Supporting Information

Synthesis and Assessment of a Maleimide Functionalized BF_2 Azadipyrromethene Near-Infrared Fluorochrome

Dan Wu,[a] Shane Cheung,[a] Marc Devocelle,[a] Lijun Zhang,[b] Zhi-Long Chen,[b]

Donal F. O'Shea*[a,c]†

[a] Department of Pharmaceutical and Medicinal Chemistry Royal College of Surgeons in Ireland,
123 St. Stephen's Green, Dublin 2, Ireland
E-mail: donalfoshea@rcsi.ie

[b] Department of Pharmaceutical Science & Technology, College of Chemistry and Biology, Donghua University, Shanghai 201620, China

[c] School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

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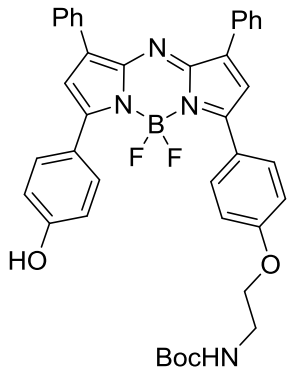
Experimental

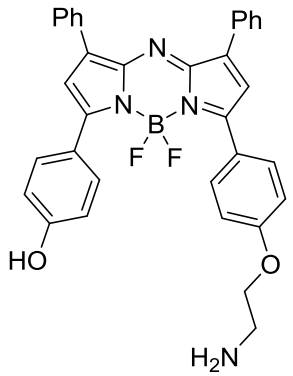
General Methods. All reactions involving air-sensitive reagents were performed under nitrogen in oven-dried glassware using syringe-septum cap technique. All solvents were purified and degassed before use. Chromatographic separation was carried out under pressure on Merck silica gel 60 using flash-column techniques. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm silica gel coated aluminum plates (60 Merck F_{254}) using UV light (254 nm) as visualizing agent. Unless it is specified, all reagents were used as received without further purifications. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature at 400 MHz or 500 MHz and 100 MHz or 125 MHz respectively, and calibrated using residual non-deuterated solvent as an internal reference. MALDI-TOF MS analysis used an α-cyano-4-hydroxy-cinnamic acid matrix. The protected thiol substituted peptide *cyclic*[Arg-Gly-Asp-D-Phe-Lys(Ac-SCH_2CO)] was purchased and used as supplied.

Synthesis of fluorophore 5.



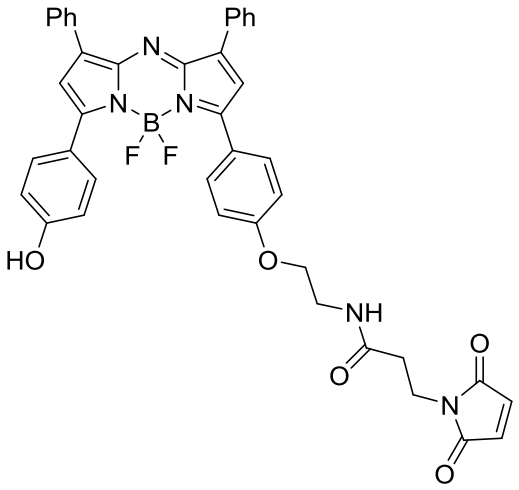
A solution of **3b** (400 mg, 0.76 mmol) in dry THF (40 mL) was treated with Ph_3P (198 mg, 0.76 mmol) and Boc-ethanolamine (184 mg, 1.14 mmol) and stirred at 0 °C under N_2 for 15 minutes. DIAD (154 mg, 0.76 mmol) was prepared in dry THF (5 mL), then was dropwise into the mixture and the solution was allowed warm to rt and stirring continued for 6 hours. The mixture was partitioned between EtOAc (30 mL) and 0.1 M HCl solution (3 × 30 mL), the organic phase washed with H_2O (3 × 30 mL), brine (30 mL), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. Crude product was purified by alumina column chromatography eluting with CH_2Cl_2 (100%) to remove bis-substituted compound. Further purification with silica gel chromatography eluting with CH_2Cl_2:ethyl acetate 90:10 gave the product as the dark blue solid **5** (204 mg, 40 %), m.p. 158-159 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.04-7.99 (m, 8H), 7.43-7.39 (m, 6H), 7.00 (s, 1H), 6.98 (s, 1H), 6.94 (d, *J* = 8.8 Hz, 2H), 6.94 (d, *J* = 9.0 Hz, 2H), 5.03 (br, s, 1H), 4.08 (t, *J* = 5.0 Hz, 2H), 3.63-3.50 (m, 2H), 1.47 (s, 9H) ppm. (OH not observed) ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.7, 158.7, 158.5, 157.7, 156.1, 145.4, 145.2, 143.3, 143.0, 132.4, 132.4, 131.9, 131.5, 129.3, 129.2, 129.2, 128.5, 128.5, 128.5, 124.5, 123.9, 118.7, 118.5, 115.9, 114.6, 79.9, 67.2, 40.0, 28.4 ppm. λ_{max} abs ($CHCl_3$): 688 nm, λ_{max} em ($CHCl_3$): 716 nm. ES-HRMS $[M-H]^-$: 671.2631, $C_{39}H_{34}N_4O_4BF_2$ requires 671.2641.

Synthesis of fluorophore 6.



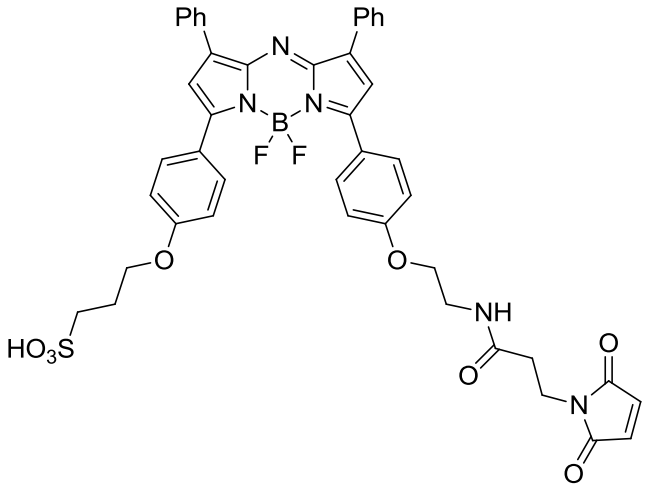
Compound **5** (100 mg, 0.15 mmol) was dissolved in dry CH_2Cl_2 (20 mL), boron trifluoride diethyl etherate (0.19 mL, 1.5 mmol) was slowly added into solution at 0 °C. Then reaction was warmed to r.t. and stirred under N_2 for 2 hours. The resulting precipitate was filtered and washed thoroughly with CH_2Cl_2 until the filtrate became colourless. The precipitate was dissolved in ethyl acetate (25 mL), washed with 2M HCl (2 x 10 mL), saturated aq. sodium bicarbonate (10 mL), dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to give the product as a dark solid (75 mg, 90%), m.p. 140-142 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.18-8.08 (m, 8H), 8.04-7.91 (brs, 2H), 7.58-7.39 (m, 8H), 7.17 (d, J = 8.9 Hz, 2H), 6.94 (d, J = 8.9 Hz, 2H), 4.30 (t, J =5.0 Hz, 2H), 3.29-3.24 (m, 2H) ppm. (OH not observed) ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.9, 160.5, 159.5, 156.0, 145.5, 144.2, 143.2, 141.5, 141.5, 132.9, 132.5, 132.1, 131.9, 130.2, 129.8, 129.6, 129.4, 129.2, 129.2, 124.7, 121.8, 119.4, 116.4, 115.4, 65.2, 38.8 ppm. λ_{max} abs (MeOH): 690 nm, λ_{max} em (MeOH): 718 nm. ES-HRMS $[M-H]^-$: 571.2121, $C_{34}H_{26}N_4O_2BF_2$ requires 571.2117.

Synthesis of fluorophore 8.



A solution of **6** (70 mg, 0.12 mmol) in dry THF (8 mL) was treated with *N*-succinimidyl 3-maleimidopropionate (47 mg, 0.18 mmol) and TEA (0.02 mL, 0.12 mmol), and stirred at rt under N_2 for 1 hour. The solvent was removed under vacuum, the residue was partitioned between EtOAc (30 mL) and 0.1 M HCl solution (30 mL), the organic phase was washed twice with the H_2O (2 × 30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. Purification by column chromatography, packed and loaded with CH_2Cl_2: ethyl acetate 70:30 and eluted with CH_2Cl_2:ethyl acetate 50:50 gave the product as a red metallic solid **8** (53 mg, 61%), m.p. 197-198 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.25 (br.s, 1H), 8.17-8.06 (m, 8H), 7.62 (s, 1H), 7.59-7.35 (m, 7H), 7.10 (d, *J* = 7.6 Hz, 2H), 6.93 (s, 2H), 6.91 (d, *J* = 7.6 Hz, 2H), 4.07 (t, *J* = 5.4 Hz, 2H), 3.61 (t, *J* = 7.2 Hz, 2H), 3.44-3.38 (m, 2H), 2.36 (t, *J* = 7.2 Hz, 2H) ppm. (tiny ethyl acetate contained) ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.2, 170.3, 162.2, 161.2, 158.9, 156.2, 145.4, 144.3, 142.8, 141.3, 134.9, 132.9, 132.5, 132.2, 131.9, 130.0, 129.7, 129.6, 129.4, 129.1, 124.1, 121.7, 120.6, 119.5, 118.4, 116.6, 115.3, 67.3, 38.5, 34.5, 34.5 ppm. λ_{max} abs (MeOH): 691 nm, λ_{max} em (MeOH): 720 nm. ES-HRMS $[M-H]^-$: 722.2390, $C_{41}H_{31}N_5O_5BF_2$ requires 722.2386.

Synthesis of fluorochrome 4.



A solution of **8** (50 mg, 0.07 mmol) in dry acetone (10 mL) was treated with 1,3-propane sultone (21 mg, 0.18 mmol) and potassium carbonate (9.6 mg, 0.07 mmol) and the mixture stirred at reflux for 6 hours. The reaction mixture was cooled to rt, precipitate filtered and washed with dry acetone (3 × 25 mL). Precipitate was further purified by Sep Pak C18 reverse phase cartridge eluting with ACN:H_2O (3:7) to give the product as a dark green solid **4** (41 mg, 70%), m.p. 256-259 °C. For NMR and HRMS analysis, the compound **4** was transformed into its sulfonic acid. ^{1}H NMR (400 MHz, CD_3OD-*d*$_4$): δ 8.20-8.08 (m, 8H), 7.52-7.37 (m, 6H), 7.32 (s, 1H), 7.28 (s, 1H), 7.12-7.00 (m, 4H), 6.61 (s, 2H), 4.32-4.17 (m, 2H), 4.17-4.04 (m, 2H), 3.85-3.70 (m, 2H), 3.64-3.43 (m, 2H), 3.11-2.92 (m, 2H), 2.55-2.39 (m, 2H), 2.40-2.20 (m, 2H) ppm, (NH not observed). λ_{max} abs (MeOH): 691 nm, λ_{max} em (MeOH): 716 nm. ES-HRMS $[M-H]^-$: 844.2401, $C_{44}H_{37}N_5O_8BF_2S$ requires 844.2424.

Synthesis of cell penetrating peptide.

Assembly of the amino acid sequence, starting from a Novabiochem® Rink Amide MBHA 100-200 mesh resin, was carried out on an automated peptide synthesizer (Applied Biosystems 433A, Warrington, UK) from Valine to Serine, according to the Fmoc-*t*Bu strategy with HATU/DIEA coupling chemistry, in NMP. Single coupling cycles, using a total 10-fold excess of Fmoc amino acid derivatives to resin-bound peptide were used. The resin was then taken from the synthesizer reactor and placed in a disposable syringe type reactor equipped with a PE frit. The addition of the β-alanine and cysteine amino acids was then carried out by manual coupling. Manual coupling was carried out with a 5-fold excess of Fmoc-amino acids, using HATU/DIEA coupling chemistry, in DMF. The reaction was monitored by the qualitative Kaiser test and a double coupling procedure was applied if the reaction did not go to completion. The peptide was deprotected and cleaved from the resin using a 5 mL cleavage cocktail made by mixing 81.5% TFA, 5% H_2O), 10% thioanisole, 2.5% 1,2-ethanedithiol and 1% triisopropylsilane. The cleavage reaction was performed for three hours at room. The peptide was precipitated and washed three times with 10 mL portions of diethyl ether. It was then dried, dissolved in distilled water and lyophilized.

The peptide was purified by reverse phase HPLC using Perseptive Biosystems BioCAD Sprint and a Gemini column (Phenomenex, 110 Å, 5 μm , C18, 100 mmd/250 mmL) semi-preparative column and analyzed by reverse phase HPLC using a Varian Prostar equipped with a reverse phase Gemini (Phenomenex, 110 Å, 5μm , C18, 4.6 mmd/250 mmL) analytical column. Buffers used were mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in acetonitrile with a gradient of 1 to 10% B initially over 20 minutes followed by 10 to 65% B over 40 minutes at a flow rate of 5 mL/min for the semi preparative column or a gradient of 3 to 97% B over 50 minutes with a flow rate of 1ml/min for the analytical column. UV detection at 214 nm was used for the Biocad Sprint, while the Varian Galaxy was equipped with a Diode Array Detector (PDA) operating from 190 nm to 950

nm.. Purified peptide was characterized by MS MALDI-TOF (M+H): 1119.5094, $C_{48}H_{95}N_{16}O_{12}S$ requires 1119.7036.

Preparation of cysteine conjugates 9a.

Cysteine (2 mg, 16.5 µmol, 1.5 equiv.) was dissolved in PBS solution (2.0 mL, pH 6.5) in a 10 mL glass vial equipped with a magnetic stir bar. The solution was purged with nitrogen for 30 min. A solution of **4** (10 mg, 11 µmol, 1 equiv.) in nitrogen-purged PBS (2.0 mL) was added dropwise, and the solution was stirred at rt for 2 hours. The reaction mixture was purified through a Sephadex G-25 column eluting with Milli-Q water. The pure fractions were combined and water removed by freeze drying to give conjugates **9a** (95%). MS ESI (M-H): 965.0018, $C_{47}H_{44}BF_2N_6O_{10}S_2$ requires 965.2621. ^{1}H NMR (400 MHz, CD_3OD-*d*$_4$): δ 8.17 - 8.06 (m, 8H), 7.48-7.35 (m, 6H), 7.29 (s, 1H), 7.27 (s, 1H), 7.09 - 7.00 (m, 4H), 4.53 (s, 2H), 4.27 - 4.17 (m, 2H), 4.17 - 4.07 (m, 2H), 3.94 - 3.81 (m, 1H), 3.80 - 3.66 (m, 2H), 3.62 - 3.51 (m, 2H), 3.51 - 3.46 (m, 1H), 3.46 - 3.33 (m, 2H), 3.16 - 3.06 (m, 1H), 3.06 - 2.91 (m, 2H), 2.54 - 2.39 (m, 2H), 2.32 - 2.16 (m, 2H), (OH not observed) ppm.

Preparation of glutathione conjugates 9b.

Glutathione (5 mg, 16.5 µmol, 1.5 equiv.) was dissolved in PBS solution (pH 6.5) in a 20 mL glass vial equipped with a magnetic stir bar. The solution was purged with nitrogen for 30 min. A solution of **4** (10 mg, 11 µmol, 1 equiv.) in nitrogen-purged PBS (2.0 mL) was added dropwise, and the solution was stirred at rt for 2 hours. The reaction mixture was purified through a Sephadex G-25 column eluting with Milli-Q water. The pure fractions were combined and water removed by freeze drying to give conjugate **9b** (92%). MS ESI (M-H): 1150.80, $C_{54}H_{54}BF_2N_8O_{14}S_2$ requires 1151.3262.

Preparation of cell penetrating peptide conjugate 9c.

Cell penetrating peptide C(β-A)SKKKKTKV-NH_2. (3.8 mg, 3.4 µmol, 1.5 equiv.) was dissolved in nitrogen-purged PBS solution (0.5 mL, pH 7.2) in a 5 mL glass vial equipped with a magnetic stir bar. A solution of **4** (2 mg, 2.3 µmol, 1 equiv.) in nitrogen-purged PBS (0.5 mL) solution was added slowly to the peptide solution, and the solution was stirred at rt for 2 hours. Purification was completed by Sephadex G-25 chromatography eluting with PBS solution to yield the conjugate **9c**. MS MALDI-TOF (M+H): 1965.2238, $C_{92}H_{133}BF_2N_{21}O_{20}S_2$ requires 1964.9538.

Preparation of cyclic RGD peptide conjugates 9d.

The thiol substituted cRGD containing peptide *cyclic* [Arg-Gly-Asp-d-Phe-Lys(Ac-SCH_2CO)] was protected by acetyl group which can be *in situ* deacetylated to generate free a thiol group peptide by including using 100 mM hydroxylamine HCl in the coupling reaction. The fluorochrome **4** (10 mg, 11 µmol, 1 equiv.) PBS solution was then added slowly to the peptide solution. The coupling was performed in PBS (pH 6.5) for 2 hours followed by Sephadex G-25 column to purify the product. MS MALDI-TOF (M-H): 1521.5619, $C_{73}H_{80}BF_2N_{14}O_{16}S_2$ requires 1521.5379.

Cell Culture and Imaging

10,000 HeLa Kyoto cells were seeded on to an eight well chamber slide, and incubated overnight in Dulbecco's modified eagles media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), and 1% L-Glutamine). Cells were then incubated for two or sixteen hours with 5µM (dissolved in DMEM media) of the conjugate **9c** Cells were fixed with 3% para-formaldehyde (PFA). The nucleus was stained with 1:5000 Hoechst 33342.

Imaging was performed on a Zeiss AxioVert 200M epi-fluorescent widefield microscope equipped with a CoolLED pE-2 solid state diode containing LEDs capable of excitation at 445nm, 488nm and 635nm. The objective was a Zeiss Plan- Apochromat 100x/1.46 Oil DIC. The camera was an Andor iXon 885 EMCCD controlled by Axiovision 2.0. Camera settings for the TFIIE- β fluorophore

conjugate fluorescence was 200ms exposure and 5X EM gain. Camera settings for the Hoechst 33342 fluorescence was 100ms exposure and 5X EM gain. 52 optical sections were made over 11.475 μm through the z-axis of the cell. Z-stacks were processed using AutoQuant deconvolution software.

***In vivo* fluorescence imaging studies.**

All *in vivo* experiments were conducted in Fudan University, Shanghai, China in compliance with relevant laws, and their institutional guidelines for animal protocols and the guidelines issued by the Ethical Committee of Fudan University which approved the experiments.

Mouse model were established by subcutaneous injection of ~ 10^7 human esophageal cancer (Eca-109) cells in 200 uL PBS into the right forelimb of five-week-old male Balb C nu/nu mice. When implanted tumor sizes were more than 10 mm in diameter, tumors were excised and small pieces of the tumor (approximately 2 mm square pieces) were implanted subcutaneously into the right forelimb of Balb C nu/nu mice (5 weeks old). When the tumors reached a size of 10-15 mm in diameter after implantation (14-21 days), the mice were used for tumor targeting studies. This method was found to more effective at successfully growing tumor than injection of Eca-109 cells.

Tumor targeting with cRGD Conjugate 9d

For tumor targeting studies, conjugate **9d** (1 mg/kg, dissolved in PBS) was intravenous tail injected in nude mice bearing Eca-109 tumors. The fluorescence images of the whole mice were obtained using the *in Vivo* Imaging System (IVIS Spectrum CT, PerkinElmer) with an NIR emission filter (680-720 nm) at 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h and 48 h after injection.

Fig. S1. HPLC monitored cysteine conjugation reaction.

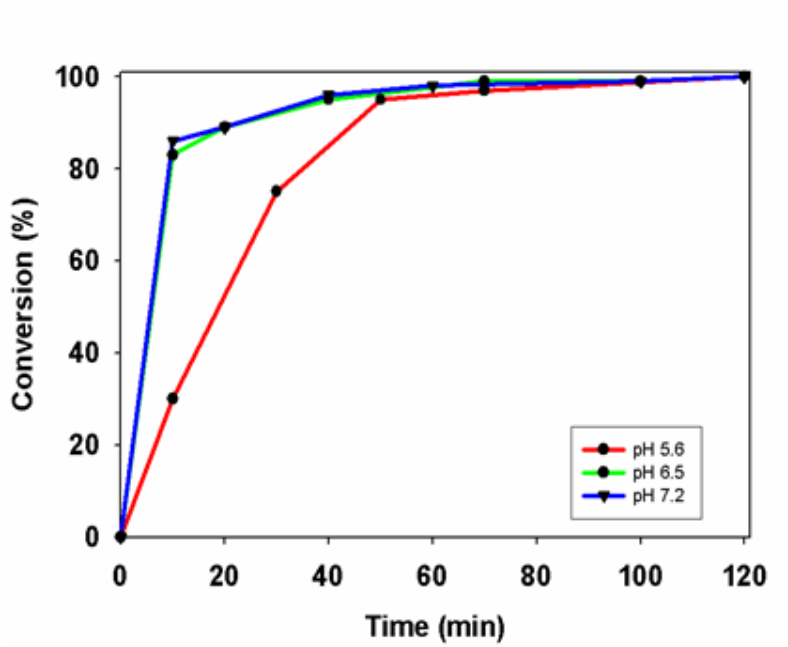
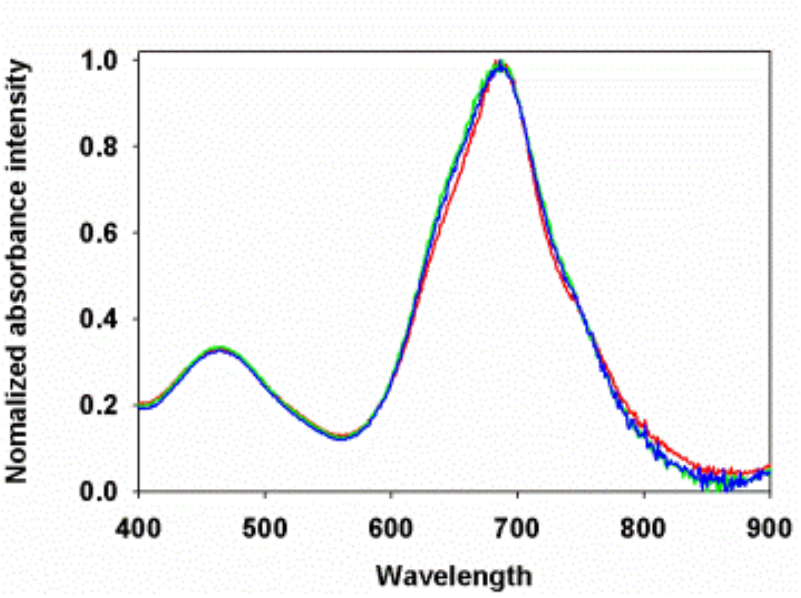
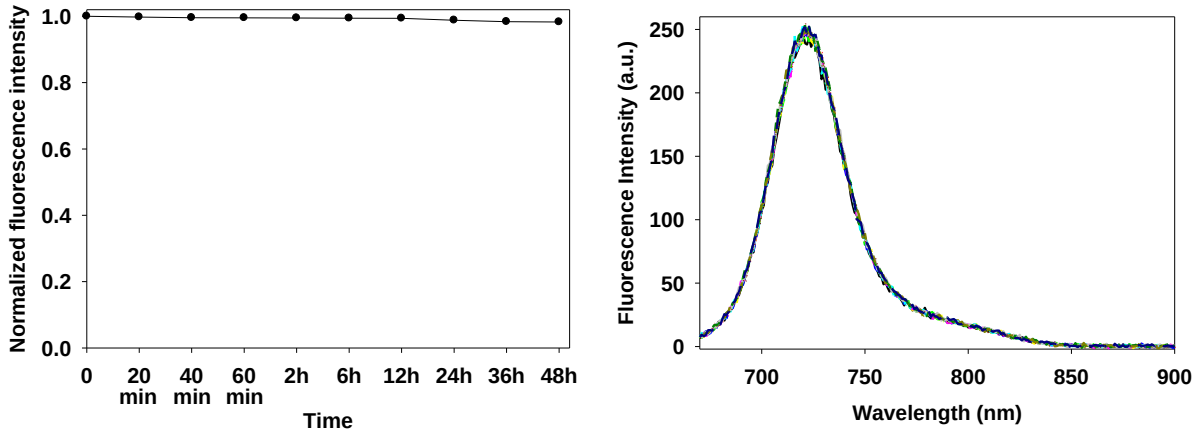


Fig. S2 UV-Visible Spectra of 4, 9a, 9b.[a]



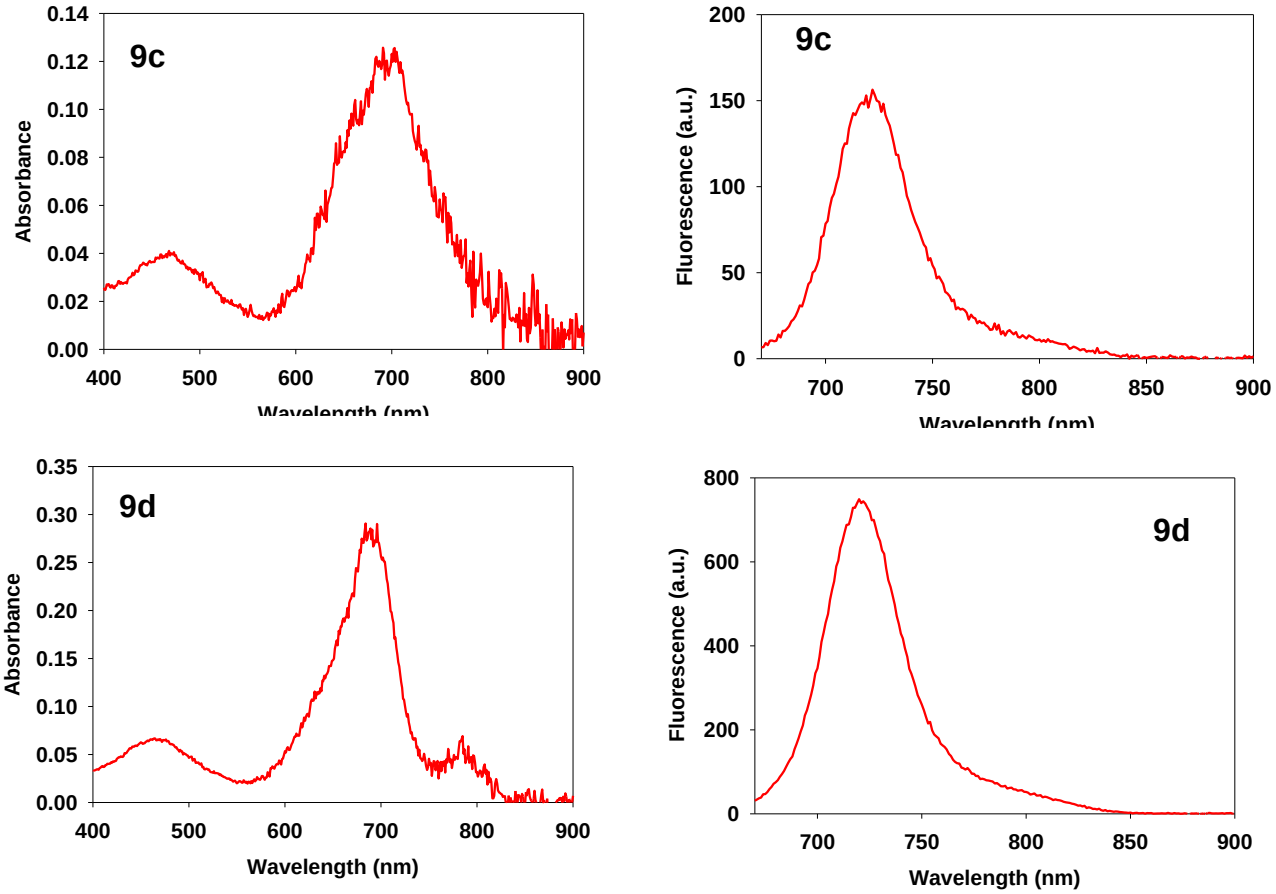
[a] Normalized spectra in MeOH/H_2O (1:1), conc =1×10^{-5} M. Red traces **4**. Green traces **9a**. Blue traces **9b**.

Fig. S2: Stability of 9c in DMEM



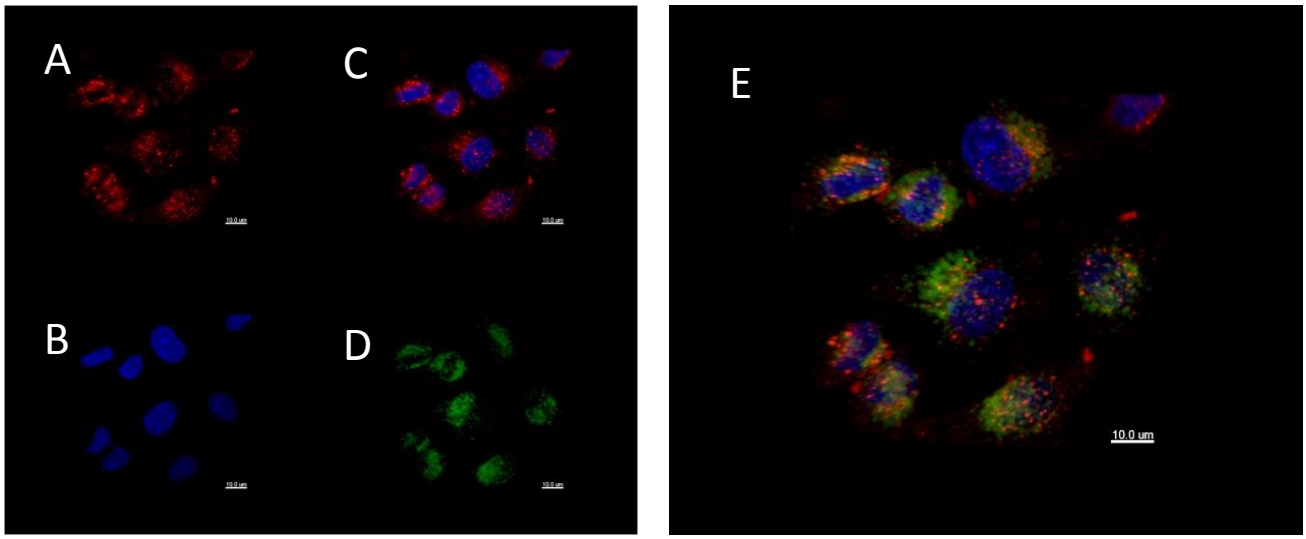
A solution of **9c** ($3x10^{-6}$ M) in DMEM with 0.5%SDS was stored on an open bench at rt for 48 h with emission spectra recorded at a series of time points.

Fig. S3: Absorption and Emission Spectra of 9c, 9d in Aqueous Buffer



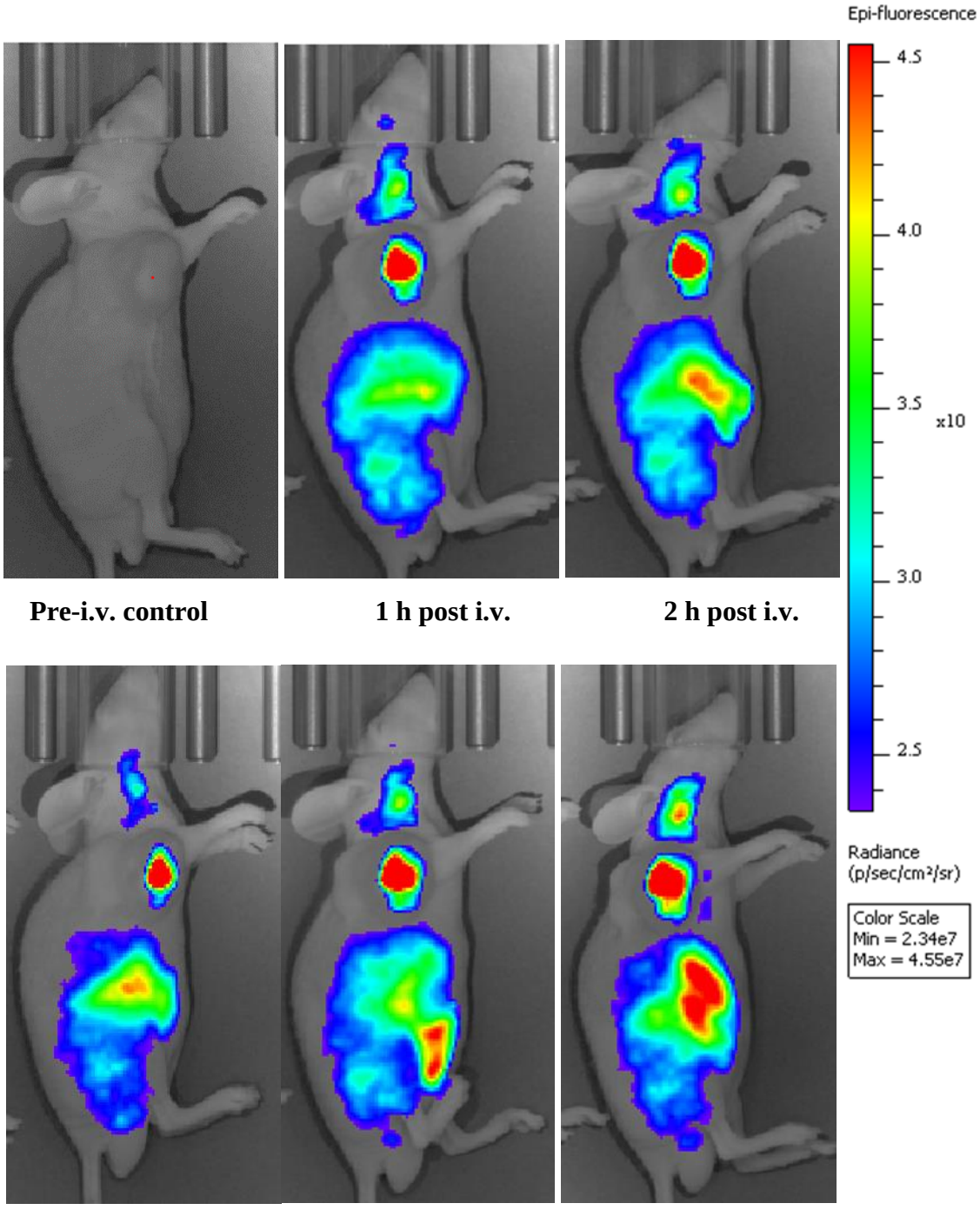
Spectra of **9c** ($2.3x10^{-6}$ M) and **9d** ($4.4x10^{-6}$ M) taken in water 0.5% SDS buffer at rt.

Fig. S4: Imaging of 9c with LAMP-1 GFP expressing HeLa cells showing partial Localization with Lysosomes



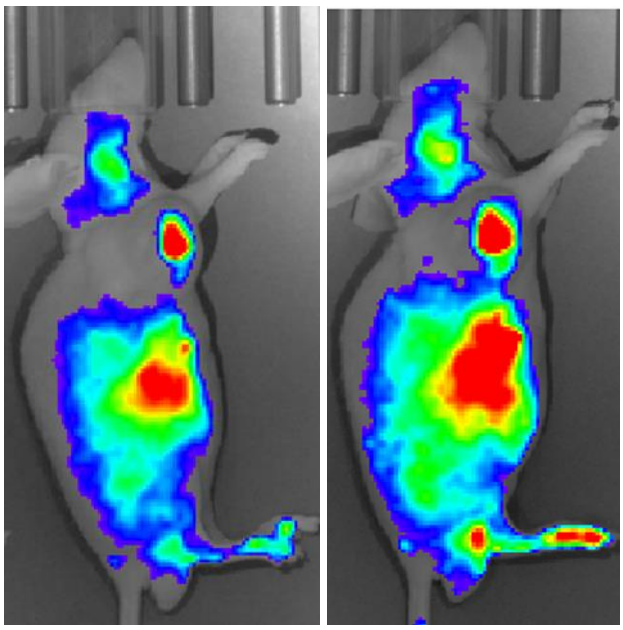
Cells imaging following 2 h incubation with **9c** (5µM). (A) Cy5 channel showing **9c**; (B) Hoechst 33342 blue nucleus stain; (C) overlay of A and B; (D) GFP channel; (E) overlay of A, B and C. Scale bars: 10 µm.

Figure S5. ***In vivo*** **imaging time course of 9d**

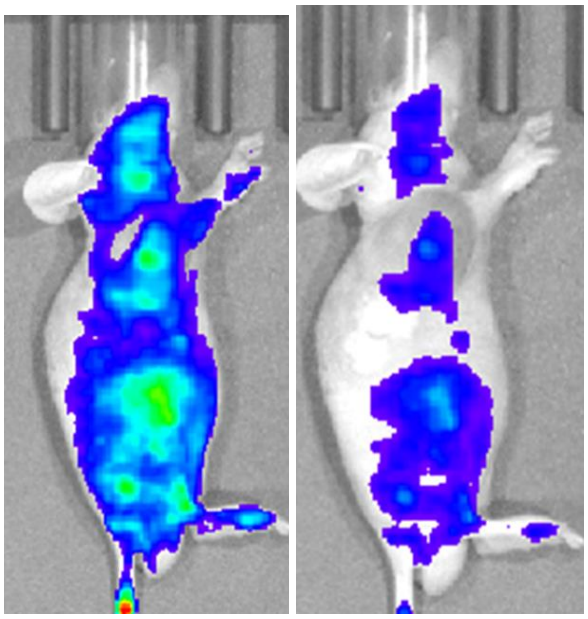


Pre-i.v. control **1 h post i.v.** **2 h post i.v.**

4 h post i.v. **6 h post i.v.** **8 h post i.v.**

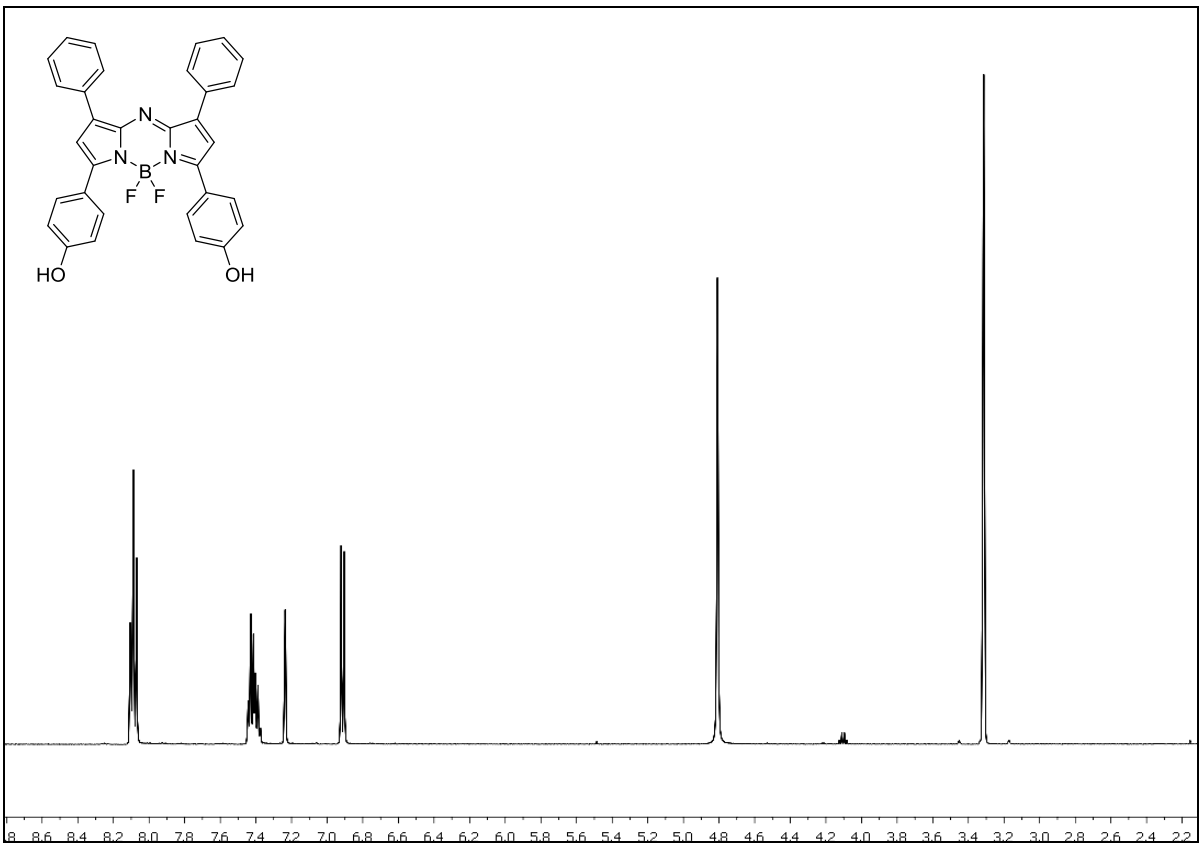


10 h post i.v. **12 h post i.v.**

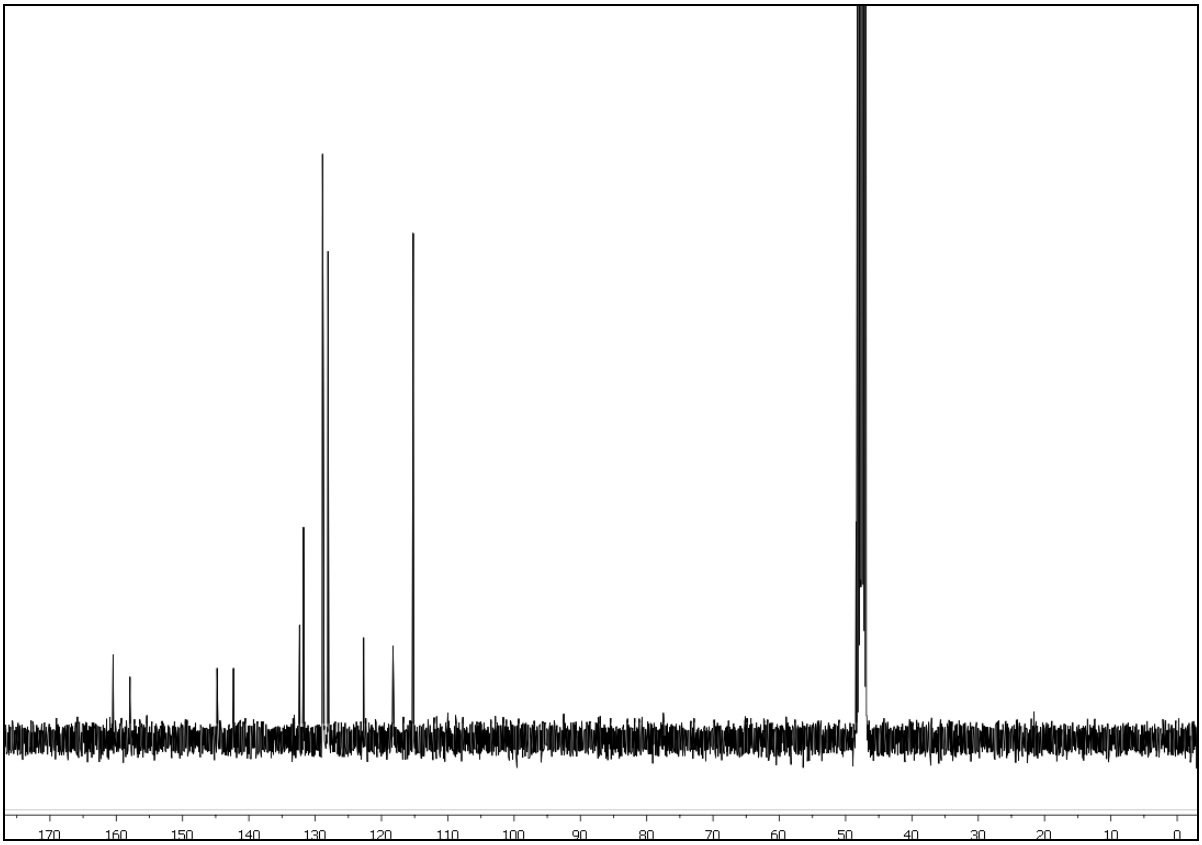


24 h post i.v. **48 h post i.v.**

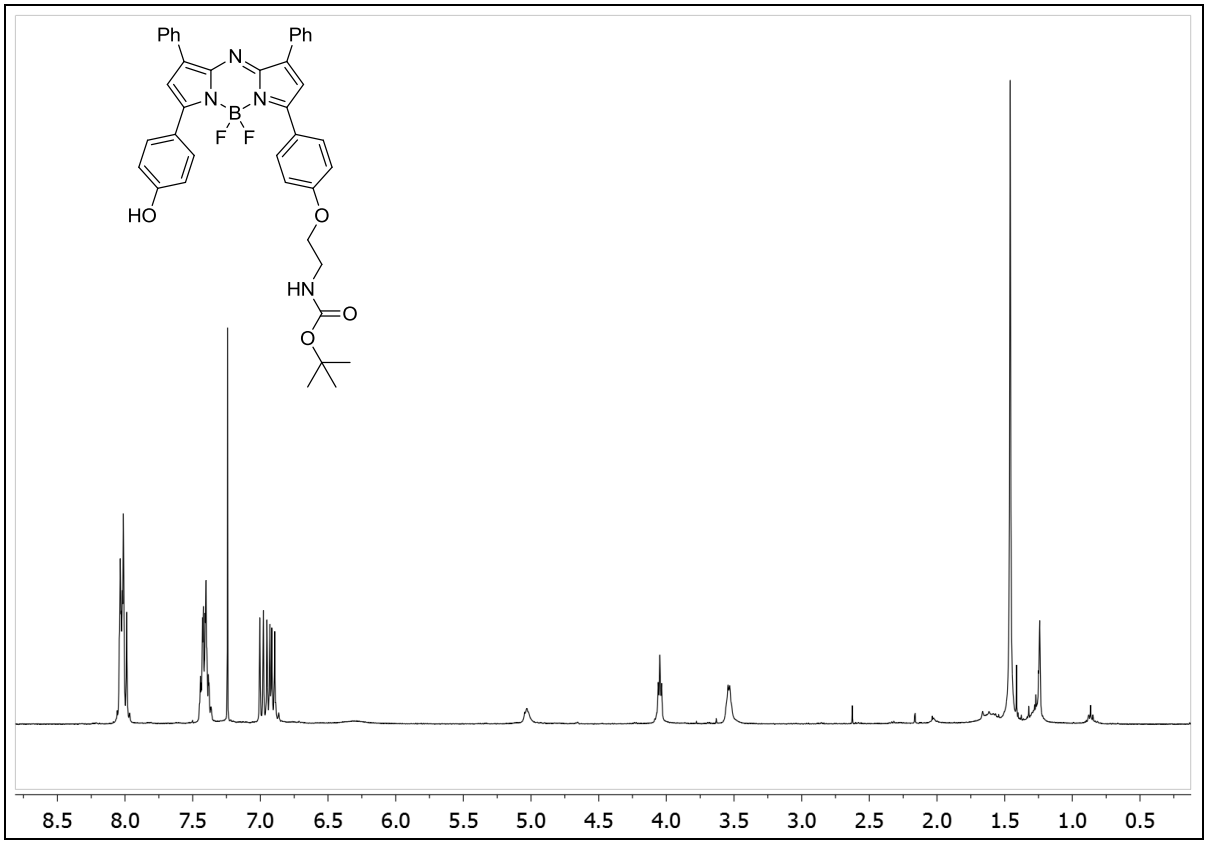
BF_2 chelate of 4-(2-((5-(4-hydroxyphenyl)-3-phenyl-1H-pyrrol-2-yl)imino)-3-phenyl-2H-pyrrol-5-yl)phenol 3b. ^{1}H NMR (500 MHz, CD_3OD-d_4)



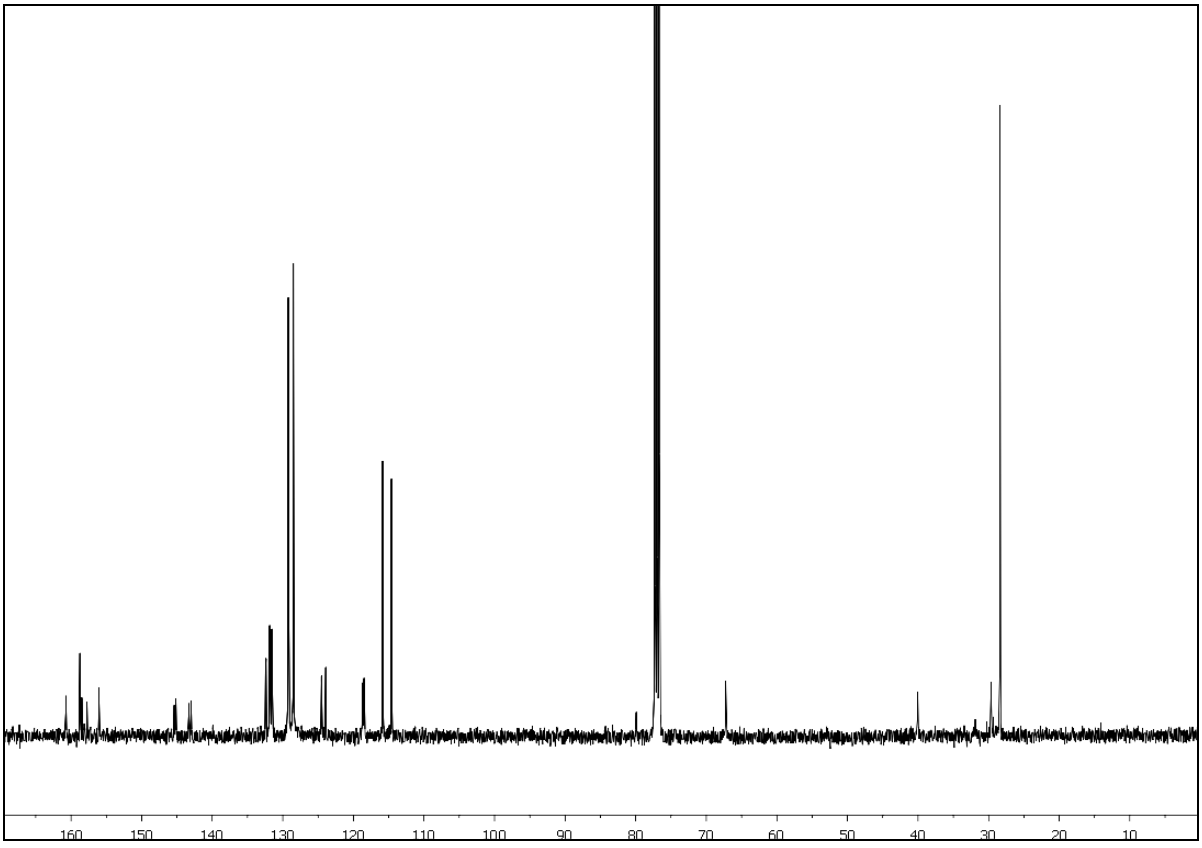
^{13}C NMR (100 MHz, CD_3OD-d_4)



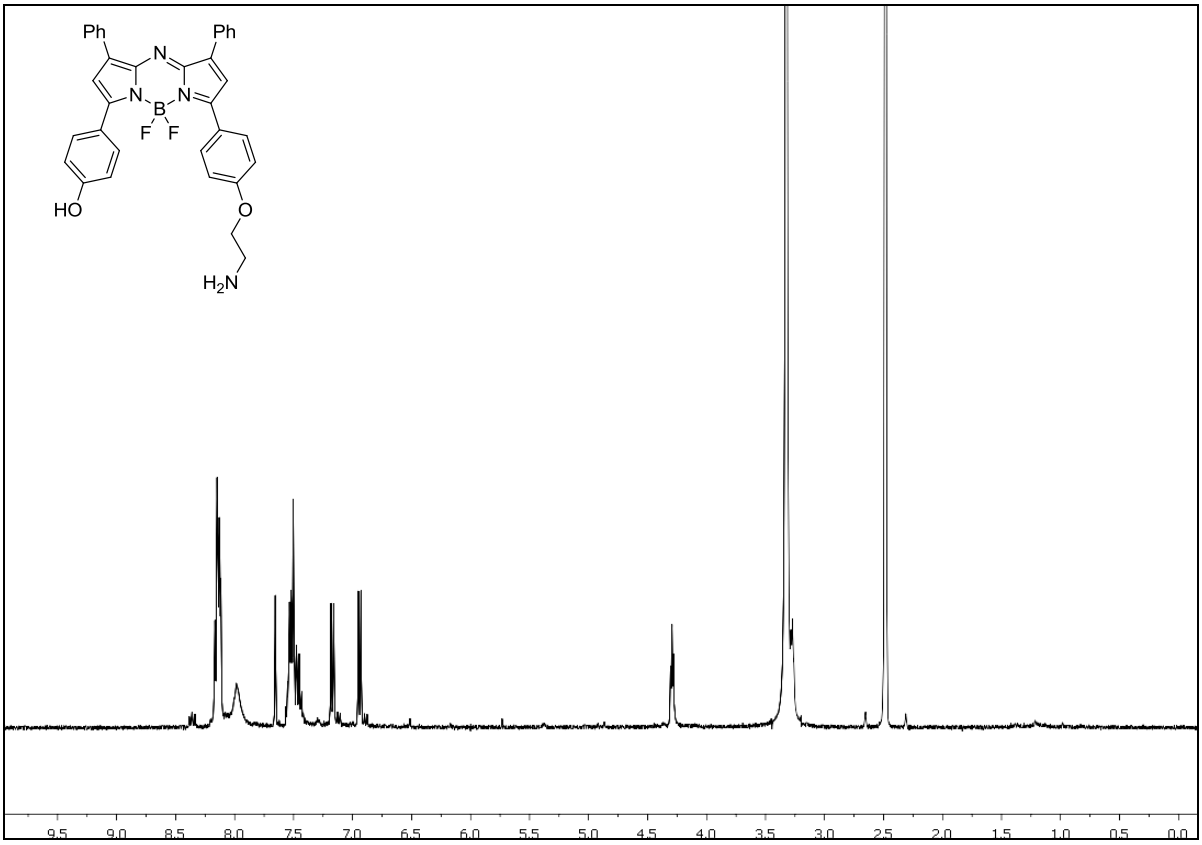
BF_2 chelate of *tert*-butyl 2-(4-(2-(5-(4-hydroxyphenyl)-3-phenyl-1H-pyrrol-2-ylimino)-3-phenyl-2H-pyrrol-5-yl)phenoxy)ethylcarbamate 5. ^{1}H NMR (400 MHz, $CDCl_3$)



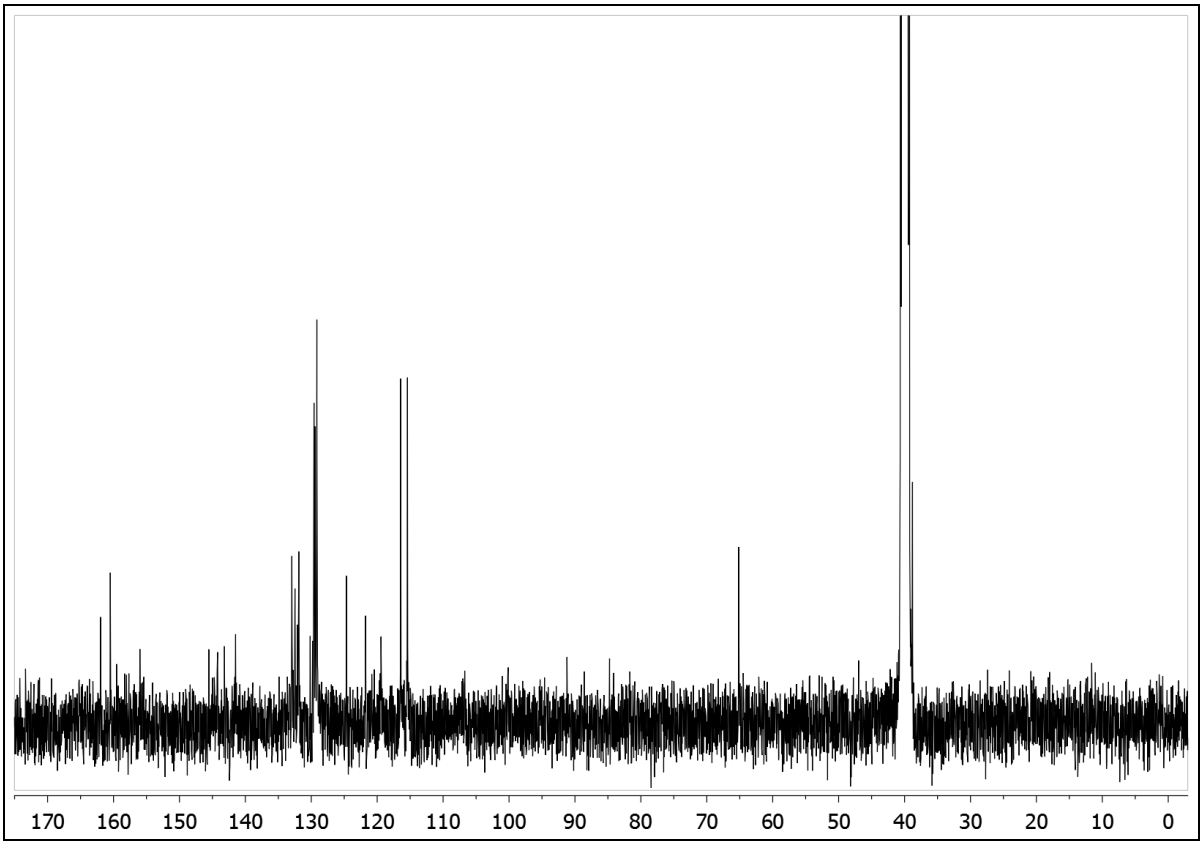
^{13}C NMR (100 MHz, $CDCl_3$)



BF_2 chelate of 4-(5-(5-(4-(2-aminoethoxy)phenyl)-3-phenyl-2H-pyrrol-2-ylideneamino)-4-phenyl-1H-pyrrol-2-yl)phenol 6. ^{1}H NMR (400 MHz, DMSO-d_6)

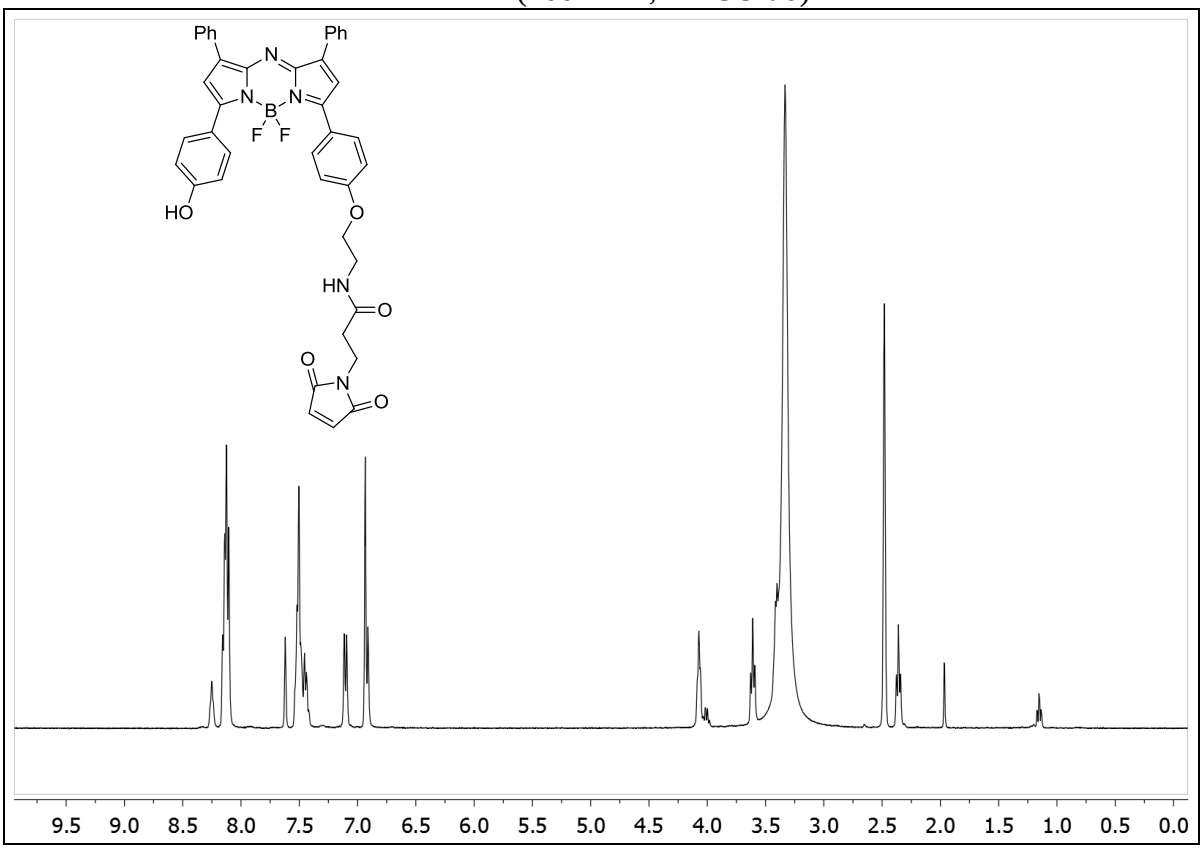


^{13}C NMR (100 MHz, DMSO-d_6)

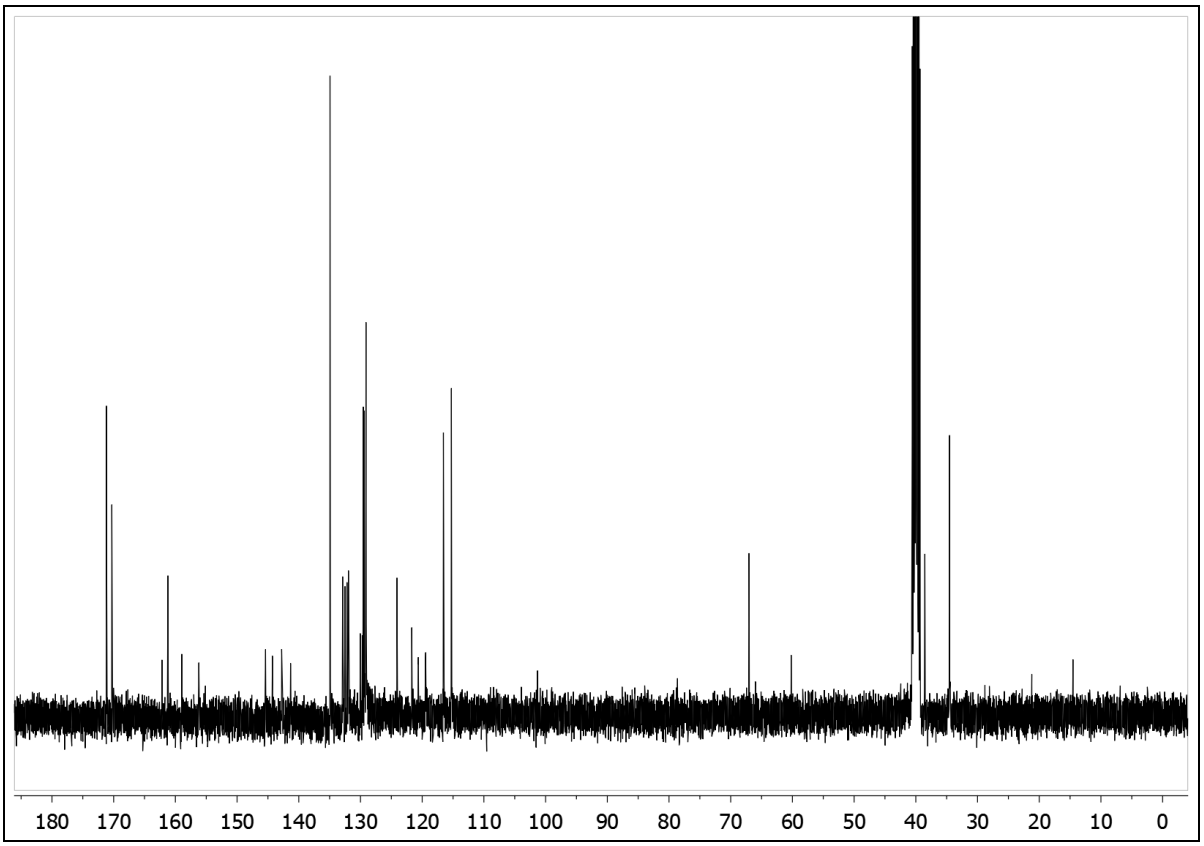


BF_2 chelate of 3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-(4-(2-((5-(4-hydroxyphenyl)-3-phenyl-1H-pyrrol-2-yl)imino)-3-phenyl-2H-pyrrol-5-yl)phenoxy)ethyl)propanamide 7.

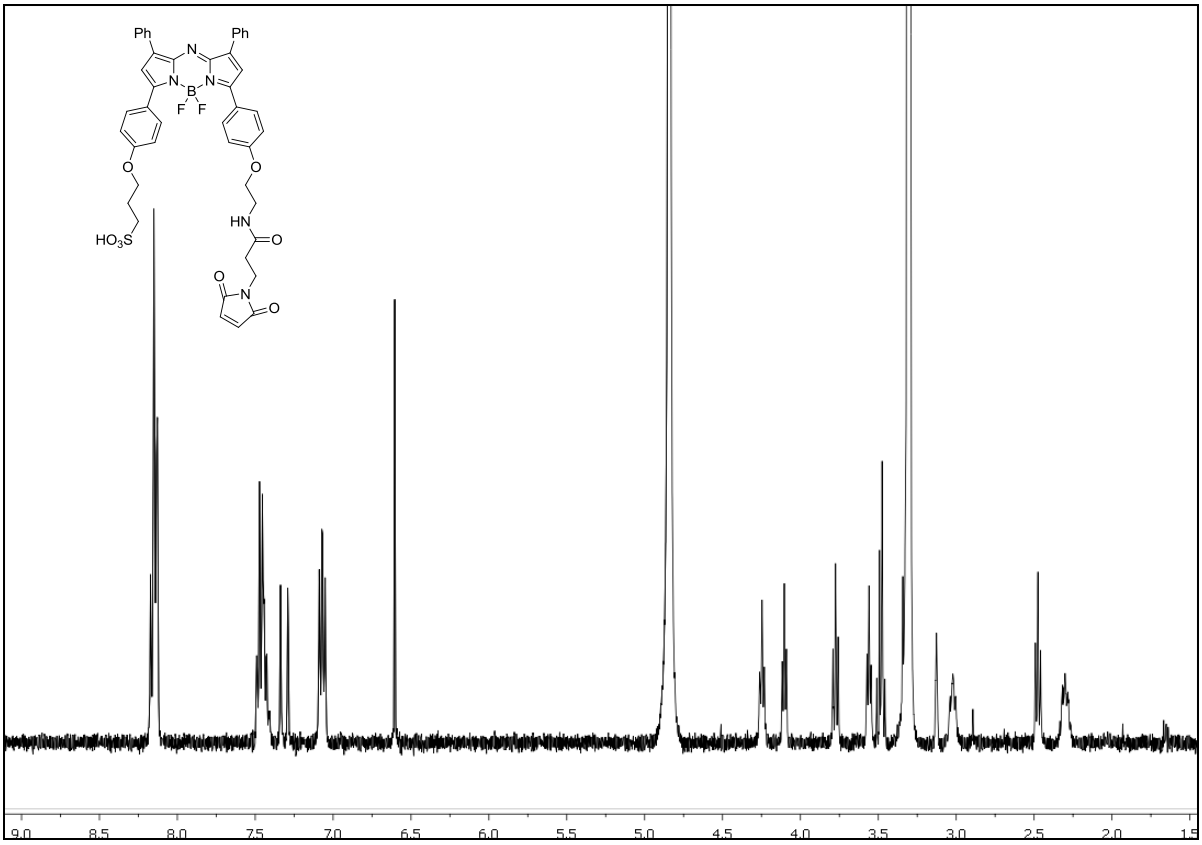
^{1}H NMR (400 MHz, DMSO-*d6*)



^{13}C NMR (100 MHz, DMSO-*d6*)



BF_2 chelate of 3-(4-(5-((5-(4-(2-(3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanamido)ethoxy)phenyl)-3-phenyl-2*H*-pyrrol-2-ylidene)amino)-4-phenyl-1*H*-pyrrol-2-yl)phenoxy)propane-1-sulfonate 4. ^{1}H NMR (400 MHz, CD_3OD-d_4)



Cysteine conjugate 9a

^{1}H NMR (400 MHz, CD_3OD-d_4)