Multi-functionalised graphene nanoflakes as tumour-targeting theranostic drug-delivery vehicles

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Methods and Materials

General

Carboxylated graphene nanoflakes (GNFs) were prepared as previously reported.[1,2] Unless otherwise stated, all other chemicals were of reagent grade and purchased from SigmaAldrich (St. Louis, MO), Merck (Darmstadt, Germany), Tokyo Chemical Industry (Eschborn, Germany), abcr (Karlsruhe, Germany) or CheMatech (Dijon, France). Water (>18.2 MΩ·cm at 25 ºC, Puranity TU 3 UV/UF, VWR International, Leuven, Belgium) was used without further purification. Solvents for reactions were of reagent grade, and where necessary, were dried over molecular sieves. Evaporation of the solvents was performed under reduced pressure by using a rotary evaporator (Rotavapor R-300, Büchi Labortechnik AG, Flawil, Switzerland) at the specified temperature and pressure.

^{1}H and ^{13}C NMR spectra were measured in deuterated solvents on a Bruker AV-400 (^{1}H: 400 MHz, ^{13}C: 100.6 MHz) or a Bruker AV-500 (^{1}H: 500 MHz, ^{13}C: 125.8 MHz) spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm) relative to the resonance of the residual solvent peaks, for example, with DMSO δ_H = 2.50 ppm and δ_C = 39.5 ppm with respect tetramethylsilane (TMS, δ_H and δ_C = 0.00 ppm). Coupling constants (*J*) are reported in Hz. Peak multiplicities are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad).

High-resolution electrospray ionisation mass spectra (HR-ESI-MS) were measured by the mass spectrometry service at the Department of Chemistry, University of Zurich.

Column chromatography was performed by using Merck silica gel *60* (63 – 200 μm) with eluents indicated in the experimental section. Standard thin-layer chromatography (TLC) for synthesis employed Merck TLC plates silica gel *60* on an aluminium base with the indicated solvent system. The spots on TLC were visualised either by UV/visible light (254 nm) or by staining with $KMnO_4$.

Electronic absorption spectra were recorded using a Nanodrop^{TM} One^{C} Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, supplied by Witec AG, Sursee, Switzerland).

Statistical analysis

Where appropriate, data were analysed by the unpaired, two-tailed Student's *t*-test. Differences at the 95% confidence level (*P*-value <0.05) were considered to be statistically significant.

Radioactivity

All instruments for measuring radioactivity were calibrated and maintained in accordance with previously reported routine quality control procedures.[3] [^{68}Ga][Ga(H_2O)$_6$]Cl_3(aq.) was obtained from ^{68}Ge/^{68}Ga-generators (Eckert&Ziegler, Model IGG100 Gallium-68 Generator), eluted with 0.1 M HCl(aq.). The eluted ^{68}Ga activity was trapped and purified by using a strong cation exchange column (Strata-XC, [SCX], Eckert&Ziegler). [^{68}Ga][Ga(H_2O)$_6$]Cl_3(aq.) was eluted from the SCX cartridge by using a solution containing 0.13 M HCl(aq.) and approx. 5 M NaCl(aq.) (SCX eluent). The generator gave molar activities in the range 197 ± 4.82 MBq/nmol. For radiolabelling experiments the ^{68}Ga stock solution was typically added as the limiting reagent to an aqueous reaction mixture buffered with NaOAc (approx. 0.2 M, pH4.4). Radioactive reactions were monitored by using instant thin-layer chromatography (iTLC). Glass-fibre iTLC plates impregnated with silica-gel (iTLC-SG, Agilent Technologies) were developed in water (>18.2 MΩ·cm) and analysed on a radio-TLC detector (SCAN-RAM, LabLogic Systems Ltd, Sheffield, United Kingdom). Radiochemical conversion (RCY) was determined by integrating the data obtained by the radio-TLC plate reader and determining both the percentage of radiolabelled [^{68}Ga]GNF-1-4 (Rf = 1.0) and 'free' 68Ga (Rf = 0.0). Integration and data analysis were performed by using the software Laura version 5.0.4.29 (LabLogic).

Samples were analysed using size-exclusion chromatography (SEC). An automated size-exclusion column (Bio-Rad Laboratories, ENrich SEC 70, 10 ± 2 µm, 10 mm ID x 300 mm) connected to a HPLC device (Rigol L-3000, Contrec AG, Switzerland) equipped with a UV/visible detector (absorption measured at 400 nm) as well as a radioactivity detector (FlowStar2 LB 514, Berthold Technologies, Zug, Switzerland). Isocratic elution with phosphate buffered saline (PBS, pH7.4) with 200 mM arginine was used.

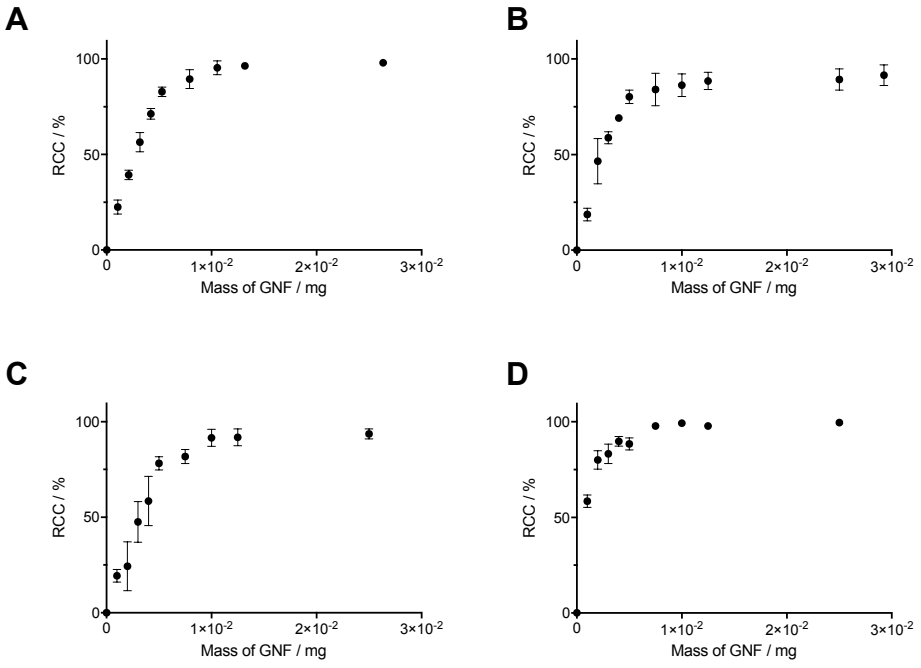
General Radiolabeling Conditions

Radiolabelling reactions to prepare [^{68}Ga]**GNF-1**, **-2**, **-3**, and **-4** were accomplished by addition of an aliquot of [^{68}Ga][Ga(H_2O)$_6$]Cl_3(aq.) stock solution (ca. 40 MBq) to an aqueous solution of GNF-1-4 (25 μg) buffered with NaOAc (0.2 M, pH 4.4). The reaction was monitored by using radio-iTLC and was found to be complete after 10 min. at 21 ^{o}C giving a radiochemical conversions (RCC) >97% (R_f [silica gel, H_2O] = 0.90-1.00).

Quantification of DFO content

[^{68}Ga]**GNF-1**, **-2**, **-3**, and **-4** samples were prepared at a variety of concentrations and buffered with NaOAc (0.2 M, pH 4.4). Aliquots of [^{68}Ga][Ga(H_2O)$_6$]Cl_3(aq.) stock solution (ca. 18 MBq) were added to each sample and after 10 min radio-iTLC was used to gain a RCC. Plots were used in conjunction with the known molar activity of the generator to calculate the amount of DFO (nmol) per mass of GNF (mg).

Figure S1. Plots showing RCY when ^{68}Ga is titrated with varying concentrations of A) **GNF-1**, B) **GNF-2**, C) **GNF-3**, D) **GNF-4**



Distribution coefficient

n-Octanol was pre-saturated with PBS for 24 h. Reactions were prepared as described above. Reaction mixture (50 μL) was added to pre-saturated octanol (400 μL) and PBS (350 μL) and shaken for 1 h at 37 °C. Samples were then centrifuged (2000 RPM, 2 min), an aliquot of 50 μL was taken from each phase and the radioactivity of aliquots quantified with the gamma counter. Experiments were performed in triplicate.

Stability Studies

Stability in saline and PBS

Reactions were prepared as previously described and then PBS or saline (300 μL) were added and samples incubated (37 °C). RCP was monitored *via* radio-TLC at time points up to 2 h (*n*=3, Table S1, Table S2).

Table S1. Percentage radiochemical purity (RCP) of [^{68}Ga]**GNF-1-4** determined from radio-iTLC following incubation with PBS up to 2 h at 37 °C.

	RCP / %			
Time / min	[^{68}Ga]**GNF-1**	[^{68}Ga]**GNF-2**	[^{68}Ga]**GNF-3**	[^{68}Ga]**GNF-4**
0	99.1 ± 0.7	98.6 ± 0.2	97.4 ± 0.6	97.3 ± 0.4
10	95.2 ± 0.3	97.0 ± 0.3	96.8 ± 0.4	88.1 ± 1.1
20	95.1 ± 1.5	95.3 ± 1.0	90.4 ± 3.5	85.8 ± 2.4
30	96.5 ± 0.6	93.0 ± 0.9	94.5 ± 0.4	88.7 ± 2.3
60	96.5 ± 0.9	92.8 ± 0.3	93.5 ± 0.4	90.7 ± 1.4
120	95.0 ± 0.4	89.3 ± 1.6	93.7 ± 0.5	89.0 ± 0.8

Table S2. Percentage radiochemical purity (RCP) of [^{68}Ga]**GNF-1-4** determined from radio-iTLC following incubation with saline up to 2 h at 37 °C.

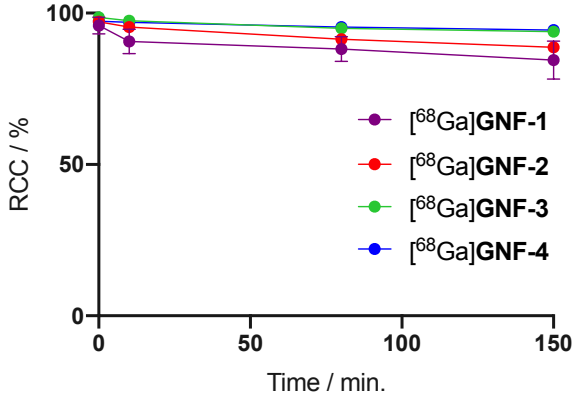
	RCP / %			
Time / min	[^{68}Ga]**GNF-1**	[^{68}Ga]**GNF-2**	[^{68}Ga]**GNF-3**	[^{68}Ga]**GNF-4**
0	96.6 ± 0.8	98.6 ± 0.2	97.4 ± 0.6	97.3 ± 0.4
10	96.0 ± 2.9	96.6 ± 0.2	93.8 ± 1.0	92.9 ± 0.6

20	91.8 ± 1.5	94.1 ± 1.1	91.5 ± 1.2	89.2 ± 2.8
30	94.7 ± 5.7	89.9 ± 2.4	92.6 ± 2.2	85.0 ± 1.9
60	95.4 ± 1.2	92.1 ± 0.5	88.6 ± 2.2	82.1 ± 1.2
120	95.1 ± 1.0	90.1 ± 0.8	87.6 ± 1.9	83.2 ± 2.2

Stability in human serum albumin

Reactions were prepared as described previously, human serum albumin (300 µL) was added and samples incubated (37 °C). Stability in human serum was monitored *via* radio-SEC (mobile phase: 200 mM arginine in PBS) for time points up to 2.5 h ([^{68}Ga]**GNF-1**: n = 3, [^{68}Ga]**GNF-2**, **-3** and **-4**: n = 1, Figure S1).

Figure S2. Plot of the change of percentage radiochemical purity (RCP) of [^{68}Ga]**GNF-1** to **-4** determined from radio-SEC following incubation at 37 °C with human serum albumin for 2.5 h.



Cell Culture

The human prostate cancer cell line LNCaP clone FCG (PSMA/positive, American Type Culture Collection (ATCC® CRL-1740™), Manassas, VA) was used. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in RPMI Medium 1640 containing [+]-L-glutamine (2.5 mM), supplemented with fetal bovine serum (FBS, 10% (v/v), ThermoFisher Scientific) and penicillin/streptomycin (P/S, 1% (v/v) of penicillin 10000 U/mL and streptomycin 10 mg/mL). Cells were grown by serial passage and were harvested by using trypsin (0.25%).

The human prostate cancer cell line PC-3 (American Type Culture Collection (ATCC® CRL-1435™), Manassas, VA) was used. Cells were cultured at 37 °C in a humidified 5% CO_2

atmosphere in DMEM/F12 (1:1) (Dulbecco's Modified Eagle Medium, F-12 Nutrient mixture (Ham), ThermoFisher Scientific, Schlieren, Switzerland) medium containing [+]-L-glutamine (2.5 mM), supplemented with fetal bovine serum (FBS, 10% (v/v), ThermoFisher Scientific) and penicillin/streptomycin (P/S, 1% (v/v) of penicillin 10000 U/mL and streptomycin 10 mg/mL). Cells were grown by serial passage and were harvested by using trypsin (0.5%).

In vitro cell proliferation assays

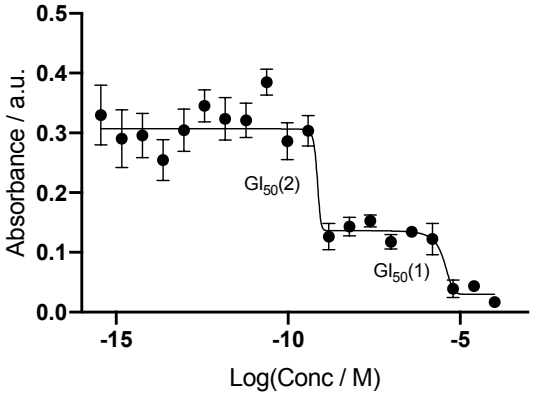
*Treatment with **GNF-2-4***

Cells were plated in 96-well plates with a density of 3000 cells/well in 150 μL media. After 24 h cells were treated with 50 μL compound (1:2 or 1:3 dilution in cell media, 0.25 mg mL^{-1} to 1 pg mL^{-1}) and plates incubated for 48 h then treated with thiazolyl blue tetrazolium bromide (50 μL, 5.0 mg mL^{-1} soln. in PBS). Following 3 h incubation in darkness, media was removed, DMSO (100 μL/well) added and after 30 min absorbance was measured at 570 nm (Hidex Sense platereader). Experiments were performed in triplicate.

Treatment with (R)-ispinesib

Experimental procedure was similar to the above with the addition of 0.2% DMSO in cell media during the treatment period.

Figure S3. Cell proliferation (MTT assay) biphasic curve with GI_{50} values calculated using GraphPad Prism.



GI_{50}(1): 3.32 ± 2.31 μM
GI_{50}(2): 0.70 ± 26.7 nM

Cell Binding Assay

Cells were harvested and distributed in Eppendorf tubes (3 x 10^6 / vial) in media (270 μL) or media with sodium azide (270 μL, 0.1%). Reactions were prepared as previously described and diluted (40-fold) in cell media and then added (30 μl, 75 kBq) to the prepared cells. After mixing for 1.5 h at 37 ^oC, the samples were centrifuged (2000 rpm, 3 min) and the cell pellet washed with ice-cold PBS (2 x 1 mL) keeping the samples on ice between washes. The radioactivity associated with each sample was quantified using the gamma counter. Experiments were performed in triplicate.

Blocking Assay

LNCaP cells were plated in 6-well plates with a density of 8 x 10^5 cells/well in 2 mL media. After 24 h cells, media was removed, cells washed with PBS (2 mL) and the media replaced (1.8 mL), with the blocked wells containing media with 5 μM PSMA binding motif. Cells were then incubated for 1 h. Reactions were prepared as described above, diluted in cell media (55-fold) and added to wells (200 μL, 1.4 MBq) then plates incubated for 1.5 h. Plates were then placed on ice and each well washed with ice-cold PBS (2 x 1 mL), 1 M NaOH (1 mL) added and plates incubated for 10 min. Cells were then collected and their associated radioactivity quantified with the gamma counter. Experiments were performed in triplicate.

Confocal Microscopy

Cells were plated in 4 well plates with a density of 8000 cells/well. After 24 h, cells were treated with 1 μg ml^{-1} GNF (**GNF**, **GNF-2**, **-3** or **-4**) and left for 24 h. The media was then removed, wells washed with PBS (3 x 600 μL) and incubated with fixative solution (600 μL, 4% formaldehyde in PBS) for 15 min. Wells then washed with PBS (3 x 600 μL), incubated with permeabilisation buffer (500 μL, x0.2) for 15 min, washed with PBS (3 x 600 μL) then incubated with 2 drops of Imageit FX. After 30 min, wells washed with PBS (3 x 600 μL), incubated with primary antibody (400 μL, mouse α-tubulin, 1:1000 dilution in blocking buffer) for 2 h, washed with PBS (3 x 600 μL) and incubated with secondary antibody (400 μL, Goat α-mouse Alexa Flour 568 α-tubulin staining, 1:2000 dilution in blocking buffer) for 1.5 h. Following washing with PBS (3 x 600 μL) cells were incubated with Hoeschst 33342 (600 μL, 1:100,000 dilution in

H_2O) for 10 min followed by a final washing with H_2O (3 x 600 µL). Slides were drained, dried and wells removed, 1 drop Prolong Gold added and cover slips held in place with nail polish.

Preparation of blocking buffer

A solution of PBS containing 1% BSA and 0.3% Tween-20 was prepared.

Preparation of permeabilisation buffer

A 10x permeabilisation buffer was prepared from 1.54 M NaCl, 15.44 mM KH_2PO_4, 28.58 mM $Na_2HPO_4.7H_2O$ and 5% Triton X-100 in 5 mL H_2O. 40 mL of H_2O added, followed by adjustment of pH to 7.2 before making up to 50 mL creating the 1x permeabilisation buffer. This was stored in the fridge and diluted when needed.

Flow Cytometry

Cells were plated onto 100 mm^2 petri dishes at a cell density of 1.0 x 10^6 cells/dish. After 24 h cells were treated with 12 ng mL^{-1} and 120 ng mL^{-1} of the compounds (**GNF**, **GNF-2, GNF-3**, **GNF-4**) for 36 h, then washed with ice-cold PBS (4 mL), detached with trypsin-EDTA (2 mL, 0.25%) and collected *via* centrifugation (100 xg, 10 min) with ice-cold PBS (10 mL). Cell pellet was washed with ice-cold PBS (5 mL) then re-suspended in fixation solution (4 mL, 70 vol% EtOH, 30 vol% H2O) and stored at -20^{o}C for 24 h. Subsequently, ice-cold PBS was added and cells centrifuged (100 xg, 10 min), washed with PBS (10 mL) and re-suspended in staining solution (1 mL) consisting of 0.1 mg/mL RNAse A (Sigma Aldrich), 50 µg/mL propidium iodide (PI) from 2.5 mg/mL stock solution and 0.05% Triton X-100 (Sigma Aldrich) in PBS. After incubation at 37 ^{o}C for 40 min the cells were collected by centrifugation (100 xg, 10 min), washed with PBS (10 mL), re-suspended in PBS (2 mL) and samples then analysed on a BD LSRFortessa cytometry system. Data were analysed by cell-cycle analysis software (Flowjo, TreeStar Inc.).

Zeta Potential

ζ potential of each GNF solution was measured by Zetasizer Nano ZS using a disposable capillary zeta cell. ζ potential measurements consist of 20 runs ($n=3$).

Xenograft models

All experiments involving mice were conducted in accordance with an animal experimentation licence approved by the Zurich Canton Veterinary Office, Switzerland (Jason P. Holland).

Experimental procedures also complied with guidelines issued in the Guide for the Care and Use of Laboratory Animals.[4] Male athymic nude mice (Crl:NU(NCr)-Foxn1nu, 27 – 35 g, 6 – 8 weeks old) were obtained from Charles River Laboratories Inc. (Freiburg im Breisgau, Germany) and were allowed to acclimatise at the University of Zurich Laboratory Animal Services Center vivarium for at least 1 week prior to implanting tumour cells. Mice were provided with food and water ad libitum. Tumours were induced on both shoulders by sub-cutaneous (s.c.) injection of approx. 2.5×10^6 cells. The cells were injected in a 200 µL suspension of a 1:1 v/v mixture of PBS and reconstituted basement membrane (Corning® Matrigel® Basement Membrane Matrix, obtained from VWR International).[5] Tumours developed after a period of between 6-8 weeks. Tumour volume (V / mm^3) was estimated by external Vernier caliper measurements of the longest axis, a / mm, and the axis perpendicular to the longest axis, b / mm. The tumours were assumed to be spheroidal and the volume was calculated in accordance with Equation S1.

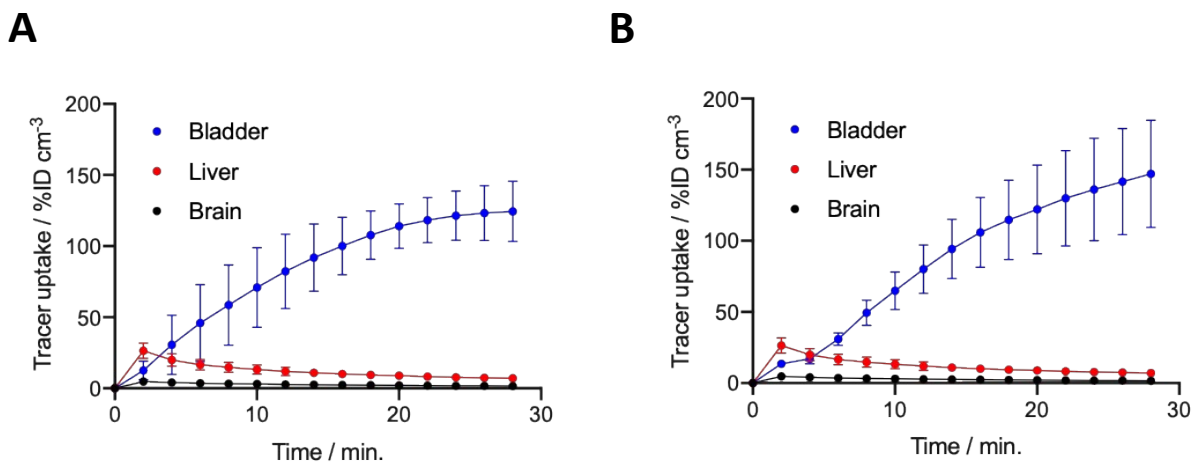
$$V = \frac{4\pi}{3} \cdot \left(\frac{a}{2}\right)^2 \cdot \left(\frac{b}{2}\right) \qquad \text{Equation S1}$$

Small-animal PET imaging

All mice injected with cancer cells developed tumours and the average volume of the LNCaP tumours was 325 ± 133 mm^3 (n = 6 mice). Mice were anesthetised by inhalation of between 2 – 4% isoflurane (AttaneTM, Piramal Enterprises Ltd, India, supplied by Provet AG, Lyssach, Switzerland)/oxygen gas mixture. PET imaging experiments were conducted on a Genesis G4 PET/X-ray scanner (Sofie Biosciences, Culver City, CA).[6] For each mouse the tail vein was warmed gently using a warm water bath, a catheter inserted in the tail vein and then placed on the scanner bed in the prone position. The dynamic scan PET scan (15x2 min scans) was commenced and at t=2 min [^{68}Ga]**GNF-3** or [^{68}Ga]**GNF-4** (~1 MBq, [~27 µCi], in 200 µL sterile PBS) was injected intravenous (i.v.) *via* the tail vein catheter. During image acquisition, the respiration rate of the animal was monitored via live video feed and anaesthesia was maintained by an experience animal experimenter by controlling the isoflurane dose between 1.5 – 2.0%. List-mode data were acquired for 15x2 min and 1x5 min using a γ-ray energy window of 150–650 keV, and a coincidence timing window of 20 ns. Images were reconstructed by iterative ordered subset maximum expectation (OSEM; 60 iterations) protocols. The reported reconstructed spatial resolution is 2.4 µL at the centre of the field-of-view (FOV). Image data

were normalised to correct for non-uniformity of response of the PET, attenuation, random events, dead-time count losses, positron branching ratio, and physical decay to the time of injection, but no scatter or partial-volume averaging correction was applied. An empirically determined system calibration factor (in units of [Bq/voxel]/[MBq/g] or [Bq/cm^3]/[MBq/g]) for mice was used to convert voxel count rates to activity concentrations. The resulting image data were normalised to the administered activity to parameterise images in terms of %ID cm^{-3} (equivalent to units of %ID/g assuming a tissue density of unity). Images were analysed by using VivoQuantTM 3.5 patch 2 software (InviCRO, Boston, MA). For image quantification and measurements of time-activity curves (TACs), 3-dimensional volumes-of-interest (VOIs) were drawn manually to determine the maximum and mean accumulation of radioactivity (in units of %ID cm^{-3} and decay corrected to the time of injection) in various tissues.

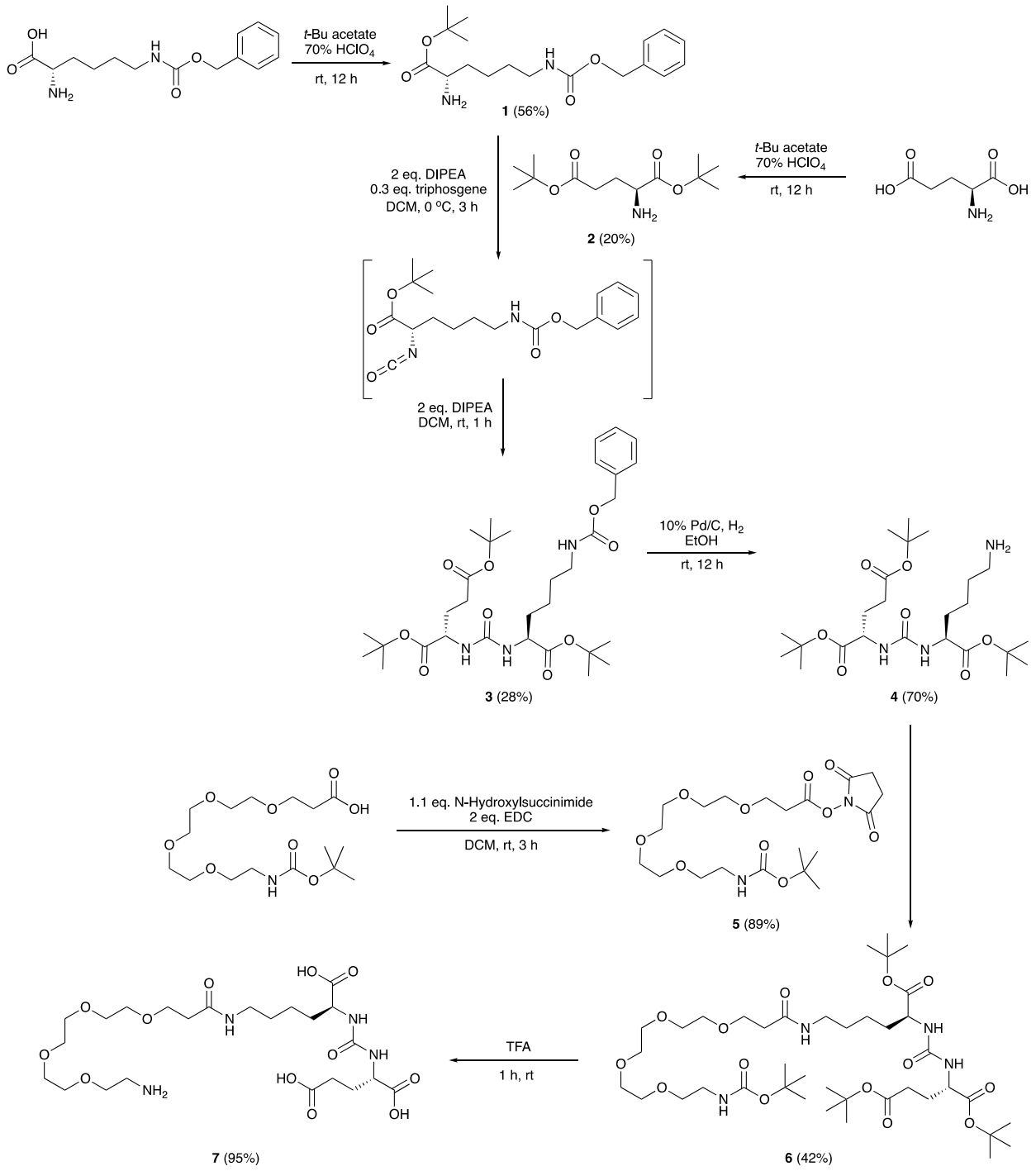
Figure S4: TACs plotted for the liver, bladder and brain with data extracted from 30 min dynamic PET analysis (15x2min scans) with injection of B) [^{68}Ga]**GNF-3** (n = 3) and C) [^{68}Ga]**GNF-4** (n = 4) at t = 2 min. VOIs were defined and data extracted using the VivoQuant™ software. See main text for kidney, tumour, muscle and heart data.



Synthesis and characterisation

Chemical synthesis of the PSMA binding motif components

Scheme S1. Chemical synthesis of PSMA binding motif (Glu-NH-C(O)-NH-Lys) and the PEG derivative (Glu-NH-C(O)-NH-Lys-NH-C(O)-PEG_4-NH_2).



Synthesis of compounds **1-4** uses modified literature procedures.[7]

*Synthesis of compound **1***

To a solution of N-CBz-L-Lysin (2.0 g, 7.1 mmol) in t-butyl acetate (20 mL), 70 % perchloric acid (1.0 mL) was added and the mixture stirred at room temperature for 20 h. Ethyl acetate (50 mL) was added and mixture extracted with water (50 mL), 0.5 M HCl (50 mL), then water (50 mL). Aqueous layers were combined followed by addition of 30% NaOH (aq.) until pH 11. Product extracted with ethyl acetate (3 x 100 mL), organic layer washed with brine, dried over anhydrous $MgSO_4$, and solvent removed under reduced pressure. Crude product was purified on silica gel (50-75% EtOAC/Hexane) to isolate compound **1** as a clear oil (1.3 g, 4.0 mmol, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.37 – 7.27 (m, 5H), 5.09 (s, 2H), 4.83 (s, 1H), 3.31 (dd, J = 7.3, 5.5 Hz, 1H), 3.20 (dd, J = 13.1, 6.6 Hz, 2H), 1.91 (s, 2H), 1.81 – 1.64 (m, 1H), 1.62 – 1.48 (m, 3H), 1.45 (s, 9H), 1.36-1.44 (m, 2H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ (ppm) 175.35, 156.47, 136.70, 128.51, 128.10, 80.99, 66.55, 54.84, 40.85, 34.50, 29.71, 28.07, 22.81. R_f (silica gel, EtOAc:Hexane 3:1) = 0.43. HRMS (ESI+) (m/z): calculated for $C_{18}H_{29}N_2O_4^+$ ($[M+H]^+$): 337.4395; found: 337.2120 (100%).

Figure S5. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **1**.

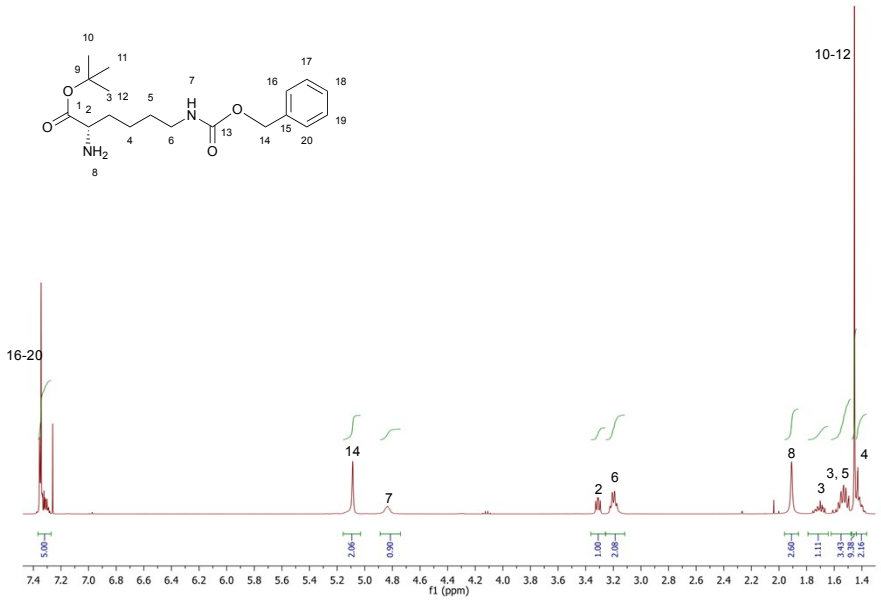
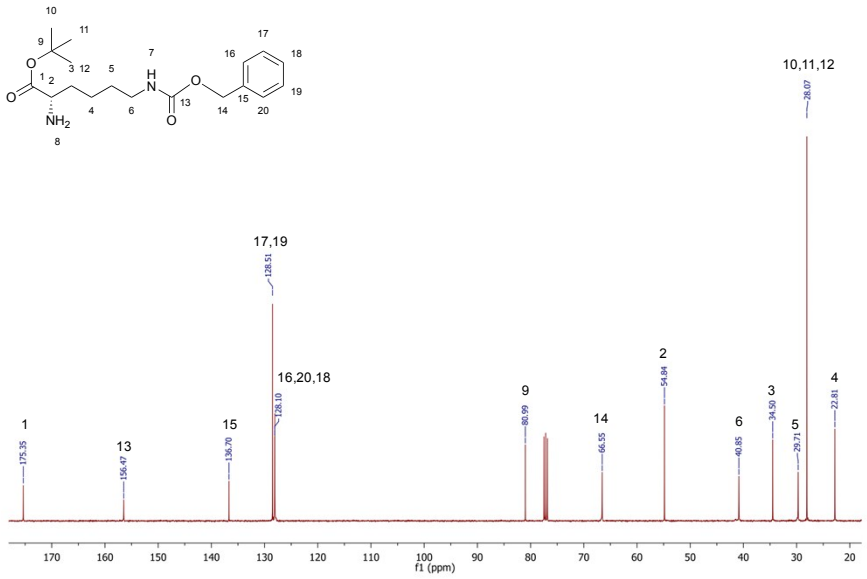


Figure S6. ^{13}C NMR{^{1}H} spectrum ($CDCl_3$, 400 MHz) of compound **1**.



*Synthesis of compound **2***

To a solution of L-glutamic acid (1.0 g, 7 mmol) in t-butyl acetate (20 mL), 70 % perchloric acid (1.0 mL) was added and the mixture stirred at room temperature for 20 h. Ethyl acetate (50 mL) was added and mixture extracted with water (50 mL), 0.5 M HCl (50 mL), then water (50 mL). Aqueous layers were combined, neutralised with saturated $NaHCO_3$ (aq.) and extracted with ethyl acetate (3 x 100 mL). The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and solvent removed under reduced pressure. Crude product was purified on silica gel (20-50% EtOAC/Hexane) to isolate compound **2** as a clear oil (350 mg, 2.3 mmol, 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 3.36 (dd, J = 8.2, 5.2 Hz, 1H), 2.35 (t, J = 7.6 Hz, 2H), 2.05 – 1.94 (m, 1H), 1.82 (s, 2H), 1.80 – 1.71 (m, 1H), 1.46 (s, 9H), 1.44 (s, 9H). $^{13}C\{^{1}H\}$ NMR (101 MHz, CDCl3) δ (ppm) 174.84, 172.71, 81.44, 80.55, 54.43, 32.06, 30.07, 28.24, 28.19. R_f (silica gel, EtOAc:Hexane 1:1) = 0.40. HRMS (ESI+) (m/z): calculated for $C_{13}H_{26}NO_4^+$ ($[M+H]^+$): 260.1817; found: 260.1854 (100%).

Figure S7. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **2**.

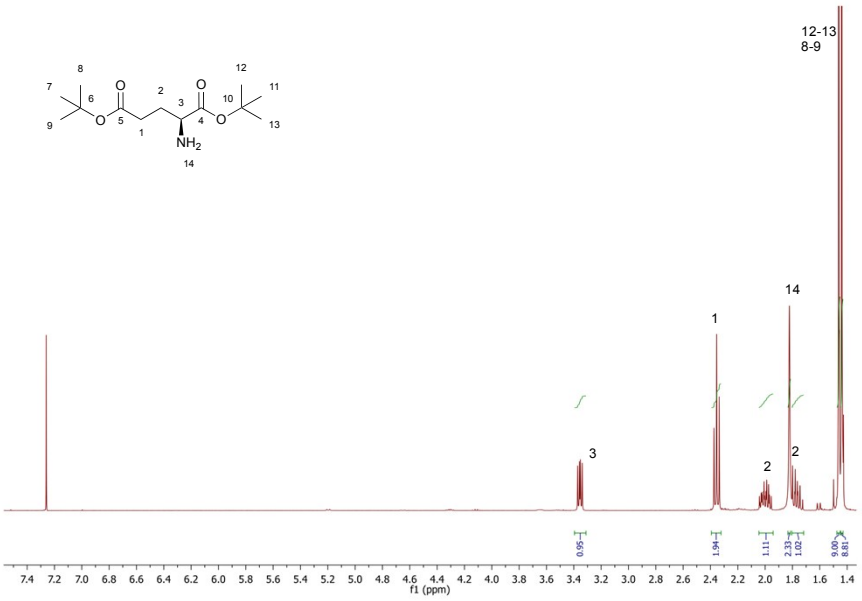
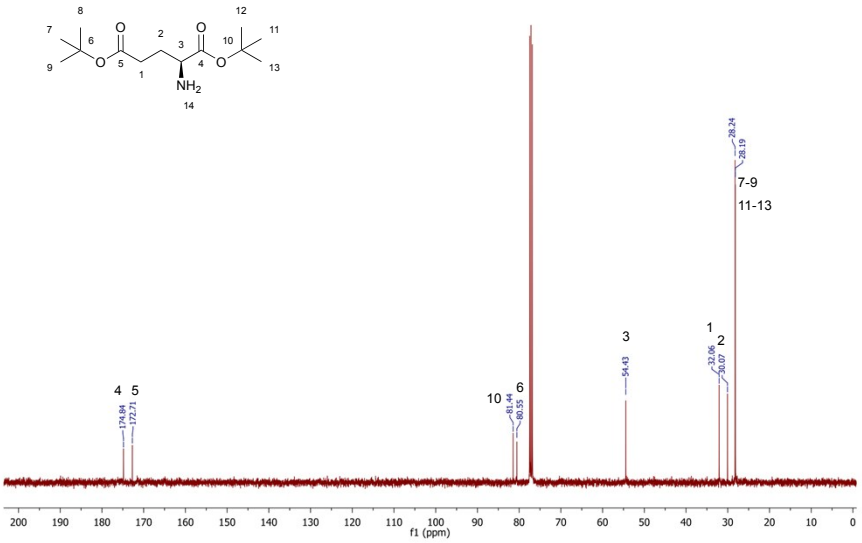


Figure S8. ^{13}C NMR{^{1}H} spectrum ($CDCl_3$, 400 MHz) of compound **2**.



*Synthesis of compound **3***

Under an inert atmosphere, a solution of triphosgene (0.2 g, 0.7 mmol) in DCM (20 mL) was cooled to 0 °C and a solution of **2** (0.7 g, 2.0 mmol) in DCM (1 mL) and DIPEA (1 mL) was added dropwise over 2 h. The reaction reached room temperature and a solution of **1** (0.5 g, 2.0 mmol) in DCM (1 mL) and DIPEA (1 mL) was added in one portion then stirred for 1 h. The mixture was concentrated to dryness, dissolved in 50 mL EtOAc then washed with 2 M $NaHSO_4$

(2x40 mL) and sat. NaCl (aq.). Organic phase was dried over anhydrous $MgSO_4$ and solvent removed under reduced pressure. Crude product was purified on silica gel (20-50% EtOAC/Hexane) to isolate compound **3** as a clear oil (0.2 g, 0.3 mmol, 28%). ^{1}H NMR (ppm) (400 MHz, $CDCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.29 (m, 5H), 5.26 – 4.92 (m, 5H), 4.32 (m, 2H), 3.25 – 3.11 (m, 2H), 2.41 – 2.19 (m, 2H), 2.16 – 1.96 (m, 1H), 1.91 – 1.71 (m, 2H), 1.60-1.71 (m, 3H), 1.51-1.59 (m, 3H), 1.45 – 1.43 (m, 27H), 1.40 – 1.24 (m, 2H). ^{13}C{^{1}H} NMR (101 MHz, $CDCl_3$) δ (ppm) 172.56, 172.19, 171.97, 156.98, 156.57, 136.66, 128.47, 128.05, 128.02, 82.25, 81.88, 80.66, 66.58, 53.38, 53.08, 40.61, 32.54, 31.53, 29.35, 28.29, 28.07, 28.01, 27.99, 22.21. R_f (silica gel, EtOAc:Hexane 1:1) = 0.58. HRMS (ESI+) (m/z): calculated for $C_{32}H_{52}N_3O_9^+$ ([M+H]$^+$): 622.2698; found: 622.3704 (100%).

Figure S9. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **3**.

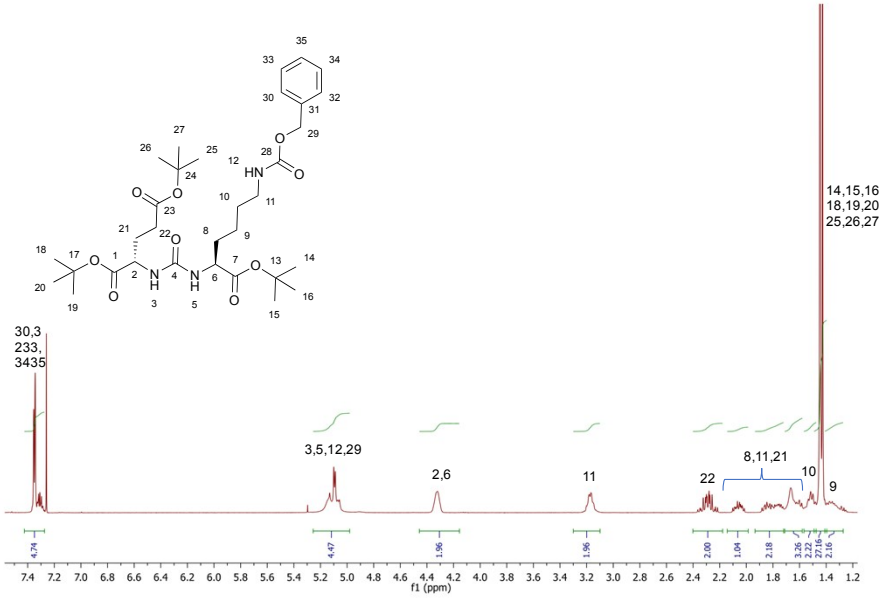
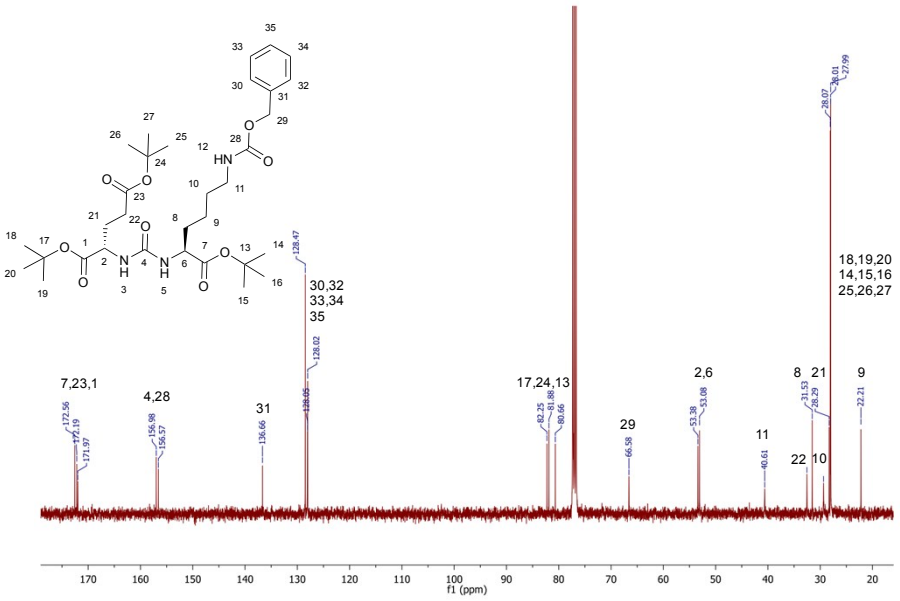


Figure S10. ^{13}C NMR{^{1}H} spectrum ($CDCl_3$, 400 MHz) of compound **3**.



*Synthesis of compound **4***

To a solution of **3** (190 mg, 0.30 mmol) in EtOH (10 mL) and Pd/C (20 mg) was added. The mixture was degassed with N_2, system flushed with H_2, and then reaction stirred for 20 h under an H_2 atmosphere. Reaction was passed through Celite® and solvent removed under reduced pressure to yield compound **4** as a yellow oil (100 mg, 0.21 mmol, 70%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.36 (s, 1H), 5.34 (s, 1H), 4.36 – 4.27 (m, 2H), 2.71 (t, J = 6.7 Hz, 2H), 2.51 (s, 2H), 2.39 – 2.19 (m, 2H), 2.11–2.00 (m, 1H), 1.91 – 1.69 (m, 2H), 1.66 – 1.53 (m, 1H), 1.53 – 1.45 (m, 2H), 1.45–1.38 (m, 27H), 1.38 – 1.26 (m, 2H). $^{13}C\{^{1}H\}$ NMR (101 MHz, $CDCl_3$) δ 172.76, 172.58, 157.12, 82.14, 81.78, 80.63, 53.56, 53.13, 41.56, 32.78, 32.37, 31.76, 28.45, 28.20, 28.14, 28.13, 22.44. HRMS (ESI+) (m/z): calculated for $C_{24}H_{46}N_3O_7^+$ ([M+H]$^+$): 488.33303; found: 488.3331 (100%).

Figure S11. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **4**.

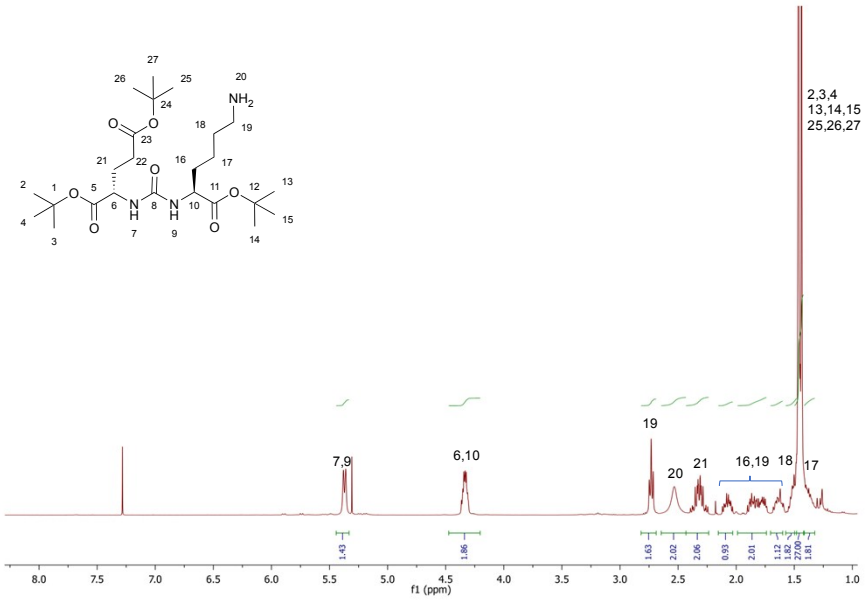
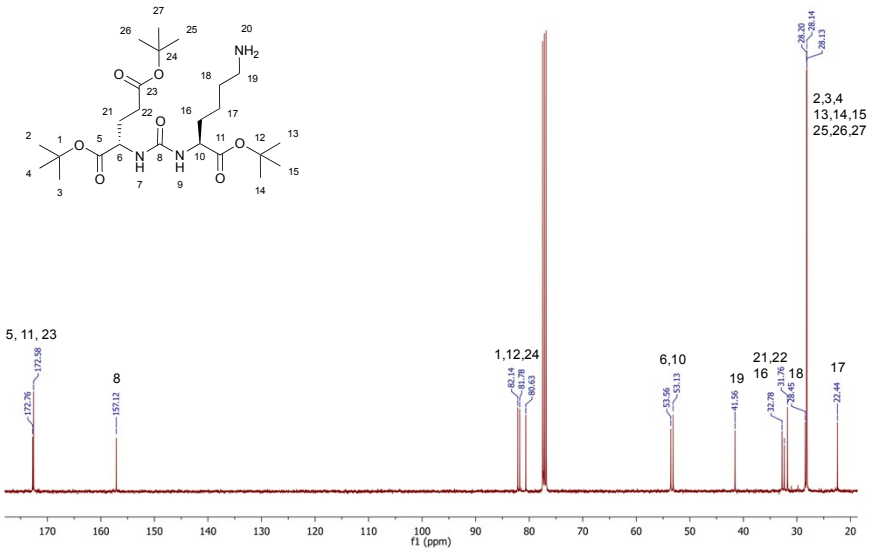


Figure S12. ^{13}C NMR{^{1}H} spectrum ($CDCl_3$, 400 MHz) of compound **4**.

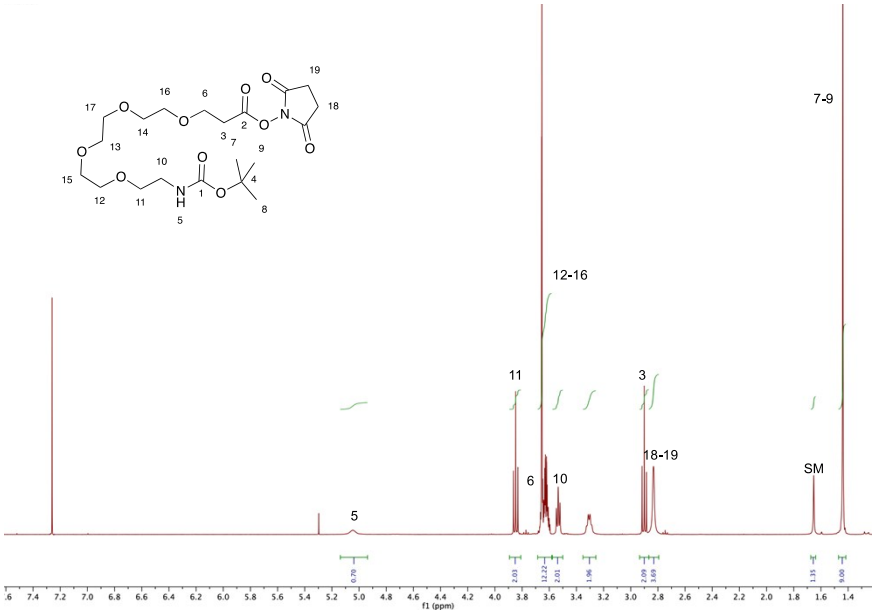


Synthesis of compound ***5***

To (Boc-amino)-PEG_4-carboxylic acid (80 mg, 0.21 mmol) in DCM (5 mL), was added NHS (29 mg, 0.25 mmol) and EDC (84 mg, 0.44 mmol) and mixture stirred until TLC showed consumption of product. Reaction was washed with 2.5% $NaHSO_4$ (aq.) (3x10 mL) and brine then the organic phase was dried over anhydrous $MgSO_4$ and solvent removed under reduced pressure to yield compound **5** as a white solid (90 mg, 0.20 mmol, 89%). ^{1}H NMR (400 MHz,

$CDCl_3$) δ (ppm) 5.05 (s, 1H), 3.85 (t, *J* = 6.5 Hz, 2H), 3.69 – 3.58 (m, 12H), 3.53 (t, *J* = 5.1 Hz, 2H), 3.35 – 3.26 (m, 2H), 2.90 (t, *J* = 6.5 Hz, 2H), 2.83 (s, 4H), 1.44 (s, 9H). R_f (silica gel, MeOH:DCM 1:20) = 0.4

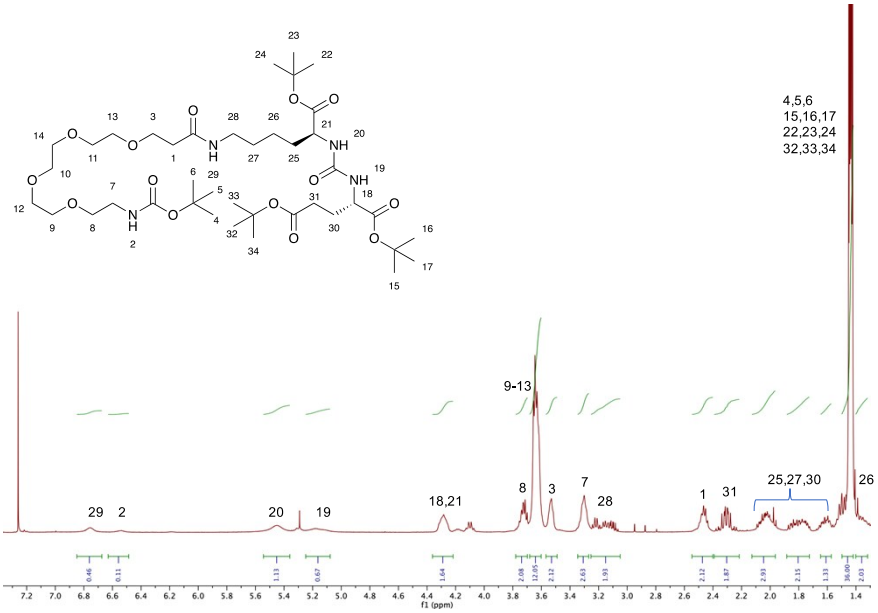
Figure S13. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **5**.



*Synthesis of compound **6***

Compound **4** (35 mg, 0.07 mmol) and ammonium formate (45 mg, 0.72 mmol) were dissolved in MeOH (3 mL), stirred for 30 mins and the solvent then removed under reduced pressure. Compound **5** (33 mg, 0.07 mmol) was then added in DCM (8 mL) and TEA (80 µL, 0.57 mmol) and reaction stirred for 16 h. Organic layer washed with water (3x10 mL) and brine (10 mL), then the organic phase was dried over anhydrous $MgSO_4$ and solvent removed under reduced pressure. Crude product was purified on silica gel (7% MeOH/DCM) to isolate compound **6** as a clear oil (0.2 g, 0.3 mmol, 42%) ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 6.78 (s, 1H), 6.56 (s, 1H), 5.47 (s, 1H), 5.20 (s, 1H), 4.38 – 4.15 (m, 2H), 3.79 – 3.72 (m, 2H), 3.70 – 3.62 (m, 12H), 3.60 – 3.52 (m, 2H), 3.40 – 3.29 (m, 2H), 3.28 – 3.08 (m, 2H), 2.55 – 2.45 (m, 2H), 2.42 – 2.27 (m, 2H), 2.13 – 2.02 (m, 3H), 1.92 – 1.74 (m, 1H), 1.70 – 1.59 (m, 2H), 1.58 – 1.49 (m, 36H), 1.48 – 1.44 (m, 2H).

Figure S14. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **6**.



Synthesis of compound 7

Compound **6** (20 mg, 0.02 mmol) was stirred in TFA (500 µL) for 1 h and then precipitated in diethyl ether (5 mL). Product collected by centrifugation and dried under reduced pressure to yield compound **7** as a white solid (13.1 mg, 0.02 mmol, 95%) ^{1}H NMR (400 MHz, MeOD) δ (ppm) 4.32 (dd, J = 8.6, 5.1 Hz, 1H), 4.26 (dd, J = 8.5, 4.9 Hz, 1H), 3.79 – 3.59 (m, 16H), 3.19 (t, J = 6.8 Hz, 2H), 3.17 – 3.11 (m, 2H), 2.45 (t, J = 6.0 Hz, 2H), 2.43 – 2.37 (m, 2H), 2.22 – 2.04 (m, 1H), 1.98 – 1.78 (m, 2H), 1.69 – 1.60 (m, 1H), 1.57 – 1.51 (m, 2H), 1.46 – 1.41 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ (ppm) 175.02, 174.96, 174.42, 172.64, 158.70, 70.01, 69.93, 69.85, 69.82, 69.41, 66.93, 66.40, 52.54, 52.10, 39.11, 38.69, 35.98, 31.79, 29.66, 28.45, 27.51, 22.52. HRMS (ESI+) (m/z): calculated for $C_{24}H_{46}N_3O_7^+$ ($[M+H]^+$): 567.6130; found: 567.2867 (100%).

Figure S15. ^{1}H NMR spectrum (MeOD, 400 MHz) of compound **7**.

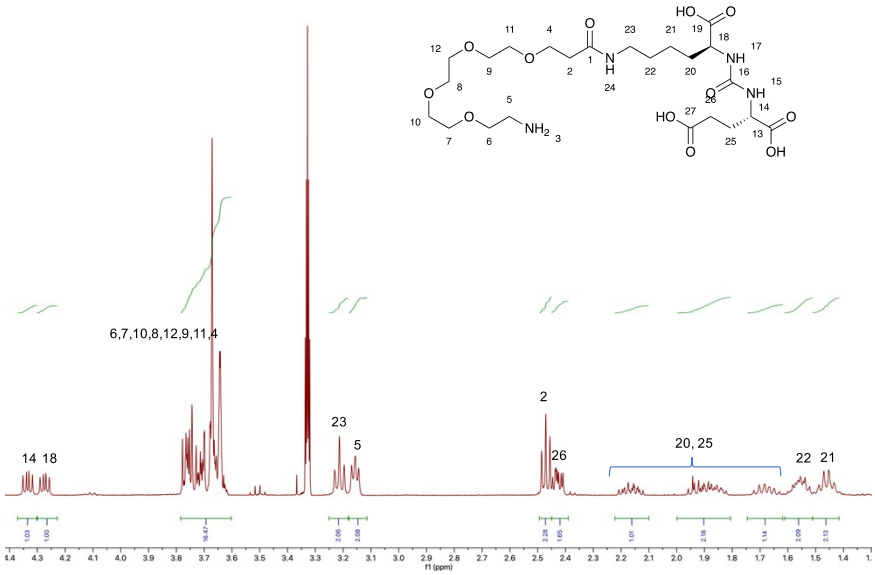
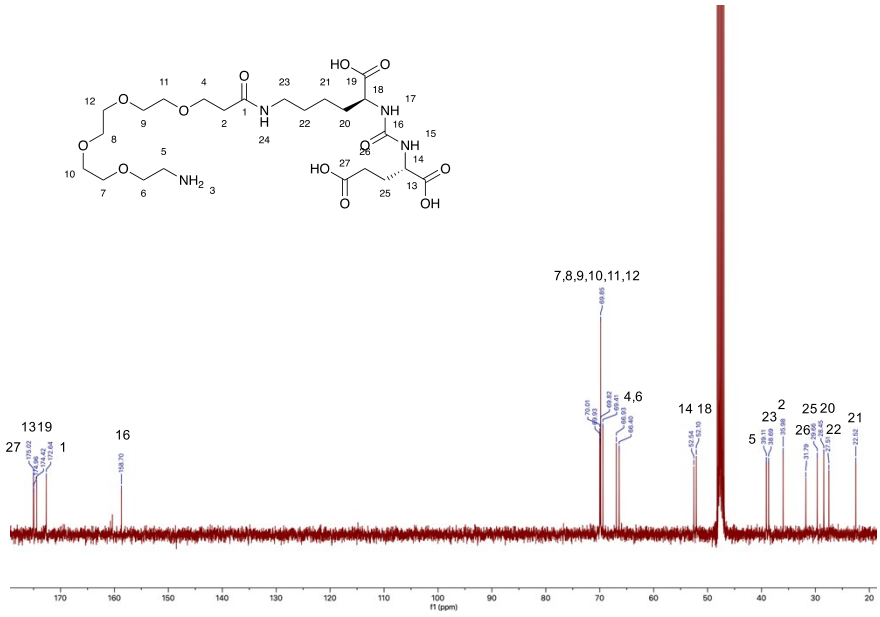
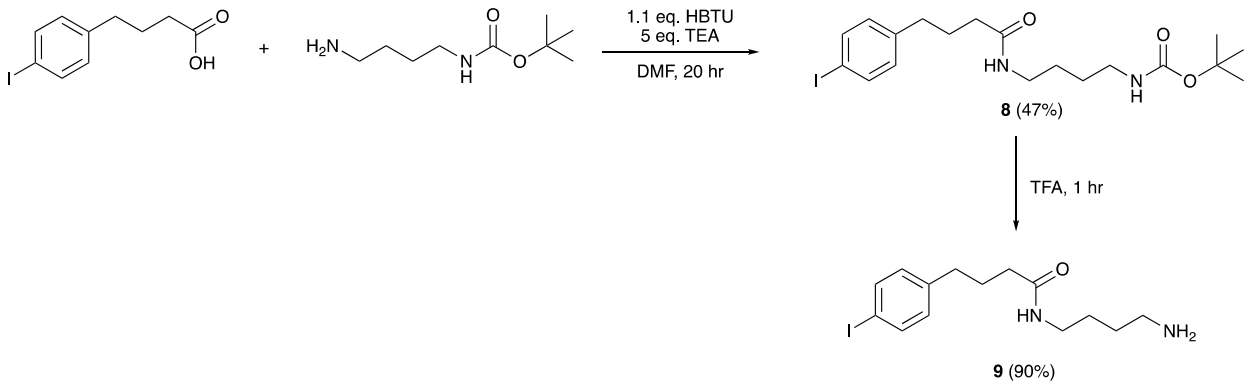


Figure S16. ^{13}C NMR{^{1}H} spectrum (MeOD, 400 MHz) of compound **7**.



Chemical synthesis of the albumin binding motif component

Scheme S2. Chemical synthesis of albumin binding motif, compound **9**.



*Synthesis of compound **8***

4-(*p*-Iodophenyl)butyric acid (300 mg, 1.03 mmol) in DMF (10 mL) was activated with HBTU (430 mg, 1.12 mol). Following the addition of N-Boc-1,4-butanediamine (194 mg, 1.03 nmol) reaction was stirred for 20 h. The solvent was removed under reduced pressure and the product re-dissolved in DCM (20 ml) which was washed with 0.1 M NaOH (3x 20 ml), 0.1 M HCl (3x20 mL), and brine (3x20 mL). Organic phase was dried over anhydrous $MgSO_4$ and solvent removed under reduced pressure. Crude product was purified on silica gel (5-10% DCM/MeOH) to isolate compound **8** as a clear oil (230 mg, 0.49 mmol, 47%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, *J* = 8.2 Hz, 2H), 6.92 (d, *J* = 8.1 Hz, 2H), 5.76 (s, 1H), 4.62 (s, 1H), 3.30 – 3.21 (m, 2H), 3.17 – 3.06 (m, 2H), 2.58 (t, *J* = 7.5 Hz, 2H), 2.15 (t, *J* = 7.5 Hz, 2H), 1.97 – 1.88 (m, 2H), 1.54 – 1.45 (m, 4H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.82, 156.34, 141.29, 137.53, 130.74, 91.11, 79.48, 39.31, 38.82, 35.80, 34.83, 28.54, 27.84, 27.04, 26.69. R_f (silica gel, MeOH:DCM 1:14) = 0.59. HRMS (ESI+) (m/z): calculated for $C_{24}H_{46}N_3O_7^+$ ($[M+Na]^+$): 483.1115; found: 483.1117 (100%).

Figure S17. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **8**.

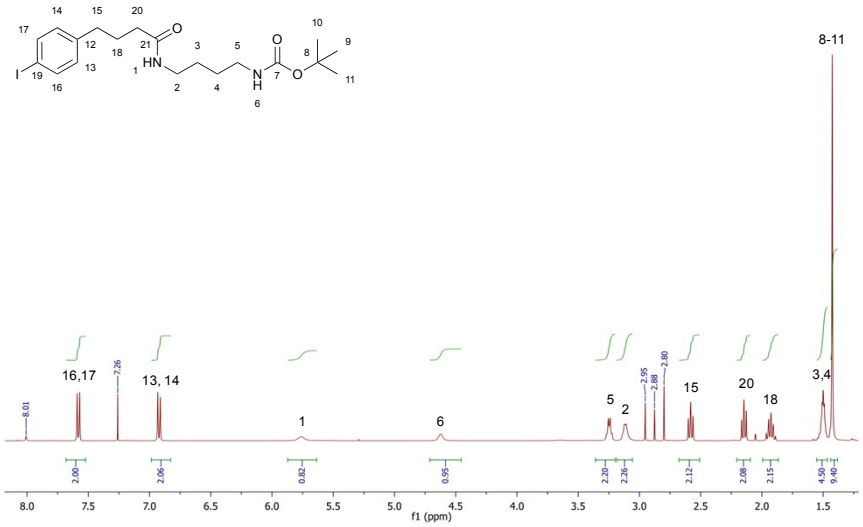
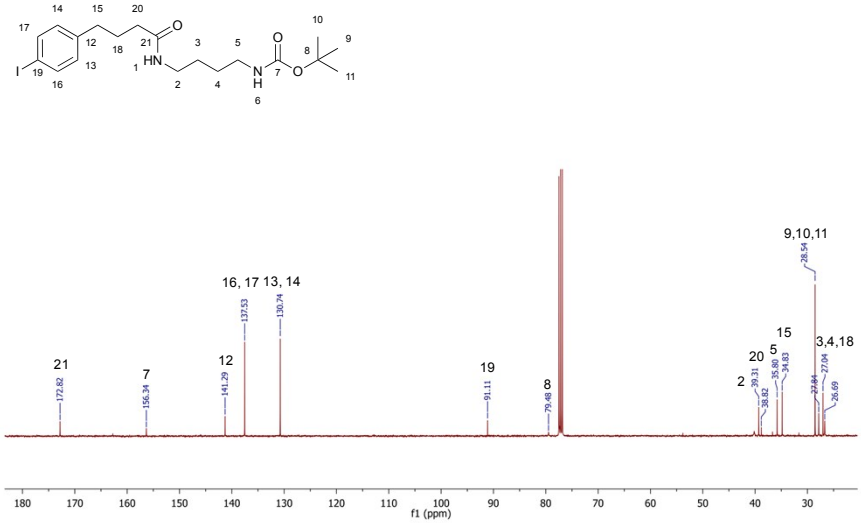


Figure S18. ^{13}C NMR{^{1}H} spectrum ($CDCl_3$, 400 MHz) of compound **8**.



*Synthesis of compound **9***

Compound **8** (230 mg, 0.48 mmol) was stirred with TFA (0.5 mL) for 1 hr. Toluene was added (10 mL) and solvent removed under reduced pressure to yield compound **9** as a yellow solid (161 mg, 0.44 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.59 (d, J = 8.2 Hz, 2H), 6.93 (d, J

= 8.1 Hz, 2H), 5.91 (s, 1H), 3.24 (m, 2H), 2.74 (t, J = 6.4 Hz, 2H), 2.58 (t, J = 7.5 Hz, 2H), 2.16 – 2.12 (m, 2H), 2.07 (s, 2H), 1.98 – 1.86 (m, 2H), 1.65 – 1.43 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 172.50, 141.21, 137.41, 130.63, 90.98, 41.45, 39.26, 35.74, 34.68, 30.27, 27.04, 26.89. HRMS (ESI+) (m/z): calculated for $C_{24}H_{46}N_3O_7^+$ ([M+Na]$^+$): 361.0777; found: 361.0772 (100%).

Figure S19. ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of compound **9**.

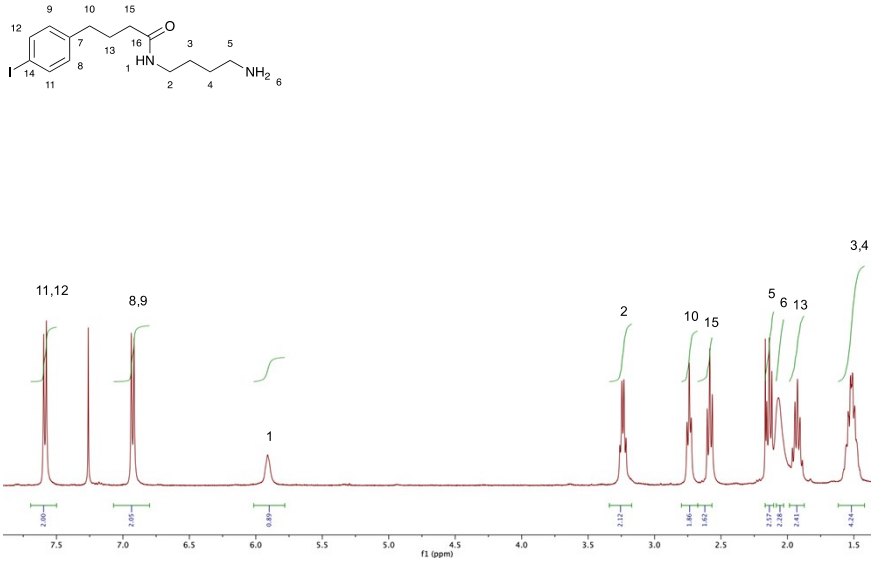
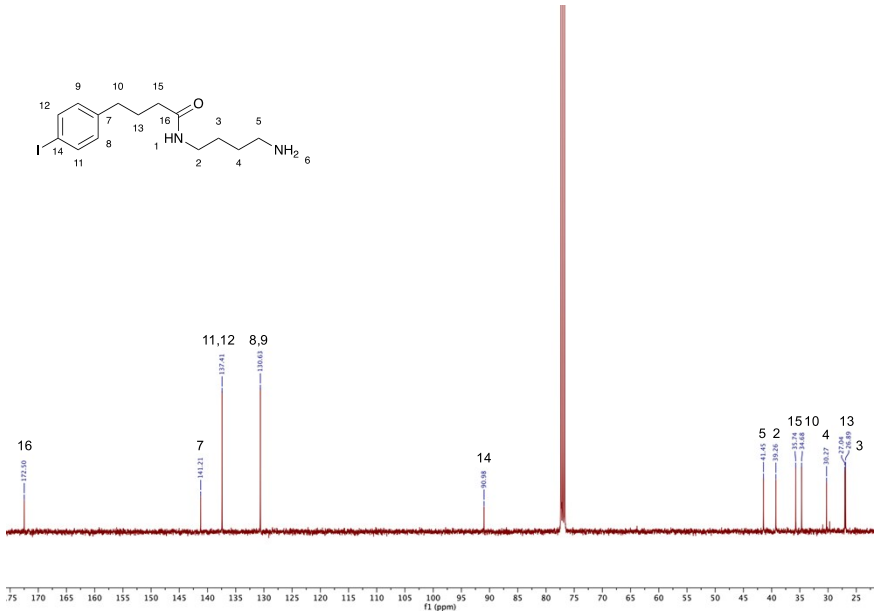


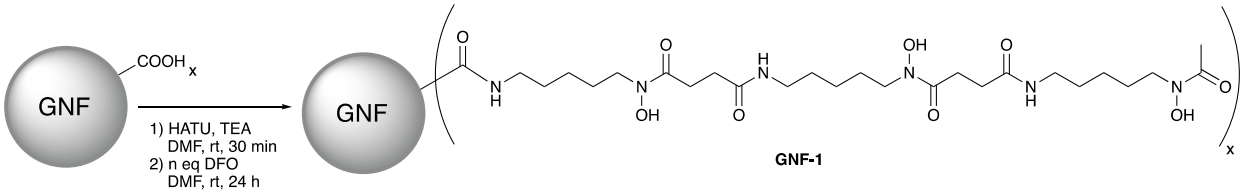
Figure S20. ^{13}C NMR{^{1}H} spectrum ($CDCl_3$, 400 MHz) of compound **9**.



Chemical synthesis of the functionalised GNFs

Synthesis of **GNF-1**

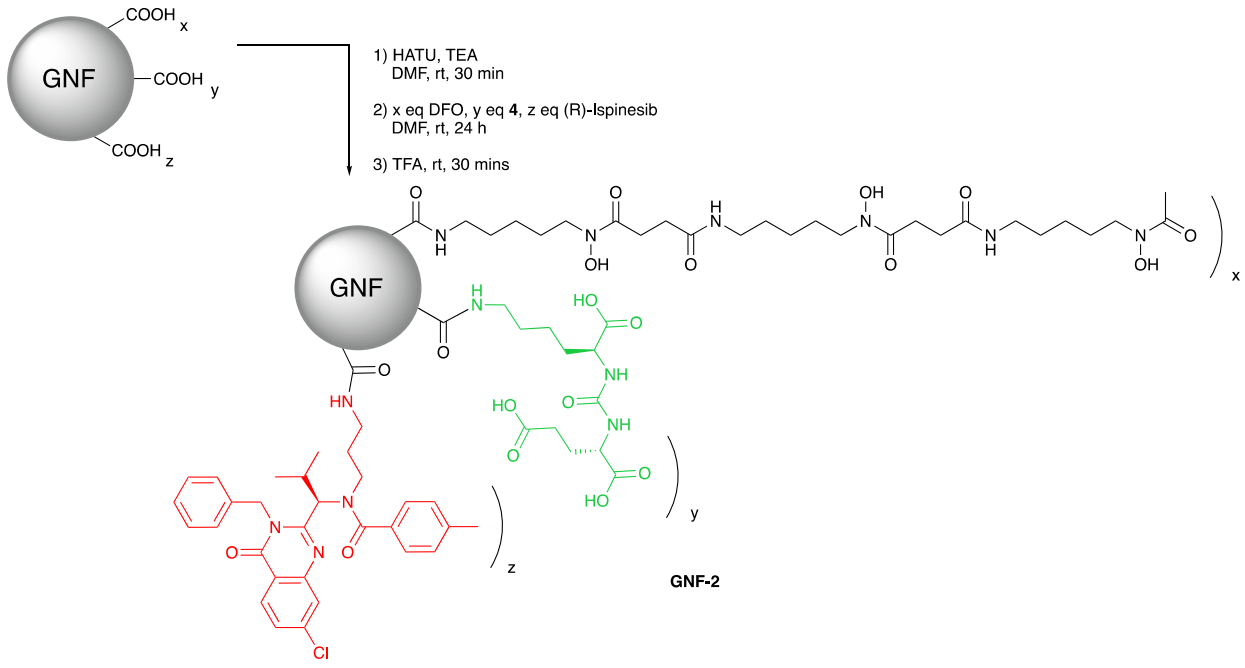
Scheme S3. Chemical synthesis of **GNF-1**.



GNFs (4.56 mg) in DMF (1 mL) and TEA (20 µL) were activated with HATU (8.24 mg, 0.02 mmol). Following the addition of DFO (0.40 mg, 0.61 µmol) reaction was vortexed for 24 h. Solvent was removed under reduced pressure and product purified on silica gel (100% H_2O), after lyophilisation, **GNF-1** was obtained as a black solid (2.13 mg, 42%).

Synthesis of **GNF-2**

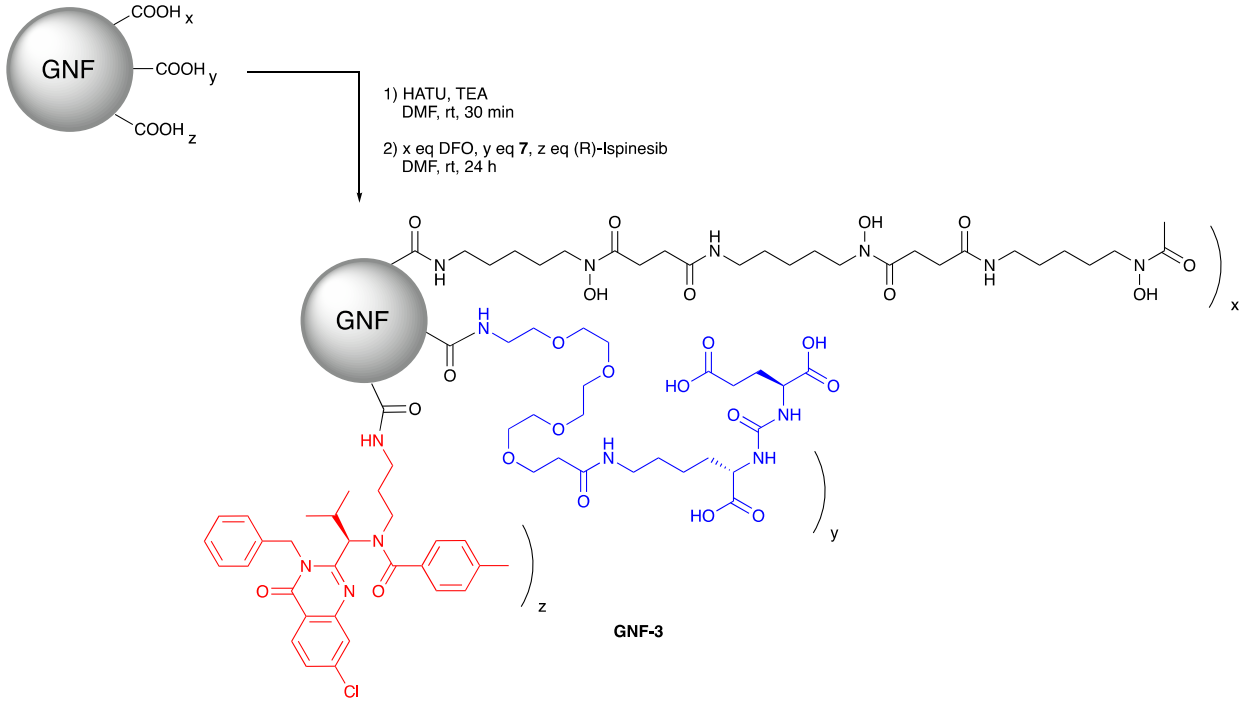
Scheme S4. Chemical synthesis of **GNF-2.**



GNFs (4.10 mg) in DMF (1 mL) and TEA (20 µL) were activated with HATU (8.01 mg, 0.02 mmol). Following the addition of DFO (0.50 mg, 0.61 µmol), (*R*)-ispinesib (0.53 mg, 1.03 µmol), and **4** (0.50 mg, 1.03 µmol) reaction was vortexed for 24 h. TFA (0.5 mL) was added and after 1 h the solvent removed under reduced pressure and product purified on silica gel (100% H_2O), after lyophilisation, **GNF-2** was obtained as a black solid (1.84 mg, 36%).

Synthesis of **GNF-3**

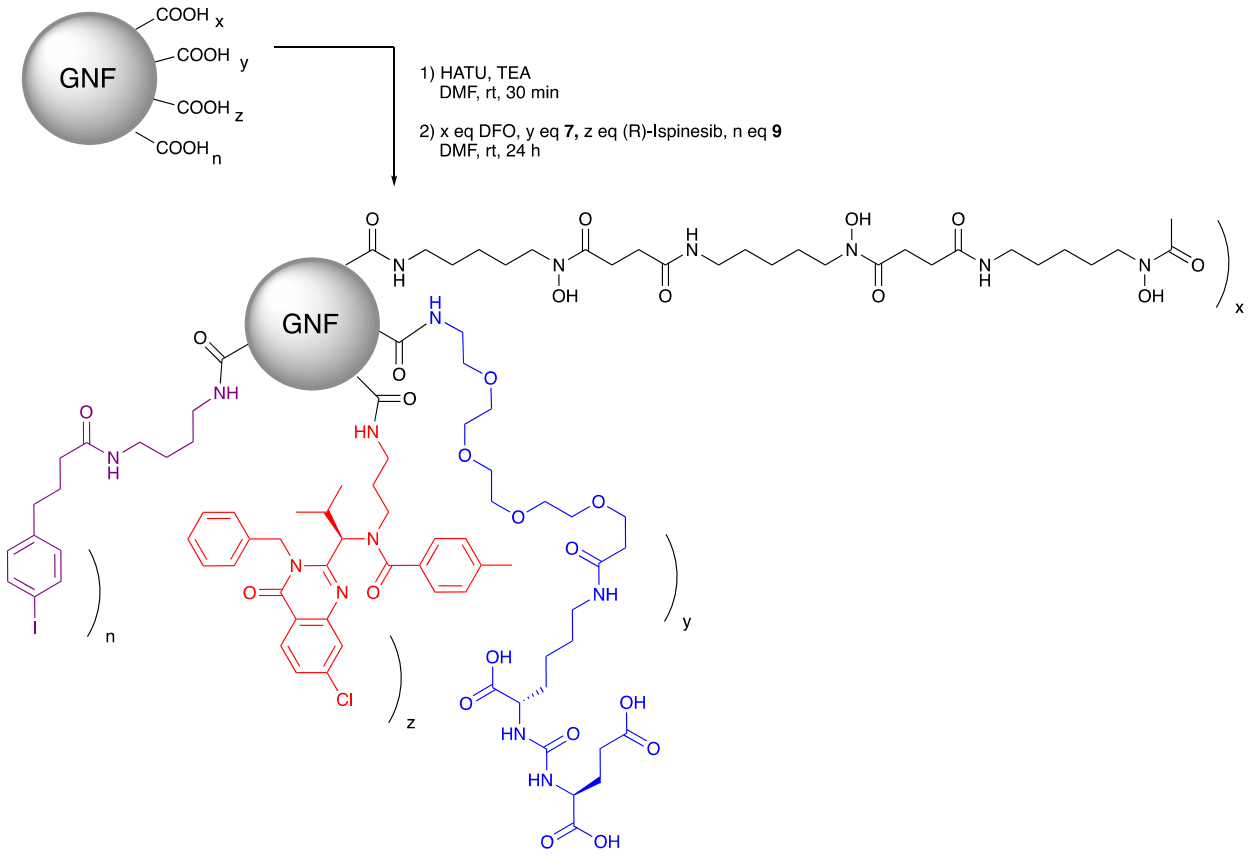
Scheme S5. Chemical synthesis of **GNF-3**.



GNFs (4.27 mg) in DMF (1 mL) and TEA (20 µL) were activated with HATU (8.07 mg, 0.02 mmol). Following the addition of DFO (0.47 mg, 0.72 µmol), (*R*)-ispinesib (0.60 mg, 1.16 µmol), and **7** (0.53 mg, 0.93 µmol) reaction was vortexed for 24 h. Solvent was removed under reduced pressure and product purified on silica gel (100% H_2O), after lyophilisation, **GNF-3** was obtained as a black solid (1.72 mg, 29%).

Synthesis of **GNF-4**

Scheme S6. Chemical synthesis of **GNF-4**.



GNFs (4.15 mg) in DMF (1 mL) and TEA (20 µL) were activated with HATU (8.06 mg, 0.02 mmol). Following the addition of DFO (0.47 mg, 0.72 µmol), (*R*)-ispinesib (0.54 mg, 1.04 µmol), **7** (0.56 mg, 0.99 µmol) and **9** (0.31 mg, 0.86 µmol) reaction was vortexed for 24 h. Solvent was removed under reduced pressure and product purified on silica gel (100% H_2O), after lyophilisation, **GNF-4** was obtained as a black solid (1.81 mg, 30%).

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