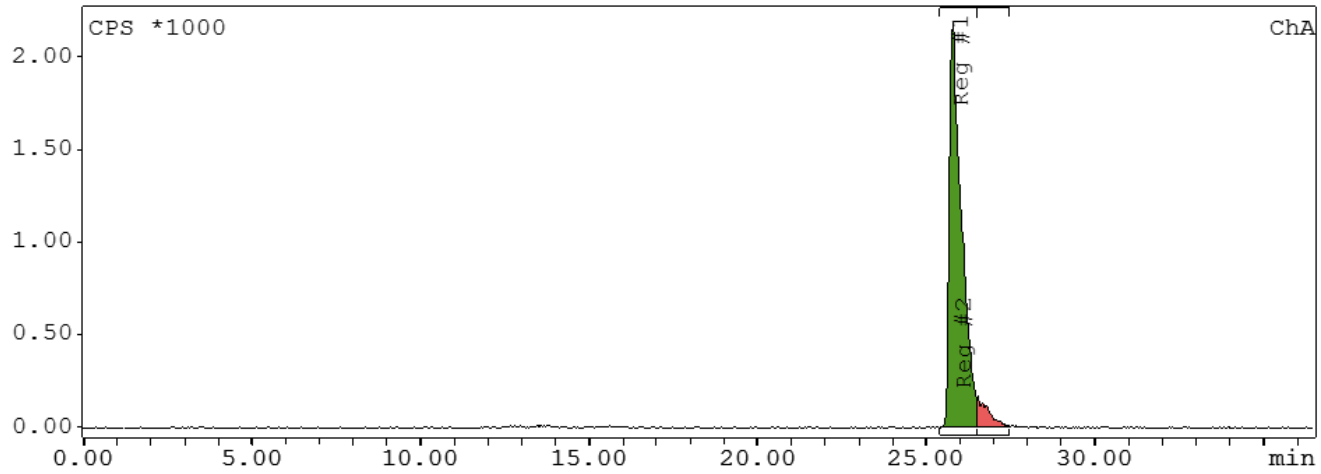
No-carrier-added [^{18}F]fluoride was produced by the (p,n) reaction of [^{18}O]H_2O (~ 97 % isotopic purity, Medical Isotopes) in a RDS-112 cyclotron (Siemens) at 11 MeV using a 1-ml tantalum target with Havar foil. Reagents and solvents were commercially available and used without further purification. TCO tosylate precursor was synthesized as described previously (*1*). Sodium ascorbate and 40% tetrabutylammonium hydroxide (TBAOH) were purchased from Sigma-Aldrich. HPLC grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) were purchased from Fisher Scientific. Anhydrous MeCN, dimethyl sulfoxide (DMSO) and tetraethylammonium bicarbonate were purchased from Simga-Aldrich. Pre-conditioned QMA Sep-Pak Light and C18 Sep-Pak Light cartridges were purchased from waters. HPLC purifications were performed on a Knauer Smartline HPLC system with inlince Knauer UV (254 nm) detector and gamma-radiation coincidence detector and counter (Bioscan Inc.). Semi-preparative HPLC was performed using a Phenomenex reverse-phase Luna column (10 × 250 mm, 5 µm) with a flow rate of 5 mL/min. Radiochemical purity and identity of compounds were determined by analytical radio-HPLC analysis performed with a Phenomenex reverse-phase Luna column (4.6 × 250 mm, 5 µm) with a flow rate of 1 mL/min. All chromatograms were collected by a GinaStar (Raytest) analog to digital converter and software.

Synthesis Protocol

^{18}F-TCO was synthesized with the ELIXYS FLEX/CHEM synthesizer (SOFIE) as reported previously (*1*). Briefly, the source vial containing [^{18}F]fluoride in [^{18}O]H_2O was pressurized and the solution transferred through the QMA Sep-Pak Light cartridge. Trapped [^{18}F]fluoride was eluted off the cartridge into a reaction vessel using 40% TBAOH (6.5 µL) in a mixture of water (0.3 mL) and MeCN (0.5 mL) which was azeotropically dried to afford activated [^{18}F]fluoride. TCO tosylate precursor (4 mg in 1 mL MeCN) was added to the activated [^{18}F]fluoride residue and the mixture was heated for 10 min at 90 °C. The reaction was cooled to room temperature and 0.5% (wt/wt) sodium ascorbate solution (2 mL) was added to the reaction vessel. The resulting mixture was transferred to the HPLC injection loop for semi-preparative HPLC purification. Gradient (% in H_2O + 0.1% TFA): 10% MeCN (0 min) to 95% MeCN (30 min). The fraction corresponding to the peak of the desired product (retention time ~26 min) was collected into a 50 mL glass conical vial that was capped with a septum and contained 0.5% (wt/wt) Na-

ascorbate solution (40 mL). ^{18}F-TCO was obtained in a decay corrected radiochemical yield of 44 ± 9 % (n = 10) within 71 min. Radiochemical purity ranged from 89% to 94%. For formulation, the product was trapped onto a C18 Sep-Pak Light cartridge (preconditioned with EtOH (5 mL) and water (10 mL)). The C18 cartridge was flipped around and eluted manually with DMSO three times (100 µL, 75 µL, 75 µL) into 3 different collection vials, each containing 0.5% (wt/wt) sodium ascorbate solution (75 µL). The contents of the vial containing the highest amount of activity (usually vial 3) were used for subsequent experiments. Activity concentrations in the final formulation ranged from to 3.6 GBq/mL to 6.7 GBq/mL.

Analytical radio-HPLC:



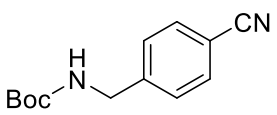
Gradient = A: H_2O + 0.1%TFA, B: ACN + 0.1%TFA. 10-95% B 0-35 min. 1.0 mL/min. 254 nm.

Synthesis of the dual modality chemical linker

Reagents and analytical methods

All chemicals and reagents were purchased from commercial sources and used without further purification. Sulfo-Cyanine5 NHS ester was purchased from Lumiprobe. Mal-amido-PEG_2-NHS was purchased from Broadpharm. α-Boc-*L*-lysine was purchased from Combi-Blocks. All deuterated solvents were purchased from Cambridge Isotope Laboratories. Solvents used for extractions and chromatography were not anhydrous. Reactions and chromatography fractions were analyzed by thin-layer chromatography (TLC) using Merck precoated silica gel 60 F254 glass plates (250 μm) and visualized by ultraviolet irradiation, potassium permanganate stain or ninhydrin stain. Flash column chromatography was performed using E. Merck silica gel 60 (230−400 mesh) with compressed air. NMR spectra were recorded on ARX 400 (400 Hz) or ARX 500 (500 MHz) spectrometers. Chemical shifts are reported in parts per million (ppm, δ) using the residual solvent peak as the reference. The coupling constants, *J*, are reported in Hertz (Hz), and the multiplicity identified as the following: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). High-resolution electrospray mass spectrometry data was collected with a Waters LCT Premier XE time-of-flight instrument controlled by MassLynx 4.1 software. Samples were dissolved in methanol and infused using direct loop injection from a Waters Acquity UPLC into the Multi-Mode Ionization source. HPLC purifications were performed on a Knauer Smartline HPLC system with inline Knauer UV (254 nm) detector. Semi-preprative HPLC was performed using Phenomenex reverse-phase Luna column (10 × 250 mm, 5 μm) with a flow rate of 4 mL/min. Final purity of compounds was determined by analytical HPLC analysis performed with a Phenomenex reverse-phase Luna column (4.6 × 250 mm, 5 μm) with a flow rate of 1 mL/min. Compounds were identified by UV absorbance at 254 nm. All chromatograms were collected by a GinaStar (Elysia-Raytest) analog to digital converter and GinaStar software (Elysia-Raytest).

Tert-butyl (4-cyanobenzyl)carbamate (1)



1

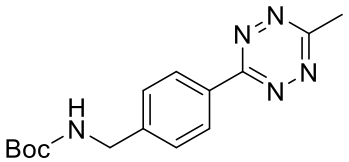
4-(Aminomethyl)benzonitrile hydrochloride (2.11 g, 12.5 mmol, 1.0 equiv), di-*tert*-butyl dicarbonate (3.00 g, 13.75 mmol, 1.1 equiv) and sodium hydroxide (1.50 g, 37.5 mmol, 3.0 equiv) were combined in

water (20 mL) at room temperature. The reaction mixture was stirred overnight, and the white precipitate was filtered off. The solvent was removed by rotary evaporation to afford **1** as a colorless powder (2.53 g, 87 % yield). ^{1}H and ^{13}C NMR spectroscopic data for **1** were identical to that previously reported (*2*).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60 (d, *J* = 8.1 Hz, 2H), 7.37 (d, *J* = 8.1 Hz, 2H), 5.04 (s, 1H), 4.35 (d, *J* = 6.3 Hz, 2H), 1.44 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 155.98, 144.80, 132.48, 127.88, 118.87, 111.18, 80.12, 44.30, 28.45.

Tert-butyl (4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamate (2)



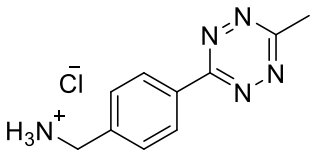
2

Compound **2** was prepared according to a literature procedure with slight modifications (*3*). A 10 mL microwave reaction tube equipped with a stir bar was charged with $Ni(OTf)_2$ (178 mg, 0.5 mmol, 0.5 equiv), *tert*-butyl (4-cyanobenzyl)carbamate (**1**) (232 mg, 1.0 mmol, 1.0 equiv), acetonitrile (0.52 mL, 10.0 mmol, 10.0 equiv) and anhydrous hydrazine (1.58 mL, 50 mmol, 50.0 equiv). The vessel was sealed, and the mixture stirred in an oil bath at 60°C overnight. The reaction solution was cooled to room temperature and the seal was removed. Sodium nitrite (1.38 g, 20.0 mmol, 20.0 equiv) in water (10 mL) was slowly added to the solution and the mixture was transferred to a 250 mL Erlenmeyer flask. While stirring, 1.0M HCl was slowly added until gas evolution ceased. Water was added so that a total volume of 50 mL was reached, and the mixture was extracted with ethyl acetate (2 x 50 mL). The combined organics were washed with water (50 mL) and 5% $NaHCO_3$ solution (50 mL). After drying over Na_2SO_4, the solvent was removed under reduced pressure, adsorbed on silica gel and then subjected to column chromatography on silica gel (25% ethyl acetate in hexanes) to afford **2** as a purple, crystalline solid (124 mg, 41 % yield). ^{1}H and ^{13}C NMR spectroscopic data for **2** were identical to that previously reported (*3*).

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.44 (d, *J* = 8.4 Hz, 2H), 7.42 (d, *J* = 8.4 Hz, 2H), 5.25 (br s, 1H), 4.36 (s, 2H), 3.03 (s, 3H), 1.43 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 167.18, 163.86, 156.06, 144.12, 130.69, 128.10, 127.99, 79.72, 44.35, 28.42, 21.13.

(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanaminium chloride (3)



3

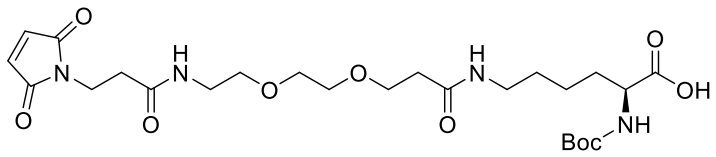
To a solution of *tert*-butyl (4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamate (**2**) (119 mg, 395 µmol, 1 equiv) in 1,4-dioxane (3 mL) was added 4 N HCl/dioxane (1.97 mL, 7.90 mmol, 20 equiv) dropwise at room temperature. The mixture was warmed to 50°C and stirred for 2 h. Consumption of (**2**) was indicated by TLC analysis. The crude mixture was frozen in a dry ice/acetone bath and then all volatiles removed *in vacuo* to afford **3** as a deep purple solid (91 mg, 97%).

^{1}H NMR (400 MHz, MeOD): δ 8.62 (d, *J* = 8.2 Hz, 2H), 7.74 (d, *J* = 8.2 Hz, 2H), 4.27 (s, 2H), 3.05 (s, 3H).

^{13}C NMR (101 MHz, MeOD): δ 169.06, 164.91, 138.74, 134.35, 130.86, 129.43, 43.95, 21.11.

ESI(+)-MS: calcd. for $C_{10}H_{11}N_5 + H^+$, 202.1087; found 202.1110.

(S)-19-((Tert-butoxycarbonyl)amino)-1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3,13-dioxo-7,10-dioxa-4,14-diazaicosan-20-oic acid (4)



4

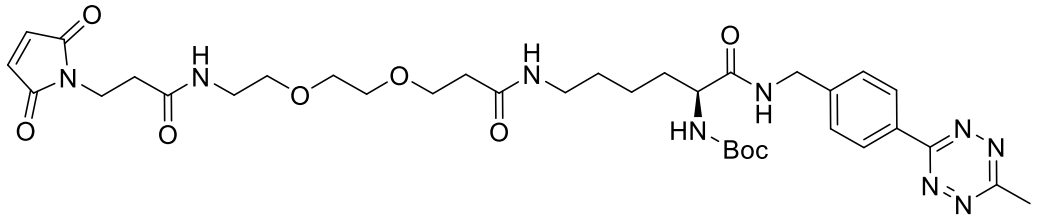
A 10 mL Schlenk flask equipped with a stir bar and filled with argon was charged with α-boc-*L*-lysine (62.8 mg, 0.25 mmol, 1.0 equiv), mal-amido-PEG_2-NHS (106.3 mg, 0.25 mmol, 1.0 equiv) and dry DMF (1 mL). To this, a solution of *N*,*N*-diisopropylethylamine (49 µL, 1.0 mmol, 1.1 equiv) in dry DMF (1 mL) was added dropwise over a period of 30 min and the mixture was stirred at room temperature overnight. DMF was removed under reduced pressure and the crude mixture was subjected to flash column chromatography on silica gel (gradient elution, 0→5% MeOH in DCM) to afford **4** as a colorless oil (98 mg, 70%). The compound was stored under argon at -20°C.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (bs, 1H), 6.99 (s, 1H), 6.75 (s, 1H), 6.67 (s, 2H), 5.78 – 5.35 (m, 1H), 4.27 – 3.99 (m, 1H), 3.77 (t, *J* = 7.2 Hz, 2H), 3.70 (t, *J* = 5.8 Hz, 2H), 3.60 – 3.53 (m, 4H), 3.50 (t, *J* = 5.2 Hz, 2H), 3.35 (dd, *J* = 10.4, 5.2 Hz, 2H), 3.19 (dd, *J* = 12.4, 6.3 Hz, 2H), 2.51 (t, *J* = 7.2 Hz, 2H), 2.43 (t, *J* = 5.8 Hz, 2H), 1.86 – 1.72 (m, 1H), 1.71 – 1.59 (m, 1H), 1.56 – 1.43 (m, 2H), 1.41– 1.30 (m, 2H), 1.38 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 174.71, 172.14, 170.75, 170.64, 155.80, 134.30, 79.85, 70.01, 69.98, 69.58, 67.11, 53.24, 50.38, 39.25, 39.15, 36.80, 34.56, 34.43, 32.00, 28.81, 28.39, 22.44.

ESI(+)-MS: calcd. for $C_{25}H_{40}N_4O_{10} + H^+$, 557.2817; found 557.2847.

Tert-butyl (S)-(22-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,20-trioxo-13,16-dioxa-2,9,19-triazadocosan-4-yl)carbamate (5)



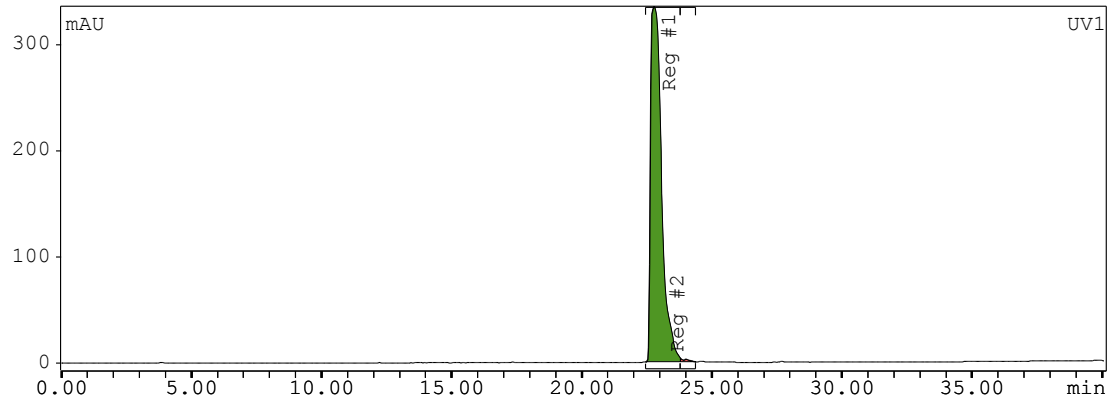
5

To a solution of (*S*)-19-((*tert*-butoxycarbonyl)amino)-1-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-3,13-dioxo-7,10-dioxa-4,14-diazaicosan-20-oic acid (**4**) (97 mg, 174 µmol, 1.0 equiv) in DMF (2 mL) was added *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (74.4 mg, 191 µmol, 1.1 equiv) and the mixture was stirred for 10 min at room temperature. To this, a solution of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanaminium chloride (**3**) (80 mg, 337 µmol, 1.93 equiv) in DMF (2.5 mL) was added and

the mixture was stirred for another 20 min. After addition of *N*,*N*-diisopropylethylamine (88 µL, 505 µmol, 2.9 equiv), the mixture was stirred at room temperature overnight. Consumption of **4** was indicated by TLC analysis. DMF was removed under reduced pressure, the crude mixture dissolved in DCM (20 mL) and washed with water (2 x 20 mL). The organics were concentrated under reduced pressure and subjected to flash column chromatography on silica gel (gradient elution, 0→10 % MeOH in DCM). Gradual decomposition of the product in column chromatography mobile phase required re-purification by semi-preparative HPLC (A: H_2O + 0.1%TFA, B: ACN + 0.1%TFA. 10% B 0-5 min, 10-75% B 5-30 min, 75-95% B 30-35 min, 95% B 35-40 min. 4 mL/min. 254 nm). Combined fractions containing **5** (21.5—23 min) were lyophilized to afford the title compound in form of a purple solid that was stored under argon at -20°C (20.4 mg, 16 %). The compound readily decomposed in $CDCl_3$, ACN-d_3 as well as MeOD leading to unidentified decomposition products visible in NMR spectra. The purity of the compound was examined by analytical HPLC.

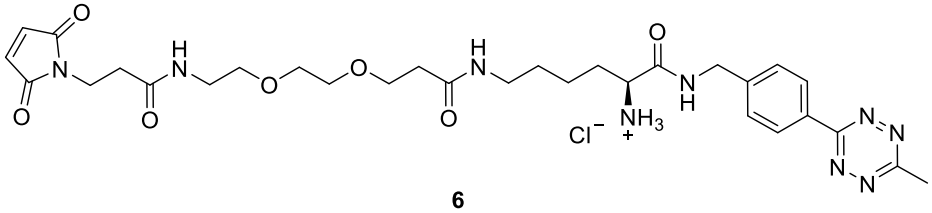
ESI(+)-MS: calcd. for $C_{35}H_{49}N_9O_9 + H^+$, 762.3545; found 762.3546.

Analytical HPLC:



Gradient = A: H_2O + 0.1%TFA, B: ACN + 0.1%TFA. 10% B 0-5 min, 10-75% B 5-30 min, 75-95% B 30-35 min, 95% B 35-40 min. 1.0 mL/min. 254 nm.

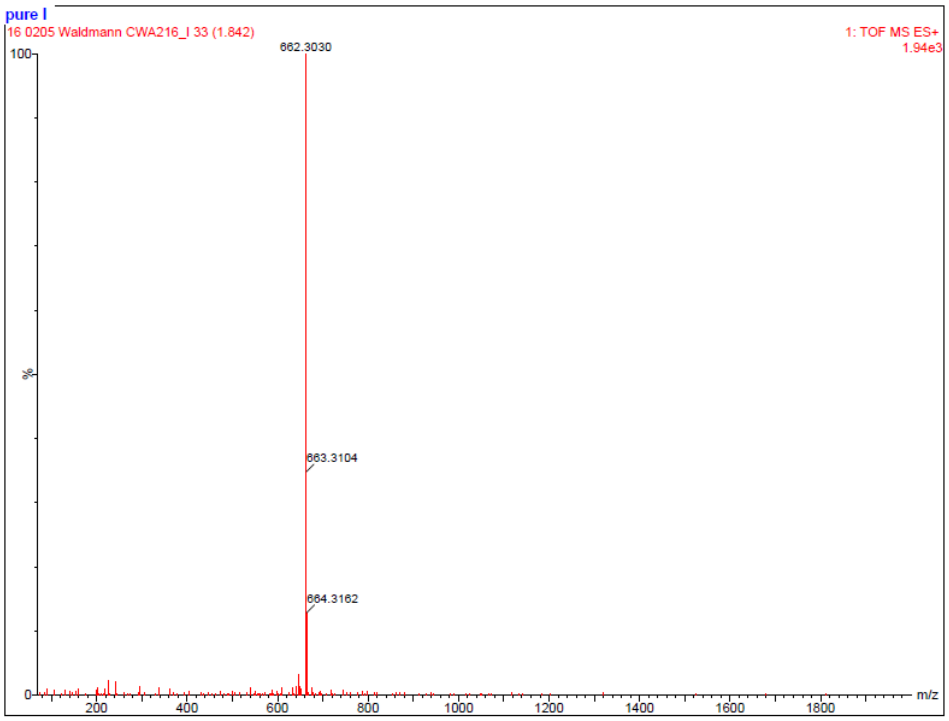
(S)-22-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,20-trioxo-13,16-dioxa-2,9,19-triazadocosan-4-aminium chloride (6)



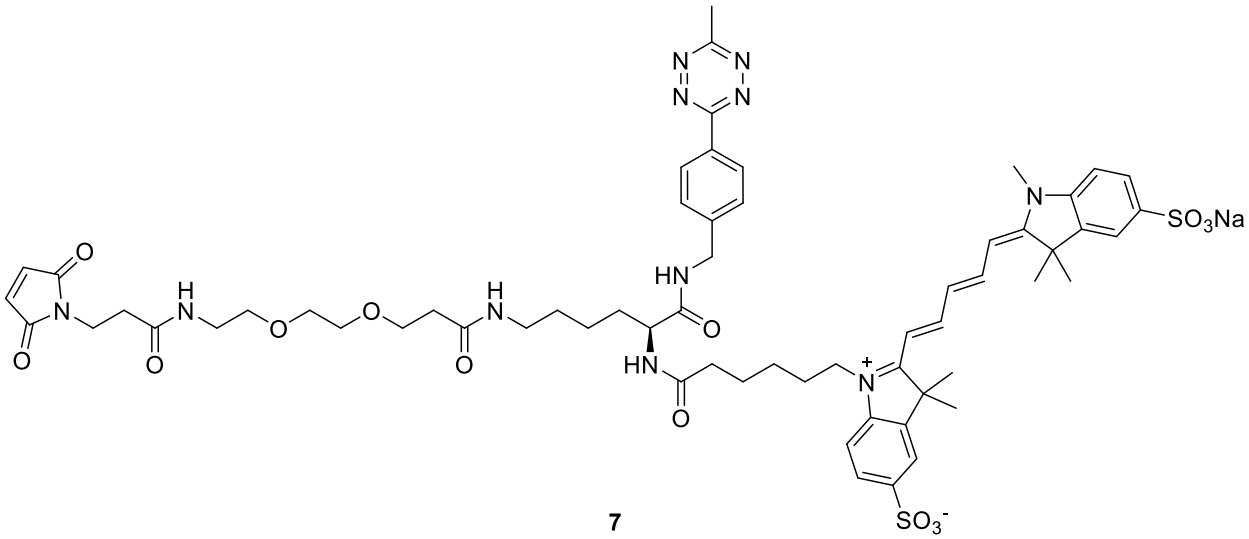
6

To an ice cold solution of *tert*-butyl (*S*)-(22-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,20-trioxo-13,16-dioxa-2,9,19-triazadocosan-4-yl)carbamate (**5**) (15 mg, 20.3 µmol, 1.0 equiv) in dioxane (300 µL) was added a solution of HCl/dioxane (4 M, 102 µL, 20 equiv) and the mixture was shaken at room temperature. After 2 h, consumption of **5** was indicated by TLC analysis (10% MeOH in DCM). The crude mixture was frozen in a dry ice/acetone bath and the solvent and other volatiles were removed *in vacuo*. The obtained residue was subjected to semi-preparative HPLC purification (A: H_2O + 0.1%TFA, B: ACN + 0.1%TFA. 10% B 0-5 min, 10-75% B 5-30 min, 75-95% B 30-35 min, 95% B 35-40 min. 4 mL/min. 254 nm). Combined fractions containing **6** (16—17 min) were lyophilized to afford the title compound as a purple solid (1.80 mg, 13 %). The compound was thermally unstable and therefore immediately used in the next step.

ESI(+)-MS: calcd. for $C_{30}H_{41}N_9O_7 + Na^+$, 662.3021; found 662.3022.



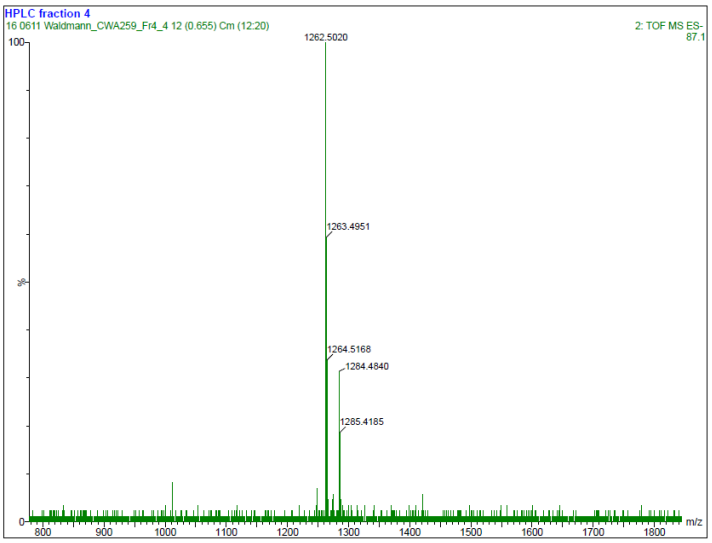
Sodium 1-((S)-1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-19-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamoyl)-3,13,21-trioxo-7,10-dioxa-4,14,20-triazahexacosan-26-yl)-3,3-dimethyl-2-((1E,3E)-5-((E)-1,3,3-trimethyl-5-sulfonatoindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium-5-sulfonate (7)



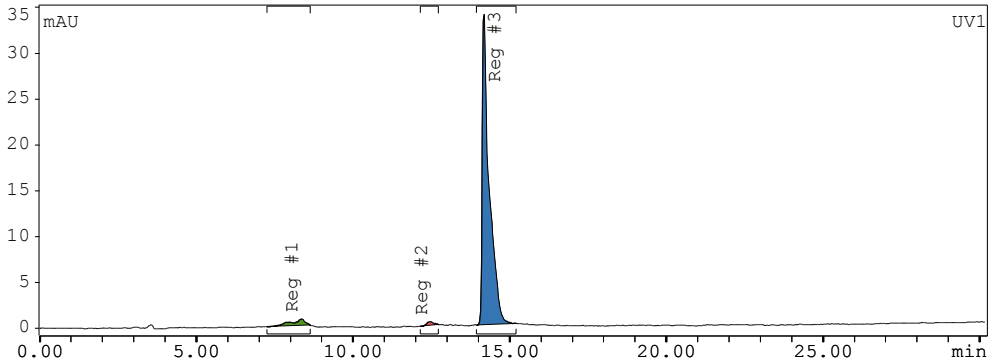
7

To a solution of (*S*)-22-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,20-trioxo-13,16-dioxa-2,9,19-triazadocosan-4-aminium chloride (**6**) (1.8 mg, 2.66 µmol, 1.1 equiv) in DMF (80 uL) was added DIPEA (1.74 µL, 13.46 µmol, 5.7 equiv) and the mixture was shaken at room temperature for 20 min. To this, a solution of sulfo-cyanine5 NHS ester in DMF (50 uL) was added and the shaking continued for 4 h. The crude material was subjected to semi-reparative HPLC purification (A: water (+0.1 TFA), B: ACN (+0.1 TFA). 0-35 min 5% B to 50% B, 35-40 min 50% B to 95% B. 4 mL min. 254 nm). Fractions containing **7** (23–24 min) were lyophilized to afford the title compound as a deep blue solid (1.59 mg, 52%). A 10 mM solution of **7** in DMF was stable for at least a month at -20 °C.

ESI(-)-MS: calcd. for $C_{62}H_{76}N_{11}O_{14}S_2^-$, 1262.5020; found 1262.5020.



Analytical-HPLC:

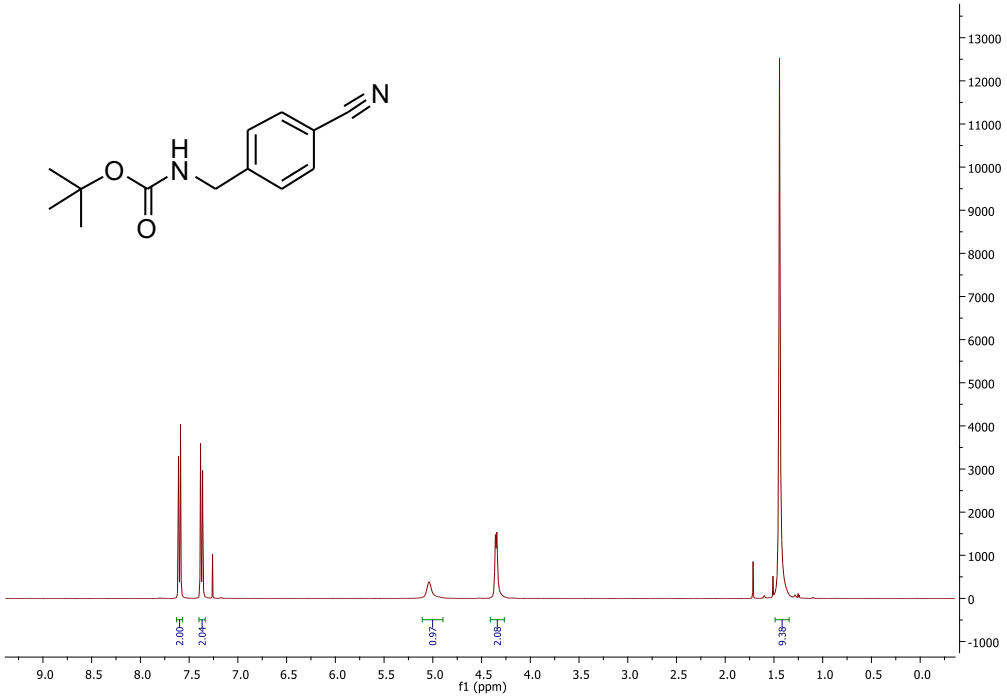


Gradient = A: H_2O + 0.1%TFA, B: ACN + 0.1%TFA. 10% B 0-5 min, 10-75% B 5-30 min, 75-95% B 30-35 min, 95% B 35-40 min. 1.0 mL/min. 254 nm. Purity = 95 %.

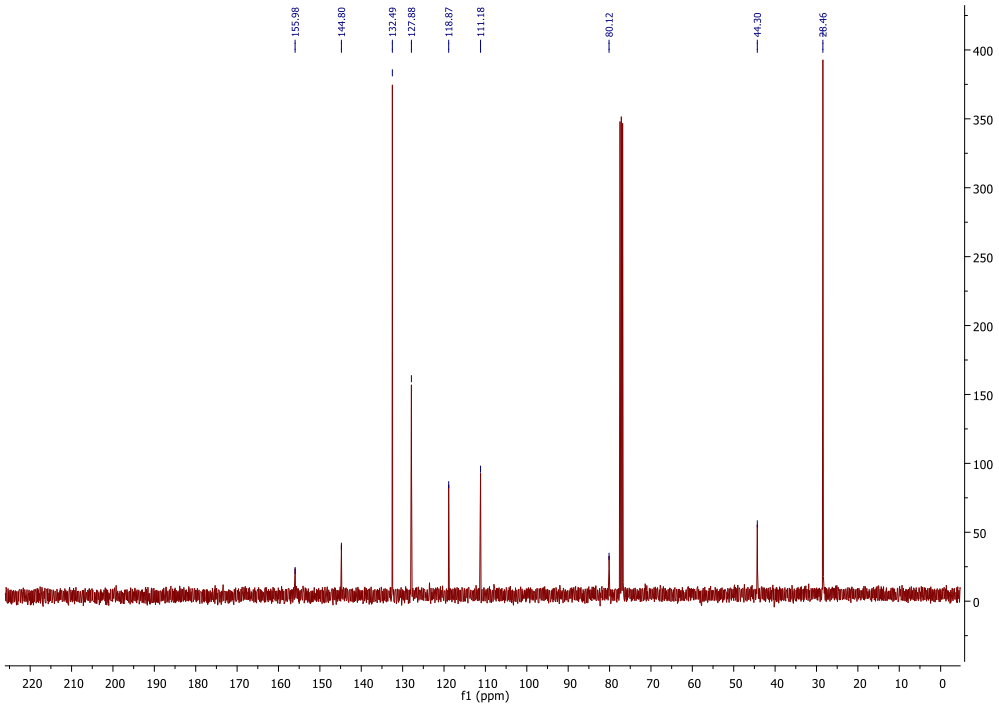
NMR Spectra

Tert-butyl (4-cyanobenzyl)carbamate (1)

^{1}H NMR

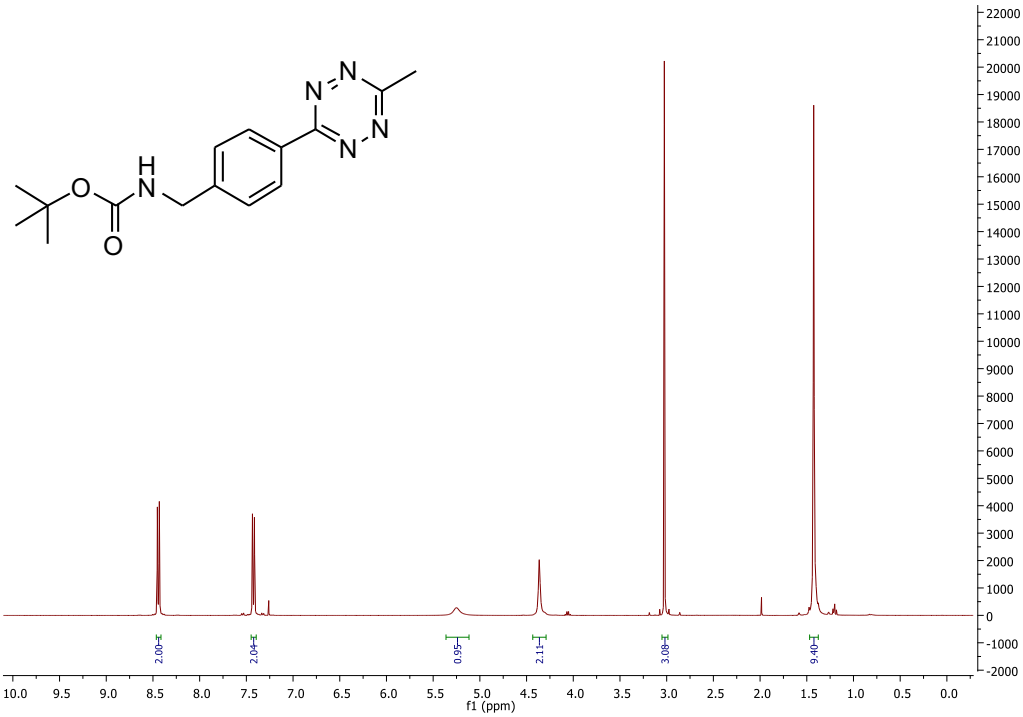


^{13}C NMR

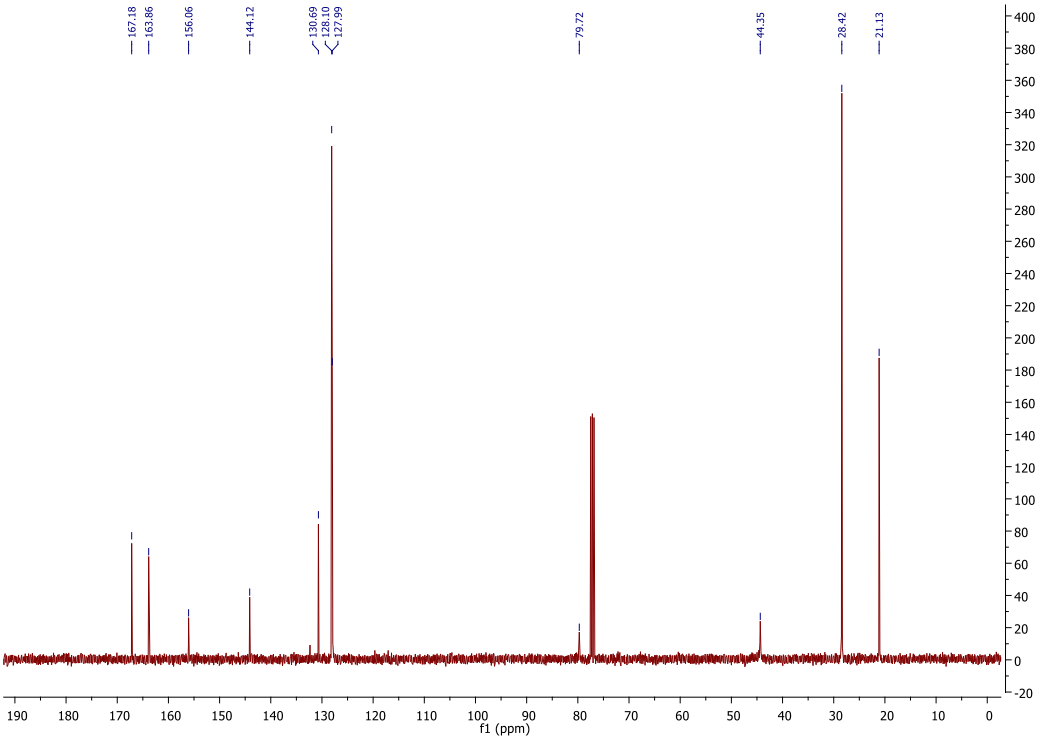


Tert-butyl (4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamate (2)

^{1}H NMR

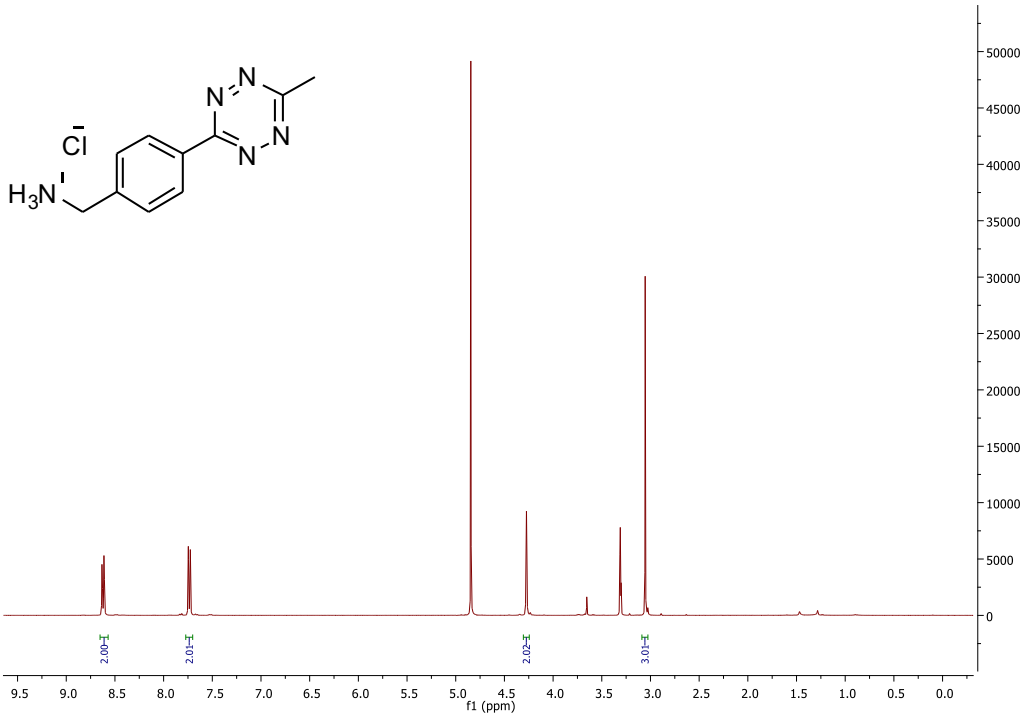


^{13}C NMR

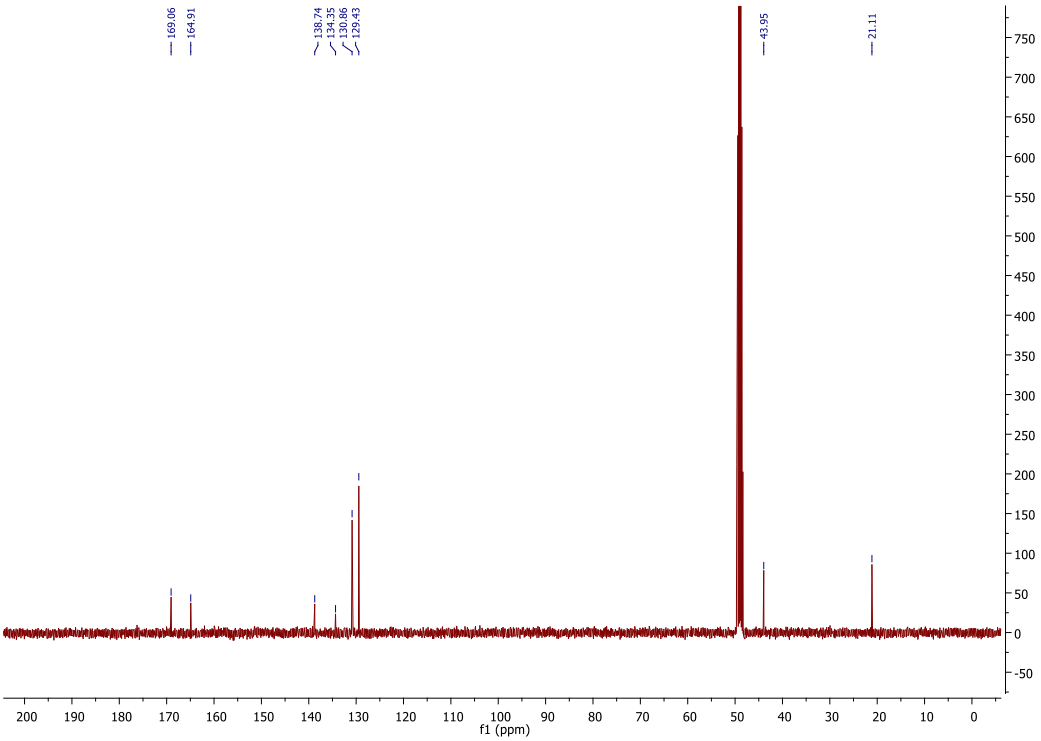


(4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanaminium chloride (3)

^{1}H NMR

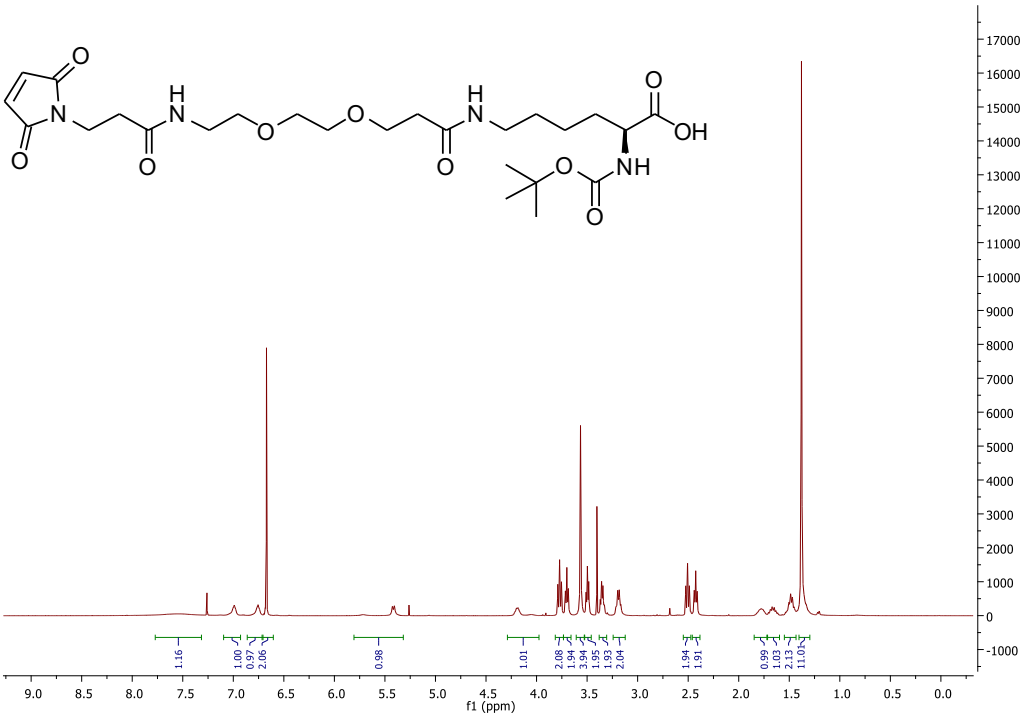


^{13}C NMR

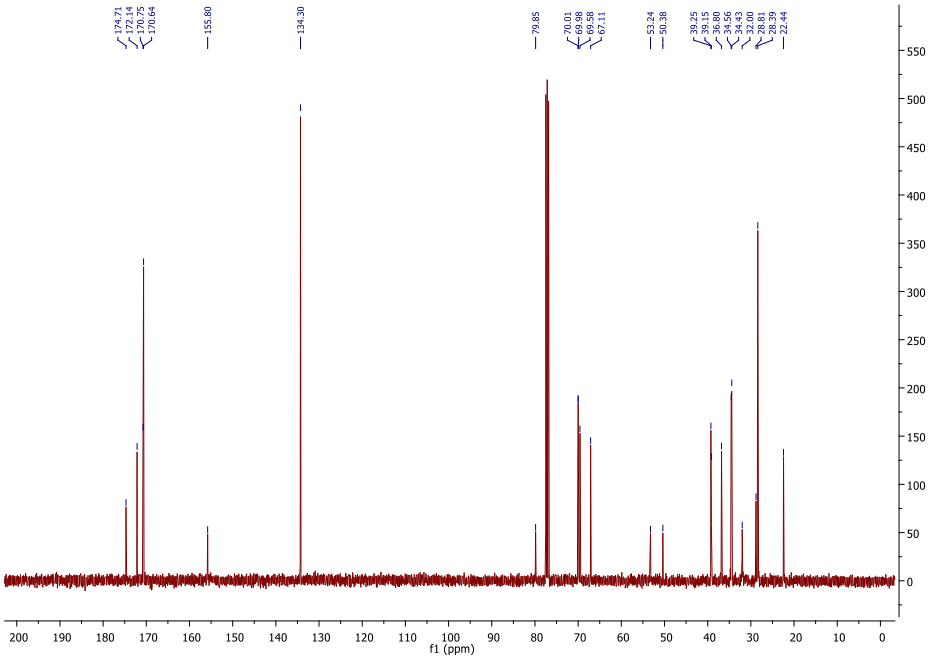


(S)-19-((tert-butoxycarbonyl)amino)-1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3,13-dioxo-7,10-dioxa-4,14-diazaicosan-20-oic acid (4)

^{1}H NMR

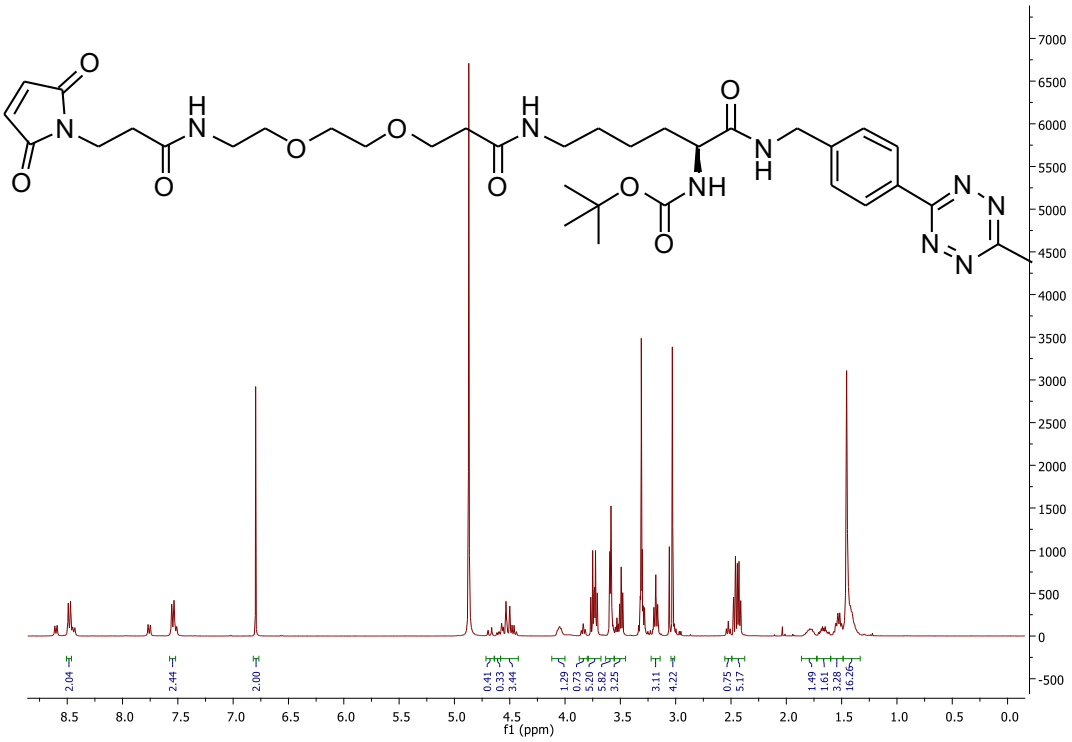


^{13}C NMR

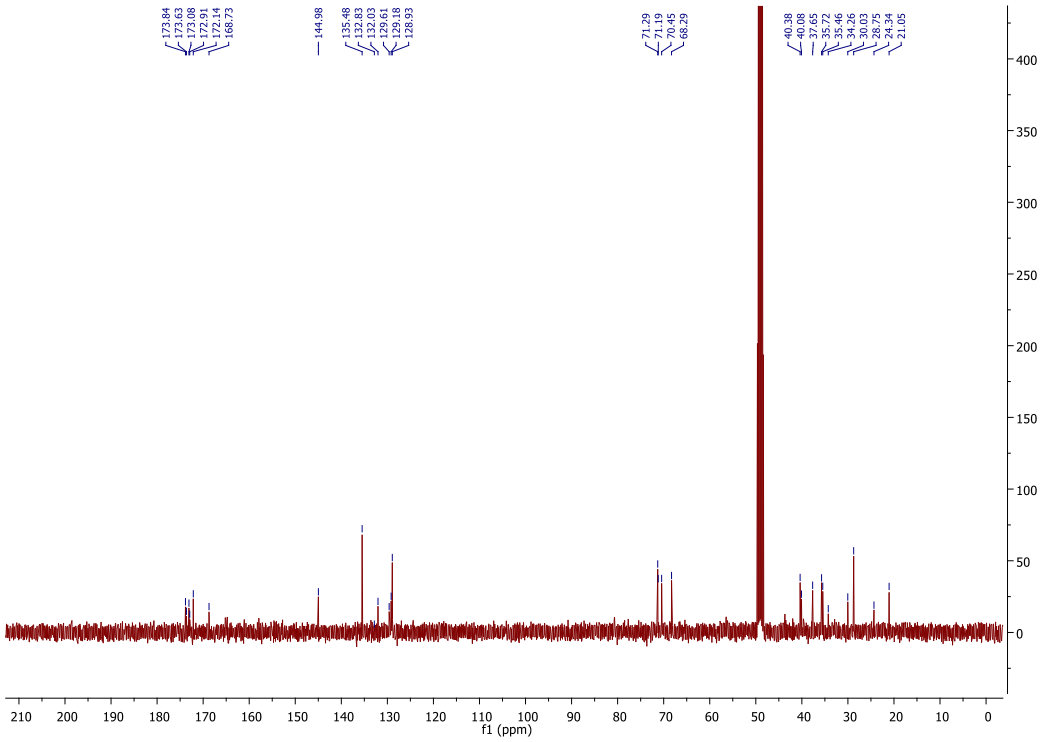


Tert-butyl (S)-(22-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3,10,20-trioxo-13,16-dioxa-2,9,19-triazadocosan-4-yl)carbamate (5)

^{1}H NMR (compound readily decomposed in MeOD; see experimental section for details)



^{13}C NMR



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