Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING INFORMATION

Bichromophoric Dyes for Wavelength Shifting of Dye-Protein Fluoromodules

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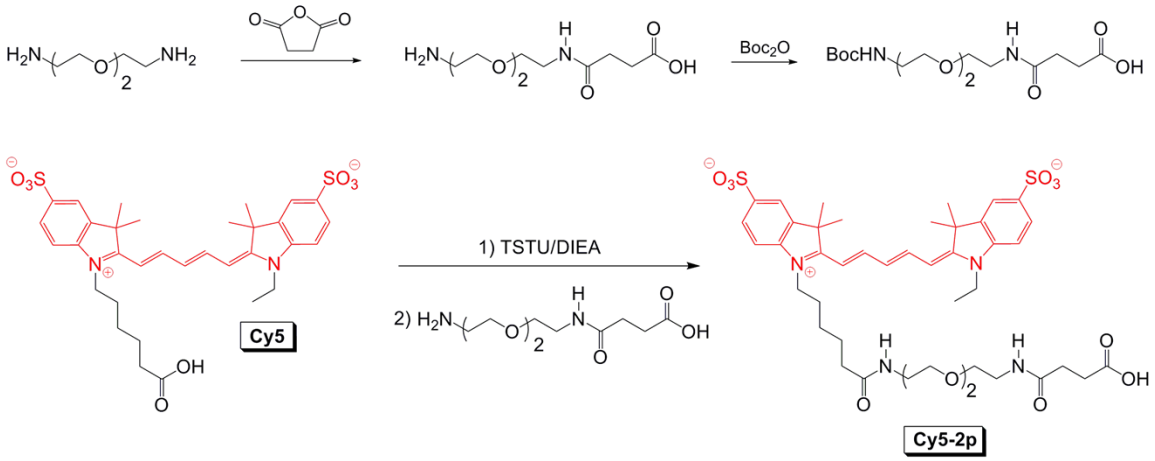
1. Synthesis of TO1-2p-Cy5 and Monochromophoric Model Compounds

Scheme S1. Synthesis of 2p-Linker and **Cy5-2p**



Experimental Section:

3-(2-(2-(2-Aminoethoxy)ethoxy)ethylcarbamoyl)propanoic acid (2p-Linker)
2,2'-(Ethylendioxy)bis(ethylamine) (2.96 g; 20 mmol) was dissolved in acetonitrile (100 mL). Succinic anhydride (2g, 20 mmol) dissolved in dry acetonitrile (50 mL) was dropwise added under vigorous stirring over a 1 hr period. Stirring was continued for 2 hrs. The product settles on the bottom as a colorless resin. The organic supernatant was poured off and discarded. The residue was dissolved in a mixture of 1 N NaOH (40 mL, 40 mmol) and acetonitrile (40 mL). Boc-anhydride (4.76 g. 20 mmol) dissolved in dry acetonitrile (50 mL) was dropwise added to the reaction mixture under stirring. Stirring was continued for 2 hrs. The acetonitrile was removed under vacuum. Water (100 mL) was added to the residue and the aqueous phase was extracted with ethylacetate (2 x 50 mL) to remove side-products. The organic phase was discarded. The aqueous phase was acidified with citric acid to pH 4. The product, the mono-boc protected linker was extracted with ethyl acetate (4 x 50 mL). The combined organic extracts were washed with water and dried over Na_2SO_4. After removal of the solvent a colorless oil was left.

^{1}H-NMR ($CDCl_3$): 7.45 (1H,s,COOH); 6.87 (1H,s,); 6.61 (1H,s,amide);
3.63 (4H,s,PEG); 3.57 (4H,m, PEG); 3.47 (2H,m,PEG); 3.35 (2H,s,PEG); 2.68 (2H,m,Succ), 2.53 (2H,m,Succ); 1.46 (9H,s.BOC);

For the synthesis of the **Cy5-2P** monochromophore (Scheme S1) the Boc-protecting group was removed from the **2p** linker by stirring the product overnight 1N HCl/ actonitrile (20 mL/20 mL). The solvent was removed under vacuum to give a colorless resin. The product was used as such in the next reaction step.

^{1}H-NMR (D_2O): 3.62 (6H.m,PEG); 3.56 (2H,m); 3.33 (2H,m,PEG); 3.13 (2H,m,PEG); 2.40 (4H,m,Succ)

2-[5-[1-[1-Carboxy-3,14-dioxo-7,10-dioxa-4,13-diazanonadecan-19-yl]-3,3-dimethyl-5-sulfonato-1,3-dihydro-2H-indol-2-ylidene]-penta-1,3-dien-1-yl]-1-ethyl-3,3-dimethyl-3H-indolium-5-sulfonate (Cy5-2p)

Cy5 (83 mg, 0.12 mmol) and TSTU (72 mg, 0.24 mmol) were dissolved in dry DMF/ 1% Diisopropylethylamine (0.5 mL) and stirred for 1 hr at rt. Conversion to the active ester was confirmed by TLC on RP-18 (20% acetonitrile/water). The **2p** linker was dissolved in 1M sodium bicarbonate (0.5 mL) and added to the reaction mixture. After stirring for 1 hr the product was precipitated by adding acetone. The organic phase was decanted and the residue dissolved in acetonitrile/water/1% TFA. The reaction mixture was separated by HPLC on a μ-Bondpak 10 μm 7.8x300 mm RP-18 column; eluent: 10-40% acetonitrile/water/0.1 TFA, linear gradient over 20 min/3 mL flow rate.

^{1}H-NMR (D_2O): 7.91 (2H,m); 7.75 (2H,d); 7.70 (2H,t); 7.20 (2H,dd); 6.42 (1H,t); 6.15 (1H,d); 6.08 (1H,d); 3.97 (2H,m); 3.93 (2H,m); 3.49 (4H,s); 3.44 (2H,t); 3.40 (2H,t); 3.23 (2H,t); 3.17 (2H,t); 2.51 (2H,t); 2.38 (2H,t); 2.12 (2H,t); 1.70 (2H,m); 1.54 (12H,s); 1.51 (2H,m); 1.25 (2H,m); 1.23 (3H,t).

MS: $C_{43}H_{57}N_4O_{12}S_2$ (886.08 g/mol) MS ESI/positive mono isotopic ion 887.47

2-[5-[3,3-Dimethyl-5-sulfonato-1-[5,8,19-trioxo-1-(4-{(3-(3-sulfonatopropyl)-1,3-benzothiazol-2(3H)-ylidene]methyl]quinolinium-1-yl)-12,15-dioxa-4,9,18-triazatetracosan-24-yl]-1,3-dihydro-2H-indol-2-ylidene}penta-1,3-dien-1-yl]-1-ethyl-3,3-dimethyl-3H-indolium-5-sulfonate (TO1-2p-Cy5)

Cy5 (83 mg, 0.12 mmol) and TSTU (72 mg, 0.24 mmol) were dissolved in dry DMF/ 1% Diisopropylethylamine (0.5 mL) and stirred for 1 hr at rt. Conversion to the active ester was confirmed by TLC on RP-18 (20% acetonitrile/water). **TO1-2p**[1] (70 mg, 12 mmol) was dissolved in DMF/1 % DIEA (0.2 mL) and added. The reaction mixture was stirred for 2hrs at rt. The product was precipitated by the addition of ether. The organic phase was decanted and the residue dissolved in acetonitrile/water/1% TFA. The reaction mixture was separated by HPLC on a μ–Bondapak 10 μm 7.8x300 mm RP-18 column; eluent: 20-60% acetonitrile/water/0.1 TFA, linear gradient over 30 min/3 mL flow rate.

^{1}H-NMR (MeOD): 8.74 (1H, d, J = 8.5 Hz/*TO*); 8.35 (1H, d, J =3.5 Hz/*TO*); 8.24 (2H, m/*CY*), 7.88 (4H,m/*CY*), 7.8 (2H, m/*TO*); 7.61 (3H,m/*TO*); 7.45 (1H, t, J =7.6 Hz/*TO*); 7.31 (1H,d, J = 8.6 Hz/*CY*); 7.26 (1H, d, J = 8.6 Hz/*CY*); 7.19 (2H,m/*TO*); 6.89 (1H,s/*TO*); 6.64 (1H, dd, J = 12.3 Hz/*CY*); 6.31 (1H,d, J =15.2 Hz/*CY*), 6.2.8 (1H, d, J = 15.2 Hz/*CY*), 4.69 (2H, m/*TO*); 4.45 (2H, m/*TO*), 4.14 (2H,m/*CY*), 4.06 (2H, m/*CY*), 3.55 (4H,s/*linker*), 3.50 (2H,m/*linker*), 3.48 (2H, m/*linker*), 3.33 (2 H,m/*linker*); 3.29 (4H,m, *TO*, *linker*), 3.09 (2H,m, *TO*); 2.54 (2H,m/*linker*), 2.51 (2H,m/*linker*), 2.31 (2H,m/*TO*), 2.16 (2H,t, J = 7.1 Hz/*CY*), 2.06 (2H,m/*CY*), 1.74 (2H,m/*CY*), 1.68 (6H,s/*CY*), 1.66 (6H,s/*CY*), 1.62 (2H,m/*CY*), 1.39 (2H,m/*CY*), 1.35 (3H,t).

MS: $C_{66}H_{82}N_7O_{14}S_4$ (1324.4802 calcd, 1324.4790 obsvd)

(1) Szent-Gyorgyi, C.; Schmidt, B. F.; Creeger, Y.; Fisher, G. W.; Zakel, K. L.; Adler, S.; Fitzpatrick, J. A.; Woolford, C. A.; Yan, Q.; Vasilev, K. V.; Berget, P. B.; Bruchez, M. P.; Jarvik, J. W.; Waggoner, A. *Nature Biotechnol.* **2007**, *26*, 235-240.

^{13}C-NMR Spectroscopy. TO1-2p-Cy5 was dissolved in MeOD for COSY, HMBC and HMQC analysis to verify the bichromophoric dye structure. (The dye could not be dissolved at sufficiently high concentration to allow 1D ^{13}C-NMR spectrum acquisition.) Peaks are assigned according to the numbering system shown in the dye structure below.

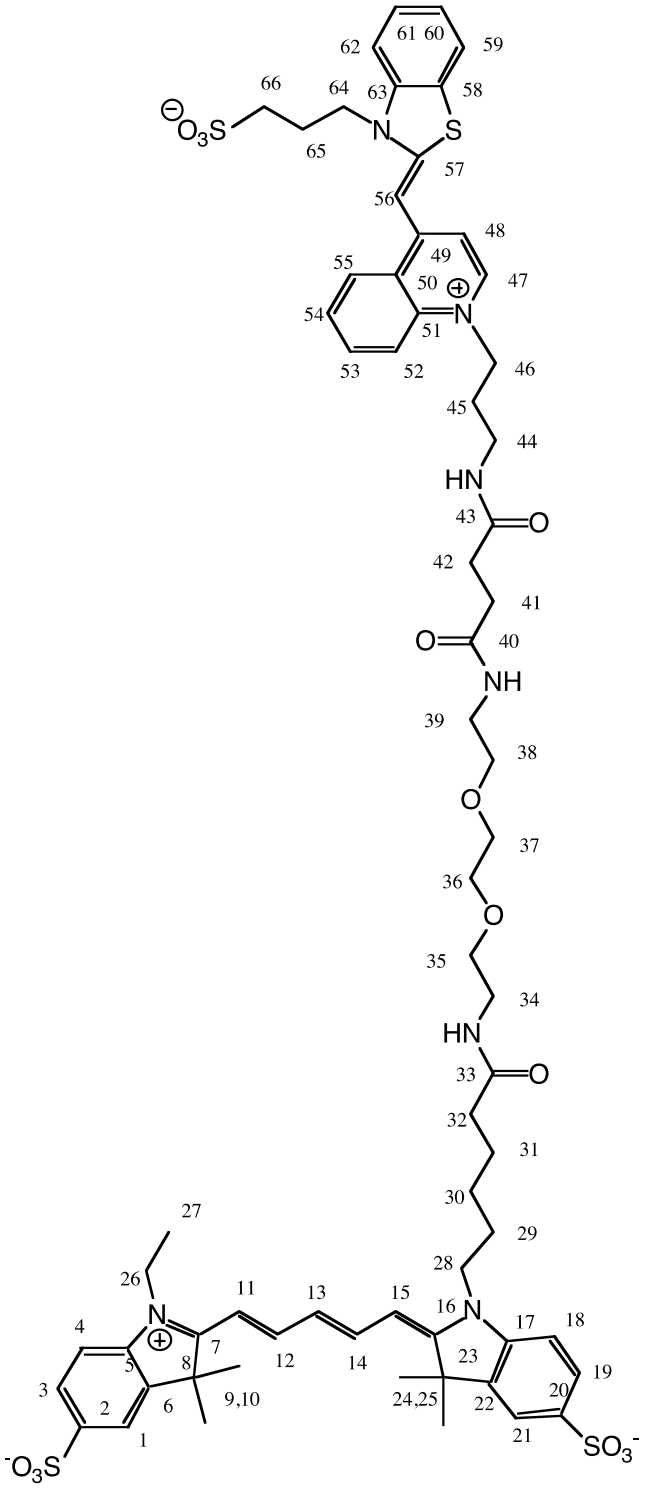


Table S1. NMR Spectroscopic Data (500 MHz, MeOD for TO1-2p-Cy5)

C/H no.	δC[a]	δH	HMBC[b]
1,21	128.1	7.87 (2H,s)	
2, 20	144.6		
3,	111.5	7.27 (1H,d)	
4,18	121.1	7.87 (2H,d)	
5,17	144.2		
6,22	144.8		
7,16	174.9		
8,23	50.6		
9,10,24,25	27.2	1.68, 1.66 (12H,s)	
11	105.1	6.28 (1H,d)	
12,14	156.2	8.24 (2H,m)	
13	127.6	6.28 (1H,t)	
15	150.0	6.31 (1H,d)	
19	111.3	7.31 (1H,d)	
26	40.3	4.14 (2H,m)	
27	12.5	1.35 (3H,t)	
28	45.1	4.04 (2H,m)	
29	27.7	1.74 (2H,m)	
30	27.2	1.39 (2H,m)	
31	26.6	1.62 (2H,m)	
32	36.5	2.16 (2H,t)	
33	175.7		
34	40.2	3.29 (2H,m)	34/32
35	70.5	3.47 (2H.m)	
36,37	71.2	3.55 (4H,s)	
38	70.5	3.50 (2H,m)	
39	40.2	3.33 (2H,m)	
40	174.5		39/40
41	32.3	2.51 (2H,m)	
42	32.9	2.54 (2H,m)	
43	174.4		41/43
44	37.3	3.29 (2H,m)	44/43
45	30.2	2.06 (2H,m)	
46	53.5	4.47 (2H,m)	
47	145.0	8.36 (1H,d)	
48	109.5	7.19 (1H,d)	
49	Unassigned [c]		
50	150.5		
51	138.2		
52	127.6	8.74 (1H,d)	
53	128.2	7.61 (1H,m)	
54	134.3	7.80 (1H,m)	
55	123.6	7.61 (1H,m)	
56	89.2	6.89 (1H,s)	
57	160.5		
58	Unassigned [c]		

59	113.6	7.61 (1H,m)	
60	129.3	7.45 (1H,m)	
61	125.6	7.19 (1H,m)	
62	118.2	7.80 (1H,m)	
63	140.8		
64	46.1	4.70 (1H,m)	
65	23.9	2.31 (2H,m)	
66	48.0	3.08 (2H,m)	

a) Chemical shifts were determined using HSQC and HMBC.
b) HMBC correlations are given from the proton(s) stated to the indicated carbon atoms.
c) Carbon 58 and 49 did not show any HMBC correlation that would allow assignment.

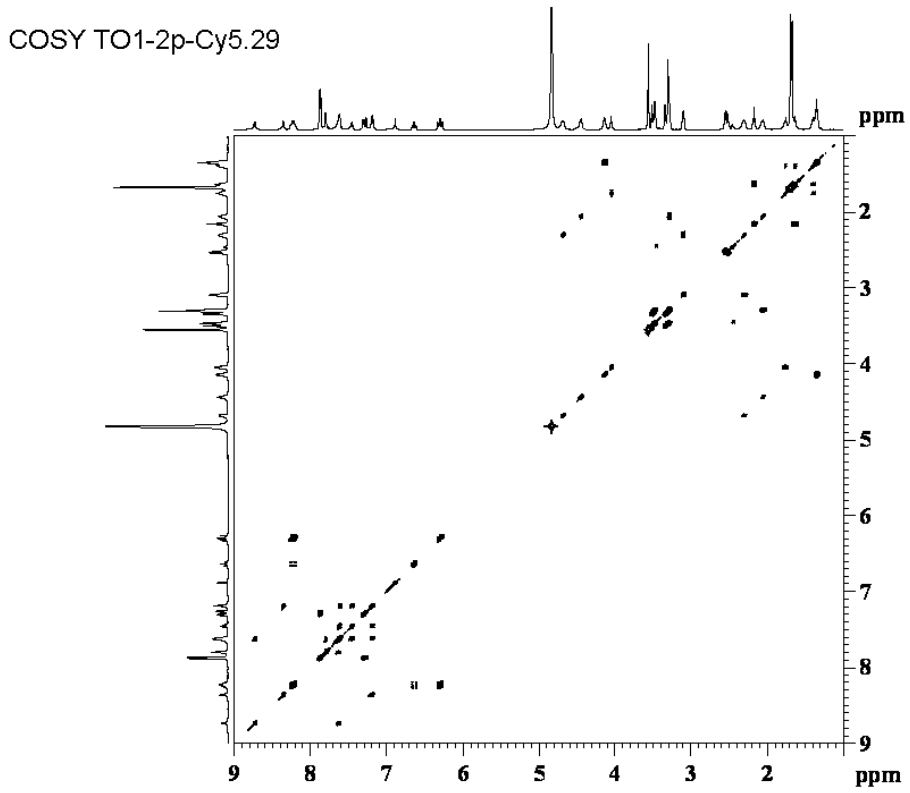


Figure S1

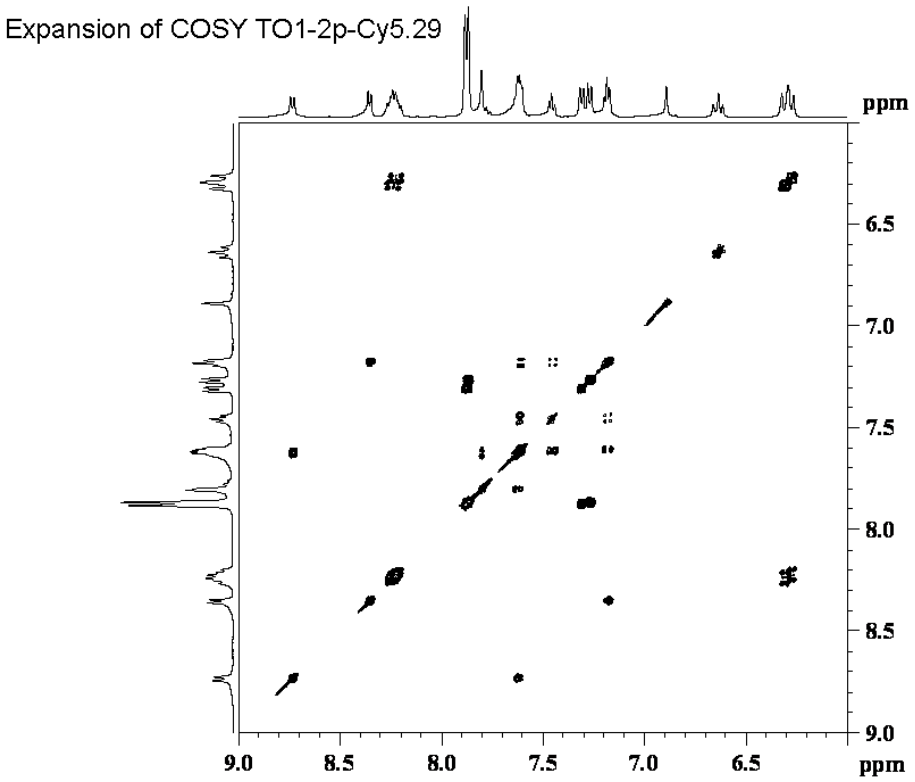


Figure S2

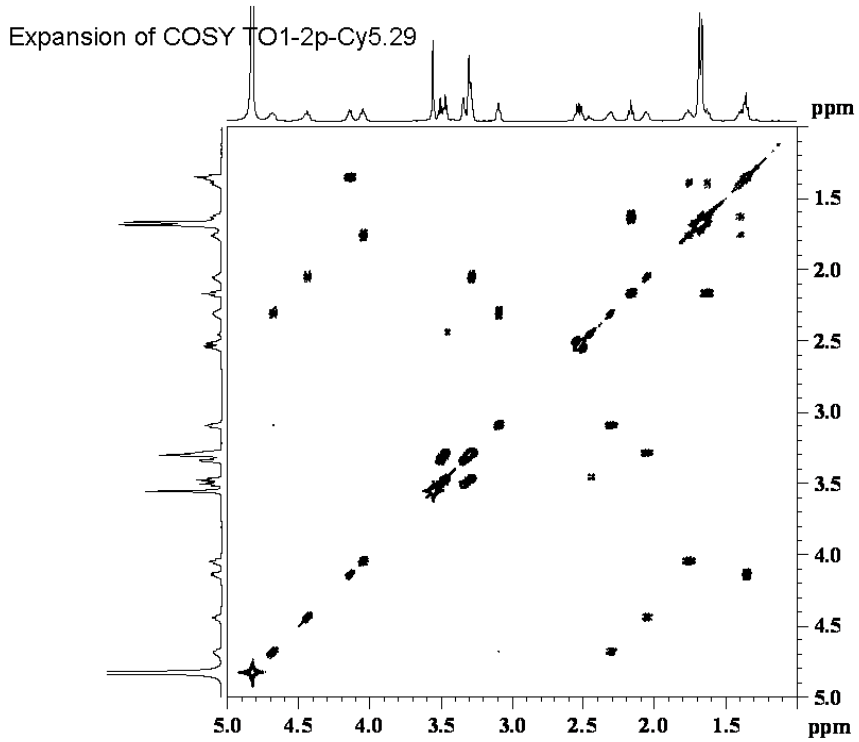
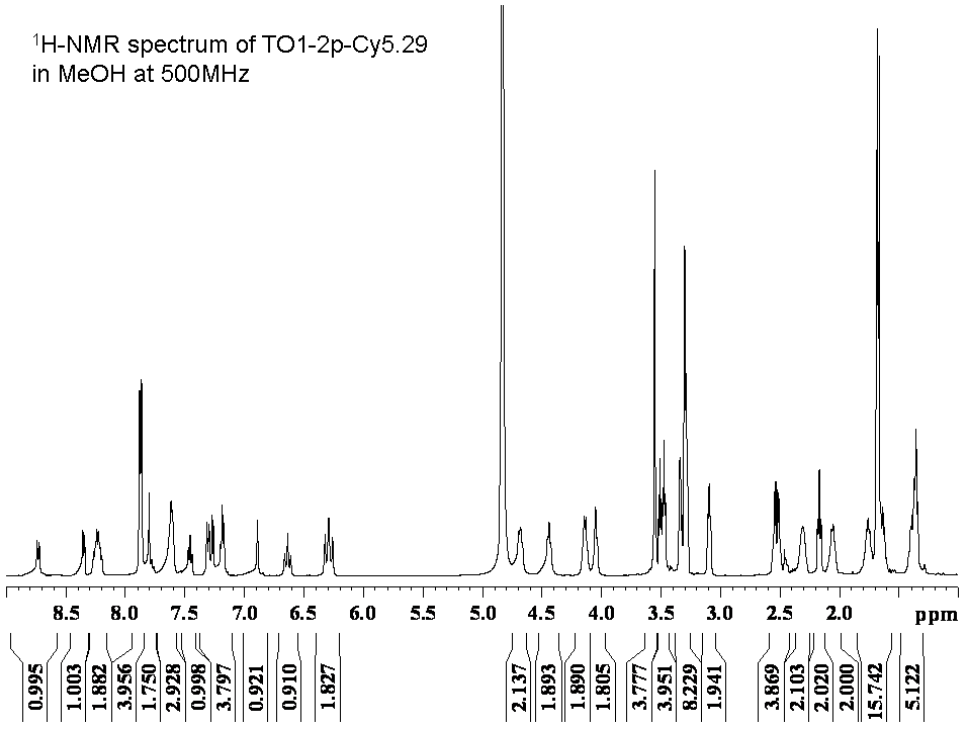


Figure S3



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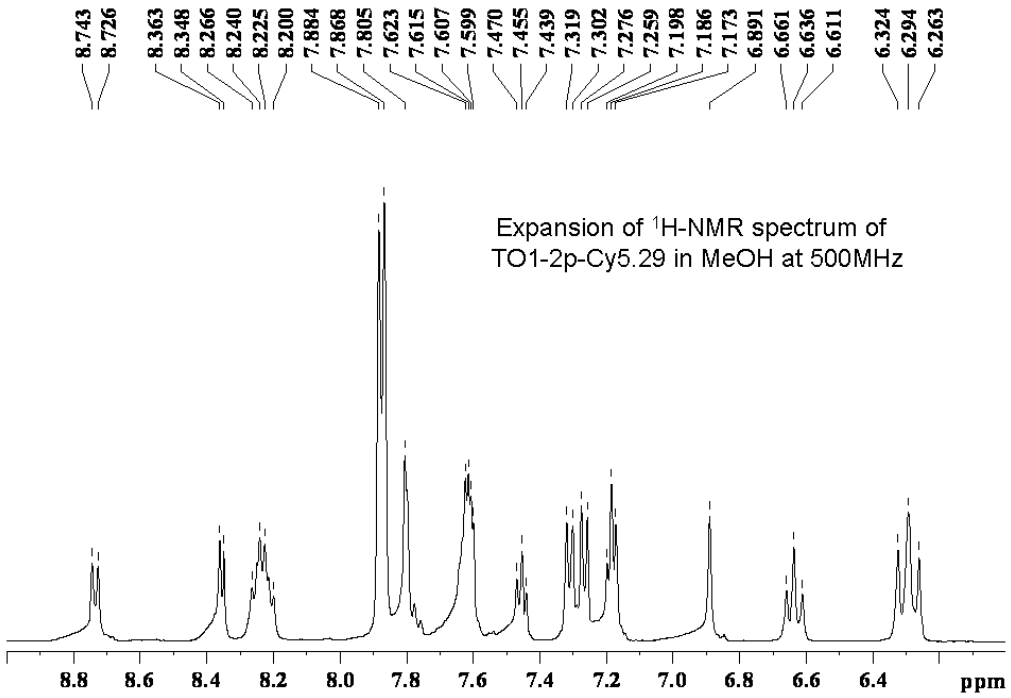


Figure S5

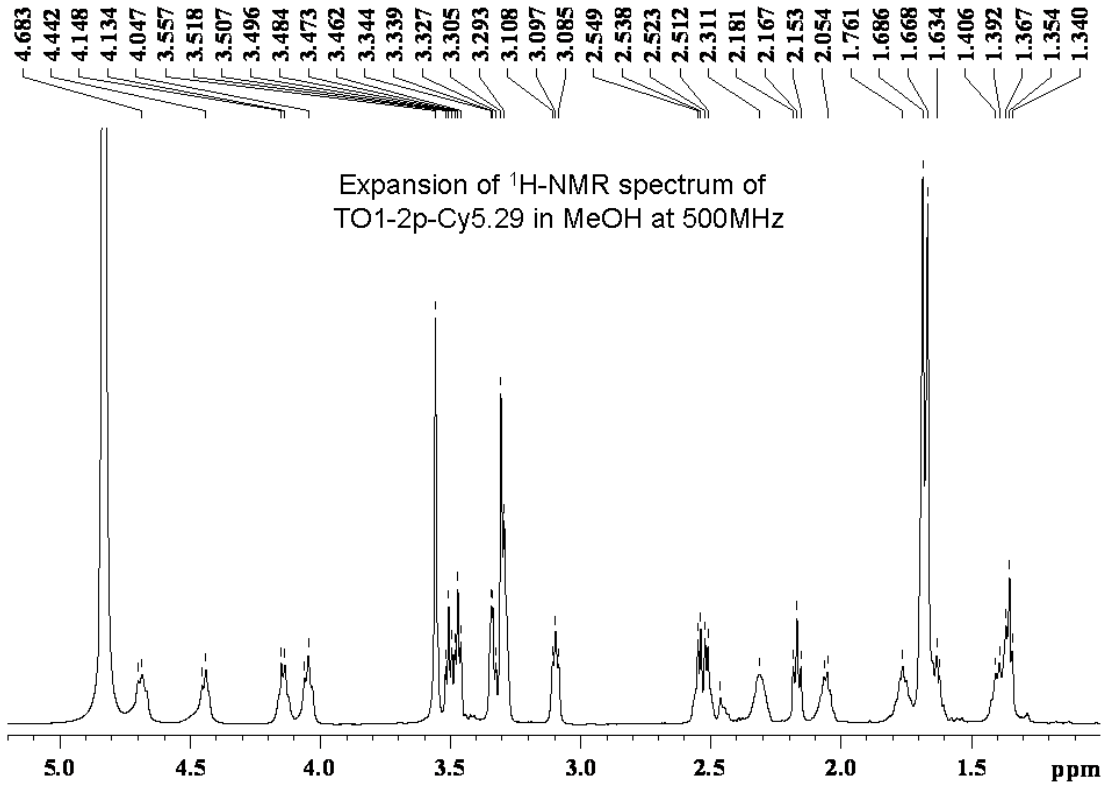


Figure S6

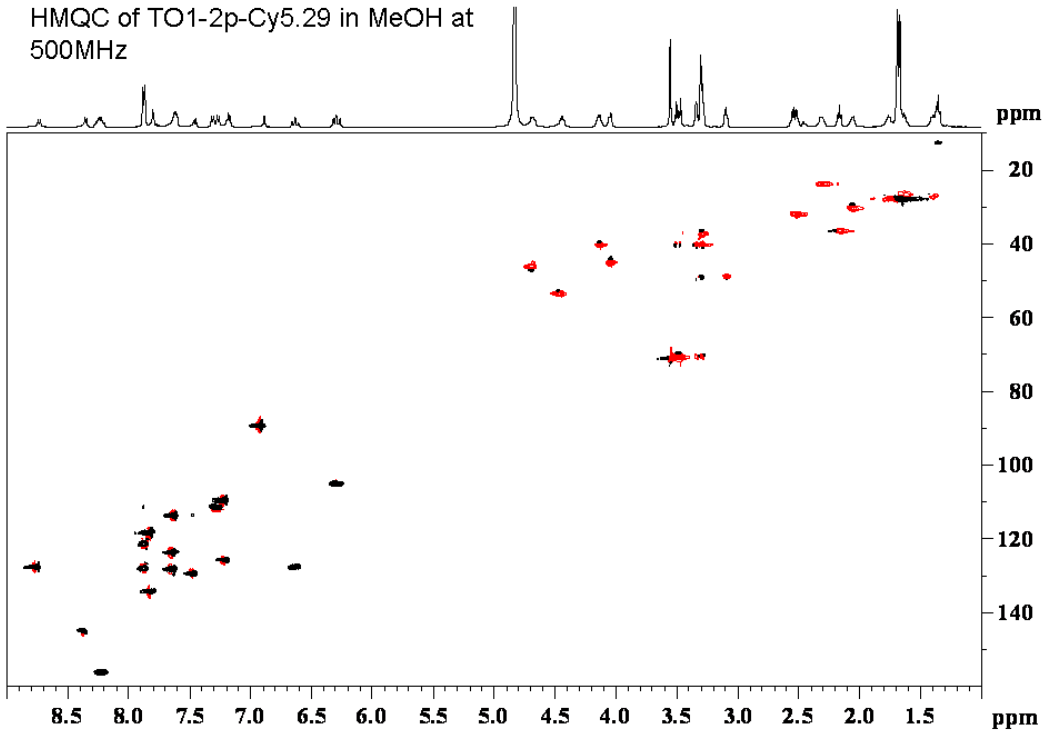


Figure S7

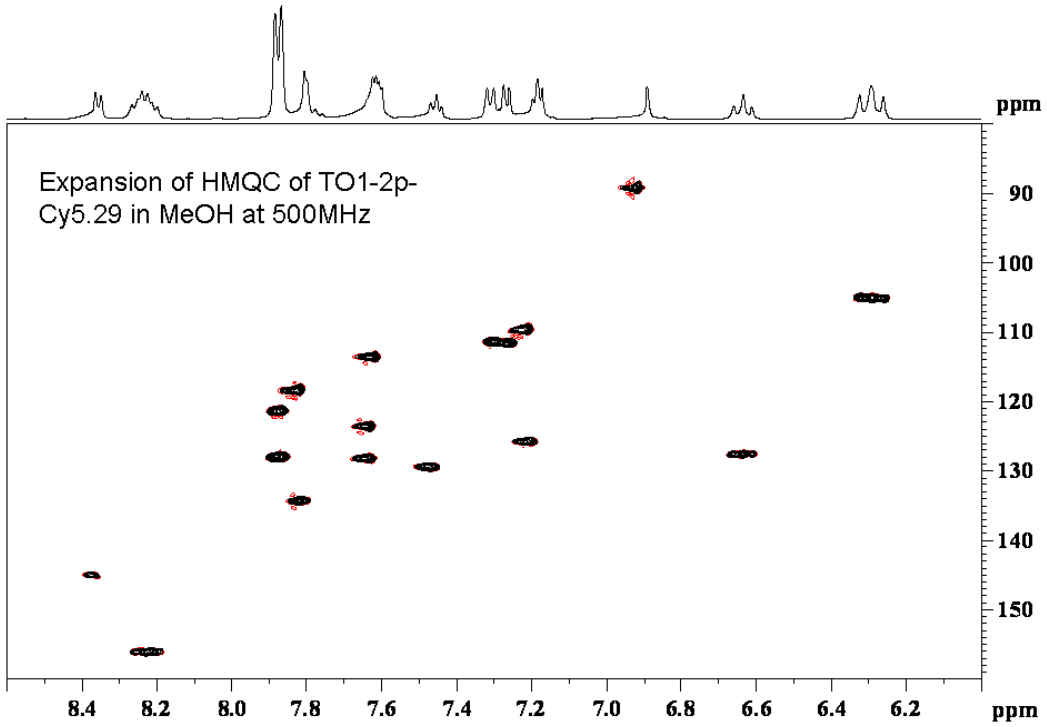


Figure S8

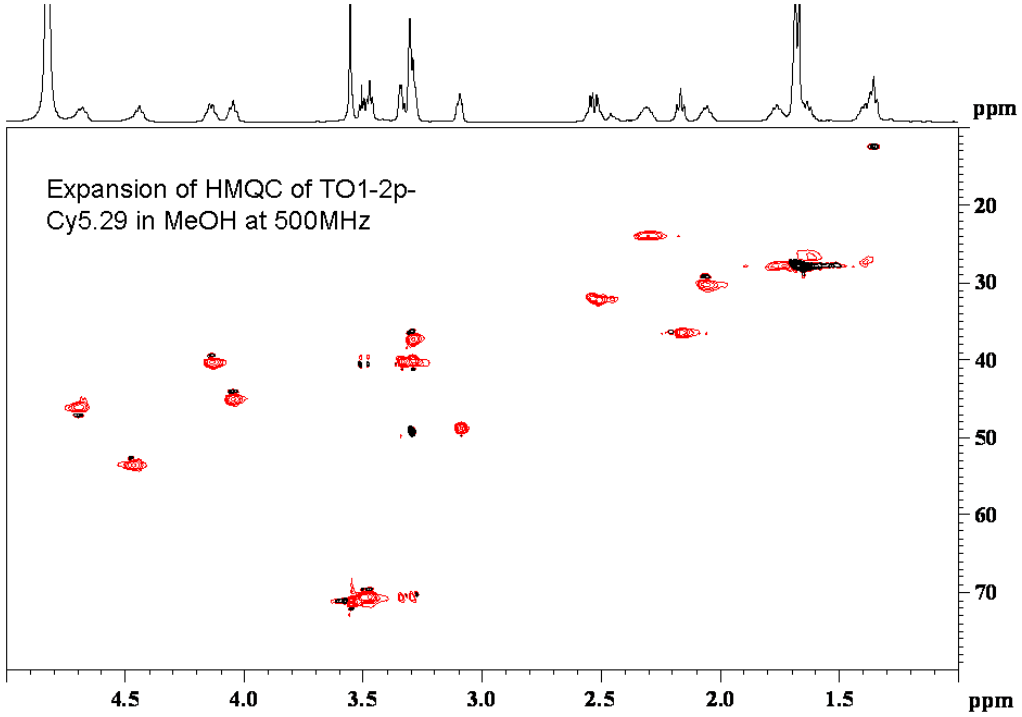


Figure S9

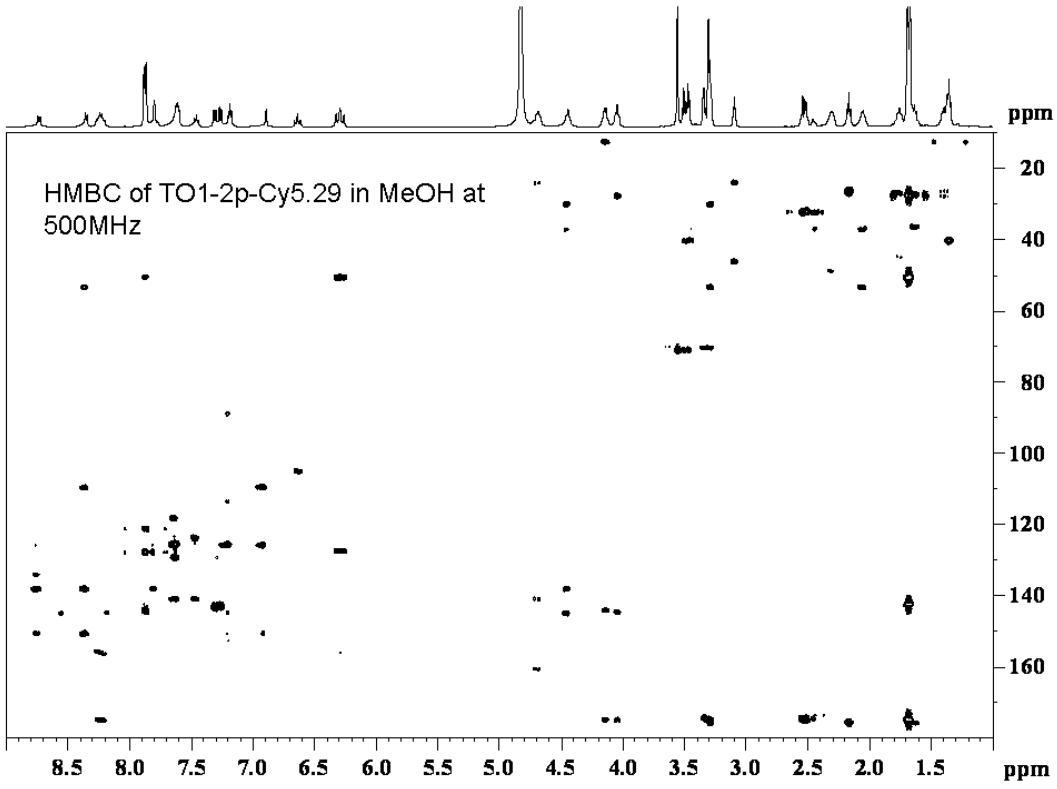


Figure S10

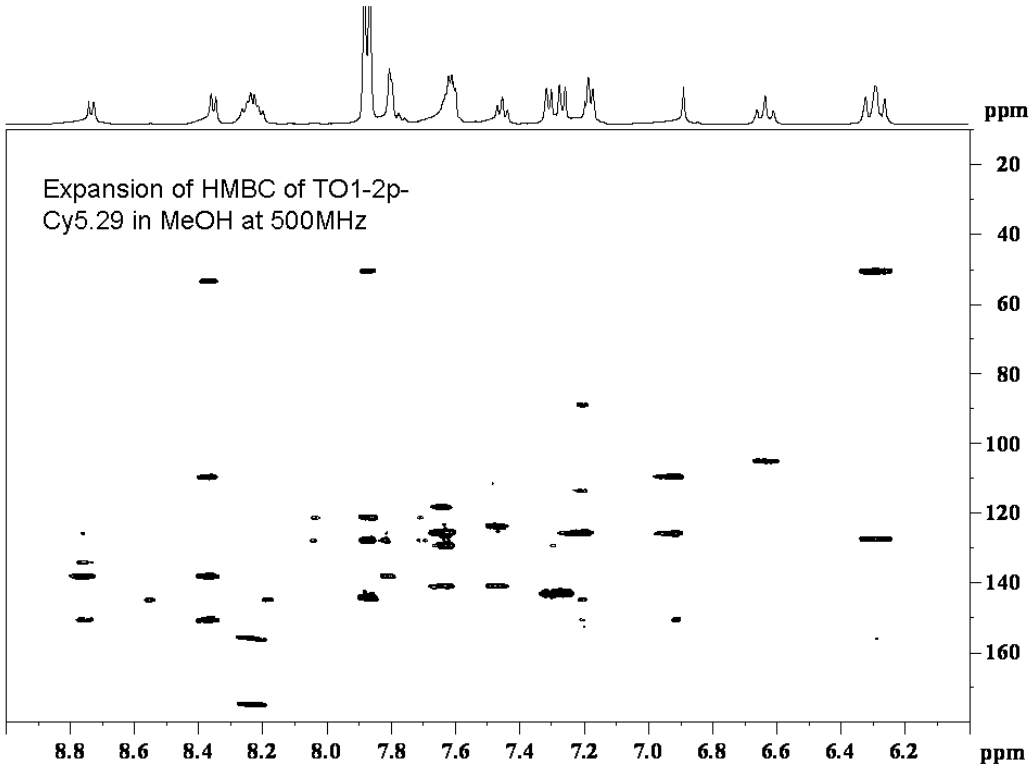


Figure S11

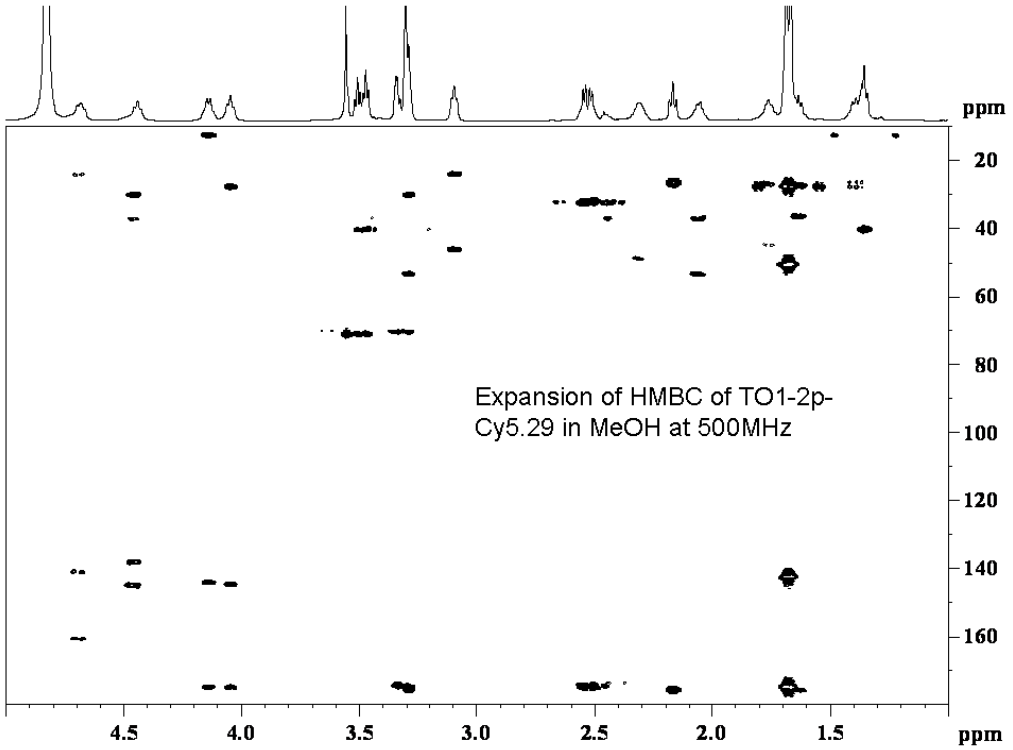
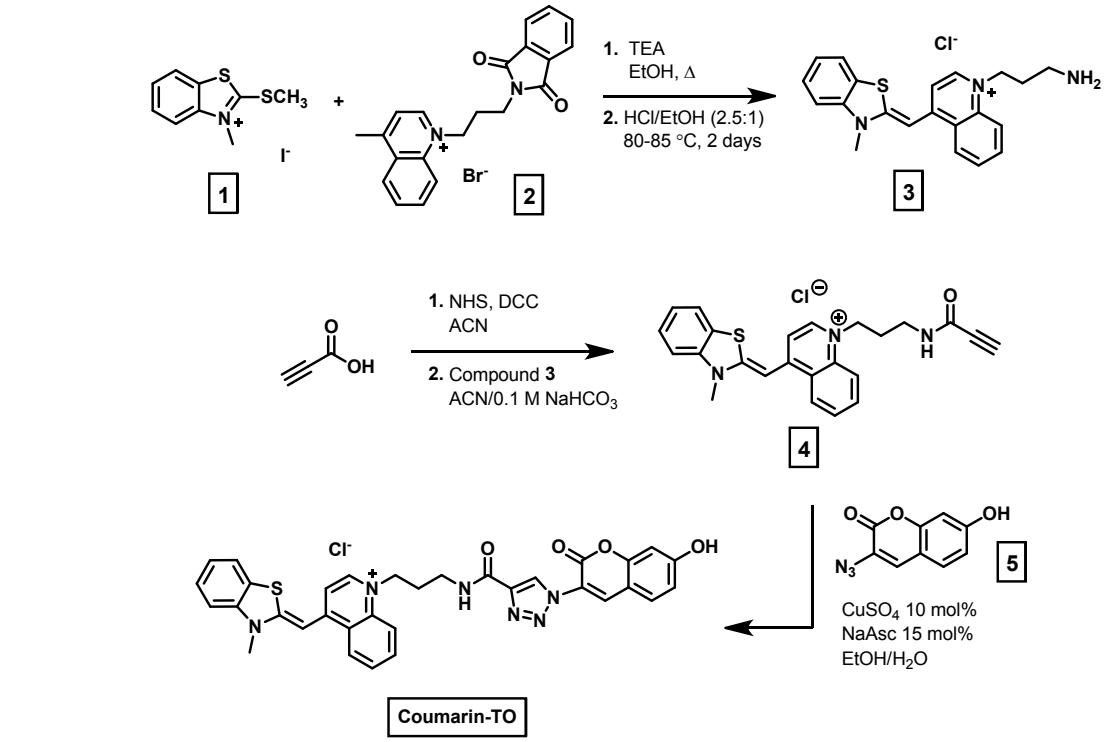
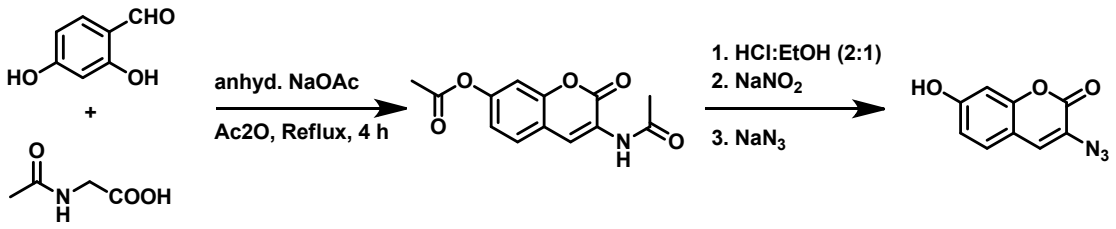


Figure S12

2. Synthesis of Coumarin-TO and Monochromophoric Model Compounds

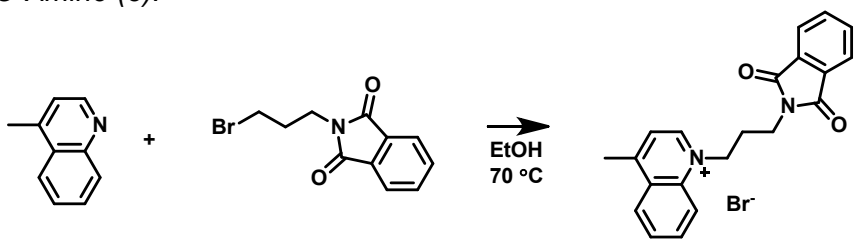
Scheme S2. Synthesis of Coumarin-TO



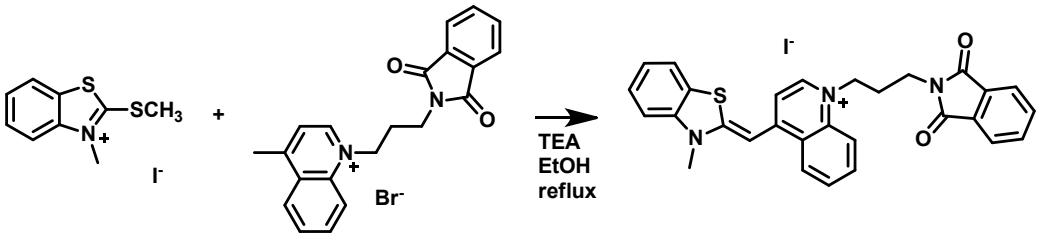


*Synthesis of 3-azido-7-hydroxycoumarin (**5**).* Following the procedure described by Wang et al.,[1] 3-azido-7-hydroxy coumarin was synthesized on a 50 mmol scale. The product was isolated as a brown solid in 13% yield (1.3 g). ^{1}H NMR (300 MHz, CD_3OD) δ: 7.38 (d/s overlapped, *J* = 8.5 Hz, 2H), 6.79 (dd, *J* = 2.2. 8.6 Hz, 1H), 6.72 (d, *J* = 2.4 Hz, 1H).

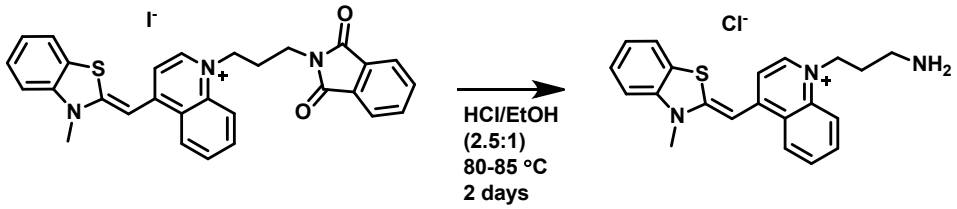
Synthesis of TO-Amine (3):



Synthesis of the lepidinium phthalimide protected amine. Lepidine (1.6 mL, 12 mmol) and bromopropylphthalimide (4.83 g, 18 mmol) were heated in a minimal amount of ethanol to 70 °C overnight. The reaction was cooled to RT and cold ether was added. The resulting crude brown solid was collected and washed several times with ether. The crude solid was dissolved with a mixture of DCM/MeOH (~1:1), triterated with ether, and placed on ice. The light beige solid was collected to give the pure product in 53% yield (2.62 g). ^{1}H NMR (MeOD, 500 MHz) δ: 9.33 (d, J = 6.0 Hz, 1H), 8.59-8.54 (m, 2H), 8.28 (ddd, J = 9.0, 7.0, 1.4 Hz, 1H), 8.07 (ddd, J = 8.9, 7.0, 0.7 Hz, 1H), 8.00 (d, J = 6.1 Hz, 1H), 7.90-7.87 (m, 2H), 7.87-7.82 (m, 2H), 5.15 (t, J = 7.4 Hz, 2H), 3.91 (t, J = 6.4 Hz, 2H), 3.06 (s, 3H), 2.56-2.49 (m, 2H).

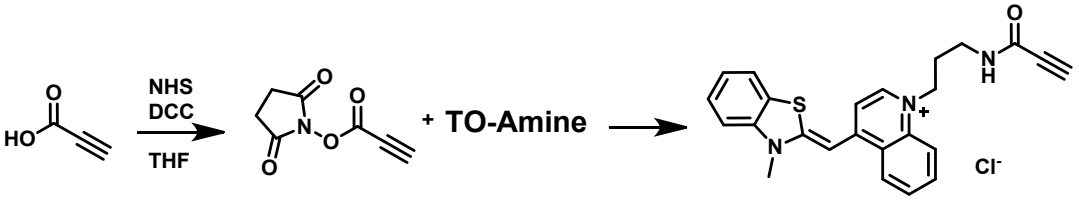


Synthesis of TO-Am intermediate. Lepidinium phthalimide protected amine (2.62 g, 6.37 mmol) and N-methyl-2(methylthio)benzothiazolium (3.95 g, 12.2 mmol) were suspended in EtOH (50 mL) and heated to reflux. Triethylamine (0.95 mL, 6.8 mmol) was added dropwise. After heating overnight, the reaction was cooled to RT and cold diethylether was added. The resulting red/orange precipitate was collected, washed several times with cold ether and dried. Analysis by 1H NMR shows this solid to be the pure product in 97% yield (3.73 g). ^{1}H NMR (MeOD, 300 MHz) δ: 8.62 (dd, J = 1.3, 8.6 Hz, 1H), 8.47 (d, J = 7.1 Hz, 1H), 8.06 (d, J = 8.7 Hz, 1H), 8.00-7.90 (m, 2H), 7.86-7.75 (m, 4H), 7.75-7.67 (m, 2H), 7.66-7.59 (m, 1H), 7.47-7.40 (m, 2H), 6.87 (s, 1H), 4.69 (t, J = 7.2 Hz, 2H), 4.01 (s, 3H), 3.86 (t, J = 6.5 Hz, 2H), 2.46-2.35 (m, 2H). LRMS (ESI$^+$, MeOH) m/z: $[C_{29}H_{24}N_3O_2S]^+$ calcd. 478.2, found 478.3.



*Removal of the phthalimide protecting group to give TO-Amine (**3**).* Phthalimide protected TO-Am (512 mg, 0.85 mmol) was dissolved in a mixture of concentrated HCl (25 mL) and EtOH (10 mL). The reaction was then heated to 80-85 °C for two days. After cooling, the solvent was removed under reduced pressure. The red/orange residue was dissolved with minimal solvent, a mixture of DCM/MeOH (~1:1), and EtOAc was added until a red/orange solid precipitated. This solid was collected, washed several times with EtOAc and allowed to dry. Analysis by ^{1}H NMR revealed this to be the pure product, **TO-Amine (3)**, in 98% yield (350 mg). ^{1}H NMR (MeOD, 500 MHz) δ: 8.66 (d, J = 8.4 Hz, 1H), 8.45 (d, J = 7.2 Hz, 1H), 8.08 (d, J = 8.7 Hz, 1H), 7.98 (t, J = 7.7 Hz, 1H), 7.90 (d, J = 7.9 Hz, 1H), 7.75 (t, J = 7.8 Hz, 1H), 7.67 (d, J = 8.2 Hz, 1H), 7.61 (t, J = 7.7 Hz, 1H), 7.48-7.40 (m, 2H), 6.93 (s, 1H), 4.69 (t, J = 7.5 Hz, 2H), 4.01 (s, 3H), 3.15 (t, J = 7.7 Hz, 2H), 2.36-2.28 (m, 2H). ^{13}C NMR (MeOD, 125 MHz) δ: 162.8, 151.0, 144.9, 142.1, 138.8, 134.8, 129.7, 128.4, 127.0, 126.3, 126.2, 126.0, 123.9, 118.8, 114.1, 109.8, 89.9, 52.8, 38.1, 34.4, 28.5. LRMS (ESI^{+}, MeOH) m/z: $[C_{21}H_{22}N_3S]^{+}$ calcd. 348.2, found 348.3.

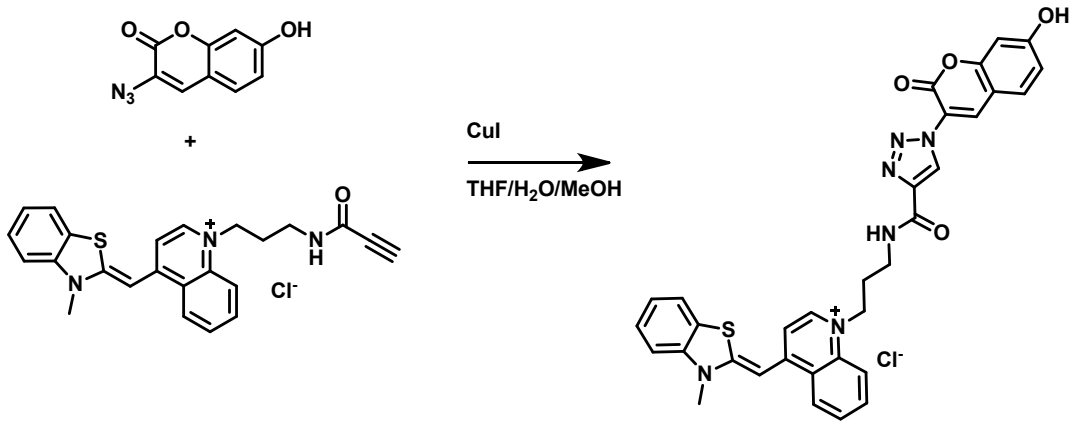
Synthesis of TO-Alkyne (4):



4

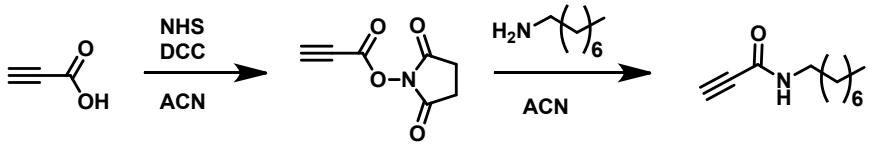
In a dry flask, propiolic acid (0.5 mL, 8 mmol) and N-hydroxysuccinimide (0.92 g, 8 mmol) were dissolved in anhydrous THF (20 mL). DCC (1.65 g, 8 mmol) was dissolved with THF (10 mL) and added dropwise to the propiolic acid/NHS solution. After stirring overnight, the white precipitate was filtered off and the solvent removed to give a light yellow oil. This oil was dissolved with acetonitrile (2 mL) and filtered through a cotton plug to remove residual solids. This solution was added to dissolved TO-Am (175 mg, 0.4 mmol) in a H_2O/ACN (1:1, 10 mL). After stirring for overnight, the solvent was removed via rotary evaporation. The reaction was then dissolved with MeOH, dried onto silica, and loaded onto a silica column with DCM. The product was eluted with increasing MeOH (up to 25%) in DCM. The fractions containing the product were identified by ESI-MS; however they also contained a higher molecular weight impurity. The tubes containing the product and this impurity were combined and the solvent removed. Once dried down, this higher molecular weight purple impurity was dissolved in acetone and decanted off to leave behind a red/orange solid. This solid was the pure TO-Alkyne product in 10% yield (21 mg). ^{1}H NMR (DMSO-d6, 500 MHz) δ: 8.92 (J = 5.3 Hz, 1H), 8.81 (t, J = 8.3 Hz, 1H), 8.64 (d, J = 7.3 Hz, 1H), 8.14 (d, J = 8.7 Hz, 1H), 8.06 (d, J = 7.8 Hz, 1H), 8.00 (t, J = 7.8 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.76 (t, J = 7.7 Hz, 1H), 7.62 (t, J = 7.8 Hz, 1H), 7.58 (t, J = 7.6 Hz, 1H), 7.37 (d, J = 7.2 Hz, 1H), 6.95 (s, 1H), 4.62 (t, J = 7.2 Hz, 2H), 4.18 (s, 1H), 4.03 (s, 3H), 3.23 (q, J = 6.4 Hz, 2H), 2.07-1.99 (m, 2H). ^{13}C NMR (DMSO-d6, 125 MHz) δ: 160.2, 151.8, 148.6, 144.4, 140.5, 137.0, 133.2, 128.2, 126.7, 125.9, 124.5, 124.3, 123.9, 122.9, 118.0, 113.0, 107.8, 88.2, 78.2, 75.9, 51.9, 36.2, 33.8, 28.2. LRMS (ESI^{+}, MeOH) m/z: $[C_{24}H_{22}N_3OS]^{+}$ calcd. 400.2, found 400.3.

Synthesis of Coumarin-TO:



TO-EDA (36.5 mg, 0.08 mmol) and 3-azido-7-hydroxycoumarin (20.3 mg, 0.1 mmol) were combined in EtOH (1.2 mL). Separately, $CuSO_4$ hexahydrate (2 mg, 0.008 mmol, 10 mol%) and sodium ascorbate (3 mg, 0.012 mmol, 15 mol%) were dissolved in water (1.2 mL) and mixed until a bright yellow color was produced. This copper(I) solution was then added to the ethanol solution containing the azide and alkyne. After stirring overnight, ESI (MeOH, pos) of an aliquot showed only the clicked product (603.2 m/z). The reaction was transferred to a larger RB with MeOH and silica gel was added and the solvent removed. The crude product being absorbed on the silica gel was loaded onto a larger silica column with DCM. Increasing MeOH in DCM (up to 20%) eluted the product. The fractions containing the product were combined and the solvent removed to give the compound in 70% yield (36 mg). ^{1}H NMR (500 MHz, DMSO-d6) δ: 11.01 (bs, 1H), 8.89 (s, 1H), 8.84 (t, J = 5.7 Hz, 1H), 8.79 (d, J = 8.0 Hz, 1H), 8.67 (d, J = 7.2 Hz, 1H), 8.62 (s, 1H), 8.15 (d, J = 8.75 Hz, 1H), 8.04-7.96 (m, 2H), 7.79-7.70 (m, 3H), 7.56 (t, J = 8.4 Hz, 1H), 7.38 (t, J = 7.6 Hz, 1H), 7.29 (d, J = 7.20 Hz, 1H), 6.93 (dd, J = 8.6, 2.2 Hz, 1H), 6.90 (s, 1H), 6.83 (d, J = 2.0 Hz, 1H), 4.70 (t, J = 6.8 Hz, 2H), 4.00 (s, 3H), 3.47 (q, J = 6.1 Hz, 2H), 2.22-2.15 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d6) δ: 163.3, 160.5, 159.9, 156.6, 155.2, 149.0, 145.0, 143.1, 140.9, 137.5, 137.2, 133.7, 131.6, 128.6, 127.5, 127.2, 126.4, 124.9, 124.8, 124.3, 123.3, 119.2, 118.6, 114.9, 113.4, 110.6, 108.3, 102.7, 88.6, 53.0, 36.7, 34.3, 28.7. ESI-MS (MeOH, pos) $[C_{33}H_{27}N_6O_4S]^+$ calcd 603.18, found 603.2.

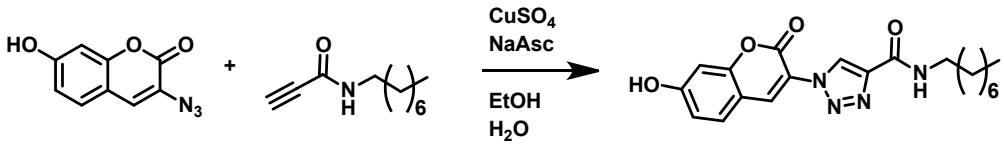
Synthesis of Coumarin Monochromophore



Synthesis of N-octyl propynamide. Propiolic acid (62 µL, 1 mmol) and N-hydroxysuccinimide (116 mg, 1 mmol) were dissolved in ACN (2 mL). *N,N'*-Dicyclohexylcarbodiimide (206 mg, 1 mmol) was separately dissolved in ACN (3 mL) and added dropwise to the propiolic acid/NHS

mixture. This mixture was stirred overnight in the dark under argon. The white solid was then filtered off and the filtrate concentrated to give a yellow oil. The propiolic NHS ester was used without further purification.

The yellow oil was dissolved in a mixture of ACN (1mL) and 0.1M $NaHCO_3$ (1mL). Octylamine (182 µL, 1.1 mmol) was added drop wise and the reaction stirred for 10 h. The solvent was then removed to give a yellow oily/solid. The crude N-octyl propynamide was then dissolved in methanol, dried onto silica gel and loaded on top of a silica column with DCM. The product was eluted with increasing EtOAc (up to 5%) in DCM. The product was visualized by TLC stained with potassium permanganate. Upon removal of the solvent, the product was isolated as a clear oil in 34% yield (61 mg). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 5.83 (bs, 1H), 3.36-3.26 (m, 2H), 2.77 (s, 1H), 1.60-1.47 (m, 2H), 1.40-1.21 (m, 10H), 0.89 (t, J = 6.7 Hz, 3H). ^{13}C NMR (CD_3OD, 300 MHz) δ: 154.8 78.4, 75.6, 40.8, 33.1, 30.5, 30.5, 30.2, 28.1, 23.8, 14.6.



CuAAC reaction of 3-azido-7-hydroxycoumarin and N-octyl propynamide. N-octyl propynamide (61 mg, 0.374 mmol) and 7-azido-3-hydroxycoumarin (69 mg, 0.34 mmol) were dissolved in ethanol (5 mL). In a separate vessel, copper sulfate pentahydrate (8 mg, 0.034 mmol, 10 mol%) and sodium ascorbate (10mg, 0.051 mmol, 15 mol%) were dissolved in H_2O (5 mL) and mixed until the solution had turned a bright yellow color. This solution was added to the ethanol mixture and allowed to stir overnight in the dark. The ethanol was then removed and addition water was added. The resulting dark yellow solid was collected and dried. This was then dissolved in MeOH, dried onto silica gel, and loaded onto a silica gel column with DCM. The product was eluted with 5% MeOH in DCM to give a pale yellow solid in 68% (89 mg). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.95 (s, 1H), 8.56 (s, 1H), 7.66 (d, J = 8.6 Hz, 1H), 6.91 (dd, J = 2.3, 8.6 Hz, 1H), 6.83 (d, J = 2.3 Hz, 1H), 3.41 (t, J = 7.2 Hz, 2H), 1.68-1.60 (m, 2H), 1.46-1.26 (m, 10H), 0.90 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ: 164.9, 162.3, 158.2, 156.9, 144.2, 137.7, 132.2, 127.9, 120.6, 115.9, 112.0, 103.6, 40.5, 33.1, 30.7, 30.6, 30.5, 28.2, 23.9, 14.6. ESI-MS (MeOH with 1% TFA): $[M+H]^+$ calc 385.2 found 385.1, $[M+Na]^+$ calc 407.2 found 407.1, $[2M+H]^+$ calc 769.4, found 769.0, (major ion) $[2M+Na]^+$ calc 791.4, found 790.9, $[3M+Na]^+$ calc 1175.5, found 1174.6.

1. Sivakumar, K.; Xie, F.; Cash, B. M.; Long, S.; Barnhill, H. N.; Wang, Q., A Fluorogenic 1,3-Dipolar Cycloaddition Reaction of 3-Azidocoumarins and Acetylenes. *Org. Lett.* **2004,** *6* (24), 4603-4606.

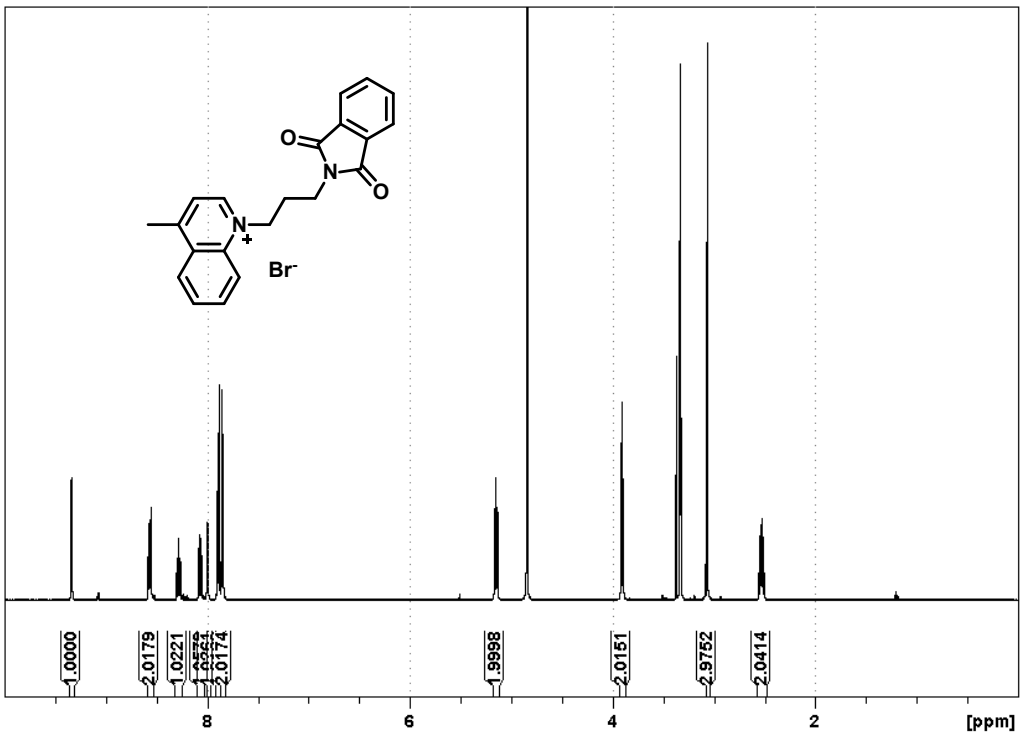


Figure S13. ^{1}H NMR (MeOD, 500 MHz) of lepidinium with the phthalimide protected amine arm.

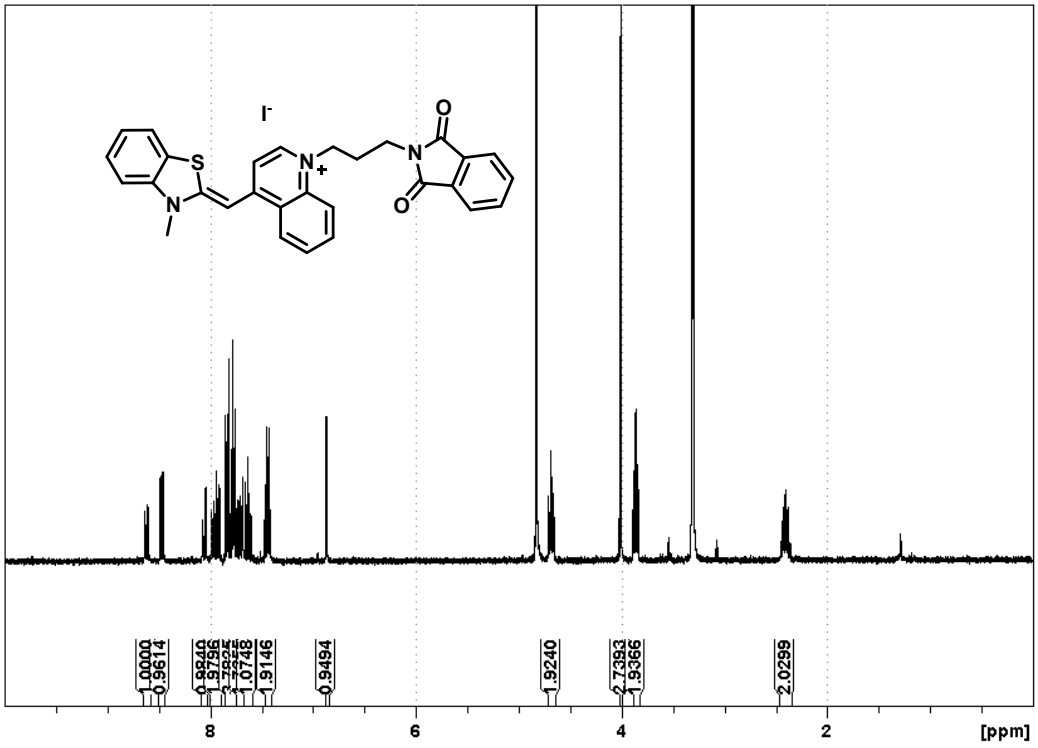


Figure S14. ^{1}H NMR (MeOD, 300 MHz) of phthalimide protected TO-Am.

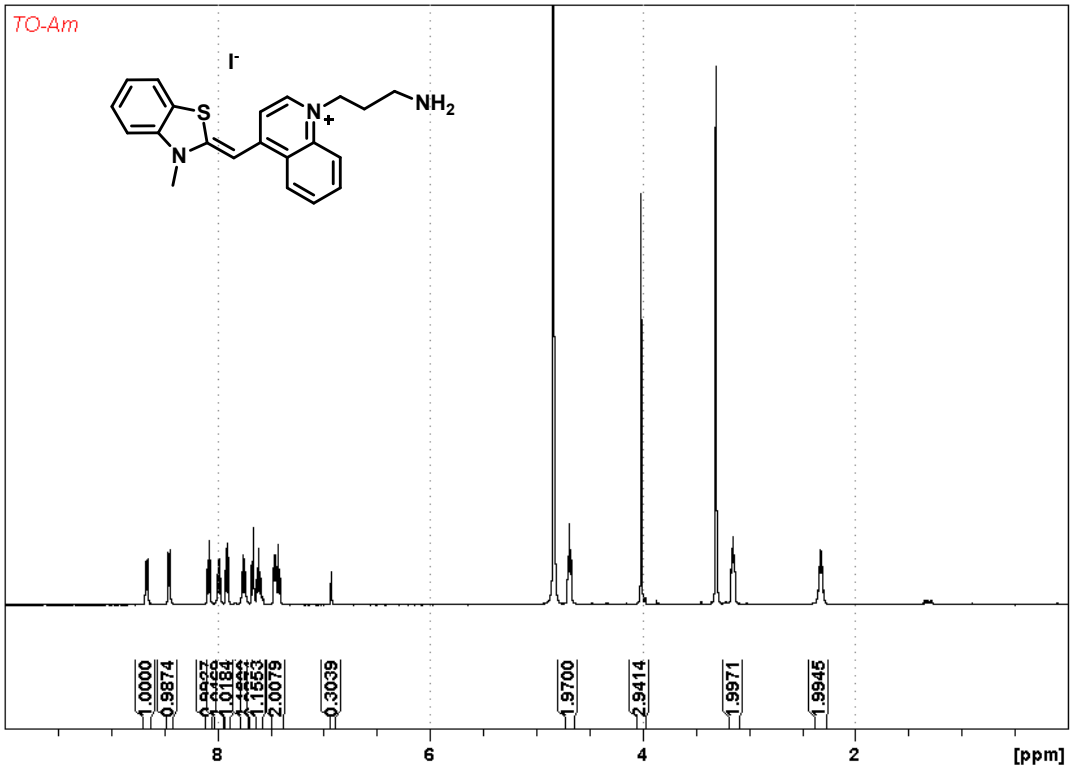


Figure S15. ^{1}H NMR (MeOD, 500 MHz) of TO-Am.

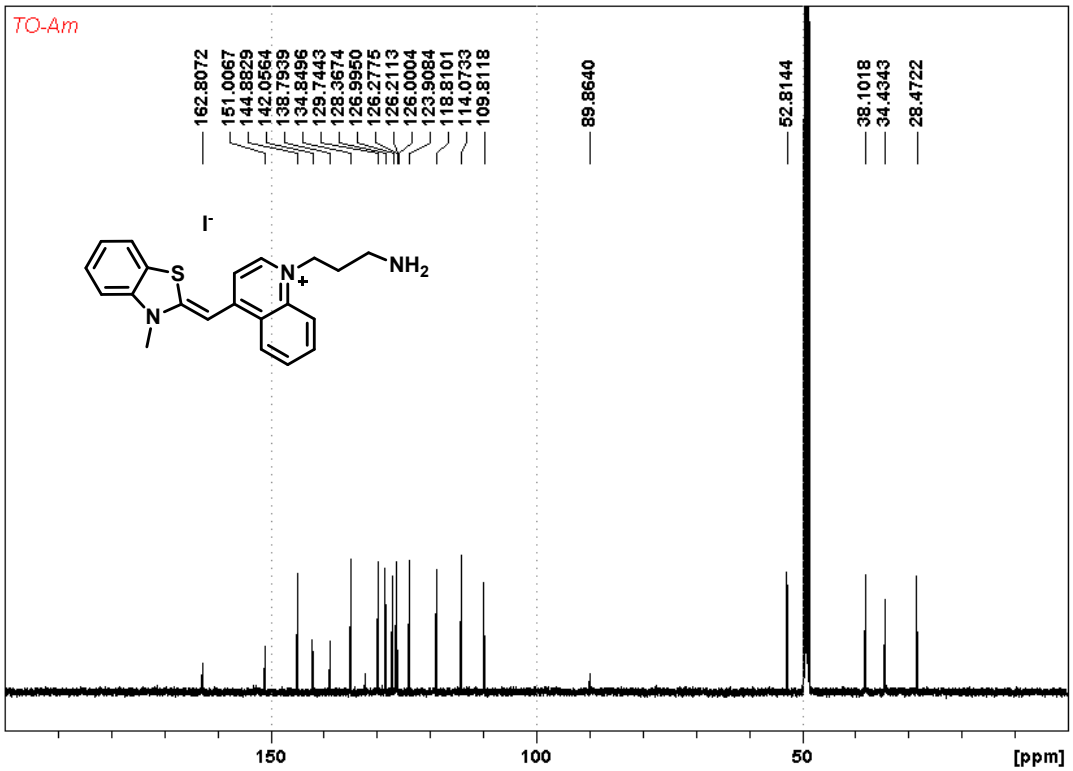


Figure S16. ^{13}C NMR (MeOD, 125 MHz) of TO-Am.

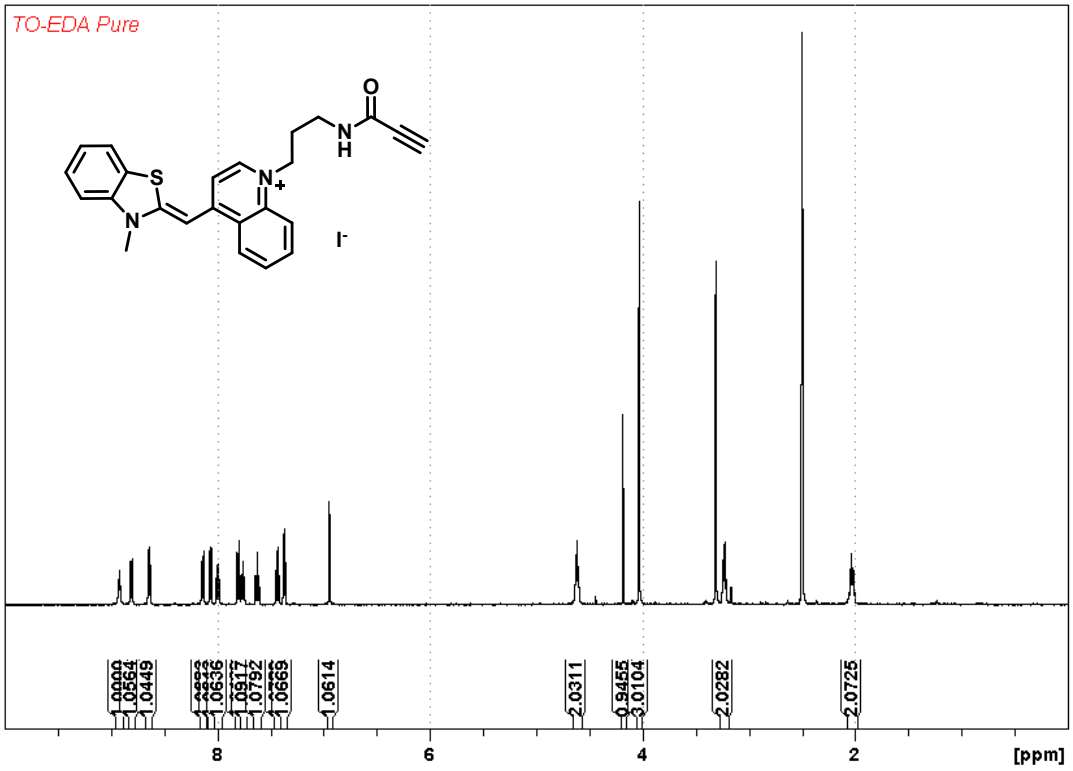


Figure S17. ^{1}H NMR (MeOD, 500 MHz) of TO-EDA.

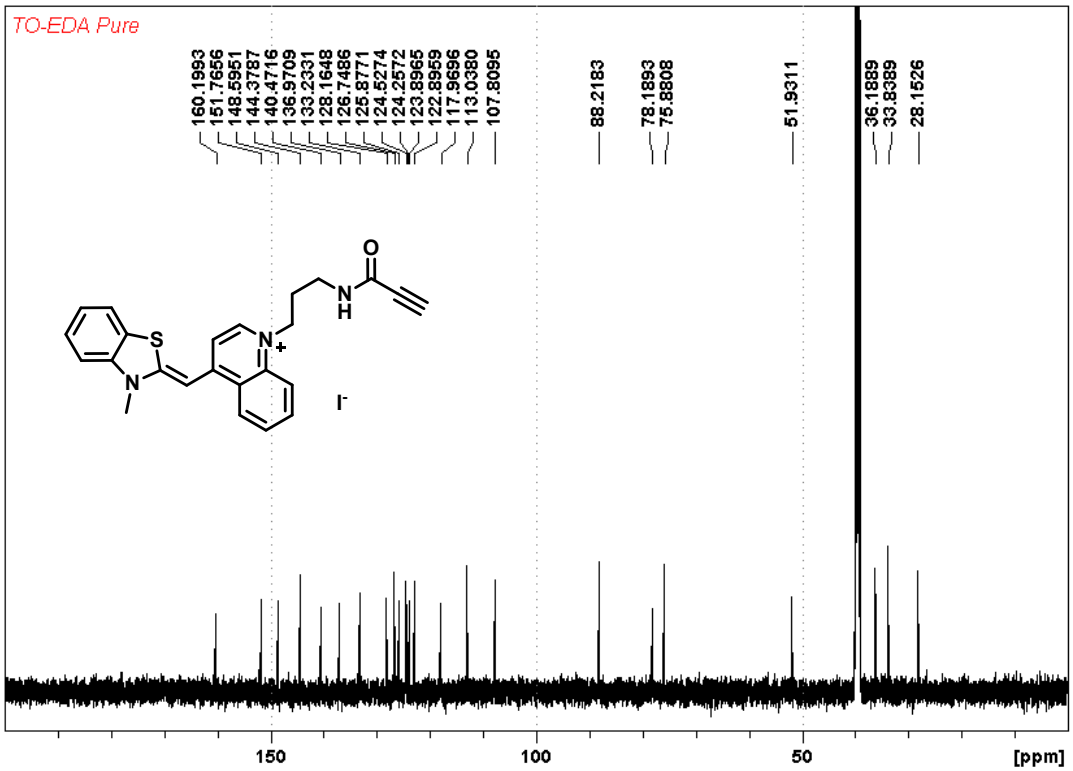


Figure S18. ^{13}C NMR (MeOD, 125 MHz) of TO-EDA.

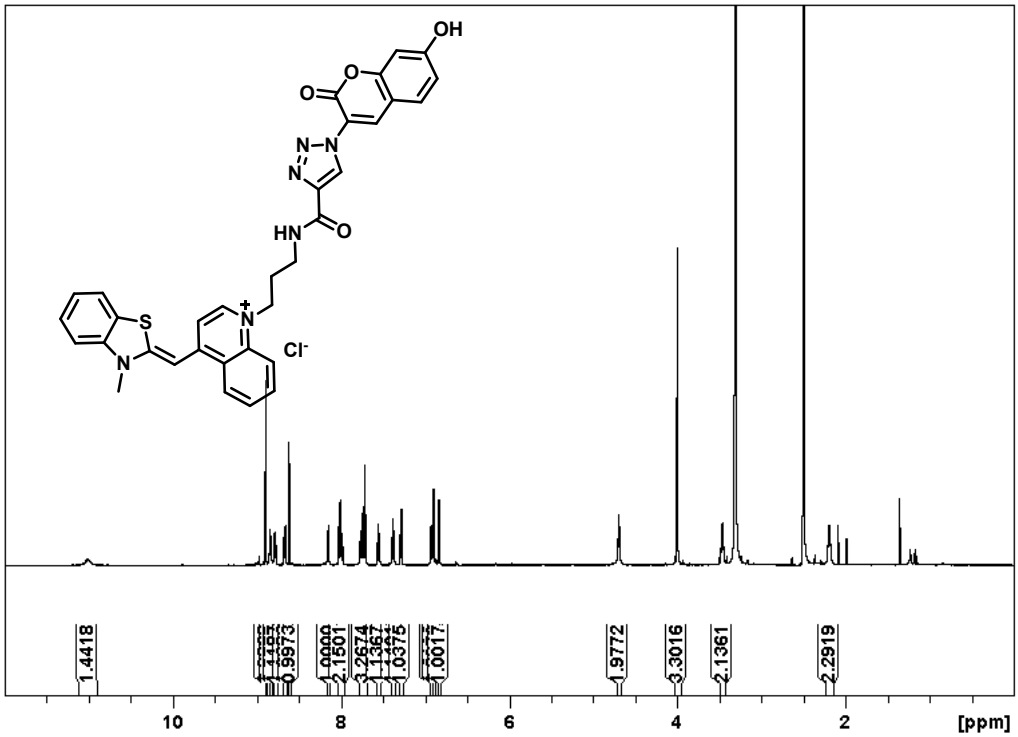


Figure S19. ^{1}H NMR (500 MHz, CD_3OD) of Coumarin_TO.

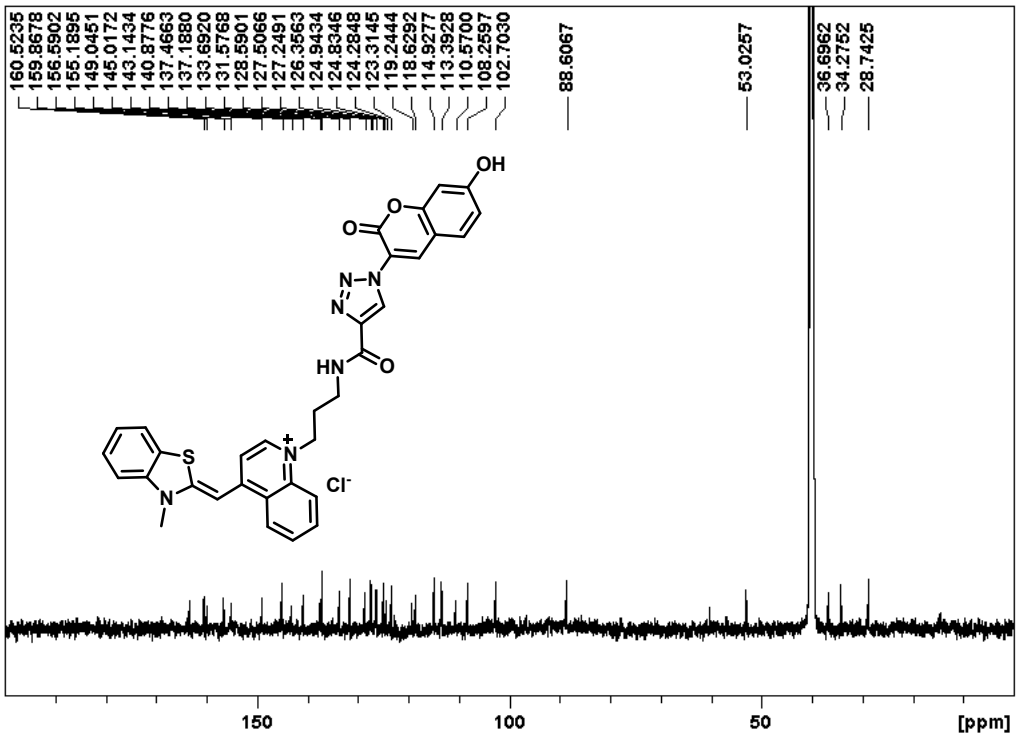


Figure S20. ^{13}C NMR (125 MHz, CD_3OD) of Coumarin_TO.

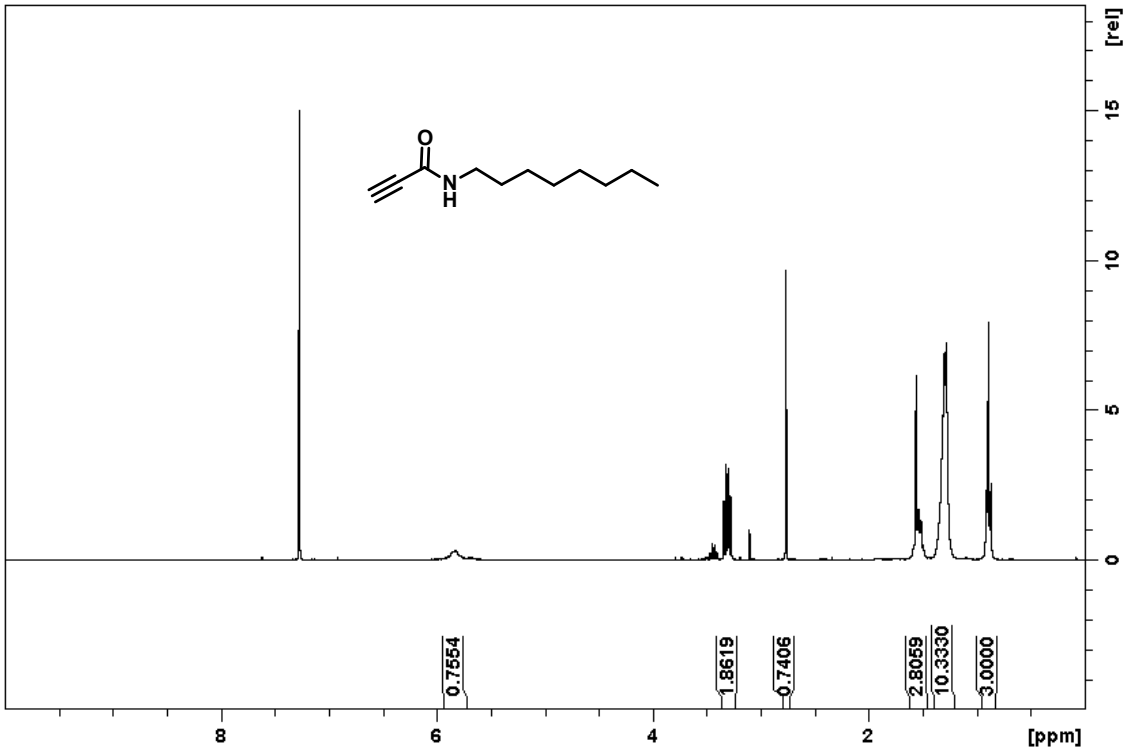


Figure S21. ^{1}H NMR (300 MHz, $CDCl_3$) of N-octyl acetylamide.

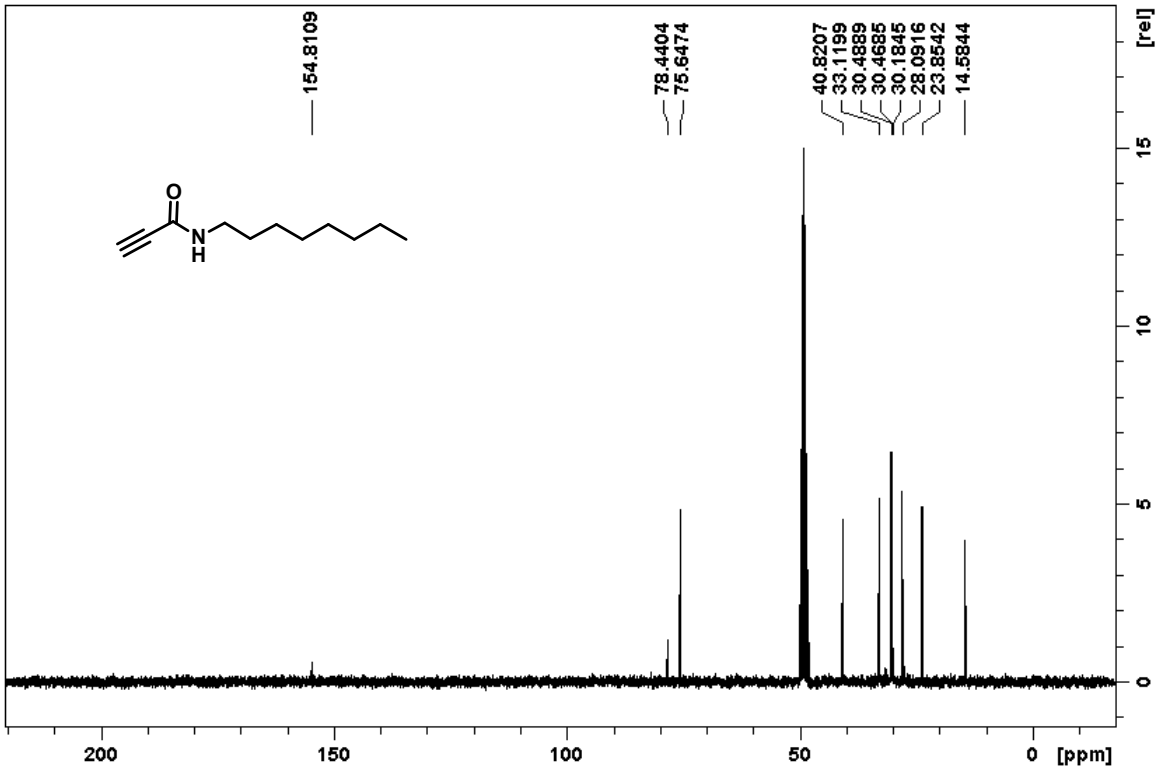


Figure S22. ^{13}C NMR (300 MHz, CD_3OD) of N-octyl acetylamide.

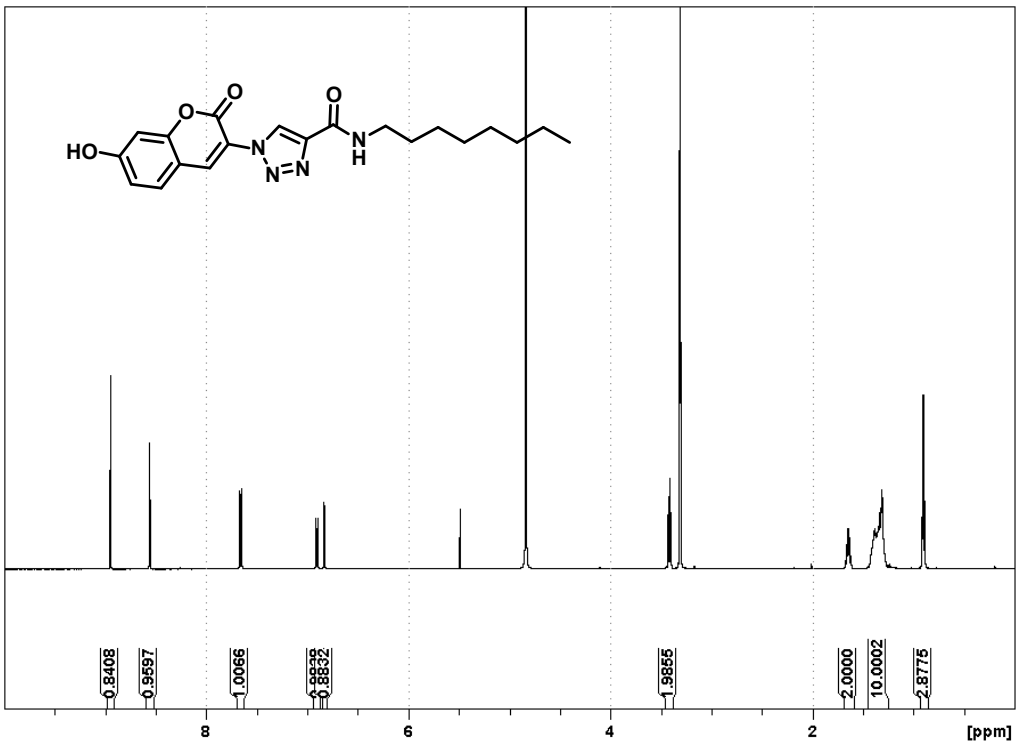


Figure S23. ^{1}H NMR (500 MHz, CD_3OD) of the coumarin control.

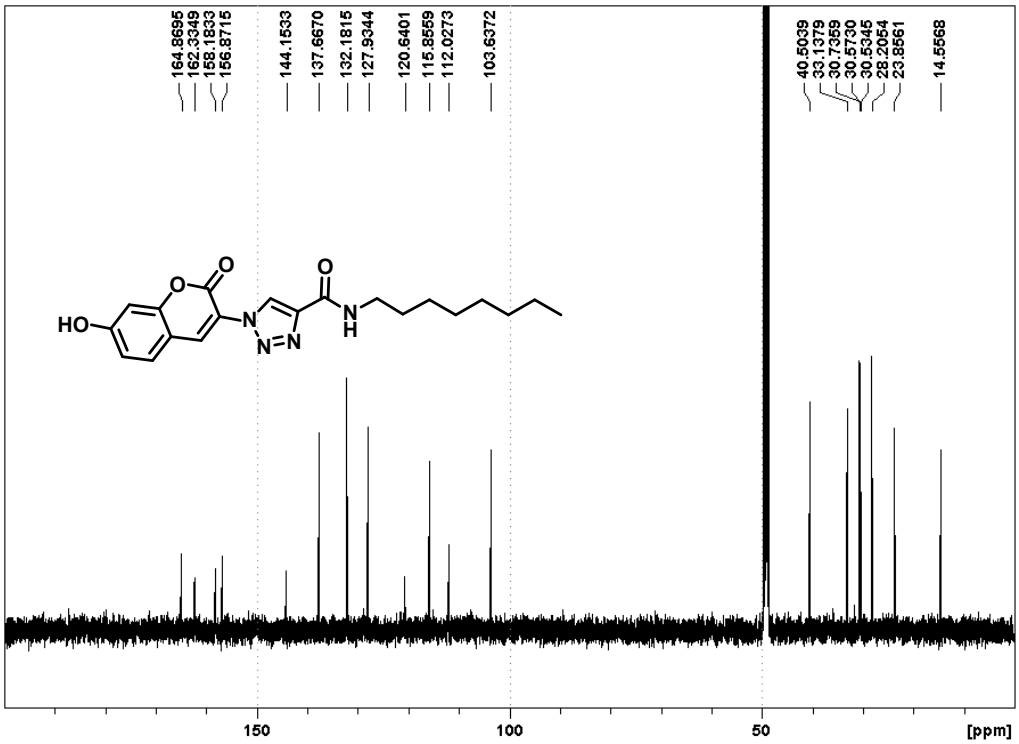


Figure S24. ^{13}C NMR (125 MHz, CD_3OD) of the coumarin control.

FAP Amino Acid Sequences

$(Gly_4Ser)_3$ linker separating V_H and V_L domains of scFv underlined.

R1

QVQLVQSGAEVKKPGASVKVSCKVSGYTPTEFSIHWVRQAPGKGFEWMGNFDPGDGETIYQ
KFQGRVALTEDTSTDTAYMELTSLTSEDTAVYYCAIDLAPGWGPGTLVTVSSGILGSGGGGSG
GGGSGGGGSNFMLTQPHSVSESPGKTVTISCTGSSGSIASDSVQWYQQRPGSAPTTVIFEDN
QRPSGVPDRFSGSIDSSSNSASLTISGLKTEDEADYYCQSYDRSNHVVFGGGTQLTVLS

HL1.0.1-TO1

QVQLVESEAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGGTIPIFGTADYAQ
EFQGRVTITTDESTSTAYMELSGLRSEDTAVYYCVLLGTTMVTGHYFDYWGQGTLVTVSSGIL
GSGGGGSGGGGSGGGGSNFMLTQPPSASGTPGQSVTISCSGSGSNIGNNKVNWYQQLPGT
APKLLIYSNNQRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDGLSGYVFGTGTK
LTVL

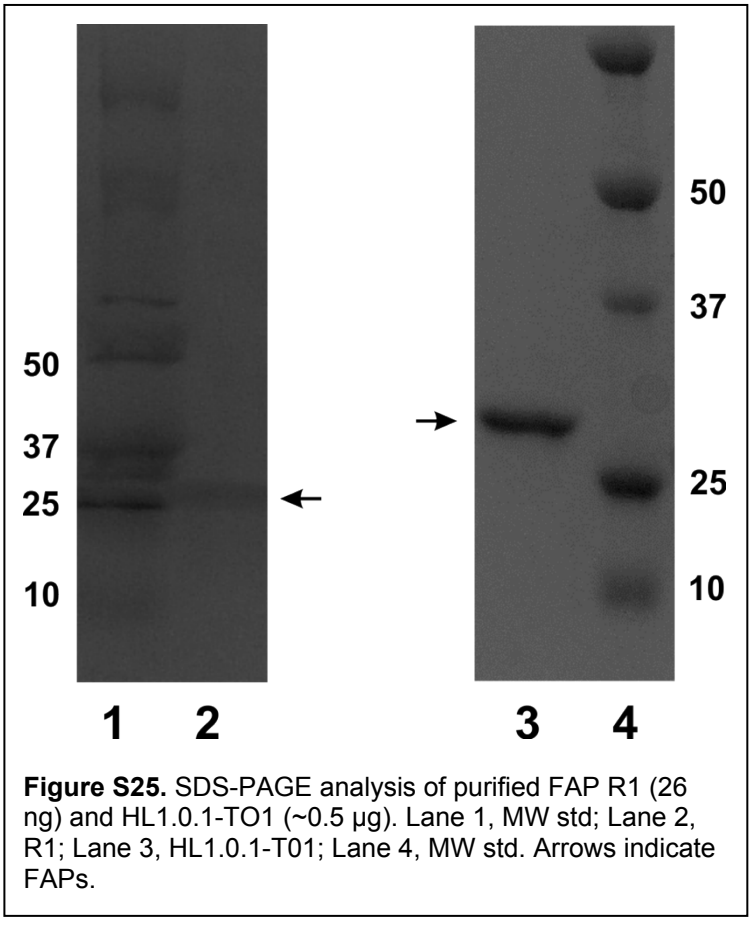


Figure S25. SDS-PAGE analysis of purified FAP R1 (26 ng) and HL1.0.1-TO1 (~0.5 µg). Lane 1, MW std; Lane 2, R1; Lane 3, HL1.0.1-T01; Lane 4, MW std. Arrows indicate FAPs.

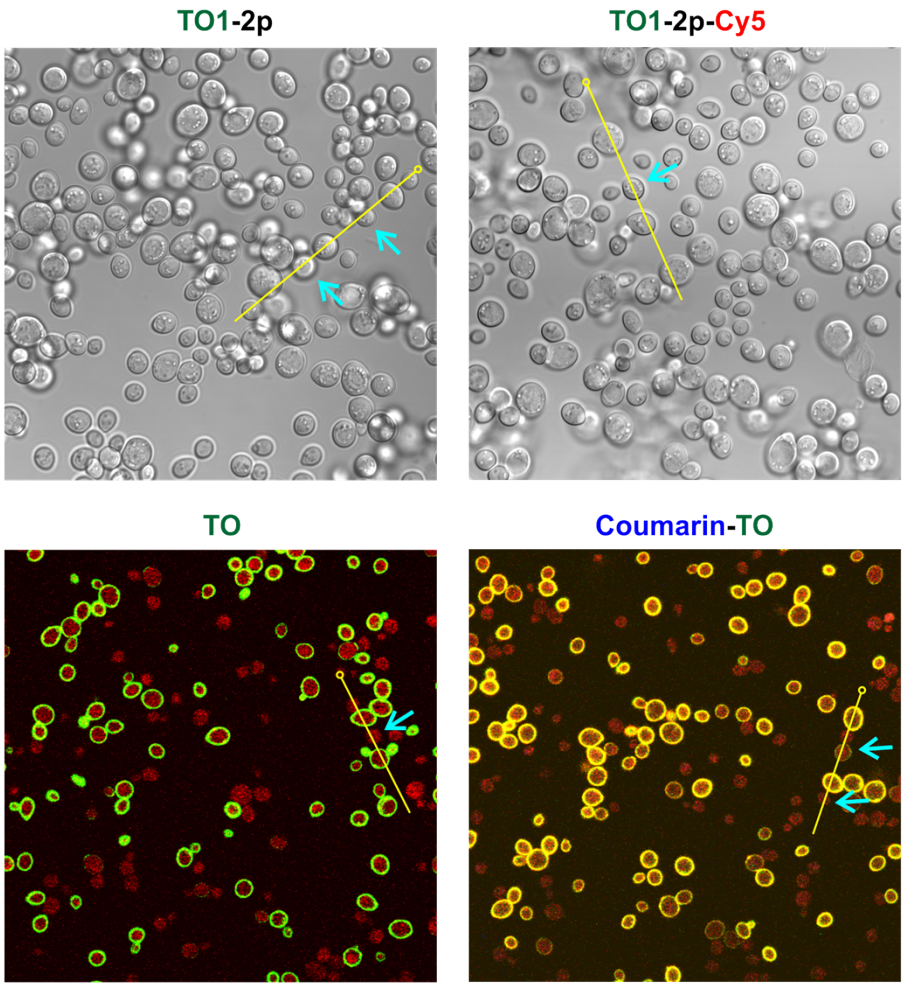


Figure S26. Localization of non-fluorescent yeast cells within confocal images. Top, DIC control panels for main text Figure 4 labeled according to fluorogen used. Blue arrows along scan profile transects from Figure 4 mark cells that are completely non-fluorescent at 100 nM fluorogen concentration due to absence of plasmid dependent expression of FAP. Bottom, control panels for main text Figure 8. Fluorogenic dyes at 10 nM generate sufficient signal to permit localization of cells that do not express FAP if images are digitally brightened. Blue arrows mark dark cells along Figure 8 scan profile transects. Note that FAP-independent background fluorescence localizes within cells rather than at the cell wall.

Table S2. Imaging Parameters on Zeiss LSM510 Confical microscope

Name	R1 FAP Coumarin_TO	R1 FAP TO	HL1.0.1 FAP TO1-Cy5	HL1.0.1 FAP TO1
Scaling X	0.279 µm	0.279 µm	0.088 µm	0.088 µm
Scaling Y	0.279 µm	0.279 µm	0.088 µm	0.088 µm
Dimensions	x: 512, y: 512, channels: 3, 16-bit	x: 512, y: 512, channels: 3, 16-bit	x: 1024, y: 1024, channels: 4, 12-bit	x: 1024, y: 1024, channels: 4, 12-bit
Image Size	x: 142.57 µm, y: 142.57 µm	x: 142.58 µm, y: 142.58 µm	x: 90.02 µm, y: 89.91 µm	x: 90.02 µm, y: 90.02 µm
Scan Mode	plane	plane	plane	plane
Zoom	1	1	1	1
Objective	Plan-Apochromat 63x/1.40 Oil DIC M27	Plan-Apochromat 63x/1.40 Oil DIC M27	Plan-Apochromat 100x/1.40 Oil M27	Plan-Apochromat 100x/1.40 Oil M27
Pixel Dwell	3.20 µs	3.20 µs	3.20 µs	3.20 µs
Average	line 2	line 2	line 2	line 2
Master Gain	405 Ch2 : 750	405 Ch2 : 750	TO1 Ch2 : 701	TO1 Ch2 : 701
	488 Ch3 : 750	488 Ch3 : 750	TO1 ChD : 280	TO1 ChD : 290
	FRET Ch3 : 750	FRET Ch3 : 750	Cy5 Ch3 : 700	Cy5 Ch3 : 700
			FRET Ch3 : 700	FRET Ch3 : 700
Digital gain	405 Ch2 : 1.00	405 Ch2 : 1.00	TO1 Ch2 : 1.00	TO1 Ch2 : 1.00
	488 Ch3 : 1.00	488 Ch3 : 1.00	TO1 ChD : 1.00	TO1 ChD : 1.00
	FRET Ch3 : 1.00	FRET Ch3 : 1.00	Cy5 Ch3 : 1.00	Cy5 Ch3 : 1.00
			FRET Ch3 : 1.00	FRET Ch3 : 1.00
Digital offset	405 Ch2 : 0.10	405 Ch2 : 0.10	TO1 Ch2 : 0.02	TO1 Ch2 : 0.02
	488 Ch3 : 0.04	488 Ch3 : 0.04	TO1 ChD : 0.10	TO1 ChD : 0.10
	FRET Ch3 : 0.04	FRET Ch3 : 0.04	Cy5 Ch3 : 0.01	Cy5 Ch3 : 0.01
			FRET Ch3 : 0.02	FRET Ch3 : 0.02
PinHole	405 Ch2 : 96 µm	405 Ch2 : 96 µm	TO1 Ch2 : 154 µm	TO1 Ch2 : 154 µm
	488 Ch3 : 96 µm	488 Ch3 : 96 µm	Cy5 Ch3 : 196 µm	Cy5 Ch3 : 196 µm
	FRET Ch3 : 96 µm	FRET Ch3 : 96 µm	FRET Ch3 : 196 µm	FRET Ch3 : 196 µm
Filters	405 Ch2 : BP 420-480	405 Ch2 : BP 420-480	TO1 Ch2 : BP 500-550 IR	TO1 Ch2 : BP 500-550 IR
	488 Ch3 : BP 505-550	488 Ch3 : BP 505-550	Cy5 Ch3 : BP 650-710 IR	Cy5 Ch3 : BP 650-710 IR
	FRET Ch3 : BP 505-550	FRET Ch3 : BP 505-550	FRET Ch3 : BP 650-710 IR	FRET Ch3 : BP 650-710 IR
Beam splitters	MBS : HFT 405/488/561	MBS : HFT 405/488/561	MBS : HFT 405/488/561/633/KP 725	MBS : HFT 405/488/561/633/KP 725
	DBS1 : Mirror	DBS1 : Mirror	DBS1 : Mirror	DBS1 : Mirror
	DBS2 : NFT 490	DBS2 : NFT 490	DBS2 : NFT 565	DBS2 : NFT 565
			FCS EF : BG 39	FCS EF : BG 39
			FCS DBS : NFT 490	FCS DBS : NFT 490
Lasers	405 405 nm : 10.0 %	405 405 nm : 10.0 %	TO1 488 nm : 8.0 %	TO1 488 nm : 8.0 %
	488 488 nm : 2.2 %	488 488 nm : 2.2 %	Cy5 633 nm : 10.0 %	Cy5 633 nm : 10.0 %
	FRET 405 nm : 10.0 %	FRET 405 nm : 10.0 %	FRET 488 nm : 8.0 %	FRET 488 nm : 8.0 %

UV-vis and Fluorescence Spectroscopy. All dye concentrations were 1 micromolar. Buffer was phosphate-buffered saline-EDTA (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 2 mM EDTA, pH 7.4). This buffer was used in combination with glycerol for the 90:10 v/v glycerol:buffer experiments but was not used for DMSO experiments. For DMSO, dyes were added from concentrated aqueous stocks, such that the final DMSO concentration v/v was 99% for TO1-2p and TO1-2p-Cy5 and 97.5% for Cy5-2p.

Corrected emission spectra were recorded on a Quantamaster fluorimeter (Photon Technology International) using 2.5 mm slits.

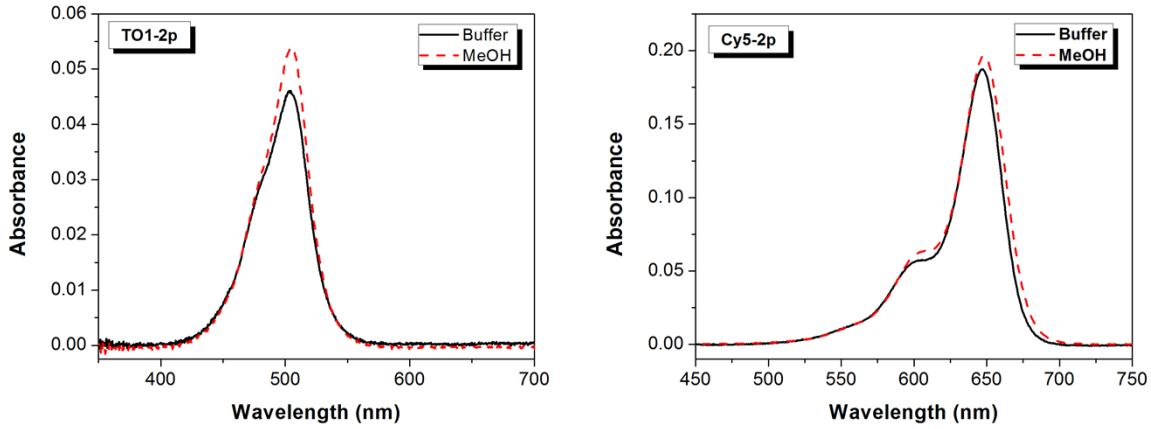


Figure S27. UV-vis spectra of monochromophoric compounds TO1-2p (left) and Cy5-2p (right) in aqueous buffer and methanol.

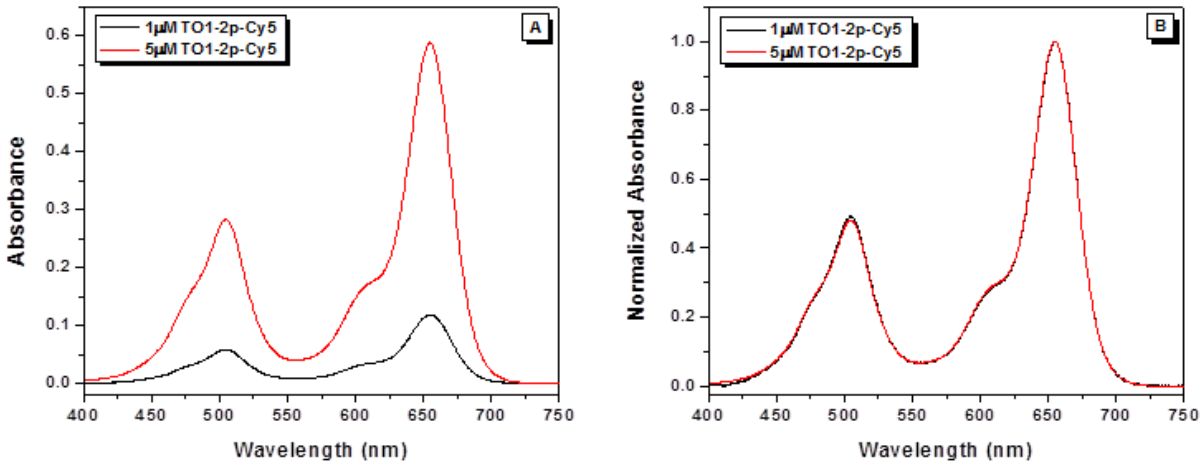


Figure S28. Raw (left) and normalized (right) UV-vis spectra of TO1-2p-Cy5 in buffer at 1 μM and 5 μM concentration in buffer solution.

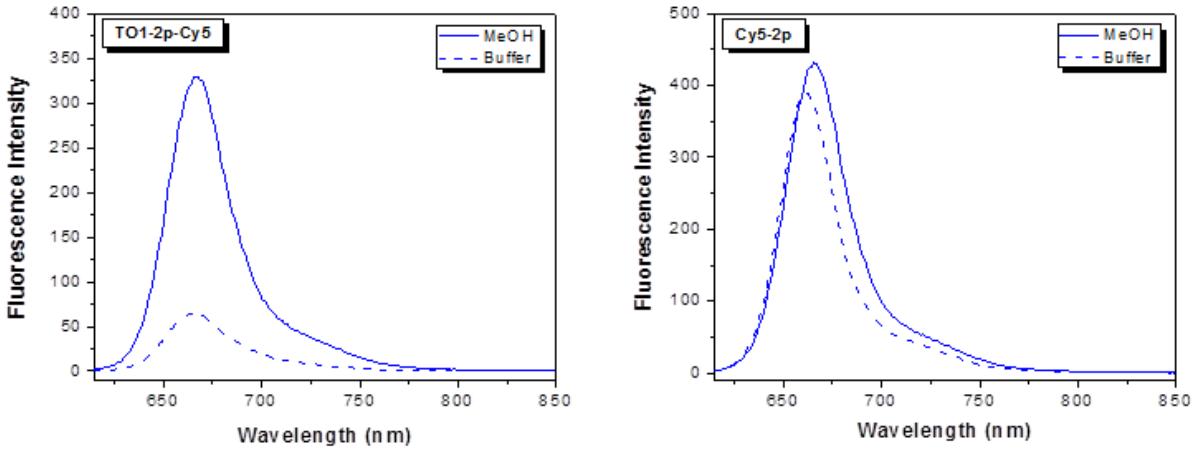


Figure S29. Emission spectra of TO1-2p-Cy5 (left) and Cy5-2p (right) in buffer with direct excitation of Cy5.

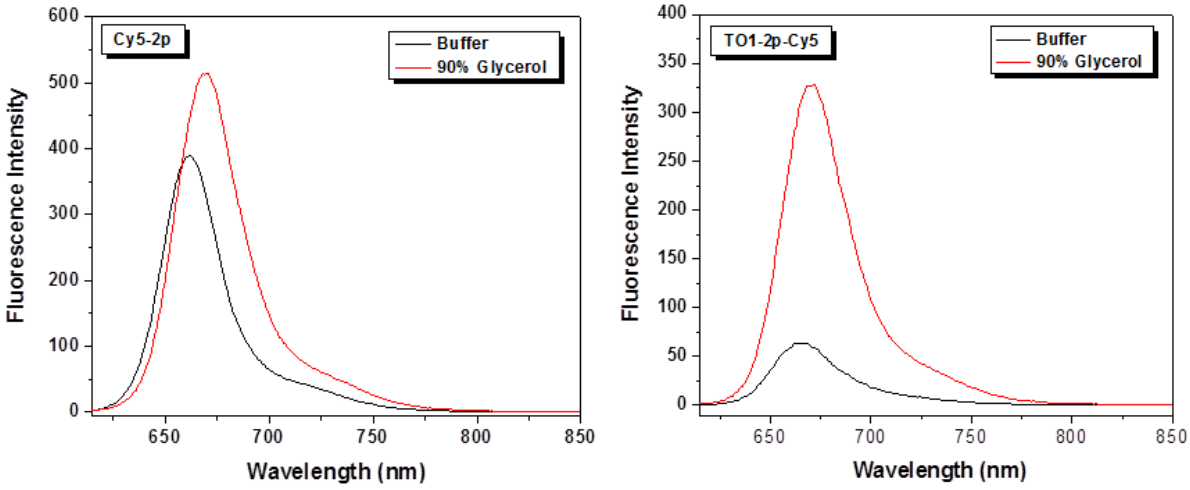


Figure S30. Emission spectra of Cy5-2p (left) and TO1-2p-Cy5 (right) in buffer versus 90% glycerol with direct excitation of Cy5 at 600 nm. The larger fluorescence enhancement for TO1-2p-Cy5 is consistent with quenching of Cy5 by TO1 in the bichromophore in aqueous buffer.

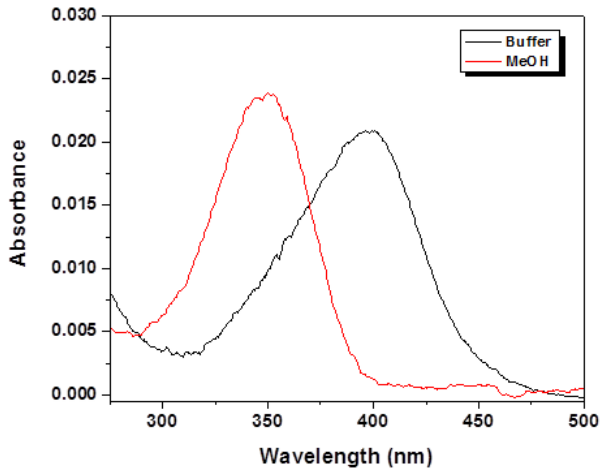


Figure S31. UV-vis spectra of coumarin monochromophore in methanol versus buffer.

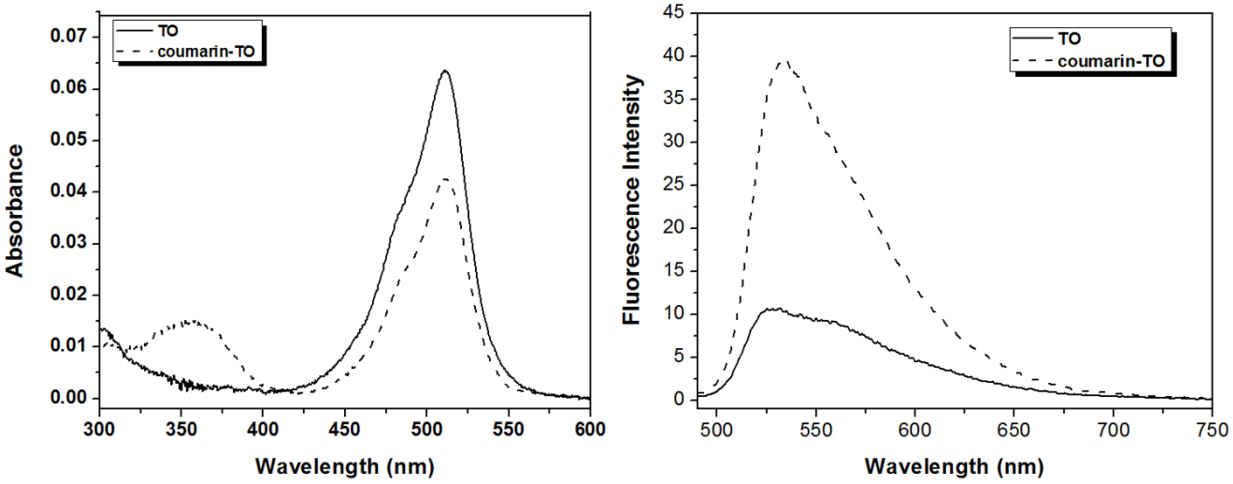


Figure S32. (Left) UV-vis absorption spectra of 1 μM TO and coumarin-TO in 90% glycerol. (Right) Fluorescence of 1 μM TO and coumarin-TO in 90% glycerol with excitation at 480 nm, i.e. direct excitation of TO. The four-fold higher fluorescence of coumarin-TO is observed in spite of the ca. 30% lower absorbance at the excitation wavelength.

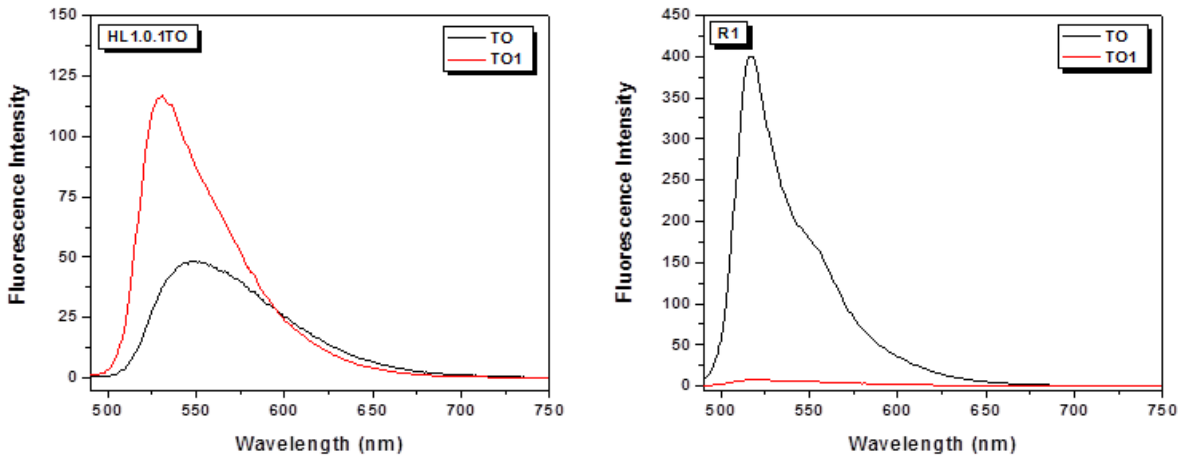


Figure S33. (Left) Fluorescence of HL1.0.1.TO for TO vs TO1. (Right) Fluorescence of R1 for TO vs TO1. Dye concentration = 300 nM, protein present in excess.

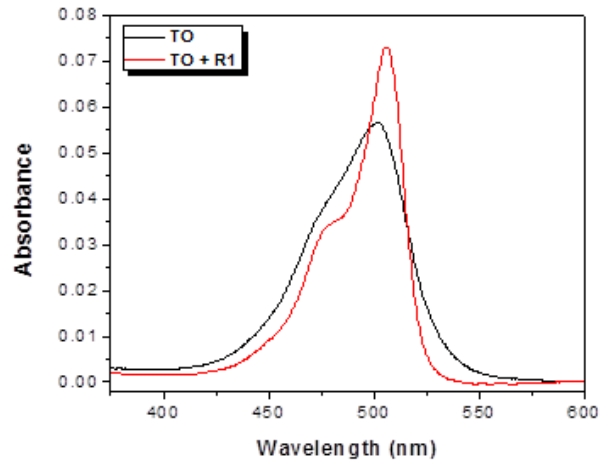


Figure S34. UV-vis spectra of 1 μM TO1 in buffer and in the presence of excess R1 protein.