Supporting Information (1)

Lipophilicity and click reactivity determine the performance of bioorthogonal tetrazine tools in pretargeted *in vivo* chemistry

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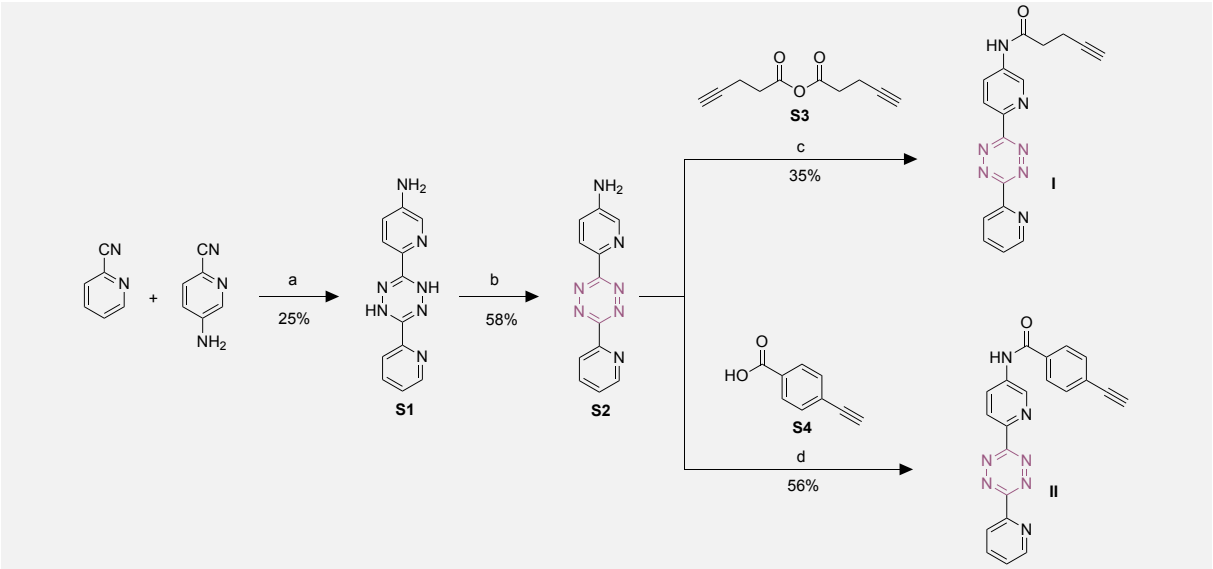
Table of Contents

1. Synthetic chemistry

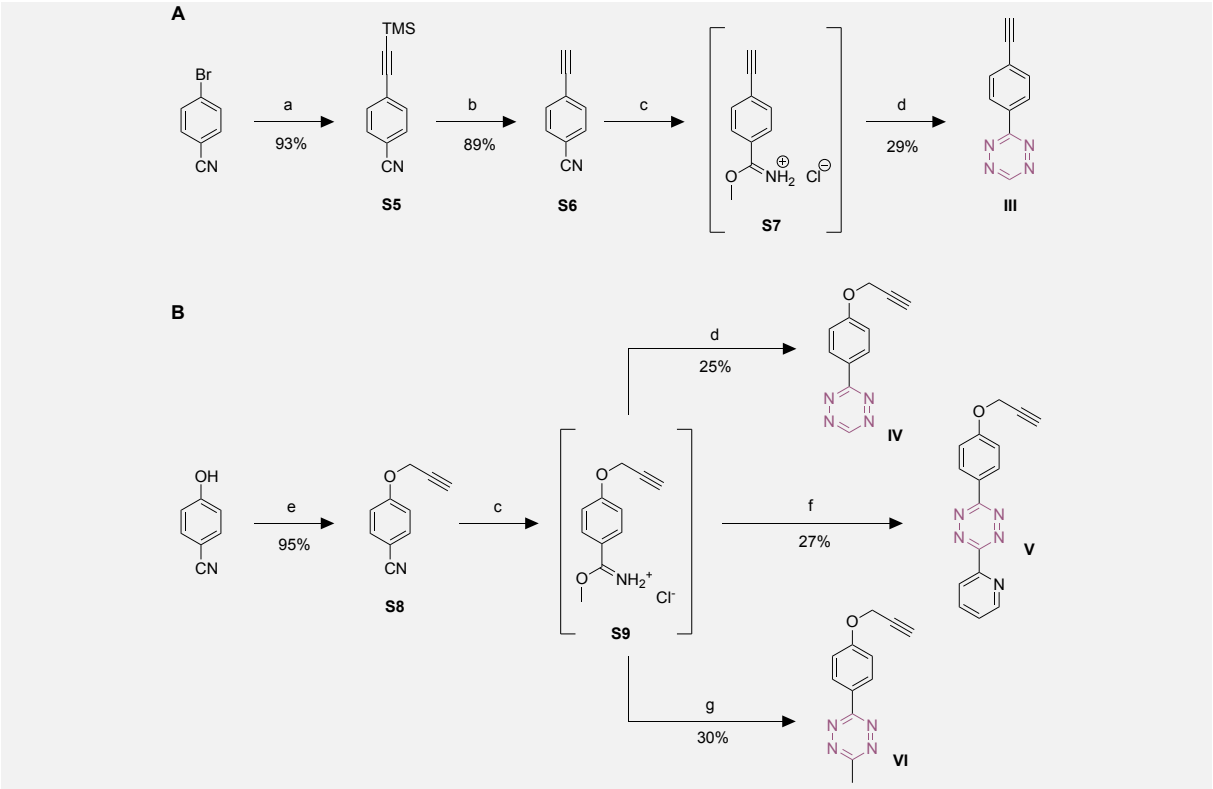
General information

All reagents and solvents were purchased from commercial suppliers and used without further purification, unless stated otherwise. Anhydrous tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were obtained from an SG Water solvent purification system (Pure Process Technology). Anhydrous dichloromethane (DCM) was obtained from the latter system or dried using PURESOLV-columns (Innovative Technology Inc.). Anhydrous dimethyl sulfoxide (DMSO), Et_2O, MeCN, pyridine and MeOH were purchased from commercial suppliers and stored under argon. Room temperature (r.t.) corresponds to a temperature interval from 18–21 ˚C. Reactions requiring anhydrous conditions were carried out under inert atmosphere (nitrogen or argon) and using oven-dried glassware. NMR spectra were acquired on a 600 MHz Bruker Avance III HD (600 MHz for ^{1}H and 151 MHz for ^{13}C), a 400 MHz Bruker Avance II (400 MHz for ^{1}H, 376 MHz for ^{19}F and 101 MHz for ^{13}C) and a 400 MHz Bruker Avance UltraShield (400 MHz for ^{1}H, 376 MHz for ^{19}F and 101 MHz for ^{13}C). Samples were measured at 300 K, except for the 400 MHz Bruker Avance UltraShield, in which samples were measured at 293 K. Chemical shifts (δ) are expressed in parts per million (ppm) relative to tetramethylsilane and calibrated using solvent residual peaks ($CDCl_3$: $\delta(^{1}H)$=7.26, $\delta(^{13}C)$=77.2; CD_3OD: $\delta(^{1}H)$=3.31, $\delta(^{13}C)$=49.0; DMSO-d_6: $\delta(^{1}H)$=2.50, $\delta(^{13}C)$=39.5). The resonance multiplicity is abbreviated as: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Spectra were analyzed with MestReNova 10.0 or ACD Labs NMR Processor Academic Edition 12.01 software. Thin-layer chromatography (TLC) was carried out using either normal phase plates (silica gel 60 coated with fluorescent indicator F254s) or reversed-phase modified silica plates (RP-18 modified silica gel 60 coated with fluorescent indicator F254s) from Merck. Spots were visualized by ultraviolet light at 254 nm, by anisaldehyde and/or by potassium permanganate staining. Preparative column chromatography was performed using a Büchi Sepacore Flash System (2 x Büchi Pump Module C-605, Büchi Pump Manager C-615, Büchi UV Photometer C-635, Büchi Fraction Collector C-660), a Reveleris Grace system or manually on silica gel (0.040–0.063 mm) from Merck. Preparative high-performance liquid chromatography (HPLC) was performed on a Thermo Scientific Dionex 3000 UltiMate instrument connected to a Thermo Scientific Dionex 3000 Diode Array Detector using a Gemini-NX 5µ RP C18 column (250 × 21.2 mm) with UV detection at 254 and 280 nm with a flow rate of 18 mL/min. Semi-preparative HPLC was performed on the same system using a Luna 5µ C18 column (250 × 10 mm) with a flow rate of 3 mL/min. Mass spectra analysis was carried out using an MS-Acquity-A Waters UPLC with QDa-detector. High resolution mass spectrometry (HRMS) was performed as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). Analyses were performed in positive ion mode with ionization on a ThermoQExactive Orbitrap mass spectrometer (Thermo Scientific) equipped with an AP-SMALDI 10 ion source (TransmitMIT) and operated with mass resolving power 140,000 at *m/z* 200 and lock-mass for internal mass calibration. Samples were dissolved in a matrix consisting of 2,5-dihydroxybenzoic acid (20 mg) in MeOH (1 mL).

Synthesis of Tz-alkynes I-VI

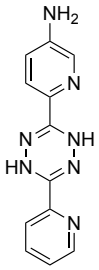


Scheme S1. Synthesis of Tz-alkynes **I** and **II**. (a) $N_2H_4{\cdot}H_2O$, 90 °C, 19 h; (b) PIDA, DCM, r.t., 4 h; (c) Et_3N, THF, 70 °C, 25 h; (d) DCC, DMAP, THF:DCM, 50 °C, 24 h.



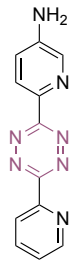
Scheme S2. Synthesis of Tz-alkynes **III-VI**. (a) Trimethylsilylacetylene, $Pd(Cl)_2(PPh_3)_2$, CuI, Et_3N, DMF, r.t.→45°C, 20 h; (b) K_2CO_3, MeOH/DCM, r.t., 1.5 h; (c) HCl (*g*), MeOH, r.t., 1 h; (d) i. Formamidine acetate, $N_2H_4{\cdot}H_2O$, 0 °C→r.t., 3-12 h; ii. $NaNO_2$, AcOH, r.t., 30 min; (e) 3-Bromopropyne, K_2CO_3, DMF, 60–75 °C, 21 h; (f) i. 2-Cyanopyridine, $N_2H_4{\cdot}H_2O$, 0 °C→r.t., 1.5 h; ii. isoamyl nitrite, AcOH, 0–5 °C, 20 min; (g) Acetamidine hydrochloride, $N_2H_4{\cdot}H_2O$, 0 °C→r.t., 5 h; ii. $NaNO_2$, AcOH, 0–5 °C, 20 min.

*6-(6-(Pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (**S1**)*



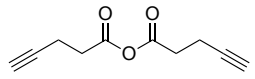
Compound **S1** was synthesized as previously described with some modifications.[1] A mixture of 5-amino-2-cyanopyridine (1.19 g, 10.0 mmol), 2-cyanopyridine (1.05 g, 10.1 mmol) and hydrazine hydrate (2.4 mL, 50 mmol) was heated in a sealed vial at 90 °C overnight. The mixture was cooled to room temperature and the orange precipitation was filtered off and washed with water. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (30→100% EtOAc) as eluent to afford **47** (621 mg, 25%) as an orange solid. TLC R_f = 0.21 (60% EtAOc in heptane). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (s, 1H), 8.64 (s, 1H), 8.62 (m, 1H), 8.05–7.83 (m, 3H), 7.65 (d, J = 8.6 Hz, 1H), 7.51 (ddd, J = 7.3, 4.9, 1.5 Hz, 1H), 7.00 (dd, J = 8.6, 2.7 Hz, 1H), 5.87 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 148.5, 147.5, 146.7, 146.61, 146.58, 137.3, 134.2, 134.1, 125.1, 121.8, 120.8, 120.3. HRMS *m/z* (MALDI-TOF) calculated for $C_{12}H_{11}N_7^+$: 254.1148, found: 254.1150 $[M+H]^+$.

*6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (**S2**)*



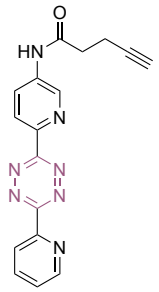
Tz **S2** was synthesized according to previously reported procedure.[1] A mixture of **S1** (620 mg, 2.45 mmol) and phenyliodonium diacetate (PIDA, 1.18 g, 3.67 mmol) in dry DCM (25 mL) was stirred at room temperature under nitrogen atmosphere for 4 h. The solvent was removed, and the crude product was purified by flash column chromatography on silica gel using MeOH in DCM (5→10%) as eluent to afford **S2** (352 mg, 57%) as a red solid. TLC R_f = 0.38 (10% MeOH in DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.90 (ddd, J = 4.7, 1.8, 0.9 Hz, 1H), 8.53 (dt, J = 7.9, 1.1 Hz, 1H), 8.36 (d, J = 8.6 Hz, 1H), 8.24 (dd, J = 2.8, 0.6 Hz, 1H), 8.12 (td, J = 7.7, 1.8 Hz, 1H), 7.69 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 7.12 (dd, J = 8.6, 2.8 Hz, 1H), 6.36 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 162.9, 162.5, 150.4, 150.4, 147.9, 137.6, 137.3, 136.0, 126.2, 125.7, 123.7, 118.9. HRMS *m/z* (MALDI-TOF) calculated for $C_{12}H_9N_7^+$: 252.0992, found: 252.0994 $[M+H]^+$.

*4-Pentynoic anhydride (**S3**)*



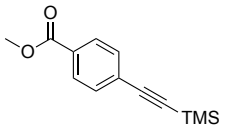
Symmetrical anhydride **S3** was synthesized with inspiration from a procedure published by Malkoch *et al*.[2] 4-Pentynoic acid (2.15 g, 21.9 mmol) and *N*,*N*'-dicyclohexylcarbodiimide (DCC) (2.25 g, 10.9 mmol) were dissolved in anhydrous DCM (40 mL). The mixture was stirred at room temperature under nitrogen atmosphere overnight. The mixture was filtered, and the filtrate was concentrated to ~15 mL under reduced pressure and thereafter cooled at -20 °C for 1 h. The formed precipitation was filtered off and the filtrate was concentrated under reduced pressure to afford **S3** (2.08 g, 53%) as a yellow oil, which was used for amide coupling to Tz **S2** directly without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.77–2.66 (m, 4H), 2.55 (tdd, J = 7.4, 2.7, 0.7 Hz, 4H), 2.01 (t, J = 2.6 Hz, 2H).^{13}C NMR (151 MHz, $CDCl_3$) δ 167.3, 81.5, 69.8, 34.5, 14.0.

*N-(6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)pent-4-ynamide (**I**)*



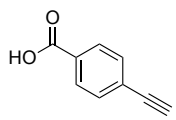
Anhydride **S3** (57.0 mg, 0.32 mmol) in anhydrous THF (2 mL) was added slowly to a suspension of **S2** (21.0 mg, 0.08 mmol) and Et_3N (11.0 µL, 0.08 mmol) in anhydrous THF (3 mL). The mixture was heated at 80 °C in a sealed vial overnight. The solvent was removed, and the residue was dissolved in DCM, washed with saturated aqueous $NaHCO_3$, water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by semi-preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA to afford **I** (9 mg, 35%) as a pink solid. R_f = 0.28 (5% MeOH in DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.65 (s, 1H), 9.06 (dd, J = 2.5, 0.7 Hz, 1H), 8.94 (ddd, J = 4.7, 1.8, 0.9 Hz, 1H), 8.63 (dd, J = 8.6, 0.6 Hz, 1H), 8.59 (dt, J = 7.9, 1.1 Hz, 1H), 8.43 (dd, J = 8.7, 2.5 Hz, 1H), 8.16 (td, J = 7.8, 1.8 Hz, 1H), 7.73 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 2.84 (t, J = 2.6 Hz, 1H), 2.66 (t, J = 7.2 Hz, 2H), 2.54–2.51 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 171.1, 163.5, 163.2, 151.1, 150.1, 144.5, 141.7, 138.8, 138.3, 127.0, 126.7, 125.4, 124.7, 83.9, 72.1, 35.7, 14.3. HRMS *m/z* (MALDI-TOF) calculated for $C_{17}H_{13}N_7O^+$: 332.1254, found: 332.1259 $[M+H]^+$.

*Methyl 4-((trimethylsilyl)ethynyl)benzoate (starting material for carboxylic acid **S4**)*



Methyl-4-iodobenzoate (503 mg, 1.92 mmol), $PdCl_2(PPh_3)_2$ (135 mg, 0.19 mmol) and CuI (37.0 mg, 0.19 mmol) were dissolved in anhydrous DMF (10 mL). Trimethylsilylacetylene (0.8 mL, 5.8 mmol) and Et_3N (2.7 mL, 19 mmol) were added and the mixture was heated at 60 °C 14 h. The mixture was diluted with EtOAc, washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (1→5%) as eluent to afford methyl ester **d** (428 mg, 96%) as a yellow oil. R_f = 0.48 (5% EtOAc in heptane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.4 Hz, 2H), 3.91 (s, 3H), 0.26 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.7, 132.0, 129.9, 129.5, 127.9, 104.2, 97.8, 52.4, -0.01. HRMS *m/z* (MALDI-TOF) calculated for $C_{13}H_{16}O_2SiNa^+$: 255.0812, found: 255.0806 $[M+Na]^+$.

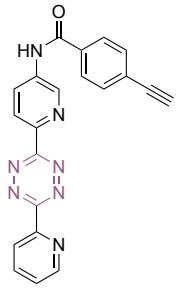
*4-Ethynylbenzoic acid (**S4**)*



A 2 M aqueous solution of NaOH (5 mL) was added drop wise to a solution of methyl 4-((trimethylsilyl)ethynyl)benzoate (541 mg, 2.33 mmol) in MeOH (16 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h, thereafter at room temperature overnight. Aqueous 2 M HCl (5 mL) was slowly added and the mixture was concentrated under reduced pressure. The residue was extracted with EtOAc and the combined organic phases were washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford **50** (337 mg, 99%) as a brown solid. R_f = 0.37 (60% EtOAc in heptane). ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.14 (s, 1H), 7.93 (d, J = 8.3 Hz, 2H), 7.59 (d, J = 8.3 Hz, 2H), 4.43 (s, 1H). ^{13}C NMR (151 MHz, DMSO) δ 166.6,

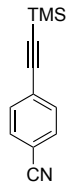
131.9, 130.9, 129.5, 126.0, 83.6, 82.7. HRMS *m/z* (MALDI-TOF) calculated for $C_9H_6O_2^+$: 147.0440, found: 147.0445 $[M+H]^+$.

*4-Ethynyl-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide (**II**)*



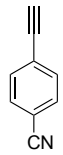
Carboxylic acid **S4** (252 mg, 1.73 mmol), dimethylaminopyridine (DMAP) (318 mg, 2.59 mmol) and DCC (535 mg, 2.59 mmol) were dissolved in anhydrous THF:DCM (1:1, 1 mL). A solution of **S2** (215 mg, 0.86 mmol) in anhydrous THF:DCM:DMF (1:1:2, 5 mL) was added and the mixture was heated at 50 °C in a sealed vial for 24 h. The solvent was removed, and the crude product was purified by flash column chromatography on silica gel using MeOH in DCM (0→5%) as eluent to afford **II** (184 mg, 56%) as a purple solid. R_f = 0.36 (5% MeOH in DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.92 (s, 1H), 9.28 (dd, J = 2.5, 0.7 Hz, 1H), 8.94 (ddd, J = 4.7, 1.8, 0.9 Hz, 1H), 8.68 (dd, J = 8.6, 0.7 Hz, 1H), 8.62–8.59 (m, 2H), 8.16 (td, J = 7.7, 1.8 Hz, 1H), 8.08–8.03 (m, 2H), 7.73 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 7.72–7.67 (m, 2H), 4.46 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.5, 163.1, 162.8, 150.6, 150.2, 144.5, 142.5, 138.4, 137.8, 134.0, 131.8, 128.2, 127.5, 126.6, 125.4, 124.7, 124.2, 83.5, 82.7. HRMS *m/z* (MALDI-TOF) calculated for $C_{21}H_{13}N_7O^+$: 380.1254, found: 380.1264 $[M+H]^+$.

*4-((Trimethylsilyl)ethynyl)benzonitrile (**S5**)*



In two equal batches 4-bromobenzonitrile (1.50 g, 8.24 mmol), $PdCl_2(PPh_3)_2$ (280 mg, 0.40 mmol) and CuI (76 mg, 0.40 mmol) were placed into oven-dried vials. For each batch, anhydrous DMF (5 mL) was added, followed by addition of trimethylsilylacetylene (3.50 mL, 24.7 mmol) and Et_3N (5.7 mL, 41.2 mmol). The mixture was stirred under nitrogen atmosphere at room temperature for 4 h. Thereafter the temperature was increased to 45 °C and the mixture was stirred overnight (16 h). Next, it was cooled to room temperature, diluted with EtOAc, washed with saturated aqueous NH_4Cl and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was co-evaporated twice with toluene in order to remove residual DMF. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (4%) as eluent to afford **51** (3.04 g, 93%) as a pale-yellow solid. R_f = 0.42 (5% EtOAc in heptane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.5 Hz, 2H), 0.26 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 132.6, 132.1, 128.2, 118.6, 111.9, 103.1, 99.7, -0.12.

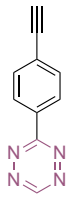
*4-Ethynylbenzonitrile (**S6**)*



TMS-protected nitrile **S5** (2.99 g, 15.0 mmol) was dissolved in MeOH/DCM (100 mL, 3:1) and K_2CO_3 (4.2 g, 30 mmol) was added. The mixture was stirred at room temperature for 1.5 h. The solvent was removed and DCM and water were added to the residue. The phases were separated, and the organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel

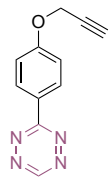
using EtOAc in heptane (1→10%) as eluent to afford **52** (1.6 g, 89%) as a pale-yellow solid. Analytical data matched that previously reported.[3] R_f = 0.38 (10% EtOAc in heptane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.62 (d, J = 8.7 Hz, 2H), 7.57 (d, J = 8.4 Hz, 2H), 3.30 (s, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 132.8, 132.2, 127.2, 118.4, 112.5, 82.0, 81.7.

*3-(4-Ethynylphenyl)-1,2,4,5-tetrazine (**III**)*



HCl (*g*), formed by slowly adding conc. H_2SO_4 to NH_4Cl, was passed through a solution of nitrile **S6** (603 mg, 4.74 mmol) in anhydrous MeOH (30 mL) for 1 h. The solvent was removed and pinner salt **S7** (2.0 g) was afforded as brown crystals and used to form the Tz without further purification. **S7** and formamidine acetate (3.2 g, 30.9 mmol) were cooled to 0 °C and 50–60% hydrazine hydrate (12.5 mL, 257 mmol) was added drop wise. The mixture was stirred under nitrogen atmosphere at room temperature overnight. Next, the mixture was poured on ice (~50 mL) and extracted with EtOAc. The combined organic phases were concentrated under reduced pressure. The residue was dissolved in AcOH (20 mL) and cooled to 0 °C. $NaNO_2$ (2.84 mg, 41.2 mmol) in water (6 mL) was added drop wise over 20 min. Thereafter, the mixture was stirred for 30 min, before it was poured on ice (~50 mL). The formed precipitate was filtered off and purified by flash column chromatography on silica gel using EtOAc in heptane (1→5%) as eluent to afford **III** (247 mg, 29%) as a pink solid. R_f = 0.19 (5% EtOAc in heptane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.23 (s, 1H), 8.60 (d, J = 8.6 Hz, 2H), 7.72 (d, J = 8.5 Hz, 2H), 3.30 (s, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.2, 157.9, 157.9, 133.2, 131.8, 128.2, 127.6, 82.9, 80.8. HRMS *m/z* (MALDI-TOF) calculated for $C_{10}H_6N_4^+$: 183.0665, found: 183.0671 $[M+H]^+$.

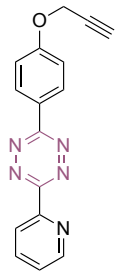
*3-(4-(Prop-2-yn-1-yloxy)phenyl)-1,2,4,5-tetrazine (**IV**)*



Nitrile **S8** (1.0 g, 6.4 mmol, synthesized as previously described by Sekiya *et al.*[4]) was dissolved in a mixture of anhydrous MeOH (1.5 mL) and anhydrous Et_2O (35 mL). HCl (*g*) was passed through the solution at room temperature for 45 min. The reaction mixture was stored at -20°C for two days to complete crystallization of the Pinner salt (**S9**). Volatiles were removed on the rotary evaporator and the residue was mixed with formamidine acetate (1.8 g, 17 mmol) and treated drop wise with hydrazine monohydrate (7 mL, 144 mmol) at 0 °C. The mixture was stirred for 3 h and allowed to reach room temperature. Thereafter, it was poured into ice-water (100 mL) and the resulting yellow solid was collected by filtration. The solid was dissolved in AcOH (20 mL) and cooled to 0–5°C. $NaNO_2$ (1.2 g, 17 mmol) was carefully added in portions during 20 min, while stirring. The red solution was poured into ice cold water (50 mL) and the pink solid was collected via filtration, dissolved in MeOH and dried under reduced pressure to afford **IV** (315 mg, 23%) as a red solid. R_f = 0.76 (33% EtOAc in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.15 (s, 1 H), 8.60 (d, J = 8.6 Hz, 2 H), 7.18 (d, J = 8.6 Hz, 2 H), 4.83 (d, J = 2.3 Hz, 2 H), 2.60 (t, J = 2.3 Hz, 1 H). ^{13}C NMR

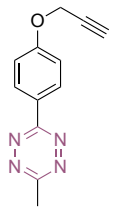
(101 MHz, $CDCl_3$) δ 166.0, 161.6, 157.4, 130.2, 124.8, 115.7, 77.7, 76.3, 55.9. FT-HRMS m/z calculated for $C_{11}H_8N_4O$+: 213.0771, found: 213.0769 $[M+H]^+$.

*3-(4-(Prop-2-yn-1-yloxy)phenyl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (**V**)*



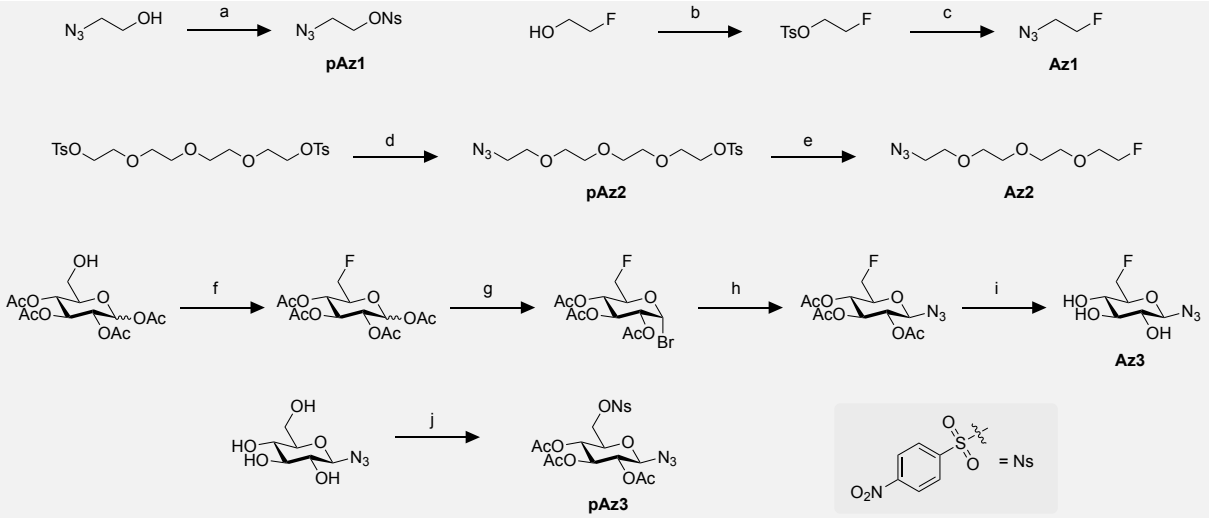
A mixture of **S9** (2.77 g, 12.29 mmol, prepared as described above) and 2-pyridinecarbonitrile (3.84 g, 36.88 mmol) was treated drop wise with hydrazine monohydrate (16 mL, 320 mmol) at 0 °C under an atmosphere of argon. The mixture was stirred for 1.5 h, while reaching room temperature and the clear brown solution turned into a yellow solid. Addition of hydrazine monohydrate (5 mL) caused a milky suspension, which was immediately poured into ice-water (100 mL). After filtration, the filtrate was stored at 4 °C overnight and the resulting orange solid was collected by filtration. The solid was dissolved in AcOH (130 mL) and cooled to 0–5°C. Isoamyl nitrite (2.2 mL, 16.3 mmol) was carefully added, while stirring. The resulting purple solution was poured into ice-cold water (600 mL) and the formed pink precipitation was filtered off, dissolved in DCM and concentrated under reduced pressure to afford **V** (948 mg, 27%) as a pink solid, which was used in the next steps without further purification. R_f = 0.41 (40% EtOAc in hexane). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (dq, J = 2.3 Hz, J = 0.8 Hz, 1 H), 8.54 (m, 3 H), 8.12 (td, J = 5.9 Hz, 1.9 Hz, 1 H), 7.69 (ddd, J = 3.5 Hz, J = 1.2 Hz, J = 1.9 Hz, 1 H), 7.30 (dt, J = 8.9 Hz, J = 3.1 Hz, 2 H), 4.98 (d, J = 2.3 Hz, 2 H), 3.67 (t, J = 2.3 Hz, 1 H). 13C NMR (101 MHz, DMSO-d_6) δ 163.1, 162.9, 161.0, 150.5, 150.3, 137.7, 129,7, 126.3, 124.6, 123.8, 115.9, 78.8, 78.7, 55.8. HRMS *m/z* calculated for $C_{16}H_{11}N_5O^+$: 290.1036, found: 290.1030 $[M+H]^+$.

*3-Methyl-6-(4-(prop-2-yn-1-yloxy)phenyl)-1,2,4,5-tetrazine (**VI**)*



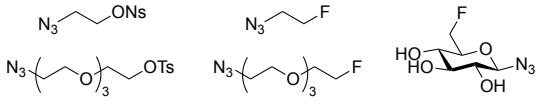
HCl (*g*) was passed through a solution of **S8** (2.1 g, 13 mmol) in anhydrous MeOH (3 mL) and anhydrous Et_2O (75 mL) at room temperature for 45 min. The mixture was stored at -20 °C for 2 days to complete crystallization of **S9**. Volatiles were evaporated and the residue was mixed with acetamidine hydrochloride (3.53 g, 37.3 mmol) and treated drop wise with hydrazine monohydrate (16 mL, 330 mmol) at 0 °C. The mixture was stirred at room temperature for 5 h, before it was poured into ice-water (100 mL). The resulting orange solid was filtered off, dissolved in AcOH (30 mL) and cooled to 0 °C. Afterward, $NaNO_2$ (2.9 g, 42 mmol) was carefully added in portions over the course of 20 min, while stirring. The red solution was poured into ice-cold water (50 mL) and the pink solid was collected via filtration, dissolved in DCM and dried under reduced pressure. Additional product was obtained by oxidation of the filtrate using $NaNO_2$ and aqueous 2 M HCl, followed by filtration of the precipitated product. Tz **VI** (890 mg, 30%) was afforded as a red-purple solid. R_f = 0.74 (33% EtOAc in hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (dt, J = 8.9 Hz, J = 2.3 Hz, 2H), 7.17 (dt, J = 8.9 Hz, J = 1.9 Hz, 2H), 4.82 (d, J = 2.3 Hz, 2H), 3.08 (s, 3H), 2.58 (t, J = 2.3 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 163.7, 161.1, 129.7, 125.1, 115.6, 77.8, 76.2, 55.9, 21.1. FT-HRMS *m/z* calculated for $C_{12}H_{10}N_4O^+$: 227.0927, found: 227.0931 $[M+H]^+$.

Synthesis of Az1-Az3 and the precursor compounds pAz1-pAz3



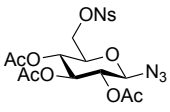
Scheme S3. Synthesis of **pAz1-pAz3** as precursors for ^{18}F-radiolabeling and **Az1**, **Az2** and **Az3** as reference compounds for the respective ^{18}F-labeled azides. (a) *p*-nitrobenzenesulfonyl (Ns) chloride, Et_3N, DCM, 0 °C, 20 min; (b) *p*-toluenesulfonyl (Ts) chloride, pyridine, 0 °C, 3 h; (c) NaN_3, DMF, 48 h, r.t.; (d) NaN_3, EtOH , reflux, 15 h; (e) tetra-*n*-butylammonium fluoride, MeCN, reflux, overnight; (f) diethylaminosulfur trifluoride (DAST), 2,4,6-collidine, -20 °C to r.t.; (g) HBr in HOAc, DCM, 0 °C to r.t.; (h) NaN_3, DMF, 65 °C; (i) K_2CO_3, MeOH, r.t.; (j) *p*-nitrobenzenesulfonyl chloride, pyridine, r.t., overnight, then Ac_2O, 0 °C, 2 h.

*2-Azidoethyl 4-nitrobenzenesulfonate (**pAz1**), 1-azido-2-fluoroethane (**Az1**), 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (**pAz2**), 1-azido-2-(2-(2-(2-fluoroethoxy) ethoxy)ethoxy)ethane (**Az2**), 6-deoxy-6-fluoro-β,D-glucosyl azide (**Az3**)*



pAz1, Az1, pAz2, **Az2** and **Az3** were prepared following known procedures.[5-7]

*2,3,4-Tri-O-acetyl-6-O-(4-nitrobenzoyl)-β,D-glucosyl azide (**pAz3**)*



A solution *p*-nitrobenzenesulfonyl chloride (4.23 g, 19.1 mmol) in anhydrous pyridine (20 mL) was added to 1-azido-1-deoxy-β,D-glucose (3.13 g, 15.3 mmol) dissolved in anhydrous pyridine (40 mL). The mixture was stirred at room temperature overnight. After cooling to 0 °C Ac_2O (9.34 g, 91.5 mmol) was added dropwise. The mixture was stirred for 2 h at 0 °C. The solvent was removed, and the residue was dissolved in EtOAc, washed with aqueous 2 M HCl, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using a gradient of EtOAc in hexanes (3→65%) to afford the product (2.5 g, 32%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (dt, J = 8.9, 2.0 Hz, 2H), 8.14 (dt, J = 8.9, 2.0 Hz, 2H), 5.19 (t, J = 9.4 Hz, 1H), 4.98 (t, J = 9.4 Hz, 1H), 4.83 (t, J = 9.8 Hz, 1H), 4.58 (d, J = 8.9 Hz, 1H), 4.28–4.34 (m, 1H), 4.20–4.26 (m, 1H), 3.81–3.91 (m, 1H), 2.03–2.07 (m, 6H), 2.00 (s, 3 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 169.3, 169.1, 151.0, 141.0, 129.5, 124.5, 87.7, 73.3, 70.1, 67.8, 67.7, 20.5 ppm.

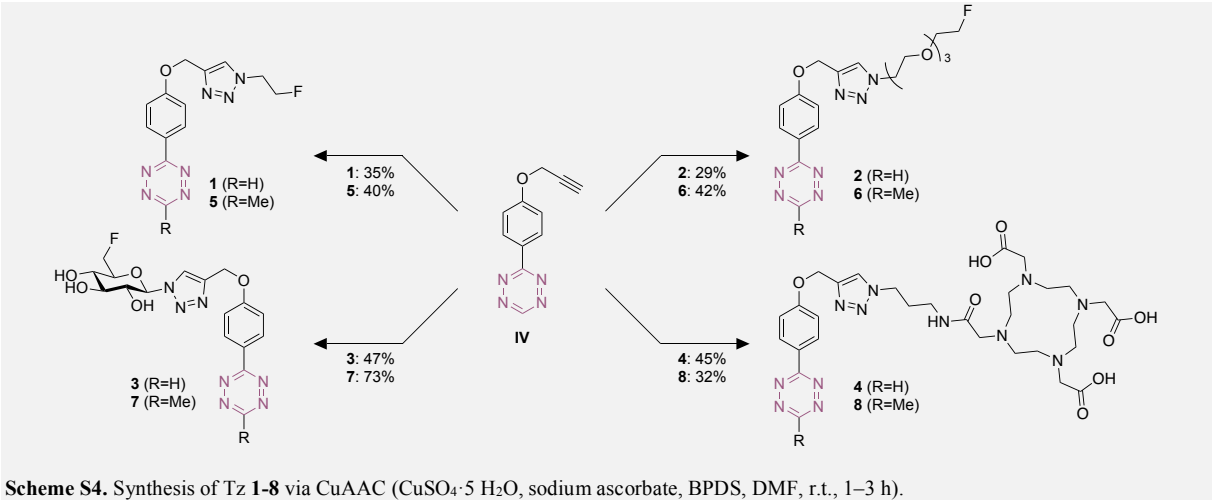
Synthesis of Tz derivatives 1-45

*General CuAAC procedure for the preparation of Tz **1–8**, **12–14** and **20***

An aqueous solution of $CuSO_4 \cdot 5H_2O$ (100 mg/mL) was mixed with an aqueous solution of sodium ascorbate (300 mg/mL). When the color of the mixture turned yellow a solution of disodium bathophenanthroline disulfonate (BPDS) in water (50 mg/mL) was added. The resulting blue/green mixture was added to a solution of the Tz in DMF. After addition of the respective azide (Az1-Az3) dissolved in DMF the mixture (total volume 0.5–2 mL DMF) was stirred at room temperature for 1–3 h, if not otherwise stated.

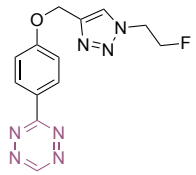
Work-up procedure A: The mixture was diluted with 1:1 solution of DMSO/water containing 0.1% TFA and directly loaded on a C18 flash column for subsequent purification.

Work-up procedure B: The mixture was diluted with water and extracted with DCM. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated. Purification was performed by preparative HPLC.



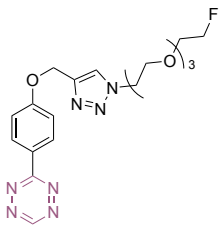
Scheme S4. Synthesis of Tz **1-8** via CuAAC ($CuSO_4 \cdot 5 H_2O$, sodium ascorbate, BPDS, DMF, r.t., 1–3 h).

*3-(4-((1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-1,2,4,5-tetrazine (**1**)*



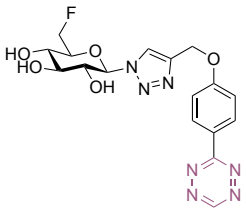
Tz **1** was synthesized according to the general CuAAC procedure from **IV** (30.0 mg, 142 µmol), **Az1** (0.66 mmol, 440 µL of a 1.5 M solution), $CuSO_4 \cdot 5H_2O$ (10 µL, 4.0 µmol), sodium ascorbate (10 µL, 15 µmol) and BPDS (40 µL, 3.4 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient (10→90%) of MeCN in water with 0.1% formic acid to afford **1** (15 mg, 35%) as a pink solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.07 (s, 1H), 8.53 (dt, J = 9.1, 2.1 Hz, 2H), 7.74 (s, 1H), 7.14 (dt, J = 9.1, 2.1 Hz, 2H), 5.28 (s, 2 H), 4.70 (dt, J = 30.2, 4.7 Hz, 2H), 4.72 (dt, J=104.46, 5.00 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 162.3, 157.4, 130.3, 124.5, 124.0, 115.6, 81.5 (d, J = 172.8 Hz), 62.1, 50.7 (d, J = 20.9 Hz). ^{19}F NMR (376 MHz, $CDCl_3$) δ -221.54. HRMS *m/z* (MALDI-TOF) calculated for $C_{13}H_{12}FN_7O^+$: 302.1160, found: 302.1161 $[M+H]^+$.

*3-(4-((1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-1,2,4,5-tetrazine (**2**)*



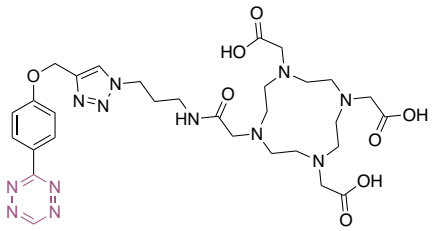
Tz **2** was synthesized according to the general CuAAC procedure from **IV** (15.0 mg, 0.08 mmol), **Az2** (27.0 mg, 0.12 mmol), $CuSO_4 \cdot 5H_2O$ (4 µL, 1.6 µmol), sodium ascorbate (4 µL, 6.4 µmol) and BPDS (16 µL, 1.3 µmol). Work-up procedure B was used. The crude product was purified by preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA to afford **2** (10 mg, 29%) as a pink solid. TLC R_f = 0.63 (10% MeOH in DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.51 (s, 1H), 8.47 (d, J = 8.9 Hz, 2H), 8.26 (s, 1H), 7.34 (d, J = 8.9 Hz, 2H), 5.31 (s, 2H), 4.56 (t, J = 5.2 Hz, 2H), 4.54–4.51 (m, 1H), 4.46–4.43 (m, 1H), 3.83 (t, J = 5.2 Hz, 2H), 3.65–3.62 (m, 1H), 3.60–3.57 (m, 1H), 3.55–3.51 (m, 4H), 3.50–3.47 (m, 4H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.1, 161.9, 157.7, 142.0, 129.6, 125.2, 124.3, 115.7, 83.0 (d, J = 165.6 Hz), 69.8–69.5, 68.6, 61.4, 49.5. HRMS *m/z* (MALDI-TOF) calculated for $C_{19}H_{24}FN_7O_4^+$: 434.1946, found: 434.1953 $[M+H]^+$.

*(2R,3R,4S,5S,6S)-2-(4-((4-(1,2,4,5-Tetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)-6-(fluoromethyl)tetrahydro-2H-pyran-3,4,5-triol (**3**)*



Tz **3** was synthesized according to the general procedure from **IV** (10 mg, 47 µmol), **Az3** (11 mg, 52 µmol), $CuSO_4 \cdot 5H_2O$ (10 µL, 4.0 µmol), sodium ascorbate (10 µL, 15 µmol) and BPDS (40 µL, 3.4 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient of MeOH in water with 0.1% formic acid (5→100%) as eluent to afford **3** (9.3 mg, 47%) as a pink solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.52 (s, 1 H), 8.53 (s, 1 H), 8.49 (dt, J = 8.9, 2.7 Hz, 2 H), 7.37 (dt, J= 8.9, 2.7 Hz, 2 H), 5.69 (d, J=9.37 Hz, 1 H), 5.41–5.54 (m, 3 H), 5.33 (s, 2 H), 4.45–4.70 (m, 2 H), 3.70–3.89 (m, 2 H), 3.43–3.48 (m, 1 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.6, 162.3, 142.7, 130.1, 124.8, 124.7, 116.2, 87.7, 82.8 (d, J = 170.20 Hz), 77.8 (d, J = 16.95 Hz), 77.2, 72.3, 68.8, 61.8, 49.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ ppm -231.99.

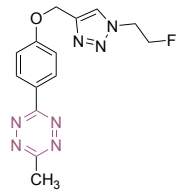
*2,2',2''-(10-((3-(4-((4-(1,2,4,5-Tetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)propyl) carbamoyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**4**), copper(II) complex*



Tz **4** was synthesized similarly to the general procedure from **IV** (19 mg, 88 µmol), 1,4,7,10-tetraazacyclododecane-1,4,7-tris (acetic acid)-10-(azidopropyl ethylacetamide) (58 mg, 97 µmol), $CuSO_4 \cdot 5H_2O$ (256 µL, 106 µmol), sodium ascorbate (23 µL, 35 µmol) and BPDS (100 µL, 8.8 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient of MeCN in water with 0.1% TFA (10→80%) as eluent to afford **4** (35 mg, 45%) as a magenta solid.

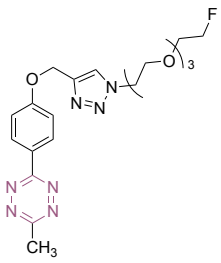
The compound was obtained as a copper complex. HPLC-MS $[M+H]^+$ m/z calculated for $[C_{30}H_{41}CuN_{12}O_8]^+$: 760.24, found: 760.31$[M+H]^+$.

*3-(4-((1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-methyl-1,2,4,5-tetrazine (**5**)*



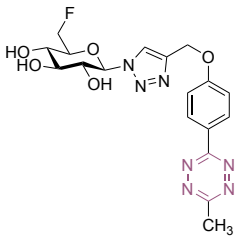
Tz **5** was synthesized according to the general procedure from **VI** (30.0 mg, 133 µmol), **Az1** (0.66 mmol, 440 µL of a 1.5M solution), $CuSO_4 \cdot 5H_2O$ (10 µL, 4.0 µmol), sodium ascorbate (10 µL, 15 µmol) and BPDS (40 µL, 3.4 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient (10→90%) of MeCN in water with 0.1% formic acid as eluent to afford **5** (17 mg, 40%) as a pink solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (dt, J = 9.4, 2.7 Hz, 2 H), 7.81 (s, 1 H), 7.20 (dt, J = 9.0, 2.2 Hz, 2 H), 5.35 (s, 1 H), 4.77 (dt, J = 9.2, 4.4 Hz, 2 H), 4.79 (dt, J = 83.0, 5.0 Hz, 2 H), 3.07 (s, 3 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 163.7, 161.8, 143.9, 129.8, 124.8, 123.9, 115.42, 81.0 (d, J = 172.8 Hz), 62.0, 50.6 (d, J = 20.9 Hz), 21.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ -221.54.

*3-(4-((1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-methyl-1,2,4,5-tetrazine (**6**)*



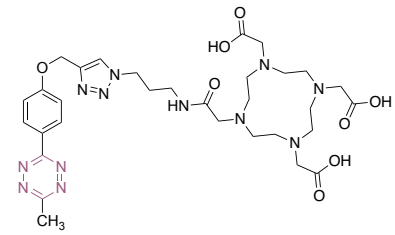
Tz **6** was synthesized according to the general procedure from **VI** (16 mg, 70 µmol), **Az2** (23 mg, 104 µmol), $CuSO_4 \cdot 5H_2O$ solution (4 µL, 1.4 µmol), sodium ascorbate (4 µL, 5.6 µmol) and BPDS (14 µL, 1.4 µmol). Work-up procedure B was used and the crude product was purified by preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA to afford **6** (13 mg, 42%) as a pink solid. TLC R_f = 0.65 (10% MeOH in DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.43 (d, J = 8.9 Hz, 2H), 8.25 (s, 1H), 7.32 (d, J = 8.9 Hz, 2H), 5.29 (s, 2H), 4.56 (t, J = 5.2 Hz, 2H), 4.54–4.51 (m, 1H), 4.46–4.43 (m, 1H), 3.83 (t, J = 5.2 Hz, 2H), 3.65–3.62 (m, 1H), 3.60–3.57 (m, 1H), 3.55–3.50 (m, 4H), 3.50–3.47 (m, 4H), 2.97 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 166.5, 162.9, 161.5, 142.0, 129.2, 125.1, 124.3, 115.6, 83.0 (d, J = 165.7 Hz), 69.8–69.5, 68.6, 61.4, 49.5, 20.7. HRMS *m/z* (MALDI-TOF) calculated for $C_{20}H_{26}FN_7O_4^+$: 448.2103, found: 448.2106 $[M+H]^+$.

(2S,3S,4S,5R,6R)-2-(Fluoromethyl)-6-(4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3,4,5-triol (7)

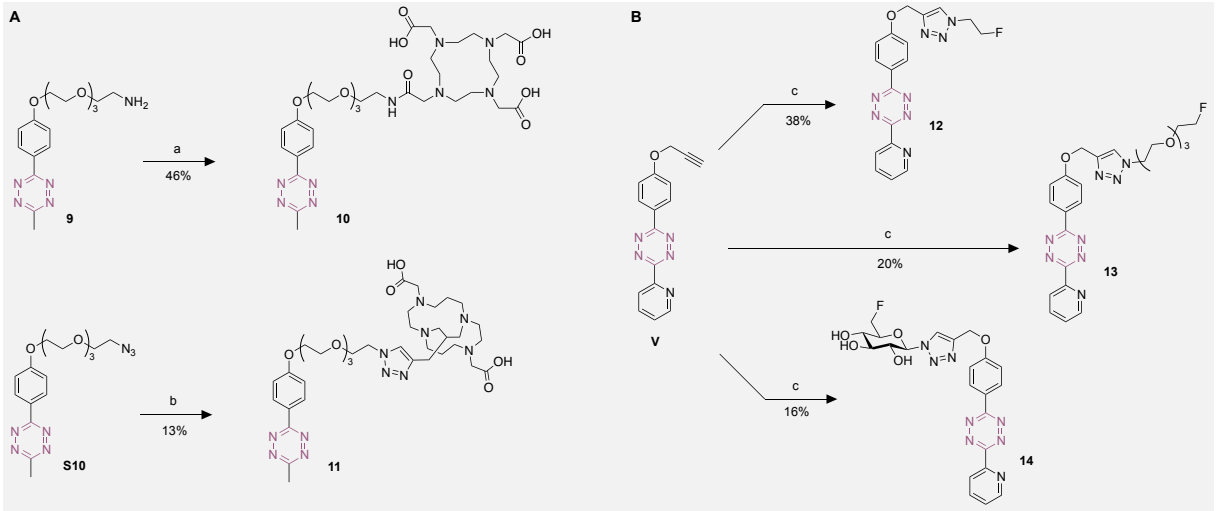


Tz **7** was synthesized according to the general procedure from **VI** (10 mg, 44 µmol), **Az3** (12 mg, 57 µmol), $CuSO_4 \cdot 5H_2O$ (5 µL, 2 µmol), sodium ascorbate (5 µL, 8 µmol) and BPDS (20 µL, 1.7 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient of MeOH in water with 0.1% formic acid (10→80%) as eluent to afford **7** (14 mg, 73%) as a pink solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.53 (s, 1 H), 8.45 (dt, J = 8.8, 2.9 Hz, 2 H), 7.35 (dt, J = 8.8, 2.9 Hz, 2 H), 5.69 (d, J = 9.7 Hz, 1 H), 5.54 (d, J = 6.2 Hz, 1 H), 5.50 (d, J = 5.6 Hz, 1 H), 5.45 (d, J = 4.9 Hz, 1H), 5.31 (s, 2H), 4.48–4.67 (m, 2H), 3.71–3.87 (m, 2H), 3.42–3.48 (m, 1H), 2.97 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.1, 163.4, 162.0, 142.7, 129.7, 124.9, 124.7, 116.1, 87.7, 82.8 (d, J = 170.6 Hz), 77.7 (d, J = 17.6 Hz), 77.7, 77.1, 72.3, 68.7, 61.7, 21.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ -232.00.

*2,2',2''-(10-(2-((3-(4-((4-(6-Methyltetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl) propyl) amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**8**), copper(II) complex*

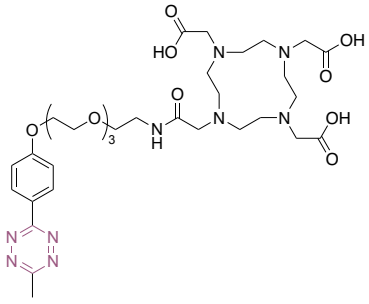


Tz **8** was synthesized similarly to the general procedure from **VI** (20 mg, 88 µmol), 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid)-10-(azidopropyl ethylacetamide) (58 mg, 97 µmol), $CuSO_4 \cdot 5H_2O$ (256 µL, 106 µmol), sodium ascorbate (23 µL, 35 µmol) and BPDS (100 µL, 8.8 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient of MeCN in water with 0.1% trifluoroacetic acid (10→80%) as eluent to afford **8** (25 mg, 32%) as a magenta solid. The compound was obtained as a copper complex. HPLC-MS $[M+H]^+$ m/z calculated for $[C_{31}H_{43}CuN_{12}O_8]^+$: 774.26, found: 774.32 $[M+H]^+$.



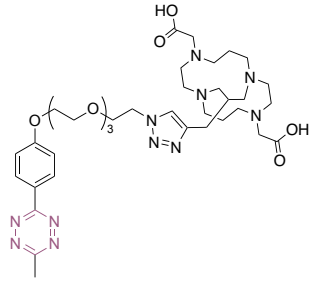
Scheme S5. Synthesis of (A) Tz **10-11** and (B) Tz **12-14**. (a) DOTA-NHS, Et_3N, DMF, r.t., 1.5–2 h (46%); (b) PCB-TE_2A-alkyne, $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., overnight (14%); (c) **Az1**-**Az3**, $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., 1–3 h.

*2,2',2''-(10-(14-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy)-2-oxo-6,9,12-trioxa-3-azatetradecyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**10**)*



Tz **9** (4.8 mg, 13.2 µmol; obtained from Combi-Blocks) and DOTA-NHS (18.6 mg, 24.4 µmol) were dissolved in anhydrous DMF (1.3 mL). Et_3N (20 µL, 144 µmol) was added and the solution was stirred at room temperature for 2 h. The mixture was diluted with water (4 mL), concentrated and the crude product was purified by semi-preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA to obtain **10** (4.6 mg, 46%) as a pink solid. ^{1}H NMR (600 MHz, D_2O) δ 8.43 (d, J = 8.8 Hz, 2H), 7.29 (d, J = 8.9 Hz, 2H), 4.41–4.36 (m, 2H), 4.02–3.99 (m, 2H), 3.93–3.75 (m, 10H), 3.74–3.68 (m, 6H), 3.64 (t, J = 5.4 Hz, 2H), 3.57–3.12 (m, 19H), 3.07 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 168.2, 165.1, 164.0, 130.7, 125.9, 116.4, 71.6, 71.6, 71.5, 71.1, 70.7, 70.4, 69.0, 55.8, 54.6, 51.5, 50.9, 40.4, 21.0. HRMS *m/z* (MALDI-TOF) calculated for $C_{33}H_{51}N_9O_{11}Na^+$: 772.3599, found: 772.3598 $[M+Na]^+$.

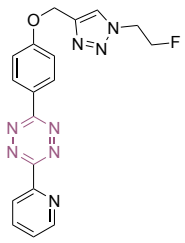
*2,2'-(16-((1-(2-(2-(2-(2-(4-(6-Methyl-1,2,4,5-tetrazin-3yl)phenoxy)ethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl)-1,4,8,11tetraazabicyclo[6.6.3]heptadecane-4,11-diyl)diacetic acid (**11**), copper(II) complex*



Tetrazine **S10** (7.6 mg, 0.0195 mmol, 1.1 eq.; obtained from Jena Bioscience) and 2,2'-(16-(prop-2-yn-1-yl)-1,4,8,11tetraazabicyclo[6.6.3] heptadecane-4,11-diyl)diacetic acid (PCB-TE_2A-alkyne, 7 mg, 0.018 mmol, 1 eq.; obtained from FutureChem) were diluted in DMF (280 µL). The catalyst solution was prepared by mixing an aqueous solution of sodium ascorbate (10 µL, 70 mg/mL, 0.0035 mmol, 0.2 eq.) and an aqueous solution

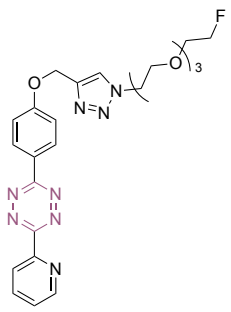
of $CuSO_4 \cdot 5H_2O$ (10 µL, 29.5mg/ 0.1 mL, 0.0009 mmol, 0.005 eq.). After approximately 1 min the black color turned yellow and BPDS solution (20 µL, 13 mg /0.5 mL, 0.00085 mmol, 0.05 eq.) was added. The color turned dark green. The catalyst solution was added immediately to the pink reaction mixture and stirring was continued at room temperature for 30 min. The reaction mixture was filtered and purified by preparative HPLC to afford **11** (2 mg, 2.55 µmol, 13%). HPLC-MS $[M+H]^+$ m/z calculated for $[C_{37}H_{56}CuN_{11}O_8]^+$: 845.36, found: 845.3 $[M+H]^+$.[8]

*3-(4-((1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (**12**)*



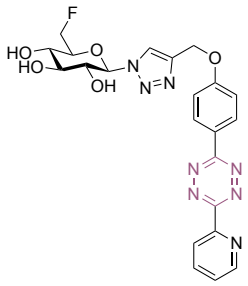
Tz **12** was synthesized according to the general CuAAC procedure (see above) from **V** (30.0 mg, 103 µmol), **Az1** (135 µmol, 90 µl of a 1.5 M solution in DMF), $CuSO_4 \cdot 5H_2O$ (10 µL, 4.0 µmol), sodium ascorbate (10 µL, 15 µmol) and BPDS (40 µL, 3.4 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient (10→90%) of MeCN in water with 0.1% formic acid as eluent to afford **12** (15 mg, 38%) as a dark purple solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ 8.88–9.08 (m, 1 H), 8.67–8.75 (m, 3 H), 7.95–8.06 (m, 1 H), 7.82 (s, 1 H), 7.51–7.64 (m, 1 H), 7.24 (d, J = 8.6 Hz, 2 H), 5.37 (s, 2 H), 4.80 (dt, J = 82.0, 4.7 Hz, 2 H), 4.77 (dt, J = 8.3, 4.2 Hz, 4 H). ^{19}F NMR (376 MHz, $CDCl_3$) δ -221.54.

*3-(4-((1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (**13**)*

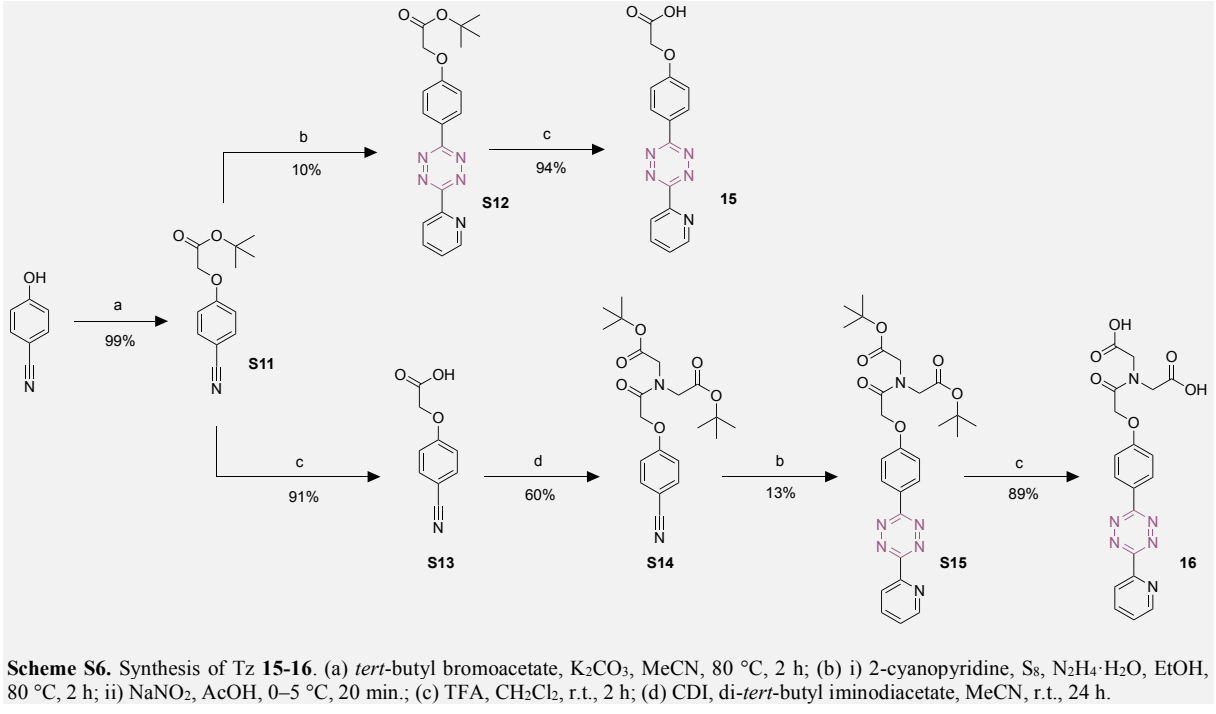


Tz **13** was synthesized according to the general CuAAC procedure (see above) from **V** (17 mg, 59 µmol), **Az2** (28 mg, 130 µmol), $CuSO_4 \cdot 5H_2O$ (13 µL, 5.2 µmol), sodium ascorbate (13 µL, 20 µmol) and BPDS (50 µL, 4.3 µmol). Work-up procedure B was used. The crude product was purified by preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA to afford **13** (6 mg, 20%) as a purple solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.92 (ddd, J = 4.7, 1.8, 0.9 Hz, 1H), 8.59–8.51 (m, 3H), 8.27 (s, 1H), 8.14 (td, J = 7.7, 1.8 Hz, 1H), 7.71 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 7.37 (d, J = 8.9 Hz, 2H), 4.56 (t, J = 5.2 Hz, 2H), 4.54–4.50 (m, 1H), 4.48–4.42 (m, 1H), 3.84 (t, J = 5.2 Hz, 2H), 3.66–3.62 (m, 1H), 3.61–3.57 (m, 1H), 3.56–3.47 (m, 8H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.1, 162.9, 161.9, 150.5, 150.3, 142.0, 137.7, 129.8, 126.3, 125.2, 124.1, 123.8, 115.8, 83.0 (J = 165.6 Hz), 69.8–69.5, 68.6, 61.4, 49.5. HRMS *m/z* (MALDI-TOF) calculated for $C_{24}H_{27}FN_8O_4^+$: 511.2212, found: 511.2236 $[M+H]^+$.

*(2S,3S,4S,5R,6R)-2-(Fluoromethyl)-6-(4-((4-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3,4,5-triol (**14**)*

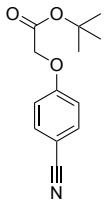


Tz **14** was synthesized according to the general CuAAC procedure (see above) from **V** (30 mg, 103 µmol), **Az3** (28 mg, 135 µmol), $CuSO_4 \cdot 5H_2O$ (10 µL, 4.0 µmol), sodium ascorbate (10 µL, 15 µmol) and BPDS (40 µL, 3.4 µmol). Work-up procedure A was used and reversed phase C18 column chromatography was performed using a gradient (10→80%) of MeOH in water with 0.1% formic acid as eluent to afford **14** (7.9 mg, 16%) as a dark purple solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.92 (d, J = 4.29 Hz, 1 H), 8.57 (s, 2 H), 8.55 (s, 2 H), 8.14 (td, J = 7.7, 1.8 Hz, 1 H), 7.71 (ddd, J = 7.5, 4.8, 0.9 Hz, 1 H), 7.39 (d, J = 8.9 Hz, 2 H), 5.70 (d, J=9.4 Hz, 1 H), 5.52 (dd, J=19.5, 5.9 Hz, 3 H), 5.34 (s, 2 H), 4.44–4.71 (m, 2 H), 3.70–3.90 (m, 2 H), 3.39–3.54 (m, 1 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.6, 163.3, 162.4, 151.0, 150.8, 142.7, 138.2, 130.3, 126.9, 124.8, 124.7, 124.3, 116.3, 87.7, 82.8 (d, J = 173.0 Hz), 77.7 (d, J = 29.0 Hz), 77.1, 72.3, 68.8 (d, J = 11.0 Hz), 61.8. ^{19}F NMR (376 MHz, DMSO-d_6) δ -231.98.



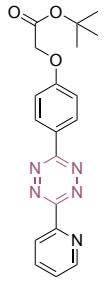
Scheme S6. Synthesis of Tz **15-16**. (a) *tert*-butyl bromoacetate, K_2CO_3, MeCN, 80 °C, 2 h; (b) i) 2-cyanopyridine, S_8, $N_2H_4 \cdot H_2O$, EtOH, 80 °C, 2 h; ii) $NaNO_2$, AcOH, 0–5 °C, 20 min.; (c) TFA, CH_2Cl_2, r.t., 2 h; (d) CDI, di-*tert*-butyl iminodiacetate, MeCN, r.t., 24 h.

*Tert-butyl 2-(4-cyanophenoxy)acetate (**S11**)*



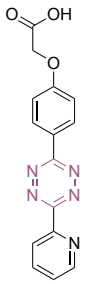
To a suspension of 4-hydroxybenzonitrile (3.0 g, 25 mmol) and K_2CO_3 (6.96 g, 50.4 mmol) in MeCN (30 mL) was added *tert*-butyl bromoacetate (5.16 g, 26.4 mmol). The mixture was heated to reflux for 4 h and then cooled down to room temperature. Water (40 mL) was added, the phases were separated and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give **S11** (5.8 g, 99%) as a yellow oil. R_f = 0.41 (20% EtOAc in heptane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 4.59 (s, 2H), 1.50 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 161.1, 134.0, 118.9, 115.3, 105.0, 83.0, 65.5, 28.0.

*Tert-butyl 2-(4-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)phenoxy)acetate (**S12**)*



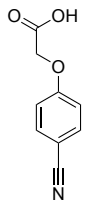
Nitrile **S11** (1.4 g, 6.0 mmol), 2-cyanopyridine (5.0 g, 48 mmol) and sulfur (0.38 g, 1.50 mmol) were suspended in EtOH (5 mL), followed by the addition of hydrazine hydrate (4.39 mL, 90.0 mmol). The reaction was heated to 90 °C for 2 h. The mixture was cooled to room temperature and the formed precipitate was removed by filtration. Water (20 mL) and a solution of $NaNO_2$ (6.20 g, 90.0 mmol) in water 30 mL were added and the mixture was cautiously acidified to pH 2 by addition of AcOH. The mixture was extracted with DCM and the combined organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography on silica gel (40% EtOAc in heptane), followed by recrystallization from EtOAc/heptane to afford **S12** (0.2 g, 10%) as a red solid. R_f = 0.38 (50% EtOAC in heptane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (dd, J = 4.7, 0.9 Hz, 1H), 8.62-8.53 (m, 3H), 7.91 (td, J = 7.8, 1.8 Hz, 1H), 7.48 (ddd, J = 7.7, 4.7, 1.1 Hz, 1H), 7.04 (d, J = 9.0 Hz, 2H), 4.58 (s, 2H), 1.44 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.3, 163.9, 163.1, 162.0, 150.9, 150.5, 137.4, 130.3, 126.1, 124.8, 123.7, 115.4, 82.9, 65.6, 28.1.

*2-(4-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)phenoxy)acetic acid (**15**)*



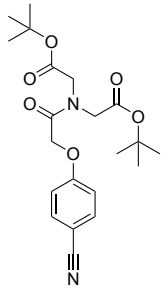
To a solution of **S12** (0.15 g, 0.41 mmol) in DCM (3 mL) was added TFA (3 mL). The mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was purified by recrystallization from MeOH to afford **15** (0.12 g, 94%) as a red solid. R_f = 0.25 (5% MeOH in DCM with 0.1% AcOH); ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.15 (s, 1H), 8.91 (dt, J = 4.7, 1.4 Hz, 1H), 8.55 (d, J = 7.9 Hz, 2H), 8.53 (d, J = 8.9 Hz, 1H), 8.14 (d, J = 1.8 Hz, 1H), 7.70 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 7.24 (d, J = 8.9 Hz, 2H), 4.87 (s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 170.2, 163.6, 163.4, 162.1, 151.0, 150.8, 138.2, 130.2, 126.9, 124.8, 124.2, 116.0, 65.1.

*2-(4-Cyanophenoxy)acetic acid (**S13**)*



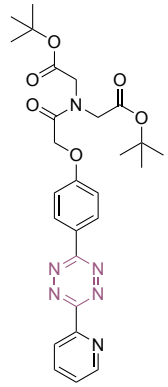
To a solution of **S11** (2.9 g, 12.4 mmol) in DCM (5 mL) was added TFA (5 mL). The mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was purified by recrystallization from EtOAc to afford **S13** (2.0 g, 91%) as a white solid. R_f = 0.21 (5% MeOH in DCM with 0.1% AcOH); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.13 (s, 1H), 7.77 (d, J = 8.8 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 4.82 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.0, 161.7, 134.6, 119.5, 116.1, 103.8, 65.0.

*Di-tert-butyl 2,2'-((2-(4-cyanophenoxy)acetyl)azanediyl)diacetate (**S14**)*



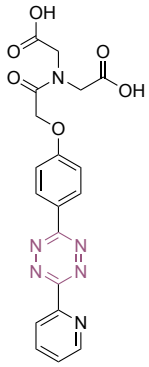
To a solution of **S13** (1.4 g, 7.9 mmol) in MeCN (30 ml) was added 1,1'-carbonyldiimidazole (CDI) (1.92 g, 11.9 mmol). After stirring at room temperature for 45 min di-*tert*-butyl iminodiacetate (1.93 g, 7.90 mmol) was added and stirring was continued for 24 h. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography (40% EtOAc in heptane) on silica gel to afford **S14** (1.9 g, 60%) as a colorless oil. R_f = 0.27 (50% EtOAc in heptane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, J = 8.9 Hz, 2H), 6.96 (d, J = 8.9 Hz, 2H), 4.76 (s, 2H), 4.05 (s, 2H), 4.02 (s, 2H), 1.43 (s, 9H), 1.40 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.7, 167.6, 167.6, 161.2, 133.9, 118.9, 104.9, 83.0, 82.1, 66.6, 50.3, 49.2, 28.0.

*Di-tert-butyl 2,2'-((2-(4-(6-(pyridin-2-yl)tetrazin-3-yl)phenoxy)acetyl)azanediyl)diacetate (**S15**)*

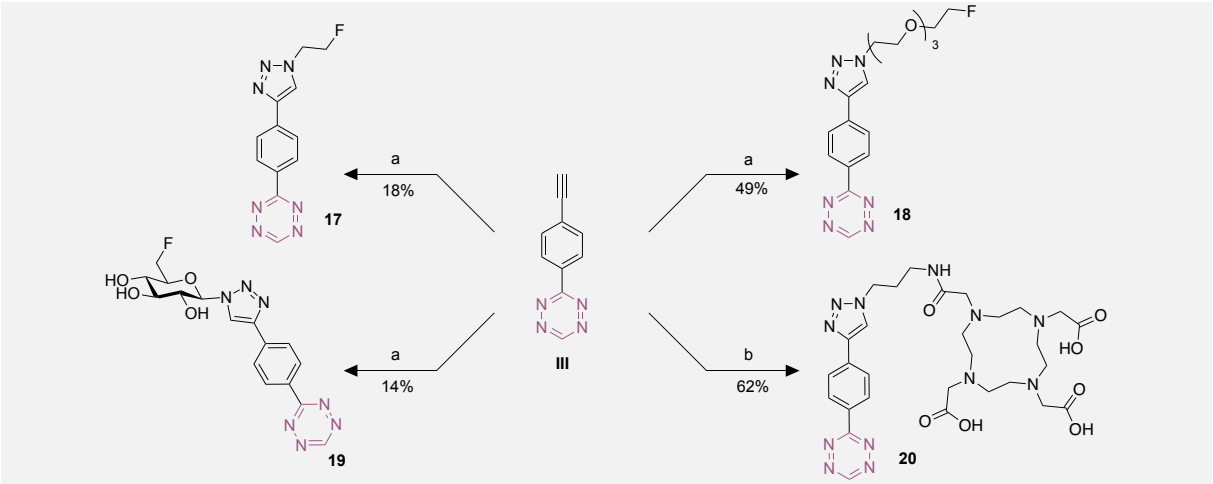


Nitrile **S15** (1.60 g, 3.95 mmol), 2-cyanopyridine (3.30 g, 31.64 mmol) and sulfur (0.25 g, 0.99 mmol) were suspended in EtOH (4 mL), followed by the addition of hydrazine hydrate (2.89 mL, 59.34 mmol). The reaction was heated to 90 °C for 2 h. The mixture was cooled to room temperature and the formed precipitate was removed by filtration. Water (20 mL) and an aqueous solution of $NaNO_2$ (5.45 g, 79.11 mmol) in water (20 mL) were added to the solution. The mixture was cautiously acidified to pH 2 with AcOH. After addition of DCM, the layers were separated and the aqueous layer was extracted with additional DCM. The combined organic layer was washed with water and brine, dried over $MgSO_4$, filtered and concentrated. Flash column chromatography (40% EtOAc in heptane) followed by recrystallization (EtOAC/heptane) afforded **S15** (0.27 g, 13%) as a red solid. R_f = 0.22 (50% EtOAC in heptane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (dd, J = 5.0, 1.6 Hz, 1H), 8.67-8.53 (m, 3H), 7.94 (td, J = 7.8, 1.8 Hz, 1H), 7.51 (dd, J = 7.6, 4.8 Hz, 1H), 7.12 (d, J = 9.0 Hz, 2H), 4.84 (s, 2H), 4.13 (s, 2H), 4.07 (s, 2H), 1.45 (s, 9H), 1.42 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.9, 167.8, 167.6, 163.8, 163.0, 161.9, 150.8, 150.4, 137.3, 130.3, 126.1, 124.9, 123.6, 115.6, 83.0, 82.2, 67.0, 50.5, 49.3, 28.03, 28.01.

*2,2'-((2-(4-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)phenoxy)acetyl)azanediyl)diacetic acid (**16**)*

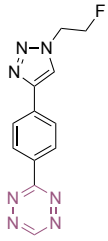


To a solution of **S15** (0.24 g, 0.44 mmol) in DCM (3 mL) was added TFA (3 mL). The mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was purified by recrystallization from MeOH/Et_2O to afford **16** (0.2 g, 89%) as a red solid. R_f = 0.19 (5% MeOH in DCM with 0.1% AcOH); ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.86 (br s, 2H), 8.92 (dt, J = 4.8, 1.3 Hz, 1H), 8.55 (dd, J = 7.9, 1.1 Hz, 2H), 8.50 (d, J = 8.9 Hz, 1H), 8.14 (td, J = 7.8, 1.8 Hz, 1H), 7.70 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 7.20 (d, J = 8.9 Hz, 2H), 5.00 (s, 2H), 4.30 (s, 2H), 4.04 (s, 2H); ^{13}C NMR (151 MHz, DMSO-d_6) δ 171.2, 170.7, 168.3, 163.6, 163.4, 162.3, 151.0, 150.8, 138.2, 130.0, 126.8, 124.7, 124.2, 116.1, 66.0, 49.6, 49.1, 48.9.



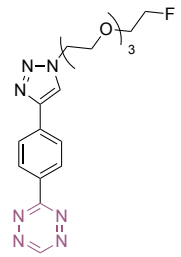
Scheme S7. Synthesis of Tz **17-20**. (a) CuI, Et_3N, DMF, r.t., 1.5–2 h.; (b) $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., 1–3 h).

*3-(4-(1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)phenyl)-1,2,4,5-tetrazine (**17**)*



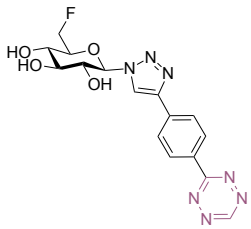
A solution of **III** (15 mg, 82 µmol) and **Az1** (250 µmol, 165 µL of a 1.5 M solution) in anhydrous DMF (2 mL) was treated with Cu(I)I (5 mg, 26 mmol) and Et_3N (40 µL, 287 µmol). The mixture was stirred under argon at room temperature for 1.5 h and thereafter concentrated under reduced pressure. The residue was dissolved in DMSO (1 mL) and directly loaded onto a C18 column. Reversed phase chromatography was performed using a gradient (10→90%) of MeCN in water containing 0.1% formic acid as eluent to afford **17** (4 mg, 18%) as a pink solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.16 (s, 1H), 8.65 (dt, J = 8.8, 2.1 Hz, 2H), 8.04 (dt, J = 8.5, 2.1 Hz, 2H), 7.99 (d, J = 0.8 Hz, 1H), 4.85 (dt, J = 185.00, 5.00 Hz, 2H), 4.76 (dt, J = 19.6, 4.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.2, 156.7, 146.1, 134.0, 130.1, 127.9, 125.5, 120.6, 80.6 (d, J = 172.2 Hz), 49.7 (d, J = 20.7 Hz). ^{19}F NMR (376 MHz, $CDCl_3$) δ -221.36.

*3-(4-(1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)phenyl)tetrazine (**18**)*



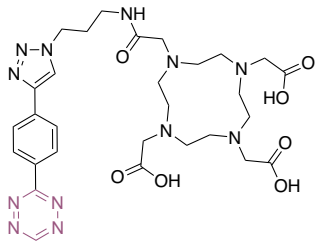
Tz **III** (31.0 mg, 0.17 mmol) and Cu(I)I (8.2 mg, 0.4 mmol) were placed in a vial. A 1.0 M solution of Et_3N in anhydrous DMF (0.43 mL, 0.43 mmol) was added. The mixture was further diluted with anhydrous DMF (1.5 mL) and stirred at room temperature under nitrogen atmosphere for 3 h. The mixture was diluted with water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (60%), followed by MeOH in EtOAc (10%) as eluent to afford **18** (34 mg, 49%) as a fuchsia-colored solid. R_f = 0.55 (10% MeOH in EtOAc). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.21 (s, 1H), 8.70 (d, J = 8.5 Hz, 2H), 8.17 (s, 1H), 8.11 (d, J = 8.4 Hz, 2H), 4.66–4.63 (m, 2H), 4.58–4.56 (m, 1H), 4.50–4.47 (m, 1H), 4.00–3.92 (m, 2H), 3.75–3.72 (m, 1H), 3.70–3.63 (m, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.4, 157.87, 157.86, 146.7, 135.7, 131.0, 129.0, 126.6, 122.2, 83.3 (d, J = 169.2 Hz), 77.4, 77.2, 77.0, 71.0–70.5, 69.6, 50.7.

*(2R,3R,4S,5S,6S)-2-(4-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-1H-1,2,3-triazol-1-yl)-6-(fluoromethyl) tetrahydro-2H-pyran-3,4,5-triol (**19**)*

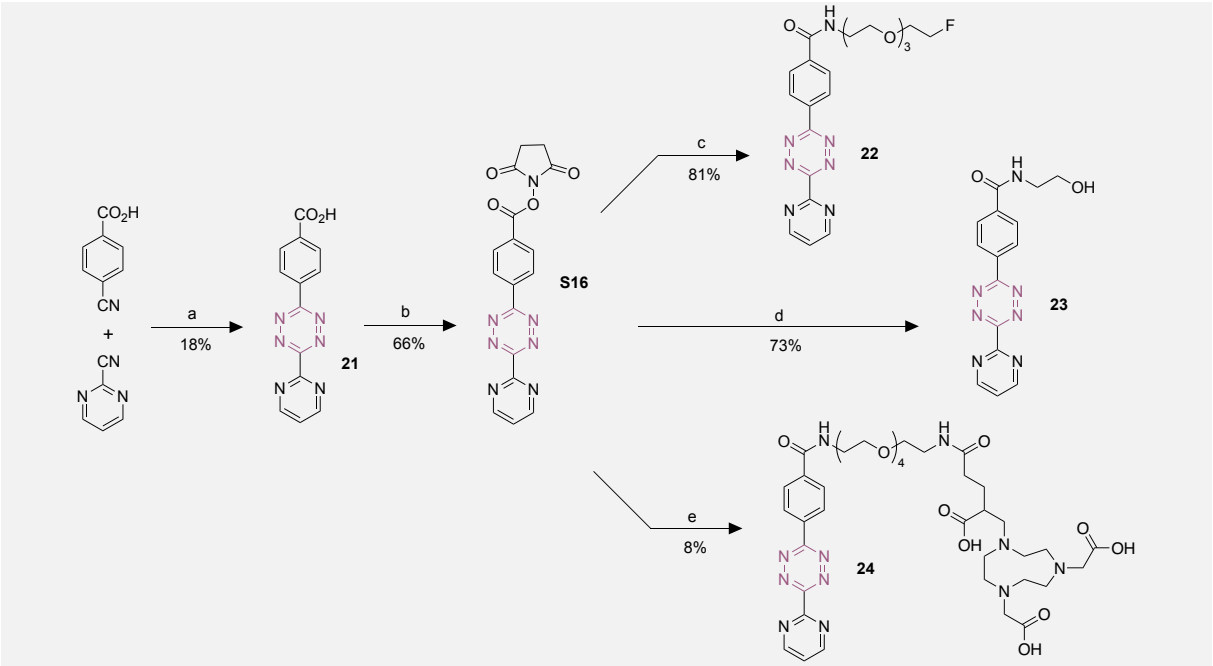


Tz **III** (9.80 mg, 0.05 mmol) and Cu(I)I (10.0 mg, 0.05 mmol) were placed in a vial. A 0.1 M solution of Et_3N in anhydrous DMF (0.54 mL, 0.05 mmol) was added. The mixture was further diluted with additional anhydrous DMF (1 mL) and stirred at room temperature under nitrogen atmosphere for 1 h. TLC indicated formation of the dihydrotetrazine of **III**. An additional equivalent of Cu(I)I (10.0 mg, 0.05 mmol) was thus added and the mixture was stirred for 1 h. Thereafter, the mixture was diluted with water and extracted with EtOAc. The combined organic phases were washed with brine and cautiously concentrated under reduced pressure. The residue was dissolved in DMSO/water (1:1) and directly purified by preparative HPLC to afford **19** (3 mg, 14%). ^{1}H NMR (400 MHz, DMSO-d_6), mixture of anomers, δ 10.60 (s, 1H), 9.08 (s, 1H), 8.61 (d, J = 8.2 Hz, 2H), 8.21 (d, J = 8.2 Hz, 2H), 5.74 (d, J = 9.3 Hz, 1H), 5.56 (d, J = 5.9 Hz, 1H), 5.48 (dd, J = 12.2, 5.2 Hz, 2H), 4.80–4.41 (m, 2H), 3.96–3.75 (m, 2H), 3.57–3.45 (m, 2H). ^{19}F NMR (376 MHz, DMSO-d_6) δ -73.41. HRMS *m/z* (MALDI-TOF) calculated for $C_{16}H_{16}FN_7O_4^+$: 390.1320, found: 390.1322 $[M+H]^+$.

*2,2',2''-(10-(2-((3-(4-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-1H-1,2,3-triazol-1-yl)propyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**20**), copper(II) complex*

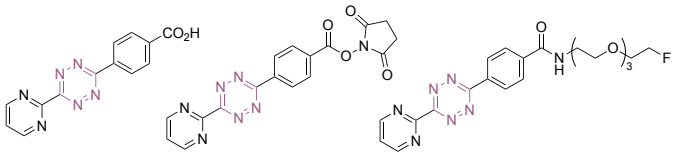


Tz **20** was synthesized similarly to the general CuAAC procedure (see above) from **III** (16 mg, 88 µmol), DOTA-NHS (58 mg, 97 µmol), $CuSO_4 \cdot 5H_2O$ (256 µL, 106 µmol), sodium ascorbate (23 µL, 35 µmol) and BPDS (100 µL, 8.82 µmol). Work-up procedure A was used and reversed phaseC18 column chromatography was performed using a gradient (10→80%) of MeCN in water with 0.1% TFA as eluent to afford **20** (45 mg, 62%) as a magenta solid. The compound was obtained as a copper complex. HPLC-MS m/z calc. for $[C_{29}H_{39}CuN_{12}O_8]^+$: 730.26; found: 730.31 $[M+H]^+$.



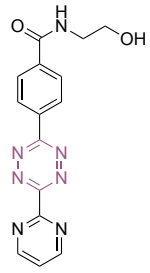
Scheme S8. Synthesis of Tz **21-24**. (a) (i) 4-cyanobenzoic acid, pyrimidine-2-carbonitrile, $N_2H_4 \cdot H_2O$, EtOH, reflux overnight, (ii) isopentyl nitrite, acetic acid; (b) N-hydroxysuccinimide, EDCI, 40 °C, 2.5 h; (c) PEG-amine, DMSO/pyridine, 4 h, 50 °C; (d) ethanolamine, DMF, Et_3N, r.t., 1 h; (e) NODAGA-PEG_4-NH_2, DMSO/pyridine, 4h at 50 °C.

*4-(6-(Pyrimidin-2-yl)tetrazin-3-yl)benzoic acid (**21**), 2,5-dioxopyrrolidin-1-yl 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoate (**S16**), N-(2-(2-(2-(2-fluoroethoxy)ethoxy)ethoxy)ethyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (**22**)*



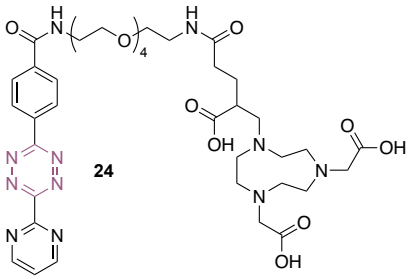
Tz **21**, NHS ester **S16**, and Tz **22** were synthesized as previously described.[6,9]

*N-(2-Hydroxyethyl)-4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzamide (**23**)*

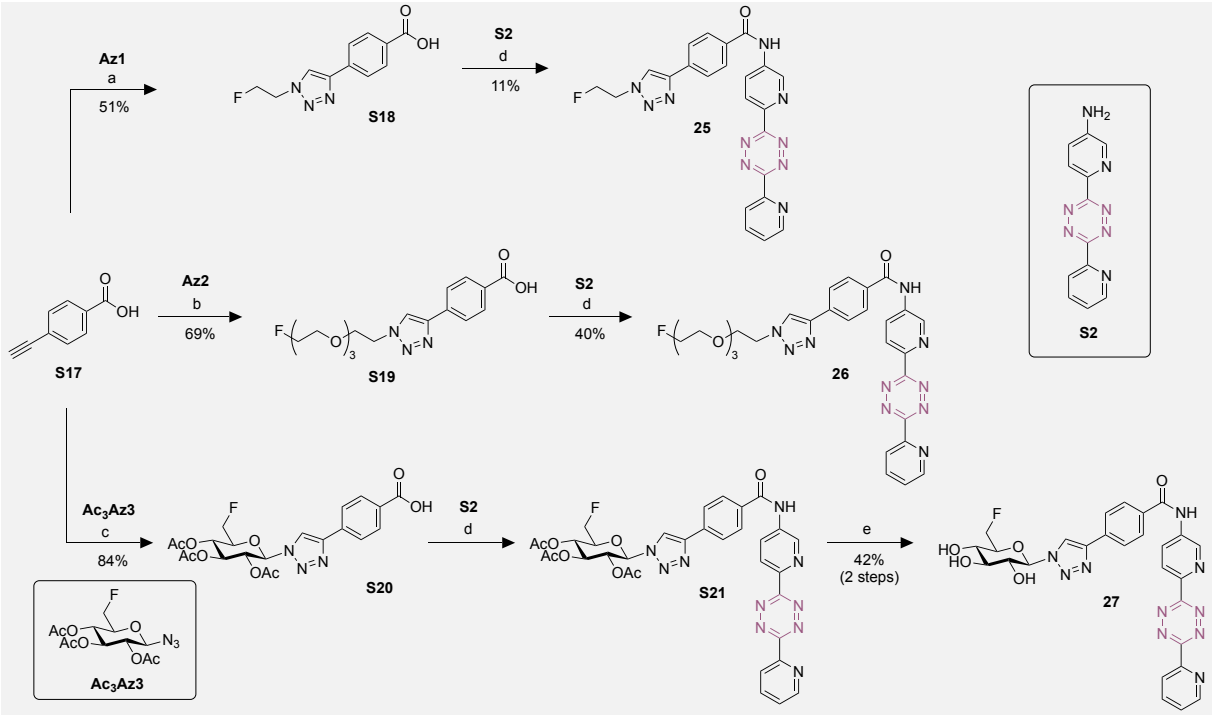


A solution of ethanolamine (7.00 µL, 0.12 mmol) in DMF (1 mL) was added to a solution of **S16** (0.04 g, 0.11 mmol) in DCM (3 mL), followed by addition of Et_3N (70.0 µL, 0.53 mmol). After a few minutes a precipitate formed and the mixture was left stirring at r.t. for 1 h. The mixture was diluted with DCM and the layers were separated. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel using MeOH in DCM (5%) as eluent to afford **23** (0.025 g, 73%) as a purple solid. R_f= 0.25 (10% MeOH in DCM); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.21 (d, J = 4.9 Hz, 2H), 8.71 (t, J = 6.9 Hz, 1H), 8.68 (d, J = 8.4 Hz, 2H), 8.17 (d, J = 8.5 Hz, 2H), 7.85 (t, J = 4.9 Hz, 1H), 4.77 (t, J = 5.6 Hz, 1H), 3.57 (q, J = 6.1 Hz, 2H), 3.40 (q, J = 5.9 Hz, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.0, 163.7, 163.4, 159.6, 159.0, 138.8, 134.3, 128.8, 128.6, 123.5, 60.1, 42.8.

*2,2'-(7-(21-Carboxy-1,18-dioxo-1-(4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl) phenyl)-5,8,11,14-tetraoxa-2,17-diazahenicosan-21-yl)-1,4,7-triazonane-1,4-diyl) diacetic acid (**24**)*

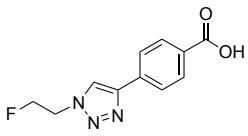


Tz-NHS **S16** (5.0 mg, 13 µmol) was dissolved in dry DMSO/pyridine (0.2 mL, 18:1) and the mixture was stirred for 30 minutes before a solution of 2,2'-(7-(1-amino-19-carboxy-16-oxo-3,6,9,12-tetraoxa-15-azanonadecan-19-yl)-1,4,7-triazonane-1,4-diyl)diacetic acid (NODAGA-PEG$_4$-NH$_2$, 7.9 mg, 13 µmol; obtained from ChemMaTech) in dry DMSO/pyridine (0.1 mL, 18:1) was added dropwise over the course of 4 h at 50 °C. The reaction mixture was left at r.t. overnight. The solvent was removed under reduced pressure and the residue was purified by preparative HPLC using a gradient of MeCN in water (10→80%) with 0.1% TFA as eluent to afford **24** (6 mg, 54%) as a pink solid. ^{1}H NMR (600 MHz, CD_3OD) δ 9.17 (d, *J* = 4.9 Hz, 2H), 8.81 (d, *J* = 8.5 Hz, 2H), 8.14 (d, *J* = 8.5 Hz, 2H,), 7.81 (t, *J* = 4.9 Hz, 1H), 3.99 (m, 2H), 3.88 – 3.56 (m, 20H), 3.53 (t, *J* = 5.6 Hz, 2H), 3.38 – 3.33 (m, 3H), 3.30 – 2.78 (m, 14H), 2.41 (t, *J* = 7.5 Hz, 2H), 2.14 – 1.94 (m, 3H). ^{13}C NMR (151 MHz, CD_3OD) δ 175.1, 175.0, 169.2, 165.6, 164.1, 160.30, 159.7, 139.9, 135.7, 129.7, 129.5, 124.5, 71.6, 71.5, 71.5, 71.4, 71.2, 70.6, 70.5, 64.4, 41.2, 40.3, 33.5, 26.6. HRMS *m/z* (MALDI-TOF) found 856.237 $[M+H]^+$ ($C_{38}H_{54}N_{11}O_{12}^+$ requires 856.39); found 811.075 $[M+H^+-CO_2H]$ ($C_{37}H_{53}N_{11}O_{10}^+$ requires 811.39); found: 767.039 $[M+H^+-2CO_2H]$ ($C_{36}H_{52}N_{11}O_8^+$ requires 766.39).



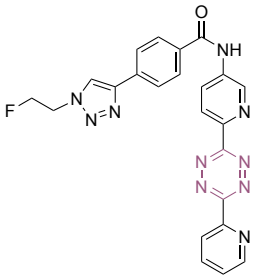
Scheme S9. Synthesis of Tz **25-27**. (a) **Az1**, $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., 23 h; (b) **Az2**, $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., 3 h; (c) **Ac$_3$Az3**, $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., 2.5 h; (d) $(COCl)_2$, DCM, 0 °C→r.t., 14 h; ii. 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (Pyr-Tz-Pyr-NH_2, **S2**), pyridine, MW, 130 °C, 10 min for **25** and **26**, 30 min for **27**; (e) K_2CO_3, MeOH/DCM, r.t., overnight.

*4-(1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)benzoic acid (**S18**)*



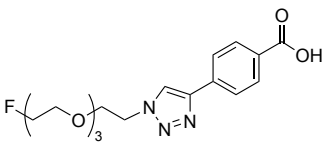
An aqueous 0.33 M $CuSO_4 \cdot 5H_2O$ solution (498 µL, 0.16 mmol) and 0.53 M sodium ascorbate solution (1.24 mL, 0.66 mmol) were mixed, when the solution turned yellow a 0.27 M aqueous solution of BPDS (593 µL, 0.16 mmol) was added. The resulting blue/green mixture was added to a solution of carboxylic acid **S17** (480 mg, 3.29 mmol) in anhydrous DMF (1 mL). A solution of **Az1** (13.3 mL, 0.37 M) in DMF was added and the mixture was stirred at room temperature for 23 h, thereafter diluted with water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (40→60%) with 1% AcOH as eluent to afford **S18** as an off-white solid (389 mg, 51%). R_f = 0.22 (60% EtOAC in heptane with 1% AcOH). ^{1}H NMR (400 MHz, CD_3OD) δ 8.49 (s, 1H), 8.10 (d, J = 8.5 Hz, 2H), 7.96 (d, J = 8.5 Hz, 2H), 4.93 (dd, J = 5.3, 4.0 Hz, 1H), 4.87–4.74 (m, 3H).

*4-(1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide (**25**)*



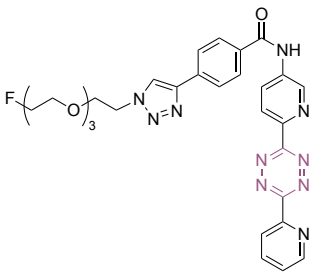
Carboxylic acid **S18** (124 mg, 0.53 mmol) was heated to reflux in thionyl chloride (2 mL) in a sealed vial overnight. The mixture was cooled to room temperature and concentrated under reduced pressure. 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine **(**Pyr-Tz-Pyr-NH_2, **S2**, 55 mg, 0.22 mmol) in dry pyridine (2.5 mL) was added to the formed acid chloride. The mixture was heated by microwave-irradiation for 45 min to 130 °C. The solvent was removed, and the compound triturated in hot methanol. Filtration afforded 28 mg (0.058 mmol, 11%) of **25**. ^{1}H NMR (400 MHz, DMSO, 100 ºC) δ 9.33 (d, *J* = 2.4 Hz, 1H), 9.13 – 8.89 (m, 1H), 8.78 – 8.64 (m, 2H), 8.64 – 8.57 (m, 2H), 8.27 – 8.13 (m, 3H), 8.08 (d, *J* = 8.3 Hz, 2H), 7.73 (dd, *J* = 7.7, 4.7 Hz, 1H), 4.98 (t, *J* = 4.7 Hz, 1H), 4.86 (q, *J* = 5.0 Hz, 2H), 4.78 (t, *J* = 4.6 Hz, 1H).

*4-(1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)benzoic acid (**S19**)*



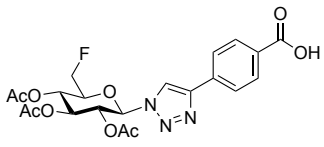
An aqueous 0.32 M $CuSO_4 \cdot 5H_2O$ solution (93.8 µL, 0.03 mmol) and 0.4 M sodium ascorbate solution (275 µL, 0.11 mmol) were mixed, when the solution turned yellow a 0.067 M aqueous solution of BPDS (448 µL, 0.03 mmol) was added. The resulting blue/green mixture was added to a solution of carboxylic acid **S17** (80.0 mg, 0.55 mmol) and **Az2** (121 mg, 0.55 mmol) in DMF (2 mL). The mixture was stirred at room temperature for 3 h, thereafter diluted with EtOAc and washed with aqueous 0.1 M HCl. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using MeOH in EtOAc (10%) with 1% AcOH as eluent to afford **S19** as an off-white solid (140 mg, 69%). R_f = 0.33 (5% MeOH in DCM). ^{1}H NMR (600 MHz, $CDCl_3$) *δ* 8.16 (d, *J* = 8.4 Hz, 2H), 8.13 (s, 1H), 7.96 (d, *J* = 8.4 Hz, 2H), 4.62 (t, *J* = 4.9 Hz, 2H), 4.58–4.54 (m, 1H), 4.50–4.46 (m, 1H), 3.93 (t, *J* = 4.9 Hz, 2H), 3.74–3.69 (m, 1H), 3.68–3.61 (m, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) *δ* 171.2, 146.7, 136.0, 130.9, 128.8, 125.7, 122.3, 83.8, 82.6, 70.9–70.4, 69.51, 50.64. HRMS *m/z* (MALDI-TOF) calculated for $C_{17}H_{22}FN_3O_5^+$: 368.1616, found: 368.1618 $[M+H]^+$.

*4-(1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide (**26**)*



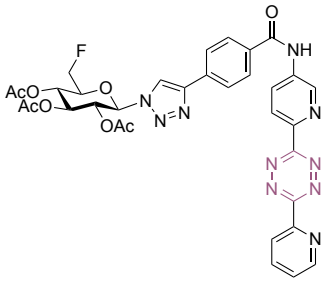
Oxalyl chloride (28 µL, 0.28 mmol) was added drop wise to a solution of carboxylic acid **S19** (51.0 mg, 0.14 mmol) in anhydrous DCM (1.5 mL) at 0 °C. The mixture was stirred overnight (14 h) under nitrogen atmosphere, while slowly reaching room temperature. The solvent was removed and anhydrous DCM (2 x ~1.5 mL) was added. 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine **(**Pyr-Tz-Pyr-NH_2, **S2**, 26 mg, 0.1 mmol) in anhydrous pyridine (1 mL) was added to the residue and the mixture was heated in a microwave reactor to 130 °C for 10 min. The solvent was removed, and the crude product was purified by flash column chromatography on silica gel using MeOH in DCM (0→5%) as eluent to afford **26** (24 mg, 40%) as a fuchsia-colored solid. R_f = 0.22 (5% MeOH in DCM).^{1}H NMR (600 MHz, DMSO-d_6) δ 10.88 (s, 1H), 9.31 (d, J = 2.5 Hz, 1H), 8.95 (ddd, J = 4.6, 1.4, 0.8 Hz, 1H), 8.73–8.67 (m, 2H), 8.63 (dd, J = 8.7, 2.5 Hz, 1H), 8.61 (dt, J = 7.9, 1.1 Hz, 1H), 8.21–8.12 (m, 3H), 8.07 (d, J = 8.4 Hz, 2H), 7.74 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 4.62 (t, J = 5.2 Hz, 2H), 4.54–4.50 (m, 1H), 4.46–4.42 (m, 1H), 3.90 (t, J = 5.2 Hz, 2H), 3.65–3.61 (m, 1H), 3.60–3.55 (m, 3H), 3.54–3.48 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 165.8, 163.1, 162.8, 150.6, 150.2, 145.3, 144.4, 142.5, 138.6, 137.8, 134.4, 133.0, 128.8, 127.4, 126.6, 125.0, 124.7, 124.2, 122.9, 82.98 (d, J = 165.7 Hz), 69.8–69.6, 68.6, 49.7. HRMS *m/z* (MALDI-TOF) calculated for $C_{29}H_{29}FN_{10}O_4^+$: 601.2429, found: 601.2434 $[M+H]^+$.

*4-(1-((2R,3R,4S,5S,6S)-3,4,5-triacetoxy-6-(fluoromethyl)tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)benzoic acid (**S20**)*



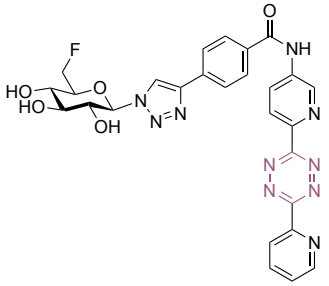
An aqueous 0.33 M $CuSO_4 \cdot 5H_2O$ solution (67 µL, 0.02 mmol) and 0.53 M sodium ascorbate solution (167 µL, 0.09 mmol) were mixed, when the solution turned yellow a 0.068 M aqueous solution of BPDS (326 µL, 0.02 mmol) was added. The resulting blue/green mixture was added to a solution of carboxylic acid **S17** (65 mg, 0.44 mmol) in dry DMF (0.6 mL). **Ac_3Az3** (148 mg, 0.44 mmol) was added in one portion and the mixture was stirred at room temperature for 2.5 h, thereafter diluted with water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford **S20** as a pale-yellow solid (178 mg, 84%). The crude product was used directly in amide coupling to **S2** without further purification. R_f = 0.14 (40% EtOAC in heptane with 1% AcOH). ^{1}H NMR (400 MHz, MeOD) δ 8.77 (s, 1H), 8.10 (d, J = 8.5 Hz, 2H), 7.97 (d, J = 8.5 Hz, 2H), 6.23 (d, J = 9.1 Hz, 1H), 5.68 (t, J = 9.3 Hz, 1H), 5.59 (t, J = 9.4 Hz, 1H), 5.34 (dd, J = 10.4, 9.3 Hz, 1H), 4.71–4.58 (m, 1H), 4.57–4.46 (m, 1H), 4.28 (dddd, J = 23.1, 10.3, 4.1, 2.2 Hz, 1H), 2.08 (s, 3H), 2.02 (s, 3H), 1.86 (s, 3H).

*(2S,3S,4S,5R,6R)-2-(Fluoromethyl)-6-(4-(4-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)carbamoyl)phenyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**S21**)*

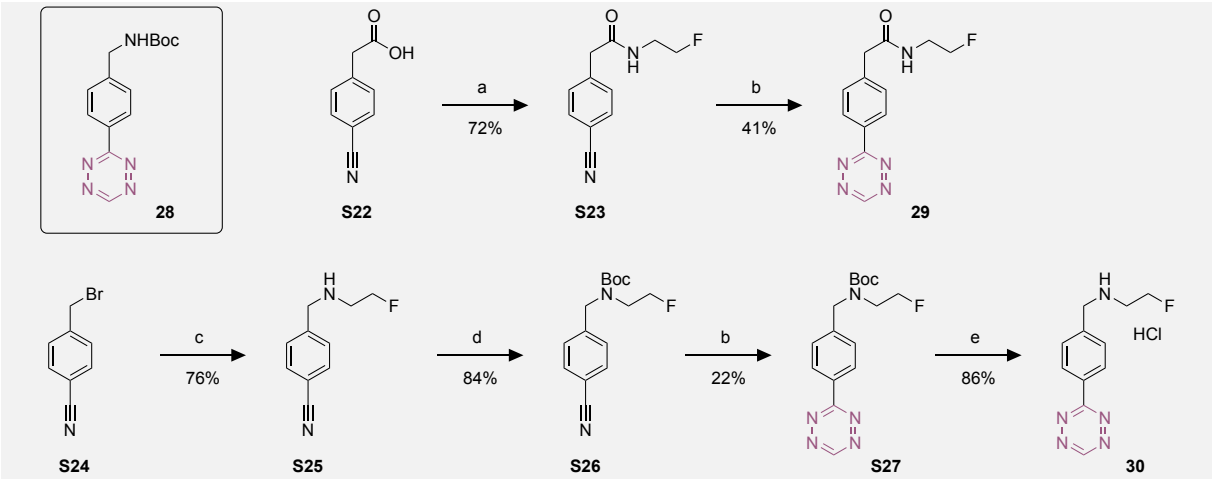


Oxalyl chloride (35 µL, 0.40 mmol) was added dropwise to a solution of carboxylic acid **S20** (129 mg, 0.27 mmol) in anhydrous DCM (3 mL) at 0 °C. The mixture was stirred overnight (14 h) under nitrogen atmosphere, while slowly reaching room temperature. The solvent was removed and anhydrous DCM (2 x ~1.5 mL) was added. 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (Pyr-Tz-Pyr-NH_2, **S2**, 61.0 mg, 0.24 mmol) in anhydrous pyridine (3 mL) was added to the residue and the mixture was heated in a microwave reactor at 130 °C for 30 min. The solvent was removed, and the crude product was washed with Et_2O to give **S21** as a fuchsia solid. The compound was used for the next step without any further purification or characterization.

*4-(1-((2R,3R,4S,5S,6S)-6-(Fluoromethyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide **(27)***

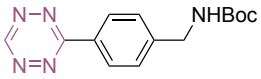


A solution of K_2CO_3 (430 µL, 0.015 M) in anhydrous MeOH was added to **S21** (46.0 mg, 0.07 mmol) dissolved in DCM/MeOH (2:3, 5 mL). The mixture was stirred at room temperature under nitrogen atmosphere overnight. The solvent was removed and the crude product was purified by preparative HPLC using a gradient (20→90%) of MeCN in water with 0.1% TFA to afford **27** (14 mg, 42%) as a purple solid. ^{1}H NMR (400 MHz, DMSO) δ 10.90 (s, 1H), 9.31 (d, *J* = 2.5 Hz, 1H), 9.05 (s, 1H), 8.99 – 8.87 (m, 1H), 8.70 (d, *J* = 8.7 Hz, 1H), 8.66 – 8.48 (m, 2H), 8.39 – 7.98 (m, 5H), 7.74 (ddd, *J* = 7.6, 4.7, 1.2 Hz, 1H), 5.74 (d, *J* = 9.2 Hz, 1H), 4.75 – 4.37 (m, 2H), 4.15 – 3.73 (m, 2H), 3.50 (t, *J* = 8.9 Hz, 1H), 3.36 (t, *J* = 9.4 Hz, 1H); ^{13}C NMR (151 MHz, DMSO) δ 166.31, 163.54, 163.31, 151.04, 150.62, 146.05, 144.81, 142.84, 139.07, 138.11, 134.59, 133.62, 129.21, 127.79, 127.07, 125.57, 125.22, 124.70, 122.04, 87.96, 82.73 (d, *J* = 170.0 Hz), 77.71 (d, *J* = 17.1 Hz), 76.95, 72.55, 68.81 (d, *J* = 7.2 Hz); ^{19}F NMR (376 MHz, DMSO) *δ* -74.79.



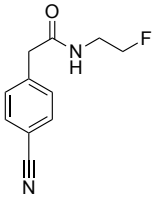
Scheme S10. Synthesis of Tz **29**, **30**. (a) Carbonyldiimidazole (CDI), 2-Fluoroethylamine hydrochloride, Et_3N, MeCN, r.t., 24 h.; (b) (i) S_8, DCM, $N_2H_4 \cdot H_2O$, 50 °C, 24 h (ii) Sodium nitrite, AcOH, 0 °C, 15 min; (c) 2-Fluoroethylamine hydrochloride, K_2CO_3, MeCN, r.t., 24 h; (d) Boc_2O, Et_3N, DCM, r.t., 12 h; (e) TFA, DCM, r.t., 2 h.

*Tert-butyl 4-(1,2,4,5-tetrazin-3-yl)benzylcarbamate (**28**)*



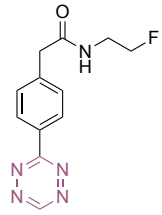
Tz **28** was synthesized as previously described by Qu *et al.*[10]

*2-(4-Cyanophenyl)-N-(2-fluoroethyl)acetamide (**S23**)*



To a solution of 2-(4-cyanophenyl)acetic acid (**S22**, 500 mg, 3.10 mmol) in MeCN (20 mL) was added CDI (650 mg, 4.03 mmol). After stirring at room temperature for 45 min 2-fluoroethylamine hydrochloride (620 mg, 6.20 mmol) was added and the reaction mixture was stirred for 24 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography using EtOAc (70%) in heptane as eluent to afford **S23** (460 mg, 72%) as a white solid. $R_f = 0.35$ (70% EtOAc in heptane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (d, $J = 8.3$ Hz, 2H), 7.34 (d, $J = 8.2$ Hz, 2H), 5.88 (s, 1H), 4.47 (t, $J = 4.8$ Hz, 1H), 4.35 (t, $J = 4.8$ Hz, 1H), 3.58-3.46 (m, 3H), 3.46 (dt, $J = 5.8$, 4.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.6 140.0, 132.6, 130.1, 118.6, 111.3, 82.5 (d, $J = 166.7$ Hz), 43.4, 40.2 (d, $J = 19.5$ Hz). ^{19}F NMR (376 MHz, $CDCl_3$) δ -75.96.

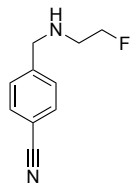
*2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-N-(2-fluoroethyl)acetamide (**29**)*



S23 (0.40 g, 1.94 mmol), CH_2Cl_2 (1.94 mmol, 0.124 mL), sulfur (0.124 g, 0.48 mmol) and ethanol (3.0 mL) were mixed in a 20 mL microwave reaction tube. Hydrazine hydrate (0.75 mL, 15.52 mmol) was added slowly and the mixture was stirred for 5 min. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. CH_2Cl_2 (3 mL) and a solution of sodium nitrite (1.34 g, 19.40 mmol) in H_2O (40 mL) was added to the mixture. Acetic acid (14 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with CH_2Cl_2. The organic layer was dried over

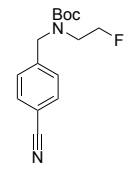
magnesium sulfate ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by column chromatography (30/70 heptane/EtOAc) to yield **29** (0.21 g, 41%) as a pink solid. R_f = 0.22 (Heptane/EtOAc 40/60); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.58 (s, 1H), 8.48 – 8.40 (m, 3H), 7.57 (d, *J* = 8.2 Hz, 2H), 4.51 (t, *J* = 5.0 Hz, 1H), 4.39 (t, *J* = 5.0 Hz, 1H), 3.62 (s, 2H), 3.43 (q, *J* = 5.3 Hz, 1H), 3.36 (q, *J* = 5.2 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.27, 165.92, 158.54, 142.02, 130.61, 130.53, 128.15, 82.90 (d, *J* = 165.1 Hz), 40.57 (d, *J* = 21.2 Hz).

*4-(((2-Fluoroethyl)amino)methyl)benzonitrile (**S25**)*



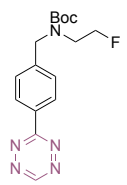
To a solution of 4-(bromomethyl)benzonitrile (**S24**, 780 mg, 4.00 mmol) in MeCN (40 mL) was added K_2CO_3 (332 mg, 24.0 mmol) and 2-fluoroethylamine hydrochloride (159 mg, 16.0 mmol). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was diluted with water (20 mL), extracted with EtOAc. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography using EtOAc (60%) in heptane to afford **S25** (540 mg, 76%) as a colorless oil. R_f = 0.24 (60% EtOAc in heptane). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.55 (d, *J* = 8.2 Hz, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 4.63 – 4.48 (m, 1H), 4.47 – 4.37 (m, 1H), 3.84 (s, 2H), 2.93 – 2.84 (m, 1H), 2.84 – 2.72 (m, 1H), 1.65 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 145.6, 132.3, 128.6, 118.9, 110.9, 83.5 (d, *J* = 165.5 Hz), 53.1, 49.1 (d, *J* = 19.7 Hz).

*Tert-butyl 4-cyanobenzyl(2-fluoroethyl)carbamate (**S26**)*



To a solution of **S25** (540 mg, 3.03 mmol) and Et_3N (1.27 mL, 9.09 mmol) in DCM (40 mL) was added Boc_2O (790 mg, 3.63 mmol) and the mixture was stirred at room temperature for 12 h. The solution was washed with water and saturated K_2CO_3 solution, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography using EtOAc (30%) in heptane to afford **S26** (710 mg, 84%) as a colorless oil (mixture of rotamers). R_f = 0.42 (20% EtOAc in heptane). ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 7.55 (d, *J* = 7.8 Hz, 2H), 7.27 (d, *J* = 7.8 Hz, 2H), 4.79 – 4.10 (m, 4H), 3.62 – 3.28 (m, 2H), 1.96 – 1.05 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 155.4, 144.2, 143.8, 132.4, 128.1, 127.5, 118.7, 111.1, 83.2 (d, *J* = 168.2 Hz), 82.7 (d, *J* = 170.5 Hz), 52.1, 51.2, 47.7, 28.3.

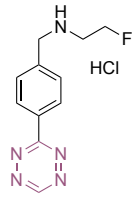
*Tert-butyl 4-(1,2,4,5-tetrazin-3-yl)benzyl(2-fluoroethyl)carbamate (**S27**)*



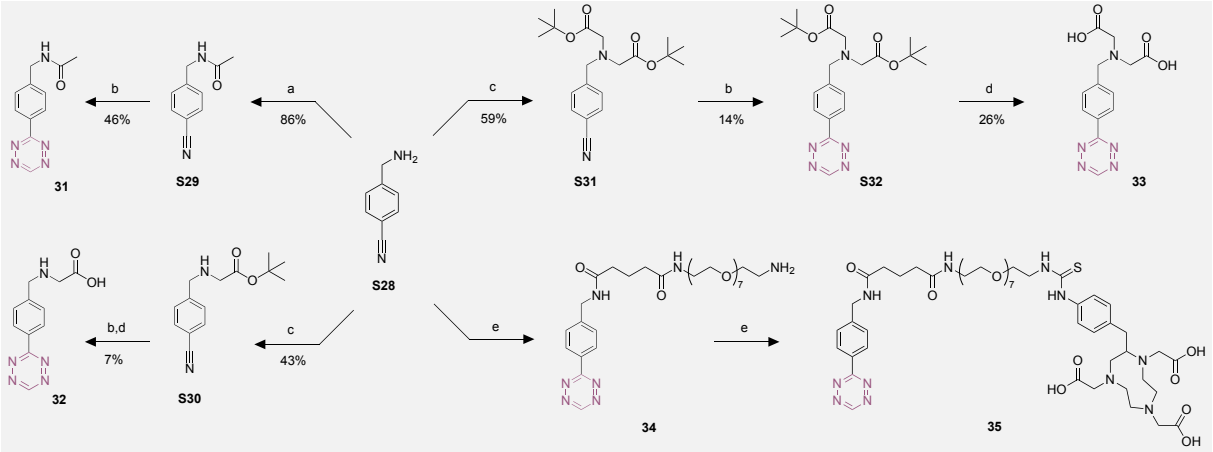
S26 (0.68 g, 2.44 mmol), CH_2Cl_2 (2.44 mmol, 0.156 mL), sulfur (0.156 g, 0.61 mmol) and ethanol (4.0 mL) were mixed together in 3 x 20 ml microwave reaction tubes. Hydrazine monohydrate (0.95 mL, 19.54 mmol) was added slowly and the mixture was stirred for 5 min. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours.

CH_2Cl_2 (3 mL) and a solution of sodium nitrite (1.68 g, 24.42 mmol) in H_2O (60 mL) were added to the mixture. Acetic acid (14 mL) was added slowly during which the solution turned bright red in color. The reaction mixture was extracted with CH_2Cl_2. The organic layer was dried over magnesium sulfate ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography (80/20 heptane/EtOAc) to afford **S27** (0.18, 22%) as a red solid (mixture of rotamers). R_f = 0.21 (heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.23 (s, 1H), 8.62 (d, *J* = 7.8 Hz, 2H), 7.49 (d, *J* = 7.8 Hz, 2H), 4.76 – 4.42 (m, 4H), 3.83 – 3.38 (m, 2H), 1.64 – 1.38 (m, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.32, 157.77, 155.51, 144.25, 132.37, 130.58, 128.55, 127.86, 83.46, 83.17 (d, *J* = 165.4 Hz), 82.67 (d, *J* = 170.4 Hz), 80.63, 52.07, 51.07, 47.49, 28.36.

*N-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)-2-fluoroethanamine hydrochloride (**30**)*

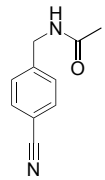


To a solution of **S27** (100 mg, 0.30 mmol) in DCM (10 mL) was added a solution of HCl in dioxane (4.0 M, 3.0 mL). The mixture was stirred at room temperature for 2 h and then concentrated under reduced pressure. The obtained solid was washed with Et_2O to afford **30** (70 mg, 86%) as a pink solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.87 (s, 2H), 8.55 (d, *J* = 8.3 Hz, 2H), 7.89 (d, *J* = 8.3 Hz, 2H), 4.89 (t, *J* = 4.6 Hz, 1H), 4.77 (t, *J* = 4.6 Hz, 1H), 4.35 (s, 2H), 3.38 (t, *J* = 4.7 Hz, 1H), 3.31 (t, *J* = 4.7 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.7, 158.7, 137.0, 132.8, 131.6, 128.4, 80.0 (d, *J* = 165.3 Hz), 50.1, 47.1 (d, *J* = 20.0 Hz).



Scheme S11. Synthesis of Tz **31**-**35**. (a) Acetic anhydride, Et_3N, DCM, r.t., 24 h; (b) (i) S_8, DCM, $N_2H_4 \cdot H_2O$, 50 °C, 24 h (ii) Sodium nitrite, AcOH, 0 °C, 15 min; (c) *tert*-Butyl bromoacetate, Et_3N, MeCN, r.t., 24 h; (d) TFA, DCM, r.t., 2 h; (e) according to Zeglis *et al.*[11]

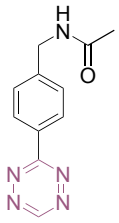
*N-(4-cyanobenzyl)acetamide (**S29**)*



To a solution of 4-(aminomethyl)benzonitrile hydrochloride (**S28**, 640 mg, 4.00 mmol) in DCM (30 mL) was added acetic anhydride (450 µL, 4.40 mmol) and Et_3N (2.23 mL, 16.0 mmol). The mixture was stirred at room temperature for 12 h. The obtained suspension was filtered and the solvent was removed under concentrated pressure. The residue was crystallized from EtOAc to afford **S29** (600 mg, 86%) as a white solid. R_f = 0.31 (40% EtOAc in

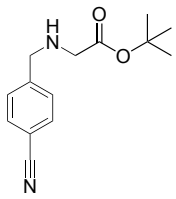
heptane). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.50 (d, J = 6.2 Hz, 1H), 7.79 (d, J = 8.2 Hz, 2H), 7.44 (d, J = 8.2 Hz, 2H), 4.32 (d, J = 6.0 Hz, 2H), 3.12 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.9, 146.1, 132.7, 128.5, 119.4, 109.9, 42.3, 23.0.

*N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)acetamide (**31**)*



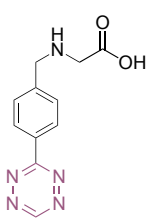
Nitrile **S29** (0.40 g, 2.29 mmol), CH_2Cl_2 (2.29 mmol, 0.15 mL), sulfur (0.15 g, 0.57 mmol) and ethanol (3.0 mL) were mixed in 20 mL microwave reaction tube. Hydrazine monohydrate (0.89 mL, 18.37 mmol). The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. CH_2Cl_2 (3 mL) and a solution of sodium nitrite (1.58 g, 22.96 mmol) in H_2O (30 mL) was added to the mixture. Acetic acid (14 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with dichloromethane. The organic layer was dried over magnesium sulfate ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography (80/20 heptane/EtOAc) to afford **31** (0.240 g, 46%) as a red solid. R_f = 0.20 (heptane/EtOAc 20/80); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (s, 1H), 8.53 – 8.41 (m, 3H), 7.55 (d, J = 8.3 Hz, 2H), 4.40 (d, J = 6.0 Hz, 2H), 1.93 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.8, 165.9, 158.6, 145.4, 130.8, 128.6, 128.3, 42.4, 23.1.

*Tert-butyl 2-((4-cyanobenzyl)amino)acetate (**S30**)*



To a suspension of 4-cyanobenzylamine hydrochloride (**S28**, 670 mg, 4.00 mmol) in DCM (20 mL) was added Et_3N (1.22 mL, 8.80 mmol) and *tert*-butylbromoacetate (640 µL, 20.0 mL). The mixture was stirred at room temperature for 12 h. The mixture was washed with water and the combined organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography using MeOH (2%) in DCM to afford **S30** (420 mg, 43%) as a colorless oil. R_f = 0.31 (40% EtOAC in heptane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.2 Hz, 2H), 3.88 (s, 2H), 3.31 (s, 2H), 1.95 (s, 1H), 1.49 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 145.3, 132.2, 128.8, 118.9, 110.9, 81.5, 52.8, 50.9, 28.1.

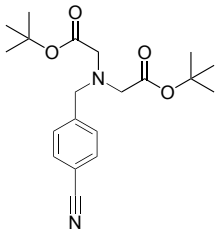
*2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)amino)acetic acid (**32**)*



Nitrile **S30** (0.42 g, 1.70 mmol), CH_2Cl_2 (1.70 mmol, 0.121 mL), sulfur (0.121 g, 0.42 mmol) and ethanol (2.0 mL) were mixed in 3 x 20 mL microwave reaction tubes. Hydrazine monohydrate (0.7 mL, 13.60 mmol) was added slowly. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. CH_2Cl_2 (3 mL) and a solution of sodium nitrite (1.21 g, 17.00 mmol) in H_2O (60 mL) was added to the mixture. Acetic acid (7 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was extracted with dichloromethane. The organic layer was dried over magnesium sulfate

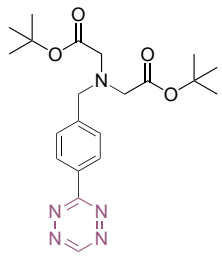
($MgSO_4$), filtered and concentrated under reduced pressure. The resulting residue was dissolved in DCM (3 mL), filtered and treated with TFA (2 mL). After 2 h at r.t. the solvent was removed under reduced pressure and the residue was purified by C18 flash column chromatography using a gradient of MeCN in water with 0.1% trifluoroacetic acid (10→80%) as eluent to afford **32** (20 mg, 7% over 2 steps) as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.24 (s, 0.6H), 10.22 (s, 0.4H), 8.52 (d, *J* = 8.4 Hz, 1.2H), 8.43 (d, *J* = 8.4 Hz, 0.8H), 7.55 (d, *J* = 8.1 Hz, 1.2H), 7.33 (d, *J* = 8.4 Hz, 0.8H), 5.49 (s, 1.2H), 4.92 (s, 0.8H), 4.89 (s, 0.8H), 4.13 (s, 1.2H); ^{13}C NMR (101 MHz, CD_3OD) δ 169.93, 167.35, 166.06, 157.95, 157.89, 139.78, 139.27, 132.23, 131.51, 129.12, 128.86, 128.25, 128.04, 55.74, 52.10, 44.96.

*Di-tert-butyl 2,2'-((4-cyanobenzyl)azanediyl)diacetate (**S31**)*



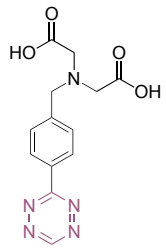
To a suspension of 4-cyanobenzylamine hydrochloride (**S28**, 670 mg, 4.00 mmol) in DCM (20 mL) was added Et_3N (2.78 mL, 20.00 mmol) and *tert*-butylbromoacetate (2.93 mL, 20.0 mL). The mixture was stirred at room temperature for 12 h, thereafter washed with water. The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography using MeOH (2%) in DCM to afford **S31** (850 mg, 59%) as a colorless oil. R_f = 0.36 (40% EtOAC in heptane). ^{1}H NMR (600 MHz, $CDCl_3$) *δ* 7.54 (d, *J* = 8.3 Hz, 2H), 7.49 (d, *J* = 8.2 Hz, 2H), 3.90 (s, 2H), 3.34 (s, 4H), 1.39 (s, 18H). ^{13}C NMR (151 MHz, $CDCl_3$) *δ* 170.2, 132.2, 129.5, 119.0, 111.1, 81.4, 57.2, 55.2, 28.2.

*Di-tert-butyl 2,2'-((4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**S32**)*



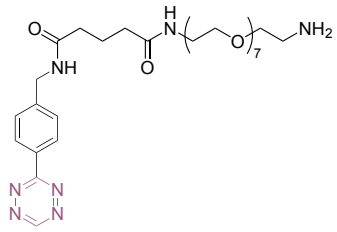
The compound was synthetized according to the literature procedure.[10] **S31** (0.85 g, 2.36 mmol), CH_2Cl_2 (0.151 mL, 2.36 mmol), sulfur (0.151 g, 0.58 mmol) and ethanol (4.0 mL) were mixed in a 20 mL microwave reaction tube. Hydrazine monohydrate (0.92 mL, 18.86 mmol) was added slowly and the mixture was stirred for 5 min. The vessel was sealed and the reaction mixture was heated to 50 °C for 24 hours. CH_2Cl_2 (3 mL) and a solution of sodium nitrite (1.63 g, 23.58 mmol) in H_2O (40 mL) was added to the mixture. Acetic acid (14 mL) was then added slowly during which the solution turned bright red in color. The reaction mixture was basified with saturated $NaHCO_3$ solution and extracted with dichloromethane. The organic layer was dried over magnesium sulfate ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography (85/15 heptane/EtoAc) to yield **S32** (0.14 g, 14%) as a red oil. R_f = 0.33 (heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.50 (d, *J* = 8.4 Hz, 2H), 7.59 (d, *J* = 8.4 Hz, 2H), 3.96 (s, 2H), 3.39 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz $CDCl_3$) δ 170.41, 166.44, 157.71, 144.70, 130.55, 129.84, 128.35, 81.12, 57.29, 55.29, 28.19.

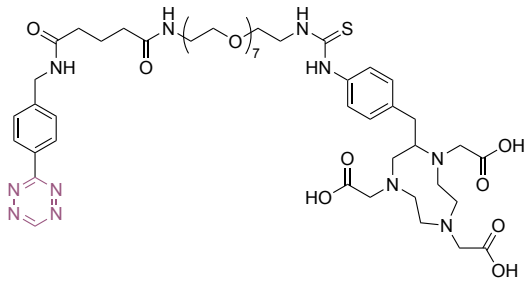
*2,2'-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)azanediyl)diacetic acid (**33**)*



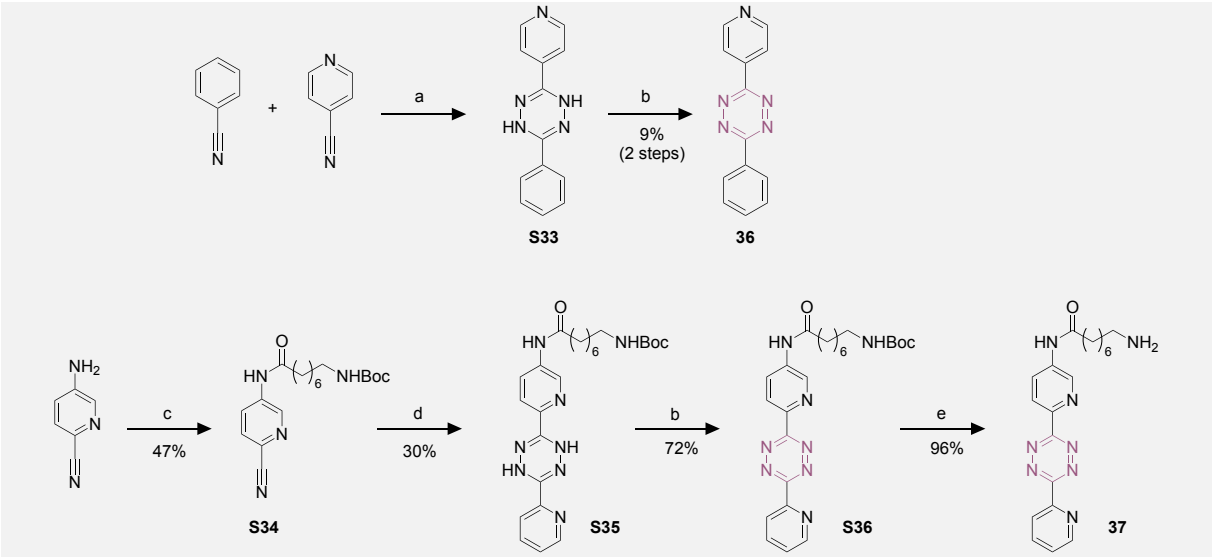
To a solution of **S32** (130 mg, 0.31 mmol) in DCM (5 mL) was added TFA (3 mL). The reaction was stirred at room temperature for 2 h. The solvent was then removed under reduced pressure. The crude product was purified by preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA as eluent afforded **33** (0.035 g, 26%) as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.27 (s, 1H), 8.55 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.4 Hz, 2H), 4.36 (s, 2H), 3.89 (s, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 169.5, 166.0, 158.0, 137.5, 132.9, 131.3, 128.2, 58.0, 53.6.

*N^1-(4-(Tetrazin-3-yl)benzyl)-N^5-(23-amino-3,6,9,12,15,18,21-heptaoxatricosyl)glutaramide (**34**) and 2,2',2''-(2-(4-(3-(1-(4-(tetrazin-3-yl)phenyl)-3,7-dioxo-11,14,17,20,23,26,29-heptaoxa-2,8-diazahentriacontan-31-yl)thioureido)benzyl)-1,4,7-triazonane-1,4,7-triyl)triacetic acid (**35**)*



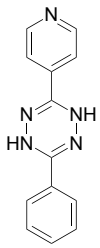


Tz **34** and Tz **35** were synthesized as previously described by Zeglis *et al*.[11]



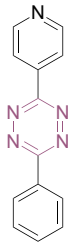
Scheme S12. Synthesis of Tz **36** and **37**. (a) S_8, $N_2H_4 \cdot H_2O$, EtOH, 90 °C, 2 h; (b) PIDA, DCM, r.t., 3 h; (c) 8-((*tert*-Butoxycarbonyl) amino)octanoic acid, HATU, Et_3N, DMF, r.t., 20 h; (d) 2-Cyanopyridine, $N_2H_4 \cdot H_2O$, EtOH, 90 °C, 17 h; (e) TFA, DCM, r.t., 30 min.

*3-Phenyl-6-(pyridin-4-yl)-1,4-dihydro-1,2,4,5-tetrazine (**S33**)*



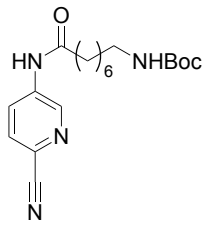
Benzonitrile (1.00 g, 9.70 mmol), 4-cyanopyridine (1.01 g, 9.70 mmol) and sulfur (249 mg, 0.97 mmol) were suspended in EtOH (5 mL), followed by the addition of hydrazine hydrate (4.73 mL, 97.0 mmol). The reaction was heated to 90°C in a sealed vail, under nitrogen atmosphere for 2h. The mixture was cooled to room temperature and concentrated under reduced pressure. The residue was extracted with DCM, washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product **S33** was used in the next step without further purification.

*3-Phenyl-6-(pyridin-4-yl)-1,2,4,5-tetrazine (**36**)*



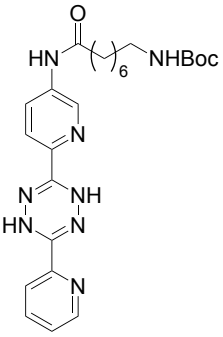
Crude **S33** (2.0 g, 8.43 mmol) was dissolved in anhydrous DCM (150 mL) and the solution was cooled to 0 °C. PIDA (3.26 g, 10.12 mmol) was added in portions over 15 min and the reaction mixture was allowed to reach room temperature, while stirring for 3 h. Celite® was added and the mixture was concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (0→25%) as eluent, followed by re-crystallization from (EtOAc/heptane) to afford **36** (182 mg, 9% over two steps) as red crystals. R_f = 0.39 (50% EtOAC in heptane). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.95–8.91 (m, 2H), 8.70–8.67 (m, 2H), 8.50–8.47 (m, 2H), 7.70–7.66 (m, 1H), 7.66–7.61 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.9, 163.0, 151.1, 139.5, 133.5, 131.5, 129.6, 128.6, 121.4. MS (ESI) m/z = 236.2 $[M + H]^+$.

*tert-Butyl (8-((6-cyanopyridin-3-yl)amino)-8-oxooctyl)carbamate (**S34**)*



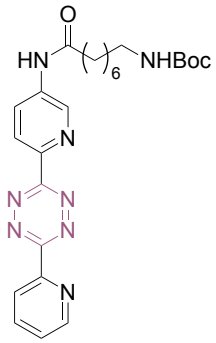
2-Cyanopyridine (1.00 g, 8.39 mmol) and 8-((*tert*-butoxycarbonyl)amino)octanoic acid were dissolved in anhydrous DMF (15 mL). Et_3N (2.34 mL, 16.8 mmol) was added, followed by (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) (3.83 g, 10.1 mmol) and the mixture was stirred for 18 h at room temperature. The mixture was treated with saturated aqueous $NaHCO_3$ solution and extracted with DCM. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (0→40%) as eluent to afford **S34** (1.41 g, 47%) as a white solid. R_f = 0.54 (60% EtOAc in heptane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (s, 1H), 8.72 (d, J = 2.5 Hz, 1H), 8.44 (dd, J = 8.6, 2.6 Hz, 1H), 7.64 (d, J = 8.5 Hz, 1H), 4.65 (s, 1H), 3.08 (q, J = 6.5 Hz, 2H), 2.42 (t, J = 7.5 Hz, 2H), 1.72 (q, J = 7.2 Hz, 2H), 1.43 (s, 15H), 1.38–1.21 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.0, 156.5, 142.1, 138.3, 129.2, 127.4, 126.2, 117.6, 79.5, 40.4, 37.3, 33.2, 29.9, 28.6, 26.3, 25.0. MS (ESI) *m/z* = 361.5 $[M + H]^+$.

*tert-Butyl (8-oxo-8-((6-(6-(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)octyl) carbamate (**S35**)*



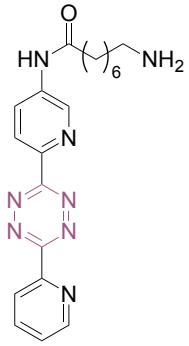
Nitrile **S34** (160 mg, 0.44 mmol) and 2-cyanopyridine (185 mg, 1.78 mmol) were dissolved in EtOH (1.5 mL), followed by the addition of hydrazine hydrate (433 µL, 8.88 mmol). The reaction was heated to 90°C in a sealed vial under nitrogen atmosphere overnight. The mixture was concentrated, suspended in water and extracted with DCM. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography using EtOAc in heptane (20→80%) as eluent to afford **S35** (66 mg, 30%) as a yellow solid. R_f = 0.55 (60% EtOAc in heptane). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, J = 2.5 Hz, 1H), 8.60 – 8.54 (m, 1H), 8.53 (s, 1H), 8.48 (s, 1H), 8.19 (dd, J = 8.7, 2.5 Hz, 1H), 8.08 – 7.97 (m, 2H), 7.75 (td, J = 7.8, 1.7 Hz, 1H), 7.34 (ddd, J = 7.5, 4.9, 1.2 Hz, 1H), 4.54 (s, 1H), 3.11 (q, J = 6.9 Hz, 2H), 2.40 (t, J = 7.4 Hz, 1H), 1.74 (p, J = 7.2 Hz, 1H), 1.53 – 1.45 (m, 3H), 1.44 (s, 9H), 1.40 – 1.25 (m, 6H), 1.28 – 1.22 (m, 1H), 1.14 (dt, J = 25.7, 7.1 Hz, 1H). MS (ESI) *m/z* = 495.4 $[M + H]^+$.

*tert-Butyl (8-oxo-8-((6-(6-(pyridin-2-yl)-tetrazin-3-yl)pyridin-3-yl)amino)octyl)carbamate (**S36**)*

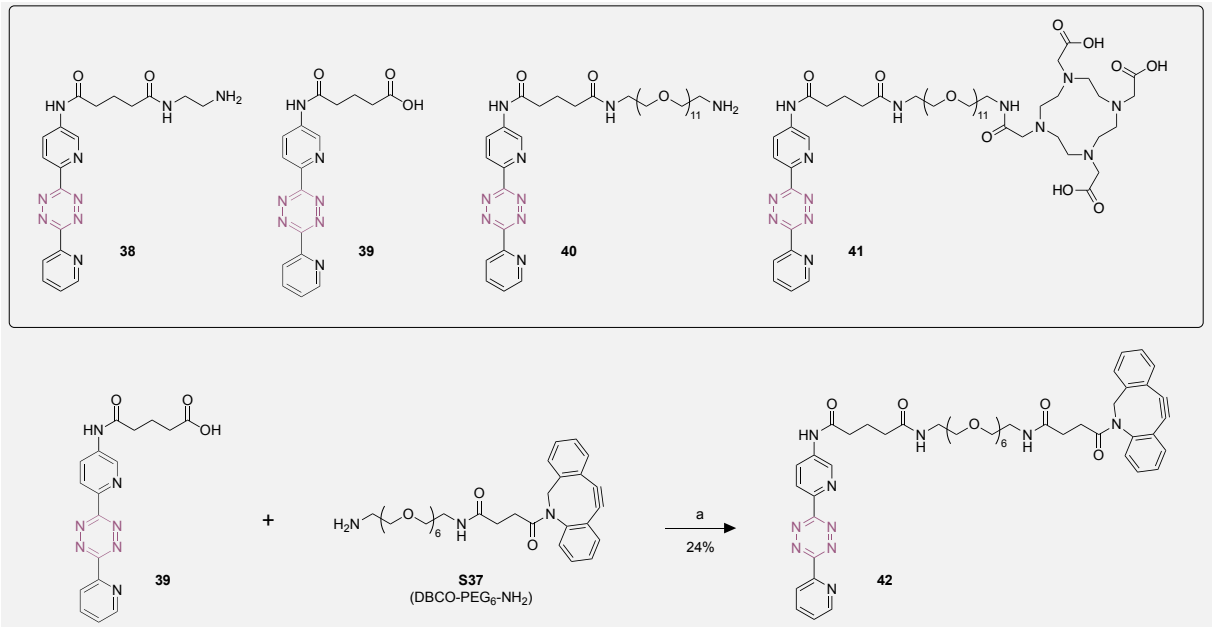


Tz **S35** (60.0 mg, 0.12 mmol) was dissolved in anhydrous DCM (5 mL) and cooled to 0°C, followed by the addition of PIDA (47 mg, 0.15 mmol) in portions over 5 min. The mixture was allowed to reach room temperature, while stirring for 3 h. Celite® was added and the mixture was concentrated. The crude product was purified by flash column chromatography using MeOH in DCM (0→10%) as eluent to afford **S36** (43 mg, 72%) as a red solid. R_f = 0.49 (10% MeOH in DCM). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.91 (dt, J = 4.7, 1.3 Hz, 1H), 8.74 (s, 1H), 8.71 – 8.67 (m, 3H), 7.99 (td, J = 7.8, 1.8 Hz, 1H), 7.56 (ddd, J = 7.7, 4.7, 1.2 Hz, 1H), 4.72 (s, 1H), 3.10 – 3.01 (m, 2H), 2.43 – 2.38 (m, 4H), 1.72 (p, J = 7.4 Hz, 2H), 1.47 – 1.42 (m, 2H), 1.40 (s, 9H), 1.37 – 1.26 (m, 5H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.4, 163.6, 163.3, 156.5, 150.9, 150.1, 143.7, 141.6, 138.9, 137.8, 127.0, 126.7, 125.4, 124.4, 79.4, 40.4, 37.2, 29.9, 28.9, 28.6, 28.5, 26.4, 25.2. MS (ESI) m/z = 493.3 $[M + H]^+$.

*8-Amino-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)octanamide (**37**), TFA salt*

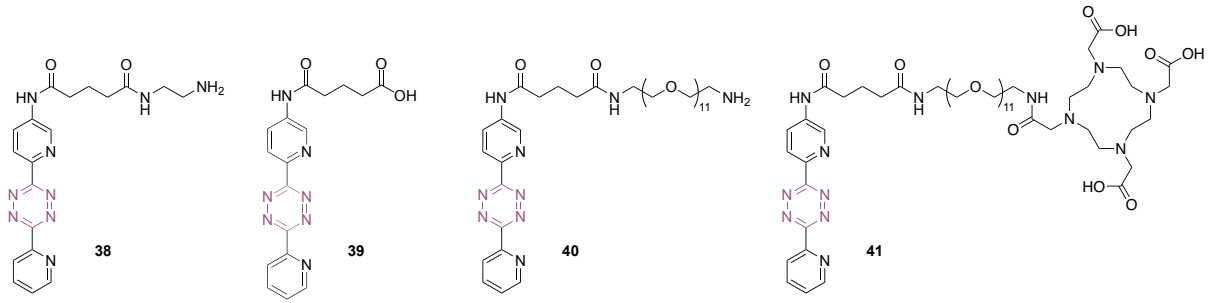


TFA (39 µL, 0.51 mmol) was added dropwise to a solution of **S36** (25.0 mg, 0.05 mmol) in anhydrous DCM (2 mL). The mixture was stirred for 2 h at room temperature. The volatiles were removed under reduced pressure and the crude product was dried in high vacuum to afford the TFA salt of **37** (24.8 mg, 96%) as a red solid. ^{1}H NMR (600 MHz, CD_3OD) δ 9.10 (d, J = 2.5 Hz, 1H), 8.93 (ddd, J = 5.0, 1.7, 0.9 Hz, 1H), 8.86 (dt, J = 8.0, 1.1 Hz, 1H), 8.80 (d, J = 8.7 Hz, 1H), 8.49 (dd, J = 8.7, 2.5 Hz, 1H), 8.31 (tt, J = 7.8, 1.3 Hz, 1H), 7.85 (ddt, J = 7.5, 4.9, 1.1 Hz, 1H), 2.93 (t, J = 7.7 Hz, 2H), 2.51 (t, J = 7.4 Hz, 2H), 1.76 (p, J = 7.5 Hz, 2H), 1.68 (p, J = 7.4 Hz, 2H), 1.48 – 1.41 (m, 7H). ^{13}C NMR (151 MHz, CD_3OD) δ 175.3, 164.3, 160.6, 160.4, 160.1, 159.8, 150.6, 142.3, 128.9, 126.5, 125.9, 117.7, 115.8, 40.7, 37.8, 30.0, 29.9, 28.5, 27.3, 26.3.



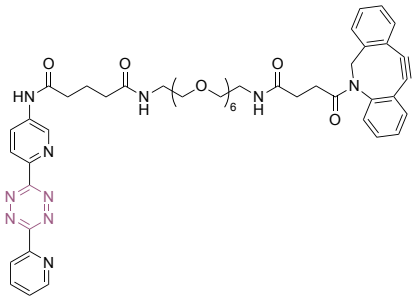
Scheme S13. Synthesis of Tz **38-42**. (a) HATU, NEt_3, then DBCO-PEG_6-NH_2 (**S37**), DMF, r.t., overnight.

*N^1-(2-Aminoethyl)-N^5-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)glutaramide (**38**), 5-Oxo-5-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)pentanoic acid (**39**), N^1-(35-Amino-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)-N^5-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)glutaramide(**40**), 2,2',2''-(10-(2,40,44-trioxo-44-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)-6,9,12,15,18,21,24,27,30,33,36-undecaoxa-3,39-diazatetratetracontyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**41**)*

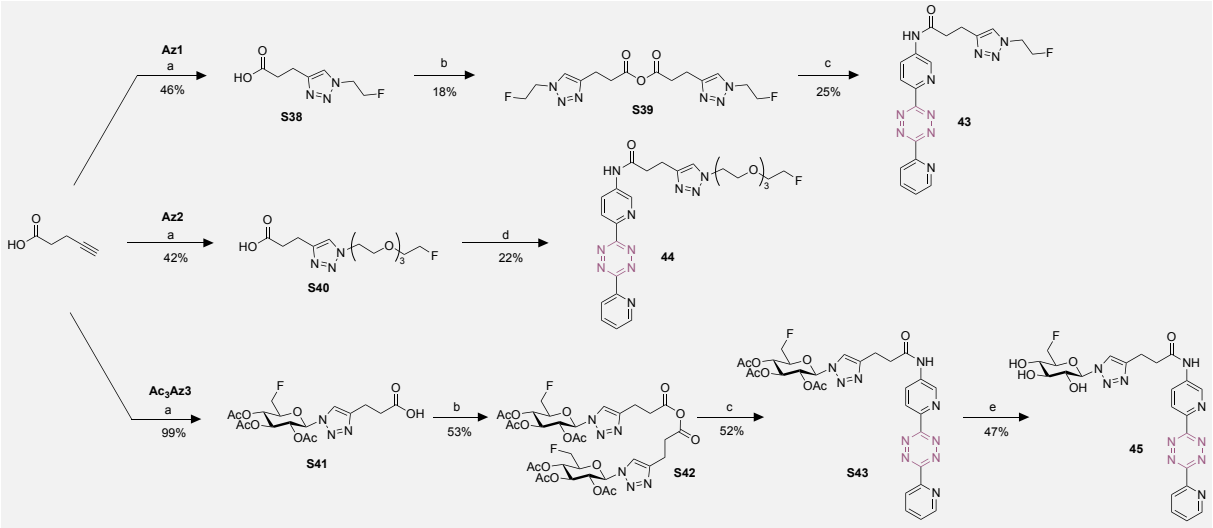


Tz **38**[12] and Tz **39-41**[13] were prepared as previously described.

N^1-(25-(11,12-didehydrodibenzo[b,f]azocin-5(6H)-yl)-22,25-dioxo-3,6,9,12,15,18-hexaoxa-21-azapentacosyl)-N^5-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)glutaramide (**42**)

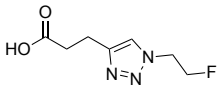


To as suspension of Tz **39** (25.0 mg, 0.07 mmol) in anhydrous DMF (1.2 mL) was added Et_3N (24.0 µL, 0.17 mmol) and HATU (31.0 mg, 0.08 mmol). After 10 min, the solution became clear and after 15 min DBCO-PEG_6-NH_2 (**S37**, 50 mg, 0.08 mmol) was added. The reaction mixture was stirred overnight at room temperature and then concentrated under reduced pressure. Water was added to the residue, followed by extraction with DCM. The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using MeOH in DCM (0→10%) as eluent to afford **42** (16 mg, 24%) as a red, highly viscous, oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (s, 1H), 9.03–8.99 (m, 1H), 8.97 (dd, J = 4.9, 1.7, 1H), 8.74–8.68 (m, 2H), 8.59 (dd, J = 8.7, 2.4, 1H), 7.99 (td, J = 7.8, 1.8, 1H), 7.62 (dd, J = 7.2, 1.6, 1H), 7.56 (ddd, J = 7.6, 4.7, 1.2, 1H), 7.52–7.45 (m, 1H), 7.40–7.33 (m, 3H), 7.32–7.27 (m, 1H), 7.22 (td, J = 6.9, 1.8, 1H), 6.81–6.72 (m, 1H), 6.54 (t, J = 5.4, 1H), 5.12 (d, J = 13.9, 1H), 3.71–3.49 (m, 23H), 3.43 (p, J = 5.0, 4H), 3.30 (q, J = 5.3, 2H), 2.79 (ddd, J = 16.7, 8.1, 6.8, 1H), 2.55–2.40 (m, 3H), 2.34 (t, J = 6.8, 3H), 2.20 (dt, J = 15.4, 6.4, 1H), 2.10–2.01 (m, 2H), 2.01–1.88 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.1, 172.6, 172.3, 172.2, 163.4, 151.3, 151.0, 150.3, 148.1, 143.8, 142.0, 138.7, 137.5, 132.2, 129.3, 128.7, 128.2, 128.1, 127.7, 127.0, 126.6, 126.4, 125.5, 125.2, 124.3, 123.2, 122.4, 114.6, 70.5, 70.4, 70.4, 70.4, 70.4, 70.1, 70.1, 69.8, 69.7, 55.6, 39.3, 39.2, 36.1, 35.0, 31.2, 30.2, 21.5. MS (ESI) m/z = 959.4 $[M + H]^+$.



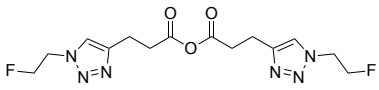
Scheme S14. Synthesis of Tz **43-45**. (a) $CuSO_4 \cdot 5\ H_2O$, sodium ascorbate, BPDS, DMF, r.t., 12-24 h; (b) DCC, DCM, r.t., overnight; (c) Tz **S2** (Pyr-Tz-Pyr-NH_2), pyridine, MW, 130 °C, 45 min; (d) (i) $(COCl)_2$, DCM, 0 °C→r.t., 12 h; (ii) Tz **S2** (Pyr-Tz-Pyr-NH_2), pyridine, MW, 130 °C, 20 min; (e) K_2CO_3, MeOH:DCM, r.t., overnight.

*3-(1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)propanoic acid (**S38**)*



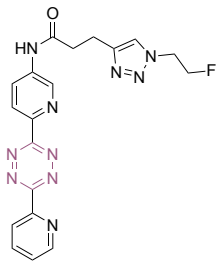
An aqueous 0.33 M $CuSO_4 \cdot 5\ H_2O$ solution (772 µL, 0.25 mmol) and 0.53 M sodium ascorbate solution (1.93 mL, 1.02 mmol) were mixed and after the solution turned yellow a 0.28 M aqueous solution of BPDS (911 µL, 0.26 mmol) was added. The resulting blue/green mixture was added to a solution of pentynoic acid (179 mg, 1.82 mmol) in dry DMF (3 mL). A solution of **Az1** (0.3 mmol) in DMF (0.5 mL) was added and stirring was continued at room temperature for 24 h. The mixture was diluted with water and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford **S38** as light brown crystals (841 mg, 4.5 mmol, 46%). The crude product was directly used in the next step without further purification.

*3-(1-(2-Fluoroethyl)-1H-1,2,3-triazol-4-yl)propanoic anhydride (**S39**)*



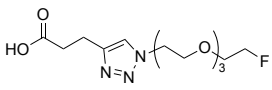
Symmetrical anhydride was synthesized as previously described for anhydride **S3** starting from carboxylic acid **S38** (680 mg, 3.64 mmol) and DCC (375 mg, 1.82 mmol) to afford **S39** (671 mg, 1,88 mmol, 18%) as a yellow oil, which was directly used in the next step without further purification or characterization.

*3-(1-(2-fluoroethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)propenamide (**43**)*



Dry pyridine (3.5 mL) was added to a vial containing Tz **S2** (69.0 mg, 0.28 mmol) and anhydride **S39** (391 mg, 1.10 mmol). The mixture was heated by MW-irradiation for 45 min to 130 °C. The solvent was removed, and the crude product was purified by preparative HPLC to afford **43** (29 mg, 25%) as a pink solid. R_f = 0.34 (10% MeOH in DCM). ^{1}H NMR (600 MHz, DMSO) δ 10.65 (s, 1H), 9.06 (d, *J* = 2.5 Hz, 1H), 9.02 – 8.88 (m, 1H), 8.62 (d, *J* = 8.6 Hz, 1H), 8.59 (d, *J* = 7.9 Hz, 1H), 8.43 (dd, *J* = 8.6, 2.4 Hz, 1H), 8.16 (td, *J* = 7.7, 1.8 Hz, 1H), 7.93 (s, 1H), 4.84 (t, *J* = 4.7 Hz, 1H), 4.76 (t, *J* = 4.7 Hz, 1H), 4.70 (t, *J* = 4.7 Hz, 1H), 4.65 (t, *J* = 4.7 Hz, 1H), 3.02 (t, *J* = 7.4 Hz, 2H), 2.84 (t, *J* = 7.5 Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 171.84, 163.52, 163.24, 151.06, 150.67, 146.31, 144.34, 141.68, 138.80, 138.26, 127.04, 126.56, 125.37, 124.66, 122.96, 82.44 (d, *J* = 168.2 Hz), 50.33 (d, *J* = 19.8 Hz), 36.09, 21.15. ^{19}F NMR (376 MHz, DMSO) δ 15.31.

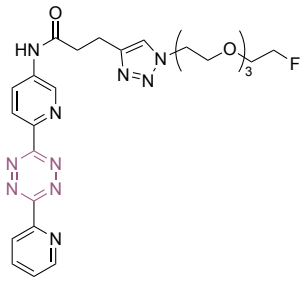
*3-(1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)propanoic acid (**S40**)*



Az2 (137 mg, 0.62 mmol) was dissolved in water/*tert*-BuOH (6 mL, 1:1). 4-Pentynoic acid (67.0 mg, 0.68 mmol), $Cu(OAc)_2$ (22.5 mg, 0.12 mmol) and sodium ascorbate (50.0 mg, 0.25 mmol) were added. The mixture was stirred at room temperature overnight (12 h). Water and EtOAc were added and the layers were separated. The aqueous layer was extracted with EtOAc and the combined organic layer was washed with brine, dried over $MgSO_4$,

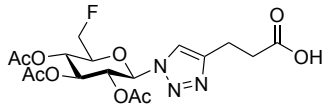
filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel using EtOAc in heptane (30%) with 1% AcOH, follwed by EtOH in EtOAc (10%) with 1% AcOH as eluent to afford **S40** (82.4 mg, 42%) a colorless oil. R_f = 0.44 (10% EtOH in EtOAc with 1% AcOH).^{1}H NMR (600 MHz, $CDCl_3$) δ 7.57 (s, 1H), 4.61–4.57 (m, 1H), 4.53–4.50 (m, 1H), 4.48 (t, J = 5.0 Hz, 2H), 3.83 (t, J = 5.0 Hz, 2H), 3.78–3.75 (m, 1H), 3.73–3.70 (m, 1H), 3.70–3.65 (m, 2H), 3.66–3.54 (m, 6H), 2.99 (t, J = 7.0 Hz, 2H), 2.64 (t, J = 7.0 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 177.2, 146.9, 122.8, 83.3 (d, J = 168.4 Hz), 70.8–70.4, 69.7, 50.3, 34.8, 21.4.^{19}F NMR (376 MHz, $CDCl_3$) δ -62.42. HRMS *m/z* (MALDI-TOF) calculated for $C_{13}H_{22}FN_3O_5^+$: 320.1616, found: 320.1616 $[M+H]^+$.

*3-(1-(2-(2-(2-(2-Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)propanamide (**44**)*



Oxalyl chloride was added drop wise to a solution of **S40** (71 mg, 0.2 mmol) in dry DCM (2 mL) at 0 °C. The mixture was stirred under nitrogen atmosphere overnight (12 h), while slowly reaching room temperature. The solvent was removed and additional dry DCM (2×2 mL) was added and subsequently evaporated to remove excess oxalyl chloride. A solution of Tz **S2** (42 mg, 0.2 mmol) in dry pyridine (2 mL) was added to the residue and the mixture was heated by microwave irradiation to 130 °C for 20 min. The solvent was evaporated and the residue was purified by flash column chromatography on silica gel using MeOH in DCM (0→10%) to afford **44** (20 mg, 22%) as a purple solid. R_f = 0.11 (5% MeOH in DCM).^{1}H NMR (600 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.06 (d, J = 2.5 Hz, 1H), 8.94 (ddd, J = 4.6, 1.5, 0.9 Hz, 1H), 8.62 (d, J = 8.6 Hz, 1H), 8.59 (dt, J = 7.9, 1.1 Hz, 1H), 8.43 (dd, J = 8.7, 2.5 Hz, 1H), 8.16 (td, J = 7.7, 1.8 Hz, 1H), 7.86 (s, 1H), 7.73 (ddd, J = 7.6, 4.7, 1.2 Hz, 1H), 4.55–4.52 (m, 1H), 4.50–4.43 (m, 3H), 3.79 (t, J = 5.3 Hz, 2H), 3.67–3.63 (m, 1H), 3.62–3.58 (m, 1H), 3.57–3.41 (m, 8H), 3.00 (t, J = 7.4 Hz, 2H), 2.82 (t, J = 7.5 Hz, 2H). ^{13}C NMR (151 MHz, DMSO) δ 171.45, 163.0, 162.8, 150.6, 150.2, 145.4, 143.8, 141.2, 138.4, 137.8, 126.6, 126.1, 124.9, 124.2, 122.4, 83.0 (J = 165.7 Hz), 69.8–69.5, 68.8, 49.2, 35.7, 20.7. HRMS *m/z* (MALDI-TOF) calculated for $C_{25}H_{29}FN_{10}O_4^+$: 553.2429, found: 553.2454 $[M+H]^+$.

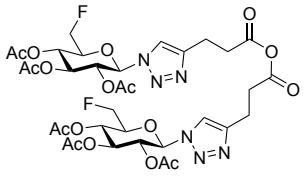
*3-(1-((2R,3R,4S,5S,6S)-3,4,5-Triacetoxy-6-(fluoromethyl)tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)propanoic acid (**S41**)*



An aqueous 0.33 M $CuSO_4 \cdot 5\ H_2O$ solution (277 µL, 0.09 mmol) and 0.53 M sodium ascorbate solution (689 µL, 0.36 mmol) were mixed, when the solution turned yellow a 0.22 M aqueous solution of BPDS (415 µL, 0.09 mmol) was added. The resulting blue/green mixture was added to a solution of pentynoic acid (179 mg, 1.82 mmol) in anhydrous DMF (0.5 mL). A solution of **Ac$_3$Az3** (674 mg, 2.02 mmol) in DMF (0.5 mL) was added and the mixture was stirred at room temperature for 24 h, thereafter diluted with

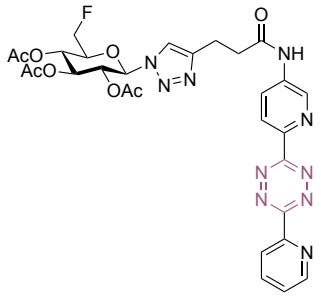
water and extracted with EtOAc. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford **S41** as a colorless oil (780 mg, 99%). The crude product was directly used in the next step without further purification.

*3-(1-((2R,3R,4S,5S,6S)-3,4,5-Triacetoxy-6-(fluoromethyl)tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)propanoic anhydride (**S42**)*



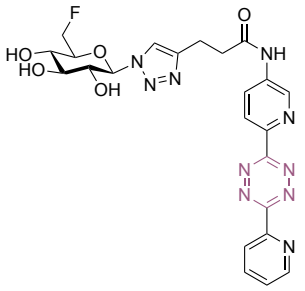
The symmetrical anhydride was synthesized as previously described for anhydride **S3** starting from carboxylic acid **S41** (792 mg, 1.84 mmol) and DCC (190 mg, 0.92 mmol) to afford **S42** (819 mg, 53%) as a white foam, which was directly used in the next step without further purification.

*(2S,3S,4S,5R,6R)-2-(Fluoromethyl)-6-(4-(3-oxo-3-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)propyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**S43**)*



Anhydrous pyridine (2.5 mL) was added to a vial containing Tz **S2** (45.0 mg, 0.18 mmol) and anhydride **S42** (600 mg, 0.71 mmol). The mixture was heated by microwave irradiation to 130 °C for 45 min. The solvent was removed and the crude product was purified by flash column chromatography on silica gel using MeOH (5%) in DCM as eluent to afford **S43** (62 mg, 52%) as a pink solid. R_f = 0.44 (10% MeOH in DCM) ^{1}H NMR (600 MHz, DMSO) δ 10.64 (s, 1H), 9.05 (d, *J* = 2.5 Hz, 1H), 8.94 (ddd, *J* = 4.7, 1.7, 0.9 Hz, 1H), 8.62 (d, *J* = 8.6 Hz, 1H), 8.59 (d, *J* = 7.9 Hz, 1H), 8.42 (dd, *J* = 8.7, 2.5 Hz, 1H), 8.21 (s, 1H), 8.16 (td, *J* = 7.8, 1.8 Hz, 1H), 7.73 (ddd, *J* = 7.6, 4.7, 1.2 Hz, 1H), 6.33 (d, *J* = 9.2 Hz, 1H), 5.63 (t, *J* = 9.4 Hz, 1H), 5.56 (t, *J* = 9.5 Hz, 1H), 5.27 – 5.08 (m, 1H), 4.55 – 4.49 (m, 1H), 4.44 – 4.38 (m, 0H), 3.01 (t, *J* = 7.4 Hz, 2H), 2.82 (td, *J* = 7.4, 2.1 Hz, 2H), 2.05 (s, 4H), 1.96 (s, 3H), 1.77 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 171.24, 169.57, 169.26, 168.37, 163.02, 162.75, 150.56, 150.16, 146.37, 143.84, 141.28, 138.43, 137.81, 126.57, 126.19, 124.87, 124.18, 121.14, 86.22, 83.72, 80.90 (d, *J* = 171.5 Hz), 73.85 (d, *J* = 18.3 Hz), 72.30, 70.00, 66.61 (d, *J* = 7.1 Hz), 35.49, 20.41, 20.25, 19.85; ^{19}F NMR (376 MHz, DMSO) *δ* -74.78.

*3-(1-((2R,3R,4S,5S,6S)-6-(Fluoromethyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)propanamide (**45**)*



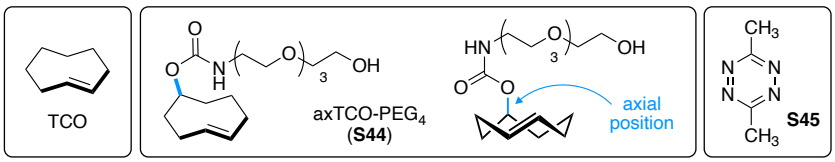
A 0.0145 M solution of K_2CO_3 (466.0 µL, 0.007 mmol) in dry MeOH was added to Tz **S43** (44 mg, 0.07 mmol) dissolved in anhydrous MeOH/DCM (1:1, 2 mL). The mixture was stirred at room tempearture under nitrogen atmosphere overnight. The solvent was removed and the residue was purified by preparative HPLC using a gradient (10→90%) of MeCN in water with 0.1% TFA to afford **45** (18 mg, 47%) as a pink solid. ^{1}H NMR (600 MHz, DMSO) δ 10.66 (s, 1H), 9.06 (d, *J* = 2.5 Hz, 1H), 8.94 (dt, *J* = 4.7, 1.4 Hz, 1H), 8.63 (d, *J* = 8.6 Hz, 1H), 8.44 (dd, *J* = 8.7, 2.5 Hz, 1H), 8.16 (td, *J* = 7.7, 1.8 Hz, 1H), 7.73 (ddd, *J* = 7.6, 4.7, 1.2 Hz, 1H), 5.59 (d, *J* = 9.3 Hz, 1H), 4.85 – 4.35 (m, 2H), 3.78 (t, *J* = 9.1 Hz, 1H), 3.43 (t, *J* = 8.9 Hz, 1H), 3.30 (dd, *J* = 10.0, 8.9 Hz, 1H), 3.03 (t, *J* = 7.5 Hz, 2H), 2.86 (dd, *J* = 8.3, 6.7 Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 171.93, 163.52, 163.25, 151.06, 150.67, 146.14, 144.35, 141.78, 138.93, 138.29, 127.05, 126.70, 125.38, 124.67, 121.69, 87.58, 82.76 (d, *J* = 169.8 Hz), 77.61 (d, *J* = 17.4 Hz), 77.20, 72.34, 68.82 (d, *J* = 6.8 Hz), 35.98, 21.14; ^{19}F NMR (376 MHz, DMSO) *δ* -74.09.

2. Reaction kinetics and physicochemical properties

Reaction kinetics

Reactivities of the Tz-scaffolds A-L in the IEDDA reaction with TCO were determined by pseudo-first order measurements in dioxane at 25 °C and in Dulbecco's phosphate buffered saline (DPBS) at 37 °C by stopped flow spectrophotometry.

Solutions of TCO[14] in anhydrous 1,4-dioxane and axTCO-PEG$_4$ (**S44**) in DPBS (10 mM) were prepared at an approximate concentration above 2 mM. The exact concentration was determined by absorbance titration with 3,6-dimethyltetrazine (**S45**)[15] (extinction coefficient 510 M^{-1}cm^{-1} at 520 nm), quantifying the decrease in tetrazine absorbance upon reaction with TCO or **S44**. These initial stock solutions were diluted before stopped-flow analysis to reach a final TCO concentration of 2 mM.



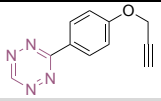
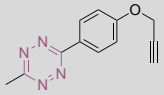
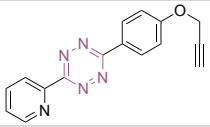
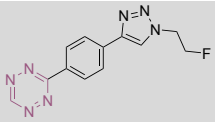
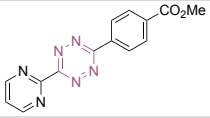
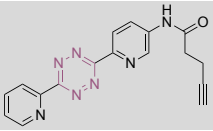
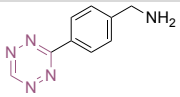
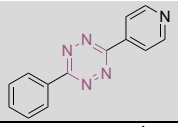
Stock solutions of tetrazines representative for the different Tz-scaffolds A-L (see manuscript, Table 1 and below, Table S1 and Table S2) were prepared in DMSO at a concentration of 10 mM. Serial dilution into 1,4-dioxane (TCO) or DPBS (**S44**) was used to prepare solutions for stopped-flow analysis at a Tz concentration of 100 µM.

Stopped-flow measurements were performed using an SX20-LED stopped-flow spectrophotometer (Applied Photophysics) equipped with a 535nm LED (optical pathlength 10mm, full width half-maximum 34nm) to monitor the characteristic tetrazine visible light absorbance (520-540 nm). The

reagent syringes were loaded with solutions of the Tz and TCO or **S44** and the instrument was primed. Subsequent data were collected in triplicate to sextuplicate for each tetrazine. Reactions were conducted at 25 °C (1,4-dioxane) or 37 °C (DPBS) and recorded automatically at the time of acquisition.

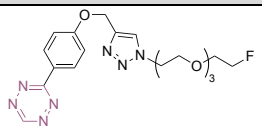
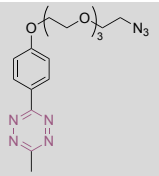
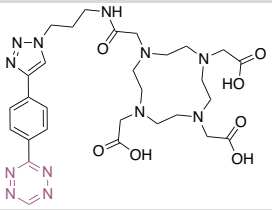
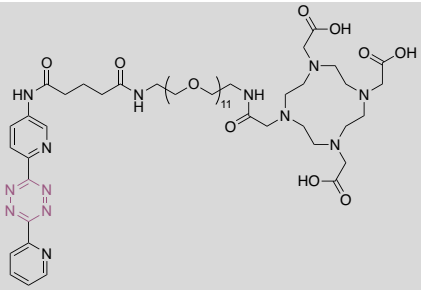
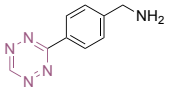
Data sets were analyzed by fitting an exponential decay using Prism 6 (Graphpad) to calculate the observed pseudo-first order rate constants that were converted into second order rate constants by dividing through the concentration of excess TCO compound.

Table S1. Second order rate constants for the reaction of selected Tz with TCO in 1,4-dioxane (25 °C).

Tz	**Structure**	**represented Tz-scaffolds**	**Second order rate constant** ($M^{-1} s^{-1}$)[a] [TCO, 1,4-dioxane, 25 °C]
IV		A	72 ± 5
VI		B, C	1.4 ± 0.1
V		D	13 ± 1
17		E	200 ± 14
S46[16]		F	83 ± 6
I		G, K, L	230 ± 16
S47[b]		H	210 ± 15
36		I	10 ± 0.5

[a] n ≥ 3, data is shown as mean ± S.D.; [b] obtained from Click Chemistry Tools.

Table S2. Second order rate constants for the reaction of selected Tz with axTCO-PEG$_4$ (**S45**) in DPBS (37 °C).

Tz	Structure	represented Tz-scaffolds	Second order rate constant (M^{-1} s^{-1})[a] [TCO, DPBS, 37 °C]
2		A	39,000 ± 2,500
S10		B, C	1,100 ± 60
20		E	60,000 ± 5,600
41		G, K, L	73,000 ± 5,500
S47[b]		H	58,000 ± 4,000

[a] n ≥ 3, data is shown as mean ± S.D.; [b] obtained from Click Chemistry Tools.

Physicochemical properties (clog$D_{7.4}$, TPSA)

Calculations of distribution coefficients at physiological pH (clog$D_{7.4}$) and topological polar surface areas (TPSA) were performed using the software Chemicalize (ChemAxon).

3. Blocking studies

Tumor xenografts in mice

All animal studies were approved by the Danish Animal Welfare Council, ministry of Justice. Five weeks old female nude BALB/c mice (Charles River, Sulzfeld, Germany) were allowed to acclimatize for one week with access to water and chow ad libitum. Human colon cancer cell line (LS174T; ATCC) was cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% *L*-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, and 1% penicillin-streptomycin at 37 °C and 5% CO_2. Cells were harvested by trypsinization at a confluence of 70–90% and subcutaneous tumors were established in the left flank of the animals by inoculation of ~5 × 10^6 LS174T cells resuspended in sterile PBS (100 µL) and allowed to grow for 7–10 days. Tumors were measured using a caliper and the volume was calculated using the formula *Volume = ½(Length x Width^2)*.

Blocking experiments

Tumor-bearing animals were matched in groups based on their tumor volume (tumor volumes of ~ 100–300 mm^3, n = 3 in each group) and were administered 100 µg/100 µL of CC49-TCO (100 µg/100 µL, ~7 TCO/mAb, axially linked TCO tags, axTCO, as described by Rossin *et al.*[17]) per mouse. After 3 days, animals were injected with non-radioactive Tz (39 nmol). After 1 h, [^{111}In]**46** (5-10 MBq/100 µL, 3.9 nmol) was administered via the tail vein. Tz [^{111}In]**46** was prepared as previously described.[13] The mice were euthanized after 22 h and tumor, blood, heart, lung, liver, spleen, kidney, and muscle were resected. All tissues were weighted and the radioactivity measured in a gamma counter (Wizard2, Perkin Elmer). Data was corrected for decay, tissue weight and injected amount of radioactivity. As a no-blocking control, a group of mice were exclusively injected with [^{111}In]**46** without any previous administration of Tz. This was used as a reference and the change in tumor uptake of [^{111}In]**46** due to blocking by the pre-administered Tz was normalized to this group of animals. In every blocking series, Tz **41**, the precursor for the synthesis of [^{111}In]**46,** was included as a positive control. Figure S1 illustrates the workflow of the blocking assay (cf. Fig. 2A in the manuscript).

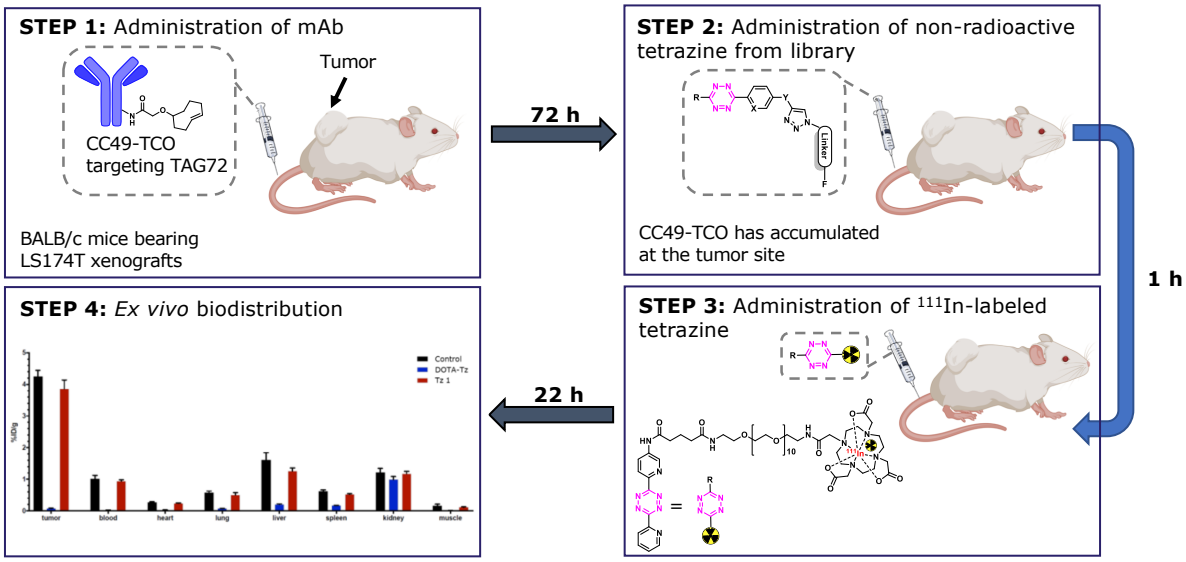


Figure S1. Workflow of the blocking assay. Tumor-bearing mice were first injected with CC49-TCO, 72 h before administration of the non-radioactive Tz. After 1 h, [^{111}In]**46** was injected and the *ex vivo* biodistribution was determined 22 h p.i., in order to measure the blocking effect of the non-radioactive Tz.

Ex vivo biodistribution data for all evaluated tetrazines (**1–45**) is shown in Figures S2-S12.

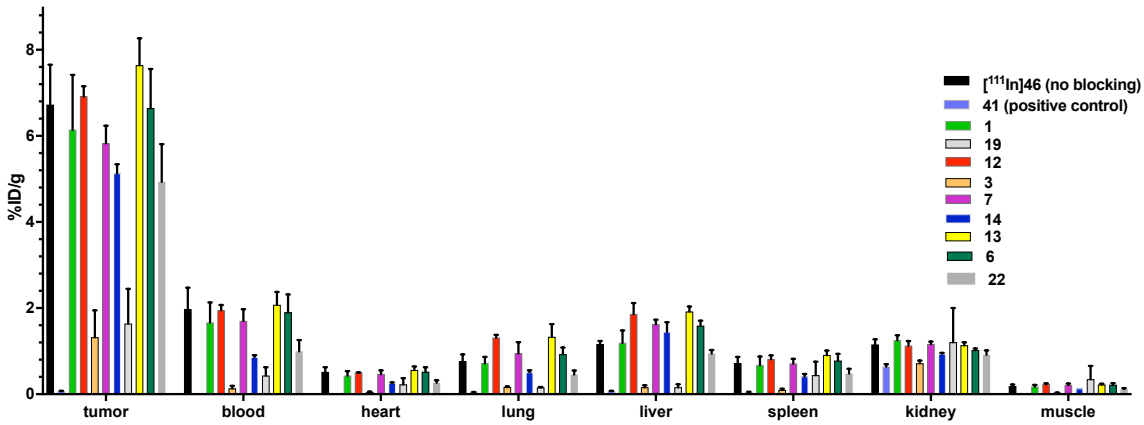


Figure S2. *Ex vivo* biodistribution after blocking (series 1).

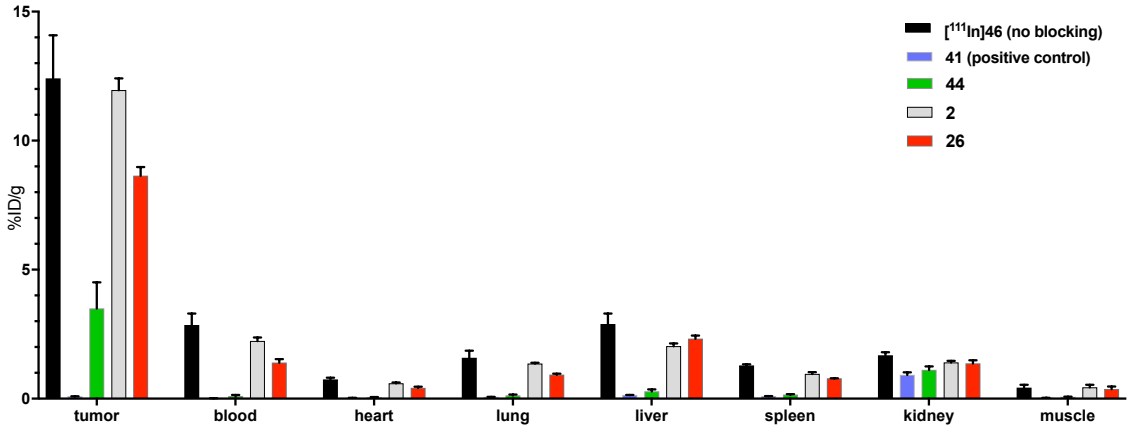


Figure S3. *Ex vivo* biodistribution after blocking (series 2).

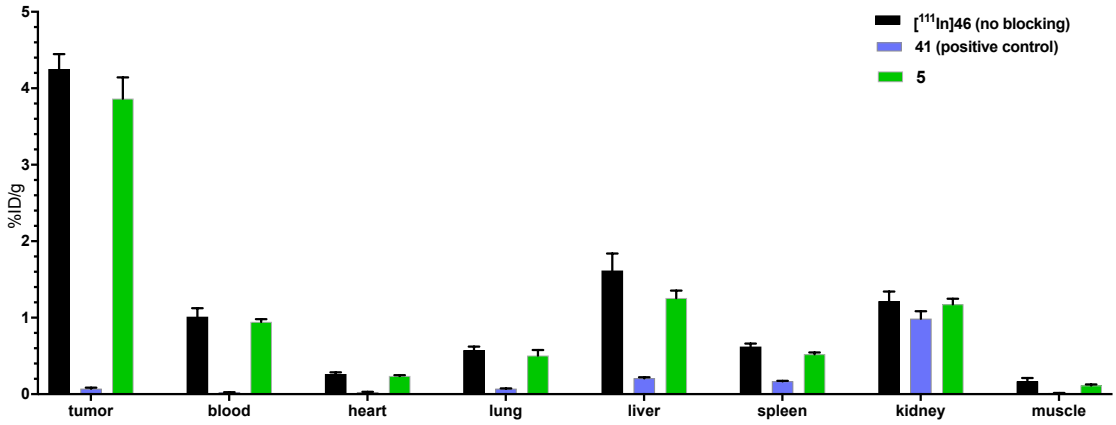


Figure S4. *Ex vivo* biodistribution after blocking (series 3).

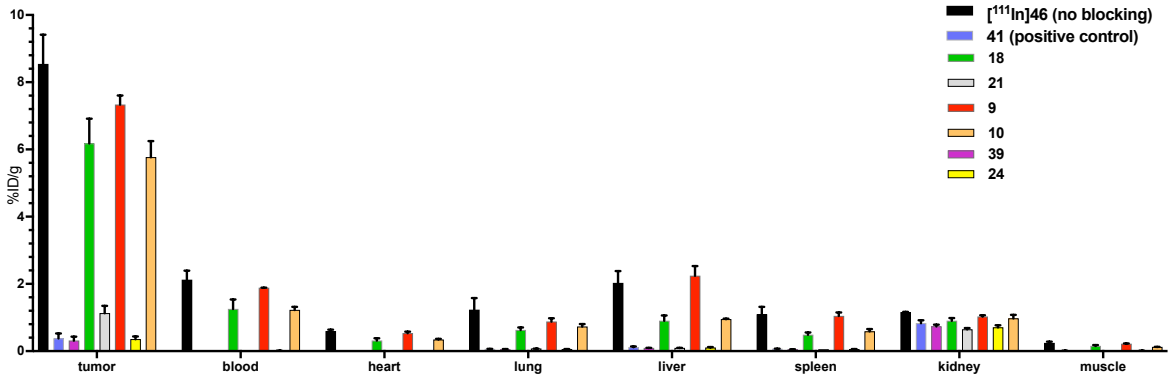


Figure S5. *Ex vivo* biodistribution after blocking (series 4).

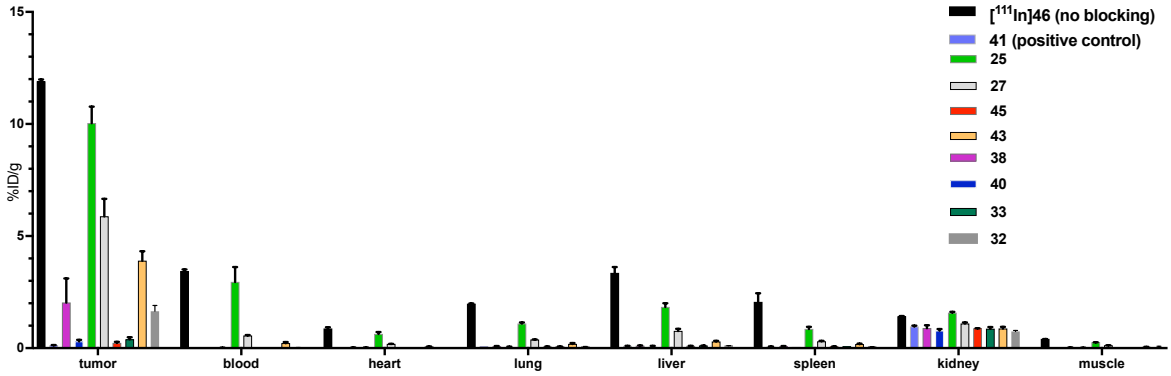


Figure S6. *Ex vivo* biodistribution after blocking (series 5).

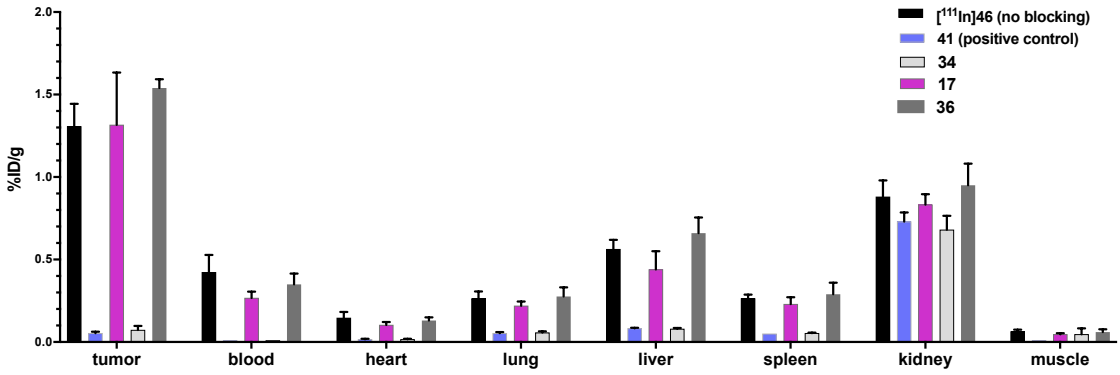


Figure S7. *Ex vivo* biodistribution after blocking (series 6).

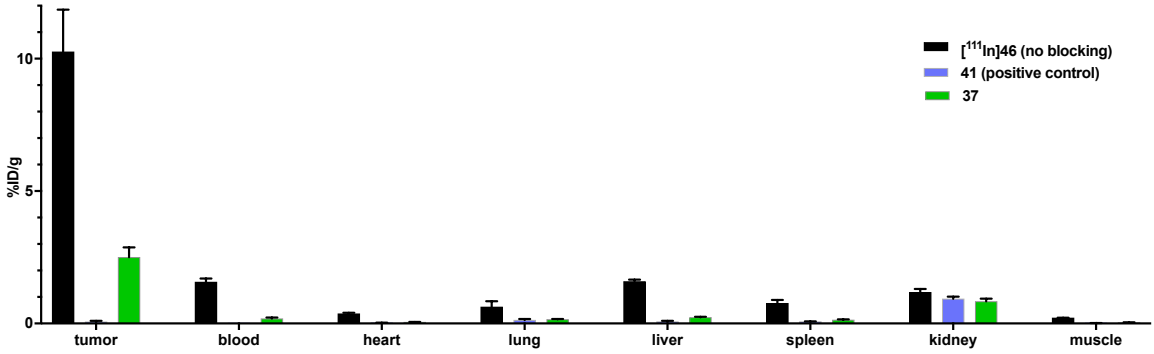


Figure S8. *Ex vivo* biodistribution after blocking (series 7).

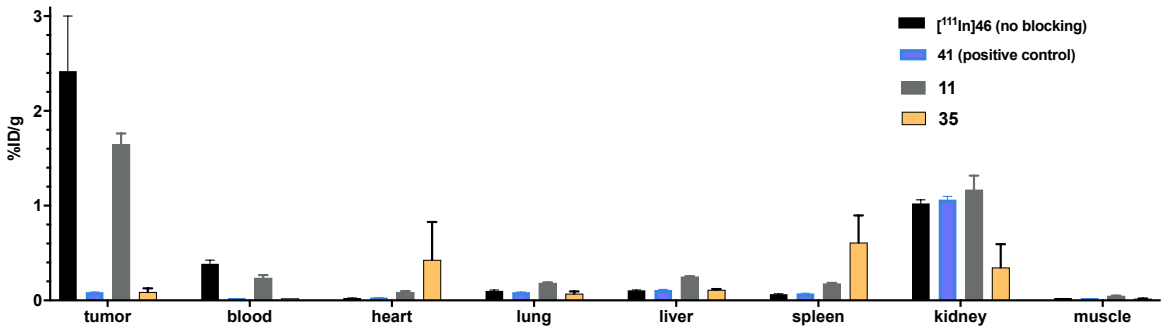


Figure S9. *Ex vivo* biodistribution after blocking (series 8).

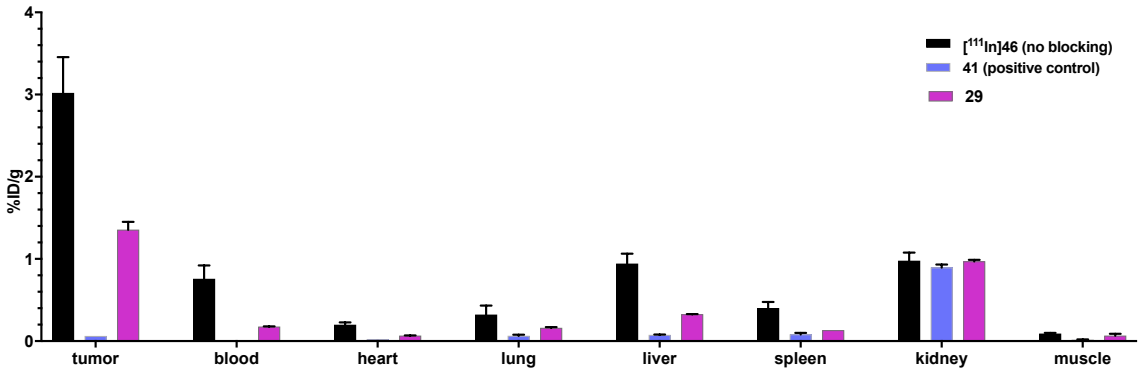


Figure S10. *Ex vivo* biodistribution after blocking (series 9).

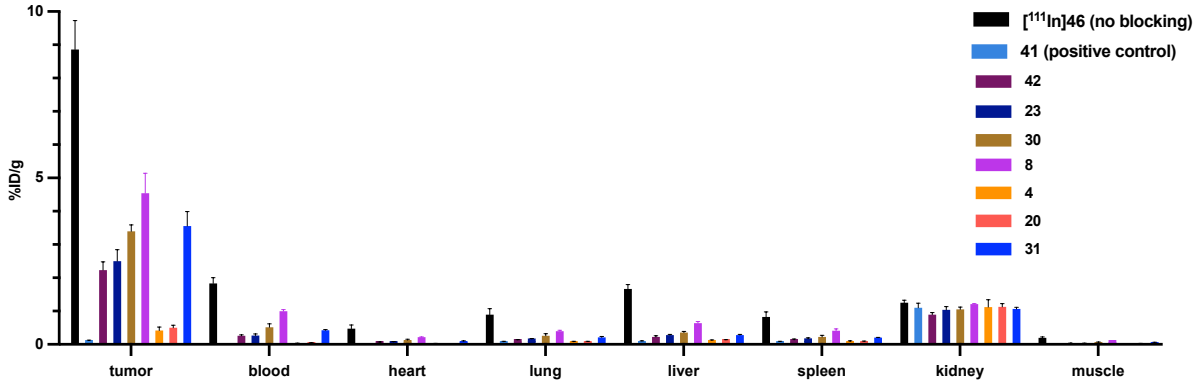


Figure S11. *Ex vivo* biodistribution after blocking (series 10).

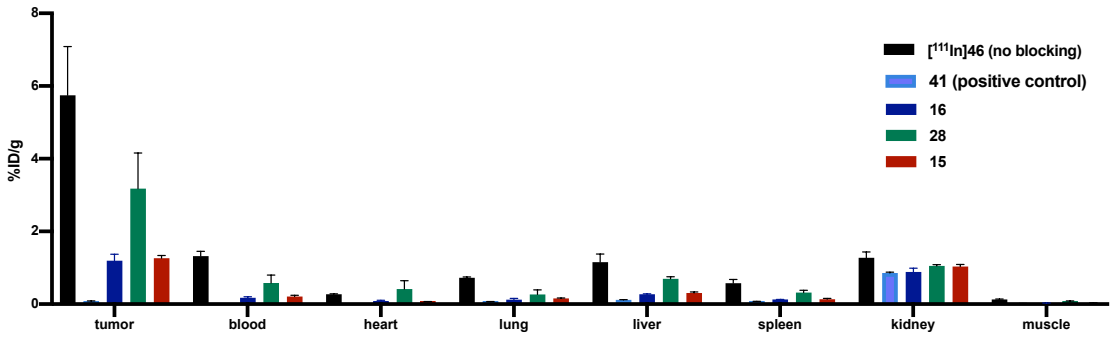


Figure S12. *Ex vivo* biodistribution after blocking (series 11).

*Investigation of different ratios between [^{111}In]**46** and non-radioactive Tz*

In order to find an appropriate ratio between non-radioactive Tz and [^{111}In]**46** to achieve blocking effect, studies were carried out using different ratios of [^{111}In]**46** to the non-radioactive DOTA-Tz **41**. Figure S13 shows the *ex vivo* biodistribution of [^{111}In]**46** after mice have been injected with **41** 2 h earlier. A significant blocking effect was observed when using a 1:10 ratio of [^{111}In]**46** to **41**. Increasing to ratios of 1:20 and 1:40 resulted in similar blocking.

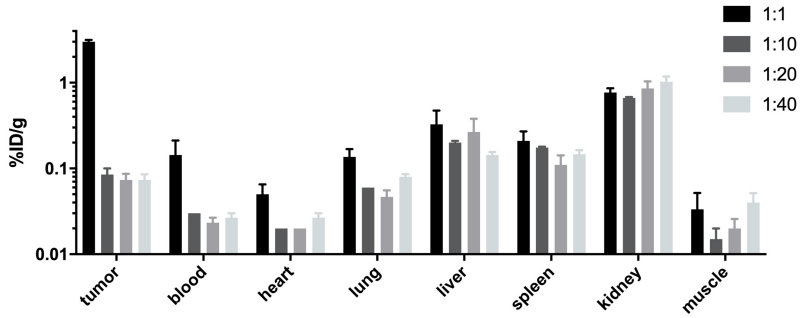


Figure S13. *Ex vivo* biodistribution of [^{111}In]**46** in LS174T-xenografted mice after pre-administration of the non-radioactive precursor, **41** in different ratios. $n = 4$ in each group. Data represents mean ± S.E.M

Blocking effect vs. topological polar surface area

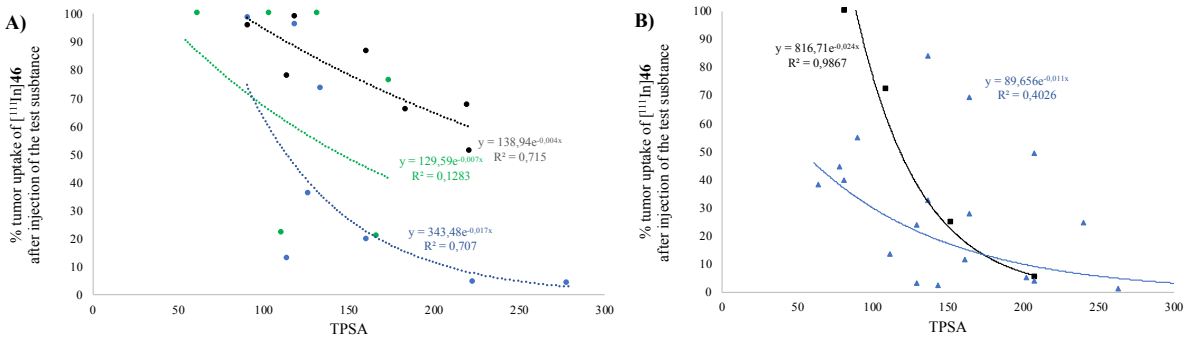


Figure S14. Correlation diagram between TPSA and blocking effect for all Tz-derivatives (**1**-**45**) evaluated in the blocking assay. Data points are colored according to the Tz-scaffold (A-L). **A**) blue = A, F; green = D, I; black = B, C. **B**) blue = G, L, K; green = H; black = E.

Blocking effect vs. clog$D_{7.4}$

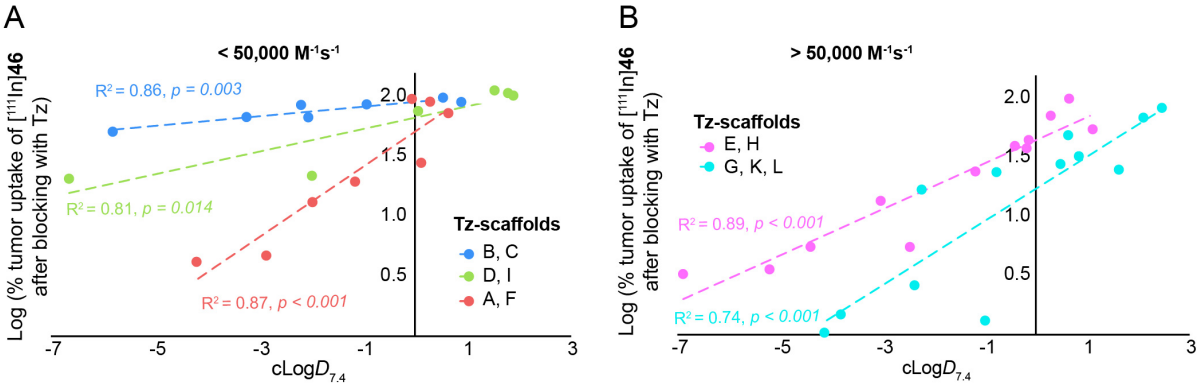


Figure S15. (A and B) Correlation of blocking effect (log transformed) and clog$D_{7.4}$ for Tz-derivatives with similar IEDDA reactivity (see Table 1). Data was fitted using linear regression (dotted line). R^2 and *p*-values describes the goodness of fit.

4. Radiochemistry

General information

Radiochemistry was performed at two different institutes:

Department of Clinical Physiology, Nuclear Medicine & PET, Rigshospitalet, Denmark: [^{18}F]Fluoride was produced via the (p,n)-reaction in a cyclotron (60 mikroA CTI Siemens or 40 mikroA Scanditronix) by irradiating [^{18}O]H_2O with a 11 MeV (CTI siemens) or 16 MeV (Scanditronix) proton beam. All QMA anion exchange cartridges (Sep-Pak Accell Plus QMA Plus Light, chloride form, Waters) and C18 cartridges (Sep-Pak C18 Plus Short types) were washed with EtOH (20 mL) and water (20 mL) and dried with air before use. Automated syntheses were performed on a Scansys Laboratorieteknik synthesis module housed in a hot cell. Analytical HPLC was performed on a Dionex system connected to a P680A pump, a UVD 170U detector and a Scansys radiodetector. The system was controlled by Chromeleon 6.8 software. Semi-preparative HPLC was performed on the built-in HPLC system in the synthesis module and the flow rate was set to 3 mL/min at all times. Radio-TLC was carried out on same plates as described for the organic chemistry. The fraction of radioactivity on the plates was measured with an instant imager from Packard and analyzed by Optiquant software.

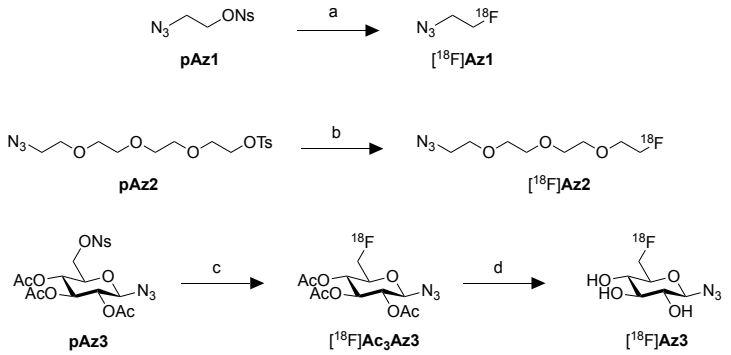
Health and Environment Department, Biomedical Systems, Austrian Institute of Technology, Austria: [^{18}F]Fluoride was produced via the (p,n) reaction by irradiating [^{18}O]H_2O using a PETtrace cyclotron equipped with high yield liquid target system (GE Healthcare). All QMA anion exchange cartridges (Sep-Pak Accell Plus QMA Plus Light, chloride form, Waters) and C18 cartridges (Sep-Pak C18 Plus Short types) were washed with EtOH (20 mL) and water (20 mL) and dried with air before use. Automated syntheses were performed on a TRACERlab™ FXFDG synthesis module from General Electric Healthcare housed in a hot cell. Analytical HPLC was performed on a 1200 series system (Agilent Technologies) using a reversed phase columns and acetonitrile/water or phosphate buffer gradients. For radio-HPLC a GABI* radioactivity detector (Raytest Isotopenmessgeraete GmbH) was

used. Semi-preparative HPLC was performed on the built-in system in the synthesis module, in combination with a K-2001 UV detector (Knauer) and radioactivity detector. The flow rate was set to 5 mL/min at all times. Radio-TLC plates were imaged in a multisensitive phosphor screen (Perkin-Elmer Life Sciences). The screens were scanned at 300 dpi resolution using a PerkinElmer Cyclone® Plus Phosphor Imager (Perkin-Elmer Life Sciences).

General procedure for the preparation of anhydrous [^{18}F]fluoride for radiolabeling (Scansys module)
Irradiated [^{18}O]water containing [^{18}F]F$^-$ was passed through an anion exchange resin cartridge (Sep-Pak Accell Plus QMA Plus Light, chloride form). [^{18}F]Fluoride trapped on the QMA was then eluted with 1 mL of a Kryptofix$_{222}$/K_2CO_3 solution (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (330 mg), K_2CO_3 (100 mg) and water (0.8 mL) in MeOH (19.2 mL)) into a 4 mL glass vial. The resulting mixture was then gently concentrated to dryness at 90–110 °C via azeotropic drying using 2x MeCN (1 mL) and a stream of helium. The procedure took 25–30 min and yielded in the ready to react [^{18}F]]FK–K_{222} complex.

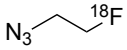
General procedure for the preparation of anhydrous [^{18}F]fluoride for radiolabeling (TRACERlab™ FXFDG module)
Irradiated [^{18}O]water containing [^{18}F]F$^-$ was passed through an anion exchange resin cartridge (Sep-Pak Accell Plus QMA Plus Light, chloride form). [^{18}F]Fluoride trapped on the QMA was then eluted with a solution of Kryptofix$_{222}$ (15 mg, 40 µmol) in MeCN (900 µL) to which a 3.5% aqueous K_2CO_3 (25 µmol, 100 µL) solution had been added. After addition of dry MeCN (500 µL) volatiles were removed in vacuo at a temperature of 60–120 °C. The procedure took 25–30 min and yielded in the ready to react [^{18}F]]FK–K_{222} complex.

Radiolabeling



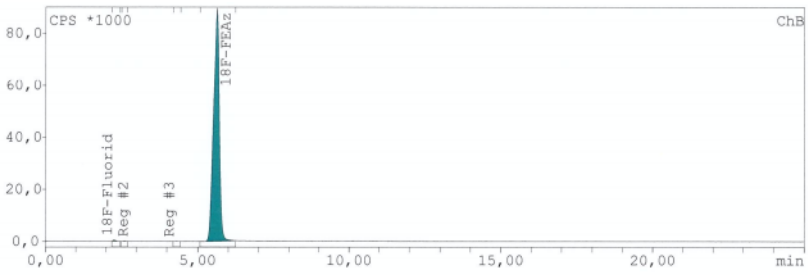
Scheme S15. ^{18}F-Labeling of azide building blocks. (a) [^{18}F]FK, K_{222}, MeCN, 110 °C, 10 min; (b) [^{18}F]FK, K_{222}, DMSO, 120 °C, 10 min; (c) [^{18}F]FK, K_{222}, MeCN, 90 °C, 5 min; (d) 2 M NaOH, 1 min on Sep-Pak cartridge.

1-Azido-2-[^{18}F]fluoroethane ([^{18}F]Az1) and subsequent CuAAC



Azide [^{18}F]**Az1** was synthesized as previously reported.[6] [^{18}F]Fluoride was dried according to the general procedures described above. To the dried residue containing [^{18}F]F$^-$, precursor **pAz1** (12.5 mg, 46 µmol) in dry MeCN (300 µL) was added. The mixture was heated at 110 °C for 5 min. All volatiles in the reactor were distilled into a vial containing the alkyne-modified Tz and additional reagents for the CuAAC. During the distillation in the TRACERlab™ FXFDG module, the temperature was gradually increased to 135 °C, and a second portion of MeCN (200 µL) was added to the reactor during distillation, to enable most efficient transfer of [^{18}F]**Az1** into the CuAAC reaction mixture. Distillation efficiencies varied between 50–80%. In the synthesis module from Scansys, the distillation was performed simultaneously as the ^{18}F-fluorination.

The following CuAAC was performed as following: An aqueous solution of $CuSO_4 \cdot 5\ H_2O$ (5 µL, 100 mg/mL) was mixed with an aqueous solution of sodium ascorbate (5 µL, 300 mg/mL), when the color of the mixture turned yellow a solution of BPDS (20 µL, 50 mg/mL) in water was added. The resulting blue/green mixture was added to the receiving vial for the distillation, which contained a solution of the Tz-alkyne (~1 mg; **I-VI**) in anhydrous DMF (100–200 µL). Following distillation and a 5 min reaction time at room temperature, the mixture was diluted with DMSO/water (1:1, 2 mL) and purified by semi-preparative HPLC. The collected HPLC fraction was trapped on a Sep-Pak C18 plus short cartridge, which was eluted with either EtOH or acetone. In the case of acetone, the solvent was removed by heating and a stream of nitrogen. The products were formulated in 0.9% saline.
For the CuAAC with azide [^{18}F]**Az1**, a starting amount of 154–225 GBq (for the TRACERlab™ FXFDG module) or ~12 GBq (for radiosynthesis of Tz [^{18}F]**1** in the Scansys module) was used. The values for radiochemical yield (RCY), radiochemical purity (RCP) and molar activity (A_m) are given as mean values. The A_m were determined by integrating the area of the UV absorbance peak corresponding to the radiolabeled product on the HPLC chromatogram. This area was converted into a molar mass by comparison with an average of integrated areas (triplet) of a known concentration for the corresponding reference compounds. This applies for all radiolabeled compounds described below.



Probenbeschreibung

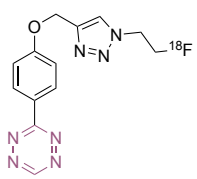
Messung:	130708.001, Injektion :	08.07.2013	11:27		
Methode:	18FTEST vom: 14.12.2012	07:25:		Position:	10
Meßstation:	33				
Inj.-Volumen (µl):	10,0				
18F-FEAz vom 08.07.2013					
Op: De					
Phase C: H2O MilliQ					
Phase D: MeCN					
Säule: Zorbax Eclipse AQ, 25cm, 5µm					
Radioaktivitätsdetektor: raytest Gabi Star				Serial Nr.: #30320 raytest GINA star 20.	

Integration ChB

Substanz	Ret min	Typ	Fläche Counts	%Fläche %
18F-Fluorid	2,27	BB	2989	0,25
Reg #2	2,61	BB	739	0,06
Reg #3	4,30	BB	467	0,04
18F-FEAz	5,63	BB	1174575	99,64
Summe in ROI			1178771	
Fläche			1397722	
Ext. BKG			0,00 CPS	

Figure S16. Analytical radio-HPLC chromatogram for azide [^{18}F]**Az1,** HPLC analysis was performed on a 1200 series system (Agilent Technologies) using a Zorbax SB-AQ (5 µm, 4.6 x 250 mm) column and H2O/MeCN gradient elution (flow rate: 1.2 mL/min, 0-2 min: 5% MeCN, 2-10 min: 5 to 20% MeCN linear gradient, 10-16 min: 20 to 90% MeCN linear gradient). For radio-HPLC a GABI* radioactivity detector (Raytest) was used.

3-(4-((1-(2-[^{18}F]Fluoroethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-1,2,4,5-tetrazine ([^{18}F]1)



[^{18}F]**1** was prepared according to the aforementioned procedure. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (70/30/0.1 v/v/v) as eluent in the Scansys module or in the TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**1** was afforded in 25% radiochemical yield (RCY), decay corrected (d.c.), over 2 steps, a radiochemical purity (RCP) of 99% and a molar activity (A_m) of 55 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 24.3 GBq.

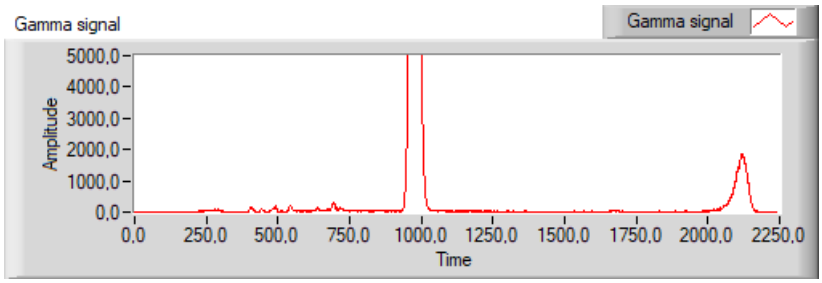


Figure S17. Semi-preparative HPLC chromatogram for [^{18}F]**1** (R_t = 2080 sec) on a Luna 5 5µ C18(2) (100Å 250 x 10 mm) column.

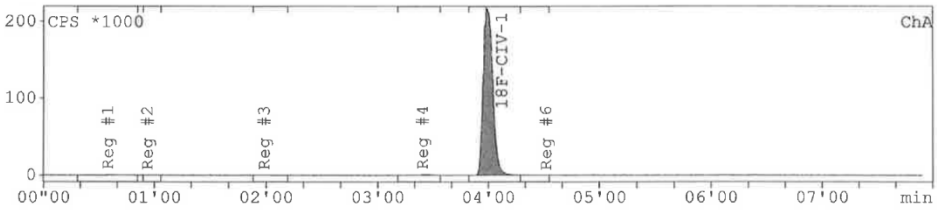


Figure S18. Analytical radio-HPLC chromatogram for Tz [^{18}F]**1** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

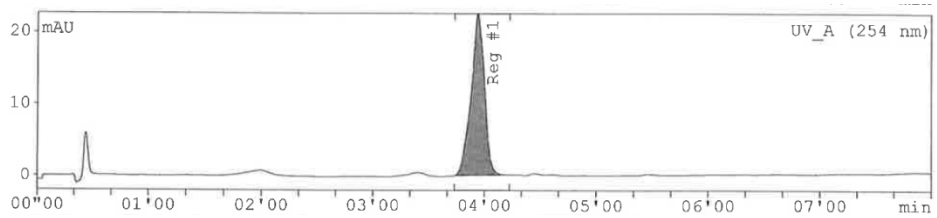
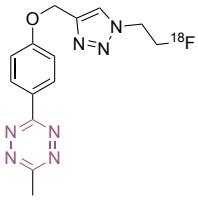


Figure S19. Analytical HPLC chromatogram for ^{19}F-reference, Tz **1** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

3-(4-((1-(2-[^{18}F]Fluoroethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-methyl-1,2,4,5-tetrazine ([^{18}F]5)



[^{18}F]**5** was prepared according to the aforementioned procedure. Semi-preparative HPLC was performed in the TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**5** was afforded in 14% RCY d.c. over 2 steps, a RCP of ≥99% and an A_m of 106 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 19.8 GBq.

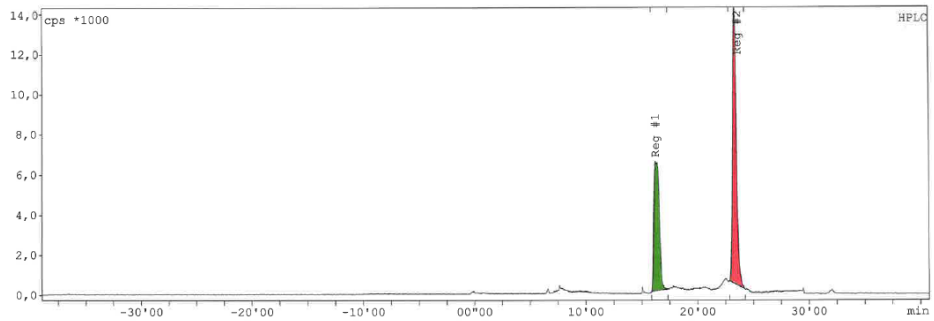


Figure S20. Semi-preparative HPLC chromatogram for [^{18}F]**5** (R_t = 24 min).

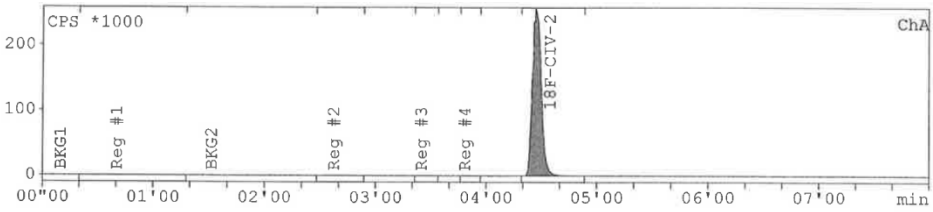


Figure S21. Analytical radio-HPLC chromatogram for Tz [^{18}F]**5** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

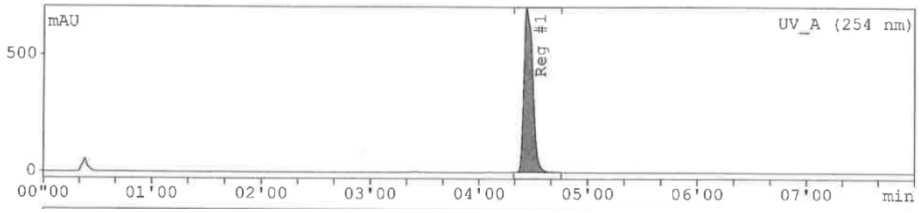
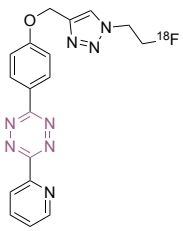


Figure S22. Analytical HPLC chromatogram for ^{19}F-reference, Tz **5** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

3-(4-((1-(2-[^{18}F]Fluoroethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine ([^{18}F]12)



[^{18}F]**12** was prepared according to the aforementioned procedure. Semi-preparative HPLC was performed in the TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**12** was afforded in 1% RCY d.c. over 2 steps, a RCP of 96% and an A_m of 107 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 1.18 GBq.

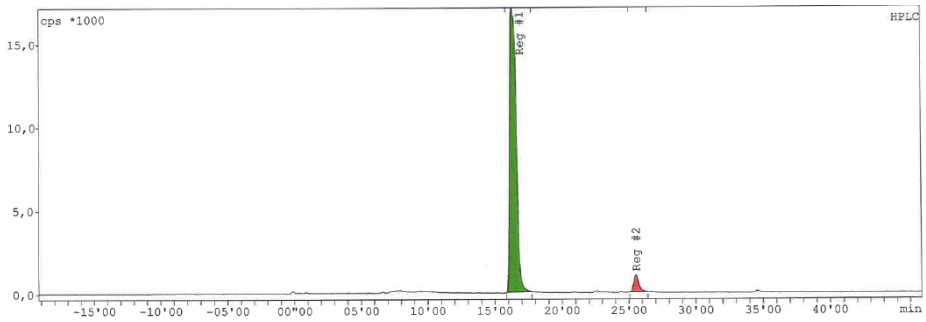


Figure S23. Semi-preparative HPLC chromatogram for [^{18}F]**12** (R_t = 16.5 min).

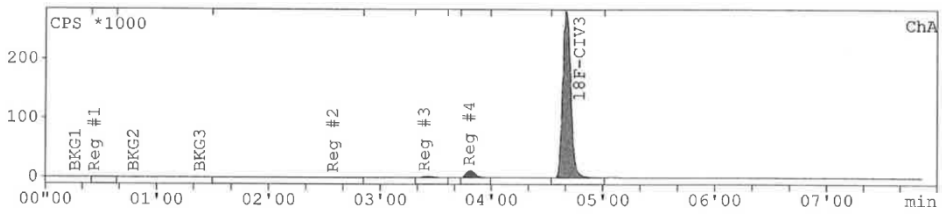


Figure S24. Analytical radio-HPLC chromatogram for Tz [^{18}F]**12** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

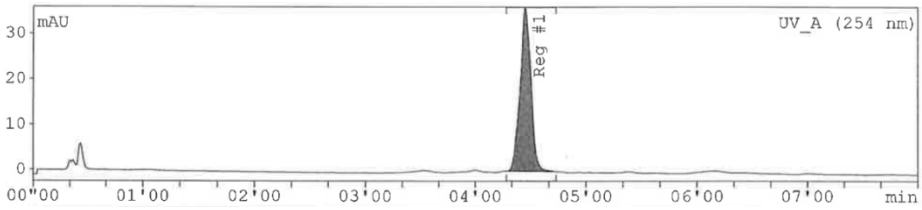
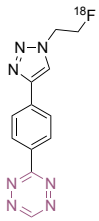


Figure S25. Analytical HPLC chromatogram for ^{19}F-reference, Tz **12** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

3-(4-(1-(2-[^{18}F]Fluoroethyl)-1H-1,2,3-triazol-4-yl)phenyl)-1,2,4,5-tetrazine ([^{18}F]17)



[^{18}F]**17** was prepared according to the aforementioned procedure. Semi-preparative HPLC was performed in the TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**17** was afforded in 8% RCY d.c. over 2 steps, a RCP of 98% and an A_m of 209 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 10.2 GBq.

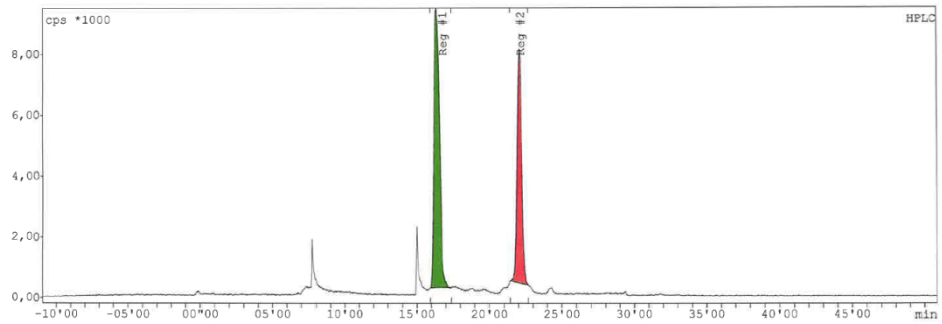


Figure S26. Semi-preparative HPLC chromatogram for [^{18}F]**17** (R_t = 22 min).

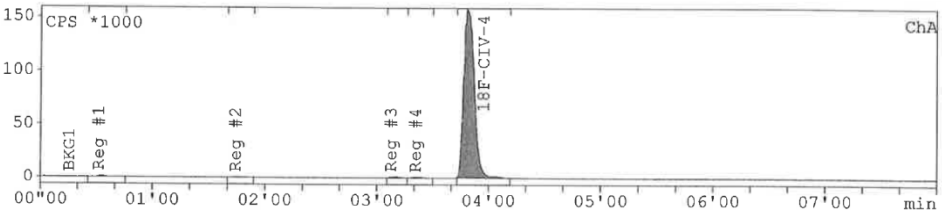


Figure S27. Analytical radio-HPLC chromatogram for Tz [^{18}F]**17** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

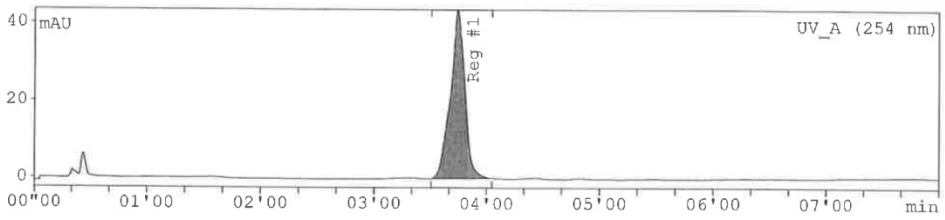
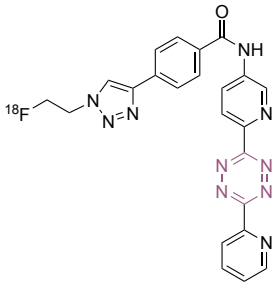


Figure S28. Analytical HPLC chromatogram for ^{19}F-reference, Tz **17** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

4-(1-(2-[^{18}F]fluoroethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide ([^{18}F]25)



An aqueous solution of copper sulfate pentahydrate (100 mg/mL, 20 µL) was mixed with a aqueous solution of sodium ascorbate (300 mg/mL, 20 µL) and the black mixture was kept at room temperature until the color changed to canary yellow. Afterwards, a solution of BPDS in 1M phosphate buffer pH = 7 (50 mg/mL, 80 µL) was added which caused a color change to green. To the catalyst was added 1 mg precursor (2.6 µmol) in 0.1 mL DMF and [^{18}F]**Az1** (3.541 GBq) in 1000 µL MeCN. The mixture was kept for 10 minutes at 100 °C in a sealed tube. After cooling the reaction mixture was diluted with 2 mL DMSO/H_2O = 1:1 and purified by HPLC. (C18, 10-80% MeCN in 10 mM phosphate buffer pH = 6.8). The collected fraction was loaded onto a Waters SepPak tc18 plus cartridge (preconditioned with 5 mL MeCN followed by 15 mL water) and eluted with 1 mL ethanol. Volatiles were removed *in vacuo,* and the product (383 MBq, 16.4% decay corrected radiochemical yield) was formulated with 0.9% saline. The product was obtained in 82.6% radiochemical purity.

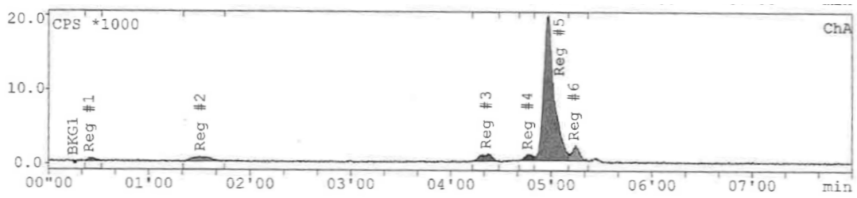


Figure S29. Analytical radio-HPLC chromatogram for Tz [^{18}F]**25**. HPLC analysis was performed on a 1200 series system (Agilent Technologies) using a Zorbax SB-AQ (5 µm, 4.6 x 250 mm) column and H2O/MeCN gradient elution (flow rate: 1.2 mL/min, 0-2 min: 5% MeCN, 2-10 min: 5 to 20% MeCN linear gradient, 10-16 min: 20 to 90% MeCN linear gradient). For radio-HPLC a GABI* radioactivity detector (Raytest) was used.

3-(1-(2-fluoroethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)propenamide ([^{18}F]43)

An aqueous solution of copper sulfate pentahydrate (100 mg/mL, 20 µL) was mixed with a aqueous solution of sodium ascorbate (300 mg/mL, 20 µL) and the black mixture was kept at room temperature until the color changed to canary yellow. Afterwards, aqueous solution of disodium bathophenanthroline disulfonate (BPDS, 50 mg/mL, 80 µL) was added which caused a color change to green. To the catalyst was added 1 mg precursor I (3.0 µmol) in 0.1 mL DMF and [^{18}F]**Az1** (137 GBq) in 800 µL MeCN/DMF mixture (1/1 v/v). Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (70/30/0.1 v/v/v) as eluent. Tz [^{18}F]**43** was afforded in 0.5% RCY d.c. for the CuAAC, a RCP of 90% and an A_m of 4.4 GBq/µmol at 2 h after the start of the synthesis. Isolated radioactivity amount was 0.9 MBq.

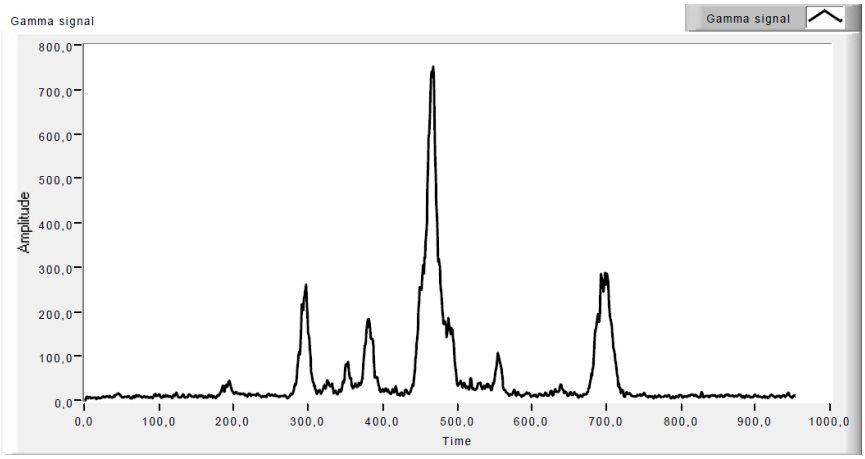
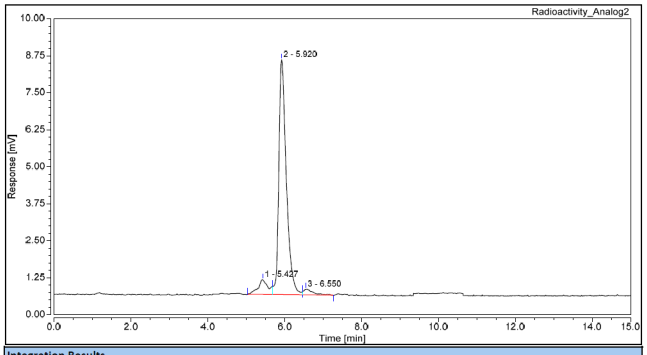
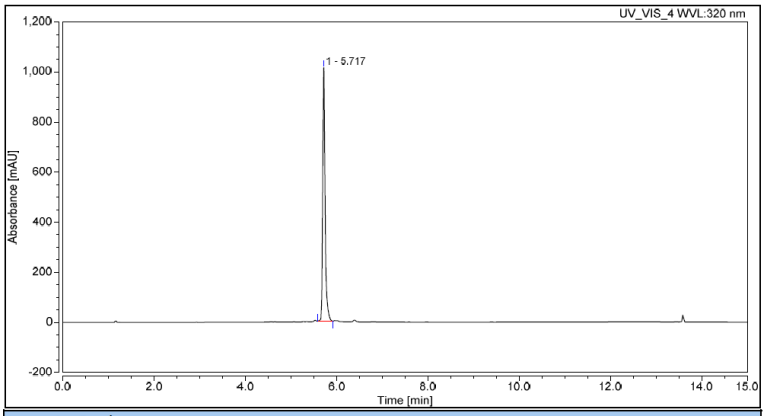


Figure S30. Semi-preparative HPLC chromatogram for [^{18}F]**43** (R_t = 460 sec).



Integration Results							
No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount n.a.
1		5.427	0.150	0.501	7.45	5.81	n.a.
2		5.920	1.802	7.937	89.79	91.94	n.a.
3		6.550	0.055	0.194	2.75	2.25	n.a.
Total:			2.007	8.632	100.00	100.00	

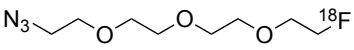
Figure S31. Analytical radio-HPLC chromatogram for Tz [^{18}F]**43**. HPLC conditions: Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. elutaed with a gradient of MeCN in water containing 0.1 % TFA; 0–12 min 0→100% MeCN; 12–13 min 100% MeCN; 13–14 min 100→0% MeCN; 14–15 min 0% MeCN) over 15 min. Flow rate: 2.0 mL/min.



Integration Results							
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		5.717	62.928	1012.757	100.00	100.00	n.a.
Total:			**62.928**	**1012.757**	**100.00**	**100.00**	

Figure S32. Analytical HPLC chromatogram for ^{19}F-reference, Tz **43**. HPLC conditions: Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. elutaed with a gradient of MeCN in water containing 0.1 % TFA; 0–12 min 0→100% MeCN; 12–13 min 100% MeCN; 13–14 min 100→0% MeCN; 14–15 min 0% MeCN) over 15 min. Flow rate: 2.0 mL/min.

1-Azido-2-(2-(2-(2-[^{18}F]fluoroethoxy)ethoxy)ethoxy)ethane ([^{18}F]Az2) and subsequent CuAAC



[^{18}F]Fluoride was dried according to the general procedure for the Scansys module described above. To the dried residue containing [^{18}F]F$^-$, precursor **pAz2** (3 mg, 8 µmol) in anhydrous DMSO (500 µL) was added. The mixture was heated at 120 °C for 10 min, thereafter cooled with air for 5 min, before it was diluted with water (2 mL) and purified by semi-preparative HPLC on a Luna 5 µ C18(2) 100 Å (250 × 10.00 mm) column using water/MeCN/TFA mixture (70/30/0.1 v/v/v) as eluent. The fraction was collected in water (20 mL) and trapped on a Sep-Pak C18 plus cartridge. The cartridge was eluted with DMF (2 mL) to afford [^{18}F]**Az2** in 41% RCY d.c. to the starting amount of activity, a RCP of ≥97% and with a A_m of 13–37 GBq/µmol.

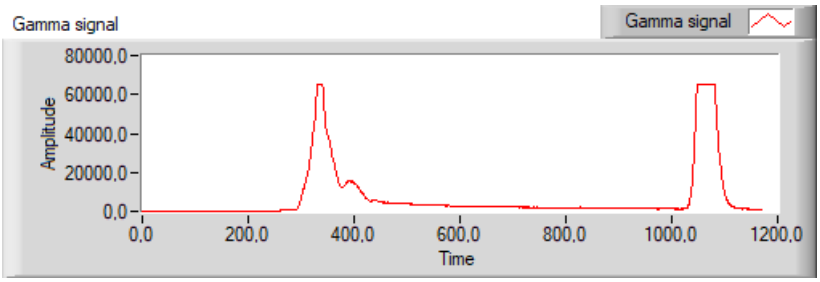
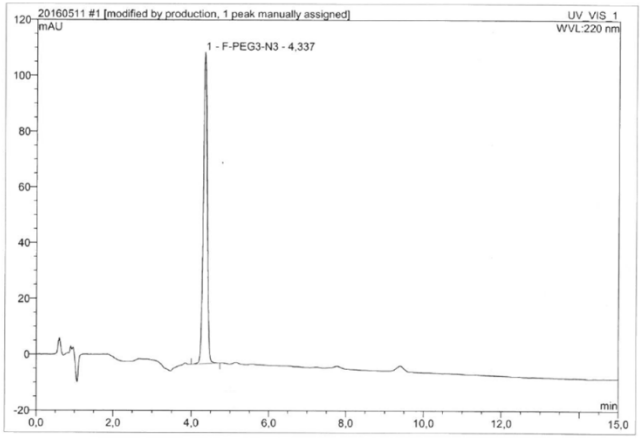
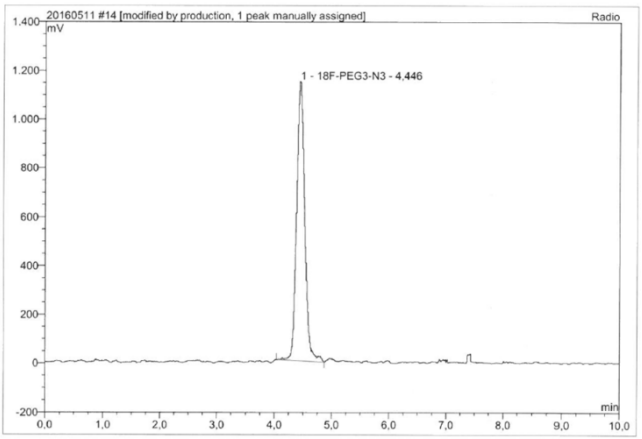


Figure S33. Semi-preparative HPLC chromatogram for [^{18}F]**Az2** (R_t = 1080 sec).



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	4,34	F-PEG3-N3	111,728	12,770	100,00	n.a.	BMB*^
Total:			111,728	12,770	100,00	0,000	

Figure S34. Analytical HPLC chromatogram for ^{19}F-reference, azide **Az2** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (75:25:0.1, *v*/*v*/*v*). Flow rate: 2 mL/min.



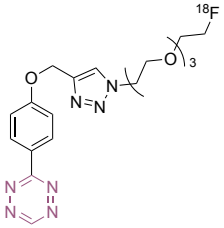
No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	4,45	18F-PEG3-N3	1144,544	188,735	100,00	n.a.	BMB*^
Total:			1144,544	188,735	100,00	0,000	

Figure S35. Analytical radio-HPLC chromatogram for azide [^{18}F]**Az2** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (75:25:0.1, *v*/*v*/*v*). Flow rate: 2 mL/min.

The following CuAAC was performed as following: An aqueous solution of $CuSO_4 \cdot 5H_2O$ (8–16 µL, 100 mg/mL) was mixed with an aqueous solution of sodium ascorbate (8–16 µL, 300 mg/mL), when the color of the mixture turned yellow a solution of BPDS (40–80 µL, 50 mg/mL) in water was added. The resulting blue/green mixture was added to a solution of the Tz-alkyne (~1 mg; **I-VI**) in DMF (100 µL). This mixture was then added to the isolated [^{18}F]**Az2**. The mixture was heated at 120 °C for 5–25 min, cooled at room temperature for 10 min and thereafter diluted with 0.1% TFA in water (~1.5 mL). Purification was performed by semi-preparative HPLC in a Scansys module. The collected HPLC fraction was trapped on a Sep-Pak C18 plus short cartridge, which was eluted with acetone. The acetone was removed by gentle heating and a stream of nitrogen. The products were formulated in 0.9% saline.

For the CuAAC with azide [^{18}F]**Az2**, a starting amount of 3–6 GBq was used. The values for RCY, RCP and A_m are stated as mean values.

3-(4-((1-(2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-1,2,4,5-tetrazine ([^{18}F]2)



[^{18}F]**2** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (8 µL), sodium ascorbate solution (8 µL) and BPDS solution (40 µL). The mixture was heated at 120 °C for 5 min. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (70/35/0.1 v/v/v) as eluent. Tz [^{18}F]**2** was afforded in 23% RCY d.c. for the CuAAC, a RCP of 96% and an A_m of 22 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 524 MBq.

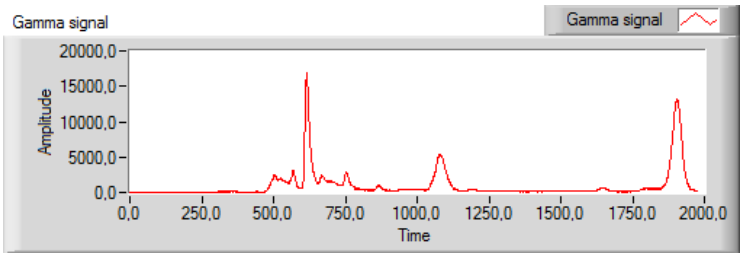
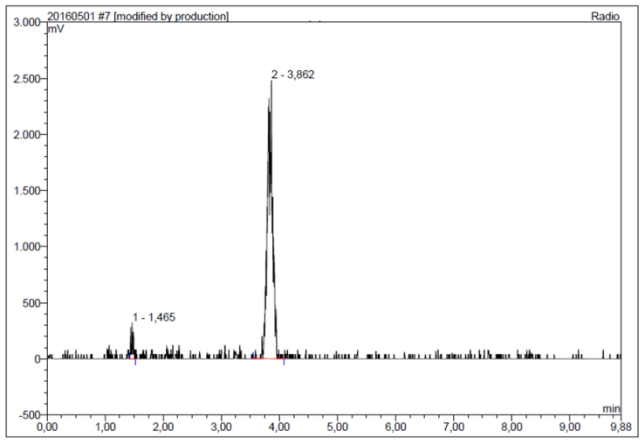
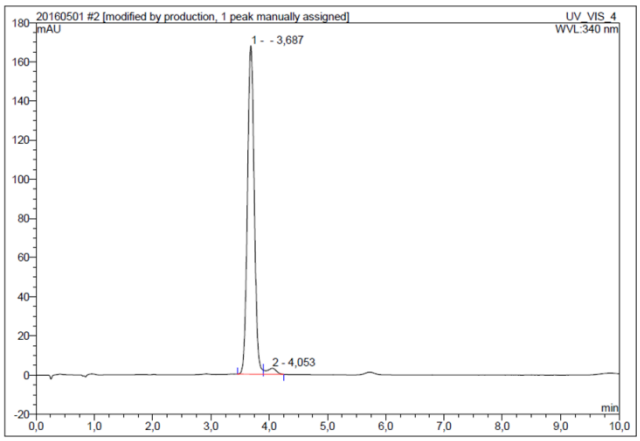


Figure S36. Semi-preparative HPLC chromatogram for [^{18}F]**2** (R_t = 1900 sec).



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	1,46	n.a.	320,166	10,852	4,20	n.a.	BMB*
2	3,86	n.a.	2480,007	247,487	95,80	n.a.	BMB*
Total:			2800,174	258,339	100,00	0,000	

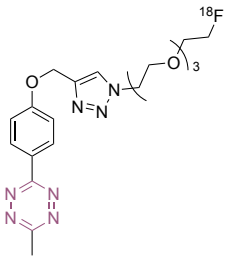
Figure S37. Analytical radio-HPLC chromatogram for Tz [^{18}F]**2** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v/v/v*). Flow rate: 2 mL/min.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3,69		167,707	22,678	97,39	n.a.	BM *^
2	4,05	n.a.	3,065	0,609	2,61	n.a.	MB*
Total:			170,772	23,286	100,00	0,000	

Figure S38. Analytical HPLC chromatogram for ^{19}F-reference, Tz **2** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v/v/v*). Flow rate 2 mL/min.

3-(4-((1-(2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-methyl-1,2,4,5-tetrazine ([^{18}F]6)



[^{18}F]**6** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (8 µL), sodium ascorbate solution (8 µL) and BPDS solution (40 µL). The mixture was heated at 120 °C for 5 min. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (70/35/0.1 v/v/v) as eluent. Tz [^{18}F]**6** was afforded in 33% RCY d.c. for the CuAAC, a RCP of ≥99% and an A_m of 100 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 854 MBq.

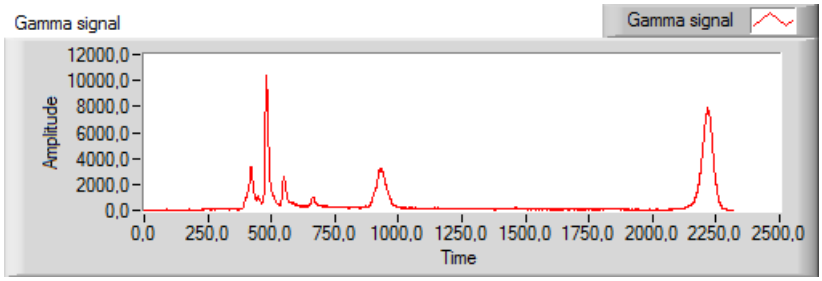
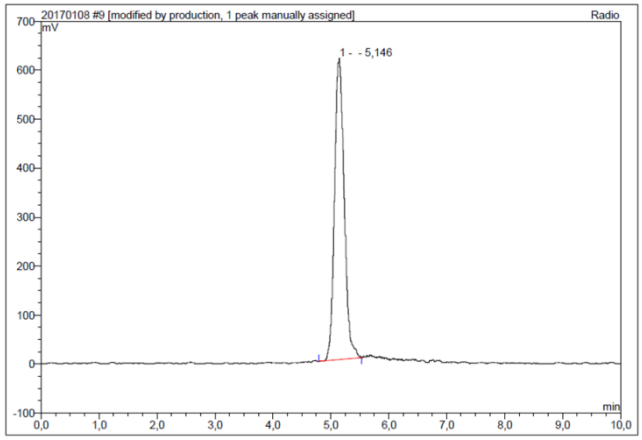
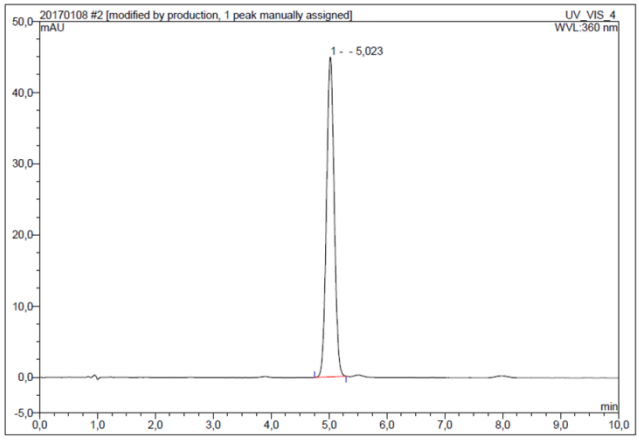


Figure S39. Semi-preparative HPLC chromatogram for [^{18}F]**6** (R_t = 2217 sec).



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	5,15		614,820	118,552	100,00	n.a.	BMB*^
Total:			614,820	118,552	100,00	0,000	

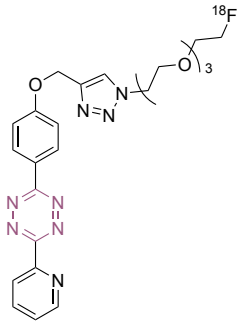
Figure S40. Analytical radio-HPLC chromatogram for Tz [^{18}F]**6** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v*/*v*/*v*). Flow rate: 2 mL/min.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	5,02		44,872	6,925	100,00	n.a.	BMB*^
Total:			44,872	6,925	100,00	0,000	

Figure S41. Analytical HPLC chromatogram for ^{19}F-reference, Tz **6** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v*/*v*/*v*). Flow rate 2 mL/min.

3-(4-((1-(2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine ([^{18}F]13)



[^{18}F]**13** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (8 µL), sodium ascorbate solution (8 µL) and BPDS solution (40 µL). The mixture was heated at 120 °C for 20 min. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (70/35/0.1 v/v/v) as eluent. Tz [^{18}F]**13** was afforded in 11% RCY d.c. for the CuAAC, a RCP of 94% and an A_m of 21 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 125 MBq.

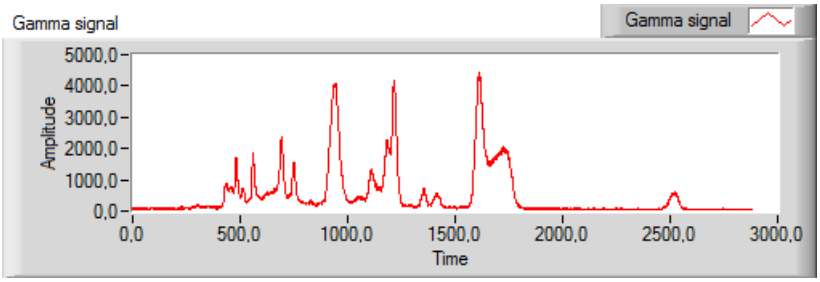
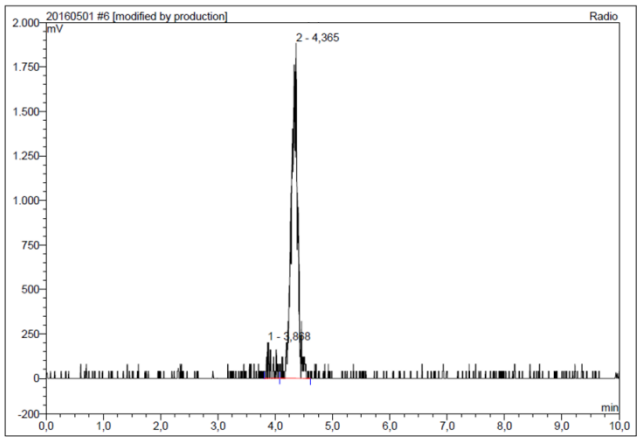
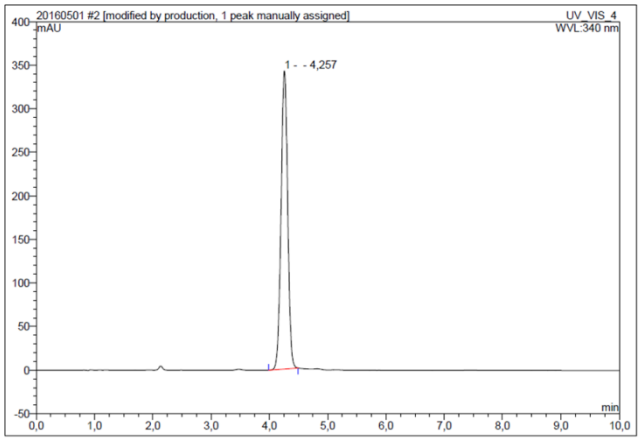


Figure S42. Semi-preparative HPLC chromatogram for [^{18}F]**13** (R_t = 2500 sec).



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	3,87	n.a.	200,154	13,947	5,81	n.a.	BMB*
2	4,36	n.a.	1881,518	226,049	94,19	n.a.	BMB*
Total:			2081,672	239,996	100,00	0,000	

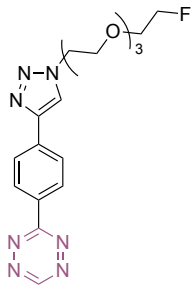
Figure S43. Analytical radio-HPLC chromatogram for Tz [^{18}F]**13** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v*/*v*/*v*). Flow rate: 2 mL/min.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	4,26		342,213	45,106	100,00	n.a.	BMB*^
Total:			342,213	45,106	100,00	0,000	

Figure S44. Analytical HPLC chromatogram for ^{19}F-reference, Tz **13** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v*/*v*/*v*). Flow rate 2 mL/min.

3-(4-(1-(2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)phenyl)-1,2,4,5-tetrazine ([^{18}F]18)



[^{18}F]**18** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (10 µL), sodium ascorbate solution (10 µL) and BPDS solution (40 µL). The mixture was heated at 120 °C for 5 min. Prior to purification, PIDA (16 mg in 300 µL MeCN) was added. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250x10 mm) column using water/MeCN/TFA mixture (70/30/0.1 v/v/v) as eluent. Tz [^{18}F]**18** was afforded in 8% RCY d.c. for the CuAAC, a RCP of ≥99% and an A_m of 60 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 123.6 MBq.

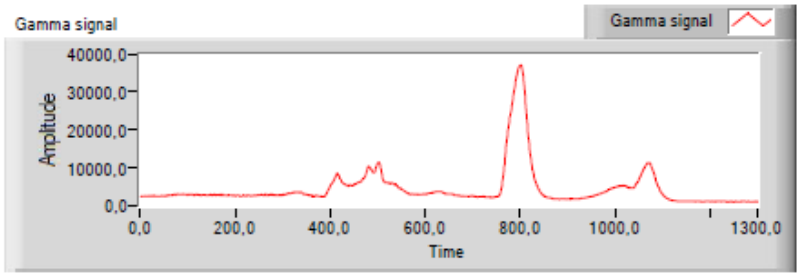
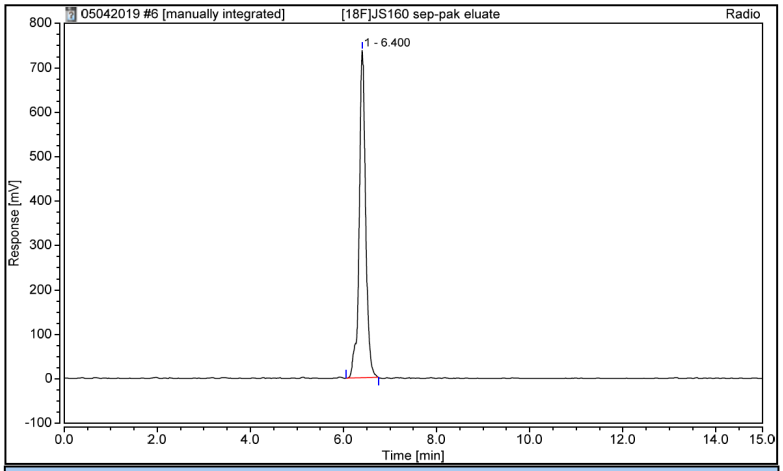


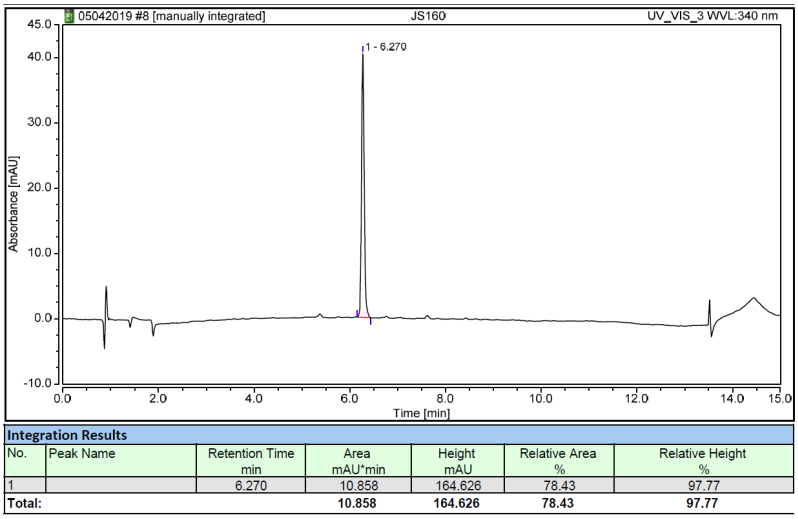
Figure S45. Semi-preparative HPLC chromatogram for [^{18}F]**18** (R_t = 1100 sec).



Integration Results

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %
1		6.400	118.119	736.586	100.00	100.00
Total:			**118.119**	**736.586**	**100.00**	**100.00**

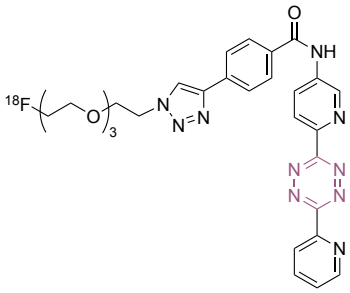
Figure S46. Analytical radio-HPLC chromatogram for Tz [^{18}F]**18** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using a gradient of MeCN in water containing 0.1 % TFA 0–12 min 0→100% MeCN; 12–13 min 100% MeCN; 13–14 min 100→0% MeCN; 14–15 min 0% MeCN) over 15 min. Flow rate: 1.5 mL/min.



Integration Results						
No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %
1		6.270	10.858	164.626	78.43	97.77
Total:			**10.858**	**164.626**	**78.43**	**97.77**

Figure S47. Analytical HPLC chromatogram for ^{19}F-reference, Tz **18** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using a gradient of MeCN in water containing 0.1 % TFA 0–12 min 0→100% MeCN; 12–13 min 100% MeCN; 13–14 min 100→0% MeCN; 14–15 min 0% MeCN) over 15 min. Flow rate: 1.5 mL/min.

4-(1-(2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide ([^{18}F]26)



[^{18}F]**26** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (16 µL), sodium ascorbate solution (16 µL) and BPDS solution (80 µL). The mixture was heated at 120 °C for 20 min. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250x10 mm) column using water/MeCN/TFA mixture (65/35/0.1 v/v/v) as eluent. Tz [^{18}F]**26** was afforded in 36% RCY d.c. for the CuAAC, a RCP of ≥85% and an A_m of 54 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 1.10 GBq.

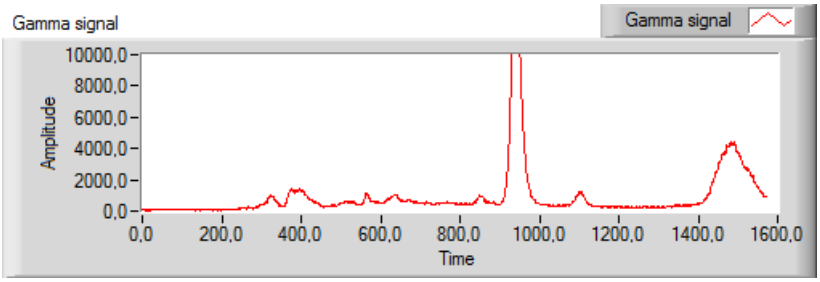
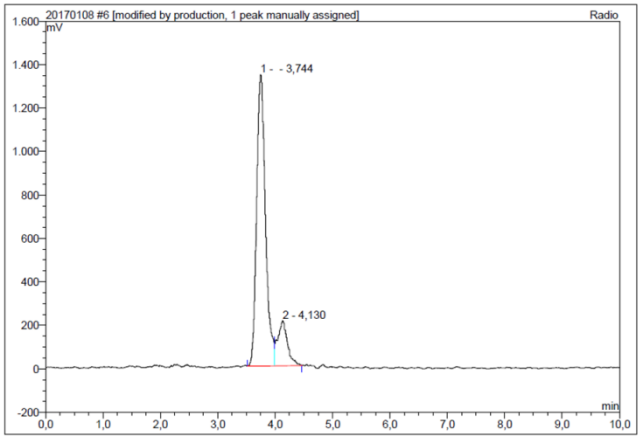
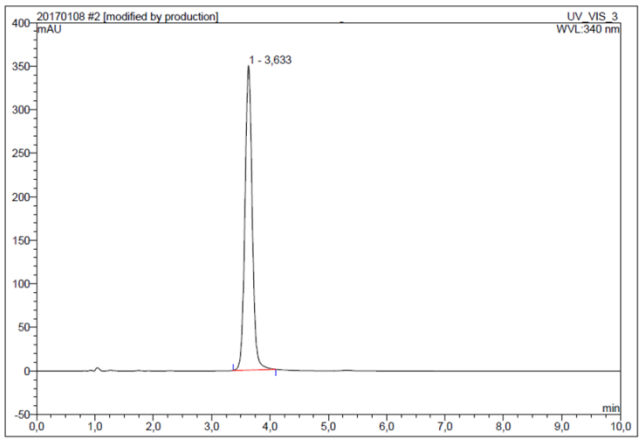


Figure S48. Semi-preparative HPLC chromatogram for [^{18}F]**26** (R_t = 1500 sec).



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	3,74		1340,238	237,378	84,85	n.a.	BM ^^
2	4,13	n.a.	207,732	42,391	15,15	n.a.	MB*
Total:			1547,970	279,768	100,00	0,000	

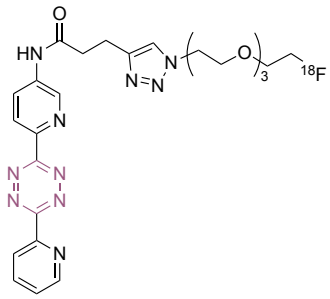
Figure S49. Analytical radio-HPLC chromatogram for Tz [^{18}F]**26** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v*/*v*/*v*). Flow rate: 2 mL/min.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	3,63	n.a.	349,634	50,249	100,00	n.a.	BMB*
Total:			349,634	50,249	100,00	0,000	

Figure S50. Analytical HPLC chromatogram for ^{19}F-reference, Tz **26** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (65:35:0.1, *v*/*v*/*v*). Flow rate 2 mL/min.

3-(1-(2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)propanamide ([^{18}F]44)



[^{18}F]**44** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (16 µL), sodium ascorbate solution (16 µL) and BPDS solution (80 µL). The mixture was heated at 120 °C for 25 min. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using a gradient of MeCN (25→40%) in aqueous ascorbic acid (25 mM) as eluent. After Sep-Pak separation, PIDA (10 mg) was added to the residue. After diluting the mixture in water (~100 mL), it was passed through a

second Sep-pak, which was later eluted with acetone and reformulated to afford Tz [^{18}F]**44** in 20% RCY d.c. for the CuAAC, a RCP of 98% and an A_m of 85 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 453 MBq.

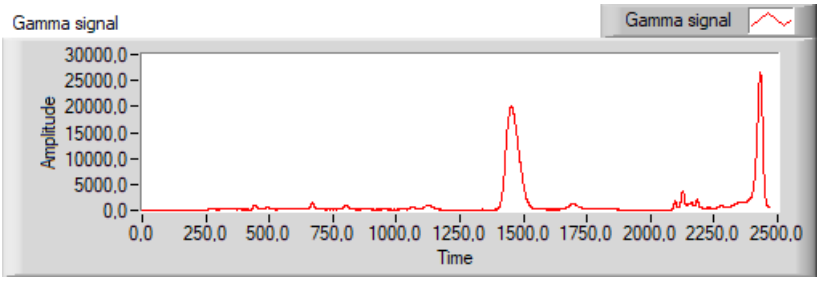
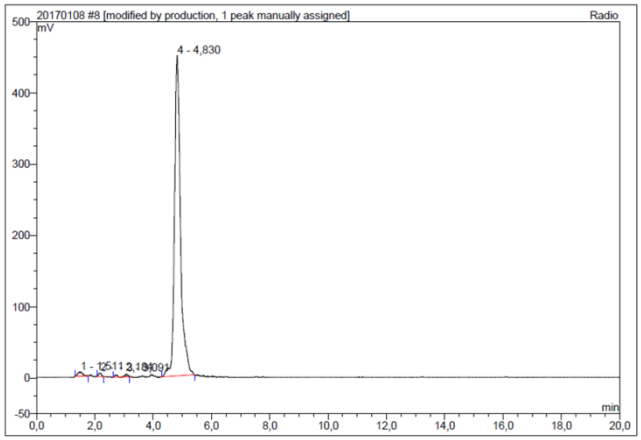
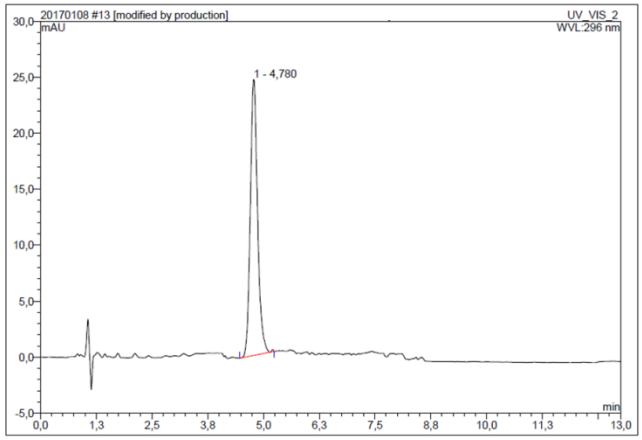


Figure S51. Semi-preparative HPLC chromatogram for [^{18}F]**44** (R_t = 2375 sec).



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	1,51	n.a.	6,114	1,169	1,08	n.a.	BMB*
2	2,18		4,662	0,579	0,53	n.a.	BMB*^
3	3,09	n.a.	3,921	0,765	0,70	n.a.	BMB*
4	4,83	n.a.	448,894	106,084	97,69	n.a.	BMB*
Total:			463,591	108,597	100,00	0,000	

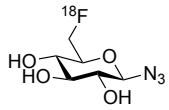
Figure S52. Analytical radio-HPLC chromatogram for Tz [^{18}F]**44** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (75:25:0.1, *v*/*v*/*v*). Flow rate: 2 mL/min.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	4,78	n.a.	24,631	4,634	100,00	n.a.	BMB*
Total:			24,631	4,634	100,00	0,000	

Figure S53. Analytical HPLC chromatogram for ^{19}F-reference, Tz **44** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using water/MeCN/TFA (75:25:0.1, *v*/*v*/*v*). Flow rate 2 mL/min.

(2R,3R,4S,5S,6S)-2-Azido-6-([^{18}F]fluoromethyl)tetrahydro-2H-pyran-3,4,5-triol ([^{18}F]Az3) and subsequent CuAAC



[^{18}F]Fluoride was dried according to the general procedures described above. To the dried residue containing [^{18}F]F$^-$, precursor **pAz3** (11 mg, 21 µmol) in anhydrous MeCN (500 µL) was added. The mixture was heated at 100 °C for 7 min, thereafter cooled with air for 5 min, before it was diluted with DMSO/water (1:1, 2 mL) for purification by semi-preparative HPLC. The latter was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using MeCN (40%) in aqueous phosphate buffer (10 mM, pH 6) as eluent in the Scansys module or in the TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 using a gradient of MeCN (5→80%) in aqueous phosphate buffer (10 mM, pH 6.8).The collected HPLC fraction was trapped on a Sep-Pak C18 plus short cartridge, which was washed with water (5 mL), followed by aqueous 2 M NaOH (1 mL). On-cartridge deprotection of [^{18}F]**Ac$_3$Az3** was carried out within 60 seconds and the product was eluted with water (2 mL) into a vial containing AcOH (150 µL) to afford [^{18}F]**Az3** in 19% RCY d.c. to the starting amount of activity, a RCP of ≥99% and with a A_m of 18 GBq/µmol.

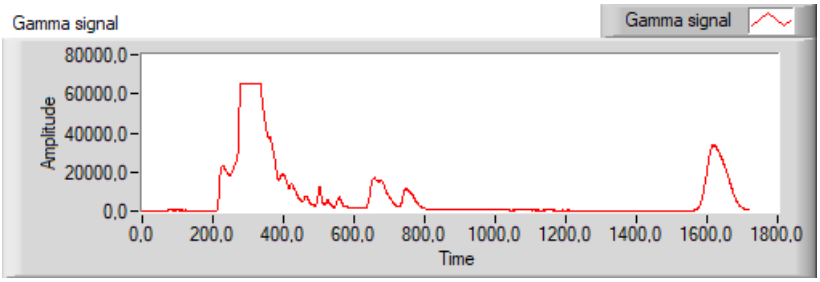


Figure S54. Semi-preparative HPLC chromatogram for [^{18}F]**Az3** (R_t = 1600 sec) on a Luna 5µ C18(2) (100Å 250 x 10 mm) column.

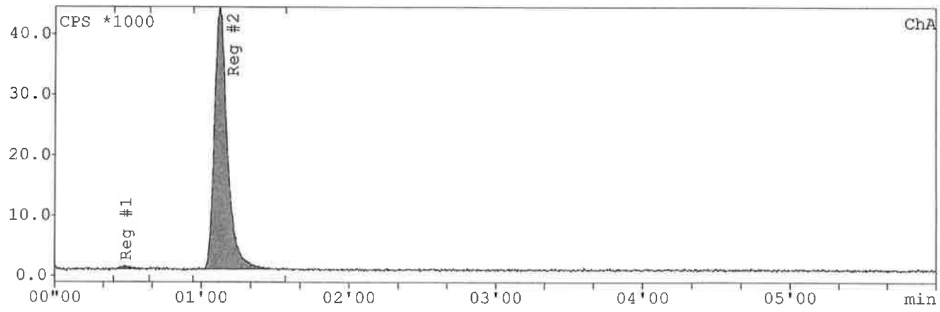
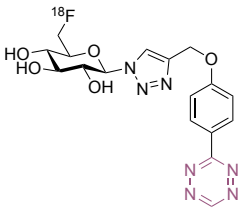


Figure S55. Analytical radio-HPLC chromatogram for azide [^{18}F]**Az3** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

The following CuAAC was performed as following: An aqueous solution of $CuSO_4 \cdot 5H_2O$ (5–10 µL, 100 mg/mL) was mixed with an aqueous solution of sodium ascorbate (5–10 µL, 300 mg/mL), when the color of the mixture turned yellow a solution of BPDS (20–40 µL, 50 mg/mL) in water was added. The resulting blue/green mixture was added to a solution of the Tz-alkyne (~1 mg; **I-VI**) in anhydrous DMF (100 µL). This mixture was then added to the isolated [^{18}F]**Az3**. The mixture was stirred at room temperature for 10–15 min and thereafter diluted with DMSO/water (1:1, 2 mL) for purification by semi-preparative HPLC. The collected HPLC fraction was trapped on a Sep-Pak C18 plus short cartridge, which was eluted with either EtOH or acetone. In case of acetone, the solvent was removed by gentle heating and a stream of nitrogen. The products were formulated in 0.9% saline. For the CuAAC with azide [^{18}F]**Az3**, a starting amount of 3–6 GBq was used. The values for RCY, RCP and A_m are given as mean values.

(2R,3R,4S,5S,6S)-2-(4-((4-(1,2,4,5-Tetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)-6-([1^{8}F]fluoromethyl)tetrahydro-2H-pyran-3,4,5-triol ([^{18}F]3)



[^{18}F]**3** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (5 µL), sodium ascorbate solution (5 µL) and BPDS solution (20 µL). Semi-preparative HPLC was performed in a TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 column using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**3** was afforded in 61% RCY d.c. for the CuAAC, a RCP of 98% and an A_m of 31 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 2.4 GBq.

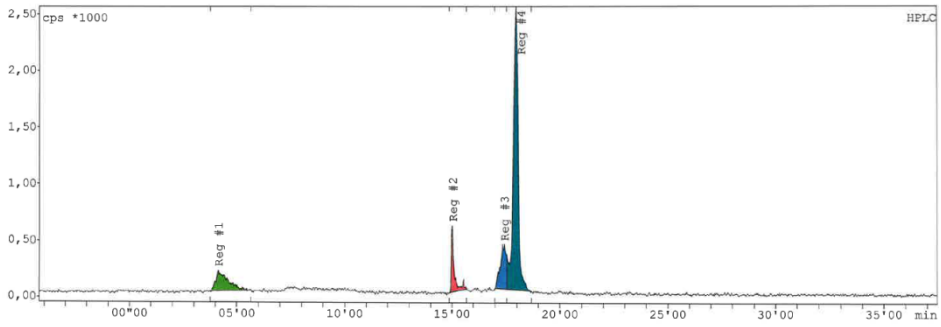


Figure S56. Semi-preparative HPLC chromatogram for [^{18}F]**3** (R_t = 18 min).

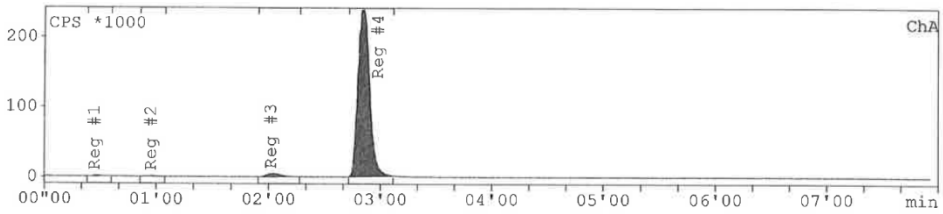


Figure S57. Analytical radio-HPLC chromatogram for Tz [^{18}F]**3** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

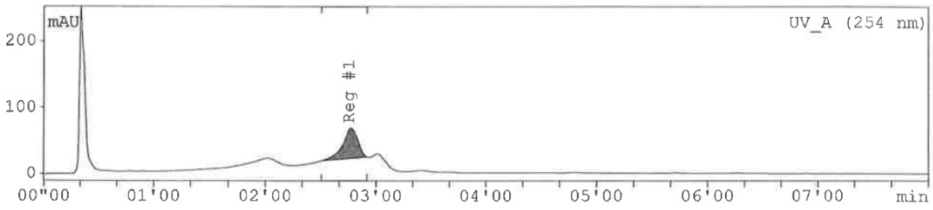
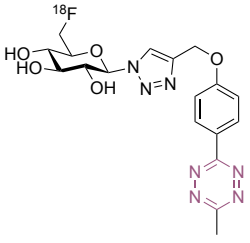


Figure S58. Analytical HPLC chromatogram for ^{19}F-reference, Tz **3** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

(2S,3S,4S,5R,6R)-2-([^{18}F]Fluoromethyl)-6-(4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3,4,5-triol ([^{18}F]7)



[^{18}F]**7** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (5 µL), sodium ascorbate solution (5 µL) and BPDS solution (20 µL). Semi-preparative HPLC was performed in a TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 column using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**7** was afforded in 52% RCY d.c. for the CuAAC, a RCP of ≥99% and an A_m of 230 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 1.3 GBq.

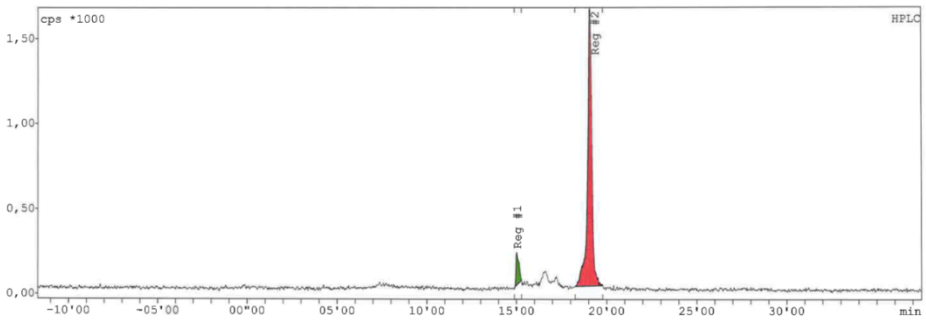


Figure S59. Semi-preparative HPLC chromatogram for [^{18}F]**7** (R_t = 19 min).

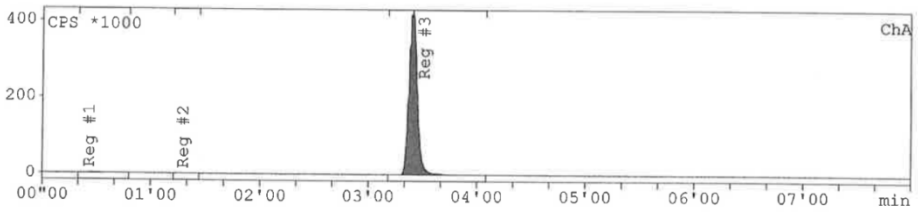


Figure S60. Analytical radio-HPLC chromatogram for Tz [^{18}F]**7** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

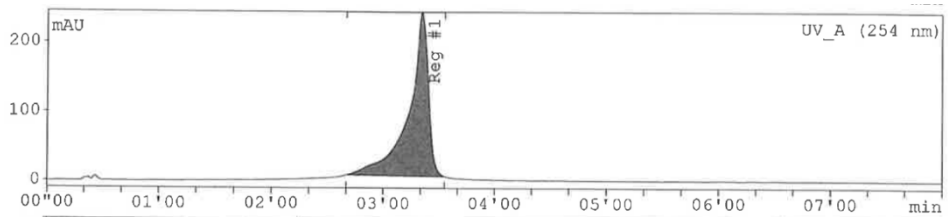
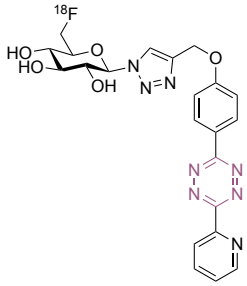


Figure S61. Analytical HPLC chromatogram for ^{19}F-reference, Tz **7** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

(2S,3S,4S,5R,6R)-2-([^{18}F]Fluoromethyl)-6-(4-((4-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)phenoxy) methyl)-1H-1,2,3-triazol-1-yl)tetrahydro-2H-pyran-3,4,5-triol ([^{18}F]14)



[^{18}F]**14** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5\ H_2O$ solution (5 µL), sodium ascorbate solution (5 µL) and BPDS solution (20 µL). Semi-preparative HPLC was performed in a TRACERlab™ FXFDG module on a Macherey-Nagel 25 x 250 mm Nucleosil 7C18 column using a gradient of MeCN (10→80%) in aqueous phosphate buffer (10 mM, pH 6.8). [^{18}F]**14** was afforded in 68% RCY d.c. for the CuAAC, a RCP of 98% and an A_m of 102 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 2.4 GBq.

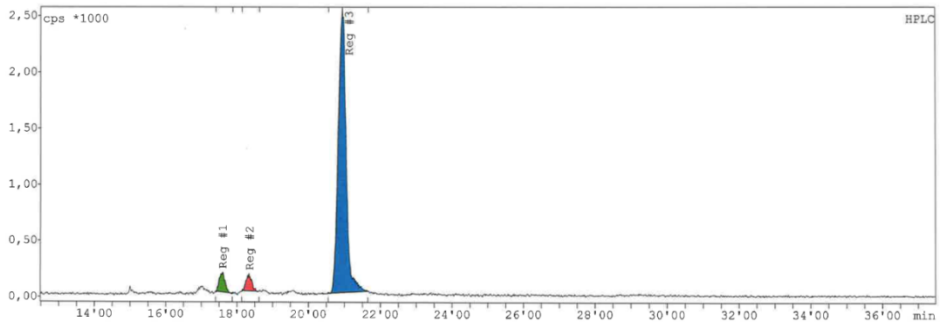


Figure S62. Semi-preparative HPLC chromatogram for [^{18}F]**14** (R_t = 21 min).

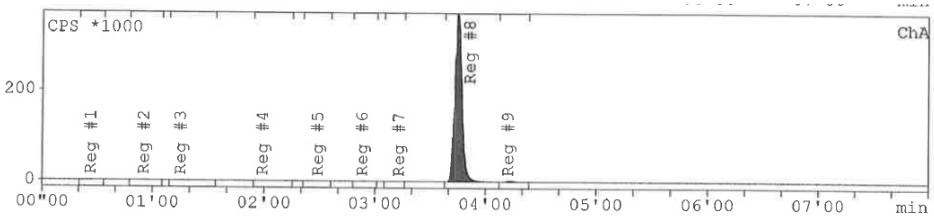


Figure S63. Analytical radio-HPLC chromatogram for Tz [^{18}F]**14** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

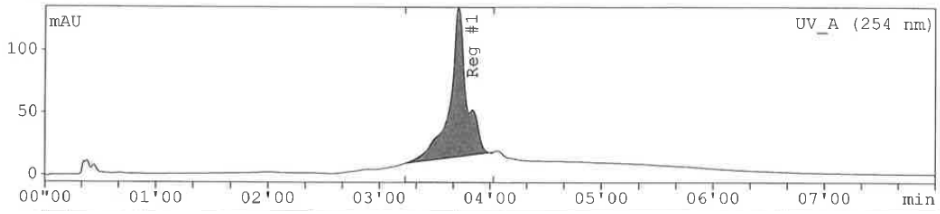
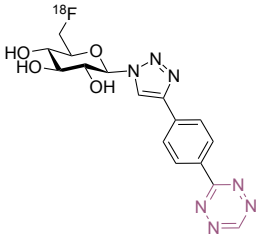


Figure S64. Analytical HPLC chromatogram for ^{19}F-reference, Tz **14** on a Zorbax Extend C18 column (100 x 4.6 mm) from Agilent using a gradient of MeCN in aqueous phosphate buffer (10 mM, pH 6.8). Flow rate: 1.4 mL/min.

(2R,3R,4S,5S,6S)-2-(4-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-1H-1,2,3-triazol-1-yl)-6-([^{18}F]fluoromethyl)-tetrahydro-2H-pyran-3,4,5-triol ([^{18}F]19)



[^{18}F]**19** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5\ H_2O$ solution (10 µL), sodium ascorbate solution (10 µL) and BPDS solution (40 µL). Semi-preparative HPLC was performed in a Scansys module on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (70/35/0.1 v/v/v) as eluent. Tz [^{18}F]**19** was afforded in 59% RCY d.c. for the CuAAC, a RCP of 98% and an A_m of 29 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 128 MBq.

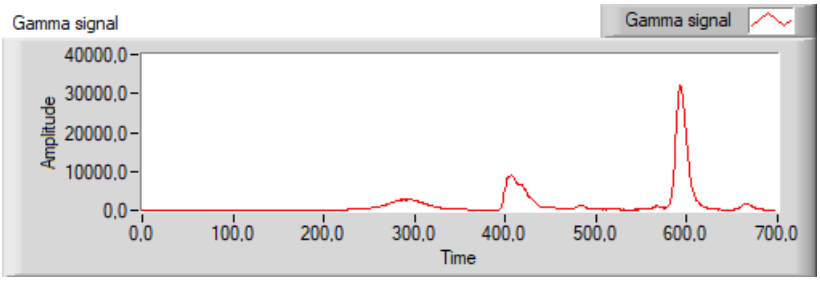
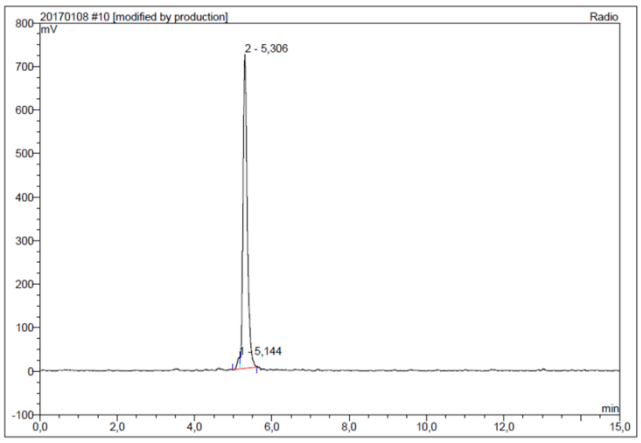
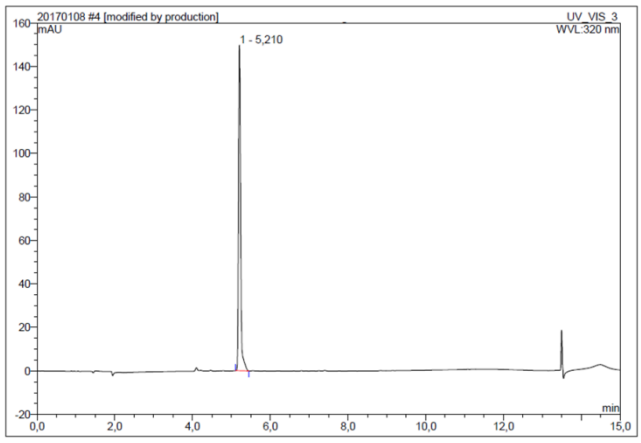


Figure S65. Semi-preparative HPLC chromatogram for [^{18}F]**19** (R_t = 600 sec).



No.	Ret.Time min	Peak Name	Height mV	Area mV*min	Rel.Area %	Amount	Type
1	5,14	n.a.	26,439	2,040	2,20	n.a.	BM *
2	5,31	n.a.	720,615	90,698	97,80	n.a.	MB*
Total:			747,054	92,738	100,00	0,000	

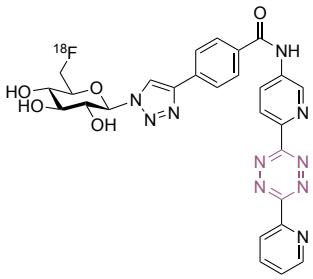
Figure S66. Analytical radio-HPLC chromatogram for Tz [^{18}F]**19** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using using a gardient of MeCN in water containing 0.1 % TFA 0–12 min 0→100% MeCN; 12–13 min 100% MeCN; 13–14 min 100→0% MeCN; 14–15 min 0% MeCN) over 15 min. Flow rate: 1.5 mL/min.



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	5,21	n.a.	149,696	9,215	100,00	n.a.	BMB*
Total:			149,696	9,215	100,00	0,000	

Figure S67. Analytical HPLC chromatogram for ^{19}F-reference, Tz **19** on a Luna 5µ C18(2) 100 Å column (150 × 4.6 mm) from Phenomenex Inc. using using a gardient of MeCN in water containing 0.1 % TFA 0–12 min 0→100% MeCN; 12–13 min 100% MeCN; 13–14 min 100→0% MeCN; 14–15 min 0% MeCN) over 15 min. Flow rate: 1.5 mL/min.

4-(1-((2R,3R,4S,5S,6S)-6-([^{18}F]fluoromethyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)benzamide ([^{18}F]27)



An aqueous solution of $CuSO_4 \cdot 5\ H_2O$ (100 mg/mL, 20 µL) was mixed with an aqueous solution of sodium ascorbate (300 mg/mL, 20 µL) and the black mixture was kept at room temperature until the color changed to canary yellow. Afterwards a solution of BPDS in water (50 mg/mL, 80 µL) was added which caused a color change to green. To the catalyst was added 1 mg Tz-alkyne **II** (2.6 µmol) in 0.1 ml DMF and [^{18}F]**Az3** (6.623 GBq) in 1000 µL sodium acetate buffer. The mixture was kept for 13 minutes at 50 °C and for additional 10 minutes at room temperature, diluted with 2 mL DMSO/H_2O = 1:1 and purified by HPLC. (C18, 10-80% MeCN in 10 mM phosphate buffer pH = 6.8). The collected fraction was loaded onto a Waters SepPak tc18 plus cartridge (preconditioned with 5 mL MeCN followed by 15 mL water) and eluted with 1 mL ethanol. Volatiles were removed *in vacuo,* and the product (818 MBq, 17.8% decay corrected radiochemical yield) was formulated with 0.9% saline.

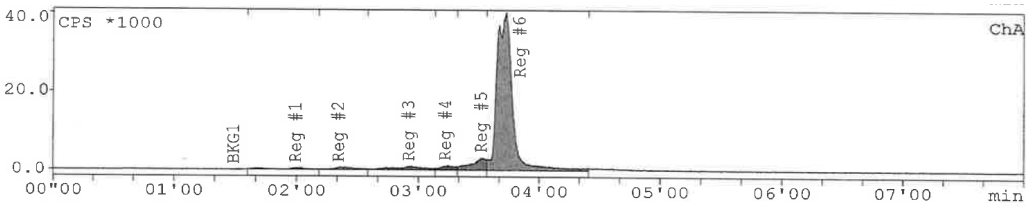
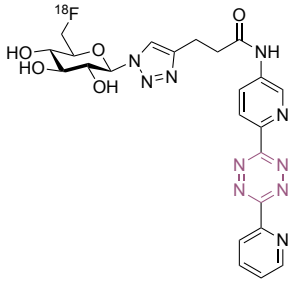


Figure S68. Analytical radio-HPLC chromatogram for Tz [^{18}F]**27.** HPLC analysis was performed on a 1200 series system (Agilent Technologies) using a Zorbax SB-AQ (5 µm, 4.6 x 250 mm) column and H2O/MeCN gradient elution (flow rate: 1.2 mL/min, 0-2 min: 5% MeCN, 2-10 min: 5 to 20% MeCN linear gradient, 10-16 min: 20 to 90% MeCN linear gradient). For radio-HPLC a GABI* radioactivity detector (Raytest) was used.

3-(1-((2R,3R,4S,5S,6S)-6-([^{18}F]Fluoromethyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)-N-(6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)propanamide ([^{18}F]45)



[^{18}F]**45** was prepared according to the aforementioned procedure using $CuSO_4 \cdot 5H_2O$ solution (10 µL), sodium ascorbate solution (10 µL) and BPDS solution (40 µL). The mixture was heated at 120 °C for 10 min and then left to cool for 3 min. Semi-preparative HPLC was performed on a Luna 5µ C18(2) (100Å 250 x 10 mm) column using water/MeCN/TFA mixture (80/20/0.1 v/v/v) as eluent. Tz [^{18}F]**45** was afforded in 14.6% RCY d.c. for the CuAAC, a RCP of 99% and an A_m of 151.4 GBq/µmol at the end of the synthesis. Isolated radioactivity amount was 428 MBq.

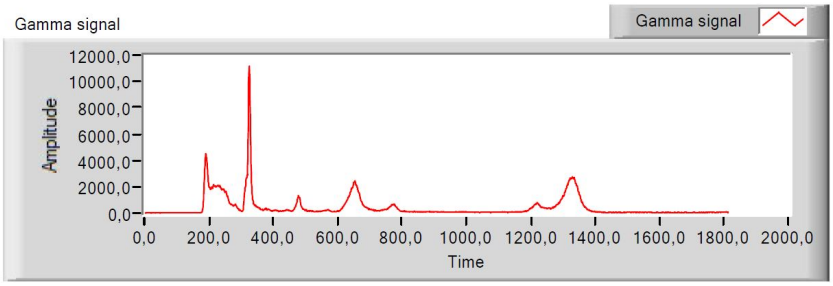


Figure S69. Semi-preparative HPLC chromatogram for [^{18}F]**45** (R_t = 1300 sec).

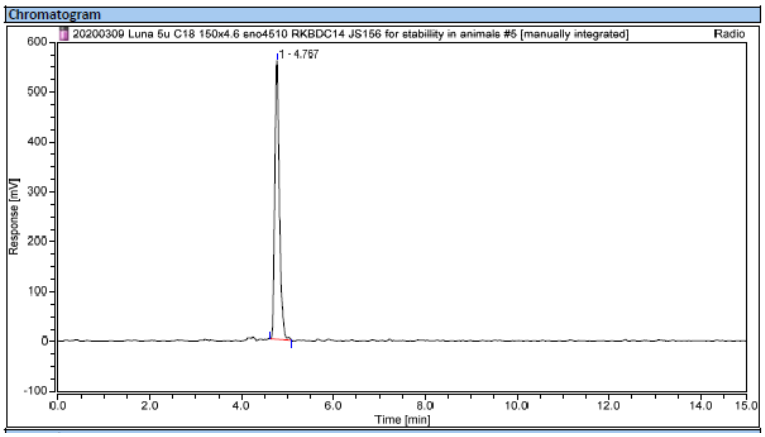


Figure S70. Analytical radio-HPLC chromatogram for Tz [^{18}F]**45**

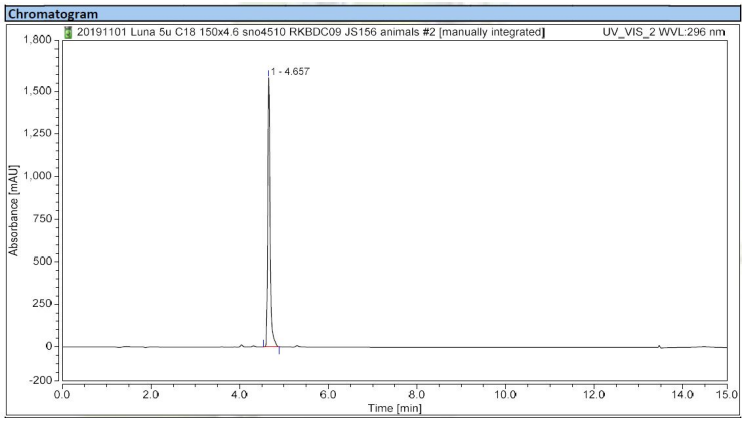


Figure S71. Analytical HPLC chromatogram for ^{19}F-reference, Tz **45**

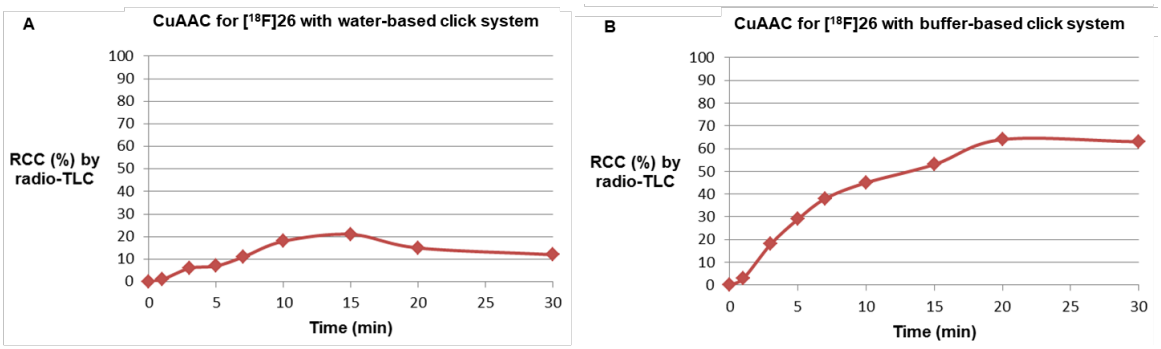


Figure S72. CuAAC-radiolabeling for the synthesis of [^{18}F]**26**; conditions: $CuSO_4 \cdot 5\ H_2O$/sodium ascorbate/BPDS. (**A**) RCC over time in water. (**B**) RCC over time in KH_2PO_4/K_2HPO_4 buffer (pH 7). RCCs were determined by radio-TLC from aliquot labeling.

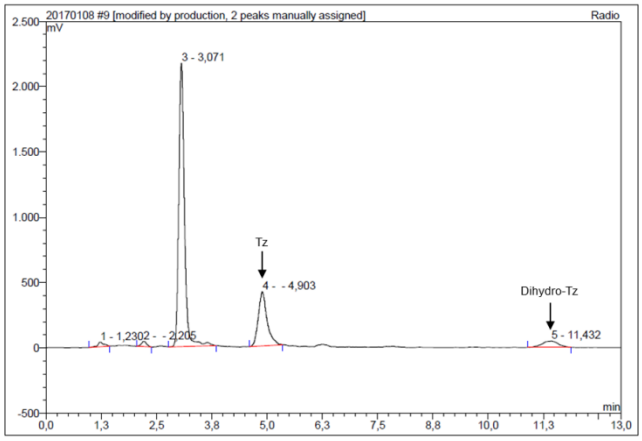


Figure S73. Radio-HPLC chromatogram for the CuAAC between Tz **I** and [^{18}F]**Az2**. Both [^{18}F]**44** and the respective dihydro-Tz are formed.

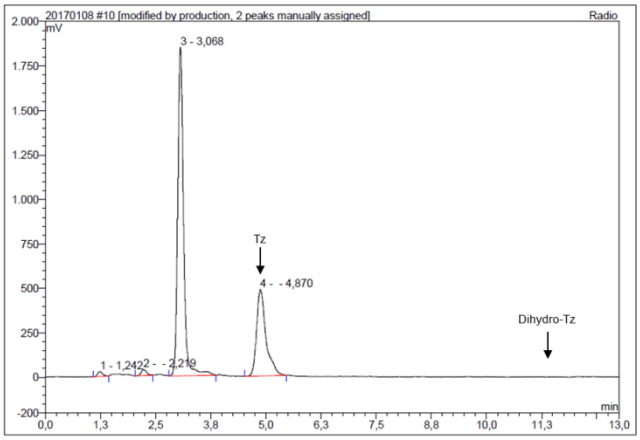


Figure S74. Radio-HPLC chromatogram for the CuAAC between Tz **1** and [^{18}F]**Az2** after addition of 4,5-dichloro-3,6-dioxocyclohexa-1,4-diene-1,2-dicarbonitrile (DDQ) confirming oxidation of the dihydro-Tz to [^{18}F]**44**.

5. *In vivo* Stability

To assess the *in vivo* stability of ^{18}F-labeled tetrazines, blood samples (n = 3–4 for each group) were taken and worked up as illustrated in Figure S75. The fraction of intact Tz was estimated by adding a TCO-modified nano-sized agent (TCO-modified polyglutamic acid[18] or TCO-modified mesoporous silica nanoparticles[16]) to the sample, and thereafter analyzed by radio-TLC. Intact Tz-derivatives, but also potential metabolites, which do contain an intact Tz-scaffold, will react with the TCO-moieties of the nano-sized agent and thus no longer appear at the according R_f value. The percentage of intact Tz-scaffold was determined by comparing the Tz-region of the TLC-plate before and after addition of TCO-modified nano-sized agent. No correlation was observed between Tz stability and blocking effect (Figure S76).

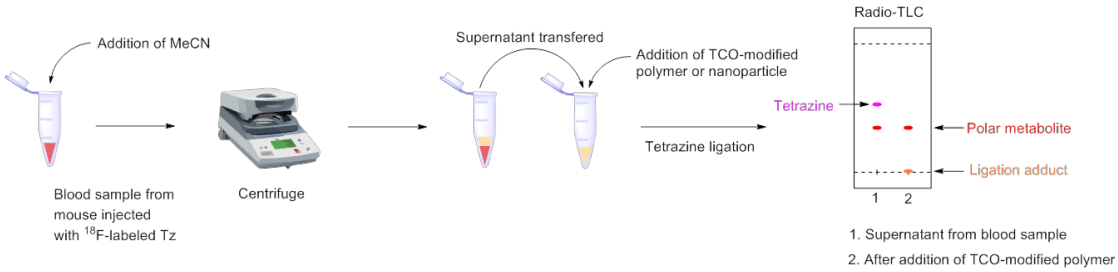


Figure S75. Schematic illustration of stability studies of ^{18}F-labeled Tz-derivatives. Blood samples were taken at 30 min p.i. (n = 3–4 for each group) of the ^{18}F-labeled Tz. Thereafter, MeCN was added and the samples were centrifuged, and the supernatant was transferred to another vial. An aliquot (5–10 µL) was spotted on a TLC-plate. To the vial with the supernatant, TCO-modified polymer or nanoparticle was added (100 µg/10 µL). After 5 min at room temperature, an aliquot (5–10 µL) was spotted on the TLC-plate. The plate was developed in an appropriate eluent (depending on the R_f of the Tz). Any intact Tz-scaffold would have been reacted with the polymer/nanoparticle and this ligation adduct will stay on the baseline of the plate. Thus, by comparing the radioactivity fraction in the Tz-region of the TLC-plate before and after addition of TCO-agent, the fraction of intact Tz-scaffold was estimated.

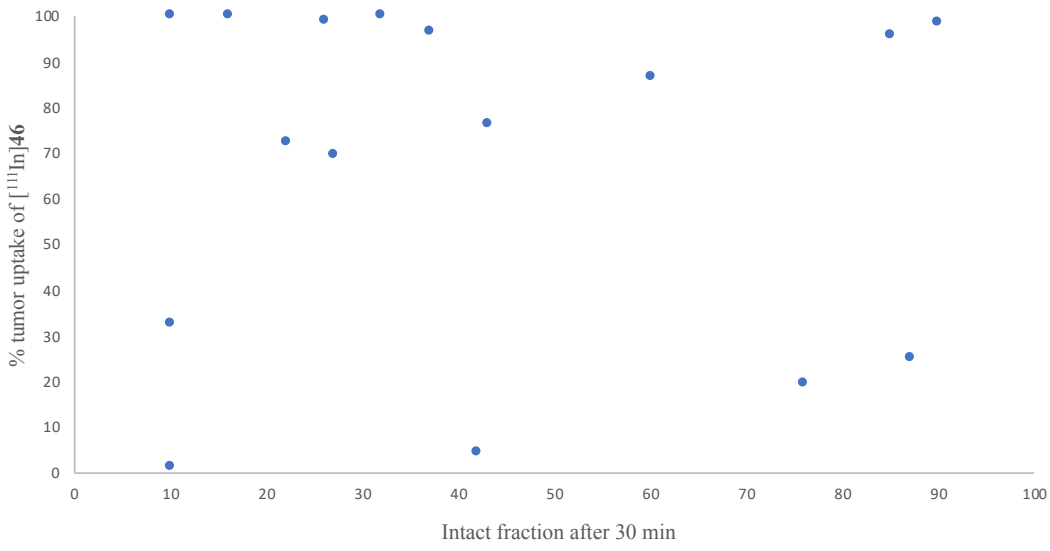


Figure S76. No correlation was observed between the measured *in vivo* stability of ^{18}F-labeled Tz and the blocking effect of the respective non-radiolabeled analog.

6. Pretargeted PET imaging

Tumor xenografts in mice were established as previously described (see blocking study). Tumor-bearing animals were matched into groups based on their tumor volume (tumor volumes of ~ 60–180 mm^3, n = 3-4 in each group) and were administered with CC49-TCO (100 µg/100 µL) per mouse. After 72 h, the animals were injected via the tail vein with ^{18}F-labeled Tz (5–10 MBq/100 µL). The tracer was allowed to distribute and the mice were PET/CT scanned (Inveon, Siemens Medical Solutions), 1 h p.i. of the ^{18}F-labeled Tz (PET acquisition: 5 min, energy window of 350–650 KeV and a time resolution of 6 ns; CT scan: 360 projections, 65 kV, 500 µA and 400 ms). During scans the animals were anaesthetized by breathing sevoflurane (3%) and the body temperature was kept stable using a heating pad. Sinograms from PET scans were reconstructed using a 3-dimensional maximum a posteriori algorithm with correction for scatter and attenuation (CT-based). PET and CT images were co-registered and analyzed using Inveon Research Workplace software (Siemens Medical Solutions). The mean percentage of injected dose per grams (%ID/g) in different tissues was extracted by manually creating regions of interest (ROI) on fussed PET/CT images.

Table S3. Uptake values (mean %ID/g ± S.D.) from tumor, heart and muscle tissue 1 h pi. Pretargeting was performed 72 h after administration of CC49-TCO and control animals were instead injected with saline. n = 4 mice/group for all compounds (except [^{18}F]**3**; n = 3)

	CC49-TCO	Tumor (% ID/g)	Heart (% ID/g)	Muscle (% ID/g)
[^{18}F]**1**	+	2.7 ± 0.4	3.6 ± 0.4	2.5 ± 0.3
	-	3.1 ± 0.2	4.1 ± 0.2	2.8 ± 0.2
[^{18}F]**3**	+	1.8 ± 1.1	2.2 ± 1.2	0.5 ± 0.2
	-	0.6 ± 0.03	0.43 ± 0.1	0.2 ± 0.1
[^{18}F]**19**	+	2.1 ± 0.5	2.8 ± 0.6	0.5 ± 0.1
	-	0.5 ± 0.2	0.5 ± 0.2	0.2 ± 0.1
[^{18}F]**26**	+	2.3 ± 0.3	3.0 ± 0.2	1.5 ± 0.2
	-	1.9 ± 0.4	3.0 ± 0.4	1.2 ± 0.1
[^{18}F]**44**	+	1.5 ± 0.5	2.5 ± 0.7	0.5 ± 0.3
	-	1.5 ± 0.2	1.5 ± 0.2	0.7 ± 0.1
[^{18}F]**45**	+	1.7 ± 0.6	2.9 ± 0.4	0.4 ±0.1
	-	0.2 ± 0.2	0.3 ±0.2	0.001 ± 0.0002

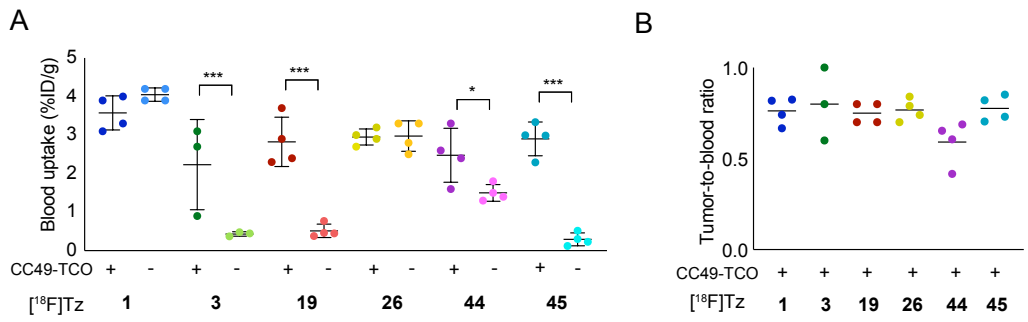


Figure S77. (A)Uptake values (mean %ID/g ± S.D.) from heart tissue (surrogate for blood) and (B) tumnot-to-blood ratios 1 h pi. Pretargeting was performed 72 h after administration of CC49-TCO or saline (control).

Table S4. Mean tumor to blood (T/B) and tumor to muscle (T/M) ratios 1 h pi from pretargeting with [^{18}F]Tzs 72 h after administration of CC49-TCO. Tumor to tumor control (T/T_c) ratios is the mean tumor uptake in mice pretreated with CC49-TCO compared to control animals without pretreatment (saline). $n = 4$ mice/group for all compounds (except [^{18}F]**3**; $n = 3$)

	T/B ratio	**T/M ratio**	**T/T$_c$ ratio**
[^{18}F]**1**	0.76	1.1	0.9
[^{18}F]**3**	0.80	3.6	3.0
[^{18}F]**19**	0.75	4.6	4.2
[^{18}F]**26**	0.77	1.5	1.2
[^{18}F]**44**	0.59	2.3	1.0
[^{18}F]**45**	0.78	4.8	8.5

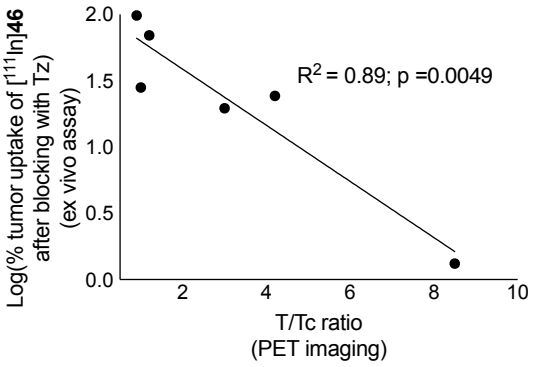


Figure S78. Correlation between the blocking effect of the unlabeled Tz-derivatives **1**, **3**, **19**, **26**, **44**, and **45** and the T/T_c-ratios for the corresponding ^{18}F-labeled compounds observed by *in vivo* pretargeted PET imaging.

Table S5. Evaluation of the effect of different Tz/TCO ratios. Uptake values (mean % ID/g ± S.D.) from tumor, heart and muscle tissue**.** tumor to blood (T/B) and tumor to muscle (T/M) ratios 1 h pi of [^{18}F]Tzs. Pretargeting was performed 72 h after administration of CC49-TCO. Image derived uptake values from the heart was used as surrogate for blood for the T/B ratio.. [^{18}F]**3**; *n* = 3 mice/group and [^{18}F]**45** *n* = 4 mice/group.

	Tz/TCO ratio	CC49-TCO	Tumor (% ID/g)	Heart (% ID/g)	Muscle (% ID/g)	T/B ratio	T/M ratio
[^{18}F]**3**	**50:1**	+	1.8 ± 1.1	2.2 ± 1.2	0.5 ± 0.2	0.80	3.6
	1:1	+	1.9 ± 0.7	2.0 ± 0.1	0.3 ± 0.1	0.90	6.3
[^{18}F]**45**	**50:1**	+	1.7 ± 0.6	2.9 ± 0.4	0.4 ± 0.1	0.78	4.8
	1:1	+	1.3. ± 0.3	1.7 ± 0.4	0.3 ± 0.1	0.76	4.3

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 (GraphPad Software). The relationship between blocking effect of tetrazines with physicochemical parameters were fitted using an exponential growth function. Persson correlation was used to evaluate the relationship. Differences in tissue uptake values between animals pretreated with CC49-TCO and controls (saline) were compared with one-way ANOVA with Sidak post-hoc test. The relationship between blocking effect of tetrazines and uptake of corresponding ^{18}F-labeled tetrazines in PET imaging was evaluated using linear regression. All results were considered statistically significant when p-value < 0.05.

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