Electronic Supplementary Material (ESI) for ChemComm.

SUPPORTING INFORMATION

A bioorthogonal ^{68}Ga-labelling strategy for rapid *in vivo* imaging

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1. GENERAL METHODS AND MATERIALS

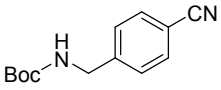
All reactions were performed under anhydrous conditions and an atmosphere of nitrogen in flame-dried glassware unless otherwise stated. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogenous materials. Solvents and reagents: All solvents were purified and dried according to standard methods prior to use. All chemicals were handled in accordance with COSHH regulations. All reagents were used as commercially supplied from Sigma Aldrich, unless otherwise specified. Flash chromatography (FC) was always performed on silica gel (Merck ASTM 60 F_{254} 230-400 mesh) according to the method of W. C. Still, unless otherwise stated.[1] Thin Layer Chromatography (TLC) was performed on Merck aluminum-backed plated pre-coated with silica (0.2 mm, 60 F_{254}) which were visualised either by quenching of ultraviolet fluorescence (λ = 254 and 366 nm) or by charring with 10% $KMnO_4$ in 1 M H_2SO_4, unless otherwise stated. ^{1}H NMR spectra: These were recorded at 400 MHz on a Bruker AV-400 instrument. Chemical shifts (δ_H) are quoted in parts per million (ppm), referenced to the appropriate residual solvent peak. Coupling constants (*J*) are reported to the nearest 0.5 Hz. ^{13}C NMR spectra: These were recorded at 100 MHz on a Bruker AV-400 instrument. Chemical shifts (δ_C) are quoted in ppm, referenced to the appropriate residual solvent peak. ^{19}F NMR: These were recorded at 400 MHz on Bruker DRX-400 instrument. Chemical shifts (δ_F) are quoted in ppm, referenced to fluorobenzene at -113.5 ppm. Mass spectra: High resolution mass spectra (*m/z*) were recorded on either a VG platform II or VG AutoSpec spectrometers, with only molecular ions (M^+, MH^+, MNa^+, MK^+, MNH_4^+) and major peaks being reported. Analytical reverse-phase HPLC was carried out on an Agilent 1100 series HPLC system (Agilent Technologies,

Stockport, UK) equipped with a UV detector (254 nm) and a LabLogic Flow-Count radio-detector, using a Phenomenex Gemini C18 column (150 mm x 4.6 mm) at a flow rate of 1 mL min^{-1}. Radio-HPLC was carried out on an Agilent 1100 series HPLC system (Agilent Technologies, Stockport, UK) equipped with a γ-RAM Model 3 gamma-detector (IN/US Systems Inc., Florida, USA) and Laura 3 software (LabLogic, Sheffield, UK). One of the following gradients was used: Gradient A; (0-95% B over 15 min) Buffer A = MeCN (0.1% TFA), Buffer B = H_2O (0.1% TFA). Semi-preparative reverse-phase HPLC was carried out on a Waters 600E system equipped with a UV detector (254 nm), using a Phenomenex Luna C18 column (100 mm x 10 mm) on Gradient A at a flow rate of 10 mL min^{-1}. Size-exclusion HPLC was carried out on either an Agilent 1100 series HPLC system (Agilent Technologies, Stockport, UK) equipped with UV detector (215 nm) or on a Waters 600E system equipped with UV detector (215 nm) using a Phenomenex BioSep-SEC-S column (300 x 7.80 mm) using sodium phosphate (pH 6.8) at a flow rate of 1 mL min^{-1}. Laura 3 software was used for processing all analytical HPLC chromatograms. $^{68}GaCl_3$ was eluted from an Eckert & Ziegler IGG100 $^{68}Ge/^{68}Ga$-Generator using a fully-automated Modular-Lab system. $^{68}GaCl_3$ was eluted in 2 mL of a 0.1 M HCl solution, which was subsequently buffered for direct use in radiolabelling experiments.

2. ORGANIC SYNTHESIS

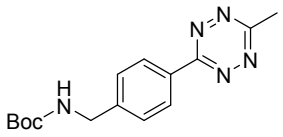
Synthesis of 4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzylamine (**3**) was carried out according to the method reported by Yang *et al.*, with some modifications:[2]

*tert-Butyl N-[4-(cyanophenyl)methyl]carbamate (**1**)*[2]



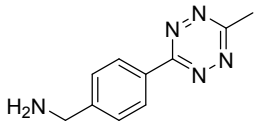
4-(Aminomethyl)benzonitrile·HCl (5 g, 30 mmol) was azeotroped with toluene (3 x 20 mL) and dissolved in CH_2Cl_2 (4 mL). Triethylamine (11 mL, 75 mmol) and Boc_2O (7.4 g, 33 mmol) were added and the resultant mixture was stirred at room temperature for 16 h. The mixture was concentrated *in vacuo* to yield a white solid, which was recrystallized from EtOAc/pet ether, to yield *carbamate* **1** as a white powder (6 g, 25.8 mmol, 87% yield). δ_H (400 MHz, $CDCl_3$) 1.43 (s, 9H); 4.33 (d, *J* = 6.0 Hz, 2H); 5.10 (bs, 1H); 7.36 (d, *J* = 8.5 Hz, 2H); 7.58 (d, *J* = 8.5 Hz, 2H). δ_C (100 MHz, $CDCl_3$) 28.3; 44.1; 79.9; 111.0; 118.7; 127.7; 132.3; 144.7; 155.8. *m/z* (ES^+) calcd for: $C_{13}H_{17}N_2O$ ($[M+H]^+$) 233.1290, found: 233.1294.

*tert-Butyl N-{[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamate (**2**)*[2]



A high pressure reaction tube was treated with *tert*-butyl *N*-[4-(cyanophenyl)methyl]carbamate (**1**) (232 mg, 1 mmol), MeCN (525 µL, 10 mmol), $Ni(OTf)_2$ (178 mg, 0.5 mmol) and hydrazine hydrate (50-60% NH_2NH_2) (3.1 mL, 50 mmol). The tube was sealed and heated to 60°C for 24 h, after which time the mixture had turned deep purple in color. $NaNO_2$ (1.4 g, 20 mmol) in H_2O (5 mL), was added to the mixture, followed by dropwise addition of HCl (1 M), until the pH reached 3 and gases stopped evolving, at which point the mixture had turned bright red. The product was extracted with EtOAc (3 x 20 mL), and the combined organic layers were washed with H_2O (2 x 10 mL) and brine (10 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield a bright pink oil as crude. The product was purified by column chromatography, eluting with 0-3% diethyl ether in CH_2Cl_2, to give *tetrazine* **2** as a bright pink solid (74 mg, 0.3 mmol, 60% yield). R_f = 0.35 (2% diethyl ether/CH_2Cl_2). δ_H (400 MHz, $CDCl_3$) 1.47 (s, 9H); 3.08 (s, 3H); 4.42 (d, *J* = 4.5 Hz, 2H); 5.04 (bs, 1H); 7.48 (d, *J* = 7.5 Hz, 2H); 8.53 (d, *J* = 7.5 Hz, 2H). δ_C (100 MHz, $CDCl_3$) 20.5; 27.8; 43.7; 79.2; 127.5 (d, *J* = 14.0 Hz); 130.1; 143.3; 155.3; 163.3; 166.6. *m/z* (ES^+) calcd for: $C_{11}H_{11}N_5O_2$ ($[M\text{-}Boc]^+$) 246.0991 found: 246.0983.

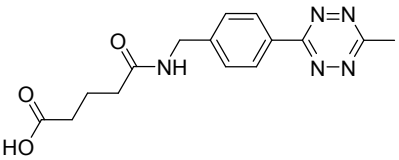
*[4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl]methanamine (**3**)*[2]



tert-Butyl N-{[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamate (**2**) (45 mg, 0.15 mmol) was treated with TFA/CH_2Cl_2 (1/1, *v/v*, 4 mL) for 1 h. The resultant

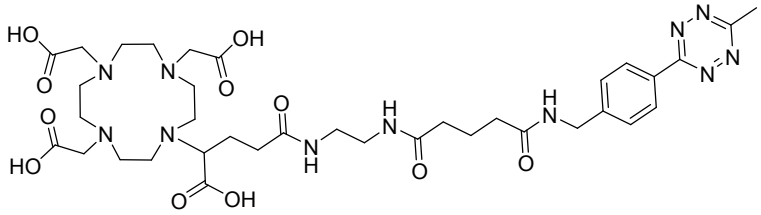
mixture was concentrated *in vacuo* to yield *amine* **3** as a bright pink solid (30 mg, 0.14 mmol, >95% yield). R_f = 0.40 (10% MeOH/CH_2Cl_2). δ_H (400 MHz, d_6-DMSO) 3.01 (s, 3H); 4.18 (bs, 2H); 7.73 (d, *J* = 8.5 Hz, 2H); 8.26 (bs, 2H); 8.52 (d, *J* = 8.5 Hz, 2H). δ_C (100 MHz, d_6-DMSO) 27.8; 41.9; 127.5; (d, *J* = 14.0 Hz); 129.7; 131.9; 138.2; 162.9; 167.2. *m/z* (CI) calcd for $C_{10}H_{12}N_5$ 202.1093 (M^+), found 202.1096. *m/z* (CI) calcd for $C_{10}H_{12}N_5$ ($[M+H]^+$) 202.1093, found 202.1096.

*4-({[4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamoyl)butanoic acid (**4**)*



A dry flask was treated with [4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methanamine (**3**) (66.4 mg, 0.33 mmol), glutaric anhydride (188 mg, 1.65 mmol) and THF (10 mL), and the resultant mixture was heated to 70°C for 4 h. The mixture was cooled to 50°C, and was stirred at this temperature for a further 16 h. The solvent was evaporated *in vacuo*, to give the crude material as a pink oil. The product was purified by column chromatography eluting with a gradient of 0-5% MeOH/CH_2Cl_2 to give *acid* **4** as a red solid (100 mg, 0.32 mmol, >95% yield). R_f = 0.3 (5% MeOH/ CH_2Cl_2). δ_H (400 MHz, d_6-DMSO) 2.24 (m, 6H); 2.99 (s, 3H); 3.36 (bs, 1H); 4.38 (d, *J* = 6.0 Hz, 2H); 7.52 (d, *J* = 8.5 Hz, 2H); 8.42 (d, *J* = 8.5 Hz, 2H); 12.08 (bs, 1H). δ_C (100 MHz, d_6-DMSO) 21.2; 21.3; 33.2; 33.5; 34.8; 42.3; 127.9; 128.5; 130.8; 145.0; 163.6; 167.5; 172.3; 174.6; 174.6. *m/z* (ES^+) calcd for $C_{15}H_{18}N_5O_3$ ($[M+H]^+$) 316.1410, found 316.1409.

*4-({2-[4-({[4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamoyl)butanamido]ethyl}carbamoyl)-2-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]butanoic acid (**5**)*

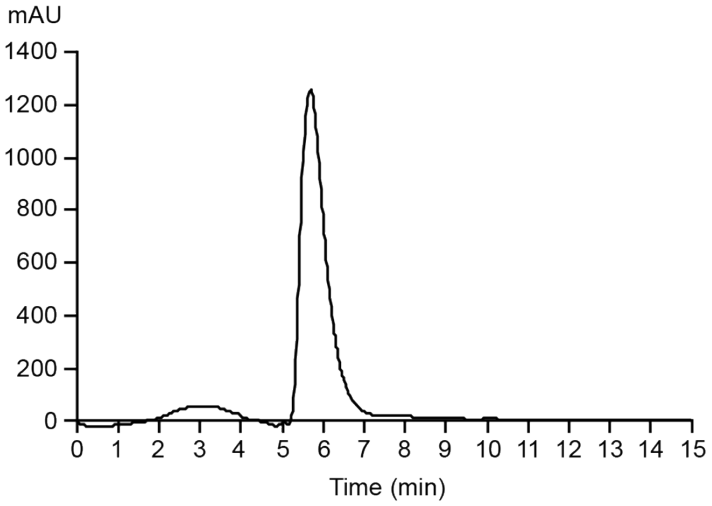


To a stirred solution of 4-({[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl} carbamoyl)butanoic acid (**4**) (63.1 mg, 0.2 mmol) in dichloroethane (2 mL), was added *N*-hydroxysuccinimide (92.1 mg, 0.8 mmol) and EDCI·HCl (153.4 mg, 0.8 mmol) and the resultant mixture was stirred for 16 h at rt. The mixture was diluted with CH_2Cl_2, and the organic layer was washed with HCl (1M, 2 x 40 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give 5-[(2,5-dioxopyrrolidin-1-yl)oxy]-N-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl]-5-oxopentanamide as a fushia-coloured solid (82 mg, 0.2 mmol, >95% yield). R_f = 0.3 (2% MeOH/CH_2Cl_2). The product was used for the next step without purification. *m/z* (ES^+) calcd for $C_{19}H_{21}N_6O_5$ ($[M+H]^+$) 413.1573, found 413.1586. To a stirred solution of this amide (95.4 mg, 0.23 mmol) in DMF (5 mL), was added DOTA-GA-NH_2 (purchased from CheMatech) (100 mg, 0.2 mmol) and triethylamine (140 µL, 1 mmol), and the resultant mixture was stirred at rt for 4 h. The solvent was evaporated *in vacuo* to give a pink oil as crude. The product was purified by preparative HPLC, to yield *DOTA-derivative* **5** as a pink solid (104.5 mg, 0.13 mmol, 64% yield). RT = 6.11 min (gradient A). δ_H (400 MHz, d_6-MeOD) 1.86-1.99 (m, 4H); 2.00 (s, 1H); 2.19-2.37 (m, 5H); 2.51-2.72 (m, 4H); 3.00 (s, 3H); 3.03-3.13 (m, 3H); 3.13-3.24 (m, 5H); 3.30-3.48 (m, 8H); 3.63-3.71 (m, 2H); 3.82-4.09 (m, 4H); 4.46 (s, 2H); 7.51 (d, *J* = 8.0 Hz, 2H); 8.48 (d, *J* = 8.5 Hz, 2H). *m/z* (ES^+) calcd for $C_{36}H_{54}N_{11}O_{11}$ ($[M+H]^+$) 816.4004, found 816.4001

Modification of Cetuximab (C225) with TCO

A 5 mg mL^{-1} stock solution of Cetuximab, as commercially supplied (3 mL, 15 mg, 1.02 x 10^{-4} mmol) was concentrated to 7 mg mL^{-1} (2.14 mL) by buffer exchange into $NaHCO_3$ (pH 8.5) *via* centrifugal filtration using Amicon Ultra® Centrifual filters (0.5 mL) with a 30K MW cut-off. TCO-PEG4-NHS (purchased from Jena Bioscience) (257 µL, 1.02 x 10^{-2} mmol, 40 mM solution in DMF) was added to the protein solution, and the mixture was sonicated for 60 sec, followed by incubation at 4°C for 16 h. The modified antibody **7** was purified from unconjugated TCO by centrifugal filtration using Amicon Ultra® Centrifual filters (30K MW cut-off), for a total spin-time of 40 min. Complete removal of unmodified TCO from the antibody mixture was confirmed by SEC HPLC. **7** was re-dissolved in PBS (pH 7.4) to a final concentration of 5 mg mL^{-1}. MALDI MS confirmed an average of 17 additions of TCO-PEG4 per C225 molecule.

SEC UV-HPLC trace of TCO-modified Cetuximab **7** at 215 nm:

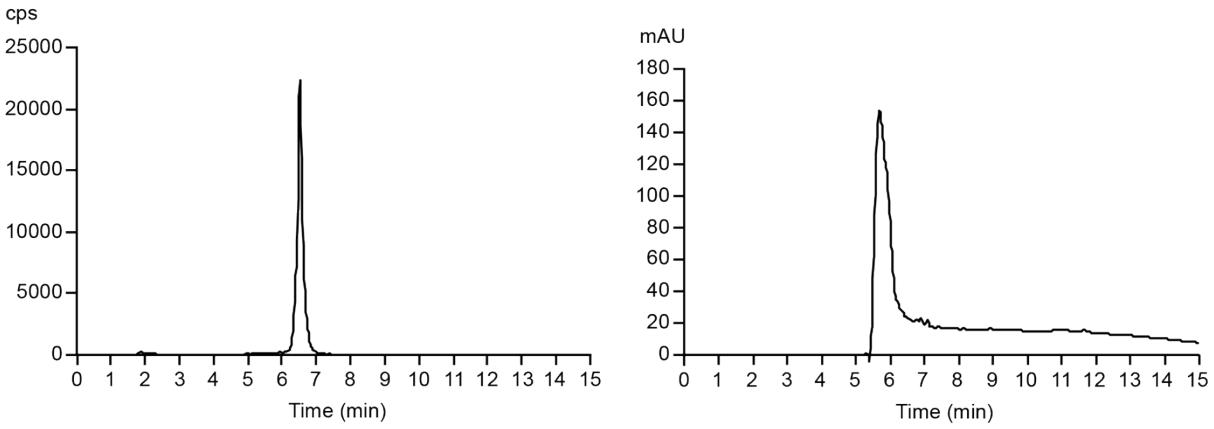


3. RADIOCHEMISTRY

*Radiosynthesis of [^{68}Ga]-**5***

A solution of **5** (25 µL, 1 mg mL^{-1} in DMSO) was diluted with NaOAc buffer solution (pH 6), and the $^{68}GaCl_3$ was eluted directly into the reaction vial (~ 222 MBq). The radiosynthesis of [^{68}Ga]-**5** was achieved in 10 min at 90°C, in >95% radiochemical conversion, and the product was obtained in >95% radiochemical purity, and in 60-70% isolated RCY (n.d.c.) by passing through a Sep-Pak light C18 cartridge, eluting in 100% EtOH (500 µL total volume).

Radio and UV (at 254 nm) HPLC traces of [^{68}Ga]-**5**:



*Radiosynthesis of [^{68}Ga]-**8***

[^{68}Ga]-**5** (~ 37 MBq) was added to a solution of **7** (50 µL, diluted to 1 mg mL^{-1} in PBS), incubated for 15 min to form [^{68}Ga]-**8**, prior to further use for PET imaging studies.

4. SUPPORTING FIGURES

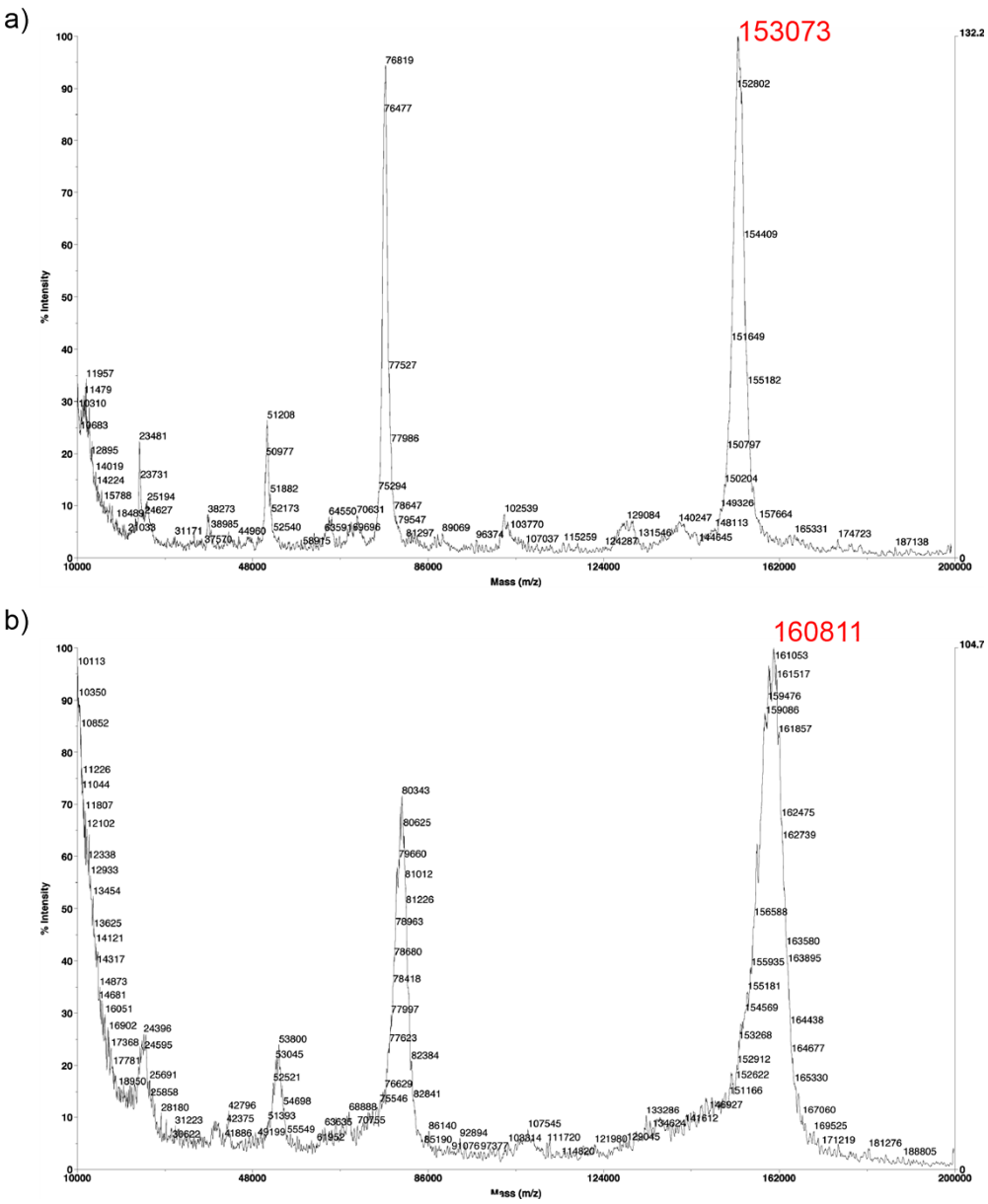


Figure S1. MALDI MS of unmodified and TCO-modified Cetuximab **7**, indicate an average MW of 153073 and 160811, respectively, suggesting the addition of an average of 17 TCO moieties per antibody (MW TCO-PEG4 = 446.54). MALDI MS of antibody samples were performed by the EPSRC National MS Facility, Swansea, UK.

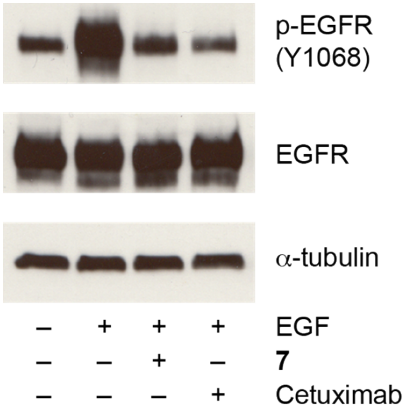


Figure S2. Immunoreactivity assessment of TCO-modified Cetuximab **7.** High EGFR expressing A431 cell lines were incubated in the absence or presence of EGF (100 ng mL^{-1}, 15min) prior to incubation with TCO-modified Cetuximab **7** (50 µg mL^{-1}) or unmodified Cetuximab (50 µg mL^{-1}) for 24 h. Whole cell lysates were prepared and samples were processed for total EGFR and phosphorylated-EGFR (p-EGFR) immunoblot analysis. α-tubulin assessment was used as loading control.

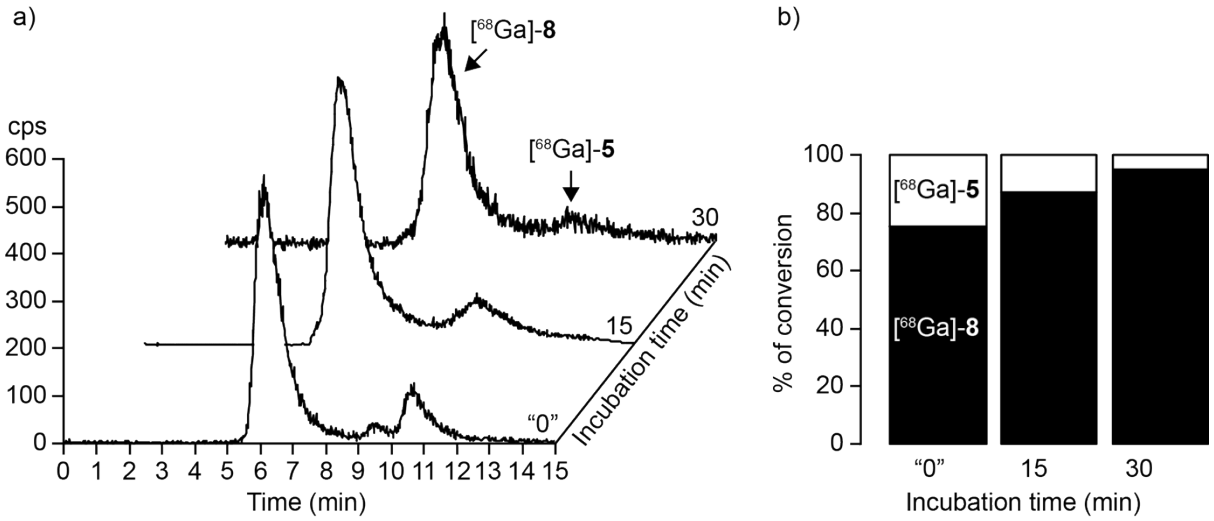


Figure S3. [^{68}Ga]-**5** (1.85MBq) was added to a solution of **7** (50 µL, diluted to 1 mg mL^{-1} in PBS), and the progress of the reaction was monitored by radio-HPLC, a) Example SEC radio-HPLC traces showing the reaction between [^{68}Ga]-**5** and **7**, injected after immediate incubation ("0"), 15 or 30 min incubation of the two reactive species, b) Percentage of radiochemical conversion to [^{68}Ga]-**8** from [^{68}Ga]-**5** over time, based on the SEC radio-HPLC traces.

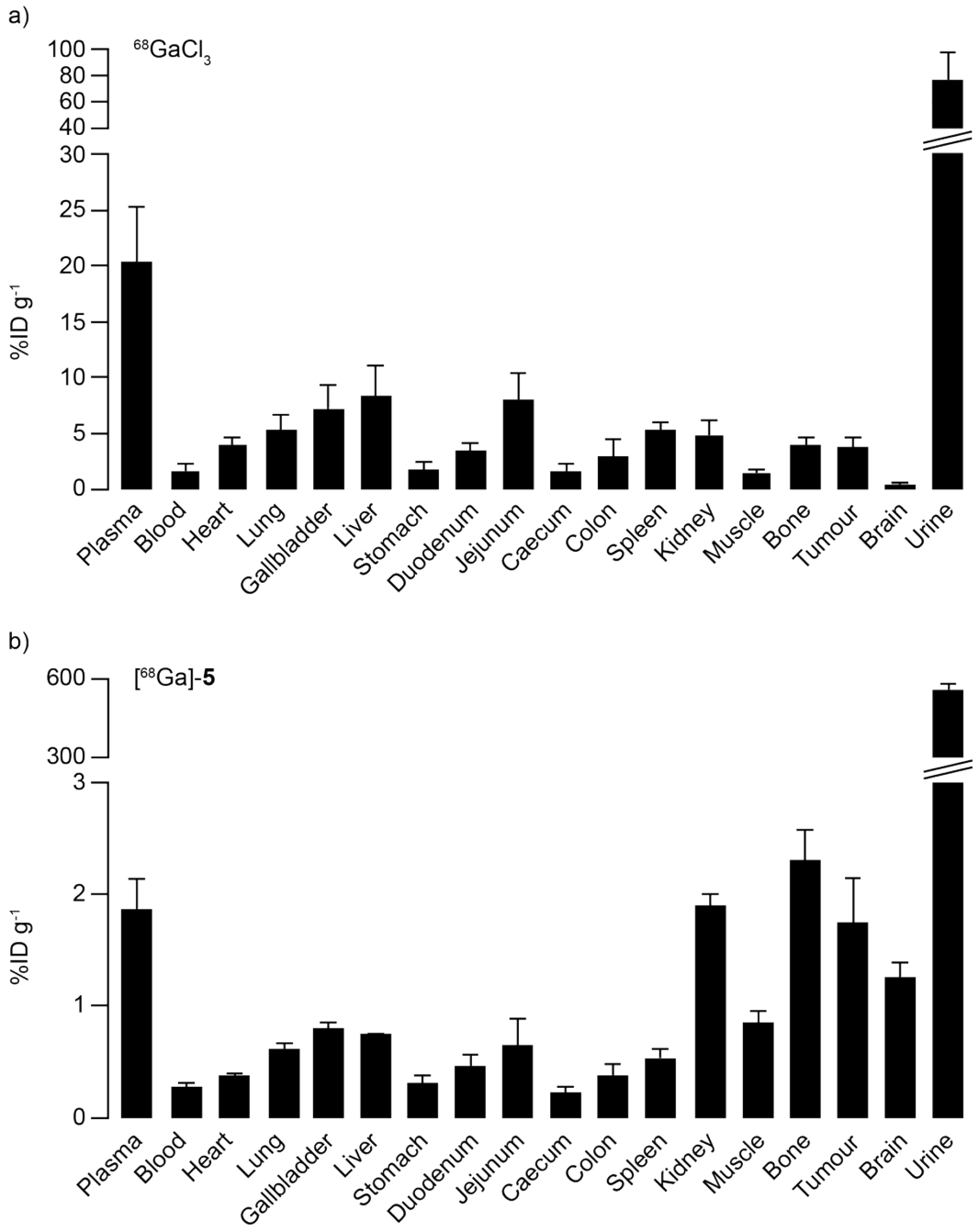


Figure S4. Tissue distribution of a) $^{68}GaCl_3$ and b) [^{68}Ga]-**5** in A431 tumour-bearing mice at 60 min post-radiotracer injection. Data are mean ± SEM.; *n* = 3 mice for each data-set.

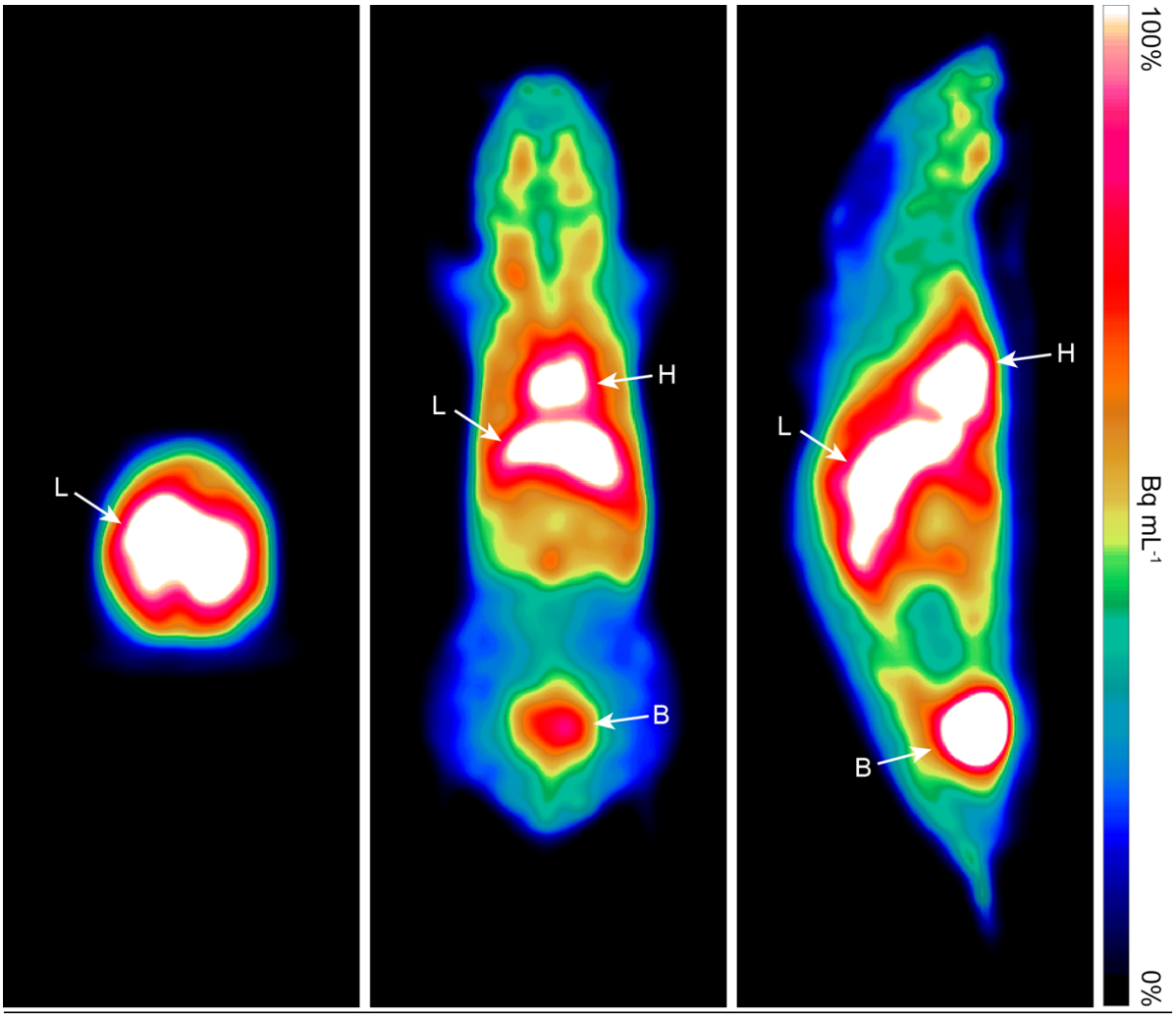


Figure S5. Representative axial, coronal and sagittal PET images of animal injected with $^{68}GaCl_3$ (*n* = 3). All scans were 60 min dynamic acquisition following a bolus injection of ~1.85 MBq of activity. White arrows indicate tissues with highest signal: H = heart, L = liver and B = bladder.

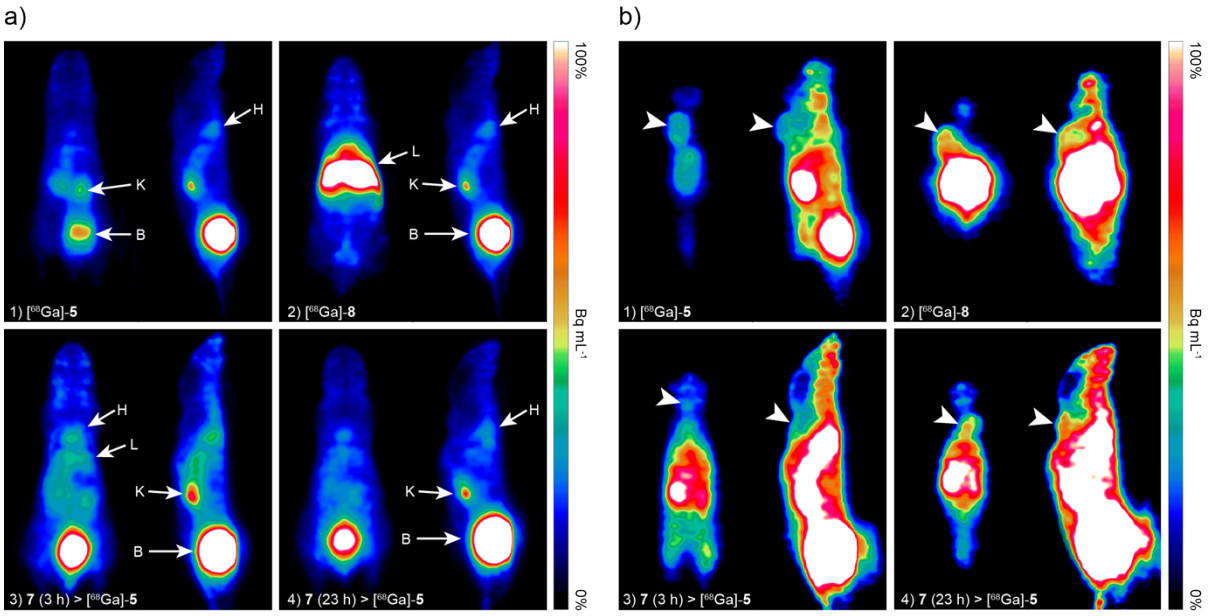


Figure S6. Representative coronal and sagittal PET images of animal injected with [^{68}Ga]-**5** (*n* = 3), [^{68}Ga]-**8** (*n* = 5), TCO-modified Cetuximab **7** for 3 or 23 hours followed by [^{68}Ga]-**5** ("pretargeting", *n* = 3 and 6, respectively). All scans were 60 min dynamic acquisition following a bolus injection of ~1.85 MBq of [^{68}Ga]-**5**, or ~7.4 MBq of [^{68}Ga]-**8**. a) The signal intensity scaling and selected slice have been chosen to display tissues with high signal, as indicated by the white arrows: H = heart, L = liver, K = kidney, and B = bladder. b) The signal intensity scaling and selected slice have been chosen to display the tumour, as indicated by the white arrowheads.

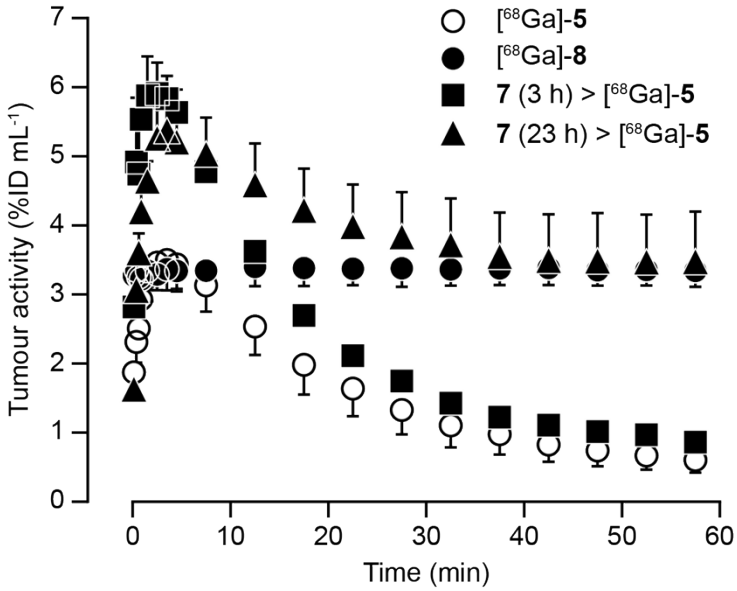


Figure S7. Tumour TACs (time versus activity curves,% injected dose per mL) derived from the PET images. Animal were injected with [^{68}Ga]-**5** (*n* = 3), [^{68}Ga]-**8** (*n* = 5), TCO-modified Cetuximab **7** for 3 or 23 hours followed by [^{68}Ga]-**5** ("pretargeting", *n* = 3 and 6, respectively). All scans were 60 min dynamic acquisition following a bolus injection of ~1.85 MBq of [^{68}Ga]-**5**, or ~7.4 MBq of [^{68}Ga]-**8**. Data are mean ± SEM.

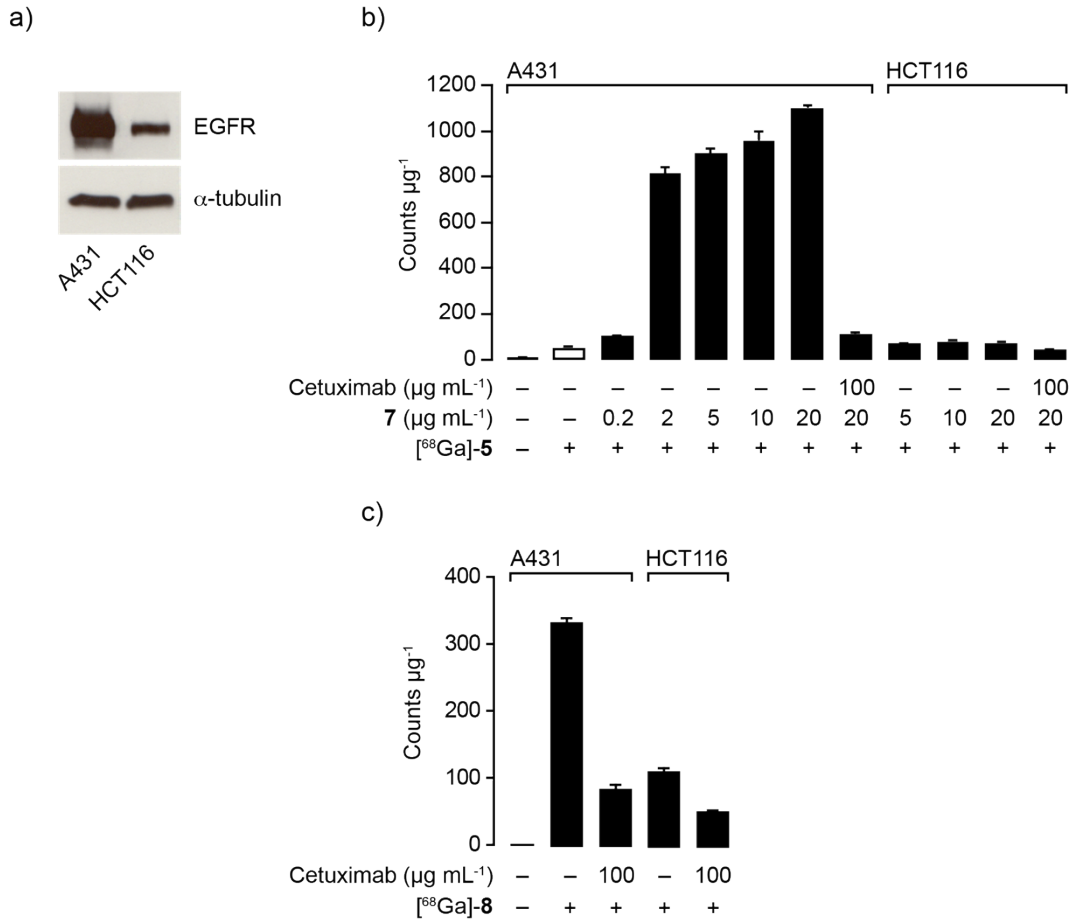


Figure S8. *In vitro* EGFR pretargeting using IeDDA bioorthogonal reaction. a) Immunoblot characterization of A431 high- and HCT116 low-EGFR expressing cells, α-tubulin assessment was used as loading control. A431 and HCT116 cells were b) pretargeted with **7** for 15 min at the indicated concentration, followed by incubation with ~ 0.74 MBq of [^{68}Ga]-**5** for 45 min at 37ºC, or c) directly incubated with ~ 0.74 MBq of [^{68}Ga]-**8** for 45 min at 37ºC. Pre-incubation with Cetuximab was used as a blocking control. The differences between absolute radioactivity in the direct versus pretargeting setting is likely due to intrinsic differences in reagents incubation sequences: the direct setting involves adding a mixture of mAbs **7** and [^{68}Ga]-**8**, which might result in competition to engage the target, whereas the pretargeting

setting is based on the two-step approach, i) addition of mAb **7**, with the cells being washed before ii) addition of [^{68}Ga]-**5.** Data shown are mean ± S.E.M. of counts mg^{-1} from triplicates of 3 independent experiments.

5. BIOLOGY EXPERIMENTAL PROCEDURES

Cell lines. The A431 epidermoid and HCT116 colon carcinoma cells were purchased from the American Type Culture Collection (ATCC), and selected based on their high and low cell surface EGFR expression, respectively. All cells were maintained in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mmol L^{-1} L-glutamine, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin (Invitrogen) and were maintained at 37ºC in a humidified atmosphere containing 5% CO_2.

Protein immunoblot. Cells were cultured in six-well plates with complete or serum-free growth media (overnight incubation) and stimulated with EGF (100 ng mL^{-1}) for 15 min, prior to incubation with TCO-modified Cetuximab **7** (50 μg mL^{-1}) or unmodified Cetuximab (50 μg mL^{-1}) for 24 h. Protein samples were subsequently prepared by lysing cells in RIPA buffer (Invitrogen Ltd.) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Equal amounts of protein (30 μg) were denatured in sample buffer, subjected to SDS–polyacrylamide gel electrophoresis on 4% to 12% gels (Bio-Rad Laboratories, Inc.), and transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences). The membranes were immunoblotted with specific primary antibodies, horseradish peroxidase–conjugated secondary antibodies, and visualised by enhanced chemiluminescence (GE Healthcare Life Sciences). The following antibodies were used: rabbit polyclonal antibody anti-phosphorylated-EGFR (Y1068, Cell signaling Technology), rabbit polyclonal antibody anti-EGFR (Cell signaling Technology), and mouse monoclonal antibody anti-α-tubulin (Santa Cruz Biotechnology) as primary antibodies. The

secondary antibodies were goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology).

***In vitro* pretargeting cell uptake.** Cells were cultured in 6-well plates with complete media, then treated with **7** for 15 min at the indicated concentration, followed by incubation with ~ 0.74 MBq of [^{68}Ga]-**5** for 45 min at 37ºC in a humidified atmosphere of 5% CO_2. Pre-incubation with Cetuximab (100 μg mL^{-1}) for 5 min was used as a blocking control. The plates were subsequently placed on ice and the cells were washed, trypsinised, and transfer in counting tubes. Decay-corrected radioactivity was determined on a gamma counter (Cobra II Auto-Gamma counter). Aliquots were snap-frozen and used for protein determination following radioactive decay by a bicinchoninic acid protein assay. Data were expressed as counts normalised for total cellular protein content (counts μg^{-1}).

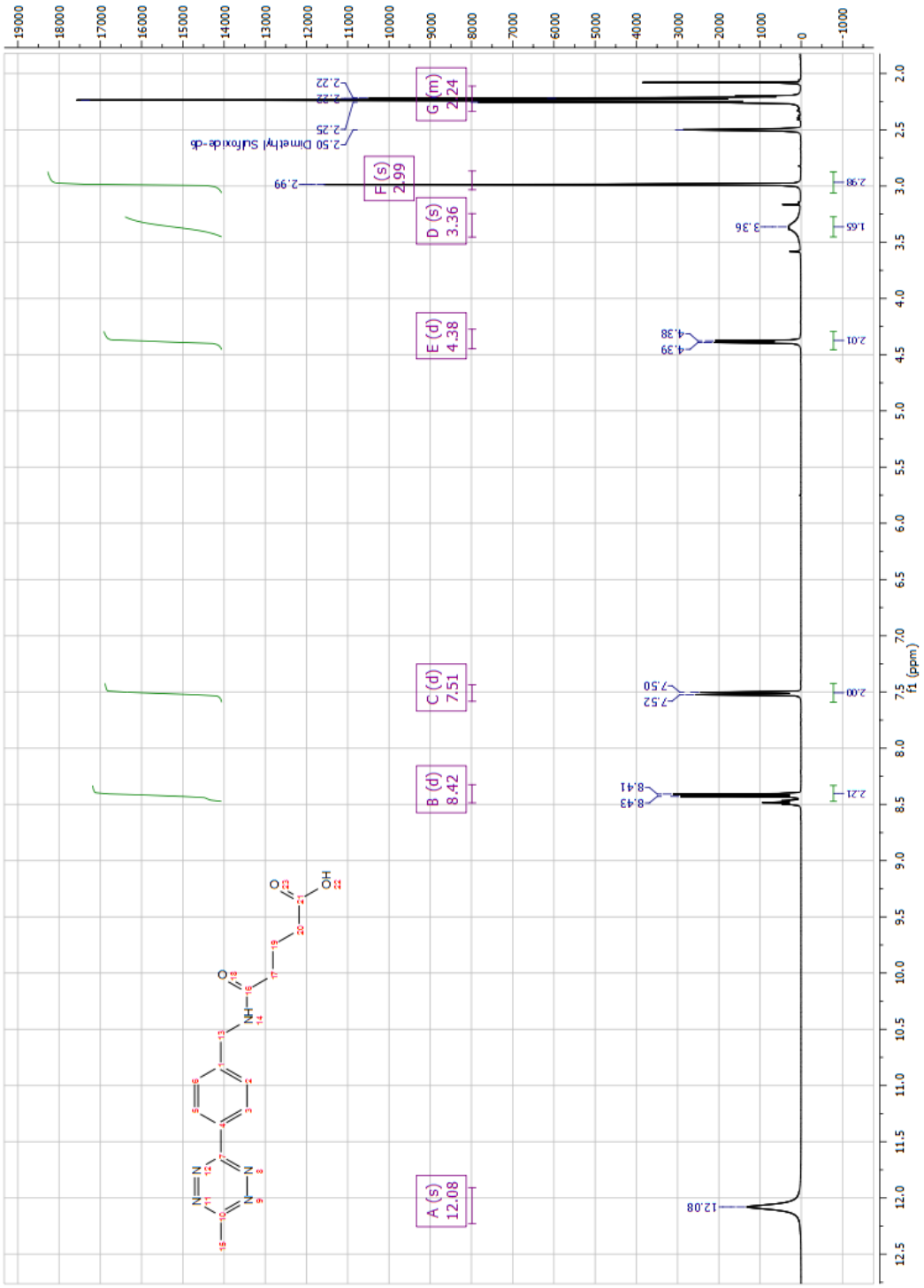
Small animal PET imaging and biodistribution. All animal experiments were done by licensed investigators in accordance with the UK Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 (HMSO, London, UK, 1990) and within guidelines set out by the UK National Cancer Research Institute Committee on Welfare of Animals in Cancer Research[3]. The *in vivo* experimental xenograft models were established by subcutaneous injection of $5x10^6$ A431 cells in BALB/c nude mice. All mice were 6- to 8-week-old females from Charles River. Animals were used when xenografts reached approximately 100 mm^3 (tumour dimensions were measured using a caliper and tumour volumes were calculated using the ellipsoid formula that is best for estimating tumour mass; volume mm3 = (π/6)x a x b x c, where a, b, and c represent 3 orthogonal axes of the tumour). At the

indicated time-points, mice were anesthetised through isoflurane inhalation and scans were carried out on the GENISYS^4 Bench-Top Preclinical PET Scanner (Sofie Biosciences) following a bolus *i.v.* injection of ~1.85 MBq ($^{68}GaCl_3$ or [^{68}Ga]-**5**) or ~7.4 MBq ([^{68}Ga]-**8**) of activity**.** Dynamic PET emission scans were acquired in list-mode format over 60 min, and corrected for decay and dead time. The acquired data were then sorted into 0.5-mm sinogram bins and 19 time frames (4 x 15, 4 x 60, and 11 x 300 sec) for image reconstruction with maximum-likelihood expectation maximization 60 iterations algorithm (matrix size = 208 (slice) x 96 (row) x 96 (column); voxel size = 0.456875 mm x 0.456875 mm x 0.456875 mm, isotropic; reconstructed axial FOV = 9.503 cm and reconstructed transaxial FOV = 4.386 cm). Siemens Inveon Research Workplace software was used for visualization of radiotracer uptake and display of representative PET images (0–60 min sums). The radioactivity concentrations within tissues were obtained from mean voxel intensity values within the volume of interest (VOI) and expressed as megabecquerels per milliliter (MBq mL^{-1}). These values were then divided by the administered activity in MBq to obtain an image VOI-derived percent injected dose per mL (%ID mL^{-1}) at each of the 19 time frames to give the time versus radioactivity curve (TAC). The normalised uptake value at 60 min and the area under the TAC (AUC) 30 to 60 min were computed for quantitative comparisons. $^{68}GaCl_3$ and [^{68}Ga]-**8** tissue biodistributions were assessed subsequent to the PET scans. For this, mice were sacrificed by exsanguination *via* cardiac puncture under general anesthesia and tissues were excised, weighed and immediately counted for gallium-68 radioactivity on a Cobra-II Auto-Gamma counter (Packard Instruments). Data were expressed as % injected dose per gram of tissue (%ID g^{-1}).

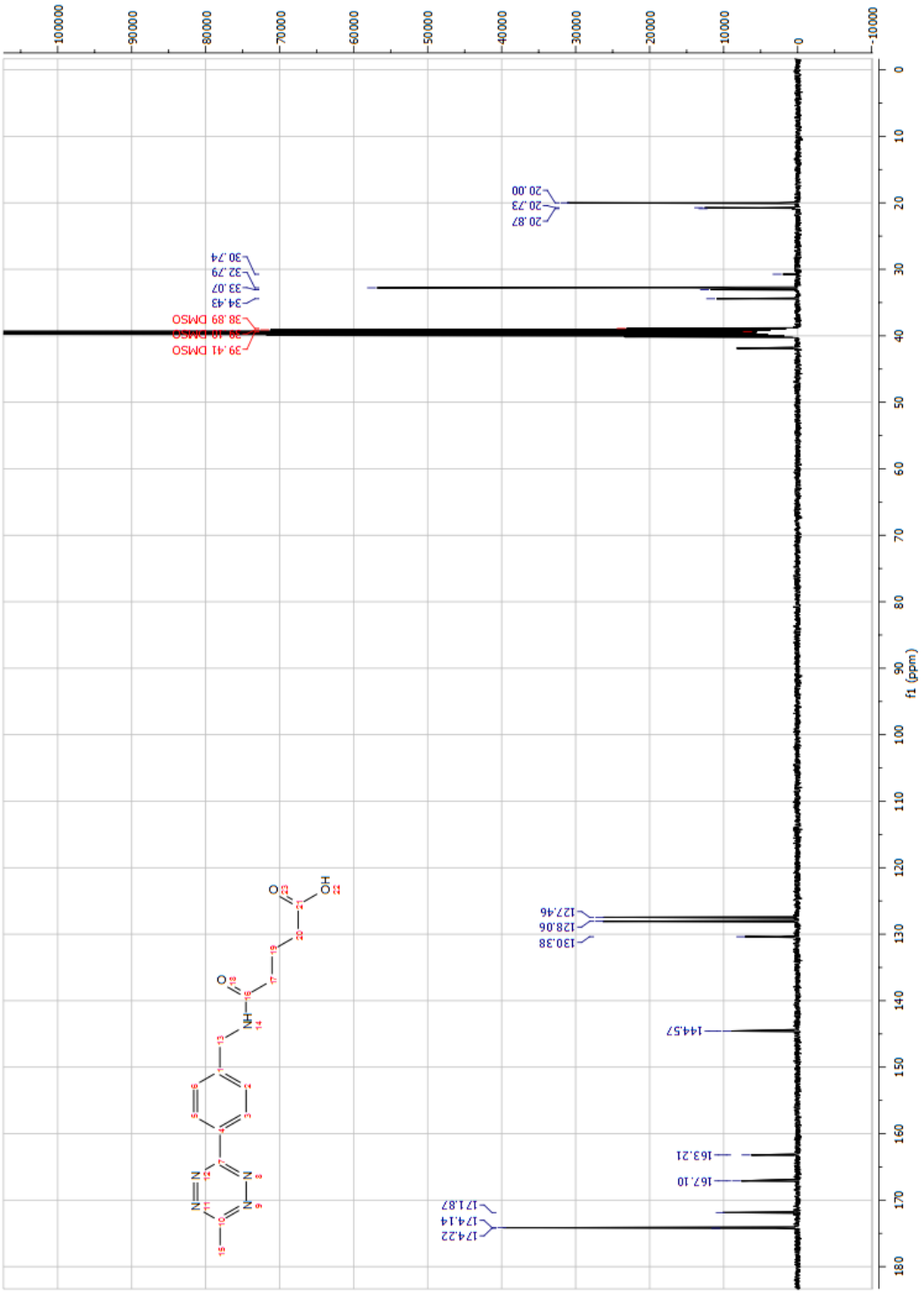
Statistical analysis. Data were expressed as mean ± standard errors of the mean (SEM) and the significance of comparison between 2 datasets was determined using Student t test (Prism v5.0 software, GraphPad) and defined as significant (*, 0.01 < P < 0.05), very significant (**, 0.001 < P < 0.01), and extremely significant (***,P < 0.001).

6. NMR SPECTRA

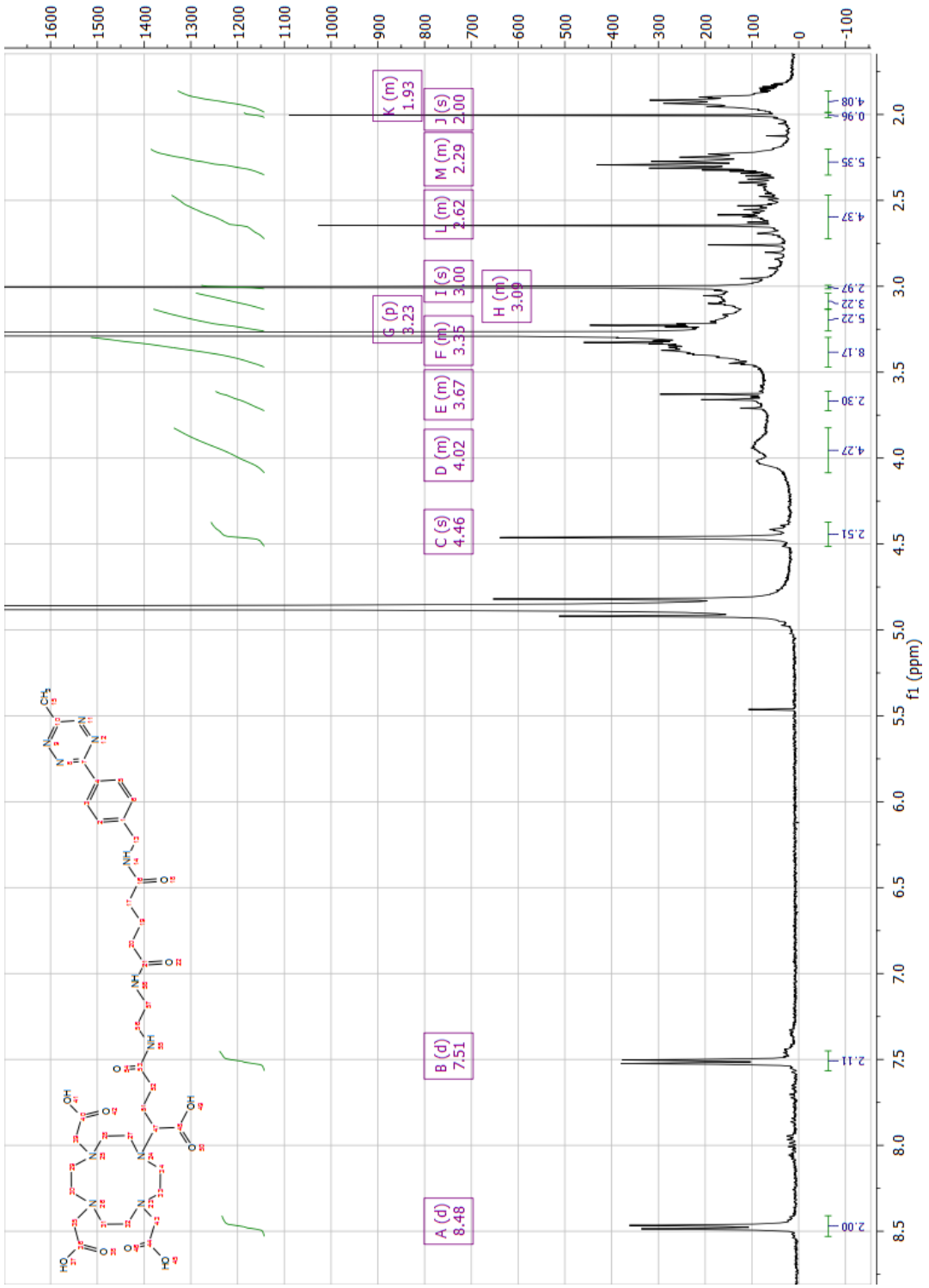
4-({[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamoyl)butanoic acid (**4**) ^{1}H NMR (400 MHz, d_6-DMSO)



4-({[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamoyl)butanoic acid (**4**) ^{13}C NMR (100 MHz, d_6-DMSO)



4-({2-[4-({[4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl]methyl}carbamoyl)butanamido]ethyl}carbamoyl)-2-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]butanoic acid (**5**) ^{1}H NMR (400 MHz, d_6-MeOD)



7. REFERENCES

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