Supporting Information for

Imaging of Tie2 with a fluorescently labeled small molecule affinity ligand

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Supplementary Figures

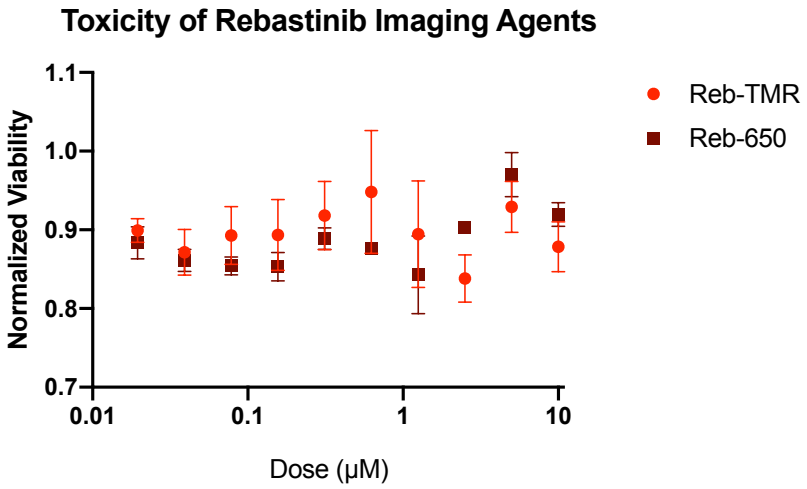


Figure S1: Toxicity of Reb-TMR and Reb-650. HUVEC cells were treated with either Reb-TMR or Reb-650 for 16 hours at the indicated doses. Minimal toxicity was seen. Data plotted as mean +/- s.d., N = 2.

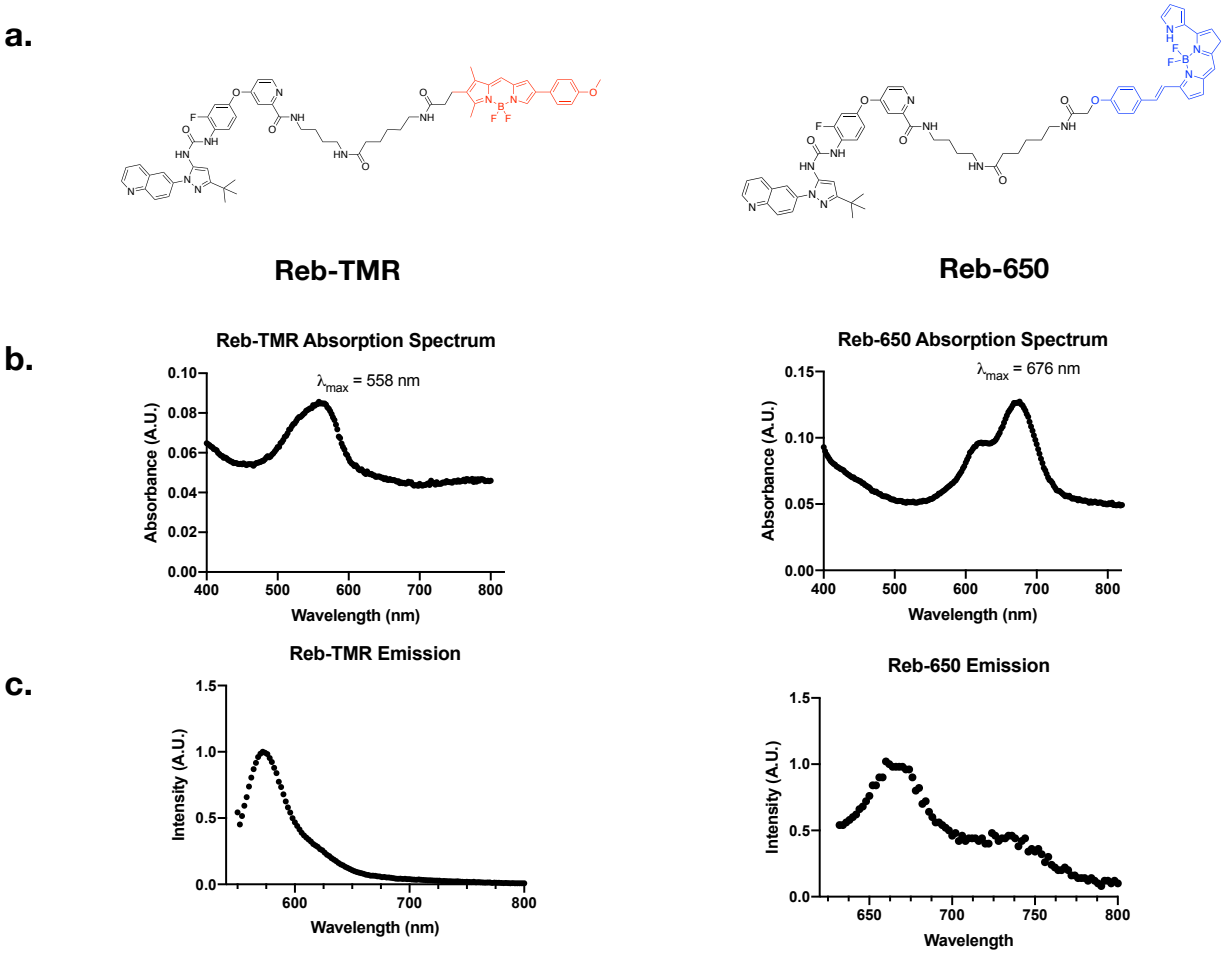


Figure S2: Photophysical properties of Reb-TMR (left) and Reb-650. a: chemical structures of Reb-TMR and Reb-650. b. Absorption spectrum of Reb-TMR and Reb-650. c. Fluorescent intensity emission spectrum of Reb-TMR and Reb-650.

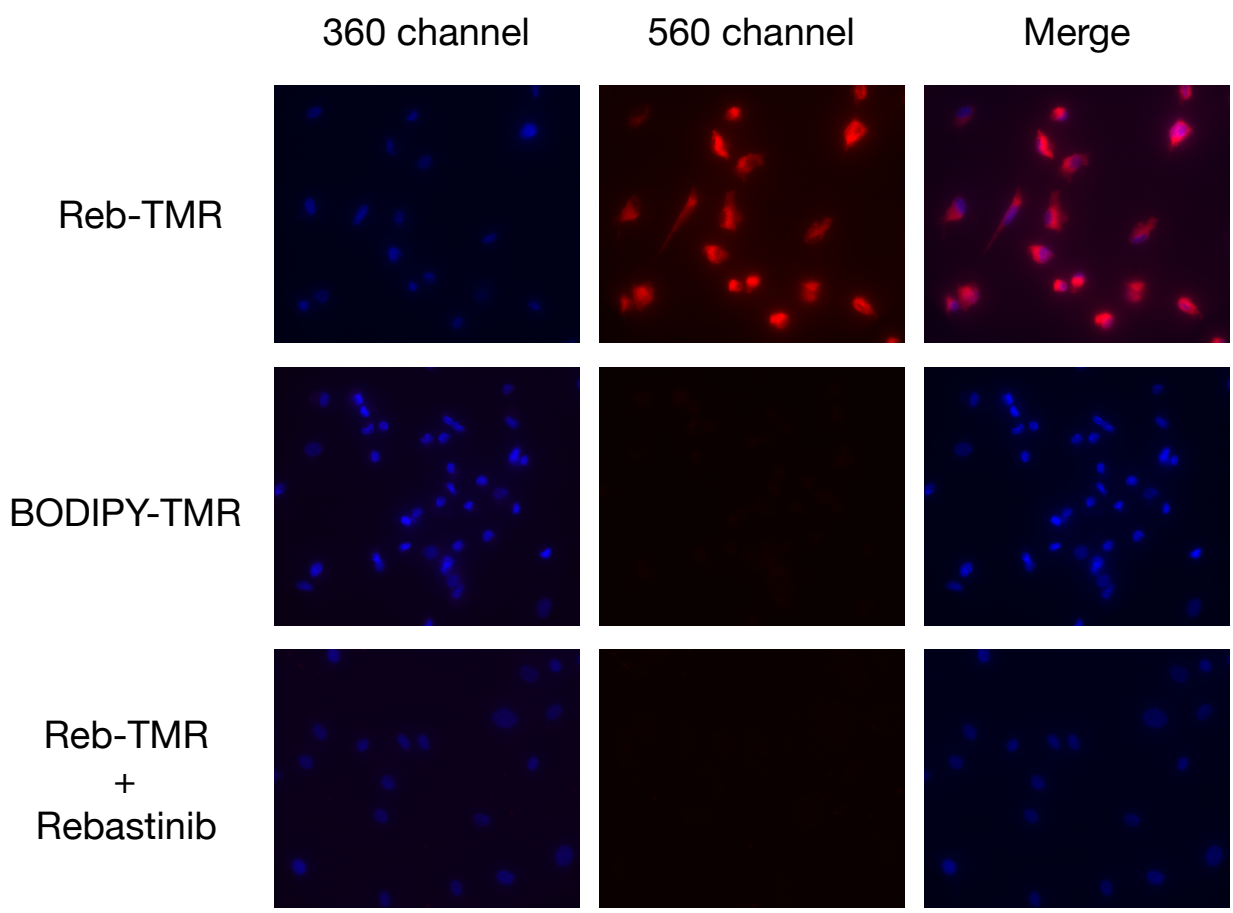


Figure S3: Treatment of HUVEC cells with either 100 nM Reb-TMR (top) or 100 nM free BODIPY-TMR dye (middle). Pre-treatment of cells with 10 μM unlabeled rebastinib eliminates fluorescent Reb-TMR signal (bottom), comparable to free dye control.

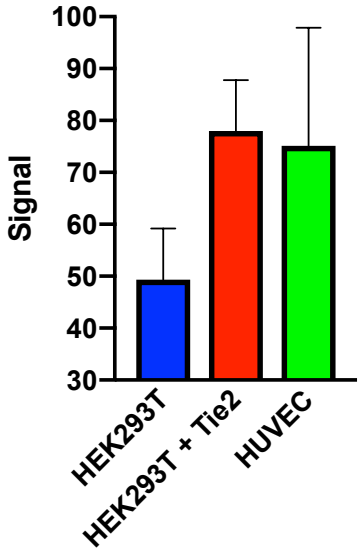


Figure S4: HUVEC (Tie2+), HEK293T (Tie2-) and HEK293T transfected with Tie2 plasmid were treated with increasing doses of Reb-TMR. Average fluorescent intensities per dose were calculated. Plotted is mean +/- S.D., N = 3

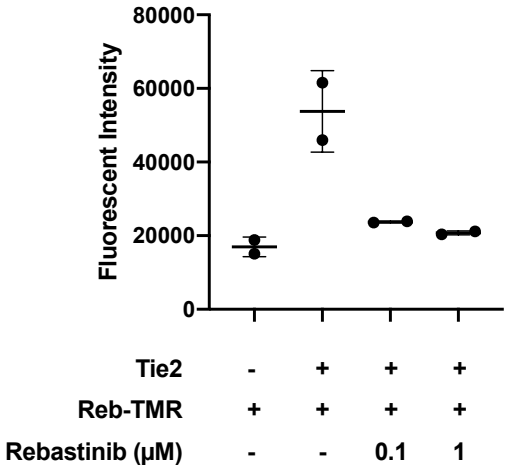


Figure S5: Biochemical fluorescent intensity of Reb-TMR (0.8 uM) in the absence or presence of recombinant Tie2. Leftmost point corresponds to intensity of Reb-TMR in PBS, and right points correspond to intensity of Reb-TMR with Tie2, and in increasing concentrations of unlabeled rebastinib (0,0.1,1,10 uM). Reported is mean over N = 2 wells. Error bars correspond to S.D.

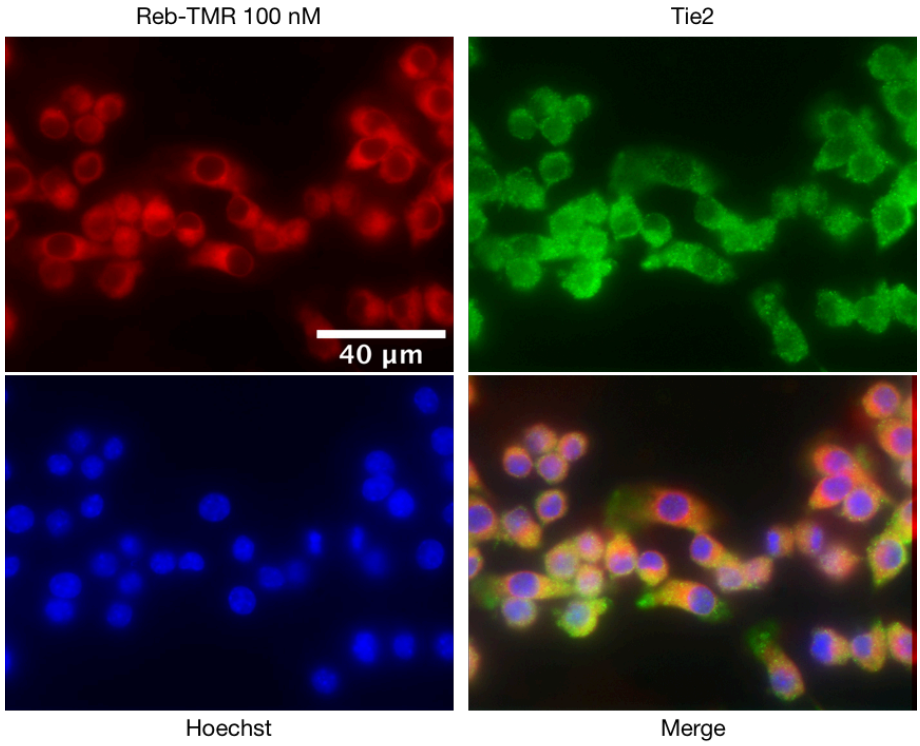


Figure S6: Co-localization in RAW264.7 cells. Staining of Reb-TMR (red) in RAW264.7 macrophages along with co-stain for Tie2 (green). Nuclei are indicated by the Hoechst stain (blue).

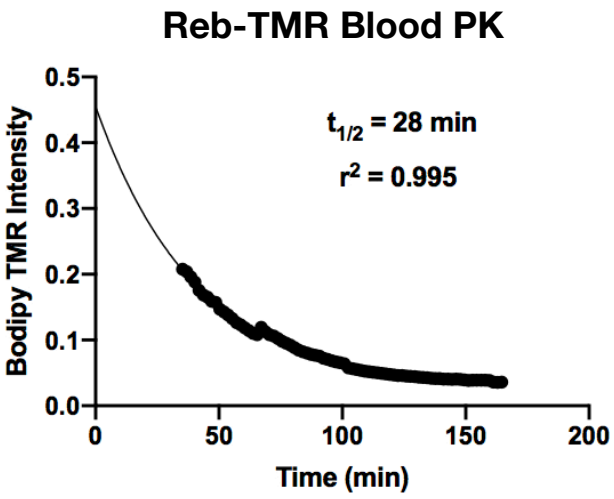


Figure S7: Measurement of blood half-life of Reb-TMR.

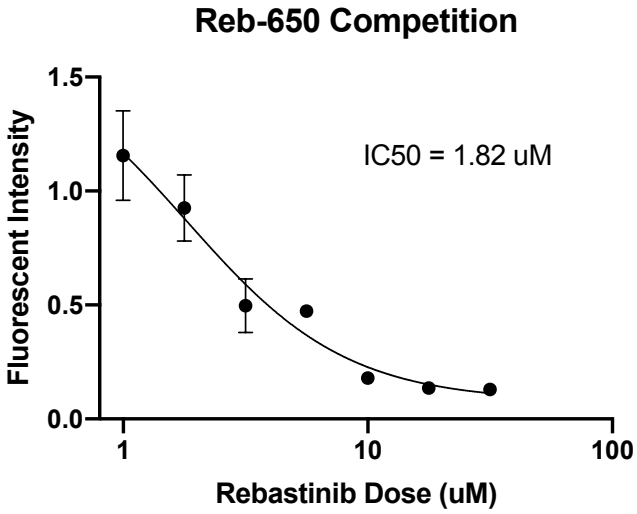


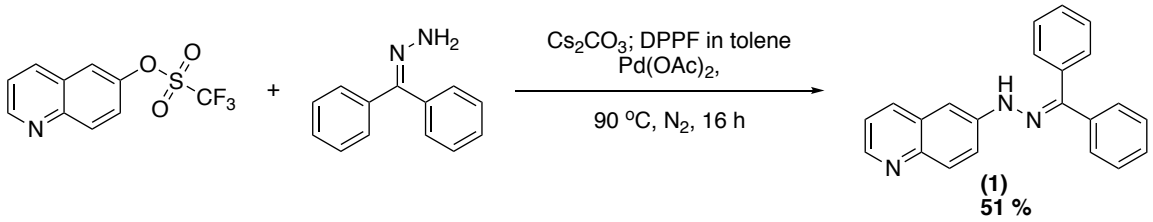
Figure S8: Competition of Rebastinib-BODIPY 650 with unlabeled rebastinib. HUVEC cells were treated with 100 nM Rebastinib-BODIPY 650, in combination with unlabeled rebastinib. Average single cell BODIPY 650 intensity per wall was calculated, N = 2 wells, error bars = S.D. Unlabeled rebastinib could compete out Rebastinib-BODIPY 650, with IC50 = 1.8 μM.

Original name in manuscript	Revised name	Ex/Em (nm)	Filter Info	Used to image	Imaging system
GFP/YFP	490 channel	488/510	Ex: 405/473/559 Dichroic Em: BA490-540	Tie2-GFP	FV1000
mAPPLE	560 channel	568/592	Ex: 405/473/559 Dichroic Em: BA575-620	Reb-TMR	FV1000
Cy3	560 channel	554/568	Ex: 520-560 Em: 560-630	Reb-TMR	Operetta, IXM
Cy5	650 channel	649/666	Ex: 620-640 Em: 650-700	Reb-650; Reb-SiR	Operetta, IXM
Hoechst	360 channel	361/497	Ex: 360-400 Em: 410-480	Hoechst, nuclear dye	Operetta, IXM
Cy3	560 channel	554/568	Ex: 511-551 Em: 573-613	Reb-TMR	BX63, Modified Olympus Deltavision
Alexa Fluor 647	650 channel	649/666	Ex: 608-648 Em: 672-712	Anti-Tie2 Antibody	BX63, Modified Olympus Deltavision
Hoechst	360 channel	361/497	Ex: 380.5-392.5 Em: 417-477	Hoechst, nuclear dye	BX63, Modified Olympus Deltavision

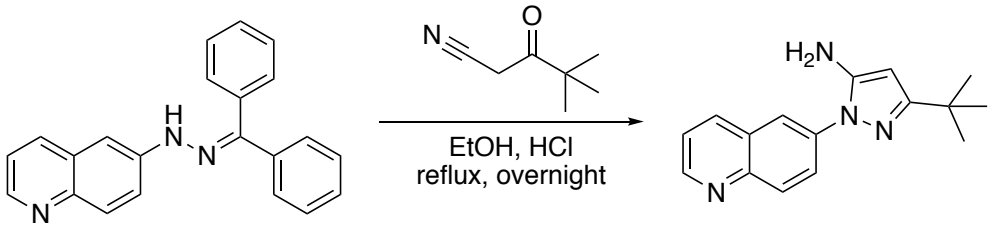
Table S1: Microscopy Information

General Considerations

All commercially available compounds were purchased and used as received. NHS Ester dyes were purchased from Thermo Fisher Scientific and SiROOH synthesis has been described in the literature[1–6]. Reaction mixtures were purified using a Biotage® SNAP Bio C18 300 Å 10 g on a Biotage® Isolera with a gradient composed of water (0.1% formic acid) and acetonitrile (0.1% formic acid) for reversed-phase chromatography, starting from 5 % to 100 % acetonitrile (0.1% formic acid). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-400 MHz spectrometer. High-performance liquid chromatography was done with a gradient of water (0.1% formic acid) and acetonitrile (0.1% formic acid) and separated with a XTerra MS C18 Column, 125Å, 5 µm, 4.6 mm X 50 mm and mass ions were detected on a Waters 3100 Mass Detector.

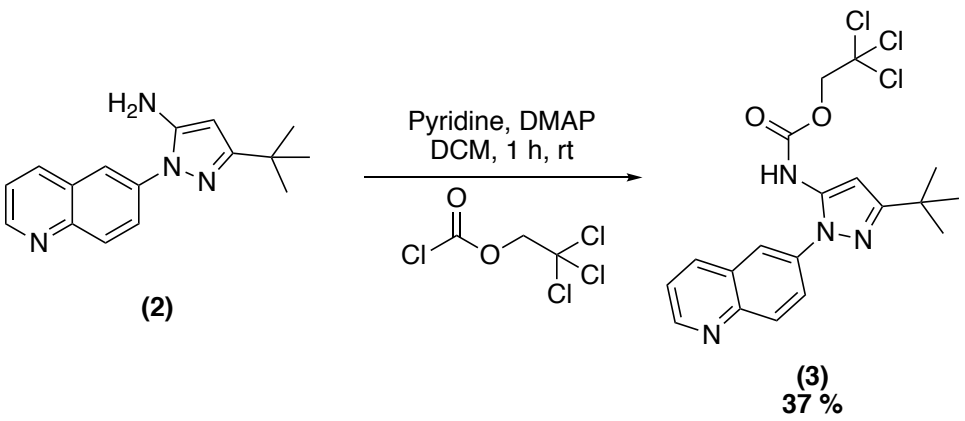


*Synthesis of **1**.* Toluene was degassed with a stream of N_2 for 3 hours prior to use. 6-Quinolinyl trifluoromethanesulfonate (2 g, 7.2 mmol, 1.0 eq), benzophenone hydrazone (1.8.g, 9.3 mmol, 1.3 eq), DPPF (126 mg, 0.23 mmol, 0.032 eq), and Cs_2CO_3 (3.8 g, 11.8 mmol, 1.6 eq) was suspended in toluene (50 mL) under a N_2 environment. To this reaction $Pd(OAc)_2$ (0.67 mg, 0.003 mmol) in 1 mL toluene was added and heated to 90 C overnight. The reaction was cooled and concentrated to a residue. The reaction residue was purified via normal phase chromatography with a gradient from hexanes to ethyl acetate. Fractions of similar purity were combined and concentrated to yield the desired product **1** (orange-red solid, 1.2 g, 51 % yield, 98 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 323.14; $^{(M)+}$ found: 325.59. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 3.5 Hz, 1H), 7.82 (dd, *J* = 8.4, 4.6 Hz, 2H), 7.68 (s, 1H), 7.51 (d, *J* = 7.6 Hz, 2H), 7.46 – 7.31 (m, 3H), 7.29 (s, 1H), 7.19 (q, *J* = 9.3, 7.8 Hz, 5H), 7.10 (dt, *J* = 6.8, 3.4 Hz, 2H), 7.02 (d, *J* = 7.7 Hz, 1H), 2.20 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 147.13, 145.64, 144.29, 142.49, 138.09, 134.46, 132.50, 130.36, 129.74, 129.43, 129.04, 128.38, 128.27, 126.68, 125.30, 121.50, 119.04, 106.09.

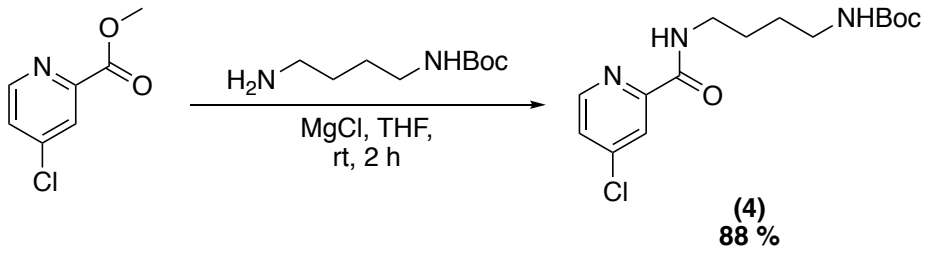


(1) **(2)** **93 %**

*Synthesis of **2**.* **1** (1.5 g, 4.6 mmol, 1.0 eq) and pivaloylacetonitrile (870 mg, 7.0 mmol, 1.5 eq) was stirred well in ethanol (20 mL). To this reaction HCl (3 mL) was added and heated to reflux overnight. The reaction was cooled and concentrated to a residue. The reaction residue was extracted with water and diethyl ether. The aqueous layer was neutralized with saturated Na_2CO_3 and extracted with ethyl acetate (3x100 mL). The organic fractions were combined and dried with Na_2SO_4 and concentrated to yield a reaction residue. The residue was purified via normal phase chromatography with a gradient of dichloromethane and methanol, ranging from 0.1 % to 2 % methanol. Fractions of similar purity were combined and concentrated to yield the desired product **2** (orange-red solid, 1.1 g, 93 % yield, 94 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 266.15; $^{(M)+}$ found: 267.02. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.89 (d, *J* = 3.8 Hz, 1H), 8.41 (d, *J* = 8.3 Hz, 1H), 8.18 (d, *J* = 1.7 Hz, 1H), 8.12 (s, 2H), 7.55 (dd, *J* = 8.4, 4.2 Hz, 1H), 5.51 (s, 1H), 1.29 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.52, 150.10, 147.66, 145.65, 137.45, 136.07, 129.78, 128.05, 125.49, 121.89, 118.96, 87.37, 31.94, 30.21.

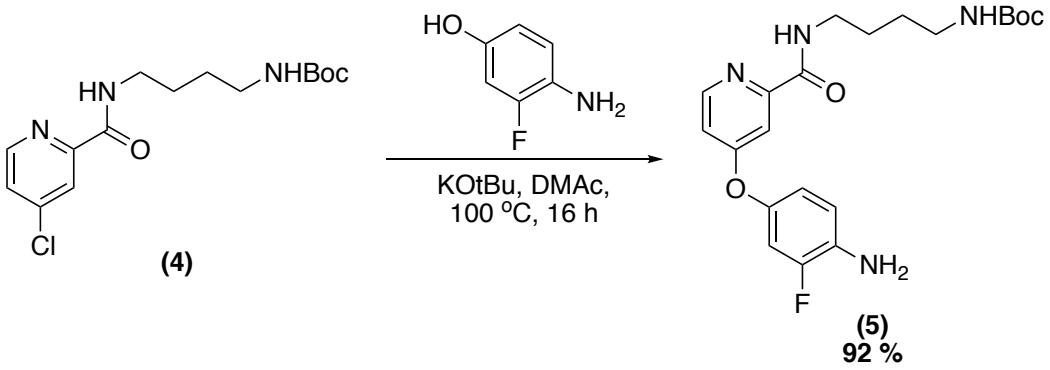


*Synthesis of **3**.* **2** (571 mg, 4.6 mmol, 1.0 eq), pyridine (0.57 mL, 7.1 mmol, 3.3 eq), and DMAP (7.9 mg, 0.065 mmol, 0.03 eq) were stirred well in dichloromethane (40 mL) and cooled to -10 C. To this 2,2,2-trichloroethyl chloroformate (0.42 mL in 12 mL DCM) was added dropwise over a period of 20 minutes. Afterwards, the reaction was stirred at room temperature for an hour. To the reaction water (40 mL) was added as stirred for 20 mins. The reaction residue was extracted with brine. The organic fractions were combined and dried with Na_2SO_4 and concentrated to yield a reaction residue. The residue was purified via reverse phase chromatography. Fractions of similar purity were combined and concentrated to yield the desired product **3** (749.0 mg, 37 % yield, 92 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 440.06; $^{(M)-}$ found: 439.38. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, *J* = 4.2 Hz, 1H), 8.61 (b, 1H), 7.92 (dd, *J* = 12.7, 8.6 Hz, 2H), 7.63 (d, *J* = 7.6 Hz, 1H), 7.29 (dd, *J* = 8.3, 4.3 Hz, 1H), 6.41 (s, 1H), 4.79 (s, 2H), 1.30 (d, *J* = 1.2 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.05, 150.87, 149.18, 144.94, 135.49, 135.33, 134.55, 129.52, 127.04, 125.55, 121.39, 120.76, 96.12, 94.02, 73.86, 31.53, 29.26.

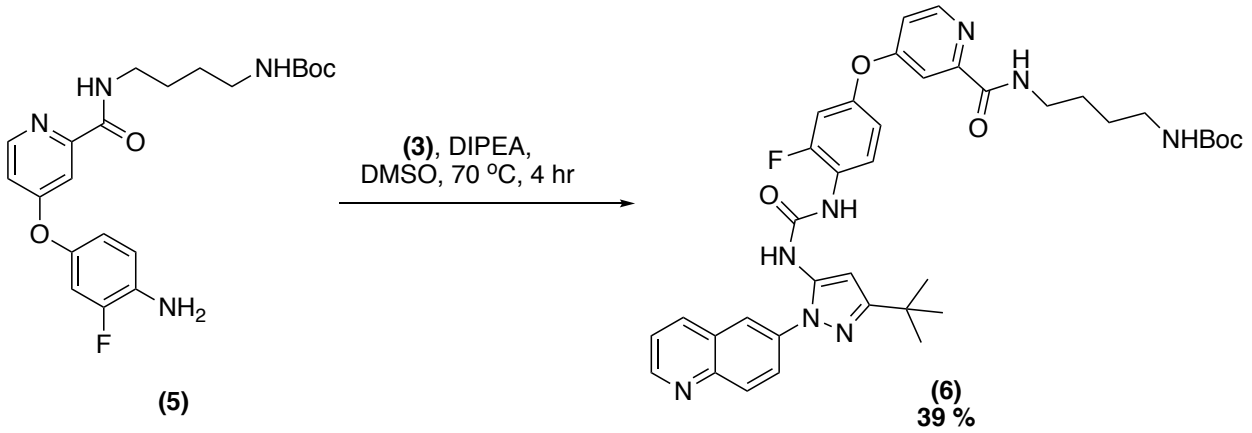


(4)
88 %

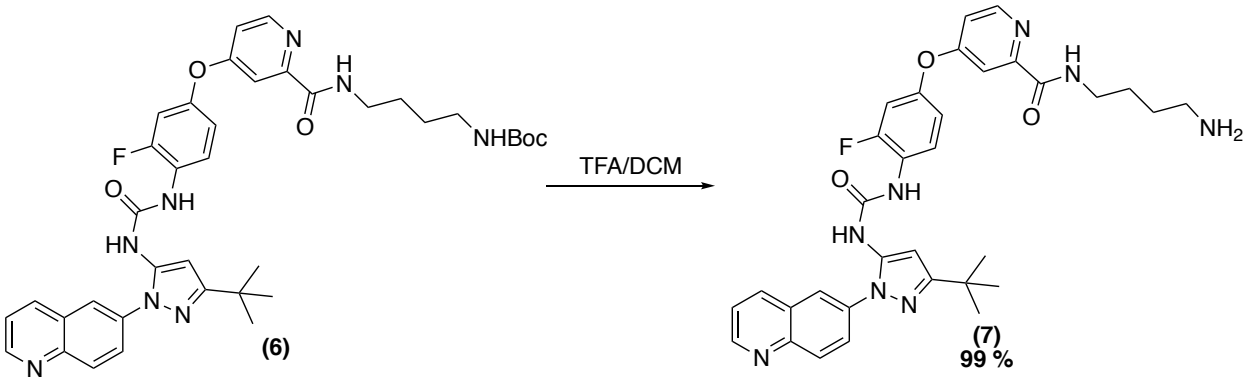
*Synthesis of **4**.* Methyl 4-chloropicolinate (1.0 g, 5.8 mmol, 2.0 eq), N-Boc-1,4-butanediamine (2.2 g, 11.7 mmol, 4.0 eq), and MgCl (279 mg, 2.9 mmol, 1.0 eq) were stirred well in THF (5 mL) for 2 hours. To the reaction water (5 mL) and 1N HCl (10 mL) were added. The reaction residue was extracted with ethyl acetate (3x20 mL). The reaction mixture was washed with brine. The organic fractions were combined and dried with $MgSO_4$ and concentrated to yield a reaction residue. The residue was purified via normal phase chromatography with a gradient of dichloromethane and methanol, ranging from 0 % to 2 % methanol. Fractions of similar purity were combined and concentrated to yield the desired product **4** (1.7 g, 88 % yield, 91 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 350.12; $^{(M + Na)+}$ found: 350.33. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (d, *J* = 5.2 Hz, 1H), 8.13 (d, *J* = 2.0 Hz, 1H), 7.96 (d, *J* = 7.3 Hz, 1H), 7.36 (dd, *J* = 5.4, 2.0 Hz, 1H), 4.52 (s, 1H), 3.42 (q, *J* = 6.7 Hz, 2H), 3.10 (d, *J* = 6.7 Hz, 2H), 1.66 – 1.45 (m, 4H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.26, 156.12, 151.48, 149.02, 146.11, 126.44, 123.04, 40.35, 39.32, 28.55, 27.70, 27.13.



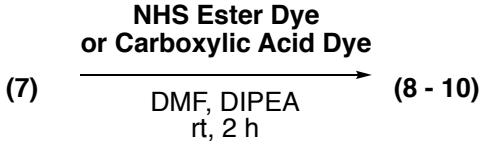
*Synthesis of **5**.* 2-Fluoro-4-methoxyaniline (1.7 g, 13.3 mmol, 1.0 eq) was suspended in DMAc (5 mL) cooled to 0 C under N_2 atmosphere. To this reaction KOtBu (1.5 g, 13.3 mmol, 1.0 eq) was added. This was stirred at 0 C for 30 minutes. After which, **4** (1.7 g, 5.1 mmol, 0.39 eq) was added and the reaction was heated to 100 C overnight. The reaction was next cooled to room temperature and a saturated solution of LiCl (10 mL)was added. The reaction mixture extracted with ethyl acetate (3x100 mL). The organic layer was washed with water (3x100 mL) and brine (3x100 mL). The organic layer was dried with $MgSO_4$ and concentrated to yield a reaction residue. The residue was purified via reverse phase chromatography. Fractions of similar purity were combined and concentrated to yield the desired product **5** (1.96g, 92 % yield, 98 % purity) which was stored under Ar at -80 C. *m/z* calculated: 417.18; $^{(M + FA)^-}$ found: 463.42 and $^{(2M + FA)}]^-$ found: 881.75. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, *J* = 5.6 Hz, 1H), 8.10 (t, *J* = 6.2 Hz, 1H), 7.58 – 7.50 (m, 1H), 6.80 (dd, *J* = 5.7, 2.2 Hz, 1H), 6.74 – 6.61 (m, 2H), 6.57 (dd, *J* = 8.5, 2.5 Hz, 1H), 5.04 (d, *J* = 6.0 Hz, 1H), 4.05 (s, 2H), 3.33 (d, *J* = 6.6 Hz, 2H), 3.02 (d, *J* = 6.5 Hz, 2H), 1.48 (dt, *J* = 32.2, 7.2 Hz, 4H), 1.30 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.67, 163.93, 156.08, 152.36, 152.02, 149.95, 149.55, 144.32, 133.10, 117.18, 113.68, 109.70, 109.03, 78.79, 40.09, 39.03, 28.32, 27.37, 26.88.



*Synthesis of **6**.* **3** (350 mg, 0.79 mmol, 1.0 eq), **5** (297 mg, 0.71 mmol, 0.9 eq), DIPEA (0.3 mL, 1.74 mmol, 2.2 eq) was suspended in DMSO (5 mL) heated to 70 C. The reaction was monitored by LCMS for 4 hours. DMSO was separated using a Waters Sep-Pak C-18. The residue was purified via normal phase chromatography with a gradient of dichloromethane and methanol, ranging from 0 % to 10 % methanol. Fractions of similar purity were combined and concentrated to yield the desired product **6** (197 mg, 39 % yield, 77 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 710.33; $^{(M)+}$ found: 711.67 and $^{(M - BoC)+}$ found: 611.62. ^{1}H NMR (400 MHz, $CDCl_3$/DMSO-d_6) δ 9.00 (s, 1H), 8.82 (s, 1H), 8.71 (d, *J* = 4.9 Hz, 1H), 8.51 (d, *J* = 8.7 Hz, 1H), 8.30 (d, *J* = 5.6 Hz, 1H), 8.24 – 8.10 (m, 3H), 8.01 (t, *J* = 6.2 Hz, 1H), 7.55 (dd, *J* = 8.6, 5.1 Hz, 1H), 7.50 (d, *J* = 2.5 Hz, 1H), 6.91 (dd, *J* = 5.7, 2.6 Hz, 1H), 6.78 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.73 – 6.63 (m, 1H), 6.54 (s, 1H), 4.54 (s, 2H), 3.29 (d, *J* = 6.6 Hz, 2H), 3.05 (d, *J* = 6.5 Hz, 2H), 1.33 (d, *J* = 18.1 Hz, 22H). (See Figure S9)



*Synthesis of **7**.* **6** (137 mg, 0.19 mmol, 1.0 eq) was suspended in dichloromethane (3 mL). To this TFA (1 mL) was added dropwise. The reaction was monitored by LCMS for 10 minutes. After which the reaction was concentrated to produce a residue. The residue was purified via reverse phase chromatography. Fractions of similar purity were combined and concentrated to yield the desired product **7** (115 mg, 99 % yield, 95 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 610.28; $^{(M)+}$ found: 610.28 and $^{(M - BoC)+}$ found: 609.54. ^{1}H NMR (400 MHz, MeOD/DMSO-d_6) δ 9.79 (1H), 9.72 (1H), 9.62 (1H), 9.53 (1H), 9.16 (2H), 8.82 (3H), 8.62 (1H), 8.29 (2H), 8.03 (1H), 7.92 (1H), 7.83 (1H), 7.67 (1H), 3.92 (2H), 3.42 (3H), 2.18 (4H), 1.95 (9H). (See Figure S10)



*Synthesis of **8**-**9**.* **7** (1.9 mg, 0.0028 mmol, 1.0 eq) and an NHS ester dye (3 eq) were suspended in DMF (0.100 mL). To this reaction DIPEA (0.030 mL, 0.0082 mmol, 3.0 eq) was added. The reaction was allowed to mix for 2 hours. After which the reaction was was purified via reverse phase chromatography. Fractions of similar purity were combined and concentrated to yield the desired product ***8***-***9*** (3 mg, 99 % yield, 90 - 95 % purity) which was stored under Ar at -80 C. *m*/*z* calculated: 1103.52; $^{(M)+}$ found: 1104.91 and $^{(M/2)+}$ found: 543.08. (See Figure S11 & S12)

*Synthesis of **10**.* **7** (7.3 mg, 0.012 mmol, 1.1 eq), HBTU (8.3 mg, 0.022 mmol, 2 eq) and SiROOH dye (5.5 mg, 0.011 mmol, 1.0 eq) were suspended in DMSO (1.00 mL). To this reaction DIPEA (4 uL, 0.022 mmol, 2 eq) was added. The reaction was allowed to mix for 2 hours. After which the reaction was was purified via reverse phase chromatography. Fractions of similar purity were combined and concentrated to yield the desired product ***10*** (79 % yield) which was stored under Ar at -80 C.

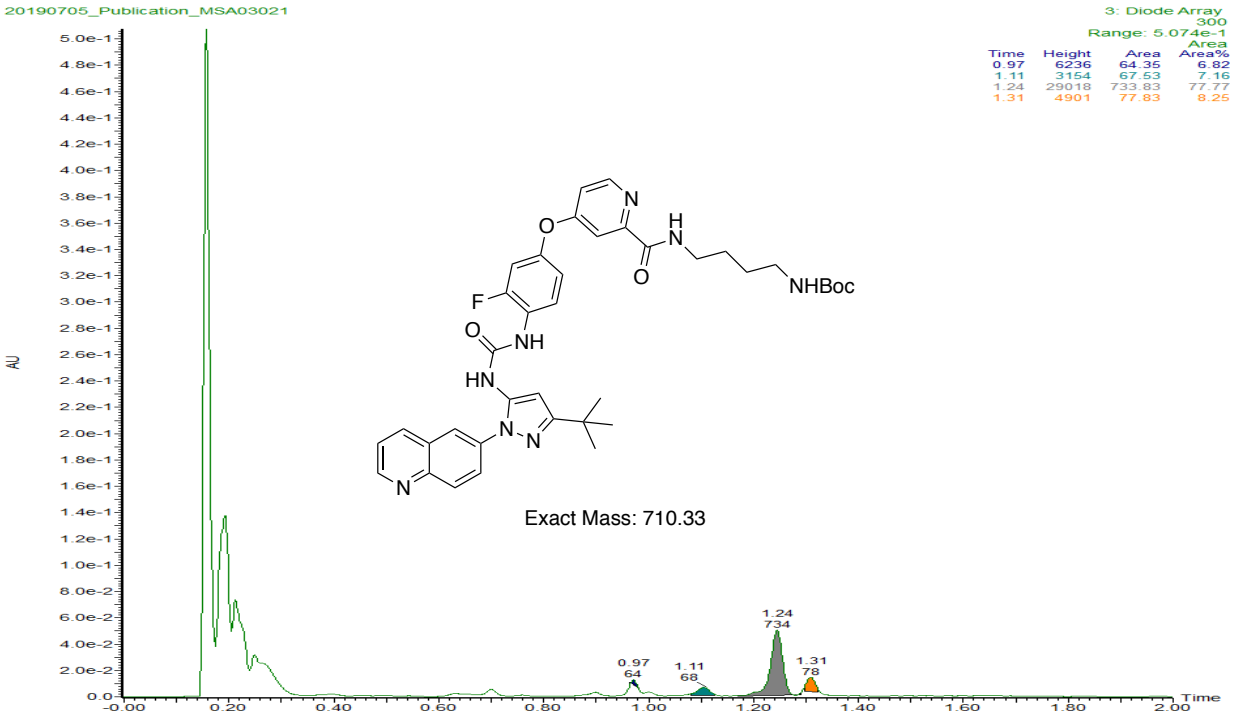


Figure S9: HPLC trace of **6** (77 % Purity).

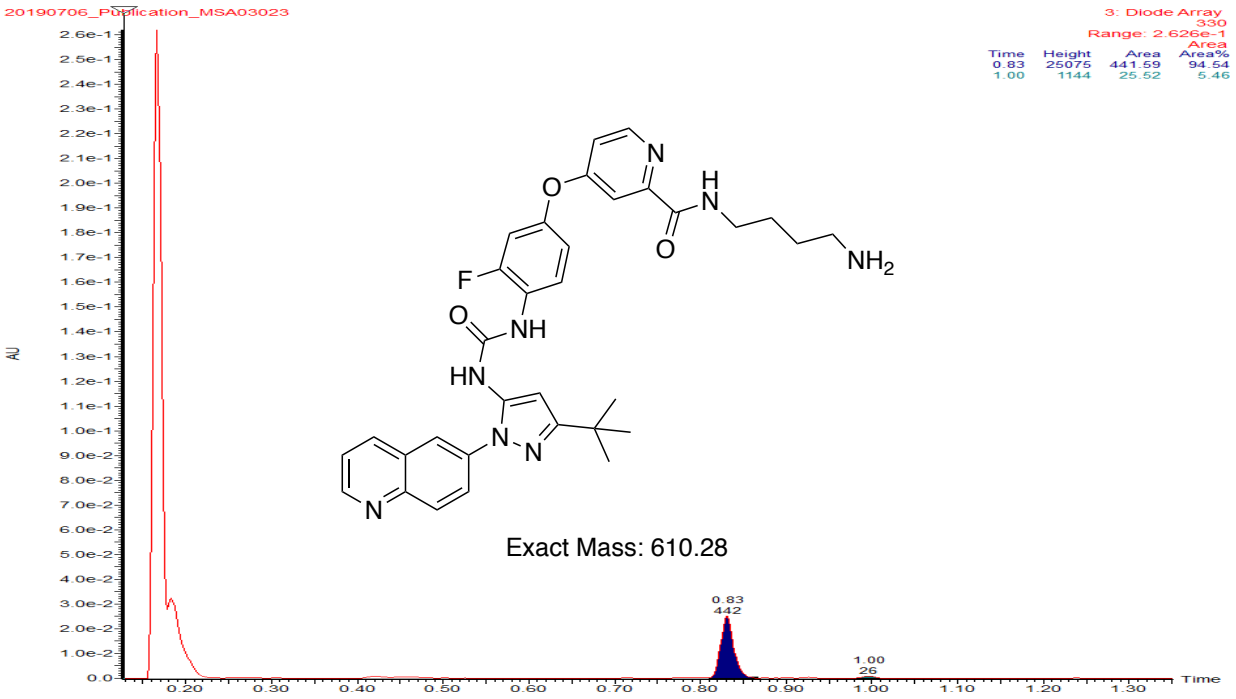


Figure S10: HPLC trace of **7** (95 % Purity).

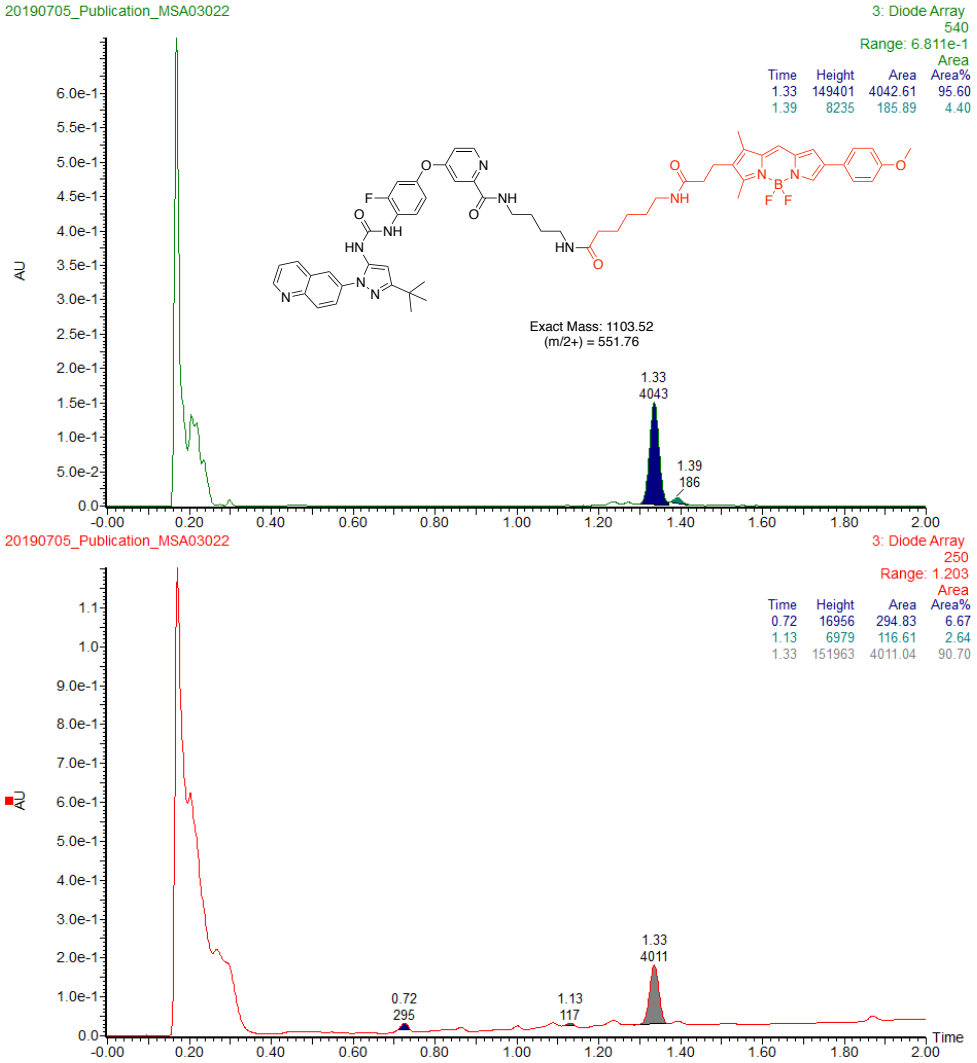


Figure S11: HPLC trace of **8** (90-95 % Purity).

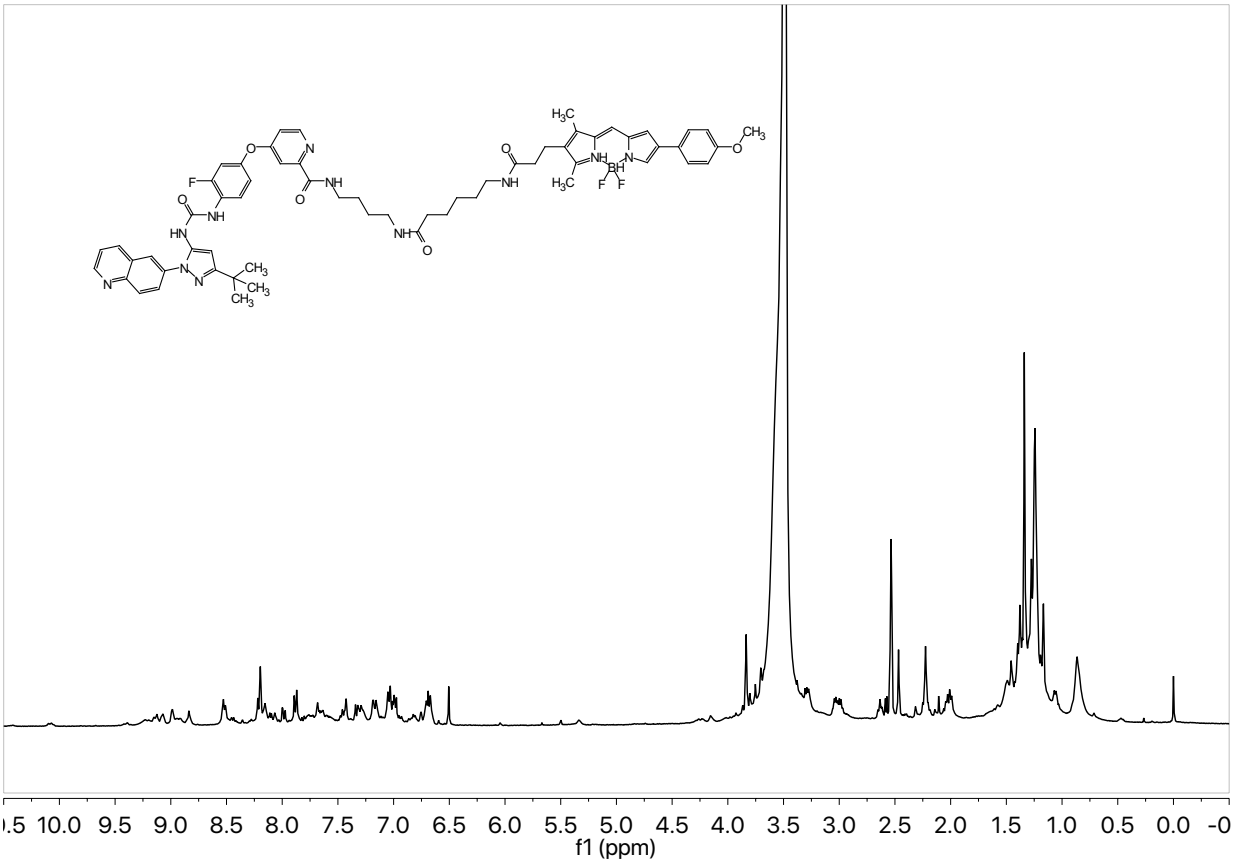


Figure S12: ^{1}H NMR of **8**.

References

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