Electronic Supplementary Material (ESI) for ChemComm.

A Matrix Metalloproteinase Activation Probe for Painting Human Tumours

Bethany Mills,[a] Dominic Norberg,[a] Kevin Dhaliwal,[a] Ahsan R Akram,[a] Mark Bradley[b] and Alicia Megia-Fernandez[b]*

DOI

Table of Contents

Supplementary results

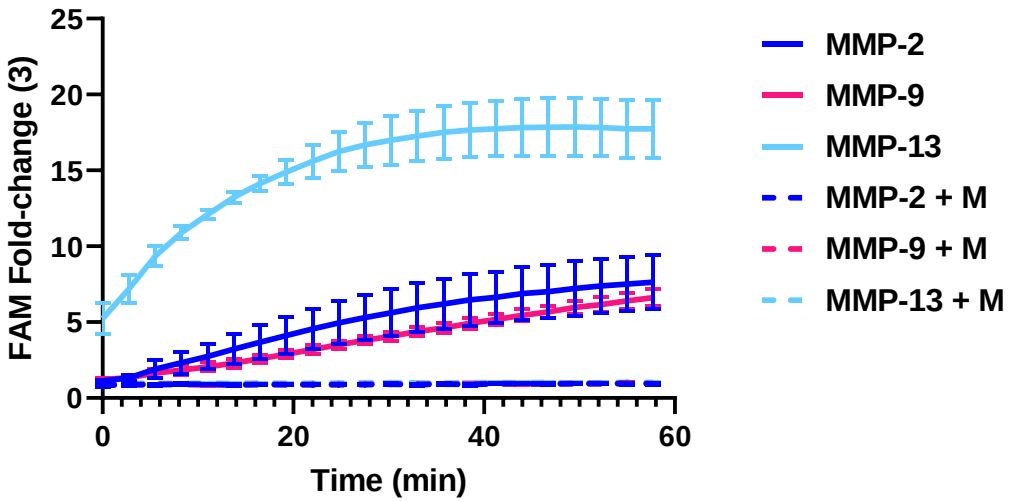


Figure S1. Relative increase in the fluorescein signal from compound **3** (5 µM) over time following incubation with MMPs (30 nM) (+/- Marimastat (M), 20 µM) in MMP buffer. Fold changes are compared to the enzyme free control. Data shows the mean of three independent repeats, performed in duplicate, error bars show s.e.m.

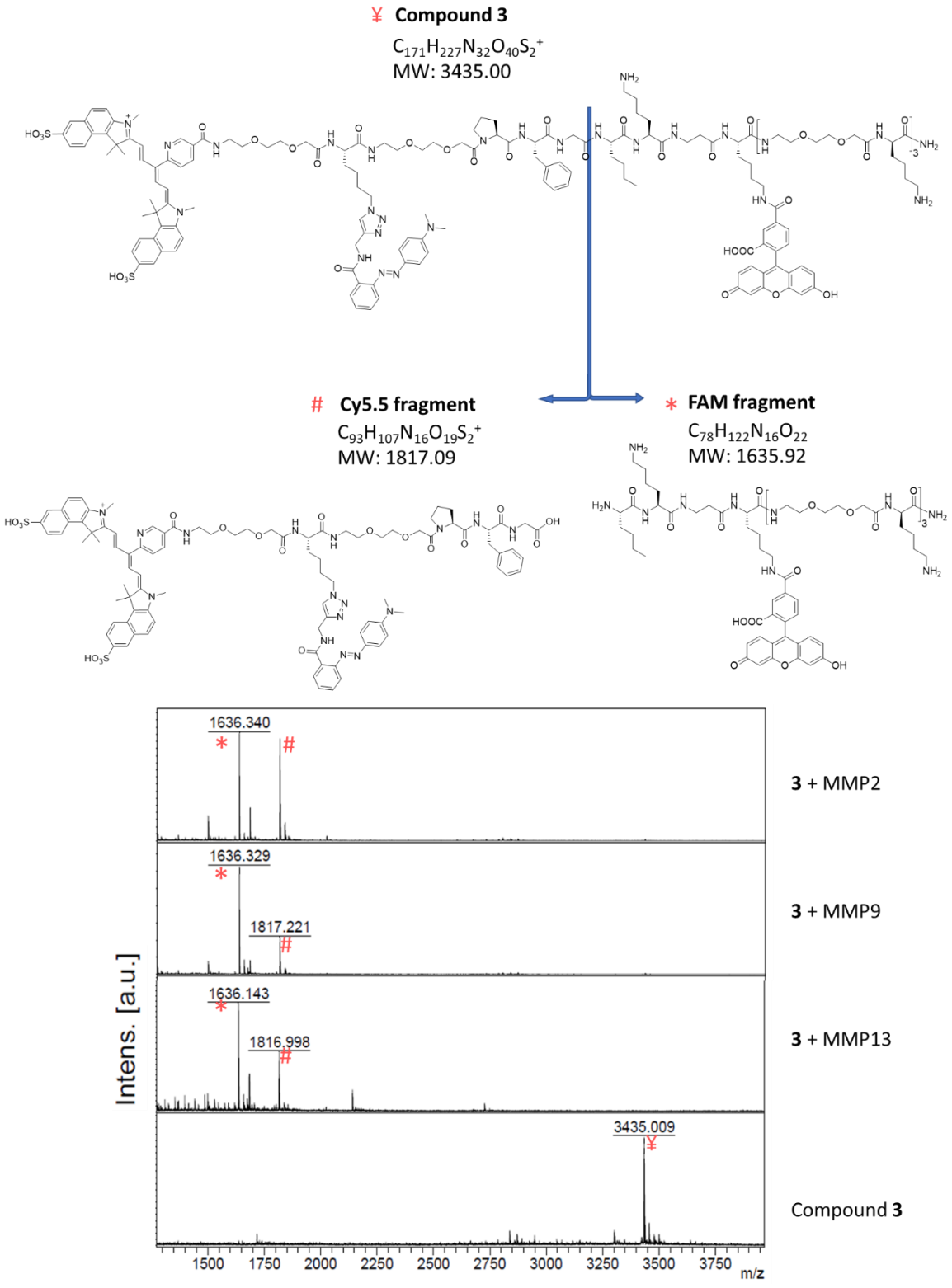


Figure S2. MALDI-TOF MS study of the enzymatic cleavage for probe **3**. Peaks obtained after treatment of compound **3** with different MMPs corresponded to the fragments expected following the specific hydrolytic cleavage between Gly-Nle in the peptide substrate.

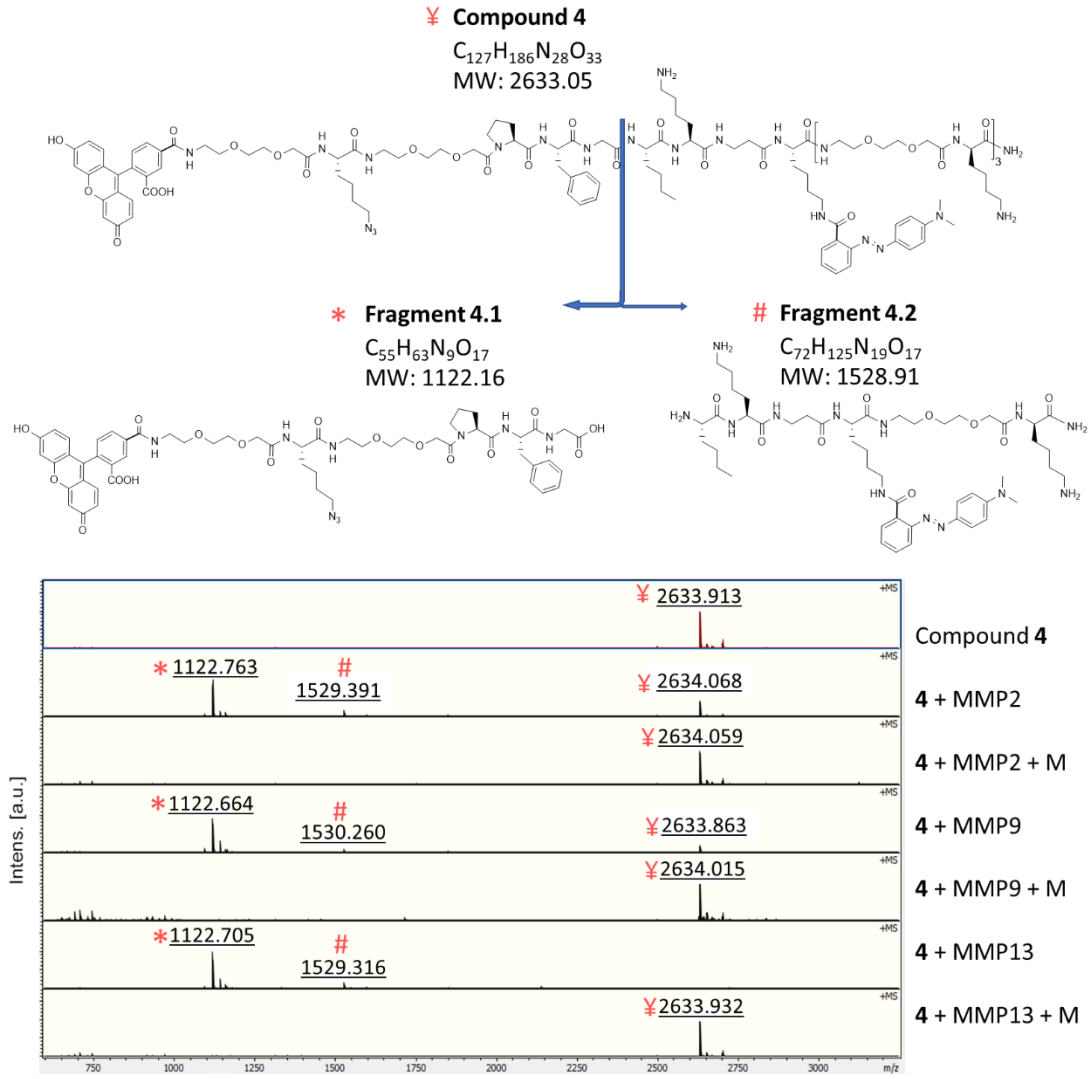
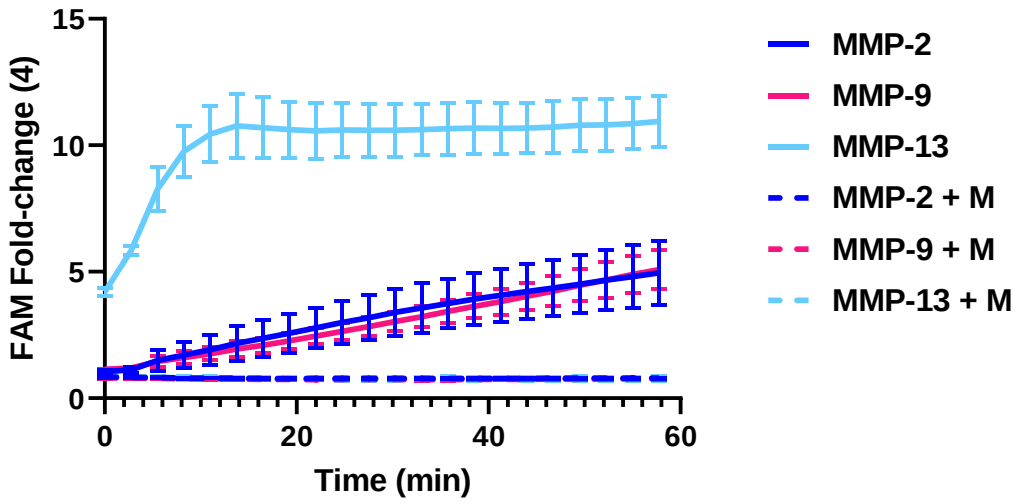


Figure S3: Compound **4** and its fragments following enzymatic cleavage. MALDI-TOF MS study of enzymatic cleavage for probe **4**. Peaks obtained after treatment of compound **4** with different MMPs (2, 9 and 13) showed two fragments corresponding to the specific cleavage between Gly-Nle in the peptide substrate. Inhibition with Marimastat (M) showed the uncleaved probe.

(A)



(B)

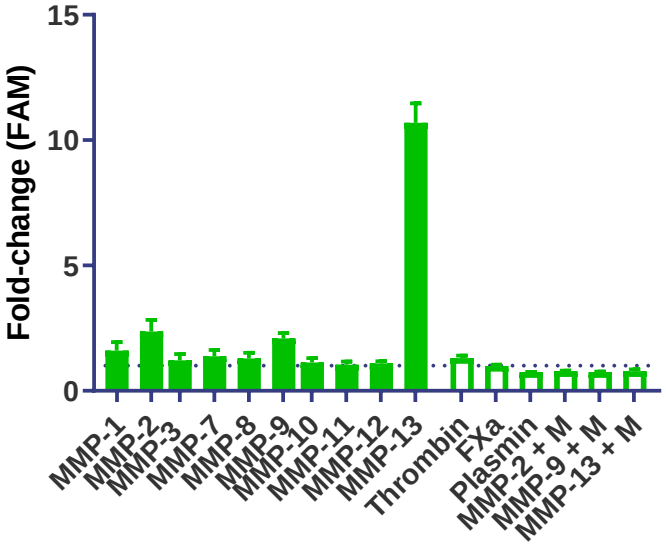


Figure S4: (**A**) Relative increase in the fluorescein signal from compound **4** (5 µM) over time following incubation with MMP's (30 nM) (+/- Marimastat (M), 20 µM). Fold changes are compared to the enzyme free control. Data shows the mean of three independent repeats, performed in duplicate, error bars show s.e.m. (**B**) Cleavage of **4** (5 µM) measured as fluorescence increase (compared to enzyme-free control) at 15 min; ex/em 485/528 nm. (M: Marimastat, MMP inhibitor). Data is the mean of three independent replicates performed in duplicate. Error bars represent s.e.m.

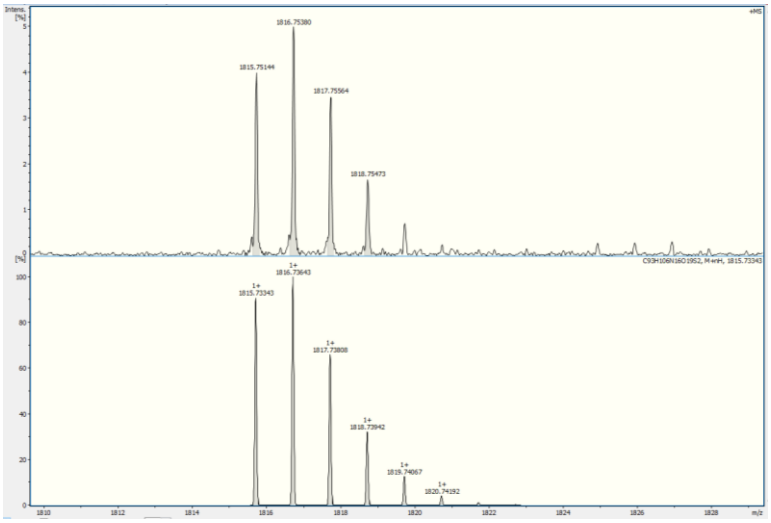


Figure S5: MALDI TOF MS study of the octanol phase after enzymatic cleavage of probe **3**. A solution of **3** (5µM) in MMP buffer was treated with MMP-13 at 37°C for 1h. The sample was diluted 1:1 with octanol. After extraction, the organic layer was lyophilised and the remaining crude dissolved in water-acetonitrile 1:1. The fragment containing Cy5.5 was observed: Top experimental HRMS: data giving a m/z of 1815.7514, bottom: theoretical distribution of ions for $C_{93}H_{107}N_{16}O_{19}S_2$ $[M]^+$: 1815.7334.

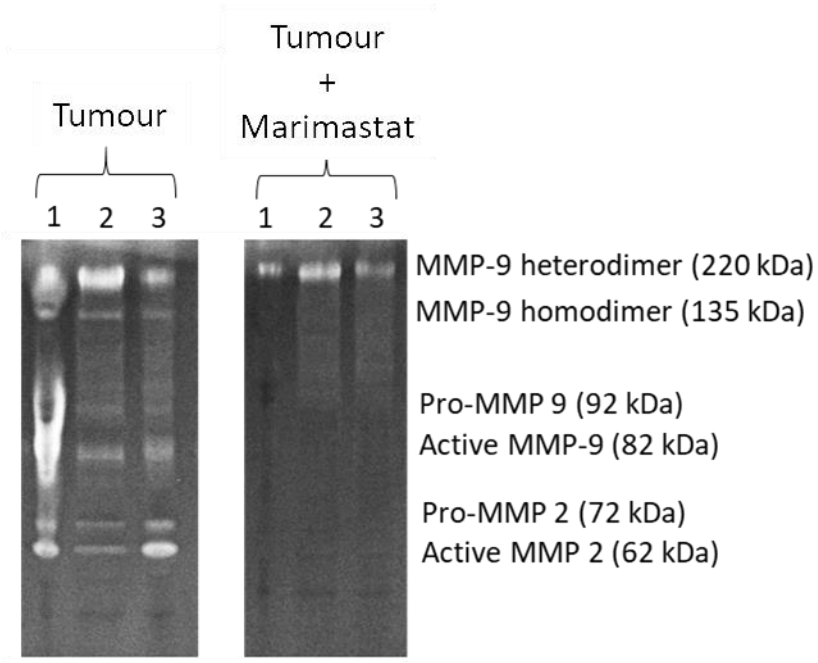


Figure S6: Activity of MMP-2 and MMP-9. Gelatin zymography of the three human lung tumour samples interrogated by fibre-based imaging (Fig. 2). Gelatin zymography demonstrates that all samples have MMP-2 and MMP-9 in pro and/or active form. Enzyme activity was blockable with the broad-spectrum inhibitor Marimastat (50 µM).

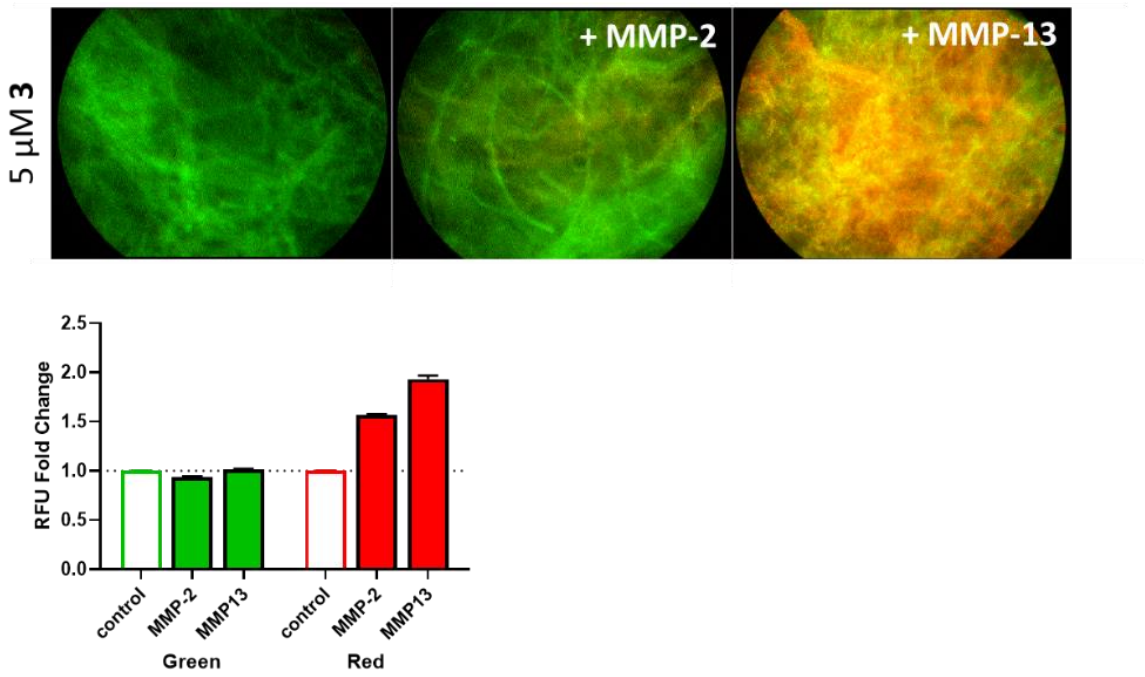


Figure S7: Incubation of healthy human lung tissue with compound **3,** with and without exogenous enzymes MMP-2 and MMP-13. Compound **3** (5 µM) was added to *ex vivo* healthy human lung tissue alone or in the presence of MMP-2 or MMP-13 (30 nM). The tissue was interrogated using an optical fibre-based imaging device with 150 frames per channel collected 15 min post addition of **3**. Individual frames are shown of an overlay of the two fluorescent channels as explained in Fig 2, and fold-change in average fluorescence (FAM: green and Cy5.5: red) after incubation with the compound was compared to base-line fluorescence from the two channels (average of 150 frames collected per channel).

Experimental Procedures. Biology

Biological methods: Ethics statement: All experiments using *ex vivo* human samples were performed following approval of the appropriate regional ethics committee (REC), NHS Lothian (references 13/ES/0126 and 15/HV/013) and NHS Lothian Bioresource (East of Scotland Research Ethics Service (REC reference 15/ES/0094)), and with informed consent of the patients. **Statistical analysis:** Statistical analyses were performed using Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Where appropriate, analyses were performed using the one-way ANOVA. Unless otherwise stated error bars show standard error of the mean (s.e.m).

Enzymatic validation: Methods were as previously reported.[1] In brief, compound **3** was incubated at a concentration of 5 µM with the recombinant MMP enzymes (Enzo Life Sciences) (active domains of MMP -1, -2, -3, -7, -8, -9, -10, -11, -12 and -13, 30 nM), thrombin (Sigma Aldrich) (5 U/ml), factor Xa (Sigma Aldrich) (500 nM) and plasmin (Sigma Aldrich) (30 nM). Where appropriate enzymes were incubated with specific inhibitors for 30 min at 37°C prior to addition of compound **3**. The pan-MMP inhibitor Marimastat (Tocris Biosciences) was used at 20 µM. Enzyme free reactions served as a control. Enzymatic reactions were performed in MMP buffer (10 mM $CaCl_2$, 6.1 g Tris-HCl, 8.6 g NaCl per litre, pH 7.5)[1] in a final volume of 20 µL. Reactions were performed in duplicate in 384 well plates (Life Technologies) with optically clear plate seals (Thermo Scientific) and with three independent repeats, unless otherwise stated.

Fluorescence signals were measured for up to 60 min, ex/em 485/528 nm (FAM), ex/em 680/710 (Cy5.5) using a microplate reader (BioTek Synergy H1 multi-mode reader). Data was normalised to buffer alone and are presented as fold-changes in signal (Relative Fluorescent Units) compared to enzyme-free controls at 15 min. Data was plotted using Prism 8 (GraphPad Software Inc., La Jolla, CA, USA).

Fibre-based lung tissue imaging: Methods were as previously reported.[1] Human lung tissue (two squamous cell carcinoma one adenocarcinoma tumours) were obtained following surgical resection and stored at -80° C until use. Healthy tissue (non-tumour) was obtained from the same patients. The tissue was sliced into 1mm x 4mm sections and placed in the wells of a 96-well plate with 100 µL of MMP buffer. Where appropriate, marimastat (50 µM) was added to the tissue and incubated at 37°C for 60 min prior to the addition of compound **3** (5 µM). MMP-2 or MMP-13 (30 nM) were added in the case of healthy tissue for the spiked experiments. The tissue was imaged over 30 min using a wide-field microendoscopy fluorescent imaging system as previously reported[2] with a commercial imaging fibre (Alveoflex, Mauna Kea Technologies). At each time point 10 s of images were taken with a 25 ms exposure time, at 10 frames per second (300 frames per recording), with LEDs chosen for optimal FAM (470 nm) and Cy5.5 (625 nm) excitation. Average fluorescence for each sample was calculated by determining the average fluorescence per frame for each time point, this was automated using an in-house designed Matlab script. All images were brightness and contrast enhanced with the same parameters. Three independent patient specimens were collected and analysed.

Lung Tissue Zymography: Lung tumour tissue was collected and stored as above. 1 mm x 4 mm sections were homogenised in 400 µL MMP buffer in Precellys Hard Tissue homogenizing (CK28)(VWR) using a Pecellys 24 homogeniser. Homogenised tissue was adjusted to 4 mg/ml protein (determined by a Pierce BCA Total Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions. Samples were mixed 1:1 with 2x Novex Tris-Glycine SDS sample buffer and loaded onto Novex® 10% Zymogram (Gelatin) Protein Gels. Zymography was run (150 kV, ~90 min), renatured (4°C, 90 min) and developed (37°C 20 h) with appropriate buffers, according to manufacturer's instructions (Thermo Fisher Scientific). Marimastat (50 µM) was added to the renaturing buffer for the control gel. A protein ladder (New England Biolabs, P7712) was loaded into the first well and used to determine the size of the protein bands. Gels were stained using Simply Blue Safe Stain (Invitrogen, LC6060).

Microscope lung tissue imaging: A lung resection (adenocarcinoma metastasis from colorectal carcinoma) was inspected macroscopically and area of transition (to include tumour and macroscopically normal lung) was dissected. This was cut to 400 µm sections and incubated in probe **3** (50 µM) for 40 minutes and imaged on an EVOS FL 2 microscope (Thermo Fisher Scientific). Images were taken with a 5 x objective lens using Cy5 and bright field filter cubes. Stitching was performed by the EVOS2 software and a separate image was taken of the

sample macroscopically. A control slice (without MMP probe) was included. Fluorescence intensity for each region of interest was determined with Fiji_ImageJ software.

Experimental Procedures. Chemistry

a. Chemistry Methods, general: Commercially available reagents were used without further purification. Methyl Red (MR) and propargylamine were purchased from Sigma, 5-Carboxyfluorescein (5-FAM) was bought from Carbosynth. Ltd., Fmoc-Lys(N_3)-OH and [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid were from Iris Biotech. Cy5.5 was synthesised as shown in the ESI. NMR spectra were recorded using a Bruker AC spectrometer operating at 500MHz for 1H. Chemical shifts are reported on the δ scale in ppm and are referenced to residual non-deuterated solvent resonances. Normal phase purifications by column chromatography were carried out on silica gel 60 (230-400 mesh). Analytical reverse-phase high-performance liquid chromatography (RP–HPLC) was performed on an Agilent 1100 system equipped with a Discovery C18 reverse-phase column (5 cm x 4.6 mm, 5 µm) with a flow rate of 1.0 mL/min and eluting with H_2O/CH_3CN/HCOOH (95/5/0.1) to H_2O/CH_3CN/HCOOH (5/95/0.1), over 6 min, holding at 95% ACN for 2 min, with detection at 254, 495 and 650 nm and by evaporative light scattering. Semi-preparative RP–HPLC was performed on an Agilent 1100 system equipped with a Zorbax Eclipse XDB-C18 reverse-phase column (250 x 9.4 mm, 5 µm) with a flow rate 2.0 mL/min and eluting with 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B), with a gradient of 5 to 95% B over 30 min and additional isocratic period of 5 min. Electrospray ionization mass spectrometry (ESI–MS) analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) in ESI mode. MALDI spectra were acquired on a Bruker Ultraflextreme MALDI-TOF MS with a matrix solution of sinapic acid (10 mg/mL) in H_2O/CH_3CN/TFA (50/50/0.1).

b. General solid-phase synthesis methods: Manual peptide synthesis was performed on aminomethyl-ChemMatrix resin using an Fmoc-protected Rink amide linker. General procedures were as follows:

Coupling of the Rink amide linker: The Fmoc-Rink linker (4-[(R,S)-a-[1-(9H-Fluoren-9-yl)-methoxy-formamido]-2,4-dimethoxybenzyl-phenoxyacetic acid) (0.54 g, 1.0 eq) was dissolved in DMF (10 mL) and Oxyma (0.14 g, 1.0 eq.) was added and the mixture was stirred for 10 min. Diisopropylcarbodiimide (DIC, 155 µL, 1.0 eq.) was then added and the solution stirred for 1 min before adding it to aminomethyl-ChemMatrix resin (1.0 g, 1.0 mmol/g). The resulting mixture was stirred at 50ºC for 45 min and washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL). Finally the resin was treated with Ac_2O:Py:DMF (2:3:15) for 30 min in order to cap any remaining free amino groups and was washed again with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL). Resin loading was calculated as ~0.58 mmol/g via a DBU Fmoc-deprotection spectrophotometric test.[3]

Fmoc deprotection: to the resin, pre-swollen in DCM, was added 20% piperidine in DMF and the mixture stirred for 2x10 min. The solution was drained and the resin washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL).

Amino acid coupling: A solution of the appropriate D or L-amino acid (3.0 eq per amine) and Oxyma (3.0 eq) in DMF (0.1M) were stirred for 10 min. DIC (3.0 eq) was added and stirred for 1 min. The pre-activated mixture was then added to the resin pre-swollen in DCM and the reaction heated at 50ºC for 30 min. The solution was drained and washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL). The completion of the coupling reactions was monitored by Kaiser or Chloranil tests (when secondary amines are involved). The side chain-protecting group used for Lysine was Boc except for addition of the fluorophore FAM when Fmoc-Lys(Dde)-OH was used as an orthogonal protecting group to allow introduction of the dyes.

Coupling of other carboxylic acids: Coupling of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (PEG), 5-Carboxyfluorescein (FAM) and Fmoc-Lys(N_3)-OH were carried out following the same procedure as described for the amino acid couplings.

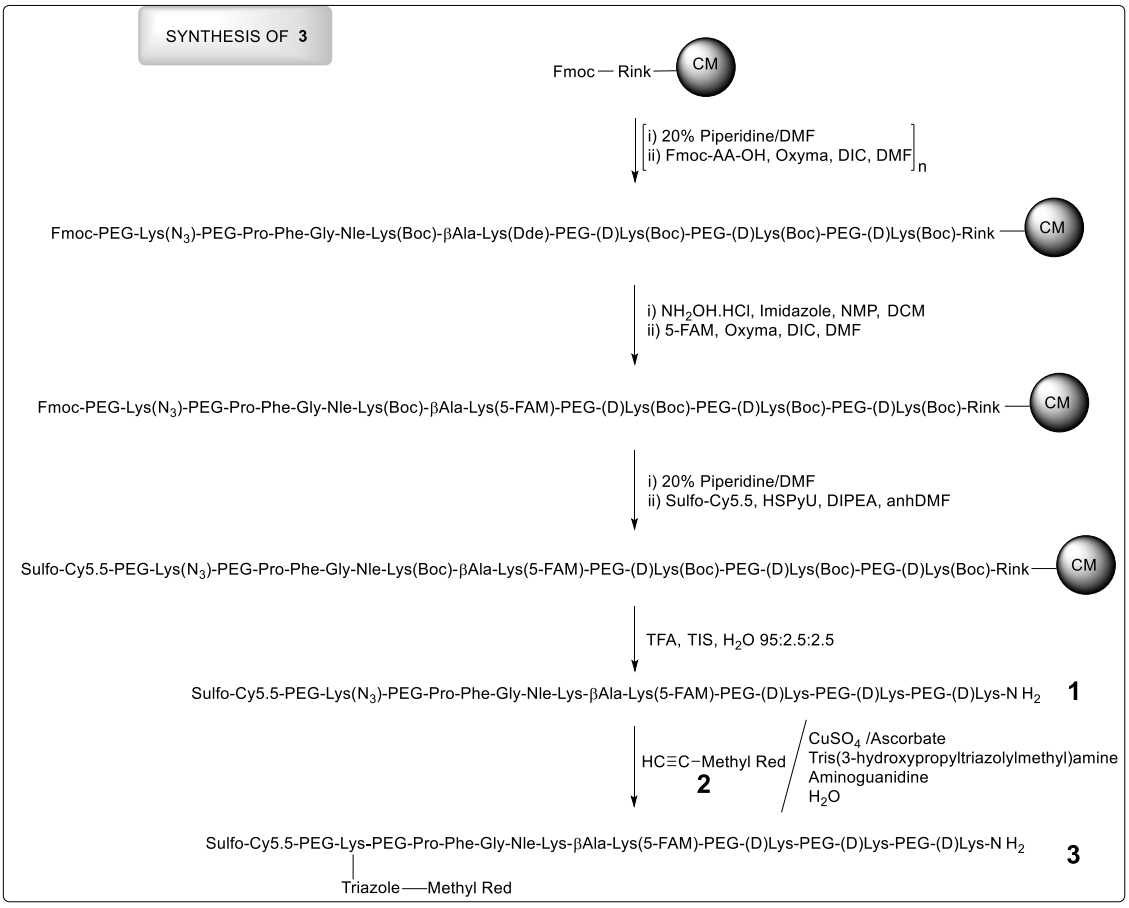
Selective Dde deprotection in presence of the Fmoc protecting group for compound **3** was achieved as previously reported[4]: 1.25 g of $NH_2OH{\cdot}HCl$ and 0.918 g of imidazole were suspended in 5 mL of NMP and the mixture was

sonicated until complete dissolution. 5 volumes of this solution were diluted with 1 volume of CH_2Cl_2 and added to the resin. After 3h the solution was drained and the resin washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL).

Coupling of Cy5.5: Cy5.5 (1.0 eq, ESI for detailed synthesis) was dissolved in anhydrous DMF. HSPyU (1.0 eq) and DIPEA (3.0 eq) were added and the mixture stirred at 40ºC for 1h. The solution was then added to the amino-functionalized resin (1.0 eq) together with DIPEA (3.0 eq) and the reaction was stirred overnight. The solution was drained and the resin washed with DMF until the washes were colourless, then DCM (3x10 mL) and MeOH (3x10 mL). The completion of the coupling reactions was monitored by a Kaiser test.

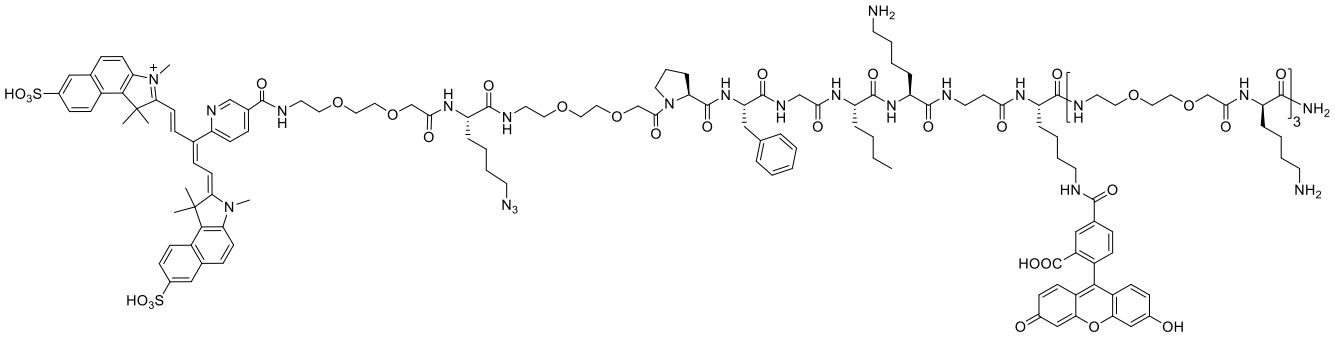
Cleavage and purification: The resin (100 mg) pre-swollen in DCM was treated with a cleavage cocktail of TFA:triisopropylsilane(TIS):water (95:2.5:2.5) (2 mL) for 3h. The reaction solution was drained and the resin washed with the cleavage cocktail (1 mL). The combined solution was precipitated against cold ether, centrifuged (x3) and used in the next step.

c. Synthesis and Characterization of 1-3:



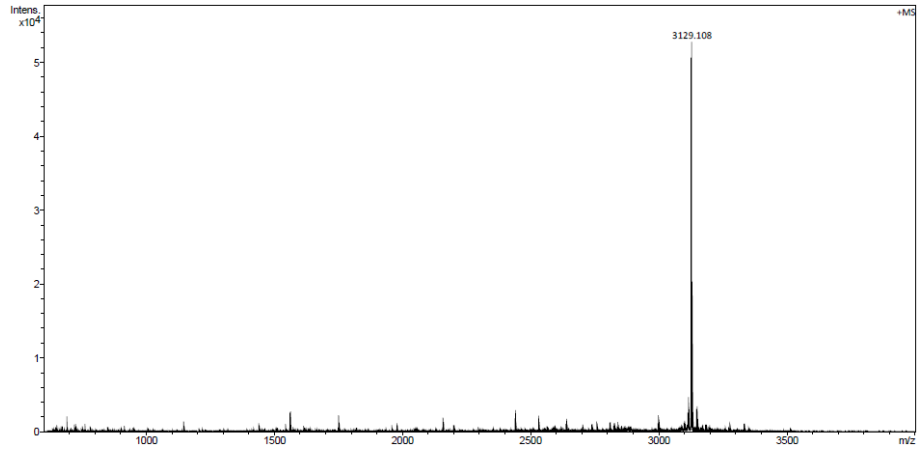
Scheme S1. Synthesis of **Compound 3**.

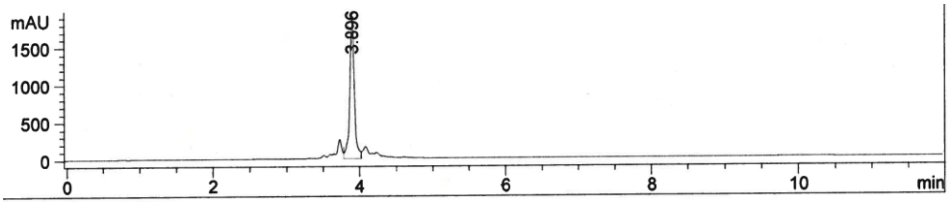
Synthesis of azide-peptide (1): Peptide (**1**) was synthesised following solid phase synthesis methods as described in general section. Characterization by MALDI-TOF MS (calc. for $C_{153}H_{209}N_{28}O_{39}S_2$ $[M]^+$: 3128.631; found: 3129.108) and analytical RP-HPLC (t_R = 3.896 min (650 nm)) See structure and MALDI-TOF MS spectra and HPLC chromatograms below.



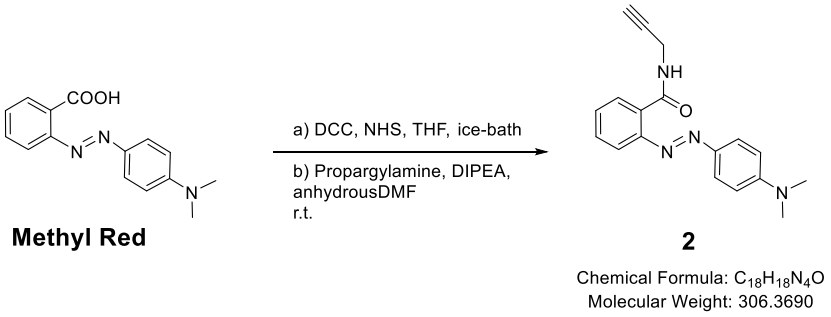
Compound 1

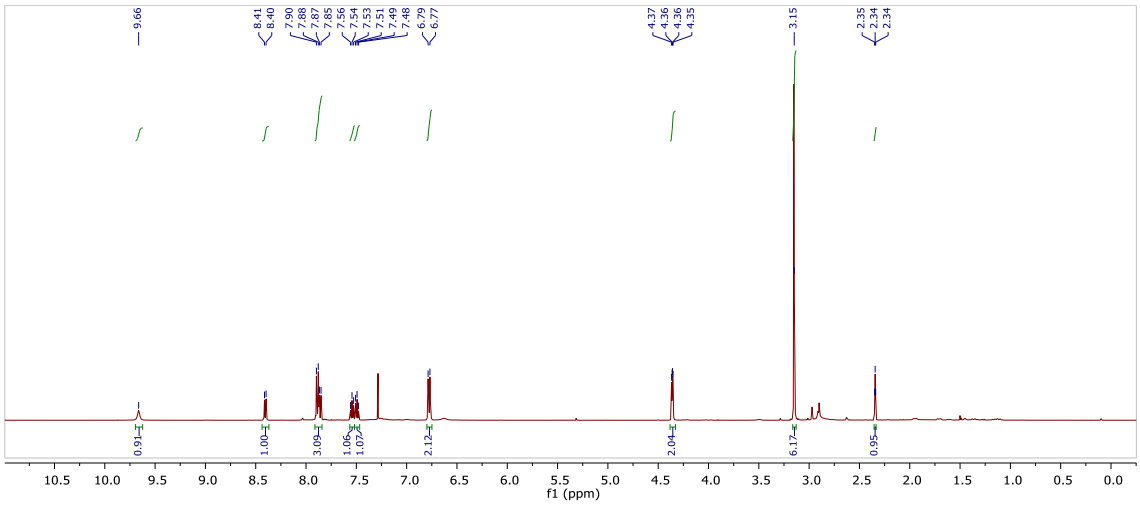
$C_{153}H_{209}N_{28}O_{39}S_2^+$
MW: 3128.6315



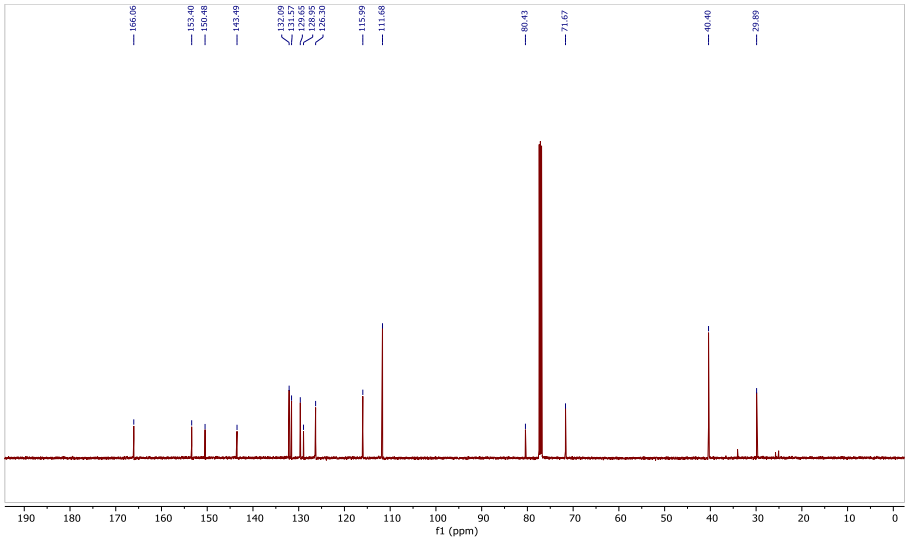


Synthesis of Methyl Red-alkyne (2): Methyl Red (2.5 g, 9.28 mmol) was dissolved in THF (60 mL) and cooled in an ice-bath. DCC (2.2 g, 10.69 mmol) was added followed by NHS (1.1 g, 9.87 mmol). The mixture was kept overnight at room temperature. After filtration, the filtrate was evaporated to give a red solid that was recrystallized with acetone/diethyl ether. The solid was then dissolved in anhydrous DMF (20 mL) and propargylamine (0.77 mL, 12.07 mmol) and DIPEA (4.8 mL, 27.85 mmol) were added and stirred overnight. The solvent was evaporated under vacuum. DCM (50 mL) was added and washed with water (90 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford the compound Methyl Red-alkyne **2** as a dark red solid (2.80 g, 98%). **^{1}H-NMR** (500 MHz, $CDCl_3$) δ: 9.66 (s, 1H), 8.41 (d, *J* = 7.8 Hz, 1H), 7.87 (m, 3H), 7.54 (t, *J* = 8.2 Hz, 1H), 7.49 (t, *J* = 7.6 Hz, 1H), 6.78 (d, *J* = 9.2 Hz, 1H), 4.36 (dd, *J* = 4.7, 2.6 Hz, 2H), 3.15 (s, 6H), 2.34 (t, *J* = 2.6 Hz, 1H); **^{13}C-NMR** (125 MHz, , $CDCl_3$) δ: 166.1, 153.4, 150.5, 143.5, 132.1, 131.6, 129.7, 129.0, 126.3, 116.0, 111.7, 80.4, 71.7, 40.4, 29.9; **HRMS** (EI) calc. for $C_{18}H_{18}N_4O$ [M]: 306.1475; found: 306.1485; **HPLC** t_R 6.871 min (495 nm).



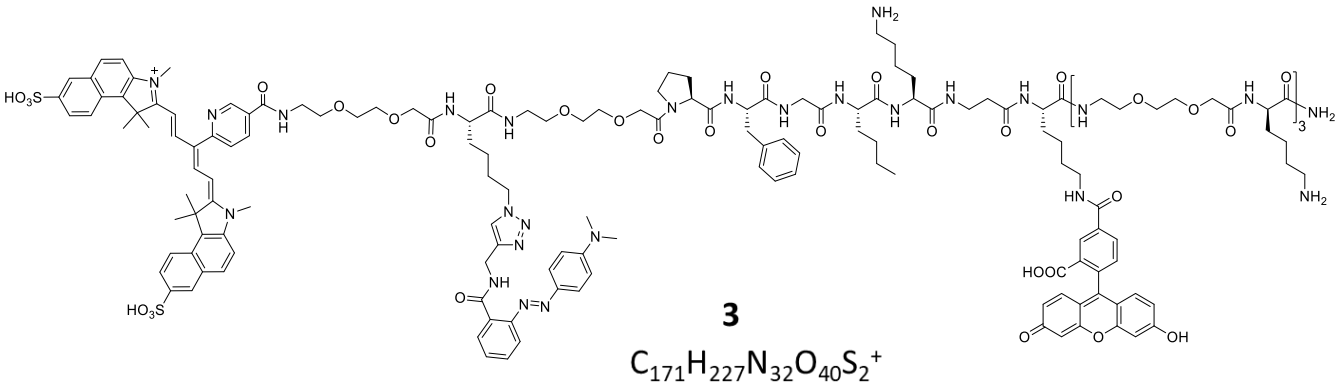


^{1}H-NMR spectra of compound **2**



^{13}C-NMR spectra of compound **2**

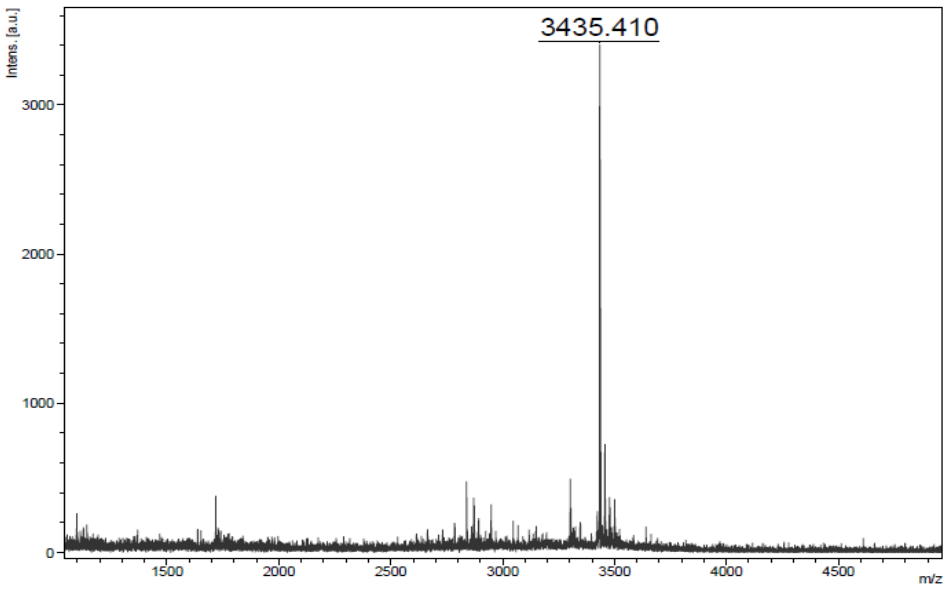
Reaction of the azide-peptide 1 with alkyne 2: The cycloaddition reaction between the azide containing peptide **1** and alkyne **2** was carried out in an Eppendorf tube mixing the following aqueous reagents: N_3-peptide (50 µL, 10 mM), alkyne (50 µL, 30 mM), premixed $CuSO_4$ and Tris(3-hydroxypropyltriazolylmethyl)amine (40 µL $CuSO_4$ 20 mM and 80 µL THPTA 50 mM), aminoguanidine hydrochloride (250 µL, 100 mM) and finally sodium ascorbate (250 µL, 100 mM). The click reaction was allowed to proceed at 30ºC for 5h, the reaction mixture was lyophilised and purified by RP-HPLC on a C-18 semi-preparative column. The desired fractions containing the product were collected and lyophilized to afford the pure compound **3**, which was characterized by MALDI-TOF MS and analytical RP-HPLC: t_R = 3.326 min (detection at 650 nm), MALDI calc. for $C_{171}H_{227}N_{32}O_{40}S_2$ $[M]^+$: 3435.001; found: 3435.410. (Structure, MALDI spectra and HPLC chromatogram below)

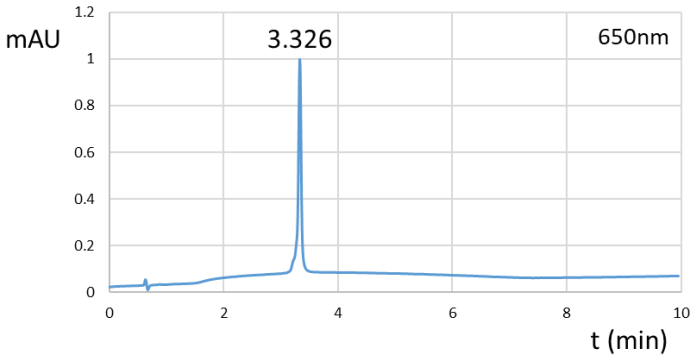


3

$C_{171}H_{227}N_{32}O_{40}S_2^+$

MW: 3435.0005





Partition coefficient octanol-water assay (Log *P*):

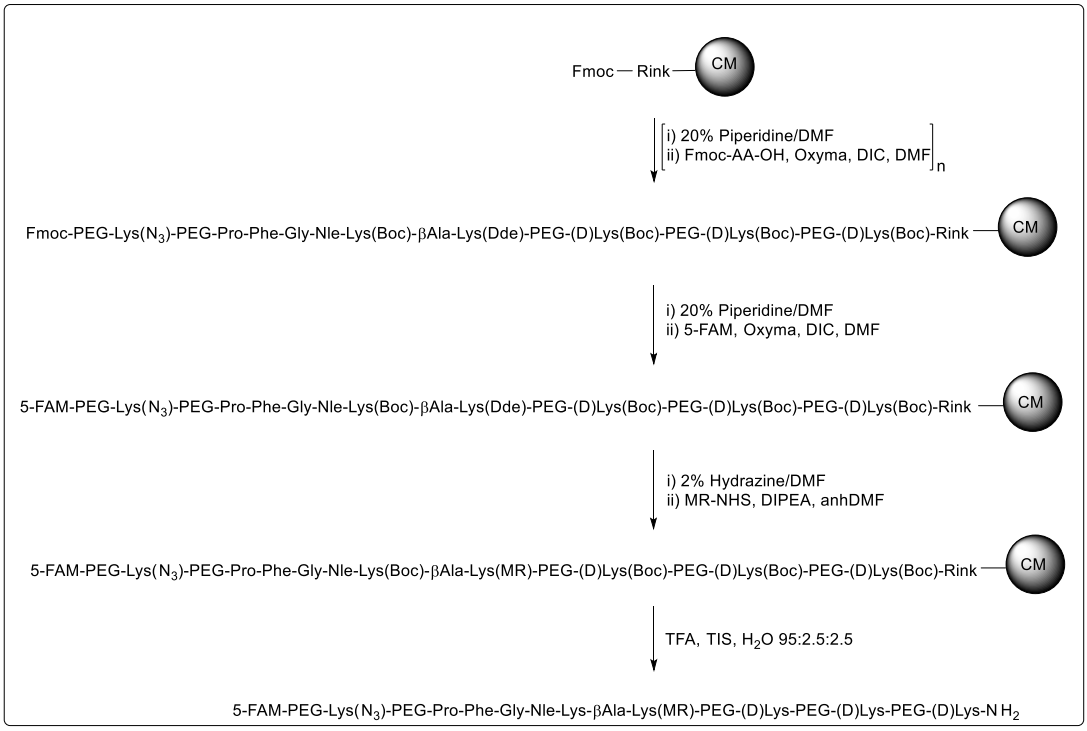
The partition coefficient (log *P*) of compound **3** was determined by the 'shake-flask' method[5]. Compound **3** (0.1 mg) was suspended in water (pre-saturated for 24 h with *n*-octanol) (400 µL) and *n*-octanol (pre-saturated for 24h with water) (400 µL), mixed by vortex and shaken at room temperature for 24h to allow the compound to partition between the two phases. After that the layers were separated by centrifugation (2000g x 5 min) and the concentration was determined by absorbance using the plate reader (680 nm). The experiment was performed in triplicate. The distribution coefficient was calculated according to the following equation obtaining a value of log *P* of -1.5 for compound **3**.

$$\log P = \log \frac{C_{n-octanol}}{C_{water}}$$

In addition, the partition assay was done in presence and absence of MMP-13 (Fig 1d), using in that case MMP buffer as the aqueous phase and adding *n*-octanol after the enzymatic reaction. Following the method described in the section "Enzymatic validation" compound **3** (5 µM) in MMP buffer (10 mM $CaCl_2$, 6.1 g Tris-HCl, 8.6 g NaCl per litre, pH 7.5) was incubated with or without recombinant MMP-13 (30 nM) at 37°C for 1h. The solution (60 µL) is mixed with *n*-octanol (60 µL) and shaken at room temperature for 24h to allow the compound to partition between the two phases. After that the two layers were separated by centrifugation (2000g x 5 min).

d. Synthesis of FRET probe 4:

Synthesis of **4** was carried out following Scheme S2 and the general methods, with the additional steps as detailed below:

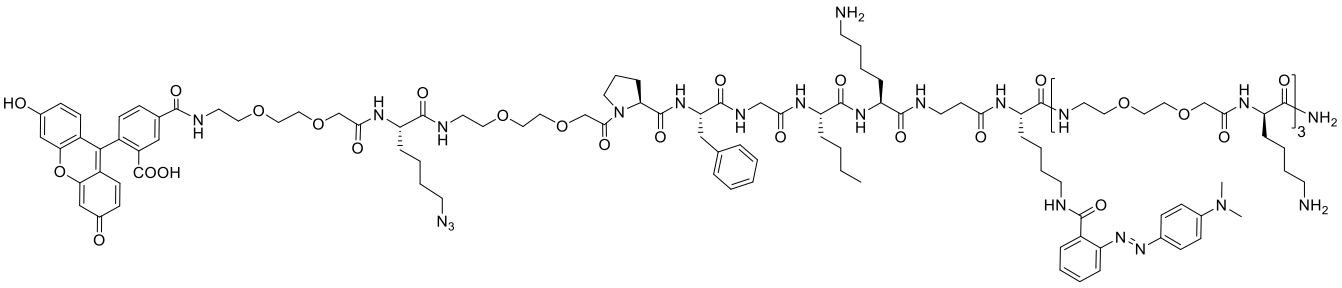


Scheme S2. Synthesis of **Compound 4**

Dde deprotection: To the resin pre-swollen in DCM was added 2% hydrazine in DMF and stirred for 10 minutes and the reaction repeated four more times. The solution was drained and the resin washed with DMF (3x10 mL), DCM (3x10 mL) and MeOH (3x10 mL).

Methyl Red-NHS coupling: Methyl Red-NHS ester (1 eq, 0.1M) coupling was carried out in anhydrous DMF containing DIPEA (3 eq) for 12 h. The solution was drained and the resin washed with DMF until the washes were colourless then DCM (3x5 mL), MeOH (3x5 mL) and finally ether (3x5 mL).

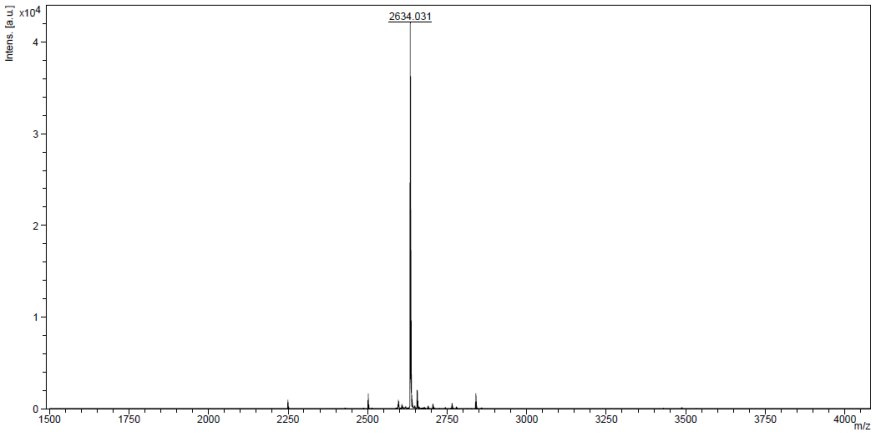
Compound **4**: RP-HPLC t_R = 4.17 min (495 nm), MALDI-TOF MS calc. for $C_{127}H_{187}N_{28}O_{33}$ $[M+H]^+$: 2634.0560; found: 2634.0310. (Structure, MALDI spectra and HPLC chromatogram below)

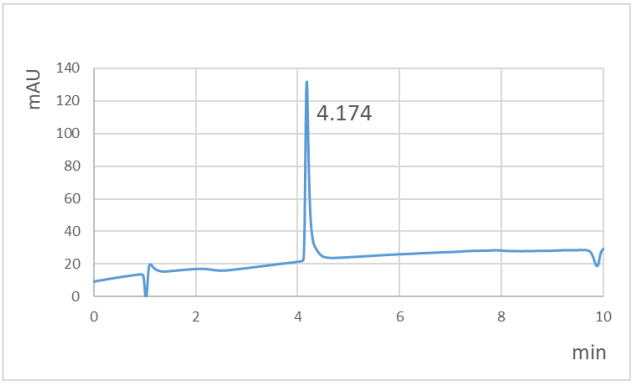


Compound 4

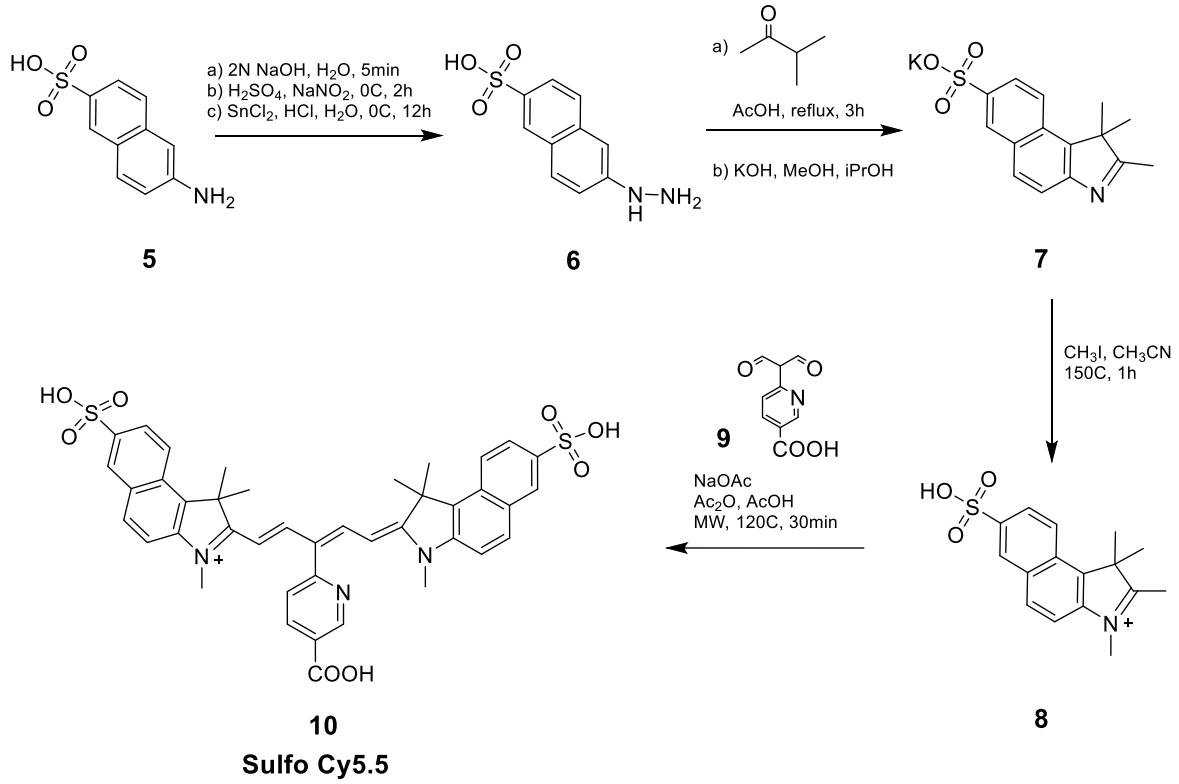
$C_{127}H_{186}N_{28}O_{33}$

MW: 2633.05



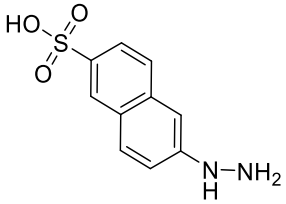


e. Synthesis of Cy5.5 (compound 10):



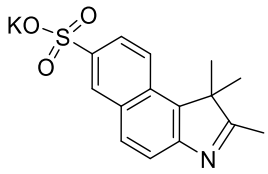
Scheme S3. Synthesis of Cy5.5 from 6-aminonaphtalene-2-sulfonic acid

2-Hydrazinonaphthalene-6-sulfonic acid (**6**):



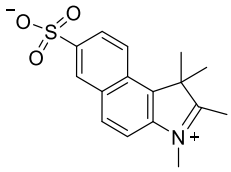
6-Aminonaphthalene-2-sulfonic acid (**5**) (1.115 g, 5.0 mmol) was dissolved in H_2O (8 mL) and 2M NaOH (0.375 mL) was added. The mixture was stirred 5 min and conc H_2SO_4 (0.34 g) was added dropwise at 0ºC. 1mL of iced water was added, followed by a solution of $NaNO_2$ (520 mg) in H_2O (1 mL). The solution was stirred at 0ºC for 2h and the diazonium salt formed was removed by filtration and washed with cold water. The salt was then added in portions to a cold solution of $SnCl_2$ (2.45 g) in conc HCl (1.6 mL) and H_2O (0.9 mL). The mixture was stirred overnight. The solid was filtered, washed with water and dried under vacuum to afford compound **6** as a white solid (1.07 g, 90%); **RP-HPLC** t_r 0.754 min; ^{1}H NMR (400 MHz, DMSO-d_6/D_2O) 8.07 (s, 1H), 7.87 (d, *J* 8.8, 1H), 7.72-7.63 (m, 2H), 7.24 (d, *J* 2.3, 1H), 7.19 (dd, *J* 8.8, 2.4, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) 144.2, 143.9, 133.8, 130.2, 128.3, 126.5, 125.3, 124.7, 117.6, 108.2; HR-MS (ESI): cal. $C_{10}H_{11}N_2O_3S$ 239.0485; found: 239.0497 $(M+H)^+$.

1,1,2-Trimethyl-1*H*-benzo[*e*]indole-7-sulfonate potassium salt (**7**):



2-Hydrazinonaphthalene-6-sulfonic acid **6** (1.0 g, 4.2 mmol) and 3-methyl-2-butanone (1.08 g, 12.6 mmol) were dissolved in AcOH (5 mL) and stirred under reflux for 3h. The solvent was removed under vacuum. The obtained residue was dissolved in MeOH and stirred with a saturated solution of KOH in isopropanol. Diethyl ether was added and the solid obtained was filtered, washed and dried under vacuum to afford compound **7** as a brown solid (1.15 g, 84%); RP-HPLC t_r 2.904 min; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.22 (s, 1H), 8.08 (d, *J* = 8.7 Hz, 1H), 7.96 (d, *J* = 8.4 Hz, 1H), 7.79 (dd, *J* = 8.7, 1.7 Hz, 1H), 7.70 (d, *J* = 8.4 Hz, 1H), 2.32 (s, 3H), 1.48 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) 190.0, 151.6, 144.7, 139.2, 131.2, 129.7, 128.4, 126.0, 125.2, 122.6, 120.3, 55.3, 22.5, 15.4; HR-MS (ESI): cal. $C_{15}H_{15}NO_3SK$ 328.0404; found: 328.0405 $(M+H)^+$.

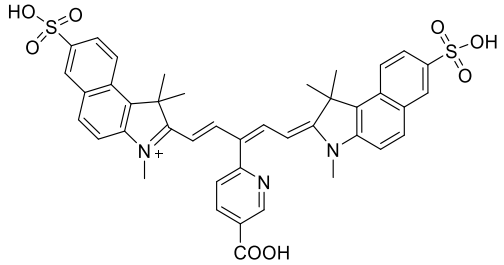
1*H*-Benz[*e*]indolium-1,1,2,3-tetramethyl-7-sulfo-, inner salt (**8**):



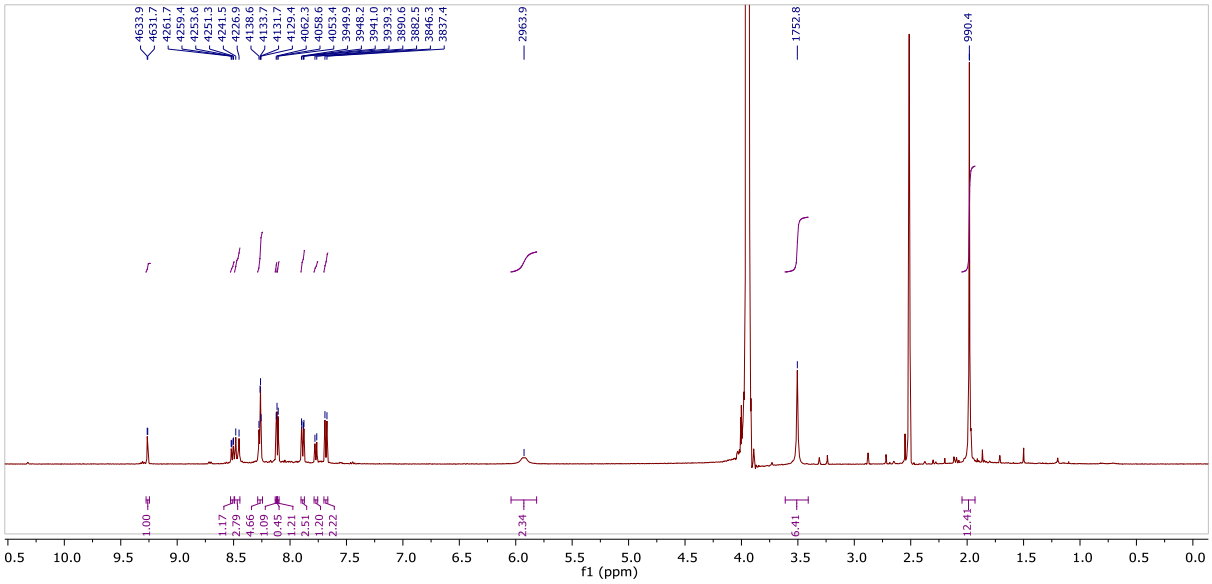
1,1,2-Trimethyl-1*H*-benzo[*e*]indole-7-sulfonate potassium salt **7** (1.0 g, 3.05 mmol) and methyl iodide (0.95 mL, 15.25 mmol) in CH_3CN (20 mL) were heated under MW irradiation at 150ºC for 1h. After cooling the solvent was removed under vacuum and ether was added. The solid was recovered by filtration, washed and dried under vacuum to obtain compound **8** as a brown solid (1.27 g, 89%); RP-HPLC t_r 0.855 min; ^{1}H NMR (500 MHz, CD_3OD) δ 8.30-8.20 (m, 2H), 8.09 (m, 1H), 8.02 (m, 1H), 7.96 (dd, *J* = 8.8, 2.2 Hz, 1H), 4.20 (s, 3H), 2.05 (s, 3H), 1.78 (s, 6H); ^{13}C NMR (125 MHz, CD_3OD) δ 197.9, 144.8, 141.8, 138.0, 134.0, 132.9, 129.0, 127.9, 126.8, 124.6, 114.6, 57.0, 35.7, 22.0; HR-MS (ESI): cal. $C_{16}H_{18}NO_3S$ 304.1002; found: 304.1015 $(M+H)^+$.

1,1,3-trimethyl-2-((1E,3Z,5E)-3-(5-carboxypyridin-2-yl)-5-(1,1,3-trimethyl-7-sulfonate-1H-benzo[e]indol-2(3H)-ylidene)penta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium-7-sulfonate (Cy5.5 **10**)

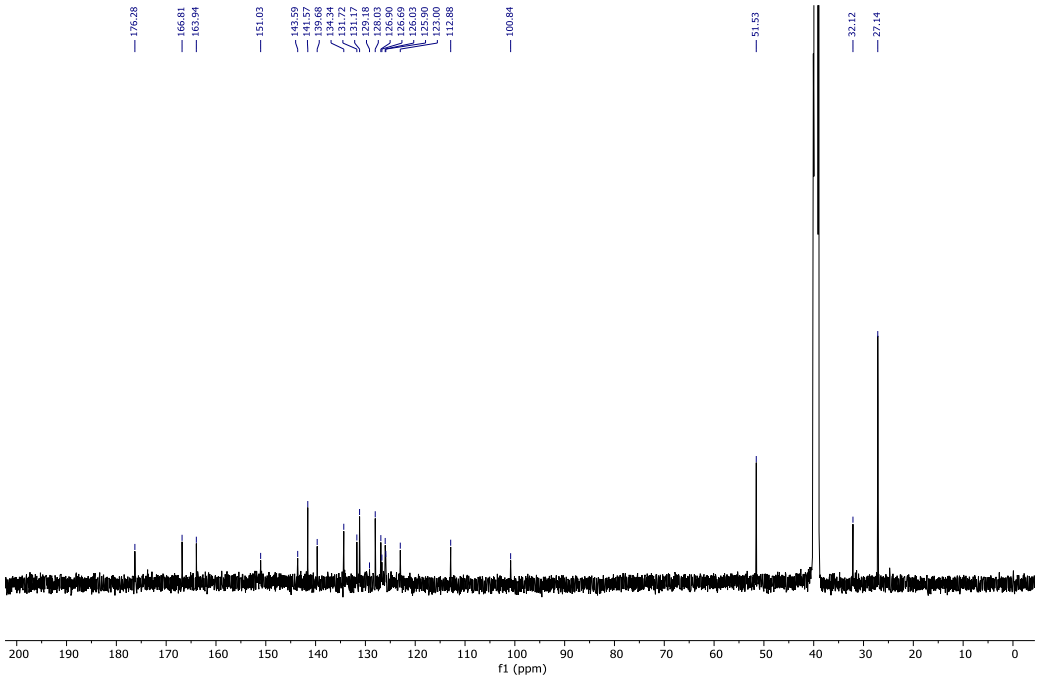
1*H*-Benz[*e*]indolium-1,1,2,3-tetramethyl-7-sulfo-, inner salt **8** (600 mg, 1.29 mmol), 2-(3-Hydroxycarbonyl-6-pyridyl)malondialdehyde **9** (111 mg, 0.57 mmol) and NaOAc (300 mg, 3.66 mmol) in Ac_2O:AcOH (1:1, 18 mL) were heated under MW irradiation at 120ºC for 30 min. After cooling the solvent was removed under vacuum. The solid obtained was dissolved in MeOH, precipitated against cold ether, centrifuged (x3) and purified by RP-HPLC to give Cy5.5 (**10**) as a dark blue solid (222 mg, 51%); RP-HPLC t_r 4.205 min; ^{1}H NMR (500 MHz, DMSO-d_6/D_2O) δ 9.26 (d, *J* = 2.2 Hz, 1H), 8.51 (dd, *J* = 8.1, 2.3 Hz, 1H), 8.47 (m, 2H), 8.26 (m, 4H), 8.12 (m, 2H), 7.89 (dd, *J* = 8.9, 1.7 Hz, 2H), 7.77 (d, *J* = 8.1 Hz, 1H), 7.68 (d, *J* = 8.9 Hz, 2H), 5.93 (br s, 2H), 3.50 (s, 6H), 1.98 (s, 12H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.3, 166.8, 163.9, 151.0, 143.6, 141.6, 139.7, 134.3, 131.7, 131.2, 129.2, 128.0, 126.9, 126.7, 126.0, 125.9, 123.0, 112.9, 100.8, 51.5, 32.1, 27.1; HR-MS (ESI): cal. $C_{41}H_{38}N_3O_8S_2^+$ 764.2106; found: 764.2122 $(M)^+$.



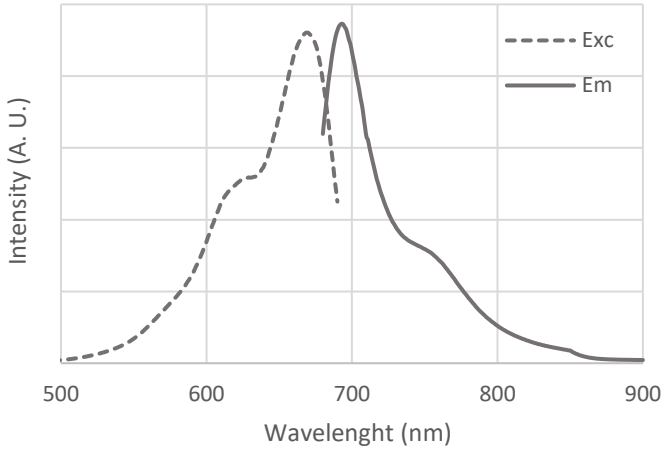
Cy5.5 (**10**)



^{1}H-NMR spectra of compound **10**



^{13}C-NMR spectra of compound **10**



Excitation and emission spectra of compound **10** with maximum at 670/693 nm

References

[1] A. Megia-Fernandez, B. Mills, C. Michels, S. V. Chankeshwara, N. Krstajić, C. Haslett, K. Dhaliwal, M. Bradley, *Org. Biomol. Chem.* **2018**, *16*, 8056-8063.
[2] N. Krstajić, B. Mills, I. Murray, A. Marshall, D. Norberg, T. H. Craven, P. Emanuel, T. R. Choudhary, G. O. S. Williams, E. Scholefield, A. R. Akram, A. Davie, N. Hirani, A. Bruce, A. Moore, M. Bradley, K. Dhaliwal, *J. Biomed. Opt.* **2018**, *23*, 076005.
[3] M. Gude, J. Ryf, P. D. White, *Int. J. Pept. Res. Ther.* **2002**, *9*, 203-206.
[4] J. J. Diaz-Mochon, L. Bialy, M. Bradley, *Org. Lett.* **2004**, *6*, 1127-1129.
[5] a) A. Leo, C. Hansch, D.Elkins. *Chem. Rev.* Partition Coefficients and their uses. **1971**, *71*, 525–616. b) OECD Guideline for the Testing of Chemicals. Partition Coefficient (n-octanol/water): Shake Flask Method. 1995. https://www.oecd.org/chemicalsafety/risk-assessment/1948169.pdf