Supplementary Information

"A bivalent small molecule-drug conjugate directed against carbonic anhydrase IX can elicit complete tumour regression in mice"

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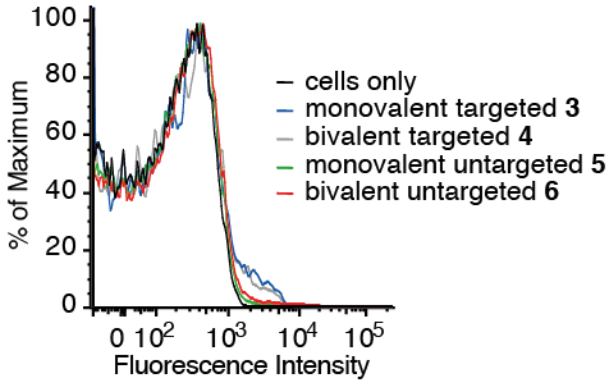
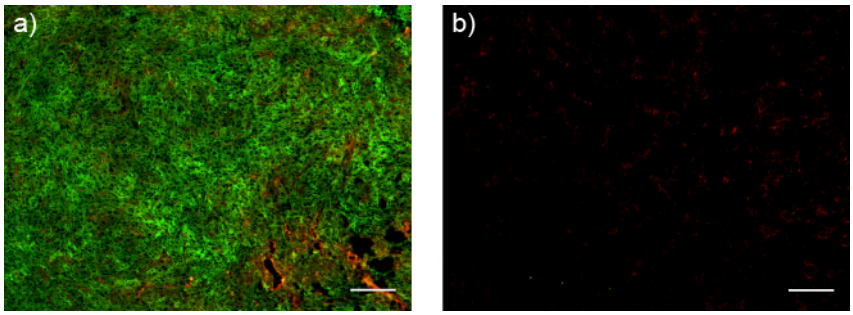
Supplementary Data

Binding of Ligand-IRDye750 Conjugates to Cells in Vitro

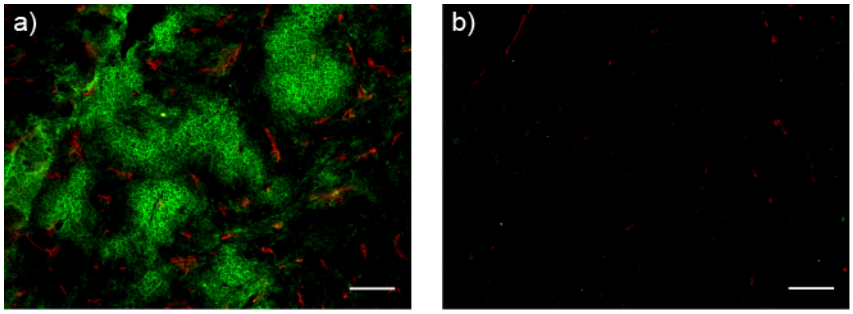


Supplementary Figure 1: Flow cytometric analysis of binding of IRDye750 conjugates **3**-**6** to CAIX-negative HEK cells. Cells were incubated with conjugates (30 nM) for 1 h at 4 °C followed by washing and analysis. All histograms are superimposable with untreated cells suggesting the absence of non-specific binding.

Antigen Expression in Tumour Models

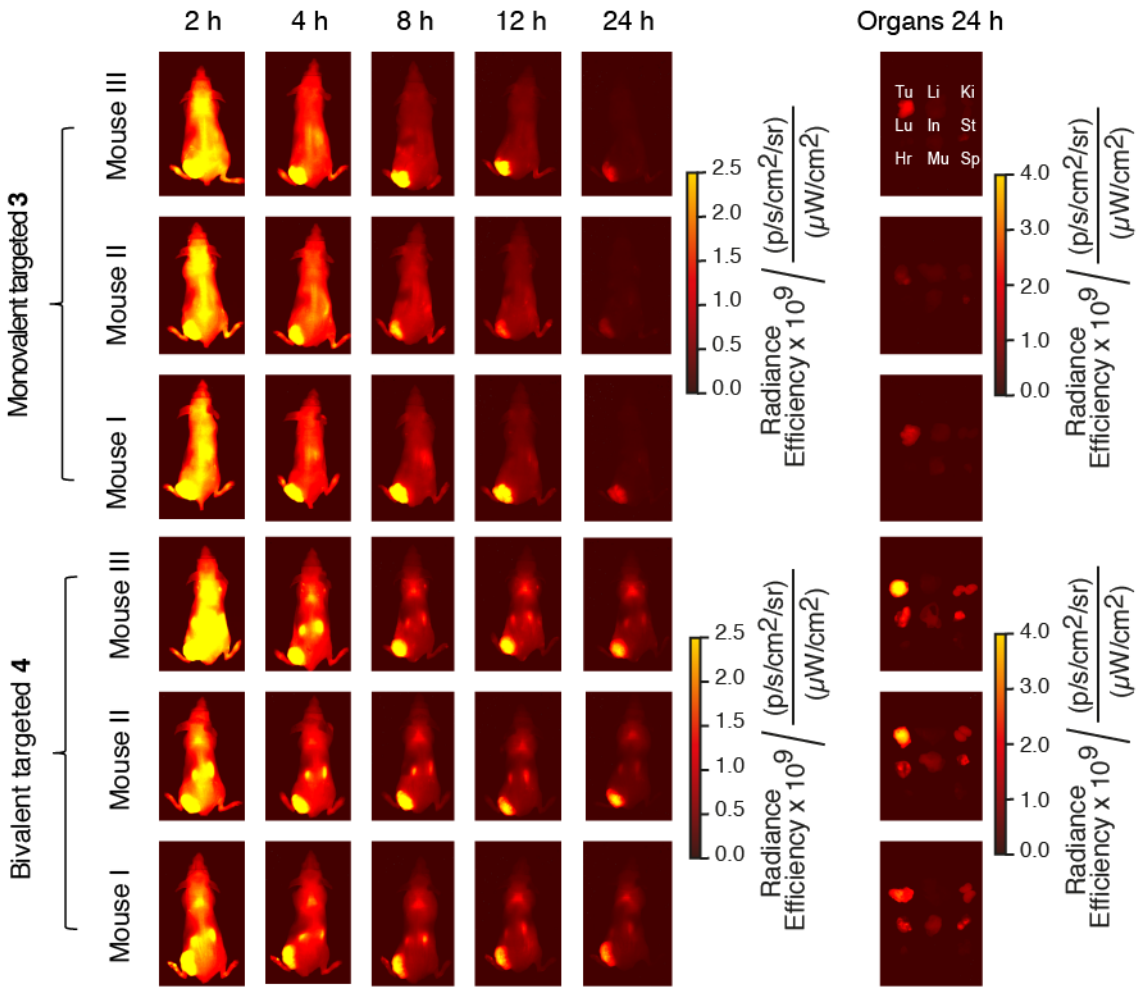


Supplementary Figure 2: a) Constitutive expression of CAIX (green) in SKRC52 tumour sections. b) Control staining with an irrelevant antibody against hen egg-white lysozyme. Scale bar indicates 200 µm.



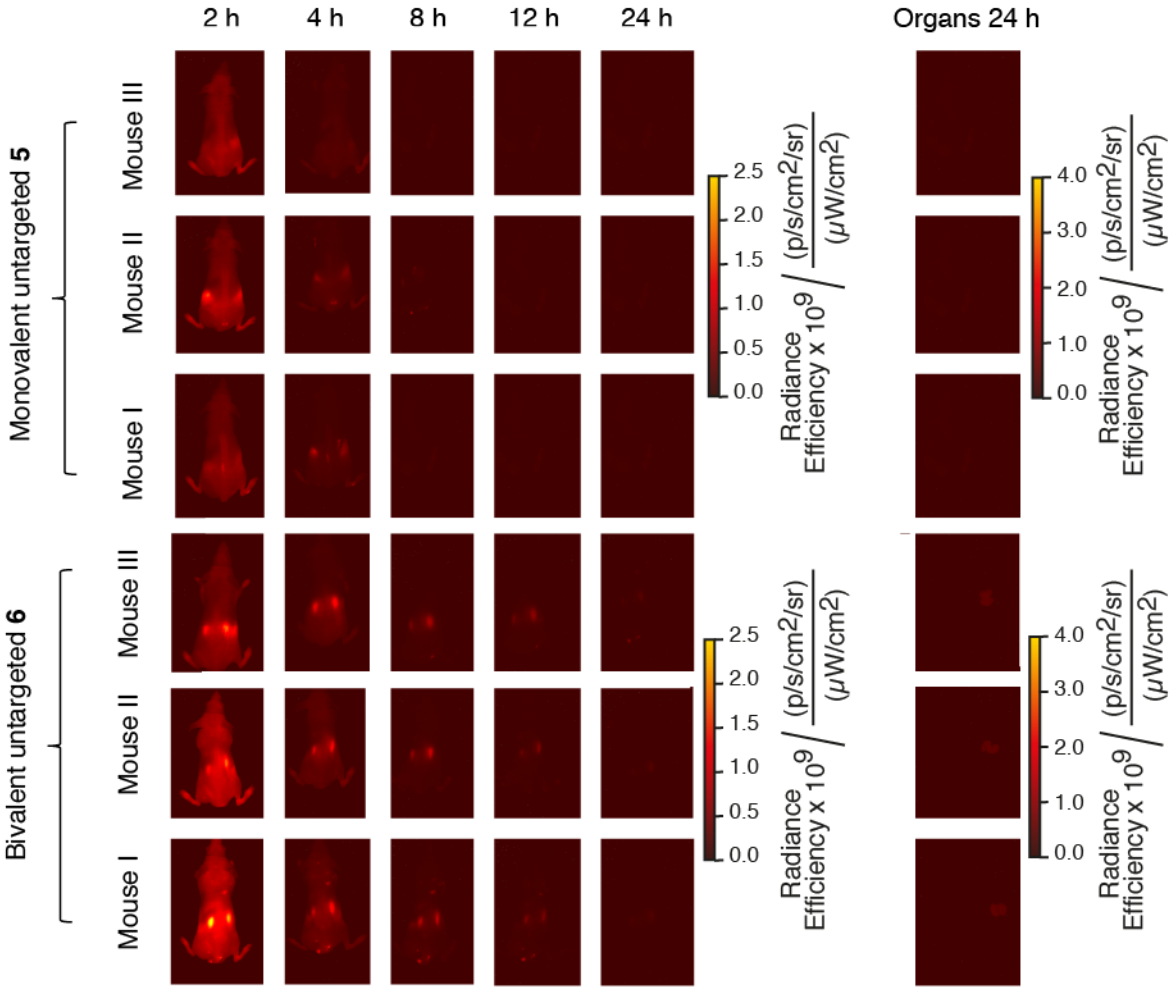
Supplementary Figure 3: a) Expression of CAIX (green) in A375 tumour sections. Antigen is located at sites distant to blood vessels (red) consistent with expression in response to hypoxia. b) Control staining with an irrelevant antibody against hen egg-white lysozyme. Scale bar indicates 200 µm.

Near Infrared Imaging

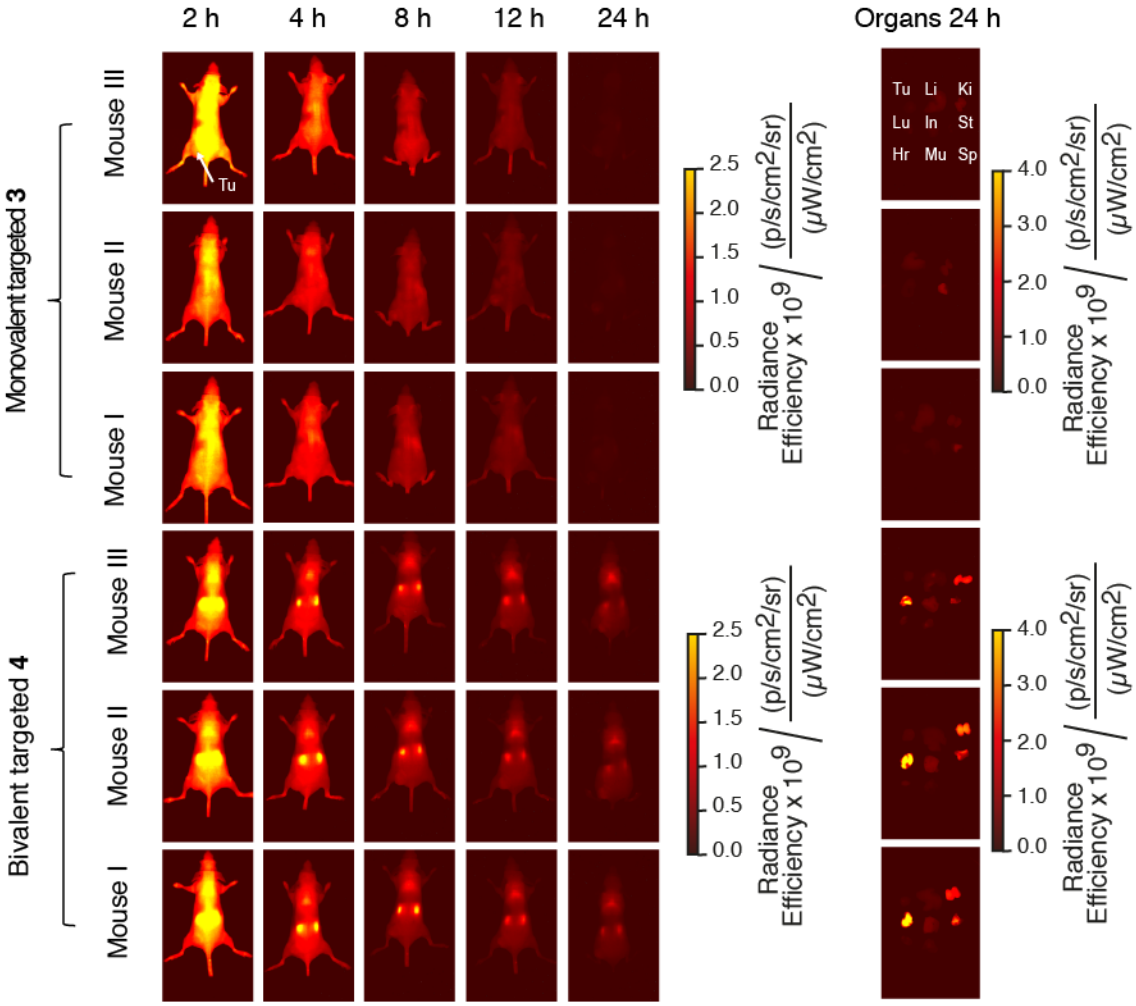


Supplementary Figure 4: Near infrared images of mice bearing SKRC52 tumours injected with 3 nmol of monovalent IRDye750 conjugate **3** or bivalent IRDye750 conjugate **4**. Images were taken 2, 4, 8, 12 and 24 h after injection. After the last time point, animals were sacrificed, organs extracted and imaged individually. Organs

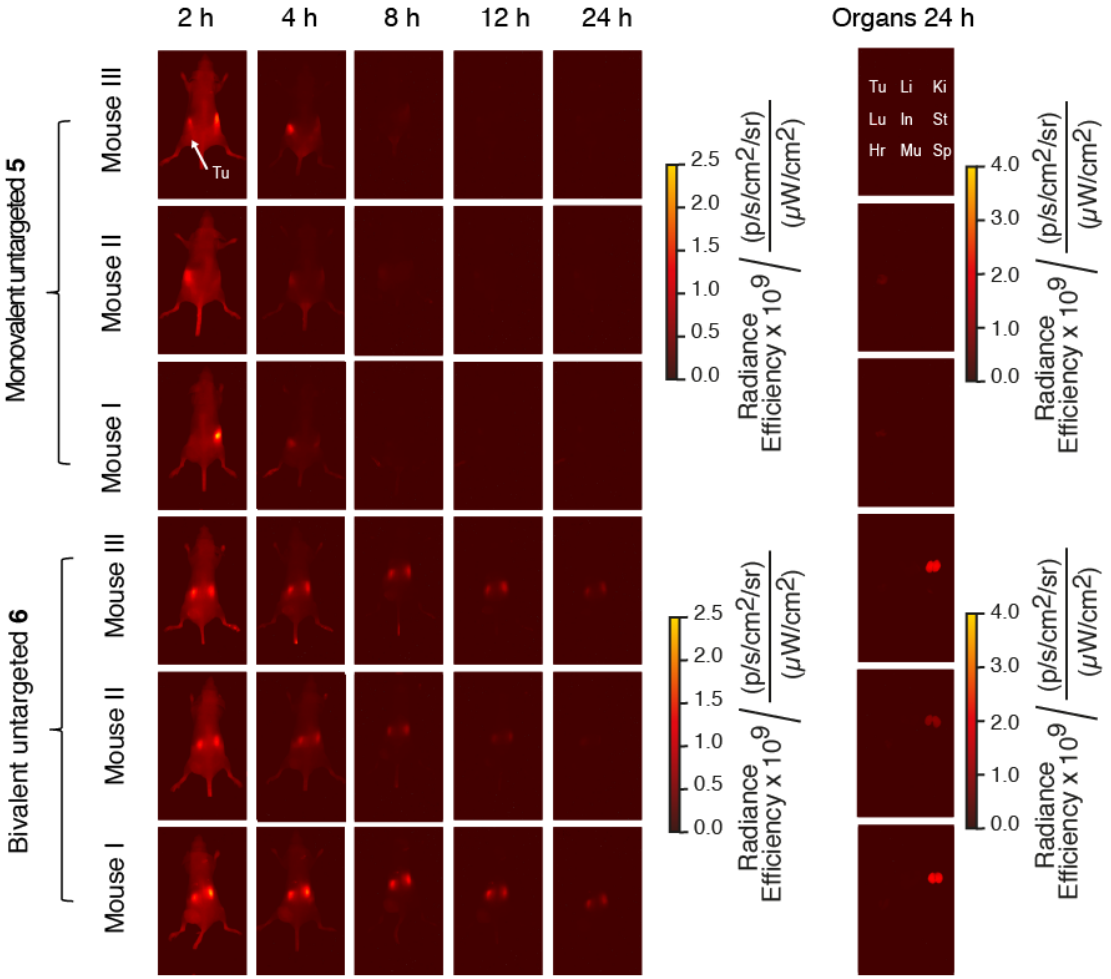
from left top to right bottom are Tu = SKRC52 tumour, Li = liver, Ki = kidneys, Lu = lungs, In = Intestine, St = stomach, Hr = heart, Mu = muscle, Sp = spleen.



Supplementary Figure 5: Near infrared images of mice bearing SKRC52 tumours injected intravenously with 3 nmol monovalent IRDye750 conjugate **5** or bivalent IRDye750 conjugate **6**. The conjugates were identical to those used in Supplementary Figure 2 but lacked the targeting ligand. Images were taken 2, 4, 8, 12 and 24 h after injection. Animals were sacrificed after the last time point, organs extracted and imaged individually. In the absence of a targeting ligand, the dye conjugate did not accumulate inside the tumour in measurable amounts.

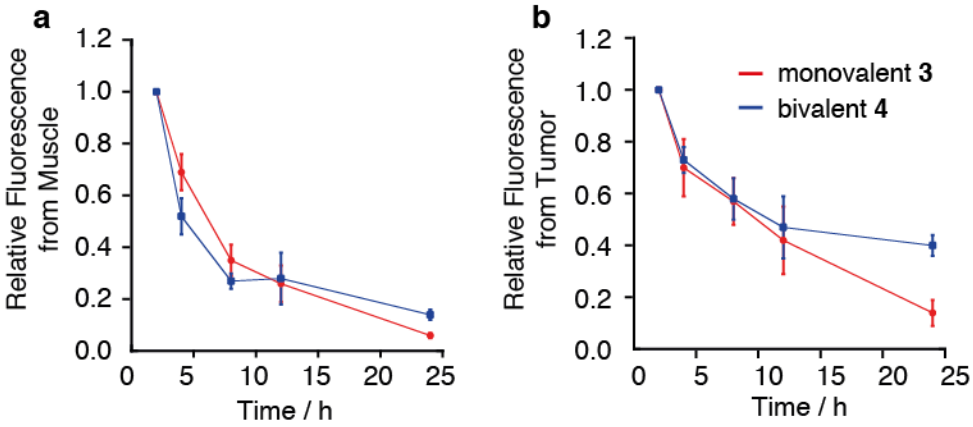


Supplementary Figure 6: Near infrared images of mice bearing A375 tumours on their lower left back injected with 3 nmol of monovalent IRDye750 conjugate **3** or bivalent IRDye750 conjugate **4**. Images were taken 2, 4, 8, 12 and 24 h after injection. After the last time point, animals were sacrificed, organs extracted and imaged individually. Organs from left top to right bottom are Tu = A375 tumour, Li = liver, Ki = kidneys, Lu = lungs, In = Intestine, St = stomach, Hr = heart, Mu = muscle, Sp = spleen. In spite of some CAIX expression at sites distant to blood vessels as evidenced by immunofluorescence, neither of the conjugates accumulates at the tumour site.

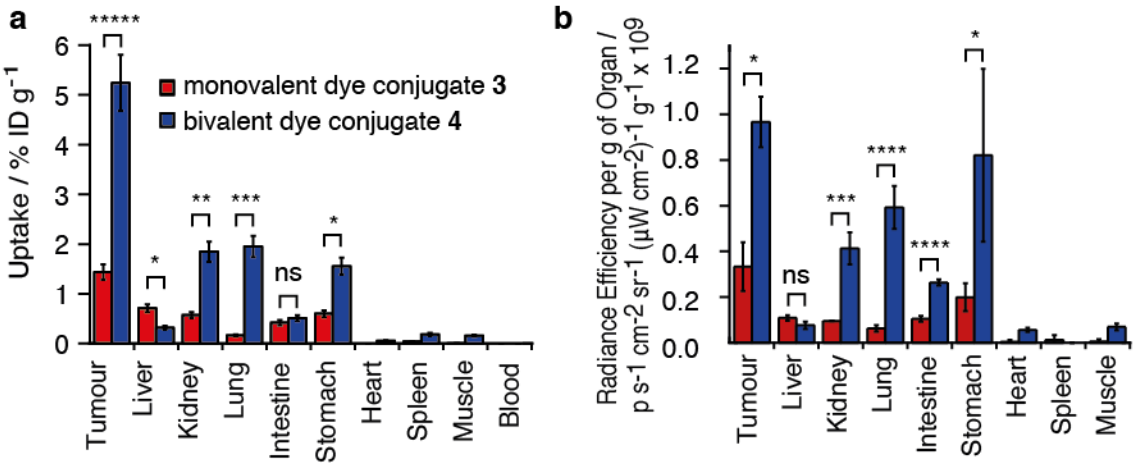


Supplementary Figure 7: Near infrared images of mice bearing A375 tumours on their lower left back injected intravenously with 3 nmol monovalent IRDye750 conjugate **5** or bivalent IRDye750 conjugate **6**. The conjugates were identical to those used in Supplementary Figure 2 but lacked the targeting ligand. Images were taken 2, 4, 8, 12 and 24 h after injection. Animals were sacrificed after the last time point, organs extracted and imaged individually.

Biodistribution Analysis

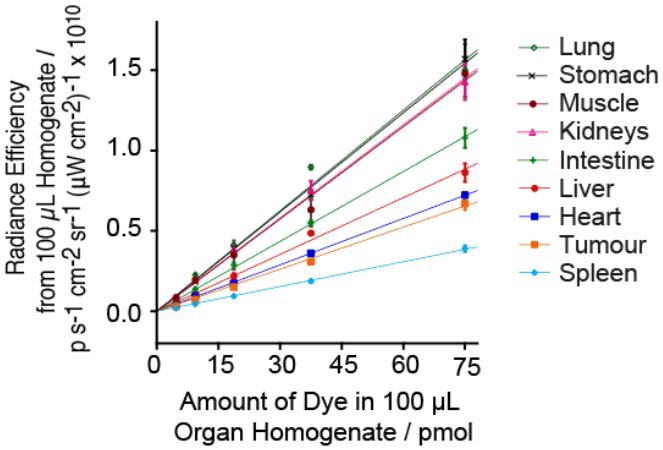


Supplementary Figure 8: (a) Change of fluorescence signal from muscle over time in SKRC52 xenograft bearing mice. Regions of interest (ROIs) were placed over the right hind leg muscle of mice in Supplementary Figure 4, integrated to obtain total fluorescence and each expressed relative to the signal from the same animal at 2 h. In the absence of antigen in muscle[1] fluorescence from this tissue was used as a surrogate marker for dye in circulation (blood + interstitium). Decay patterns are comparable suggesting that clearance from circulation is broadly the same for monovalent and bivalent conjugates. Data points are averages of three animals. Error bars give standard deviations. (b) Fluorescence emanating from a region of interest (ROI) laid over the SKRC52 tumour relative to the signal from the corresponding ROI at 2 h after injection of 3 nmol monovalent **3** or bivalent dye conjugate **4**. Data points are averages of three animals. Error bars give standard deviations. Difference at 24 h is statistically significant ($p = 0.002$; unpaired two-sided t-test).



Supplementary Figure 9: (a) Biodistribution of monovalent IRDye750 conjugate **3** and bivalent IRDye750 conjugate **4** in SKRC52 xenograft bearing balb/c nu/nu mice 24 h after intravenous administration of 3 nmol conjugate. Uptake levels are given as the percentage of the injected dose resident in each organ normalised to

organ weight in g (% ID g^{-1}) and were obtained by comparison of fluorescence from organ homogenate with organ-specific calibration curves (Supplementary Figure 10). (b) Total fluorescence normalised to organ weight originating from the same organs as described in the left part of the figure. Fluorescence was determined by integration over near infrared images of intact organs and is given as radiance efficiency in units of (photons s^{-1} cm^{-2} sr^{-1} (μW cm^{-2})$^{-1}$) × 10^9 normalised to organ weight in g. Values are averages of three experiments. Error bars give standard deviations. A 2-sided unpaired t-test was used to test for statistically significant differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, ***** $p < 0.0005$.



Supplementary Figure 10: Calibration curves for the estimation of IRDye750 uptake in different organs. Organ homogenates were spiked with different amounts of IRDye750 and fluorescence emanating from homogenates was measured on an IVIS imaging system as radiance efficiency in units of (photons s^{-1} cm^{-2} sr^{-1} (μW cm^{-2})$^{-1}$) × 10^9. With the exception of heart, data points are averages of three organ samples. Error bars give standard deviations. Tumours are SKRC52 xenografts.

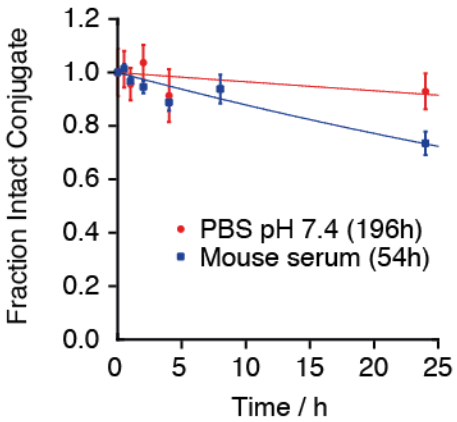
	Liver	Kidney	Lung	Intestine	Stomach	Heart	Spleen	Muscle	Blood
3	2.0 ± 0.3	2.5 ± 0.4	8.8 ± 1.4	3.4 ± 0.6	2.4 ± 0.4	ND	34 ± 6	285 ± 61	ND
4	16 ± 3	2.8 ± 0.4	2.7 ± 0.4	10 ± 2	3.4 ± 0.5	91 ± 16	29 ± 6	33 ± 5	ND

Supplementary Table 11: Selectivity of uptake of monovalent IRDye750 conjugate **3** and bivalent IRDye750 conjugate **4** into SKRC52 tumours relative to other organs 24 h after intravenous administration of 3 nmol as determined from organ uptake levels in % ID g^{-1} (Supplementary Figure 9a). Tumour:organ ratios are given as averages of three animals ± standard deviation.

	Liver	Kidney	Lung	Intestine	Stomach	Heart	Spleen	Muscle	Blood
3	3.0 ± 0.8	3.5 ± 1.1	5.2 ± 1.0	3.2 ± 0.9	1.9 ± 0.9	ND	ND	ND	ND
4	12.8 ± 1.1	2.4 ± 0.2	1.6 ± 0.1	3.7 ± 0.3	1.3 ± 0.4	17.5 ± 3.7	ND	14.2 ± 0.0	ND

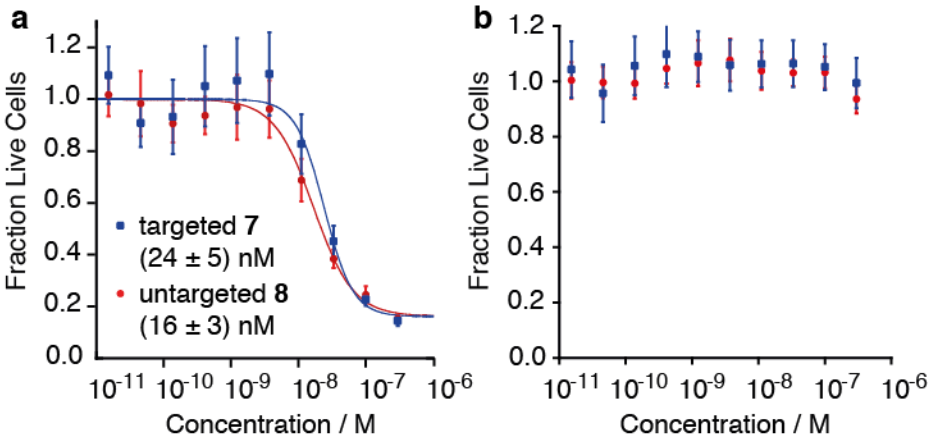
Supplementary Table 12: Selectivity of uptake of monovalent IRDye750 conjugate **3** and bivalent IRDye750 conjugate **4** into SKRC52 tumours relative to other organs 24 h after intravenous administration of 3 nmol as determined from total fluorescence signal integrated over organs and normalised to organ weight (Supplementary Figure 9b). Tumour:organ ratios are given as averages of three animals ± standard deviation.

Stability of Drug Conjugates in Vitro



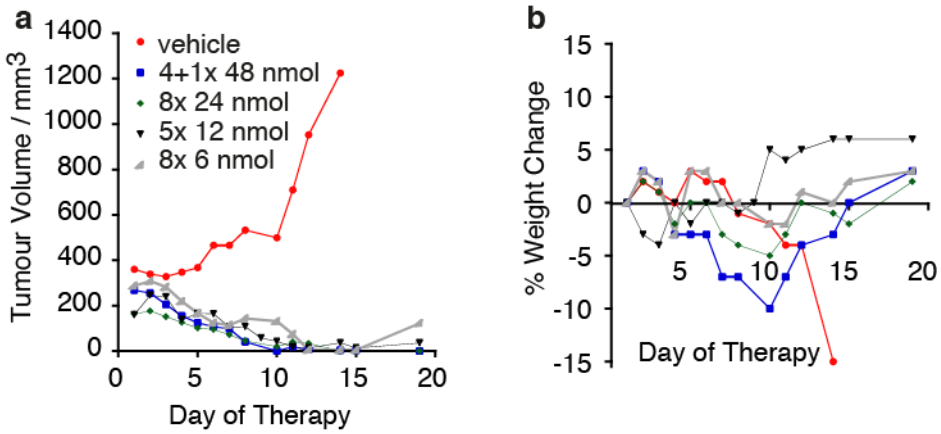
Supplementary Figure 13: Stability of bivalent DM1 conjugate **7** in PBS pH 7.4 and mouse serum at 37 °C as determined by high performance liquid chromatography (HPLC). Data points are averages of 3 experiments. Error bars give standard deviations. Stability half-lives are given in brackets. Errors of fit were < 3 min and are therefore not given explicitly.

Cytotoxicity of Drug Conjugates in Vitro



Supplementary Figure 14: In vitro cytotoxicity of targeted DM1 conjugate **7** and untargeted DM1 conjugate **8** towards CAIX-expressing SKRC52 cells. Cell killing EC_{50} values are given in brackets ± error of fit. (a) Cells were exposed to toxin over 72 h. (b) Cells were exposed to toxin for 1 h, washed and then incubated for 71 h. Targeted and untargeted conjugates exhibit similar cytotoxicity suggesting that receptor-mediated internalisation followed by intracellular activation of the conjugate is inefficient. If bivalent conjugates were actively internalised by receptor-mediated mechanisms and activated intracellularly, targeted conjugate **7** would be expected to be more toxic than conjugate **8** lacking the targeting ligand. Similar results had been observed with monovalent drug conjugates.[3] Internalising peptide ligands of CAIX have previously been described[2] suggesting that the observed behaviour is related to the nature of our conjugate rather than a general inability of the antigen to internalise.

Dose Finding with Bivalent DM1 Conjugate in Vivo



Supplementary Figure 15: Dose finding study with targeted bivalent DM1 conjugate **7**. a) Tumour growth curves b) Weight change over course of therapy experiment. A single animal was used for each condition. Conjugates were administered daily for the indicated number of days at the given dose in PBS containing 5% DMSO v/v with the exception of 48 nmol which was given on 4 consecutive days followed by 1 day of pause and then the final

dose. Doses of 8× 6 nmol, 5× 12 nmol and 8× 24 nmol were well tolerated as indicated by only temporary weight loss < 5%. A dose of (4+1)× 48 nmol led to a delayed toxicity on day 10 (weight loss < 10%) and was thus deemed to be above the maximum tolerated dose. The mouse, which had received 8× 24 nmol, was still tumour free 90 days after start of therapy and was thus deemed cured. The tumours of all other mice regrew after the end of therapy.

Experimental Methods

Propagation of Errors

Standard errors and standard deviations were propagated during data analysis using the method recommended by the National Institute of Standards and Technology.[3] In general, the standard deviation σ_f of $f = f(x_1, x_2, \ldots x_n)$ is given by formula (1) where σ_i is the standard deviation of x_i.

$$\sigma_f = \sqrt{\sum_{i=1..n} \left(\frac{\partial f}{\partial x_i} \sigma_i \right)^2} \quad (1)$$

Affinity determination of CAIX ligands by Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were performed on a Biacore T200 instrument (GE Healthcare) at room temperature. For all measurements, CM5 chips and flow buffer of PBS pH 7.4 with DMSO (5 % v/v) and P20 surfactant (0.05 % v/v, GE Healthcare) were used. CAIX protein was immobilised on the chip to 3,000 – 4,000 response units (RU) using EDC·HCl and NHS following manufacturer instructions. Serial dilutions of monovalent ligand **1** or bivalent ligand **2** (640 to 0.63 nM in steps of 1:4) in running buffer at a flow rate of 25 µL min^{-1} were used as analytes. After each cycle, the sensor surface was regenerated by a short treatment with DMSO (50 % v/v) in H_2O. For bivalent ligand **2** an additional washing step with aqueous HCl (50 mM) for 30 sec at 25 µL min^{-1} was required to remove bound ligand. Sensorgrams were solvent corrected and the binding kinetics were analyzed with the Biacore T200 evaluation software (version 2.0) using a 1:1 Langmuir or bivalent binding model. Matlab (R2010b) was used for generation of sensogram plots from exported data.

Cell Culture

SKRC52 and HEK cells were cultured in RPMI medium as described previously.[4] A375 cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS, Invitrogen) and antibiotic-antimycotic (Invitrogen) at 37 °C with 5% CO_2. When reaching 90% confluence, cells were detached using 0.05% EDTA-Trypsin (Invitrogen) and re-seeded at a dilution of 1:4.

Ligand Binding Analysis by Flow Cytometry

Binding of ligand-IRDye750 conjugates **3**, **4**, **5** or **6** to CAIX-expressing SKRC52 human kidney cancer cells and CAIX-negative HEK cells was analyzed by flow cytometry as described previously.[4] In brief, cells were incubated with dye conjugates in PBS pH 7.4 containing 1% FCS v/v for 1h at 0 °C, washed, resuspended in PBS pH 7.4 containing 1% FCS v/v and propidium iodide as a viability dye and analyzed by flow-cytometry.

Immunofluorescence Microscopy

Tumor specimens were shock frozen in NEG 50 (Thermo Scientific) and stored at -80 °C for up to 2 days before use. Organs were warmed to -20 °C and 10 µm sections prepared in a cryostat. Tumor sections were fixed for 10 min in ice-cold acetone, dried and incubated in blocking buffer consisting of 10% FCS v/v and 3% BSA in PBS pH 7.4 for 45 min at room temperature. Slides were washed twice for 5 min in PBS pH 7.4 and incubated with the PHB11 proprietary fully human anti CAIX antibody in IgG format (Philochem, 5 µg mL^{-1}) or the KSF fully human negative control antibody specific for hen egg-white lysozyme in IgG format (Philochem, 5 µg mL^{-1}) in blocking buffer for 90 min at room temperature. After washing (2× 5min with PBS pH 7.4), slides were incubated with a mixture of rat anti-mouse CD31 IgG (BD Pharmigen, 1:500) and rabbit anti-human IgG (Bethyl Laboratories, 1:250) in blocking buffer for 60 min at room temperature. Slides were washed again (2× 5min with PBS pH 7.4), stained with goat anti rabbit Alexa488 conjugates IgG (Invitrogen, 1:200) and donkey anti rat Alexa594 IgG (Invitrogen, 1:200) in blocking buffer for 60 min at room temperature, washed again (2× 5min with PBS pH 7.4), stained with Hoechst 33342 (Invitrogen, 1:1000) in PBS pH 7.4 for 1 min at room temperature, washed (5 min with PBS pH 7.4) and mounted with fluorescence mounting medium (Dako). After drying at room temperature over night, images were taken on a Zeiss Axioskop 2 (Carl Zeiss).

In Vitro Cytotoxicity Assay

In vitro cytotoxicity assays were carried out as described previously.[4] In brief, cells were incubated with medium containing varying concentrations of drug conjugates **7** or **8** for 72 h and cell viability determined with MTS reagent (Promega). Alternatively, cells were incubated with drug conjugates for 1 h, washed and then incubated with fresh medium for 71 h followed by viability assessment. Viability was plotted as fraction of cells alive relative to conjugate concentration and the dose of the half-maximum effect (EC_{50}) determined by non-linear curve fitting to the four-parameter logistic curve.

Stability Determination by High-Performance Liquid Chromatography (HPLC)

Stability of drug conjugate **7** was determined by High-Performance Liquid Chromatography (HPLC) using a previously described method.[4] In brief, drug conjugate **7** was incubated in PBS pH 7.4 or mouse serum (Invitrogen) at 37 °C. Aliquots were withdrawn at different time points, serum proteins precipitated with MeCN and samples analyzed by HPLC. The fraction of intact conjugate was determined by integration over the corresponding intact conjugate peak and divided by the integral at time zero. Etodolac was used as an internal standard.

Animal Studies

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 42/2012 granted by Veterinaeramt des Kanton Zurich.

Implantation of Subcutaneous Tumours

10^7 SKRC52 or A375 cells per animal were implanted subcutaneously into the lower backs of balb/c nu/nu mice (Charles River) according to previously established procedures.[4]

Near Infrared Imaging of Tumour Bearing Mice

A modified version of a previously described protocol was used.[4] As reported, mice bearing subcutaneous SKRC52 or A375 tumours (200 – 300 mm^3 in sise) were injected with IRDye750 (Licor) labeled CAIX ligands **3-6** (3 nmol) dissolved in 5% v/v DMSO in PBS pH 7.4 (150 μL) through the tail vein. Fluorescence images were acquired at 2 h, 4 h, 8 h, 12 h and 24 h after injection on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning 'small', excitation at 745 nm, emission filter at 800 nm, *f* number 2, field of view 'C', sample height 1.50 cm). The temperature in the imaging chamber was set to 37 °C and mice were under isoflurane anesthesia during all imaging procedures. Between time points, mice were kept in standard IVC rack cages at 21-24 °C. Food and water were freely available during the course of the entire experiment. After the last time point, mice were sacrificed using CO_2. Organs were extracted (heart, lung, kidney, liver, spleen, a section of the small intestine of 100 - 150 mg, skeletal muscle weighing in total 100 – 150 mg, stomach, 100 uL of blood and tumour). Organs were weighed and imaged individually using the IVIS parameters described above. Total fluorescence originating from tumour, muscle or extracted organs was measured by integrating over organ areas on near infrared images using the built in ROI tools of Living Image software version 4.3.1 (Caliper Life Science) and corrected for background fluorescence by subtracting a background ROI of equal sise and shape.

Biodistribution Analysis

A previously described protocol was used with minor modifications.[4] Organs from imaging experiments (24 h time point) were cut into small pieces and ice-cold homogenisation buffer containing EDTA (40 mM), trypsin (6 mg/ml), Triton X-100 (1.6 µl/ml) and trace amounts of DNase 1 in PBS pH 7.4 (100 µL per 100 mg of tissue) was added. After treatment on a TissueLyser organ homogeniser (Quiagen, 25 Hz, 15 min), homogenates were incubated for 2 h at room temperature and 100 µL of each sample was transferred to black 96-well plates. Organ-specific calibration series were prepared by spiking organ homogenates from previously untreated mice with different amounts of IRDye750 (75 pmol – 4.7 pmol dye in 100 µL of organ homogenate in steps of 1:2) and spotted alongside organ homogenates with unknown dye content. Fluorescent images of plates were recorded on an IVIS Spectrum imaging system (Xenogen, parameters as above except for height which was set to 0.50 cm) and the fluorescence originating from individual wells was quantified using the built-in plate region of interest (ROI) tool of Living Image software version 4.3.1 (Caliper Life Science). Organ-specific calibration curves were obtained by plotting fluorescence intensity from calibration wells against dye concentration and linear regression analysis. Dye concentrations in organ samples were inferred by comparison of fluorescence intensities from the corresponding well with the calibration curve from the correct organ and then converted to % of injected dose per gram of tissue (% ID g^{-1}, 100 % ID = 3 nmol).

Therapy Experiments

SKRC52 xenograft bearing balb/c nu/nu mice (Charles River) were randomly assigned into therapy groups of 5 or 6 animals 10 days after tumour implantation. Starting with the day of randomisation, animals were treated with once-daily intravenous injections of 35 nmol **2**, **7** or **8** in PBS pH 7.4 (150 µL) containing 5% DMSO for 8 consecutive days. One group was treated with vehicle (5% DMSO in PBS pH 7.4). In the case of the untargeted conjugate an equimolar amount of AAZ was added to the injection solution to control for a possible antitumour activity of CAIX inhibitors. Since sorafenib and sunitinib had previously been shown to lack any measurable anti-tumour effect in this model, these drugs were not included as further controls.[4] Animal weight and tumour dimensions were monitored on a daily basis. Tumour volume was calculated according to the formula (long side of tumour) × (short side of tumour)^2 × 0.5. Animals were euthanised when the body weight fell by > 15% relative to the first therapy day or when tumours exceeded a volume of 2000 mm^3. Prism 6 (GraphPad Software) was used for statistical data analysis (regular two-way ANOVA with Bonferroni multiple comparison test).

General Chemical Procedures

Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV400 (400 MHz) or a Bruker AVIII500 (500 MHz) spectrometer. Carbon (^{13}C) NMR spectra were recorded on a Bruker AV400 (100 MHz)

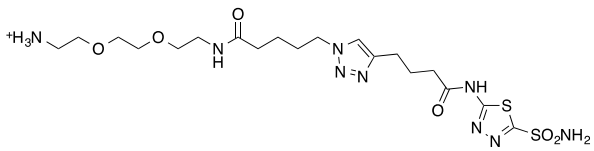
spectrometer or on a Bruker AVIII500 (125 MHz) spectrometer. Chemical shifts are given in ppm using residual solvent as the internal standard. Coupling constants (*J*) are reported in Hz with the following abbreviations used to indicate splitting: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

High-resolution mass spectrometry (HRMS) spectra were recorded at ETH's mass spectrometry service using a Bruker Daltronics maXis ESI-QTOF mass spectrometer. Chemdraw (Perkin Elmer) was used to calculate exact masses. Calculated and exact m/z values are reported in Daltons.

Analytical and preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on an Waters Alliance HT RP-HPLC with a PDA UV detector, using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of solvents A and B (A = Millipore water with 0.1% trifluoroacetic acid [TFA], B = MeCN) at a column temperature of 30 °C.

Anhydrous solvents for reactions were purchased from Acros or Fluka. Peptide grade dimethyl formamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Aldrich in HPLC or analytical grade. IRDye750 *N*-hydroxysuccinimidyl (NHS) ester was purchased from Licor, DM1 was purchased from Concortis Biosystems. All other reagents were purchased from Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under an atmosphere of argon.

Synthesis of AAZTL - 1

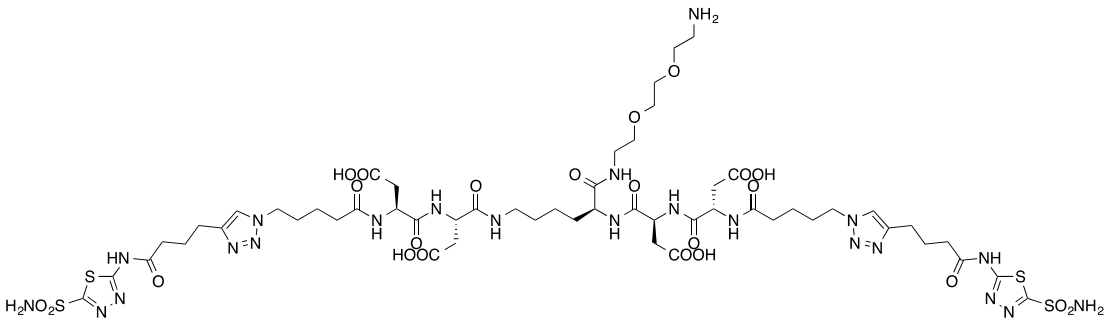


Commercially available polystyrene Wang *p*-nitrophenyl carbonate resin (250 mg, 0.15 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (250 µL), DIPEA (500 µL) and DMAP (2.5 mg) in DMF (4.5 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of 5-azido valeric acid (65 mg, 0.45 mmol), HATU (171 mg, 0.45 mmol) and DIPEA (148 µL, 0.9 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) a solution of CuI (2.9 mg, 0.015 mmol), TBTA (8 mg, 0.015 mmol) and alkyne **13** (123 mg, 0.45 mmol) in a mixture of DMF (1 mL) and THF (1 mL) was prepared and reacted with the resin for 24 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (2.2 mL), TIS (50 µL), H_2O (50 µL), *m-cresol* (100 µL) and thioanisol (100 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 2.5 mL) and the combined cleavage and washing

solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (78 mg, 0.14 mmol, 95%).

^{1}H-NMR (500 MHz, DMSO-d_6) δ [ppm] = 13.01 (s, 1H), 8.32 (s, 2H), 7.89-7.82 (m, 5H), 4.28 (t, *J* = 7.0 Hz, 2H), 3.58-3.50 (m, 6H), 3.38 (t, *J* = 6.1 Hz, 2H), 3.18 (m, 2H), 3.00 (m, 2H), 2.65 (t, *J* = 7.5 Hz, 2H), 2.59 (t, *J* = 7.4 Hz, 2H), 2.09 (t, *J* = 7.4 Hz, 2H), 1.94 (m, 2H), 1.75 (m, 2H), 1.42 (m, 2H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ [ppm] = 172.5, 172.4, 164.8, 161.5, 146.4, 122.4, 70.1, 69.8, 69.6, 67.1, 49.4, 39.1, 38.8, 35.0, 35.7, 29.8, 24.8, 24.6, 22.6; HRMS: (m/z) $[M + H]^+$ calcd. for $C_{19}H_{34}N_9O_6S_2$ 548.2068; found 548.2071.

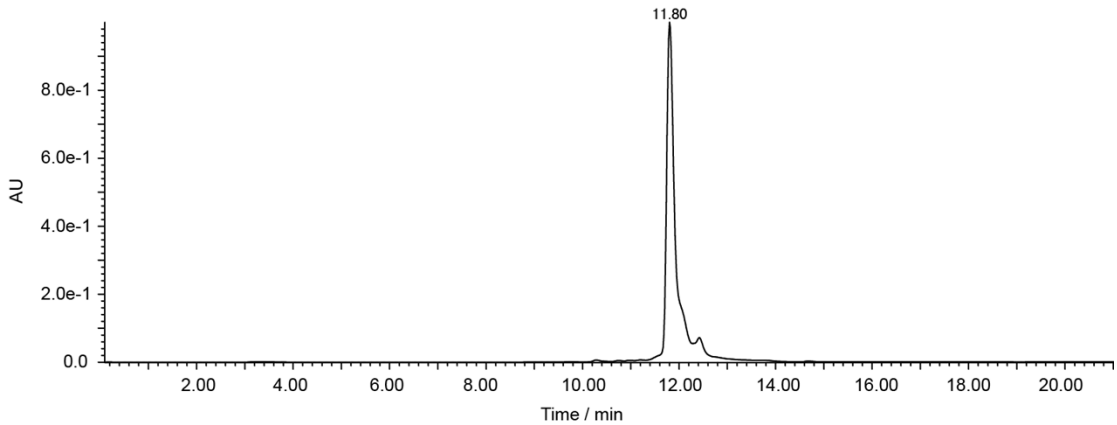
Bivalent targeted amine - 2



Commercially available polystyrene Wang *p*-nitrophenyl carbonate resin (500 mg, 0.3 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (500 µL), DIPEA (500 µL) and DMAP (5 mg) in DMF (4 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of Fmoc-Lys(Fmoc)-OH (532 mg, 0.9 mmol), HBTU (341 mg, 0.9 mmol), HOBt (138 mg, 0.9 mmol) and DIPEA (298 µL, 1.8 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.2 mmol), HATU (465 mg, 1.2 mmol) and DIPEA (397 µL, 2.4 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of CuI (76 mg, 0.12 mmol), TBTA (21 mg, 0.12 mmol) and alkyne **13** (329 mg, 1.2 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H_2O (100 µL), *m*-cresol (200 µL) and thioanisol (200 µL) for

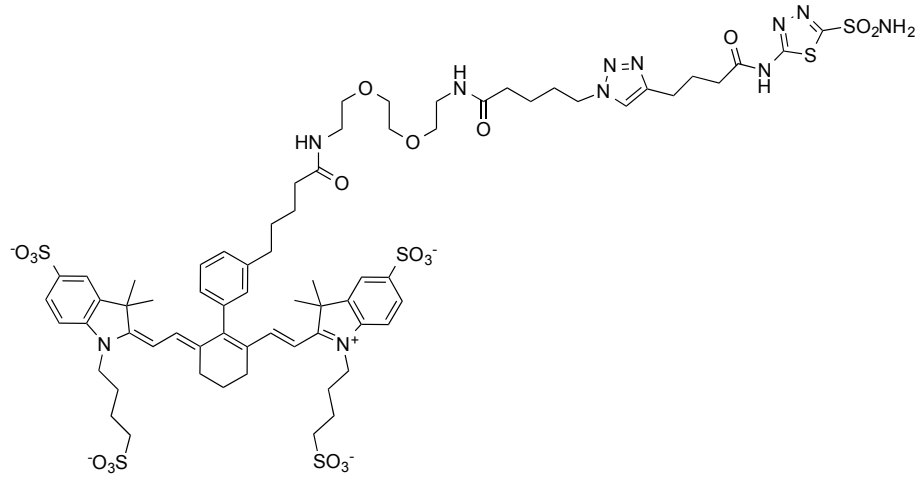
2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation, dissolved in aq. MeCN and lyophilised to yield the title compound as an off-white powder (468 mg, 0.3 mmol, quant.).

^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm] = 13.09 (s, 2H), 8.37 (s, 4H), 8.29-8.26 (m, 3H), 8.14 (d, *J* = 8.0 Hz, 1H), 7.91 (s, 2H), 7.80-7.78 (m, 3H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.65 (t, *J* = 5.4 Hz, 1H), 4.60-4.48 (m, overlaps with broad H_2O peak), 4.33 (t, *J* = 7.0 Hz, overlaps with broad H_2O peak), 4.19-4.13 (m, overlaps with broad H_2O peak), 3.64-3.59 (m, 6H), 3.44 (t, *J* = 6.3 Hz, 2H), 3.27-3.23 (m, 2H), 3.05-3.00 (m, 4H), 2.77-2.48 (m, overlaps with solvent peak), 2.20 (t, *J* = 7.2 Hz, 4H), 2.04-1.96 (m, 4H), 1.86-1.78 (m, 4H), 1.74-1.63 (br m, 1H), 1.61-1.16 (br m, 9H); HRMS: (m/z) $[M + H]^+$ calcd. for $C_{54}H_{83}N_{22}O_{23}S_4$ 1535.4879; found 1535.4868.



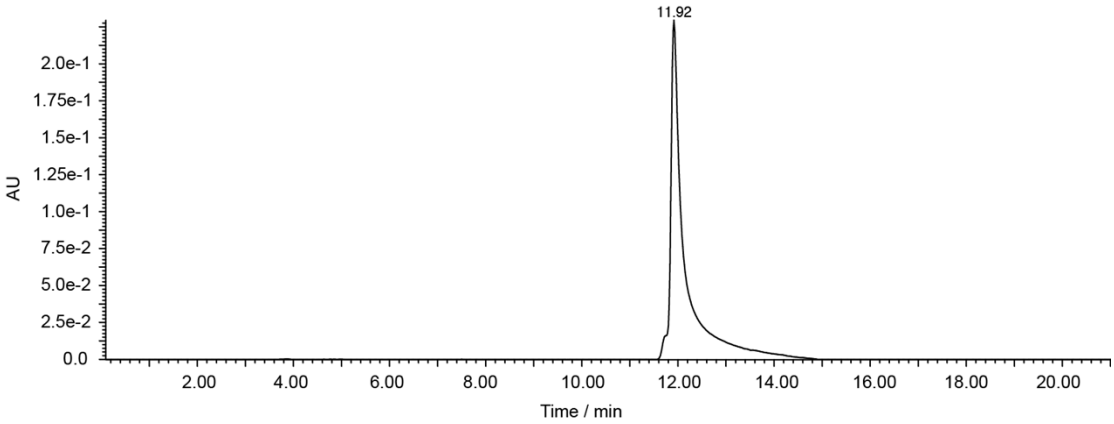
Supplementary Figure 16: Analytical HPLC trace of **2** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

Monovalent targeted IRDye conjugate - 3



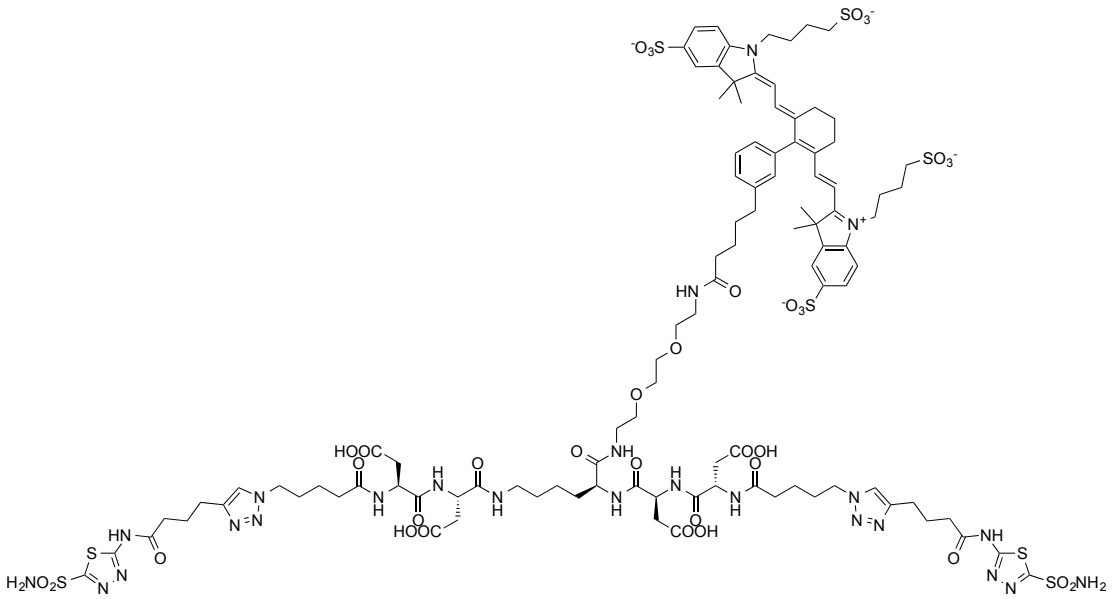
To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added acetazolamide derivative **1** (200 µg, 366 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_{max} = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M^{-1} cm^{-1}) of stock samples diluted 1:200 into PBS pH 7.4 (640 µM, 32 nmol, 38%).

HRMS: (m/z) $[M + 4H]^+$ calcd. for $C_{68}H_{92}N_{11}O_{19}S_6$ 1558.4890; found 1558.4844.



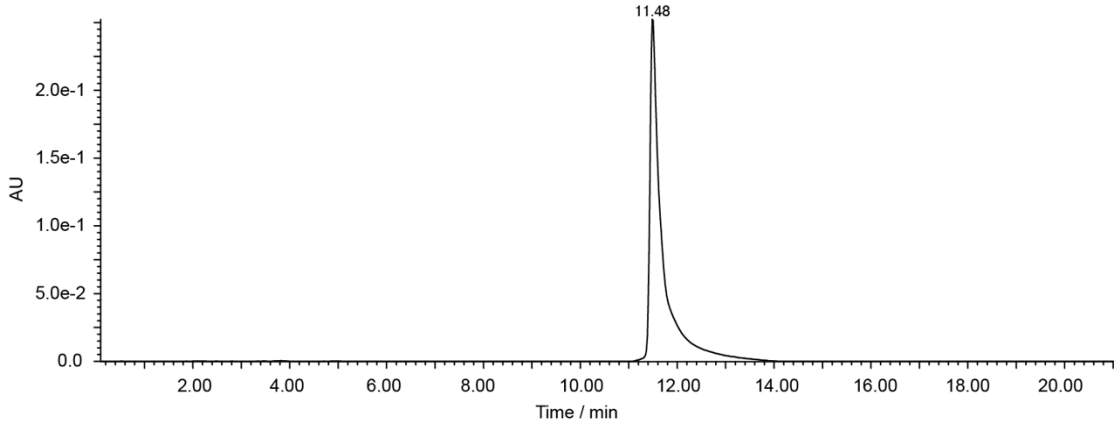
Supplementary Figure 17: Analytical HPLC trace of **3** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

Bivalent targeted IRDye conjugate - 4



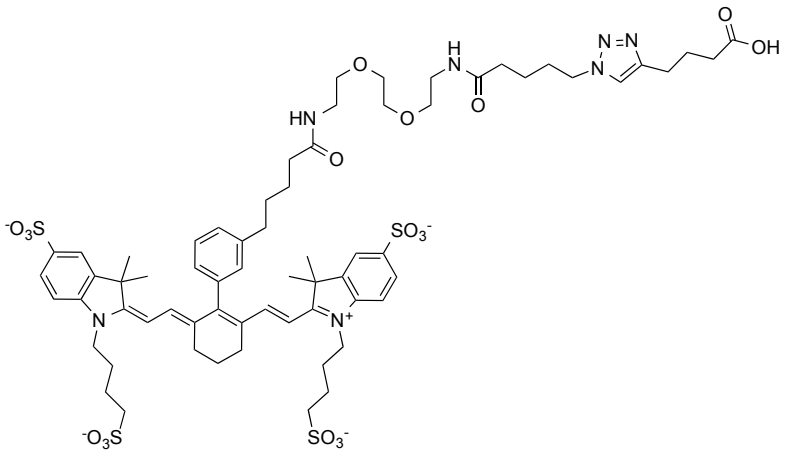
To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added **2** (200 µg, 130 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_{max} = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M^{-1} cm^{-1}) of stock samples diluted 1:200 into PBS pH 7.4 (287 µM, 14 nmol, 17%).

HRMS: (m/z) $[M + 4H]^+$ calcd. for $C_{103}H_{141}N_{24}O_{36}S_8$ 2545.7700; found 2545.7703.



Supplementary Figure 18: Analytical HPLC trace of **4** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

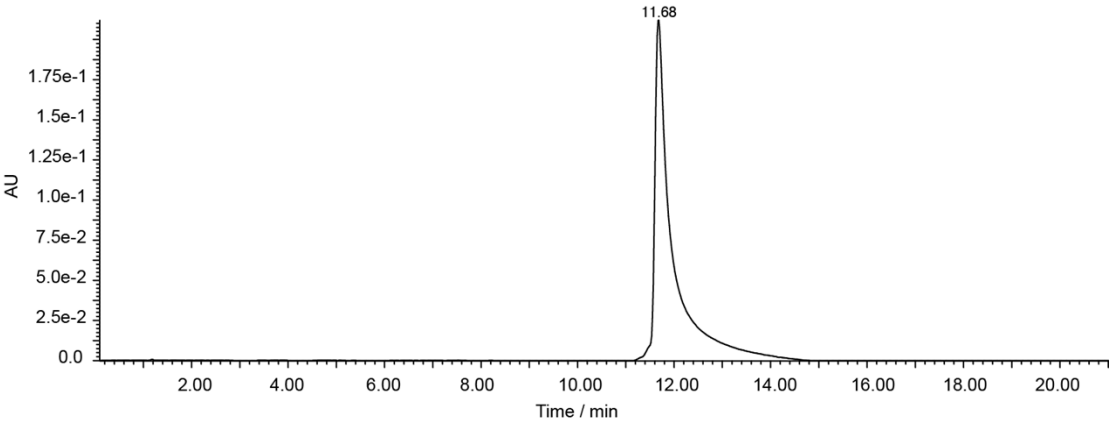
Monovalent untargeted IRDye conjugate - 5



To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added **1** (200 µg, 520 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2 h and

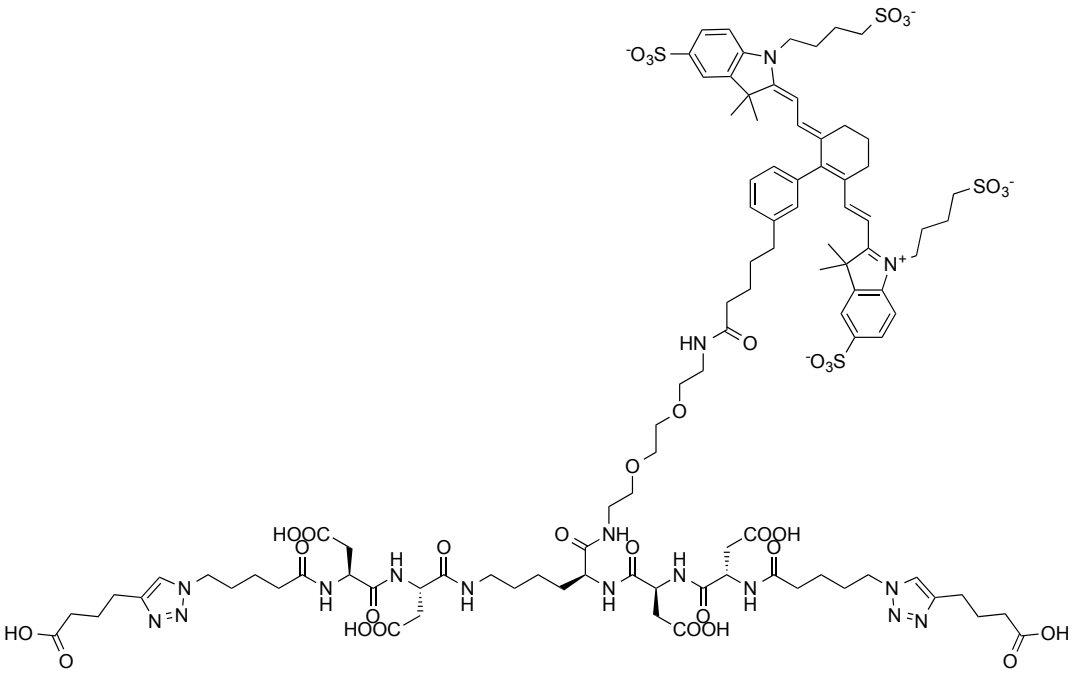
then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_{max} = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M^{-1} cm^{-1}) of stock samples diluted 1:200 into PBS pH 7.4 (608 µM, 30 nmol, 36%).

HRMS: (m/z) $[M + 4H]^+$ calcd. for $C_{66}H_{90}N_7O_{18}S_4$ 1396.5220; found 1396.5218.



Supplementary Figure 19: Analytical HPLC trace of **5** using a Synergi 4µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

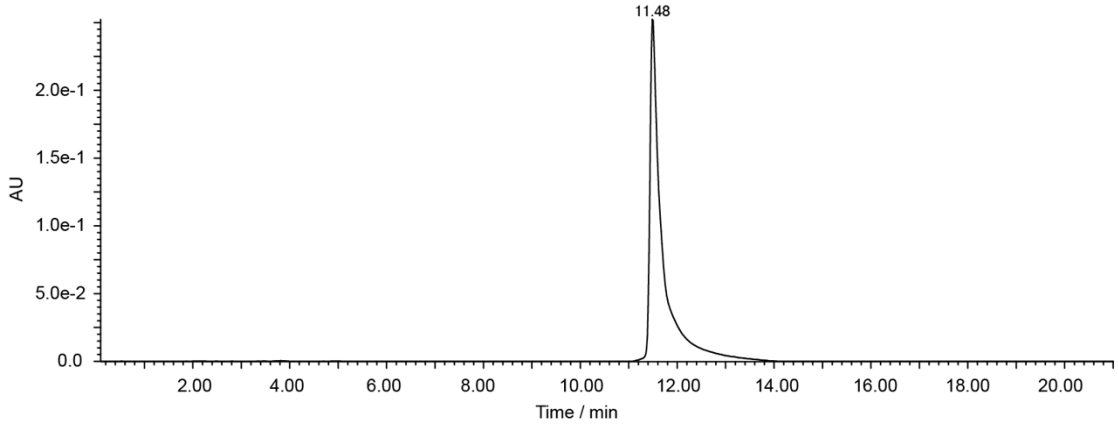
Bivalent untargeted IRDye conjugate - 6



To IRDye750 NHS ester (100 µg, 84 nmol) in DMSO (10 µL) and DMF (100 µL) was added **10** (200 µg, 165 nmol) in DMSO (20 µL) and DIPEA (2 µL, 12 µmol). The mixture was allowed to stand at room temperature for 2

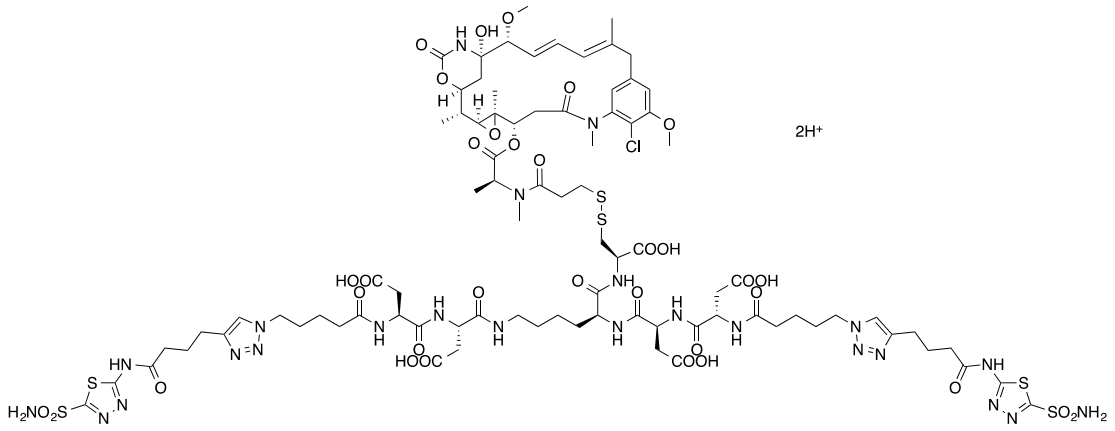
h and was then directly purified over reversed-phase HPLC (95% A / 5% B to 40% A / 60% B over 30 min). Fractions containing dye conjugate were identified through their characteristic UV/VIS spectrum (λ_{max} = 750 nm), pooled, lyophilised and dissolved in DMSO (50 µL) to give a dark green stock solution. Its concentration and the reaction yield were determined by measuring the absorbance at 750 nm (ε_{750} = 260,000 M^{-1} cm^{-1}) of stock samples diluted 1:200 into PBS pH 7.4 (518 µM, 26 nmol, 31%).

HRMS: (m/z) $[M + 4H]^+$ calcd. for $C_{99}H_{137}N_{16}O_{34}S_4$ 2221.8360; found 2221.8363.



Supplementary Figure 20: Analytical HPLC trace of **6** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 40% A / 60% B over 30 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 750 nm.

Bivalent targeted DM1 conjugate - 7

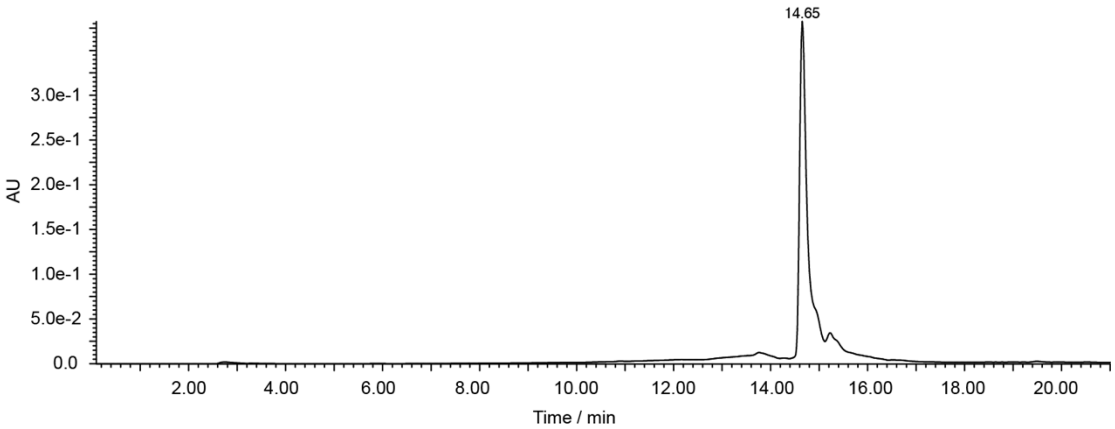


Bivalent targeted linker **11** (20 mg, 13 µmol), TCEP·HCl (7.6 mg, 27 µmol) and DIPEA (2 µL) were dissolved in degassed DMF (500 µL). After 1 h 2,2'-dipyridyldisulphide (11.7 mg, 53 µmol) was added. The mixture was stirred at room temperature for 12 h, diluted with NMP (500 µL) and was added drop wise to ice cold diethyl ether (40 mL). The precipitate was collected by centrifugation, re-dissolved in DMF (200 µL) and NMP (200 µL) and

precipitated again with ice cold diethyl ether (40 mL) and dried under vacuum to give the activated disulphide as a white residue (18 mg, 11 μmol, 85%). An aliquot of the activated disulphide (15 mg, 9 μmol) was dissolved in DMF (400 μL) and DM1 free thiol (7 mg, 9 μmol) added. The reaction was allowed to stand at room temperature for 48 h after which the product was recovered by reversed phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilised to yield the title compound as an off white powder (9.5 mg, 4 μmol, 47%).

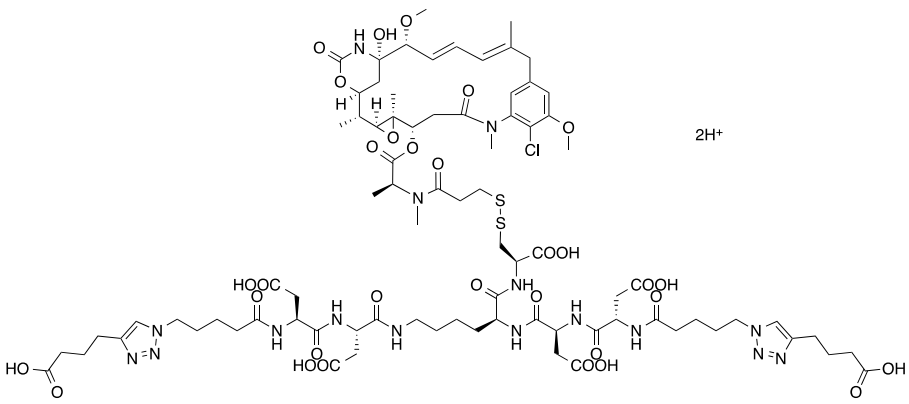
^{1}H-NMR (500 MHz, DMSO-d_6) δ [ppm] = 12.98 (s, 2H), 8.31 (s, 4H), 8.22-8.15 (m, 4H), 8.07 (d, *J* = 8.2 Hz, 1H), 7.85 (s, 2H), 7.69-7.59 (m, 2H), 7.12 (s, 1H), 6.89 (s, 1H), 6.61-6.52 (m, 3H), 5.92 (br s, 1H), 5.57-5.52 (m, 1H), 5.30-5.29 (m, 1H), 4.52-4.43 (m, 5H), 4.39-4.34 (m, 1H), 4.27 (t, *J* = 6.9 Hz, 4H), 4.19-4.16 (m, 1H), 4.08-4.03 (m, 1H), 3.92-3.90 (m, 3H), 3.53-2.41 (m, overlap with solvent peak), 2.13-2.12 (m, 4H), 2.04-2.01 (m, 1H), 1.97-1.91 (m, 4H), 1.79-1.73 (m, 4H), 1.67-1.54 (m, 4H), 1.51-1.10 (m, 21H), 0.77 (s, 3H);

HRMS: (m/z) $[M + 2H]^{2+}$ calcd. for $C_{86}H_{119}ClN_{24}O_{33}S_6$ 1122.3270; found 1122.3279.



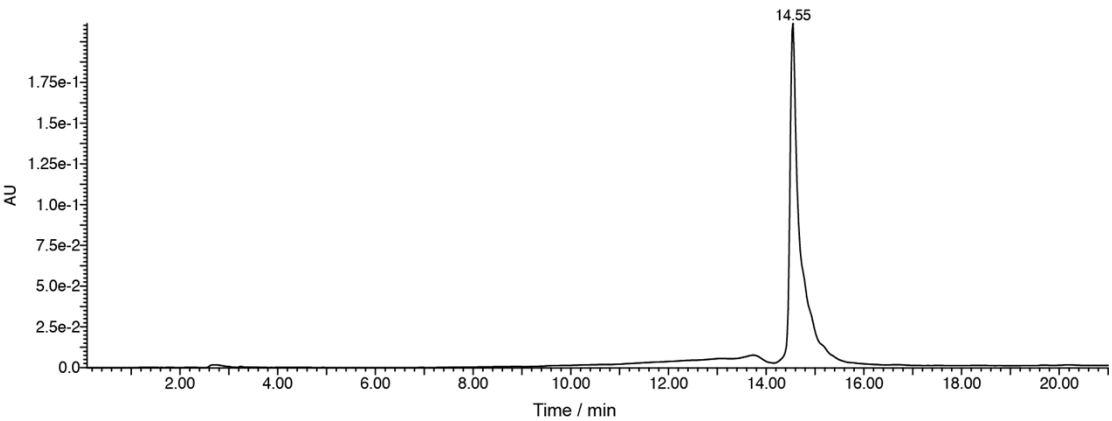
Supplementary Figure 21: Analytical HPLC trace of **7** using a Synergi 4 μm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

Bivalent untargeted DM1 conjugate - 8



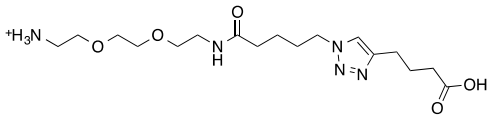
Bivalent untargeted linker **12** (20 mg, 17 μmol), TCEP·HCl (19 mg, 68 μmol) and DIPEA (10 μL) were dissolved in degassed DMF (1 mL). After 1 h 2,2'-dipyridyldisulphide (22 mg, 100 μmol) was added. The mixture was stirred at room temperature for 12 h, diluted with NMP (500 μL) and was added drop wise to ice cold diethyl ether (40 mL). The precipitate was collected by centrifugation, re-dissolved in DMF (200 μL) and NMP (200 μL) and precipitated again with ice cold diethyl ether (40 mL) and dried under vacuum to give the activated disulphide as a white residue (45 mg, product + side products). An aliquot of the residue (15 mg) was dissolved in DMF (400 μL) and DM1 free thiol (7 mg, 9 μmol) was added. The reaction was allowed to stand at room temperature for 48 h after which the product was recovered by reversed phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). Fractions containing the desired product by MS were pooled and lyophilised to yield the title compound as an off white powder (7.4 mg, 3.9 μmol, 42%).

^{1}H-NMR (500 MHz, DMSO-d_6) δ [ppm] = 8.22-8.09 (m, 5H), 7.83 (s, 2H), 7.64-7.58 (m, 2H), 7.12 (s, 1H), 6.89 (s, 1H), 6.61-6.52 (m, 3H), 5.93 (s, 1H), 5.55 (dd, *J* = 9.1, 14.8 Hz, 1H), 5.32-5.28 (m, 1H), 4.56-4.43 (m, 6H), 4.27 (t, *J* = 6.85 Hz, 4H), 4.20-4.17 (m, 1H), 4.05 (t, *J* = 12.2 Hz, 1H), 3.91 (s, 3H), 3.49-2.41 (m, overlap with solvent peak), 2.25 (t, *J* = 7.4 Hz, 4H), 2.15 (m, 4H), 2.04-2.02 (br m, 1H), 1.80-1.73 (m, 8H), 1.62-1.10 m, 24H), 0.77 (s, 3H); HRMS: (m/z) $[M + H]^+$ calcd. for $C_{82}H_{116}ClN_{16}O_{31}S_2$ 1919.7117; found 1919.7098.



Supplementary Figure 22: Analytical HPLC trace of **8** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

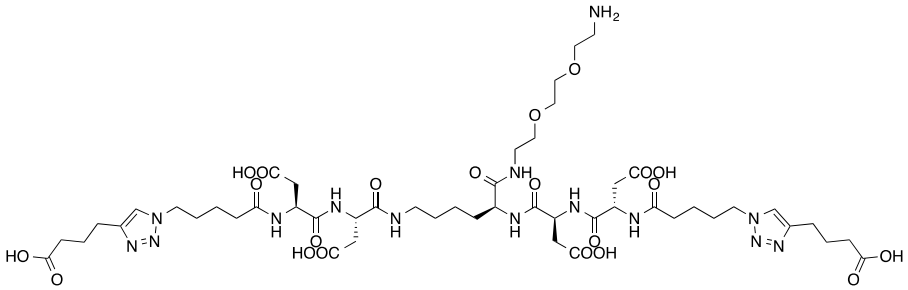
Monovalent linker - 9



Commercially available polystyrene Wang *p*-nitrophenyl carbonate resin (250 mg, 0.15 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (250 µL), DIPEA (500 µL) and DMAP (2.5 mg) in DMF (4.5 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of 5-azido valeric acid (65 mg, 0.45 mmol), HATU (171 mg, 0.45 mmol) and DIPEA (148 µL, 0.9 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) a solution of CuI (2.9 mg, 0.015 mmol), TBTA (8 mg, 0.015 mmol) and 5-hexynoic acid (51 mg, 50 µL, 0.45 mmol) in a mixture of DMF (1 mL) and THF (1 mL) was prepared and reacted with the resin for 24 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (2.2 mL), TIS (50 µL), H_2O (50 µL), *m*-cresol (100 µL) and thioanisol (100 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 2.5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (21 mg, 54 µmol, 36%).

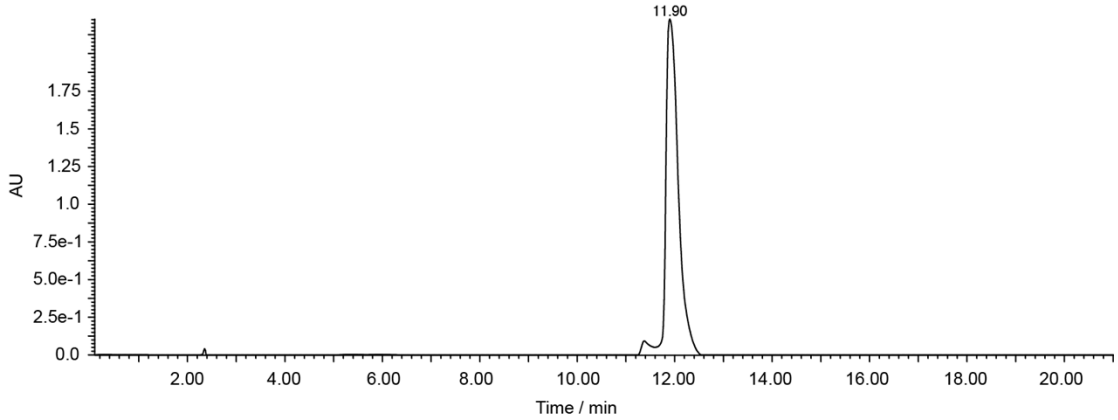
^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm] = 7.90-7.86 (m, 5H), 4.29 (t, J = 7.0 Hz, 2H), 3.60-3.51 (m, 6H), 3.40 (t, J = 6.1 Hz, 2H), 3.20 (q, J = 5.8 Hz, 2H), 3.00-2.96 (m, 2H), 2.62 (t, J = 7.6 Hz, 2H), 2.26 (t, J = 7.4 Hz, 2H), 2.10 (t, J = 7.4 Hz, 2H), 1.85-1.74 (m, 4H), 1.46-1.42 (m, 2H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ [ppm] = 174.8, 172.4, 146.7, 122.3, 70.1, 69.8, 69.5, 67.2, 49.4, 39.0, 38.9, 35.0, 33.6, 29.8, 24.9, 24.8, 22.7; HRMS: (m/z) $[M + H]^+$ calcd. for $C_{17}H_{32}N_5O_5$ 386.2398; found 386.2403.

Bivalent linker - 10



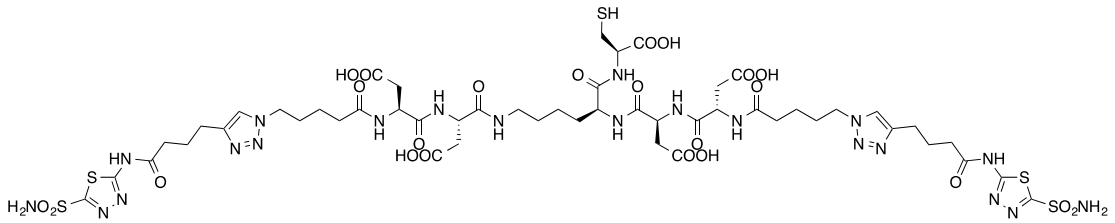
Commercially available polystyrene Wang *p*-nitrophenyl carbonate resin (500 mg, 0.3 mmol) was swollen in DMF (5 mL for 5 min) and reacted with a solution of 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (500 µL), DIPEA (500 µL) and DMAP (5 mg) in DMF (4 mL) for 12 h at room temperature under shaking. The resin was washed with DMF (3× 5 mL for 1 min), MeOH (3× 5 mL for 1 min) and again DMF (3× 5 mL for 1 min). A solution of Fmoc-Lys(Fmoc)-OH (532 mg, 0.9 mmol), HBTU (341 mg, 0.9 mmol), HOBt (138 mg, 0.9 mmol) and DIPEA (298 µL, 1.8 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.2 mmol), HATU (465 mg, 1.2 mmol) and DIPEA (397 µL, 2.4 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of CuI (76 mg, 0.12 mmol), TBTA (21 mg, 0.12 mmol) and 5-hexyonic acid (440 µL, 1.2 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H_2O (100 µL), *m*-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (64 mg, 53 µmol 17%).

^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm] = 8.25-8.22 (m, 3H), 8.09 (d, *J* = 8.1 Hz, 1H), 7.85 (s, 2H), 7.78-7.73 (br m, 3H), 7.66 (d, *J* = 7.9 Hz, 1H), 7.59 (t, *J* = 5.3 Hz, 1H), 4.55-4.44 (m, 4H), 4.29 (t, *J* = 7.0 Hz, 4H), 4.14-4.09 (m, 2H), 3.60-3.55 (m, 6H), 3.40 (t, *J* = 6.2 Hz, 2H), 3.22-3.19 (m, 2H), 3.01-2.92 (m, 4H), 2.73-2.44 (m, overlap with solvent peak), 2.26 (t, *J* = 7.4 Hz, 4H), 2.15 (t, *J* = 7.2 Hz, 4H), 1.85-1.74 (m, 7H), 1.70-1.60 (br m, 1H), 1.55-1.14 (br m, 9H); HRMS: (m/z) $[M + H]^+$ calcd. for $C_{50}H_{79}N_{14}O_{21}$ 1211.5539; found 1211.5515.



Supplementary Figure 23: Analytical HPLC trace of **10** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 50% A / 50% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 210 nm.

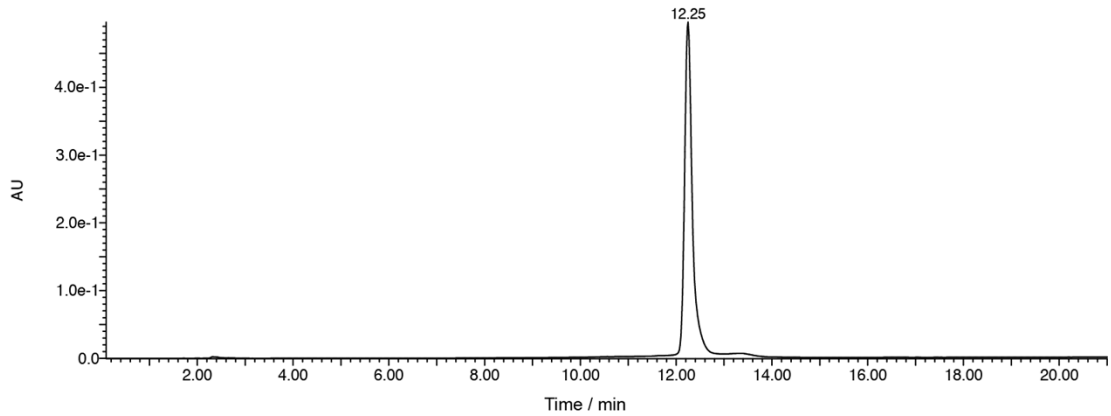
Bivalent targeted Cys ligand derivative - 11



Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). A solution of Fmoc-Lys(Fmoc)-OH (736 mg, 1.25 mmol), HBTU (472 mg, 1.25 mmol), HOBt (191 mg, 1.25 mmol) and DIPEA (412 µL, 2.5 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.7 mmol), HATU (643 mg, 1.7 mmol) and DIPEA (549 µL, 3.3 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of CuI (106 mg, 0.17 mmol), TBTA (29 mg, 0.17 mmol) and alkyne **13** (455 mg, 1.7 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the

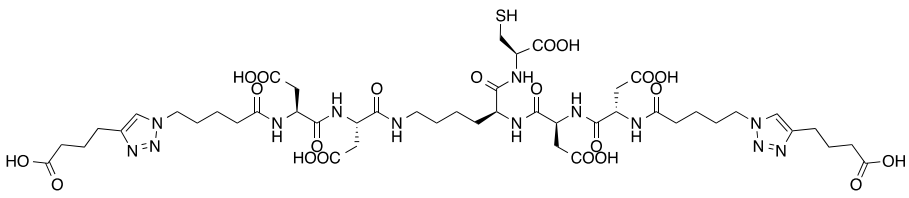
resin with a mixture of TFA (4.4 mL), TIS (100 µL), H_2O (100 µL), *m*-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (68 mg, 45 µmol, 10%).

^{1}H-NMR (400 MHz, DMSO-d_6) δ [ppm] = 13.01 (s, 2H), 8.32 (s, 4H), 8.21 (t, *J* = 7.5 Hz, 3H), 8.09 (d, *J* = 8.1 Hz, 1H), 8.05 (d, *J* = 7.9 Hz, 1H), 7.87 (s, 2H), 7.74 (d, *J* = 7.84 Hz, 1H), 7.61 (t, *J* = 5.4 Hz, 1H), 4.55-4.45 (m, overlap with broad water peak), 4.40-4.34 (m, overlap with broad water peak), 4.29 (t, *J* = 7.0 Hz, overlap with broad water peak), 4.24-4.22 (m, overlap with broad water peak), 3.07-2.94 (br m, 2H), 2.90-2.41 (m, overlap with solvent peak), 2.15 (t, *J* = 7.1 Hz, 4H), 1.99-1.92 (m, 4H), 1.82-1.74 (m, 4H), 1.71-1.24 (br m, 10H); HRMS: (m/z) $[M + H]^+$ calcd. for $C_{51}H_{74}N_{21}O_{23}S_5$ 1508.3864; found 1508.3861.



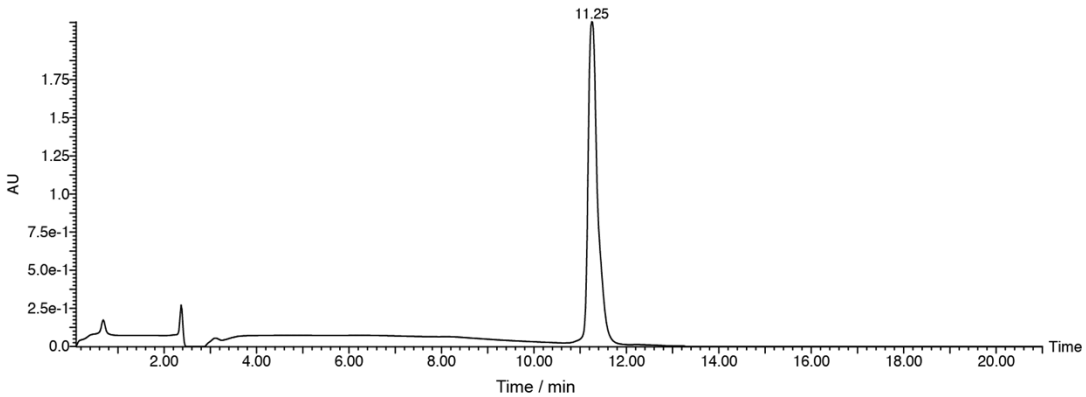
Supplementary Figure 24: Analytical HPLC trace of **11** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 260 nm.

Bivalent untargeted Cys linker - 12



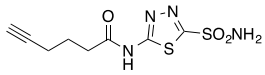
Commercially available pre-loaded Fmoc-Cys(Trt) on Tentagel resin (500 mg, 0.415 mmol, RAPP Polymere) was swollen in DMF (3 × 5 min × 5 mL), the Fmoc group removed with 20 % piperidine in DMF (1 × 1 min × 5 mL and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL). A solution of Fmoc-Lys(Fmoc)-OH

(736 mg, 1.25 mmol), HBTU (472 mg, 1.25 mmol), HOBt (191 mg, 1.25 mmol) and DIPEA (412 µL, 2.5 mmol) was prepared and immediately reacted with the resin for 1 h at room temperature under shaking. After washing with DMF (6 × 1 min × 5 mL) the Fmoc group was removed with 20 % piperidine in DMF (1 × 1 min × 5 min and 2 × 10 min × 5 mL) and the resin washed with DMF (6 × 1 min × 5 mL) before the next coupling step was initiated. In the following, the peptide was extended with Fmoc-Asp(OtBu)-OH twice followed by 5-azido-valerate. For this purpose, a solution of acid (1.7 mmol), HATU (643 mg, 1.7 mmol) and DIPEA (549 µL, 3.3 mmol) was prepared in DMF (5 mL) and reacted with the resin for 1 h at room temperature under gentle agitation. Each coupling was followed by a washing step with DMF (6 × 1 min × 5 mL) and Fmoc deprotection as described above. After coupling of the azide, a solution of CuI (106 mg, 0.17 mmol), TBTA (29 mg, 0.17 mmol and 5-hexyonic acid (609 µL, 1.7 mmol) in a mixture of DMF (2.5 mL) and THF (2.5 mL) was prepared and reacted with the resin for 48 h at room temperature. After washing with DMF (3 × 1 min × 5 mL), 50 mM aq. EDTA solution (3 × 1 min × 5 mL), DMF (3 × 1 min × 5 mL) and DCM (3 × 1 min × 5 mL), the compound was cleaved by agitating the resin with a mixture of TFA (4.4 mL), TIS (100 µL), H_2O (100 µL), *m*-cresol (200 µL) and thioanisol (200 µL) for 2 h at room temperature. The resin was washed with TFA (1 × 5 min × 5 mL) and the combined cleavage and washing solutions added drop-wise to ice cold diethyl ether (100 mL). The precipitate was collected by centrifugation and the product purified by reversed-phase HPLC (95% A / 5% B to 20% A / 80% B over 20 min). After lyophilisation the title compound was collected as a white powder (147 mg, 0.12 mmol, 30%). ^{1}H-NMR (500 MHz, DMSO-d_6) δ [ppm] = 8.22-8.19 (m, 3H), 8.08 (d, J = 8.9 Hz, 1H), 8.02 (d, J = 7.8 Hz, 1H), 7.83 (s, 2H), 7.72 (d, J = 7.8 Hz, 1H), 7.59-7.56 (m, 1H), 4.56-4.43 (m, 3H), 4.37-4.34 (m, 1H), 4.27-4.20 (m, 4H), 3.03-2.92 (m, 2H), 2.87-2.39 (m, overlap with solvent peak), 2.25 (t, J = 7.35 Hz, 4H), 2.13 (t, J = 7.0 Hz, 4H), 1.83-1.21 (br m, 16H); HRMS: (m/z) $[M + H]^+$ calcd. for $C_{47}H_{70}N_{13}O_{21}S$ 1184.4524; found 1184.4508.



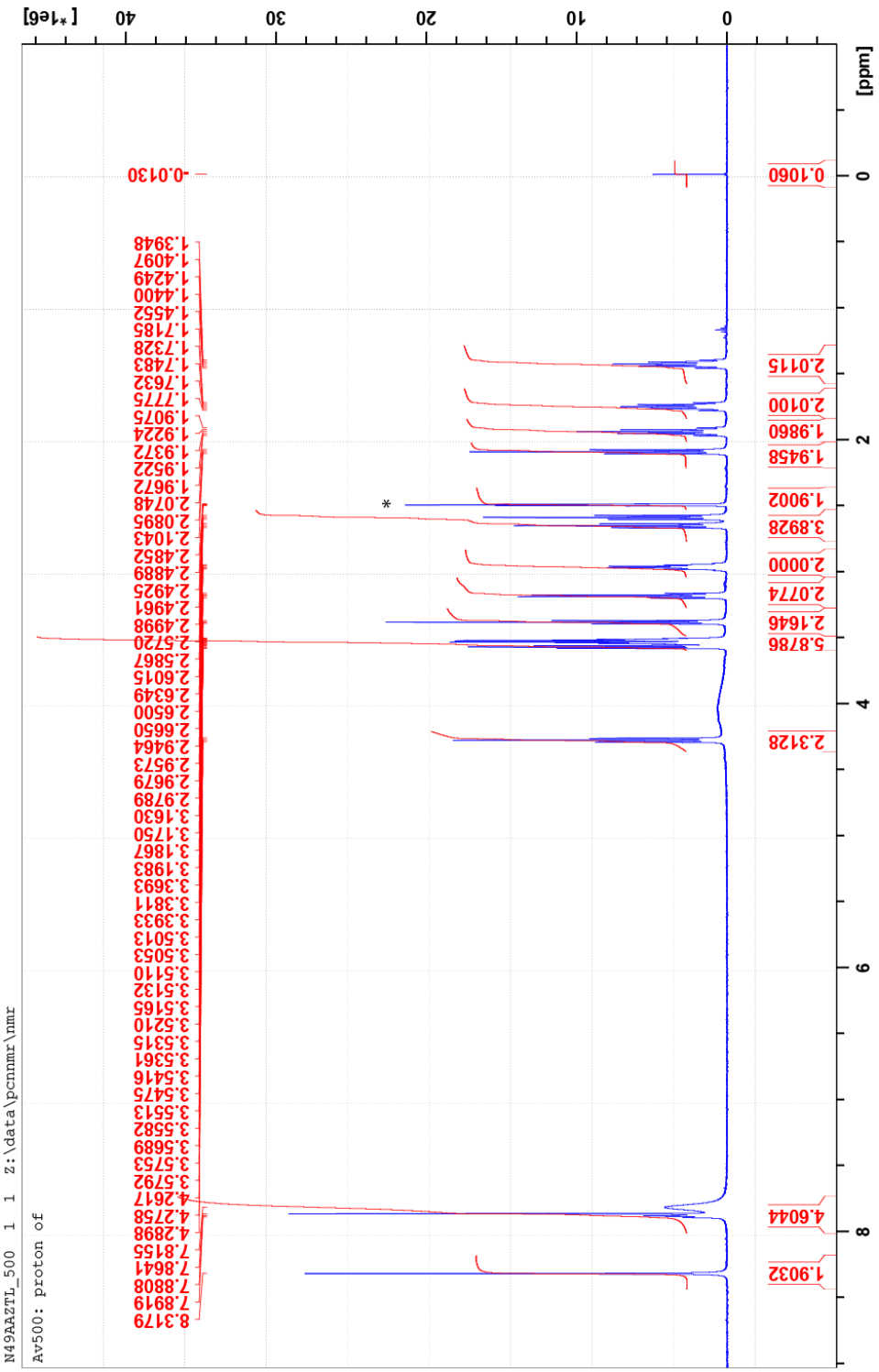
Supplementary Figure 25: Analytical HPLC trace of **12** using a Synergi 4 µm, Polar-RP 150 × 10 mm column at a flow rate of 4 mL min^{-1} with linear gradients of 95% A / 5% B to 20% A / 80% B over 20 min (A = Millipore water with 0.1% TFA, B = MeCN) detecting absorbance at 210 nm.

Acetazolamide derivative - 13

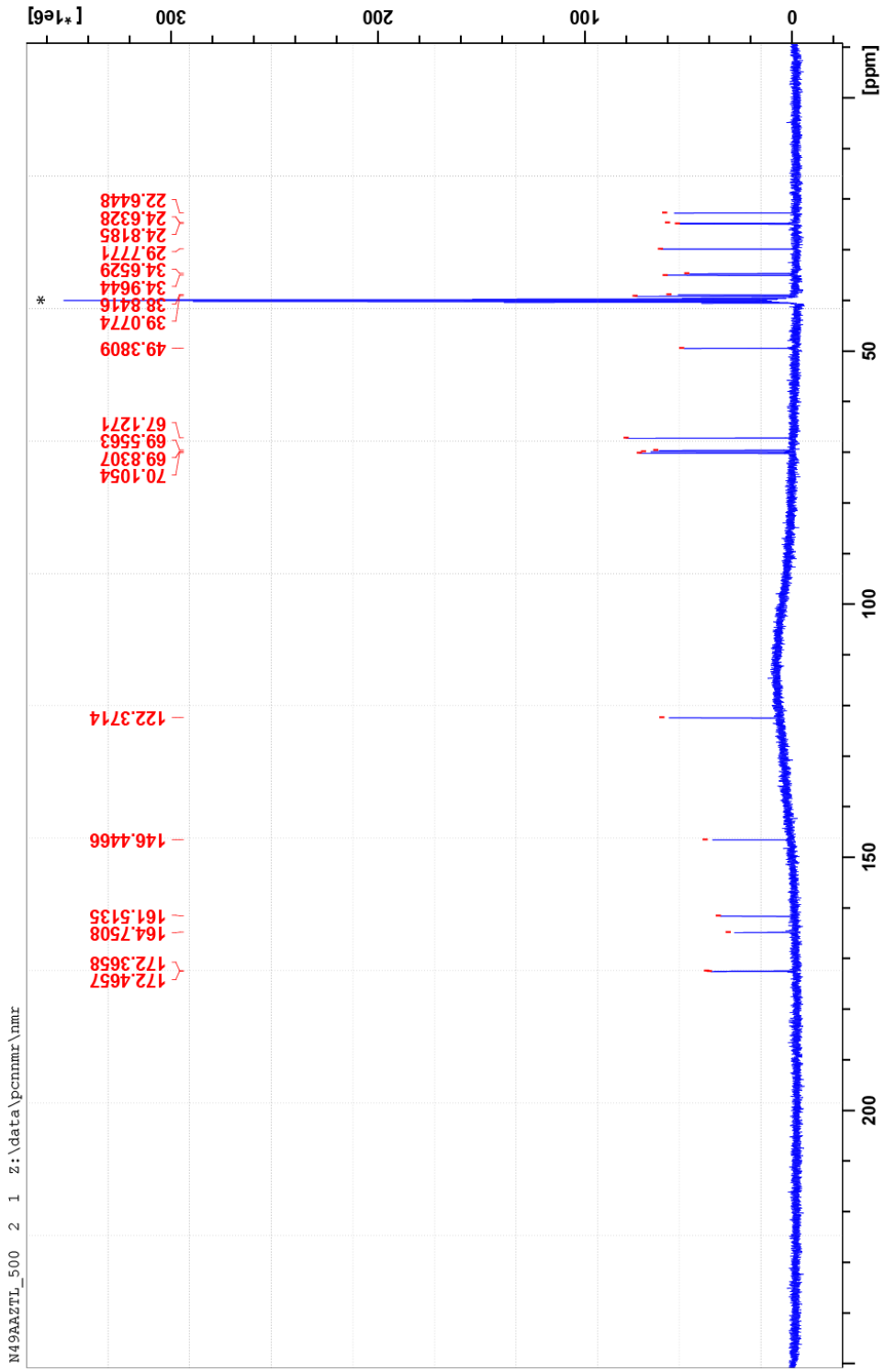


Compound **13** was prepared according to previously described methods.[4]

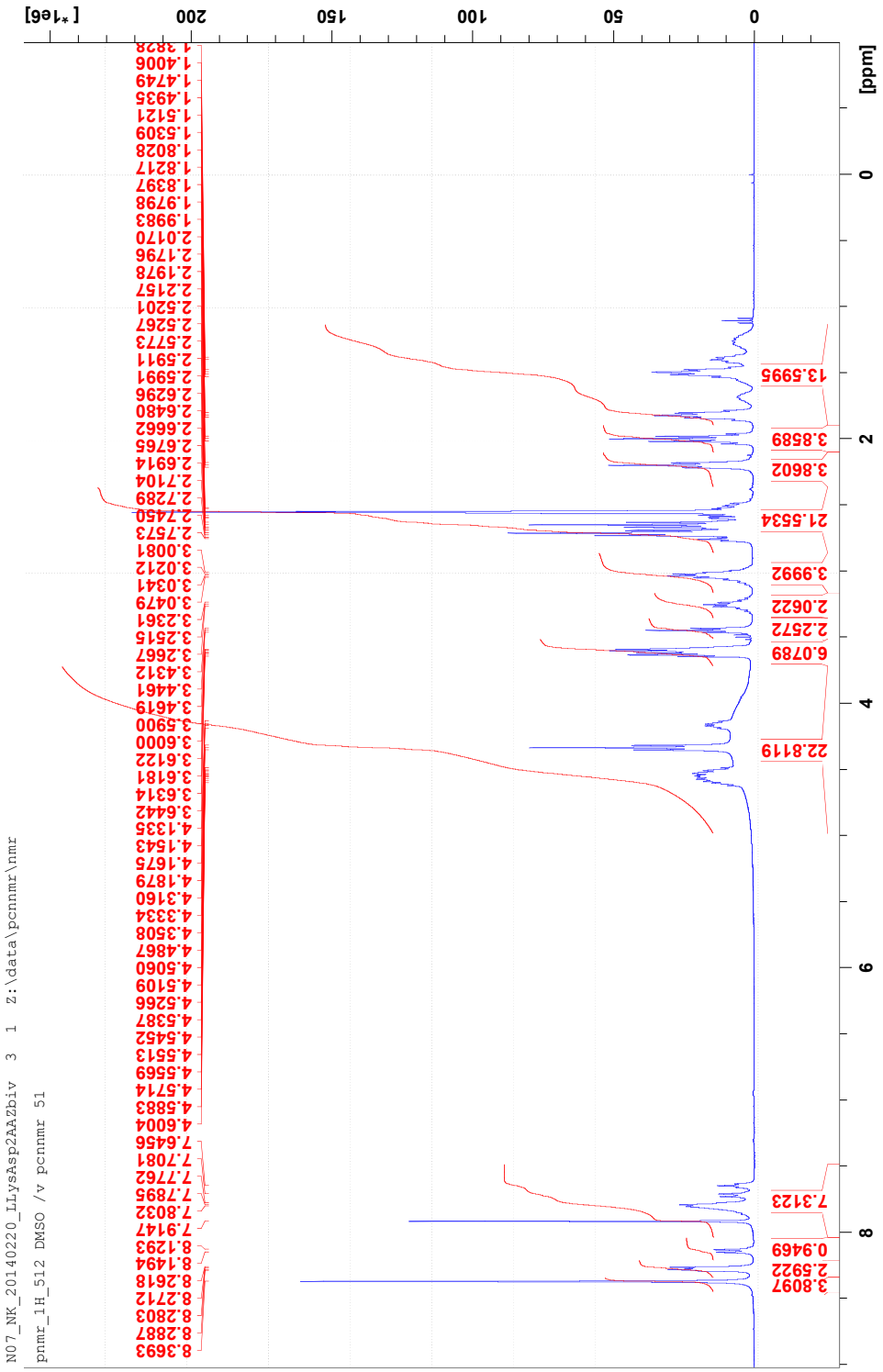
NMR Spectra



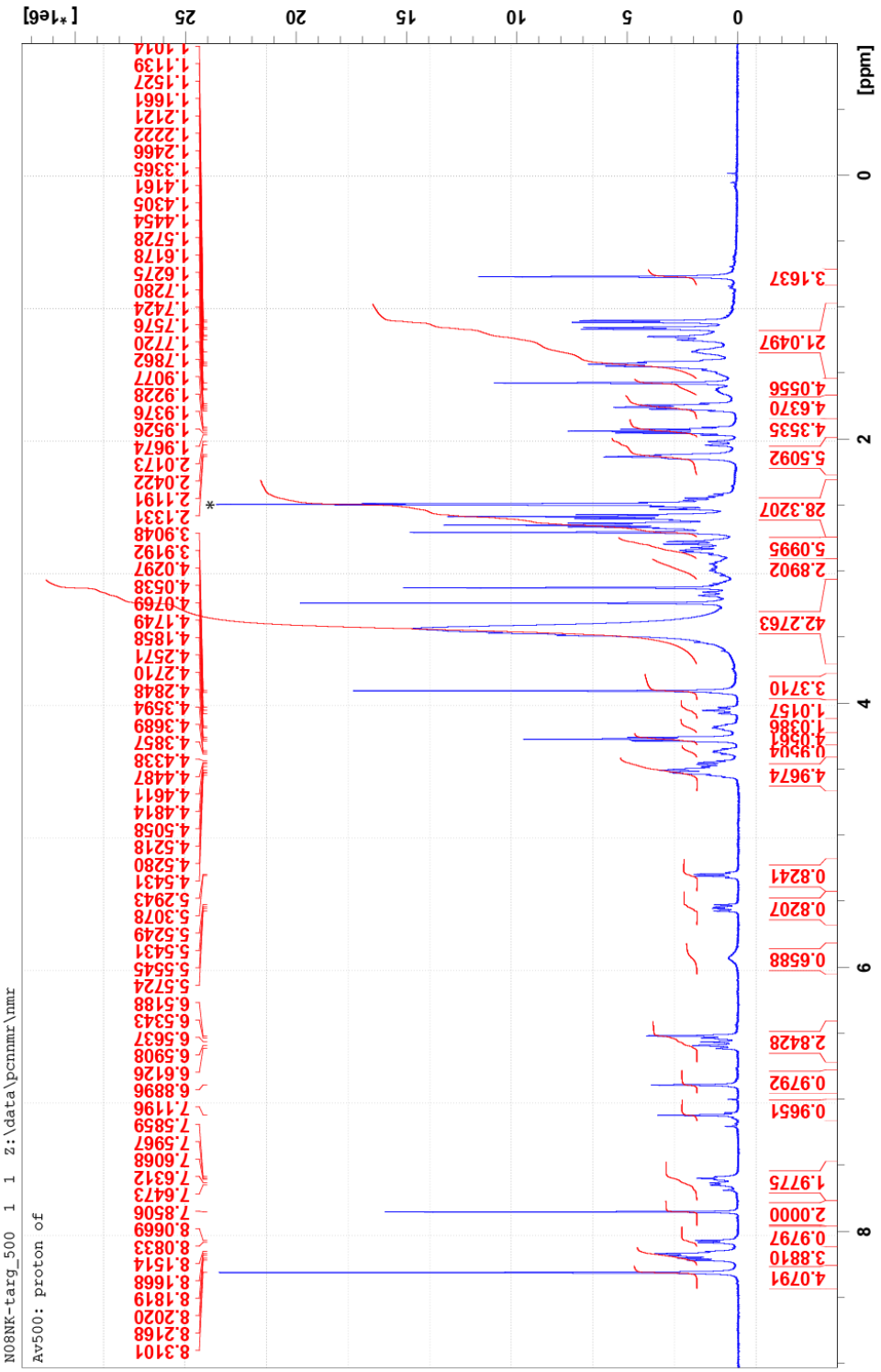
Supplementary Figure 26: 500 MHz 1H spectrum of **1** in DMSO-d_6. Residual DMSO peak is marked with an asterisk. TMS peak is at 0 ppm.



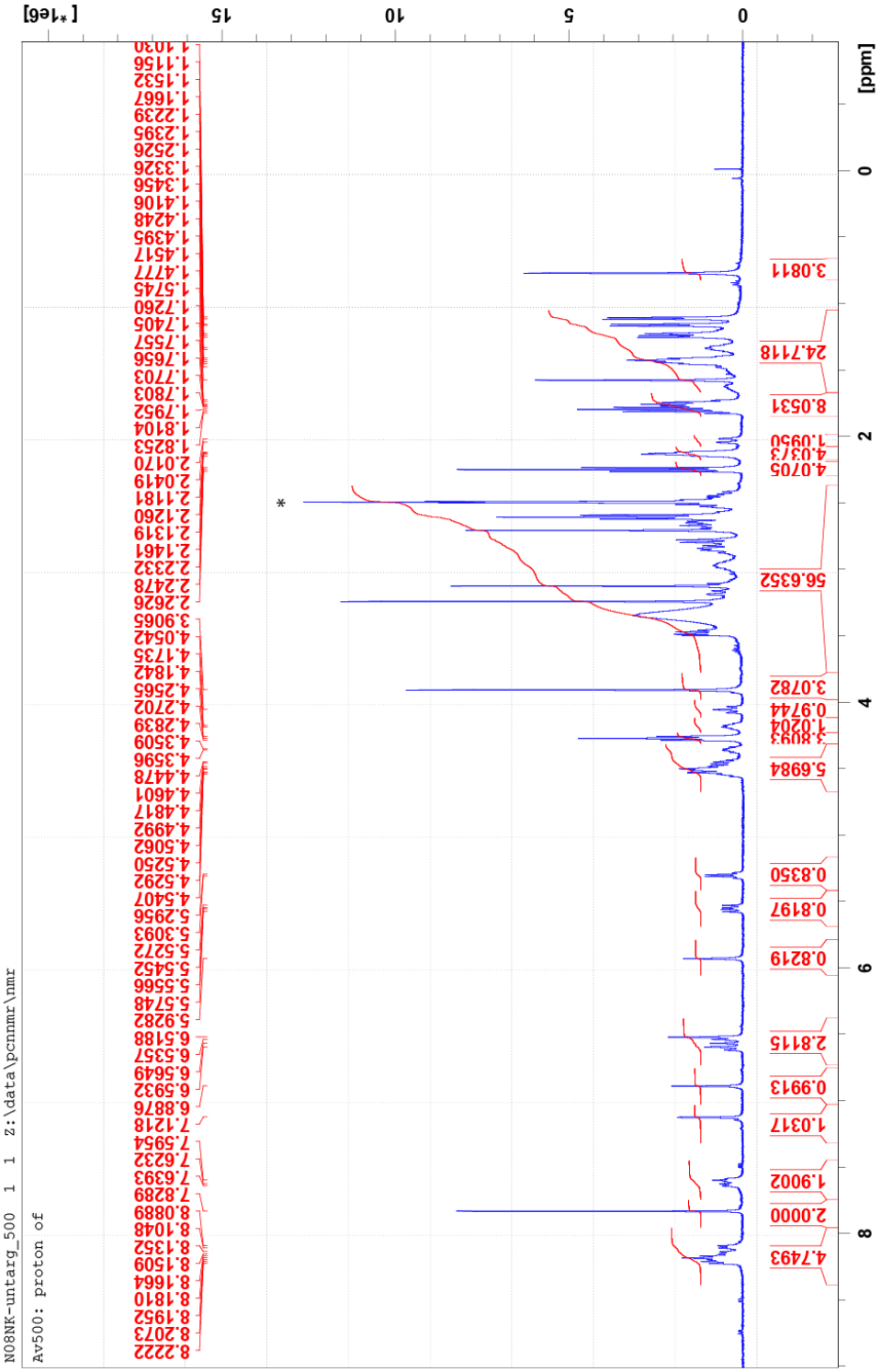
Supplementary Figure 27: 500 MHz ^{13}C spectrum of **1** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



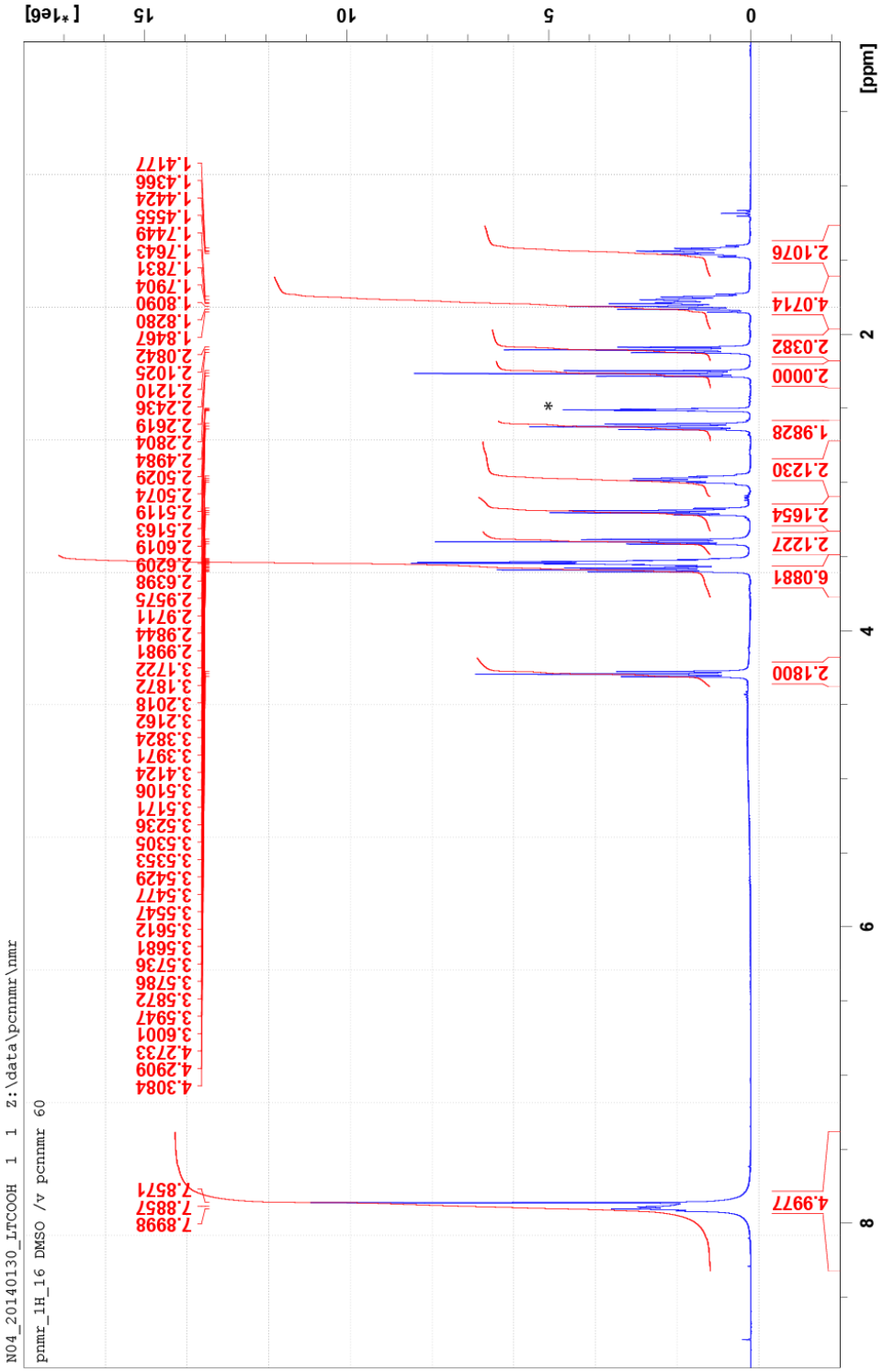
Supplementary Figure 28: 500 MHz ^{1}H spectrum of **2** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



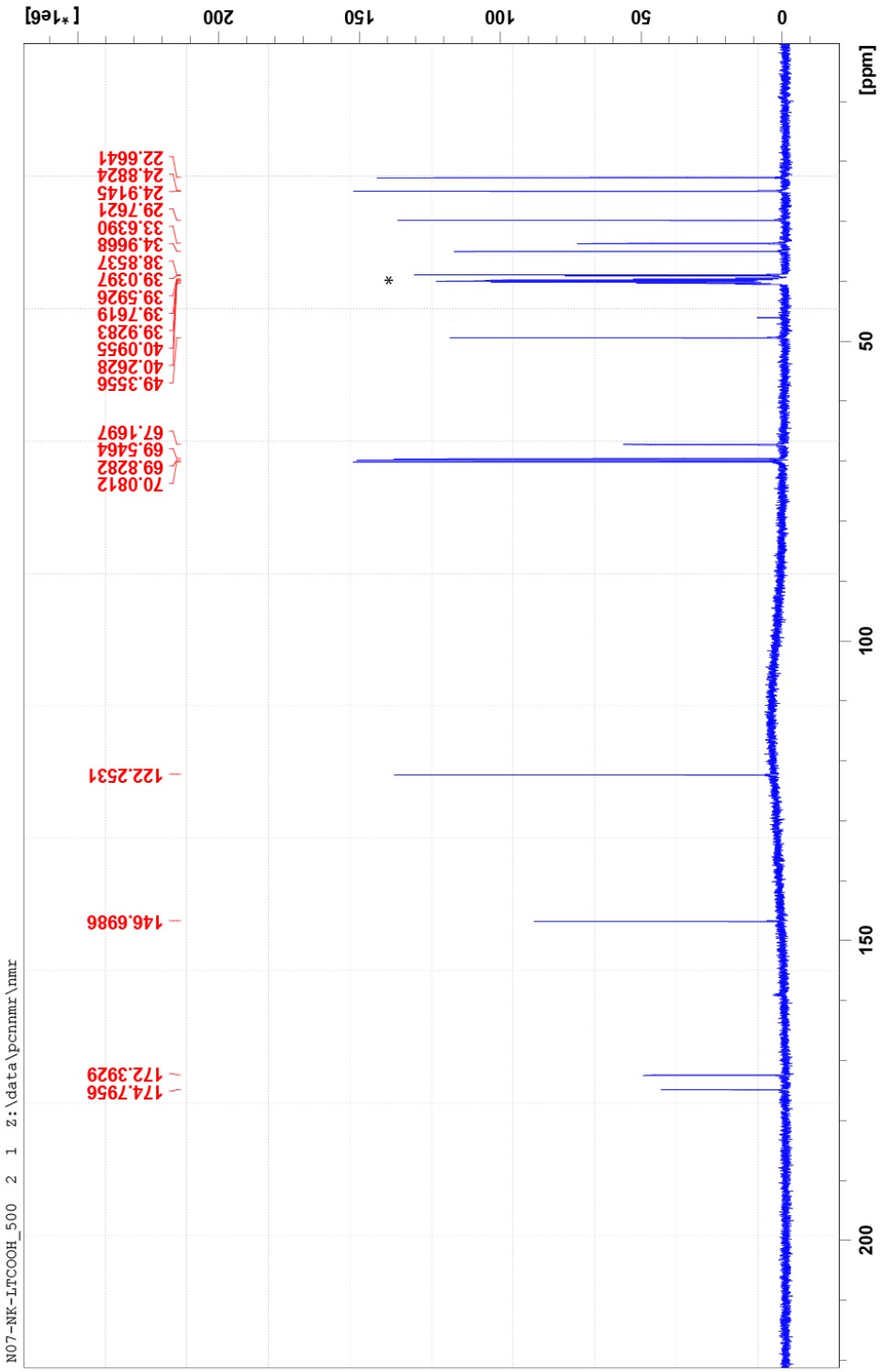
Supplementary Figure 29: 500 MHz ^{1}H spectrum of **7** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



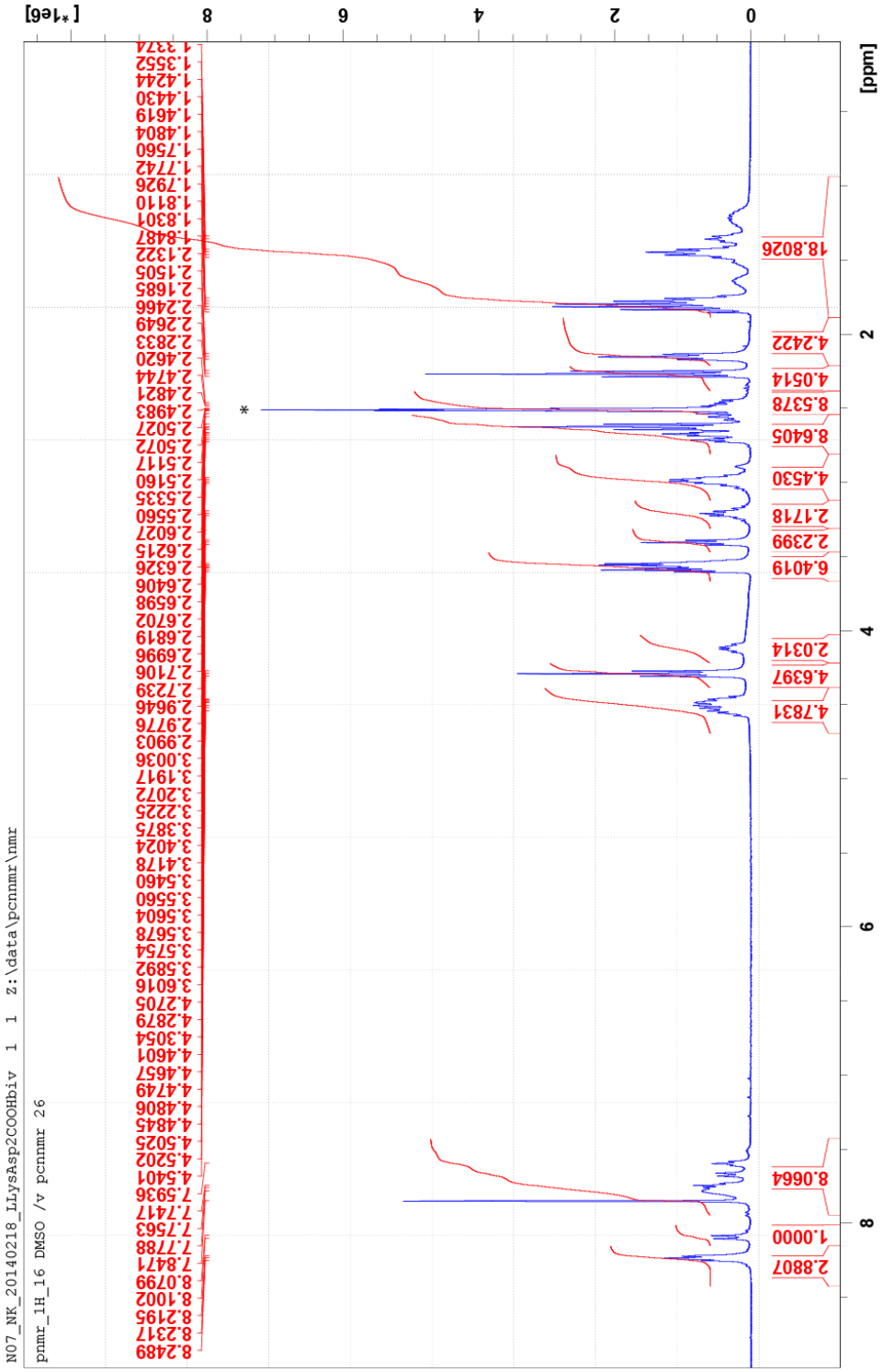
Supplementary Figure 30: 500 MHz ^{1}H spectrum of **8** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



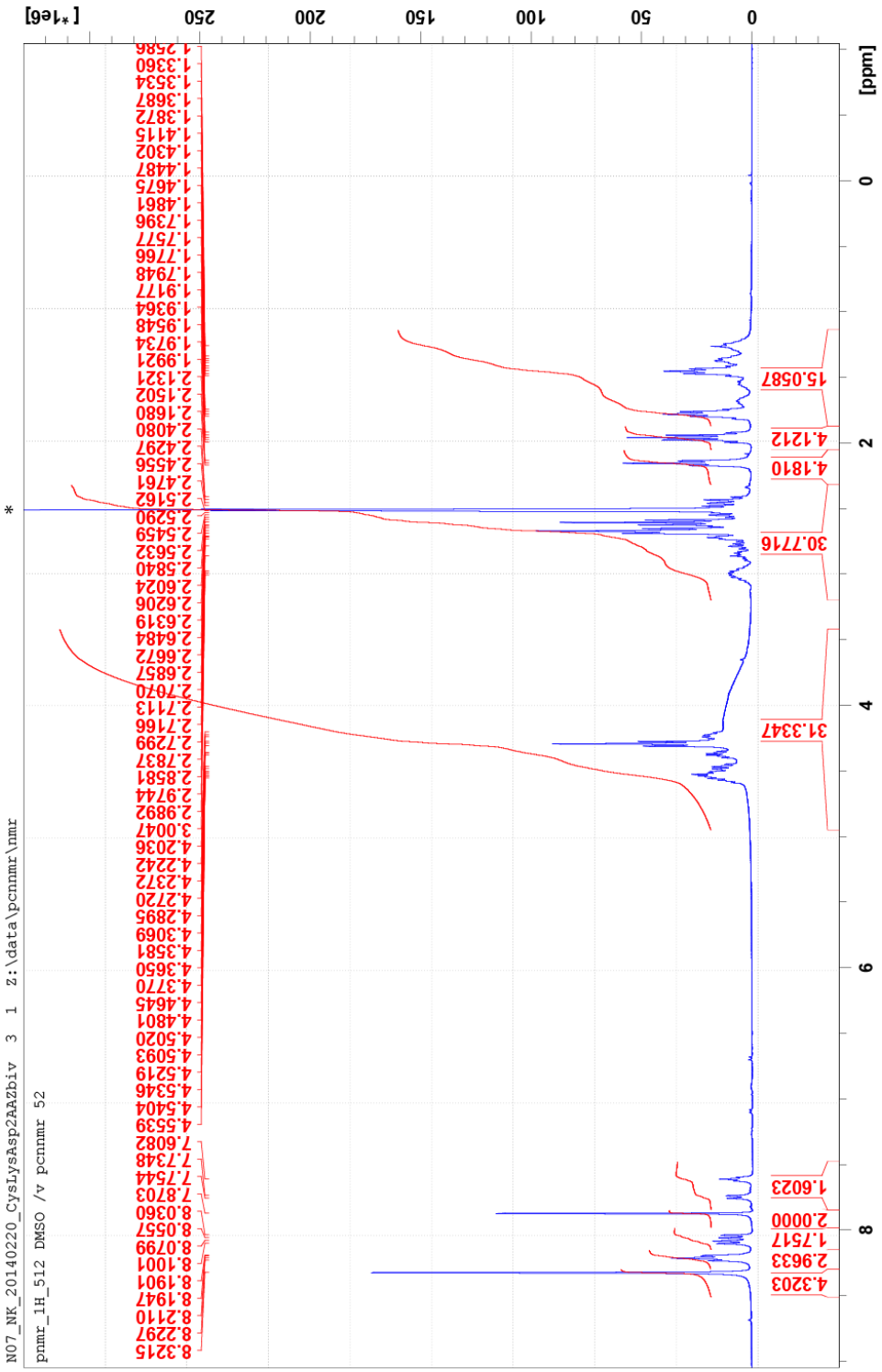
Supplementary Figure 31: 500 MHz ^{1}H spectrum of **9** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



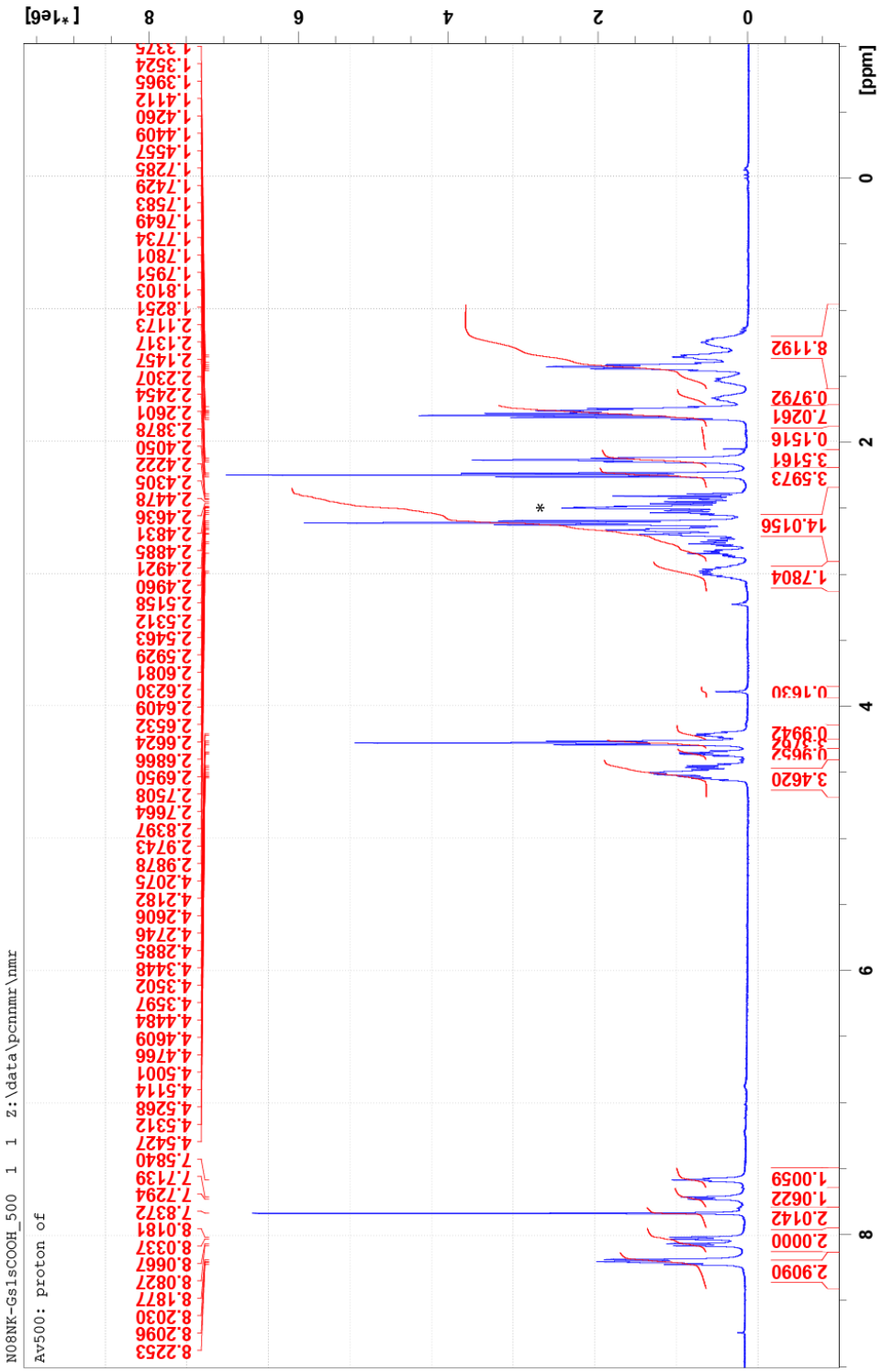
Supplementary Figure 32: 500 MHz ^{13}C spectrum of **9** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



Supplementary Figure 33: 400 MHz ^{1}H spectrum of **10** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



Supplementary Figure 34: 500 MHz ^{1}H spectrum of **11** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.



Supplementary Figure 35: 500 MHz ^{1}H spectrum of **12** in DMSO-d_6. Residual DMSO peak is marked with an asterisk.

References

1. M. Hilvo, M. Rafajova, S. Pastorekova, J. Pastorek and S. Parkkila, *J Histochem Cytochem*, 2004, 52, 1313-1322.
2. V. Askoxylakis, R. Garcia-Boy, S. Rana, S. Kramer, U. Hebling, W. Mier, A. Altmann, A. Markert, J. Debus and U. Haberkorn, *PloS one*, 2010, 5, e15962.
3. H. H. Ku, *J Res Nat Bur Stand Sec C: Eng Inst*, 1966, 70C, 263.
4. N. Krall, F. Pretto, W. Decurtins, G. J. Bernardes, C. T. Supuran and D. Neri, *Angew Chem Int Ed Engl*, 2014, 53, 4231-4235.