

Supporting Information

Quantification of Small Molecule–Protein Interactions using FRET between Tryptophan and the Pacific Blue Fluorophore

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Determination of the K_d of **16 for SA by Fluorescence Polarization (FP):** The change in polarization of **16** was calculated by subtracting the average ($n=3$) polarization of the free ligand (P_f), plotted against [SA]. The maximum polarization of the fully bound complex was estimated (B_{max}) based on a one-site-specific binding model (GraphPad Prism 6.0). This polarization of the complex (P_b) was used in the following equation to calculate the apparent fraction bound (F_a):

$$F_a = \frac{P - P_f}{P_b - P_f}$$

Background-subtracted fluorescence signals ($I_{ad,400}$) were calculated as described in the experimental section of the main text. To correct for fluorescence enhancement or quenching, a fluorescence enhancement factor (Q) was calculated using the following equation, where $I_{ad,400}$ and $I_{a,400}$ are the fluorescence intensity ($\lambda_{ex}= 400$ nm, $\lambda_{em}= 460$ nm) of the sample and free ligand, respectively:

$$Q = \frac{I_{ad,400} - I_{a,400}}{I_{a,400}}$$

To incorporate fluorescence enhancement or quenching into binding assays, the corrected fraction bound (f_a) was calculated using the following equation, plotted against the concentration of SA, and the K_d analyzed by non-linear regression (Figure S2):

$$f_a = \frac{F_a}{1 + Q(1 - F_a)}$$

(A) Determination of extinction coefficients (B) Determination of the quantum yield of **12**

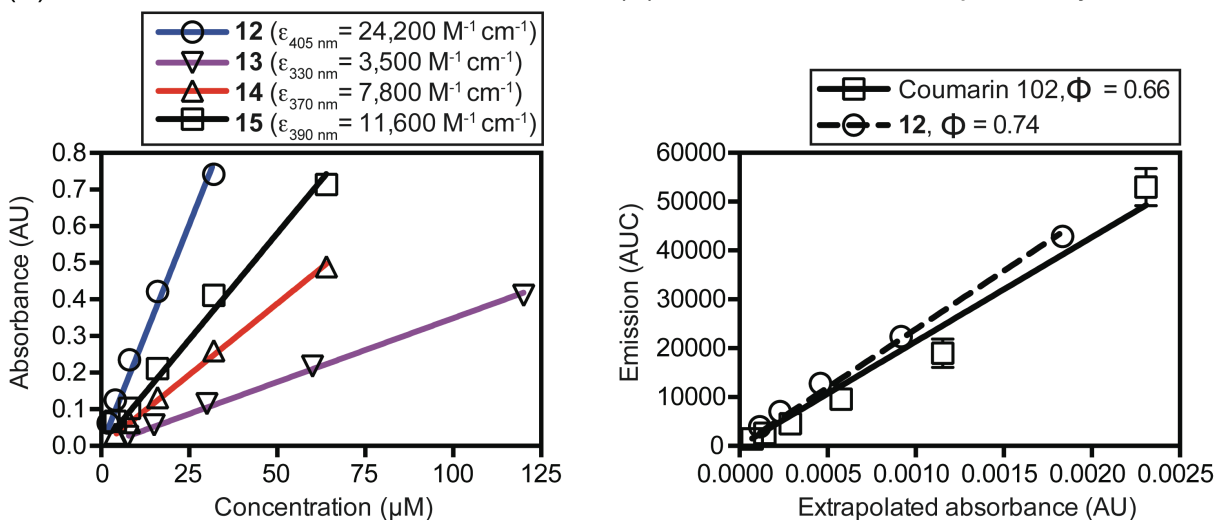


Figure S1. Analysis of compounds in PBS containing DMSO (0.5%). A: Determination of extinction coefficients of **12-15**. B: Measurement of the quantum yield of **12** relative to coumarin 102.

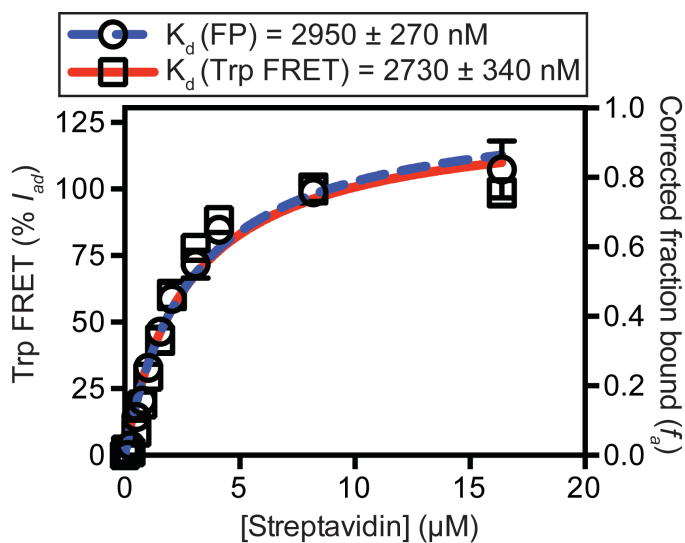


Figure S2. Quantification of binding of **16** to SA by FP compared with Trp-FRET. [**16**] = 25 nM.

Table S1. Data for direct binding of PB-thiobiotin (**17**, 5 nM, PBS pH = 7.4, 25 °C) to SA by Trp-FRET. $\lambda_{ex.} = 290\text{--}300$ nm, $\lambda_{em.} = 455\text{--}465$ nm.

[Streptavidin (monomer)] (nM)	Raw FRET ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm), I_{295}			Fluorescence from SA alone ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm), $I_{d,295}$			Background-subtracted FRET (minus SA ($I_{d,295}$) and free ligand ($I_{a,295}$)), I_{FRET}		
0	4.25	4.22	4.96	3.39	3.15	2.44	-0.22	-0.25	0.48
0.1	4.56	4.95	5.17	2.57	3.44	2.06	0.39	0.78	1.00
1	4.85	4.66	6.40	4.04	3.24	2.70	0.03	-0.15	1.59
2	6.99	4.63	6.25	3.97	4.92	2.50	1.72	-0.64	0.98
4	5.50	5.31	5.59	3.02	2.92	3.73	0.80	0.61	0.88
8	6.54	6.25	6.98	2.85	4.10	3.33	1.64	1.34	2.08
12	8.13	8.70	8.64	3.63	4.30	3.20	2.94	3.50	3.45
16	8.19	8.59	8.72	3.14	3.57	3.17	3.42	3.82	3.95
24	9.32	9.02	9.39	3.54	3.81	3.10	4.35	4.06	4.42
32	10.93	10.30	9.26	3.56	3.38	3.52	5.96	5.32	4.29
64	12.30	13.05	12.38	3.94	3.67	3.33	7.17	7.92	7.25
128	15.05	15.21	15.30	5.05	4.57	5.07	8.67	8.83	8.92
256	25.90	24.41	26.44	8.49	7.80	7.49	16.49	15.00	17.03

[Streptavidin (monomer)] (nM)	FRET corrected for quenching, I_{ad}			Normalized Trp-FRET, I_{ad}			Average background fluorescence ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm) of the free ligand, $I_{a,295}$		
0	-0.23	-0.26	0.45	-1.7%	-1.9%	3.3%	1.48		
0.1	0.40	0.74	0.92	2.9%	5.4%	6.7%			
1	0.04	-0.17	1.70	0.3%	-1.2%	12.4%			
2	2.65	-0.85	1.25	19.4%	-6.2%	9.1%			
4	1.67	1.10	1.90	12.2%	8.0%	13.9%			
8	4.41	3.21	5.15	32.3%	23.5%	37.8%			
12	8.53	10.97	8.95	62.5%	80.4%	65.5%			
16	11.04	9.60	10.18	80.9%	70.3%	74.6%			
24	11.17	9.67	13.28	81.8%	70.9%	97.3%			
32	13.90	12.62	12.63	101.8%	92.5%	92.5%			
64	14.28	13.74	11.93	104.6%	100.6%	87.4%			
128	14.02	12.56	13.92	102.7%	92.0%	102.0%			
256	13.80	12.83	14.32	101.1%	94.0%	104.9%			

Fluorescence of Pacific Blue of **17**: $\lambda_{ex.} = 395\text{--}405$ nm, $\lambda_{em.} = 455\text{--}465$ nm.

[Streptavidin (monomer)] (nM)	Fluorescence intensity, I_{400}			Fluorescence of SA alone, $I_{d,400}$			Background-subtracted fluorescence intensity (minus fluorescence of SA ($I_{d,400}$)), $I_{ad,400}$		
0	20.92	21.23	22.35	6.73	6.42	6.40	14.52	14.83	15.95
0.1	21.27	22.22	22.83	6.67	6.25	6.48	14.87	15.82	16.43
1	19.85	19.79	20.52	6.82	6.30	6.50	13.45	13.39	14.12
2	16.20	17.88	18.23	6.77	6.37	6.38	9.80	11.48	11.83
4	13.60	14.73	13.43	6.67	6.29	6.52	7.20	8.33	7.03
8	11.99	12.71	12.48	6.57	6.28	6.40	5.59	6.31	6.08
12	11.60	11.22	12.22	6.87	6.19	6.32	5.20	4.82	5.82
16	11.07	12.41	12.26	6.50	6.23	6.22	4.67	6.01	5.86
24	12.28	12.73	11.43	6.54	6.19	6.19	5.88	6.33	5.03
32	12.87	12.77	11.53	6.38	6.31	6.30	6.47	6.37	5.13
64	13.98	15.11	15.58	6.47	6.28	6.09	7.58	8.71	9.18
128	15.73	17.01	16.07	6.48	6.25	6.15	9.33	10.61	9.68
256	24.45	24.06	24.36	6.46	6.10	6.24	18.05	17.66	17.96

[Streptavidin (monomer)] (nM)	Average fluorescence intensity of the free ligand, $I_{a,400}$	Quenching Ratio, Q_r ($I_{ad,400}/I_{a,400}$)		
0	15.098	0.96	0.98	1.06
0.1		0.98	1.05	1.09
1		0.89	0.89	0.94
2		0.65	0.76	0.78
4		0.48	0.55	0.47
8		0.37	0.42	0.40
12		0.34	0.32	0.39
16		0.31	0.40	0.39
24		0.39	0.42	0.33
32		0.43	0.42	0.34
64		0.50	0.58	0.61
128		0.62	0.70	0.64
256		1.20	1.17	1.19

Table S2. Data for direct binding of PB-imidazolidinethione (**18**, 25 nM, PBS pH = 7.4, 25 °C) to SA by Trp-FRET. $\lambda_{\text{ex.}} = 290\text{--}300$ nm, $\lambda_{\text{em.}} = 455\text{--}465$ nm.

[Streptavidin (monomer)] (nM)	Raw FRET ($\lambda_{\text{ex.}} = 295$ nm, $\lambda_{\text{em.}} = 460$ nm), I_{295}			Fluorescence from SA alone ($\lambda_{\text{ex.}} = 295$ nm, $\lambda_{\text{em.}} = 460$ nm), $I_{d,295}$			Background-subtracted FRET (minus SA ($I_{d,295}$) and free ligand ($I_{a,295}$)), I_{FRET}		
0	13.80	15.72	13.31	3.00	5.11	2.72	-0.47	1.44	-0.97
3.75	16.71	15.28	14.84	1.98	4.74	2.13	3.09	1.66	1.22
7.5	18.02	20.41	15.22	1.87	4.15	3.15	4.29	6.68	1.50
15	18.44	20.54	17.87	1.53	4.42	2.66	4.91	7.00	4.34
27.5	20.89	20.68	18.97	1.74	4.74	2.61	7.20	6.99	5.28
55	27.89	22.27	24.14	1.39	6.43	4.40	13.15	7.53	9.40
87.5	28.78	25.22	31.57	2.63	8.00	5.45	12.75	9.20	15.54
175	47.08	45.04	51.79	3.92	12.24	10.04	27.68	25.64	32.39
275	67.33	61.93	62.45	12.8	14.96	14.04	42.72	37.32	37.83
550	71.28	72.05	70.90	19.5	17.38	18.58	42.11	42.87	41.73
1000	85.12	82.59	82.94	24.6	26.65	22.75	49.79	47.25	47.60
2000	110.32	114.8	107.28	54.4	54.84	49.83	46.62	51.16	43.58

[Streptavidin (monomer)] (nM)	FRET corrected for quenching, I_{ad}			Normalized Trp-FRET, I_{ad}			Average background fluorescence ($\lambda_{\text{ex.}} = 295$ nm, $\lambda_{\text{em.}} = 460$ nm) of the free ligand, $I_{a,295}$		
0	-0.47	1.53	-0.92	-0.9%	2.9%	-1.7%	10.67		
3.75	3.86	2.26	1.67	7.3%	4.3%	3.2%			
7.5	5.29	9.29	1.87	10.0%	17.6%	3.5%			
15	6.35	9.44	5.62	12.0%	17.8%	10.6%			
27.5	9.12	9.61	6.51	17.2%	18.2%	12.3%			
55	17.47	9.79	11.15	33.0%	18.5%	21.1%			
87.5	16.68	12.20	17.57	31.5%	23.1%	33.2%			
175	33.70	31.73	35.93	63.7%	60.0%	67.9%			
275	49.43	44.91	42.15	93.4%	84.9%	79.7%			
550	45.32	47.97	43.26	85.7%	90.7%	81.8%			
1000	54.33	51.87	52.53	102.7%	98.0%	99.3%			
2000	49.28	56.18	44.92	93.1%	106.2%	84.9%			

Fluorescence of Pacific Blue of 18: $\lambda_{\text{ex.}} = 395\text{--}405$ nm, $\lambda_{\text{em.}} = 455\text{--}465$ nm.

[Streptavidin (monomer)] (nM)	Fluorescence intensity, I_{400}			Fluorescence of SA alone, $I_{d,400}$			Background-subtracted fluorescence intensity (minus fluorescence of SA ($I_{d,400}$)), $I_{\text{ad},400}$		
0	182.62	171.8	191.84	6.22	6.06	6.41	176.39	165.61	185.6
3.75	146.94	134.9	134.73	6.84	5.69	5.64	140.88	128.90	128.6
7.5	148.55	132.4	146.51	5.82	5.76	5.99	142.69	126.58	140.6
15	142.05	136.5	141.69	6.60	5.61	5.85	136.03	130.50	135.6
27.5	144.85	133.8	148.52	6.22	6.18	5.73	138.80	127.82	142.4
55	139.29	142.2	155.27	7.18	7.24	6.40	132.35	135.30	148.3
87.5	142.46	140.5	163.54	7.58	9.11	7.27	134.47	132.55	155.5
175	152.91	150.5	166.97	8.13	9.24	8.00	144.45	142.12	158.5
275	158.59	152.7	164.44	7.06	6.41	6.32	151.99	146.14	157.8
550	171.38	165.1	177.62	8.20	8.32	7.43	163.40	157.16	169.6
1000	175.34	174.4	173.56	13.7	14.78	13.99	161.16	160.22	159.3
2000	186.44	180.2	190.72	20.5	20.00	19.71	166.37	160.17	170.6

[Streptavidin (monomer)] (nM)	Average fluorescence intensity of the free ligand, $I_{a,400}$	Quenching Ratio, Q_r ($I_{\text{ad},400}/I_{a,400}$)		
0	175.87	1.00	0.94	1.06
3.75		0.80	0.73	0.73
7.5		0.81	0.72	0.80
15		0.77	0.74	0.77
27.5		0.79	0.73	0.81
55		0.75	0.77	0.84
87.5		0.76	0.75	0.88
175		0.82	0.81	0.90
275		0.86	0.83	0.90
550		0.93	0.89	0.96
1000		0.92	0.91	0.91
2000	0.95	0.91	0.97	

Table S3. Data for direct binding of PB-iminobiotin (**16**, 25 nM, PBS pH = 7.4, 25 °C) to SA by Trp-FRET. $\lambda_{ex.} = 290\text{--}300$ nm, $\lambda_{em.} = 455\text{--}465$ nm.

[Streptavidin (monomer)] (nM)	Raw FRET ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm), I_{295}			Fluorescence from SA alone ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm), $I_{d,295}$			Background-subtracted FRET (minus SA ($I_{d,295}$) and free ligand ($I_{a,295}$)), I_{FRET}		
0	16.89	20.17	19.71	2.16	3.83	4.74	-2.04	1.25	0.79
64	18.91	21.03	23.67	2.33	3.92	2.30	0.71	2.83	5.48
128	18.57	21.57	23.35	3.66	3.89	2.11	0.00	3.00	4.78
256	18.84	23.04	24.91	3.35	6.59	5.56	-1.67	2.53	4.40
512	42.21	43.38	35.01	10.49	14.98	14.56	13.52	14.69	6.32
768	55.80	62.30	51.95	13.49	16.55	16.73	24.86	31.37	21.02
1024	70.36	77.45	76.04	18.59	18.61	21.73	35.37	42.46	41.05
1536	99.86	90.94	88.50	21.55	19.04	24.18	62.92	54.00	51.56
2048	120.49	120.12	111.0	22.81	22.61	25.48	81.51	81.14	72.08
3072	151.87	152.38	148.9	27.95	38.39	33.64	103.20	103.71	100.2
4096	176.07	174.56	172.5	37.97	50.88	45.28	116.01	114.50	112.4
8192	223.00	216.10	215.1	70.62	78.59	81.50	130.75	123.85	122.9
16384	258.84	250.16	255.9	109.15	125.05	115.08	127.07	118.39	124.2

[Streptavidin (monomer)] (nM)	FRET corrected for quenching, I_{ad}			Normalized Trp-FRET, I_{ad}			Average background fluorescence ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm) of the free ligand, $I_{a,295}$		
0	-2.07	1.22	0.79	-1.5%	0.9%	0.6%	15.35		
64	0.77	2.83	5.23	0.6%	2.0%	3.8%			
128	0.00	3.01	4.67	0.0%	2.2%	3.4%			
256	-1.75	2.48	4.29	-1.3%	1.8%	3.1%			
512	14.31	14.31	6.47	10.4%	10.4%	4.7%			
768	26.83	30.67	21.51	19.4%	22.2%	15.6%			
1024	38.11	42.02	42.08	27.6%	30.4%	30.5%			
1536	67.47	55.98	55.56	48.9%	40.5%	40.2%			
2048	90.23	83.55	76.02	65.3%	60.5%	55.0%			
3072	110.99	107.13	104.4	80.4%	77.6%	75.6%			
4096	125.99	120.50	119.4	91.2%	87.2%	86.5%			
8192	146.48	134.13	133.7	106.1%	97.1%	96.8%			
16384	143.05	129.68	135.6	103.6%	93.9%	98.2%			

Fluorescence of Pacific Blue of **16**: $\lambda_{ex.} = 395\text{--}405$ nm, $\lambda_{em.} = 455\text{--}465$ nm.

[Streptavidin (monomer)] (nM)	Fluorescence intensity, I_{400}			Fluorescence of SA alone, $I_{d,400}$			Background-subtracted fluorescence intensity (minus fluorescence of SA ($I_{d,400}$)), $I_{ad,400}$		
0	322.26	334.38	327.0	6.72	6.44	7.32	315.43	327.55	320.2
64	303.91	328.48	342.9	6.33	6.46	6.83	297.37	321.94	336.3
128	304.10	326.69	335.0	6.42	6.48	6.52	297.63	320.21	328.6
256	313.76	334.44	335.9	6.58	6.53	6.78	307.13	327.81	329.2
512	310.27	336.53	320.7	6.76	6.57	7.61	303.29	329.56	313.7
768	304.35	335.23	320.5	6.83	6.58	7.10	297.51	328.39	313.6
1024	305.84	332.36	321.0	7.11	9.14	7.35	297.98	324.49	313.2
1536	306.78	317.08	305.3	7.28	7.26	7.55	299.41	309.72	297.9
2048	297.58	319.37	311.9	7.28	7.58	7.76	290.04	311.83	304.4
3072	307.54	319.85	317.3	8.10	9.76	9.19	298.52	310.84	308.3
4096	307.65	317.08	314.3	9.29	15.04	11.67	295.65	305.08	302.3
8192	308.66	318.54	317.1	19.11	24.33	22.75	286.60	296.47	295.0
16384	318.57	326.47	327.2	29.01	35.53	35.52	285.22	293.12	293.9

[Streptavidin (monomer)] (nM)	Average fluorescence intensity of the free ligand, $I_{a,400}$	Quenching Ratio, $Q_r (I_{ad,400}/I_{a,400})$		
0	321.08	0.98	1.02	1.00
64		0.93	1.00	1.05
128		0.93	1.00	1.02
256		0.96	1.02	1.03
512		0.94	1.03	0.98
768		0.93	1.02	0.98
1024		0.93	1.01	0.98
1536		0.93	0.96	0.93
2048		0.90	0.97	0.95
3072		0.93	0.97	0.96
4096		0.92	0.95	0.94
8192		0.89	0.92	0.92
16384		0.89	0.91	0.92

Table S4. Data for direct binding of PB-iminobiotin (**16**, 25 nM, PBS pH = 7.4, 25 °C) to SA by FP.

Fluorescence Polarization of PB: $\lambda_{ex.} = 395\text{--}405\text{ nm}$, $\lambda_{em.} = 455\text{--}465\text{ nm}$.

[Streptavidin (monomer)] (nM)	Fluorescence Polarization, P			Apparent Fraction Bound, F_a			Corrected Fraction Bound, f_a		
0	109.23	117.30	102.14	0.00	0.03	-0.03	0.00	0.03	-0.03
64	97.71	112.56	99.55	-0.04	0.01	-0.04	-0.05	0.01	-0.04
128	109.29	108.95	111.57	0.00	0.00	0.01	0.00	0.00	0.01
256	120.48	118.00	112.89	0.04	0.03	0.01	0.04	0.03	0.01
512	145.20	140.33	129.71	0.13	0.12	0.08	0.14	0.11	0.08
768	152.47	154.80	143.26	0.16	0.17	0.13	0.17	0.17	0.13
1024	174.27	179.88	170.32	0.24	0.27	0.23	0.26	0.26	0.23
1536	203.59	200.64	196.60	0.35	0.34	0.33	0.37	0.35	0.35
2048	223.40	230.88	220.35	0.43	0.46	0.42	0.45	0.47	0.43
3072	257.67	258.89	240.40	0.56	0.56	0.49	0.58	0.57	0.50
4096	276.56	270.69	285.69	0.63	0.61	0.66	0.65	0.62	0.68
8192	312.71	297.44	307.21	0.77	0.71	0.75	0.79	0.73	0.76
16384	347.88	302.91	320.68	0.90	0.73	0.80	0.91	0.75	0.81

Average Polarization of the free ligand, P_f	Polarization of the ligand-protein complex (calculated with GraphPad Prism), P_b
109.6	374.6

Fluorescence of Pacific Blue of **16**: $\lambda_{ex.} = 395\text{--}405\text{ nm}$, $\lambda_{em.} = 455\text{--}465\text{ nm}$.

[Streptavidin (monomer)] (nM)	Fluorescence intensity, I_{400}			Fluorescence of SA alone, $I_{d,400}$			Background-subtracted fluorescence intensity (minus fluorescence of SA ($I_{d,400}$)), $I_{ad,400}$		
0	322.26	334.38	327.09	6.72	6.44	7.32	315.43	327.55	320.26
64	303.91	328.48	342.92	6.33	6.46	6.83	297.37	321.94	336.39
128	304.10	326.69	335.07	6.42	6.48	6.52	297.63	320.21	328.60
256	313.76	334.44	335.90	6.58	6.53	6.78	307.13	327.81	329.27
512	310.27	336.53	320.77	6.76	6.57	7.61	303.29	329.56	313.79
768	304.35	335.23	320.52	6.83	6.58	7.10	297.51	328.39	313.68
1024	305.84	332.36	321.08	7.11	9.14	7.35	297.98	324.49	313.21
1536	306.78	317.08	305.33	7.28	7.26	7.55	299.41	309.72	297.96
2048	297.58	319.37	311.97	7.28	7.58	7.76	290.04	311.83	304.43
3072	307.54	319.85	317.38	8.10	9.76	9.19	298.52	310.84	308.36
4096	307.65	317.08	314.35	9.29	15.04	11.67	295.65	305.08	302.35
8192	308.66	318.54	317.14	19.11	24.33	22.75	286.60	296.47	295.08
16384	318.57	326.47	327.28	29.01	35.53	35.52	285.22	293.12	293.93

[Streptavidin (monomer)] (nM)	Average fluorescence intensity of the free ligand, $I_{a,400}$	Fluorescence enhancement factor, Q ($I_{ad,400}/I_{a,400}$)		
0	321.08	-0.02	0.02	0.00
64		-0.07	0.00	0.05
128		-0.07	0.00	0.02
256		-0.04	0.02	0.03
512		-0.06	0.03	-0.02
768		-0.07	0.02	-0.02
1024		-0.07	0.01	-0.02
1536		-0.07	-0.04	-0.07
2048		-0.10	-0.03	-0.05
3072		-0.07	-0.03	-0.04
4096		-0.08	-0.05	-0.06
8192		-0.11	-0.08	-0.08
16384		-0.11	-0.09	-0.08

Table S5. Data for competitive binding of imidazolidinethione-OH (**20**, PBS pH = 7.4, 25 °C) to SA by Trp-FRET. $\lambda_{ex.} = 290\text{--}300$ nm, $\lambda_{em.} = 455\text{--}465$ nm.

[20] (nM)	Raw FRET ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm), I_{295}			Background-subtracted FRET (minus SA ($I_{d,295}$) and free ligand ($I_{a,295}$)), I_{FRET}			FRET corrected for quenching, I_{ad}		
0	157.55	158.20	158.90	153.90	154.55	155.25	155.25	151.99	156.51
1	154.62	161.82	155.38	150.97	158.17	151.73	151.72	156.01	153.82
10	142.77	157.29	150.33	139.12	153.64	146.68	144.18	153.74	145.94
100	139.22	147.44	143.44	135.58	143.79	139.79	141.30	146.83	143.45
200	122.82	137.78	135.49	119.17	134.13	131.84	130.03	137.86	135.90
400	125.22	128.64	128.14	121.58	124.99	124.49	130.44	131.99	135.95
800	101.62	105.25	104.27	97.97	101.60	100.62	109.16	112.67	108.42
1600	71.14	78.76	78.94	67.49	75.11	75.29	80.34	87.09	85.67
3200	62.37	63.11	63.34	58.72	59.46	59.70	71.08	72.42	72.40
6400	52.62	54.18	50.97	48.98	50.53	47.32	62.44	63.86	60.99
10000	40.44	41.58	40.29	36.79	37.93	36.64	48.90	51.60	48.93
100000	24.86	26.31	27.67	21.21	22.66	24.02	26.56	28.84	30.52
1000000	47.93	48.24	50.00	44.28	44.59	46.35	32.53	32.82	33.92

[20] (nM)	Normalized Trp-FRET, I_{ad}			Background fluorescence ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm) of the free ligand, $I_{a,295}$		
0	100.53%	97.94%	101.53%	10.96		
1	97.72%	101.13%	99.39%			
10	91.74%	99.33%	93.13%			
100	89.45%	93.84%	91.16%	Average background fluorescence ($\lambda_{ex.} = 295$ nm, $\lambda_{em.} = 460$ nm) of SA alone, $I_{a,295}$		
200	80.50%	86.72%	85.16%	0.77		
400	80.82%	82.06%	85.20%			
800	63.93%	66.72%	63.35%			
1600	41.05%	46.41%	45.28%	Background fluorescence of ($\lambda_{ex.} = 400$ nm, $\lambda_{em.} = 460$ nm), of the free ligand $I_{a,400}$		
3200	33.70%	34.76%	34.74%	186.03		
6400	26.84%	27.97%	25.69%			
10000	16.09%	18.23%	16.11%			
100000	-1.65%	0.15%	1.50%	Average fluorescence from SA alone ($\lambda_{ex.} = 400$ nm, $\lambda_{em.} = 460$ nm), $I_{d,400}$		
1000000	3.09%	3.32%	4.19%	1.67		

Fluorescence of Pacific Blue of 18: $\lambda_{ex.} = 395\text{--}405$ nm, $\lambda_{em.} = 455\text{--}465$ nm.

[20] (nM)	Fluorescence intensity, I_{400}			Background-subtracted fluorescence intensity (minus fluorescence of SA ($I_{d,400}$)), $I_{ad,400}$			Quenching Ratio, Q_r ($I_{ad,400}/I_{a,400}$)		
0	224.08	229.70	224.23	217.89	223.50	218.04	0.99	1.02	0.99
1	224.93	229.04	223.02	218.73	222.85	216.83	1.00	1.01	0.99
10	218.29	225.86	227.13	212.10	219.67	220.93	0.96	1.00	1.01
100	217.10	221.46	220.40	210.91	215.26	214.20	0.96	0.98	0.97
200	207.64	220.06	219.44	201.45	213.87	213.25	0.92	0.97	0.97
400	211.08	214.35	207.47	204.88	208.16	201.28	0.93	0.95	0.92
800	203.48	204.41	210.20	197.28	198.21	204.00	0.90	0.90	0.93
1,600	190.86	195.76	199.37	184.66	189.57	193.18	0.84	0.86	0.88
3,200	187.77	186.66	187.44	181.58	180.47	181.25	0.83	0.82	0.82
6,400	178.60	180.12	176.74	172.41	173.93	170.55	0.78	0.79	0.78
10,000	171.58	167.79	170.81	165.38	161.60	164.62	0.75	0.74	0.75
100,000	181.72	178.95	179.18	175.53	172.76	172.99	0.80	0.79	0.79
1,000,000	305.44	304.81	306.58	299.24	298.62	300.39	1.36	1.36	1.37

Table S6. Data for competitive binding of imidazolidinethione-OBn (**22**, PBS pH = 7.4, 25 °C) to SA by Trp FRET. $\lambda_{\text{ex.}} = 290\text{--}300$ nm, $\lambda_{\text{em.}} = 455\text{--}465$ nm.

[22] (nM)	Raw FRET ($\lambda_{\text{ex.}} = 295$ nm, $\lambda_{\text{em.}} = 460$ nm), I_{295}			Background-subtracted FRET (minus SA ($I_{d,295}$) and free ligand ($I_{a,295}$)), I_{FRET}			FRET corrected for quenching, I_{ad}		
0	174.76	170.49	165.41	166.86	162.58	157.51	163.42	162.78	160.70
1	153.91	129.46	138.55	146.01	121.56	130.65	157.99	150.60	150.49
6	136.08	129.23	126.50	128.18	121.33	118.60	151.21	148.65	146.29
13	131.30	140.70	145.53	123.40	132.79	137.63	149.95	153.77	152.90
25	135.13	137.61	138.78	127.23	129.71	130.88	141.37	150.37	147.43
50	126.10	123.74	120.80	118.20	115.84	112.89	139.70	133.91	121.93
100	112.35	117.67	114.78	104.45	109.77	106.88	119.90	124.80	123.02
200	100.86	105.01	100.63	92.96	97.11	92.73	108.80	106.29	106.49
400	72.62	72.96	70.92	64.72	65.06	63.02	76.12	74.80	76.58
800	60.81	62.88	60.96	52.91	54.98	53.06	66.07	67.69	67.65
1,600	52.25	52.34	53.10	44.35	44.44	45.20	58.24	59.26	58.38
10,000	43.38	42.22	32.69	35.48	34.32	24.79	49.30	48.20	35.28
100,000	36.89	43.04	43.57	28.99	35.14	35.67	39.33	49.42	50.96

[22] (nM)	Normalized Trp-FRET, I_{ad}			Background fluorescence ($\lambda_{\text{ex.}} = 295$ nm, $\lambda_{\text{em.}} = 460$ nm) of the free ligand, $I_{a,295}$		
0	100.95%	100.41%	98.64%	6.15		
1	96.35%	90.09%	89.99%			
10	90.60%	88.44%	86.44%			
100	89.54%	92.77%	92.04%	Average background fluorescence ($\lambda_{\text{ex.}} = 295$ nm, $\lambda_{\text{em.}} = 460$ nm) of SA alone, $I_{a,295}$		
200	82.27%	89.89%	87.40%	1.75		
400	80.85%	75.95%	65.80%			
800	64.08%	68.23%	66.72%			
1,600	54.68%	52.55%	52.72%	Background fluorescence of ($\lambda_{\text{ex.}} = 400$ nm, $\lambda_{\text{em.}} = 460$ nm), of the free ligand $I_{a,400}$		
3,200	26.99%	25.87%	27.38%	169.41		
6,400	18.48%	19.85%	19.82%			
10,000	11.84%	12.71%	11.96%			
100,000	4.27%	3.34%	-7.61%	Average fluorescence from SA alone ($\lambda_{\text{ex.}} = 400$ nm, $\lambda_{\text{em.}} = 460$ nm), $I_{d,400}$		
1,000,000	-4.18%	4.37%	5.68%	2.37		

Fluorescence of Pacific Blue of 18: $\lambda_{\text{ex.}} = 395\text{--}405$ nm, $\lambda_{\text{em.}} = 455\text{--}465$ nm.

[22] (nM)	Fluorescence intensity, I_{400}			Background-subtracted fluorescence intensity (minus fluorescence of SA ($I_{d,400}$)), $I_{\text{ad},400}$			Quenching Ratio, Q_r ($I_{\text{ad},400}/I_{a,400}$)		
0	257.63	252.37	247.98	240.65	235.39	231.00	1.02	1.00	0.98
1	234.78	207.21	221.59	217.80	190.23	204.61	0.92	0.81	0.87
10	216.76	209.34	208.05	199.78	192.37	191.07	0.85	0.82	0.81
100	210.93	220.51	229.12	193.95	203.53	212.14	0.82	0.86	0.90
200	229.09	220.27	226.19	212.11	203.29	209.21	0.90	0.86	0.89
400	216.38	220.85	235.20	199.41	203.87	218.22	0.85	0.87	0.93
800	222.28	224.28	221.74	205.30	207.30	204.76	0.87	0.88	0.87
1,600	218.33	232.30	222.20	201.35	215.32	205.22	0.85	0.91	0.87
3,200	217.34	221.96	210.93	200.36	204.98	193.95	0.85	0.87	0.82
6,400	205.70	208.40	201.83	188.72	191.42	184.85	0.80	0.81	0.78
10,000	196.43	193.70	199.46	179.45	176.72	182.49	0.76	0.75	0.77
100,000	186.61	184.79	182.59	169.63	167.81	165.61	0.72	0.71	0.70
1,000,000	190.68	184.55	181.93	173.70	167.57	164.95	0.74	0.71	0.70

NMR Spectra

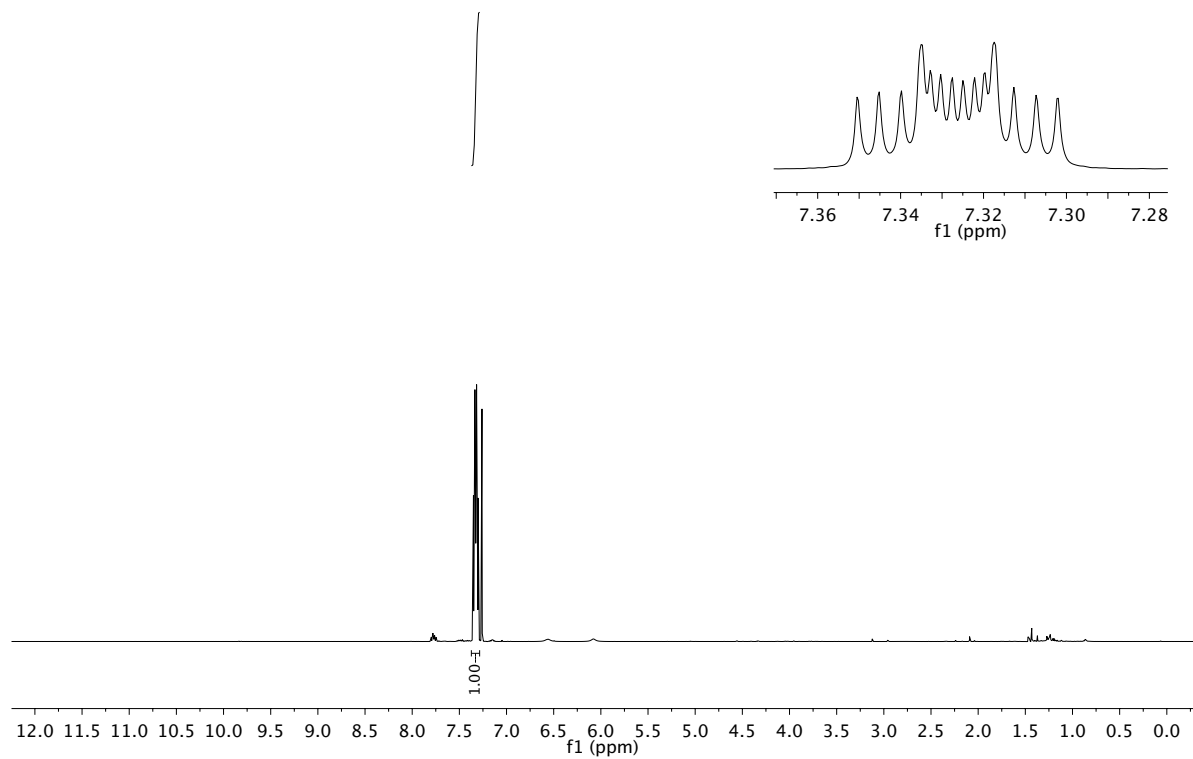
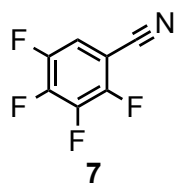


Figure S3. ^1H NMR (500 MHz, CDCl_3) spectrum of **7**.

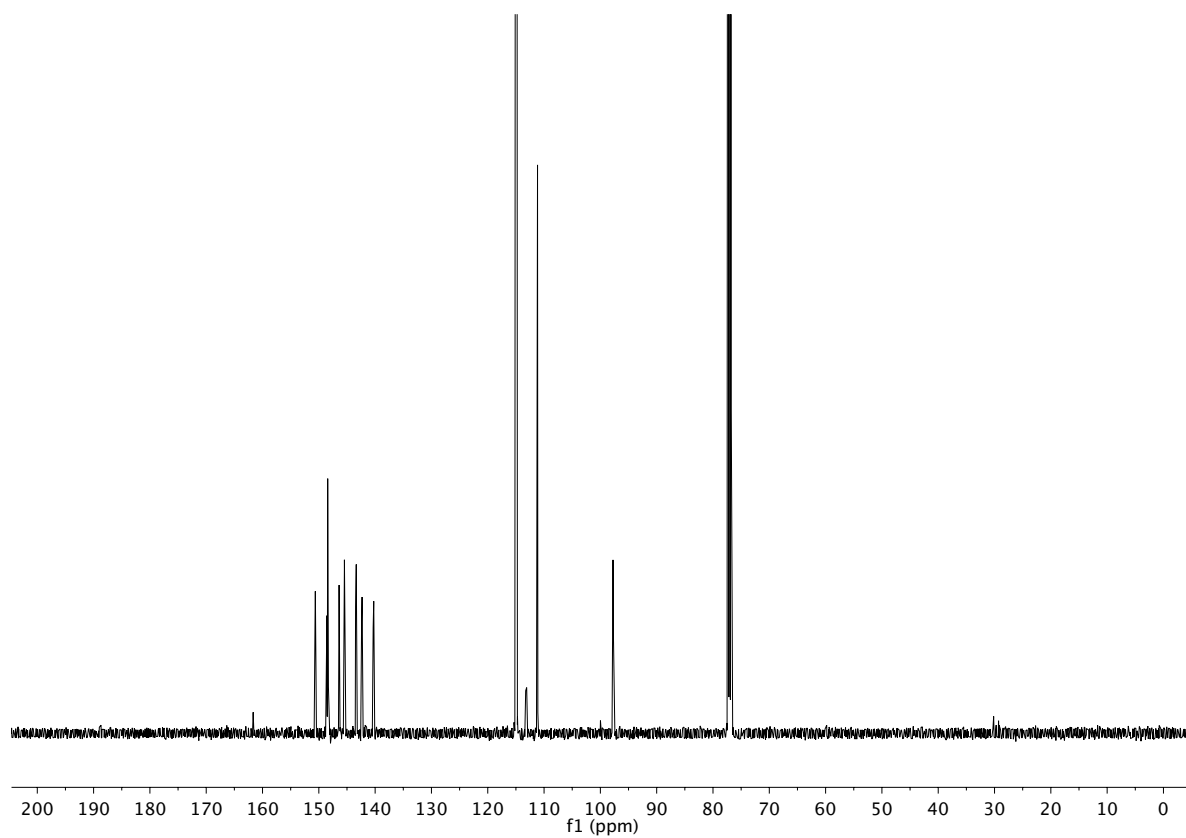
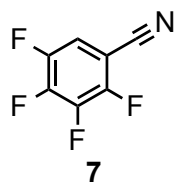


Figure S4. ^{13}C NMR (126 MHz, CDCl_3) spectrum of 7.

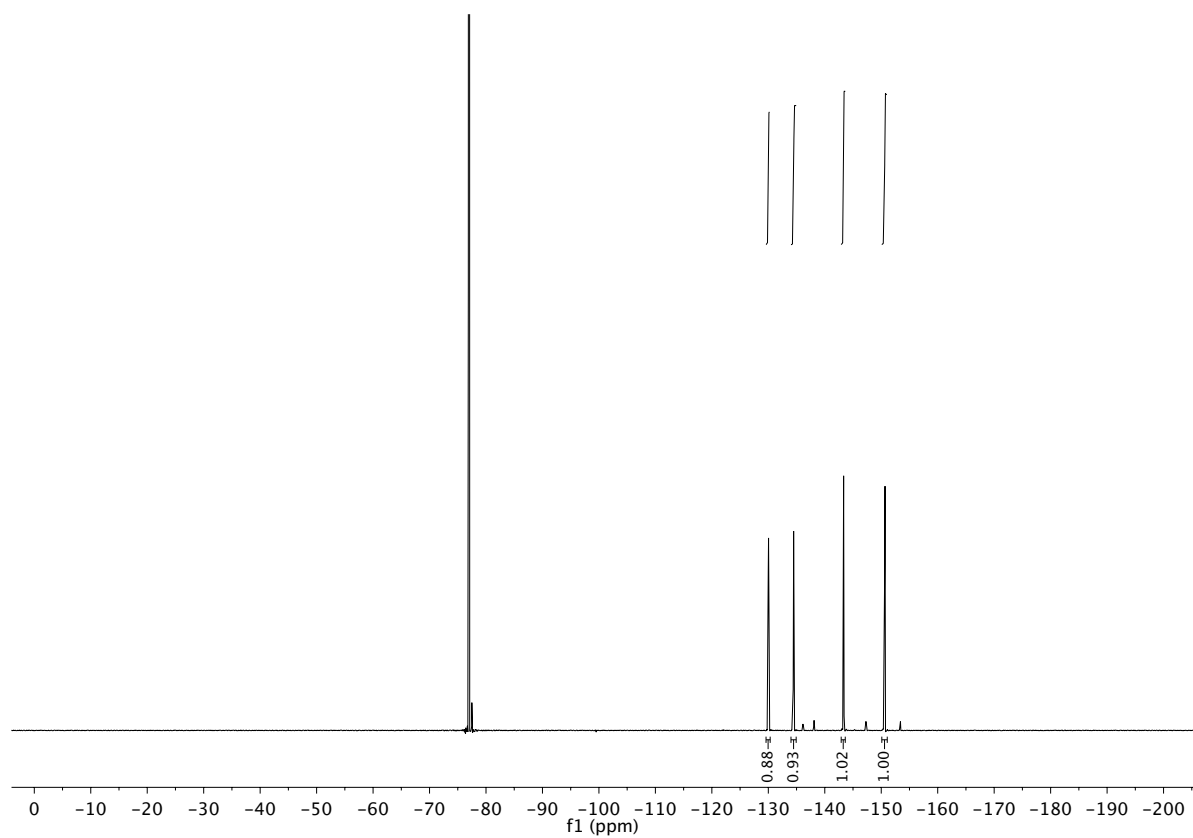
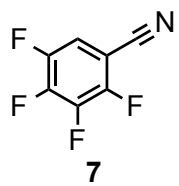


Figure S5. ^{19}F NMR (376 MHz, CDCl_3 , decoupled) spectrum of **7**.

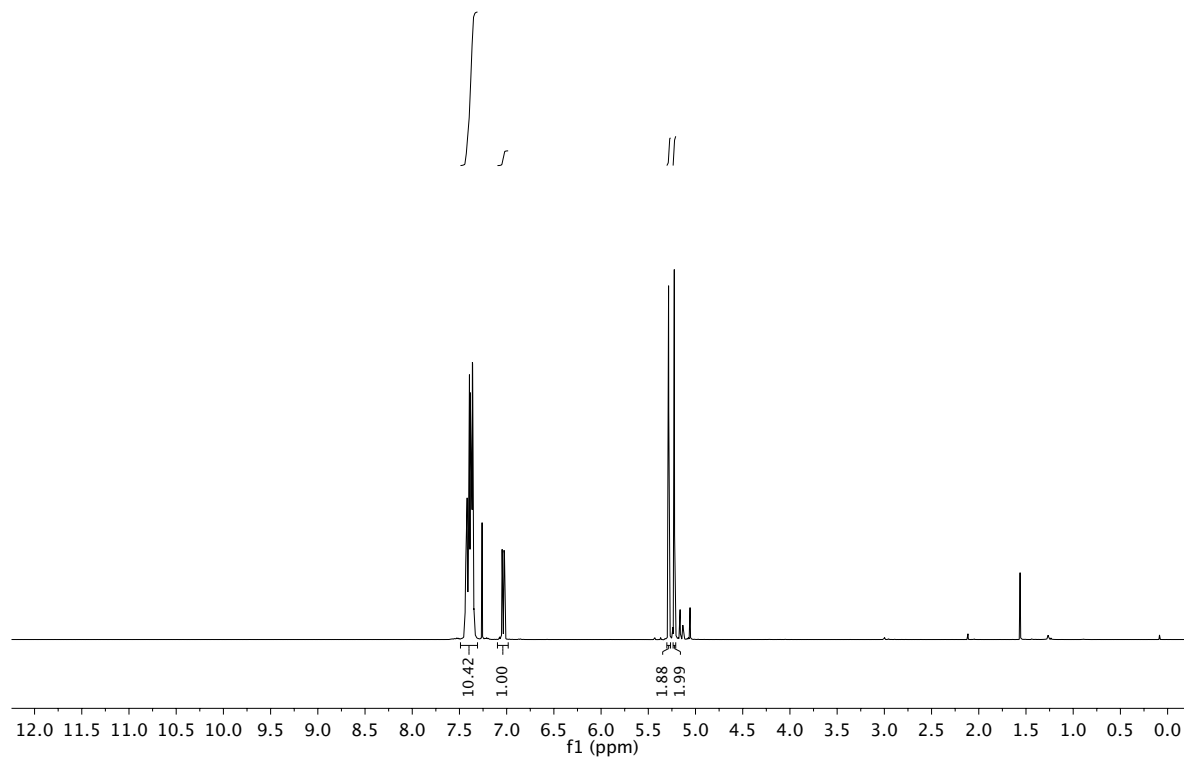
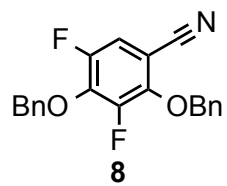


Figure S6. ^1H NMR (500 MHz, CDCl_3) spectrum of **8**.

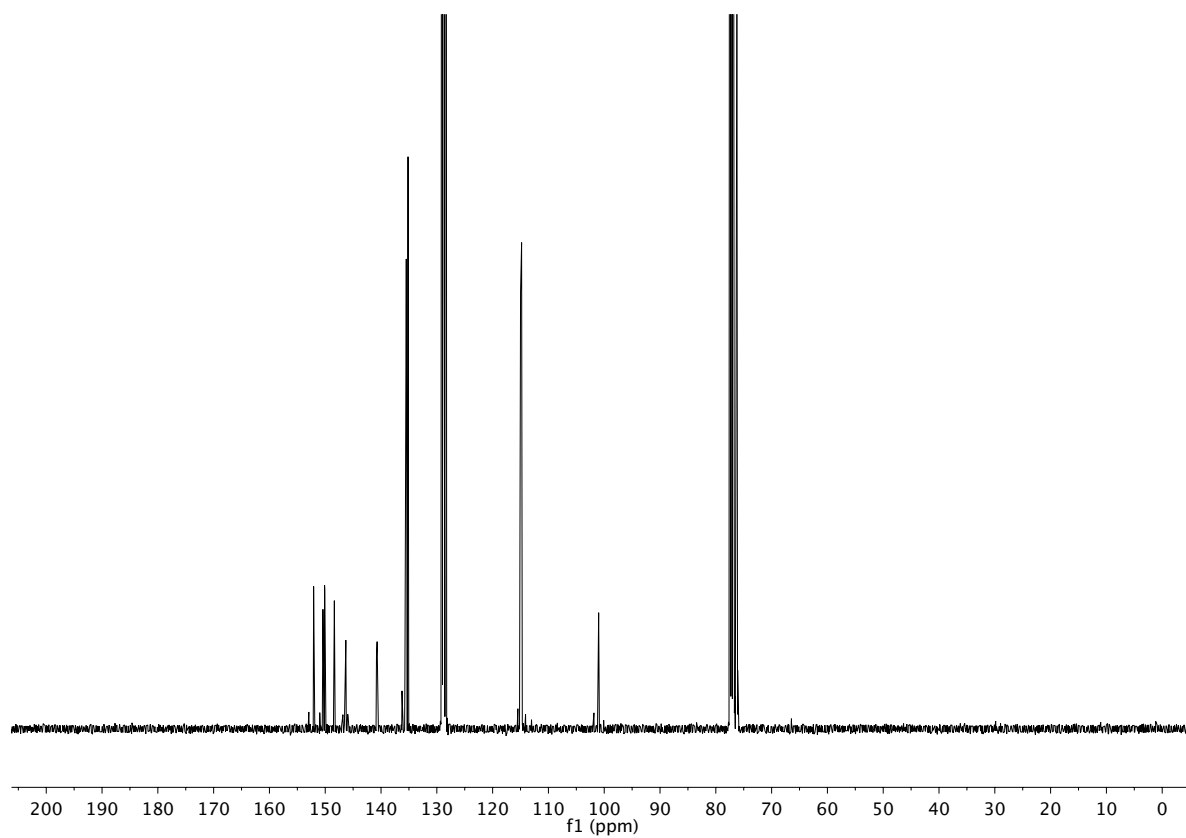
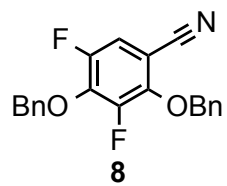


Figure S7. ^{13}C NMR (126 MHz, CDCl_3) spectrum of **8**.

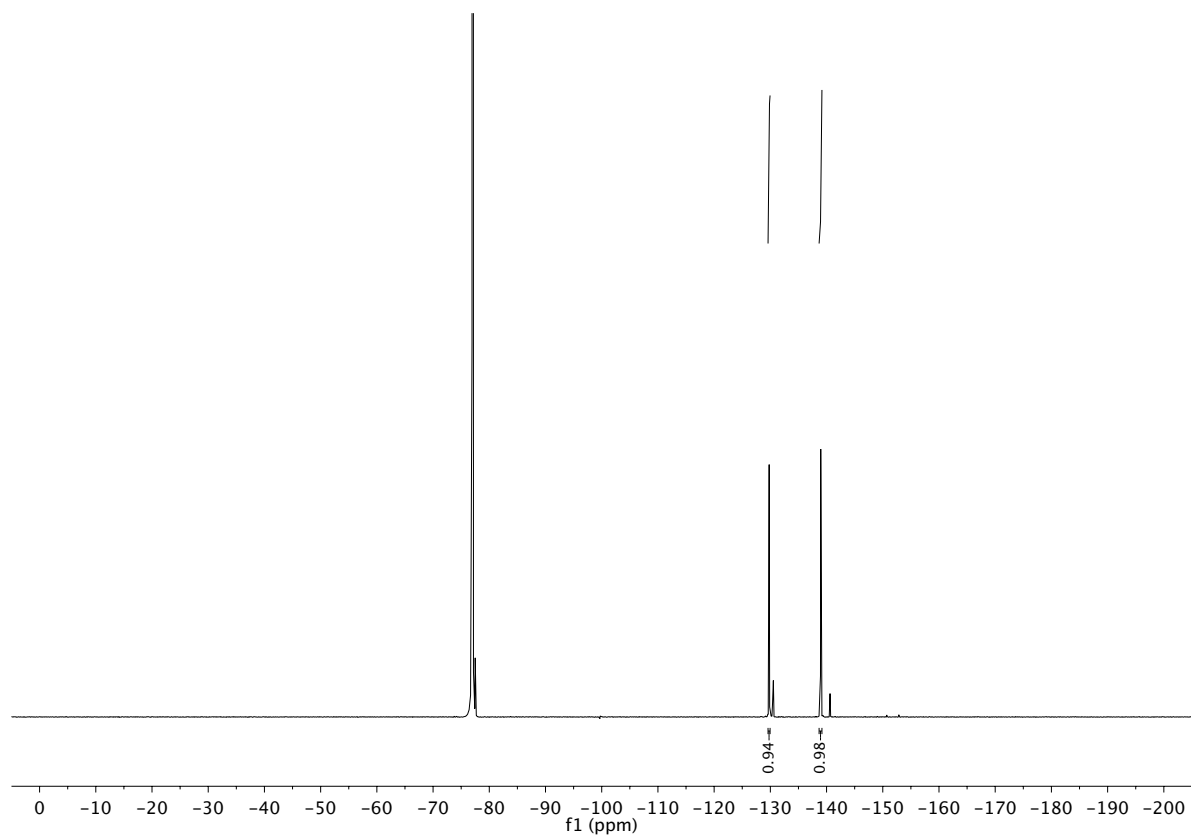
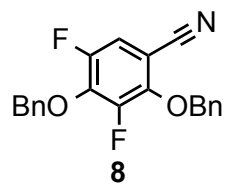


Figure S8. ^{19}F NMR (376 MHz, CDCl_3 , decoupled) spectrum of **8**.

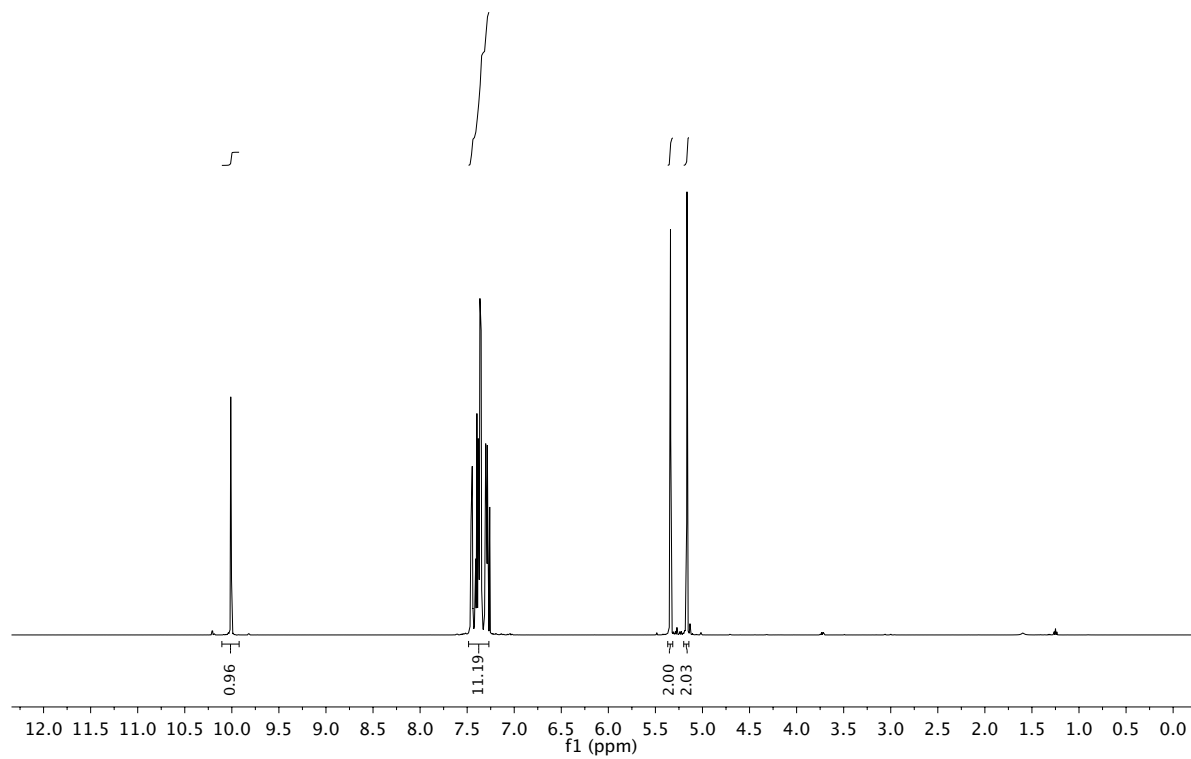
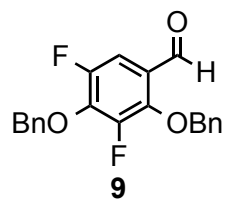


Figure S9. ¹H NMR (500 MHz, CDCl₃) spectrum of **9**.

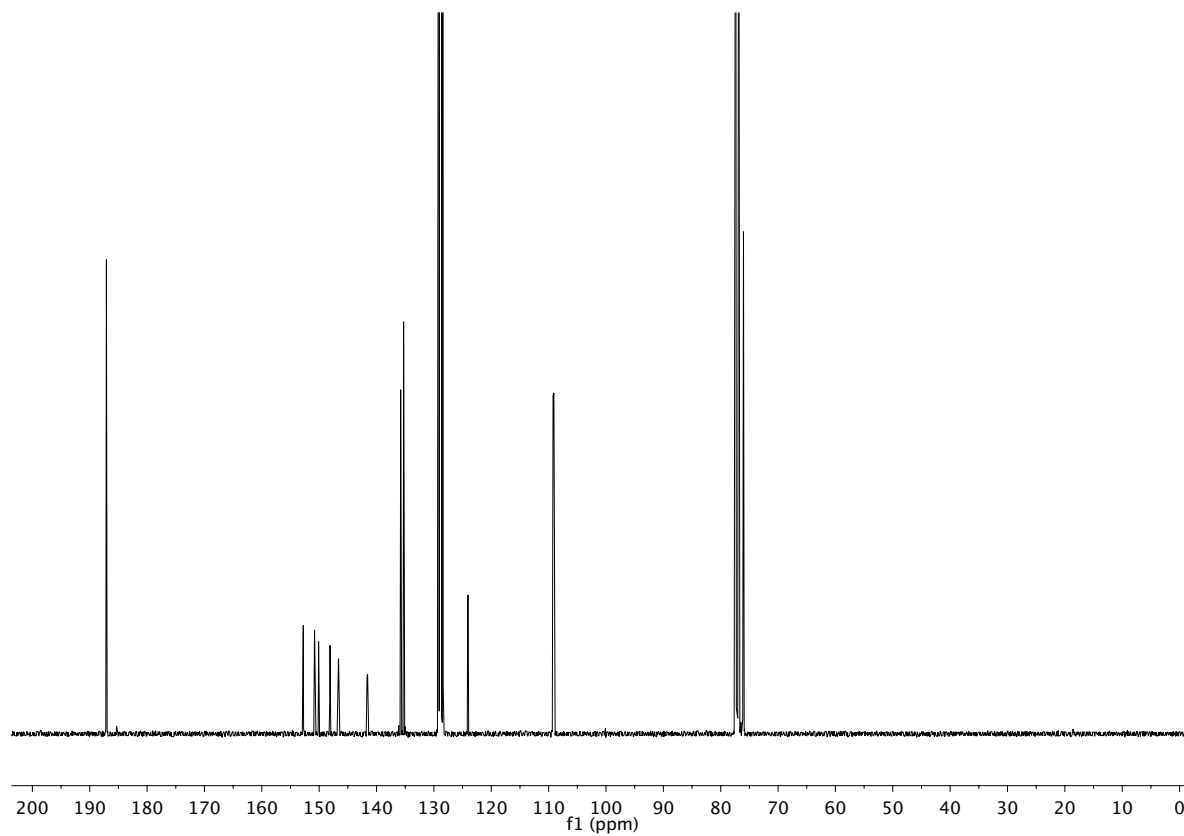
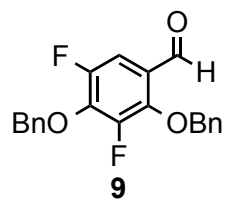


Figure S10. ^{13}C NMR (126 MHz, CDCl_3) spectrum of **9**.

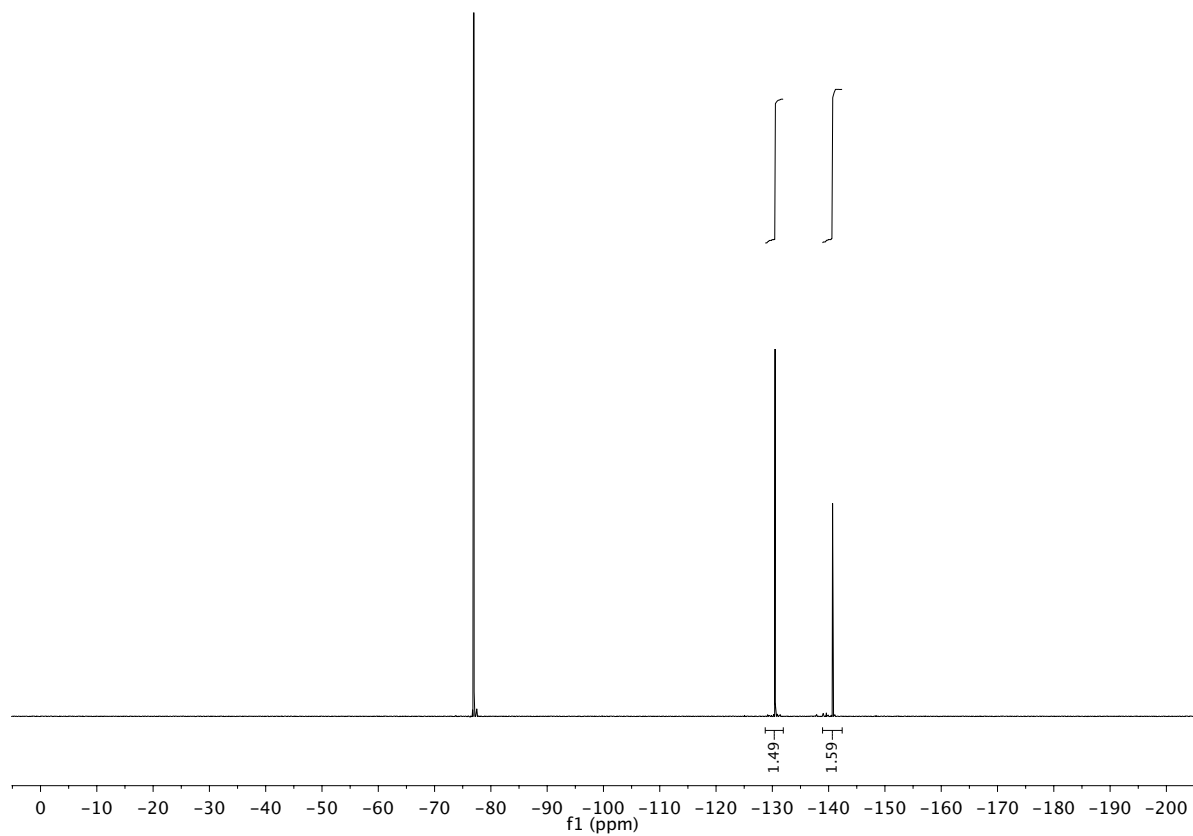
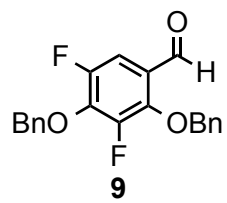


Figure S11. ^{19}F NMR (376 MHz, CDCl_3 , decoupled) spectrum of **9**.

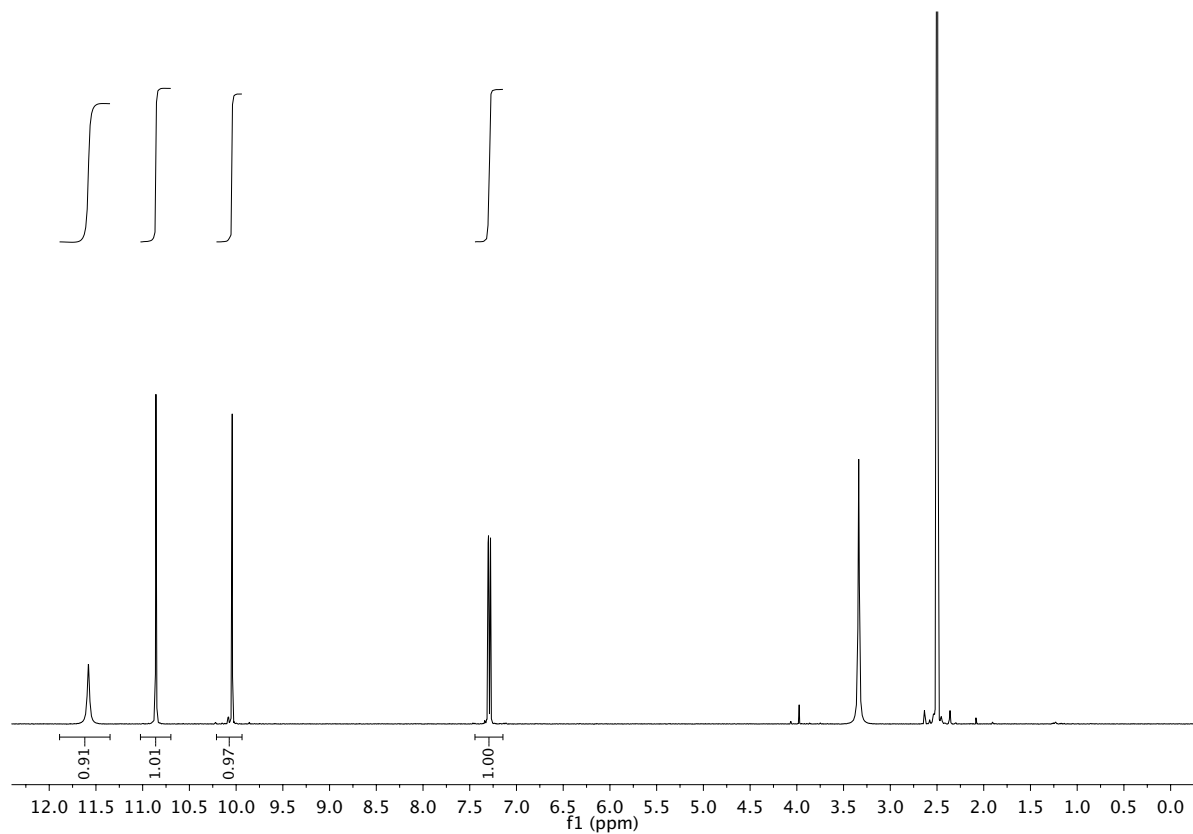
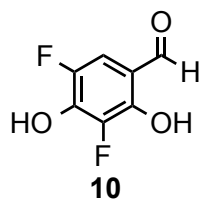


Figure S12. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **10**.

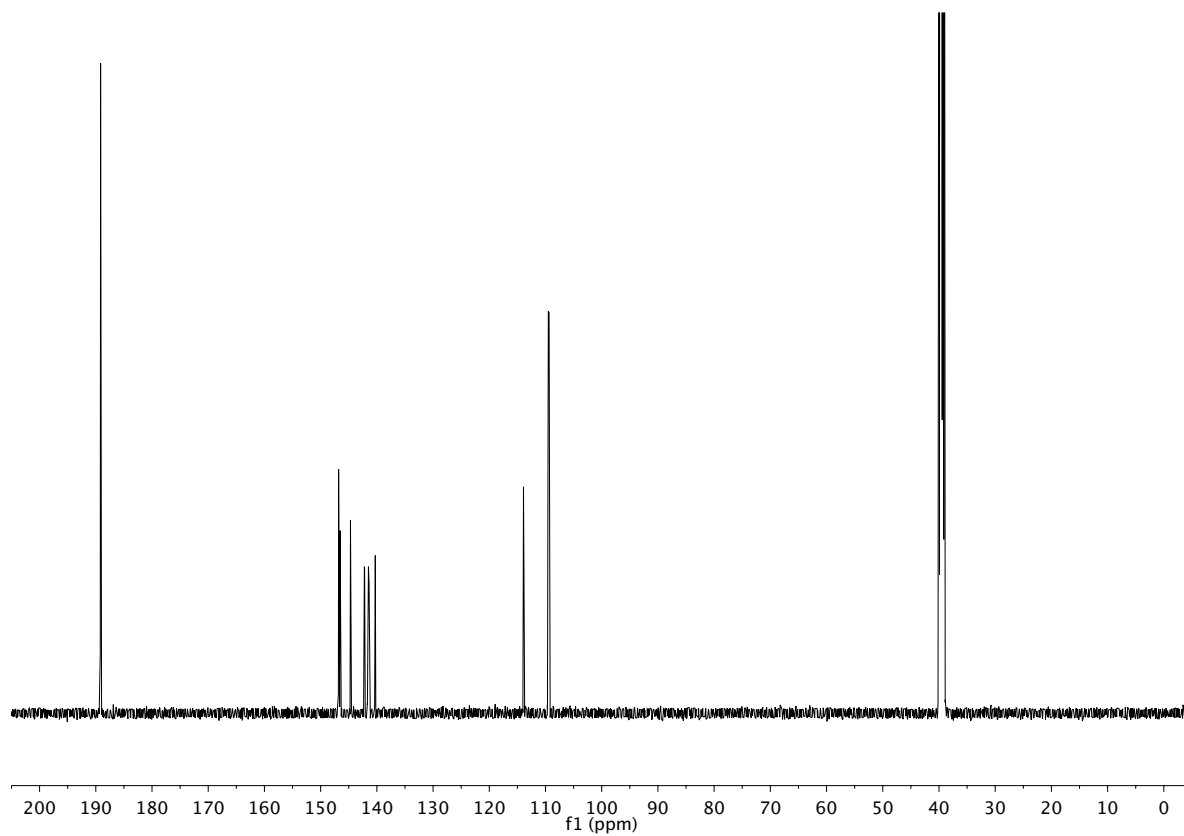
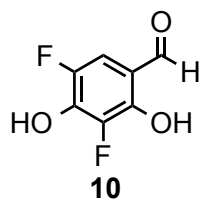


Figure S13. ^{13}C NMR (126 MHz, DMSO- d_6) spectrum of **10**.

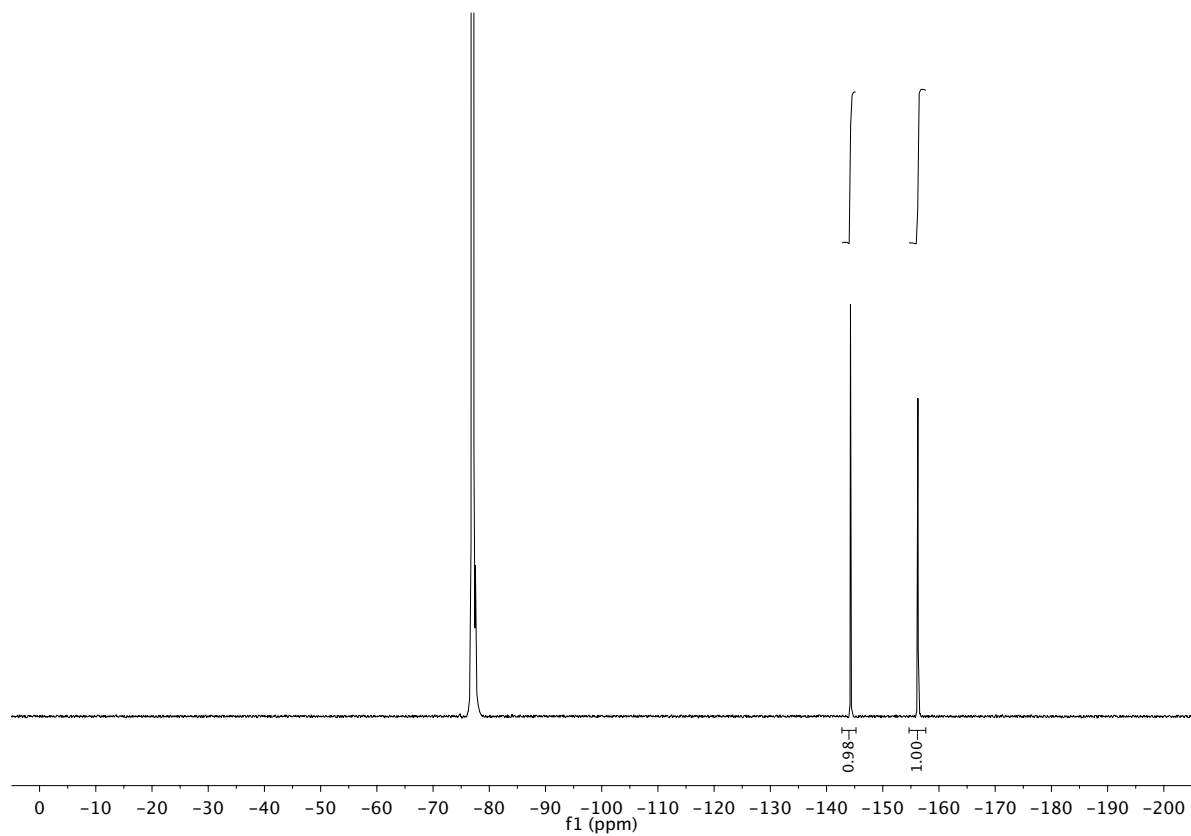
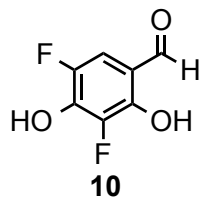


Figure S14. ^{19}F NMR (376 MHz, $\text{DMSO-}d_6$, decoupled) spectrum of **10**.

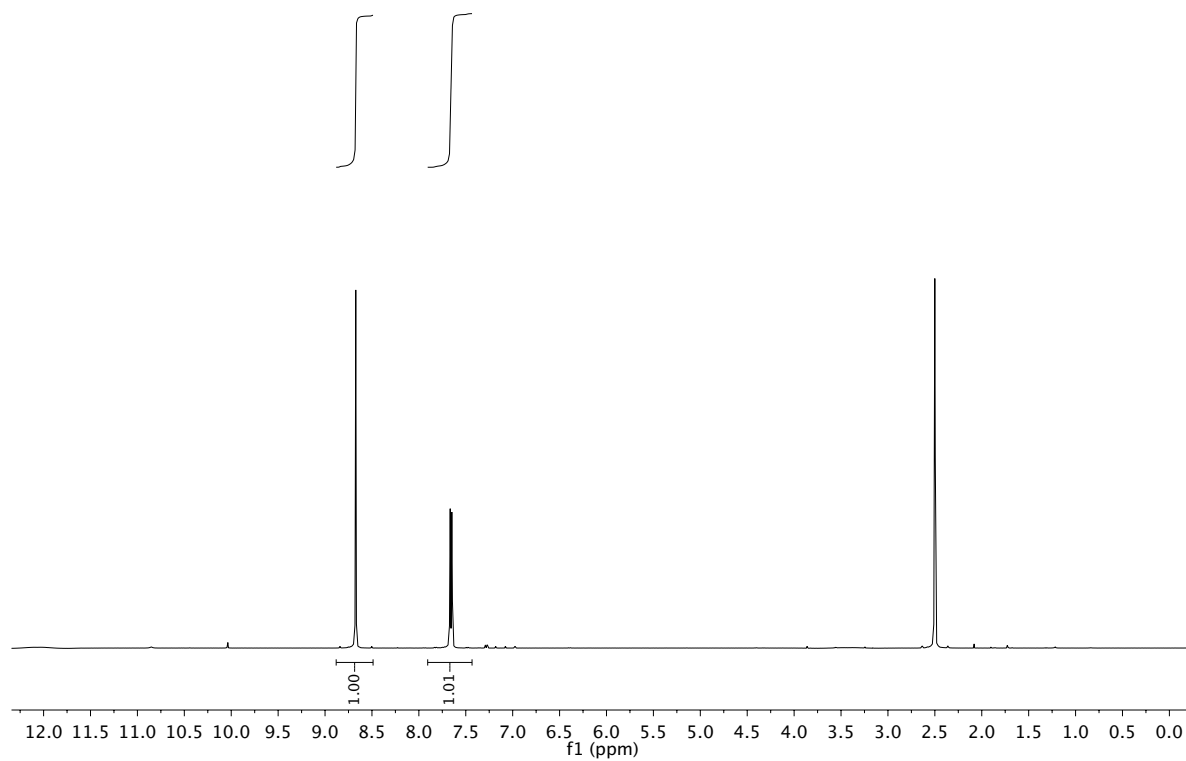
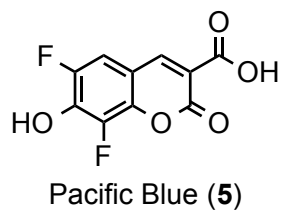


Figure S15. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **5**.

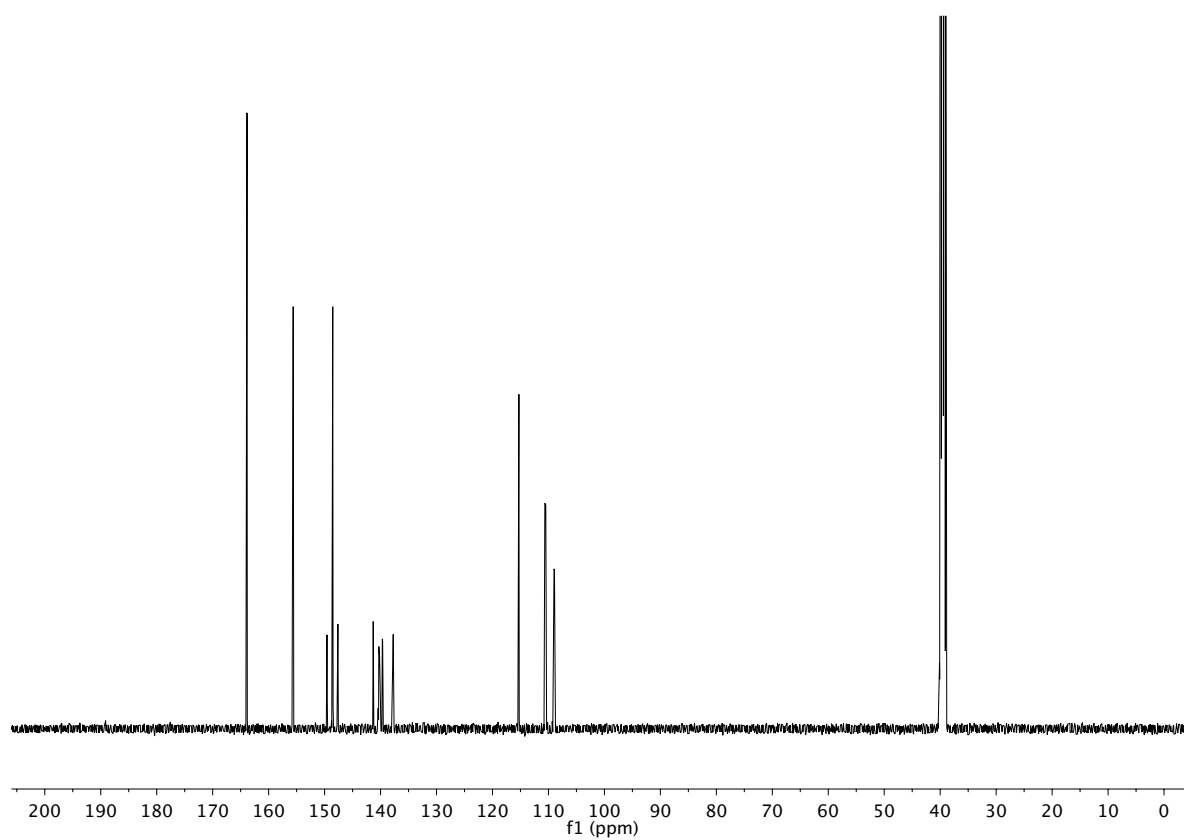
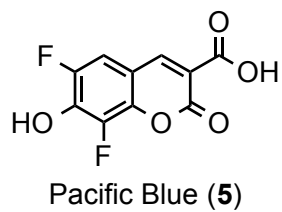


Figure S16. ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) spectrum of **5**.

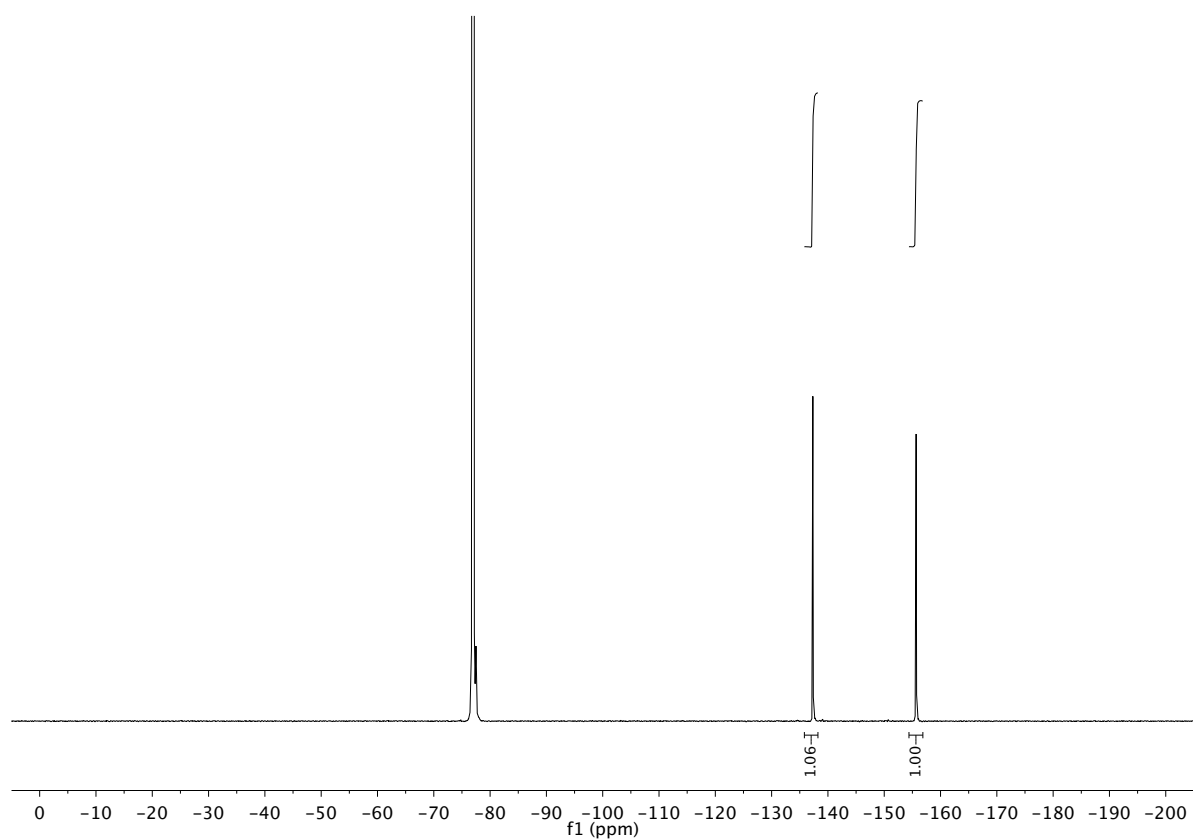
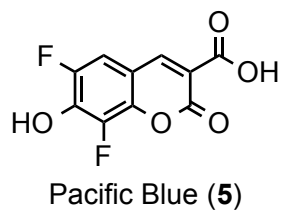


Figure S17. ^{19}F NMR (376 MHz, $\text{DMSO-}d_6$, decoupled) spectrum of **5**.

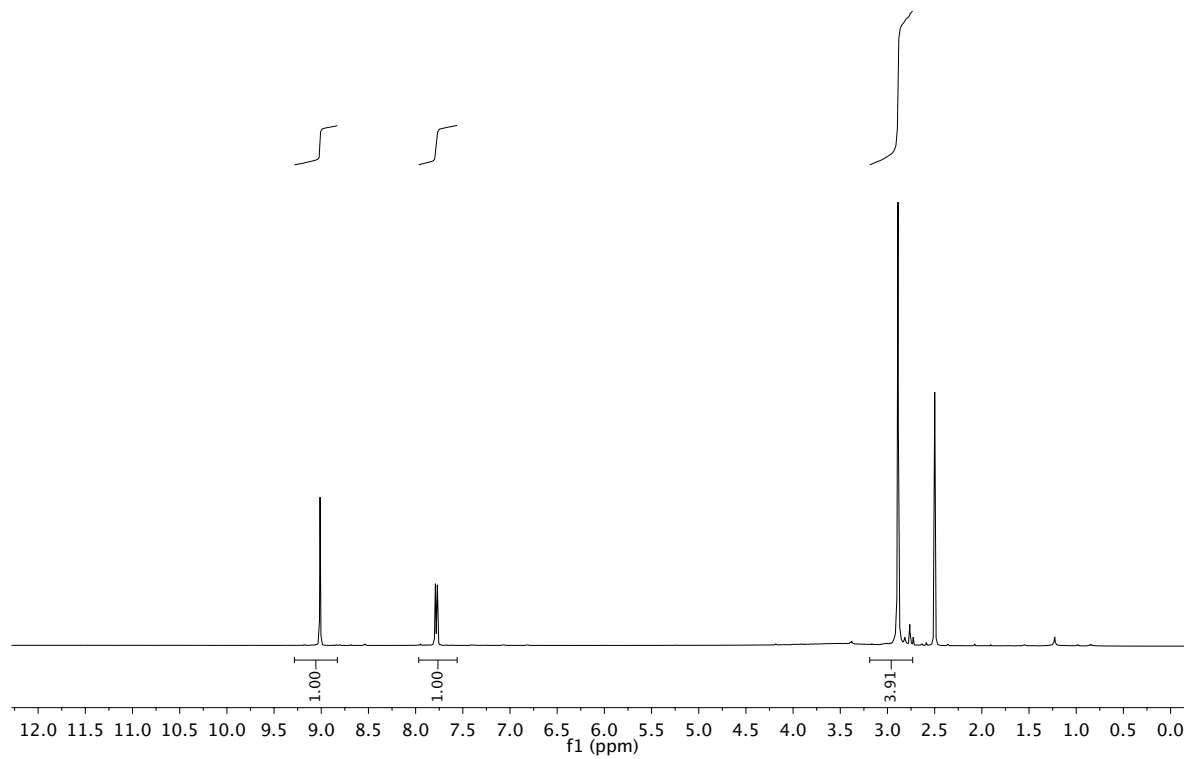
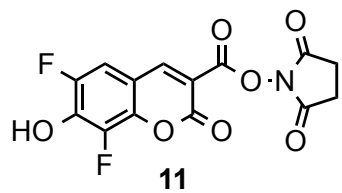


Figure S18. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **11**.

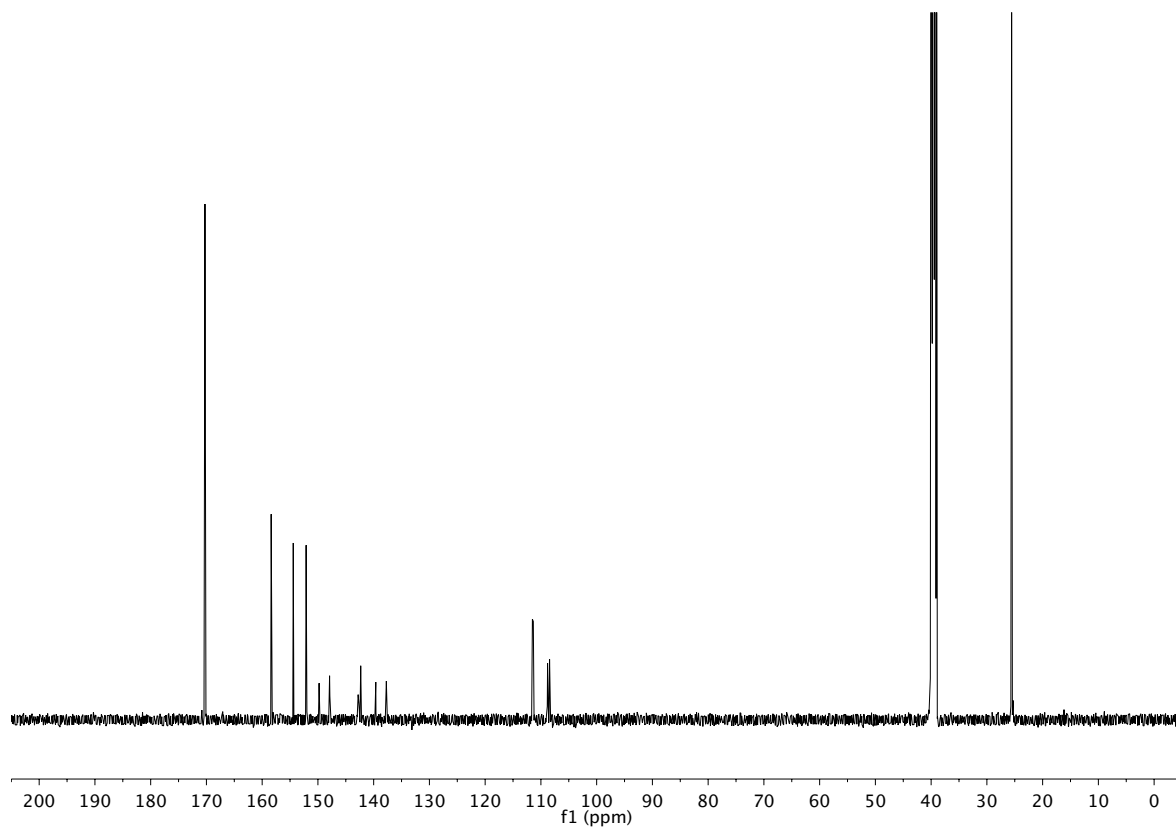
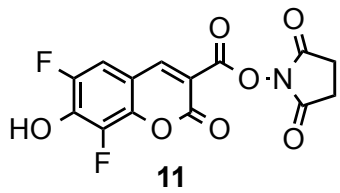


Figure S19. ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) spectrum of **11**.

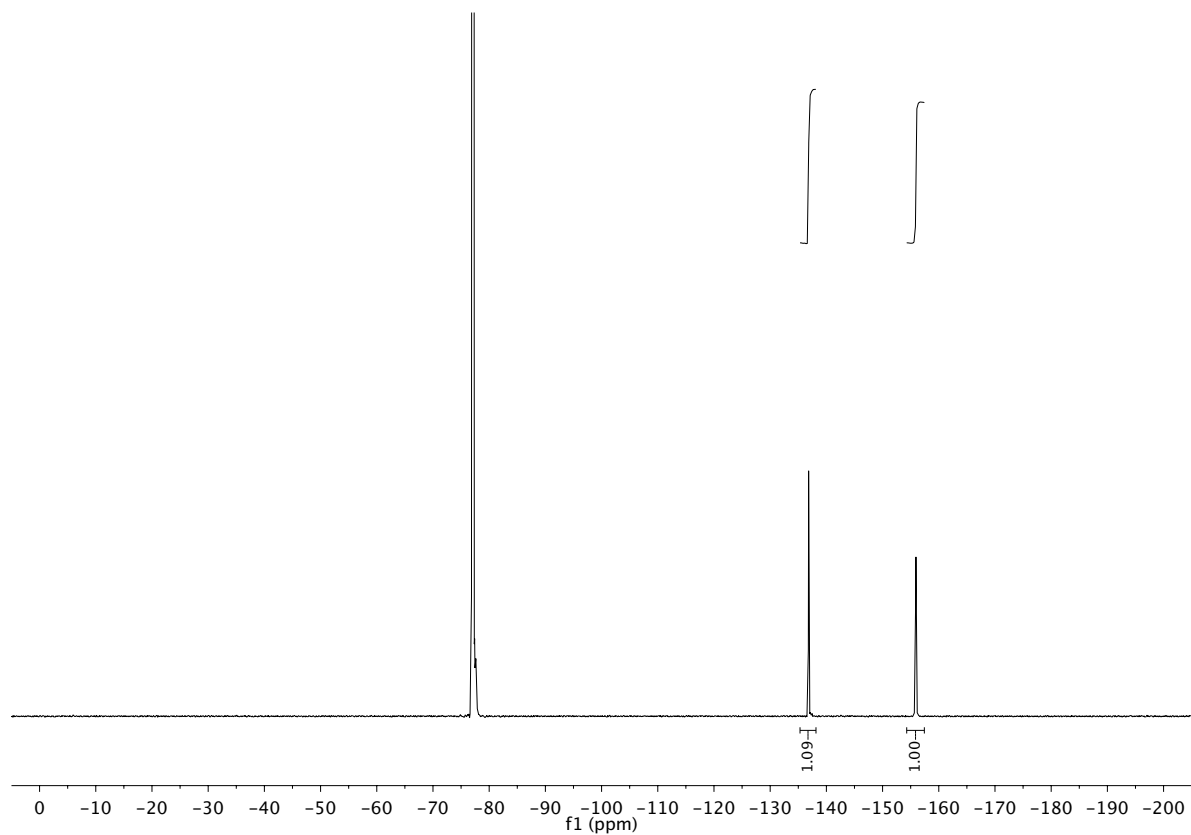
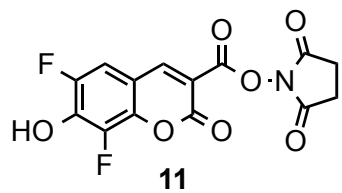


Figure S20. ^{19}F NMR (376 MHz, DMSO- d_6 , decoupled) spectrum of **11**.

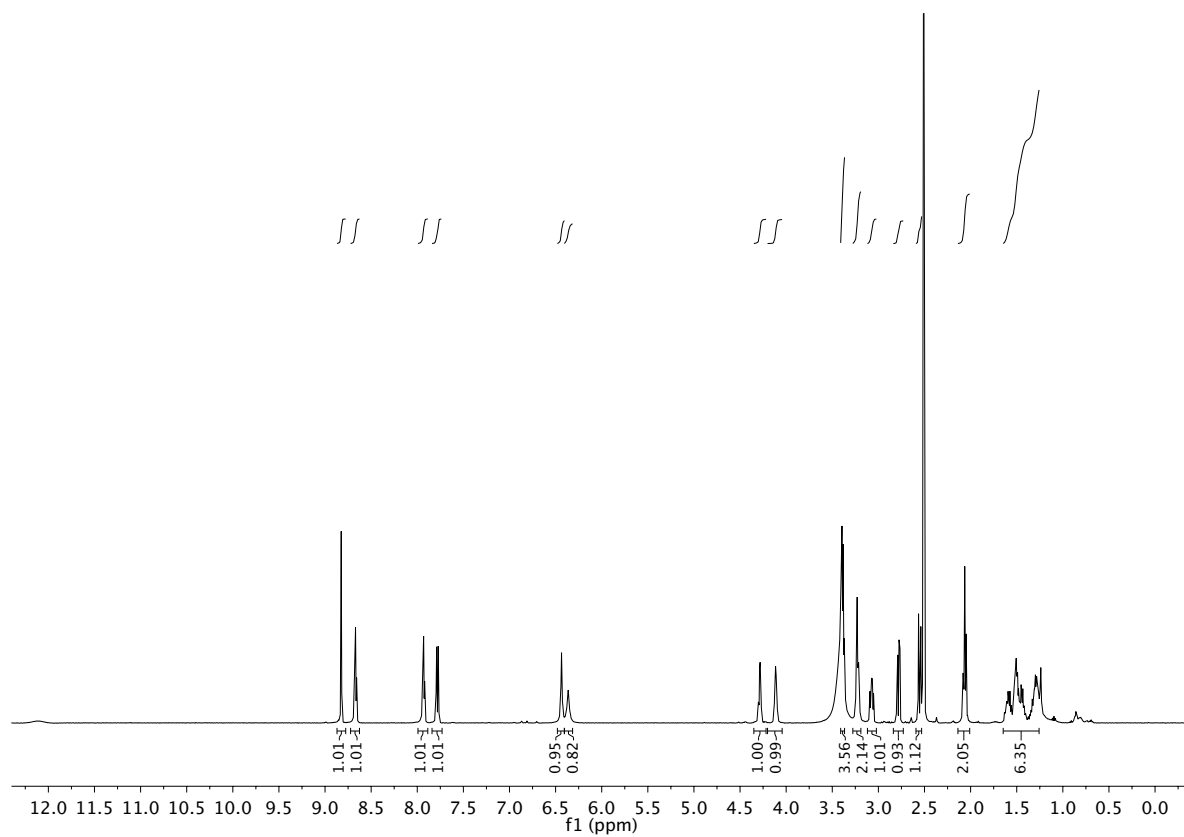
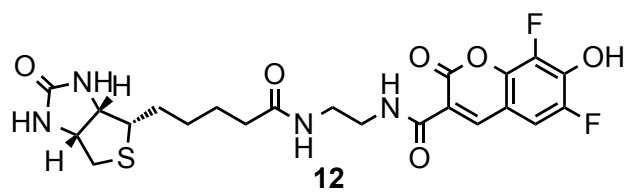


Figure S21. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **12**.

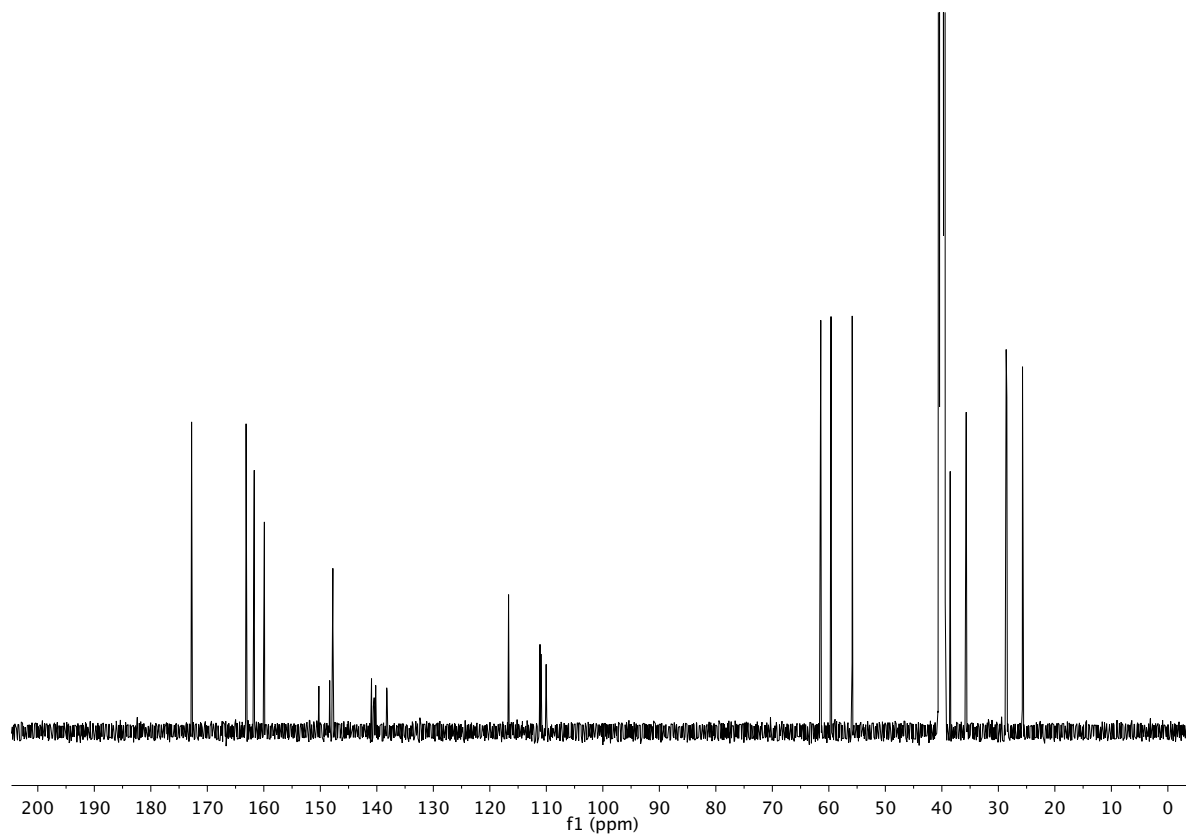
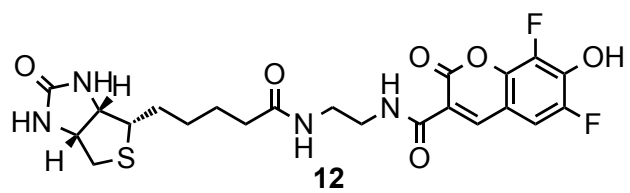


Figure S22. ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) spectrum of **12**.

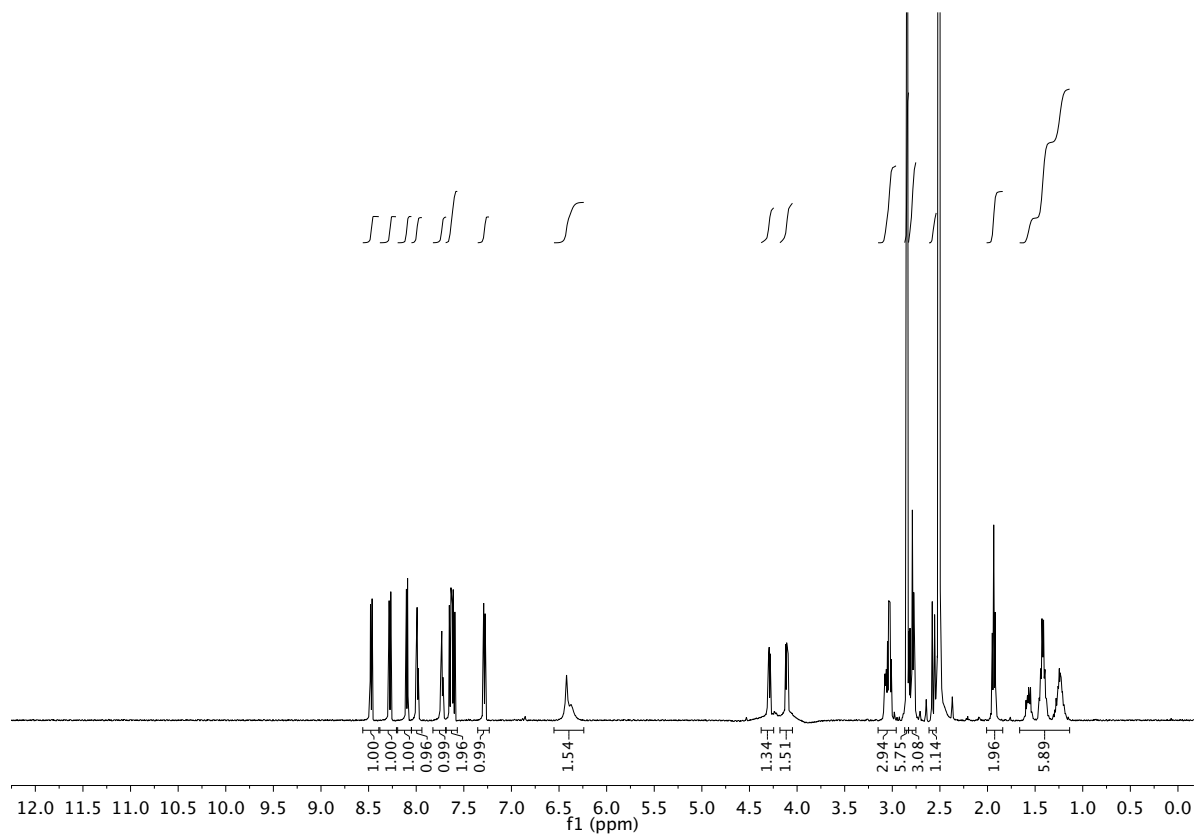
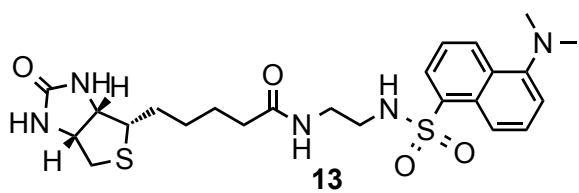


Figure S23. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **13**.

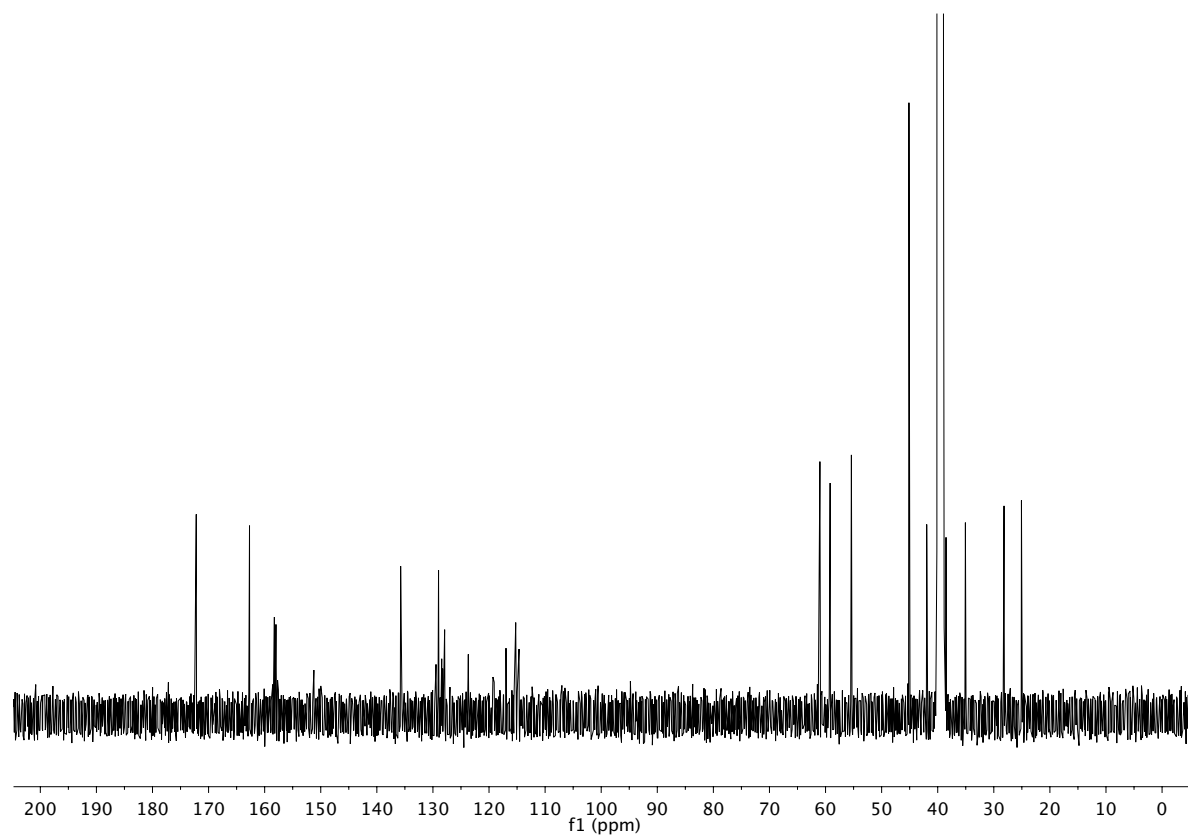
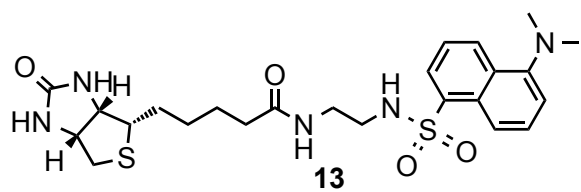


Figure S24. ^{13}C NMR (126 MHz, DMSO- d_6) spectrum of **13**.

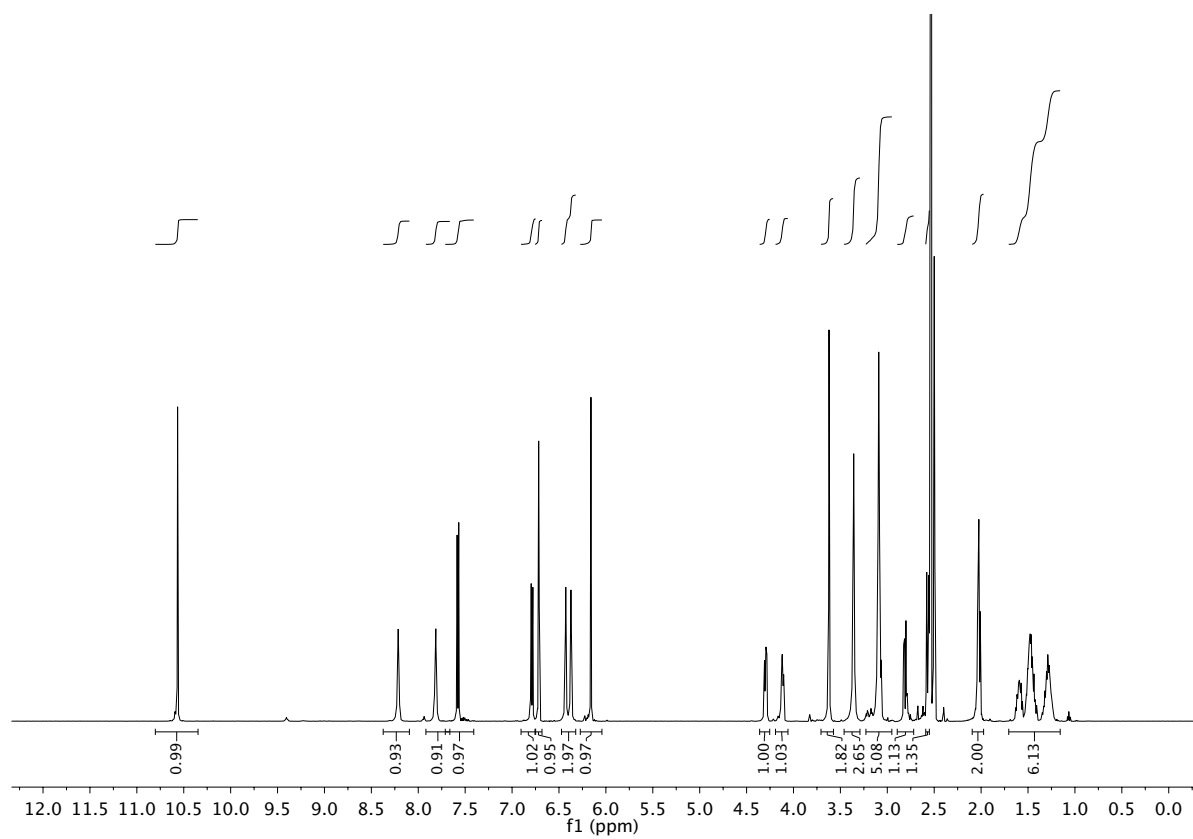
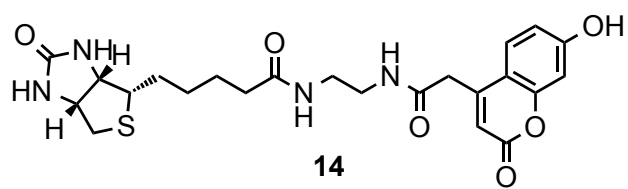


Figure S25. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **14**.

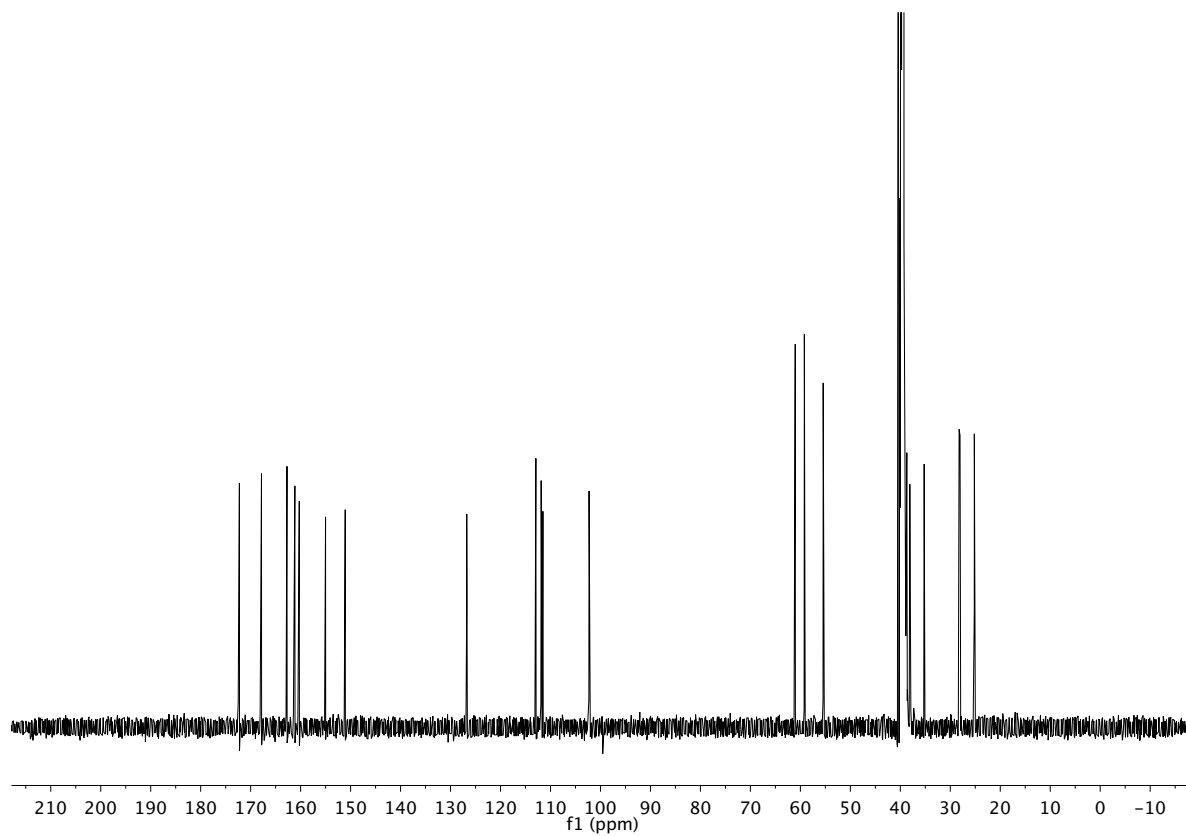
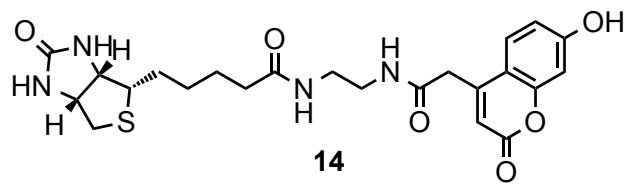


Figure S26. ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) spectrum of **14**.

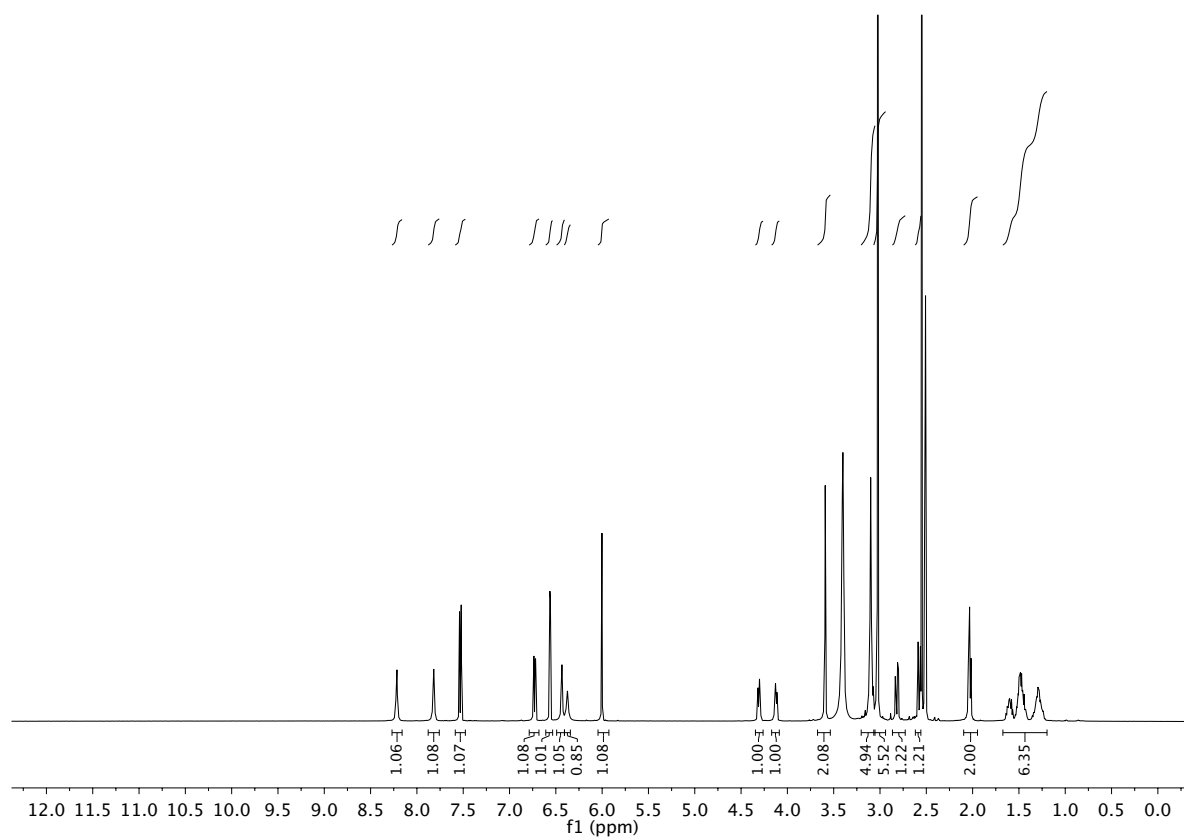
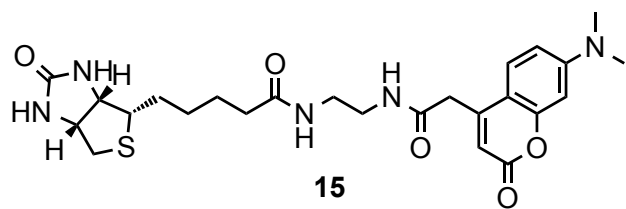


Figure S27. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **15**.

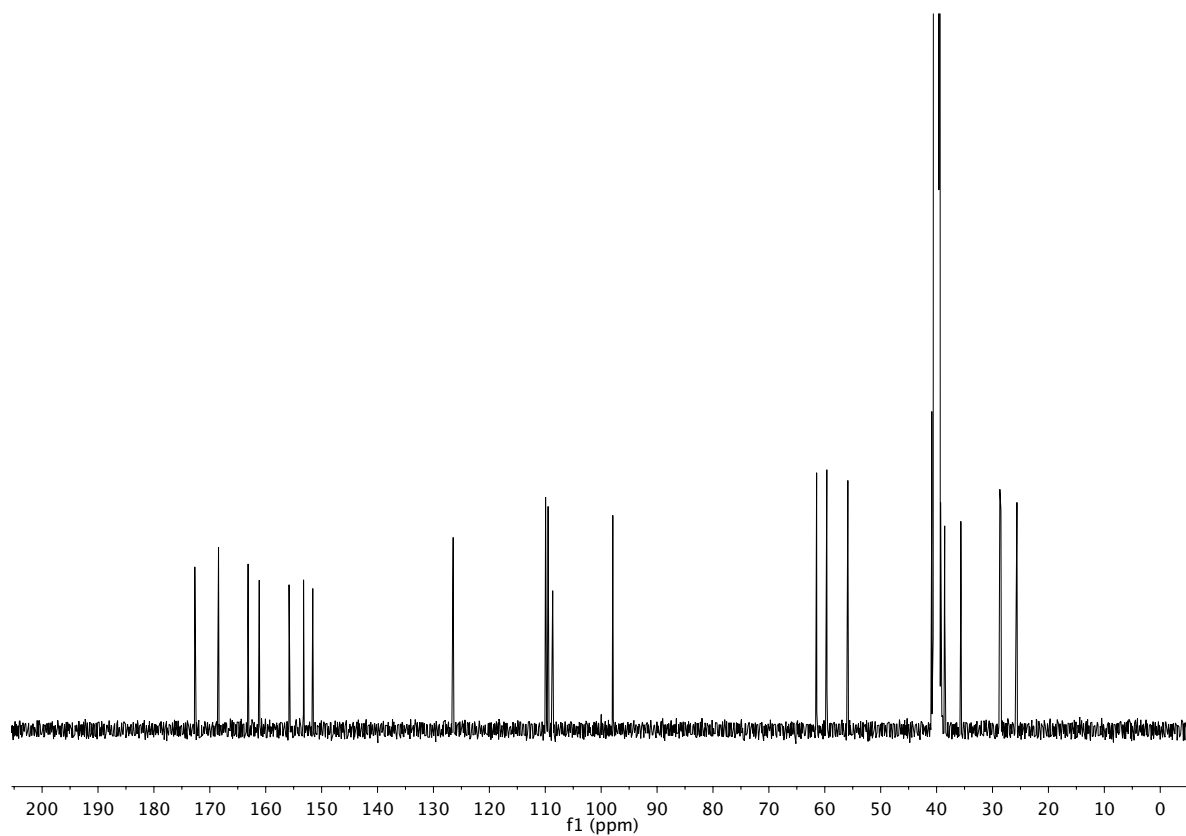
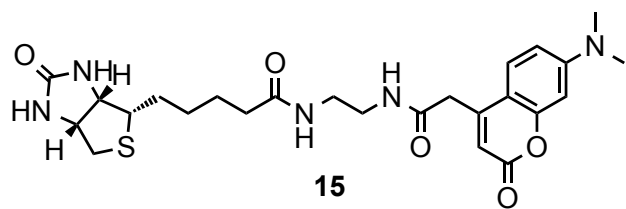


Figure S28. ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) spectrum of **15**.

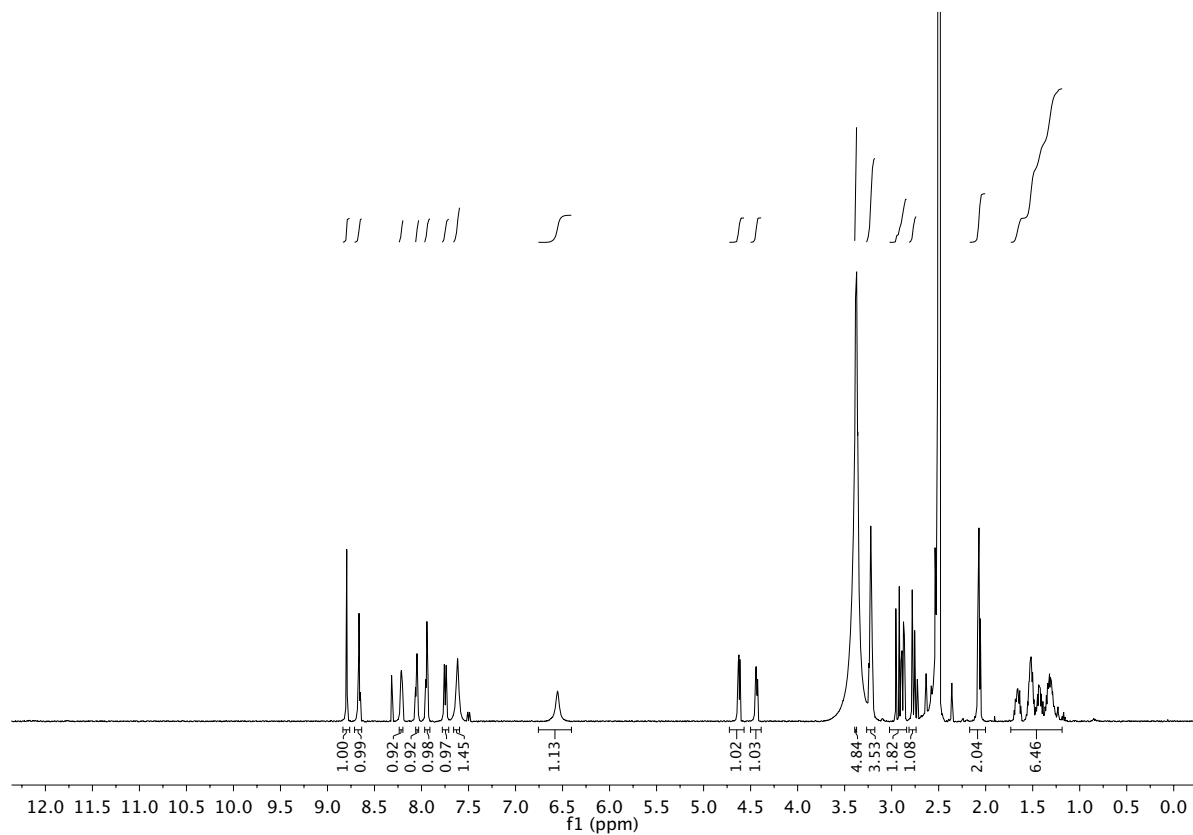
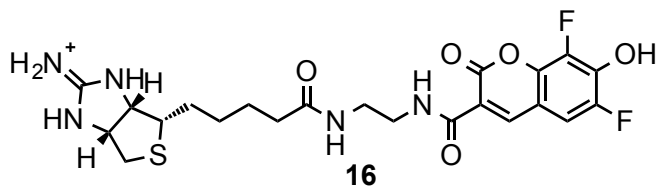


Figure S29. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **16**.

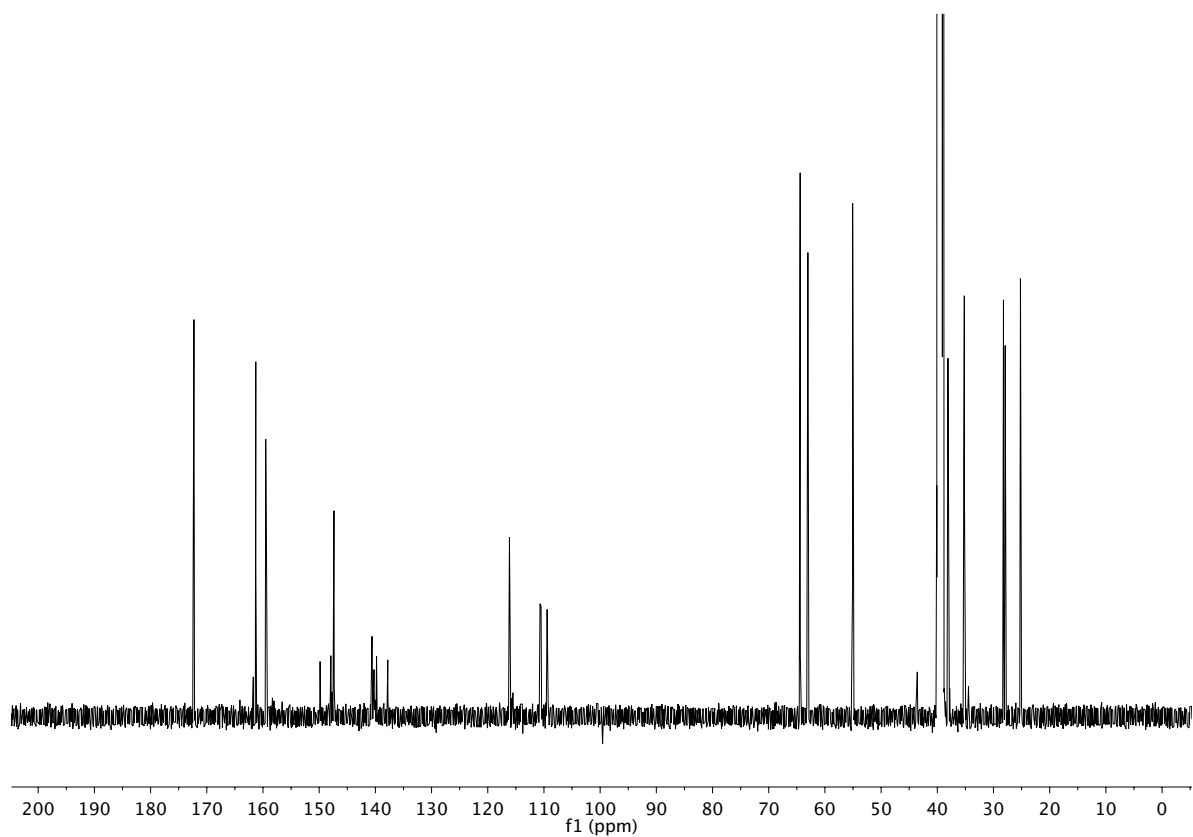
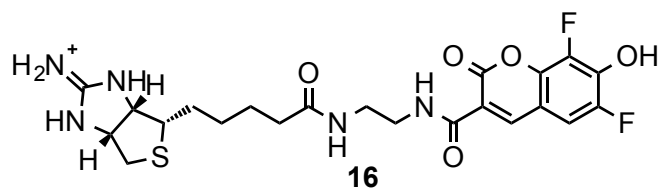


Figure S30. ¹³C NMR (126 MHz, DMSO-*d*₆) spectrum of **16**.

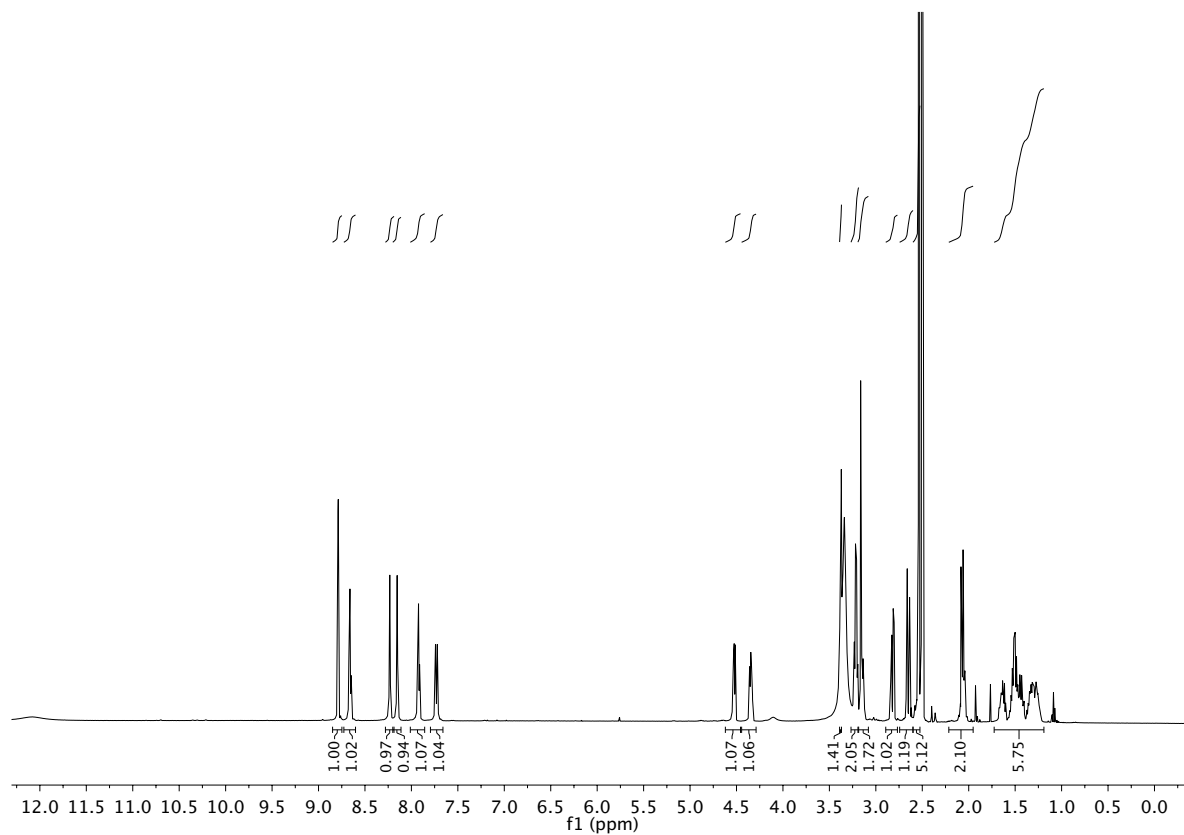
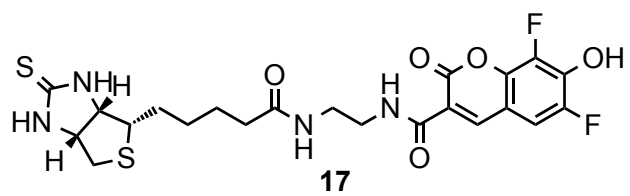


Figure S31. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **17**.

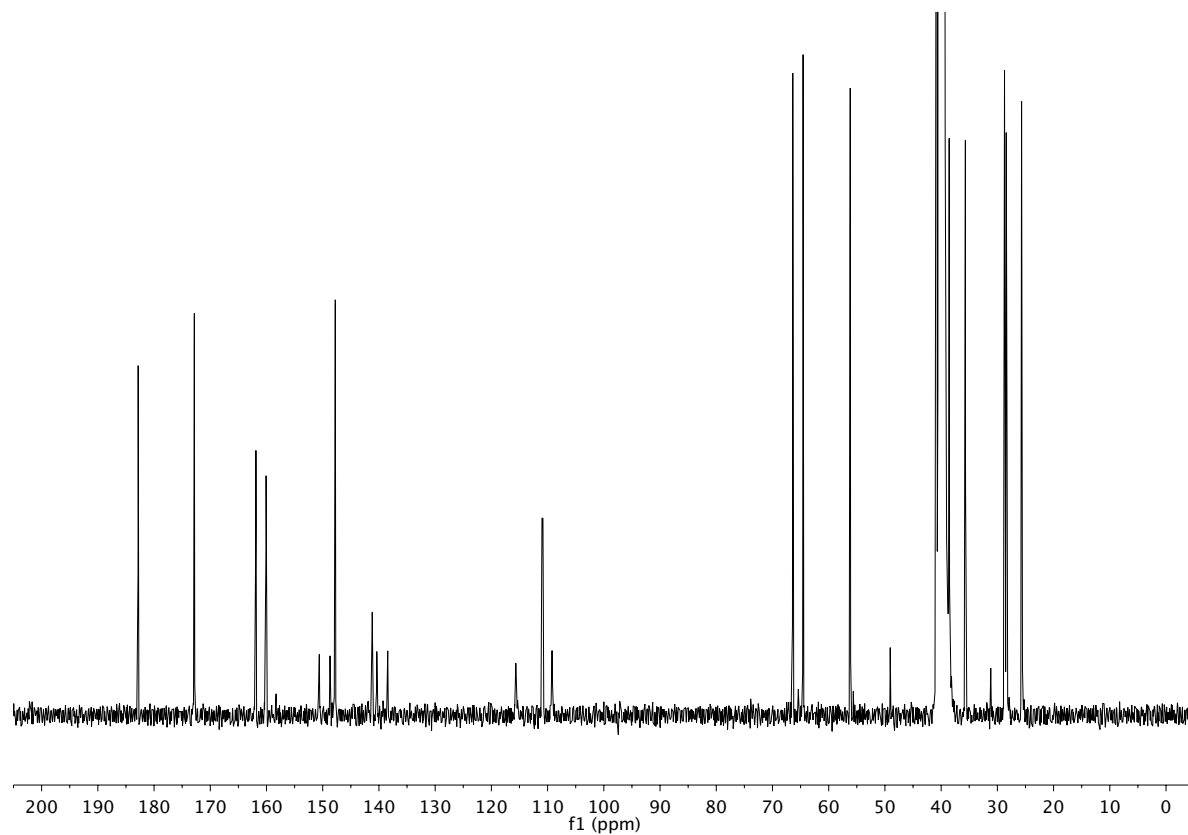
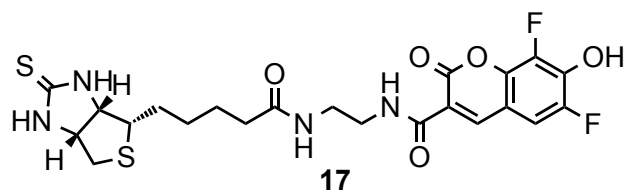


Figure S32. ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) spectrum of **17**.

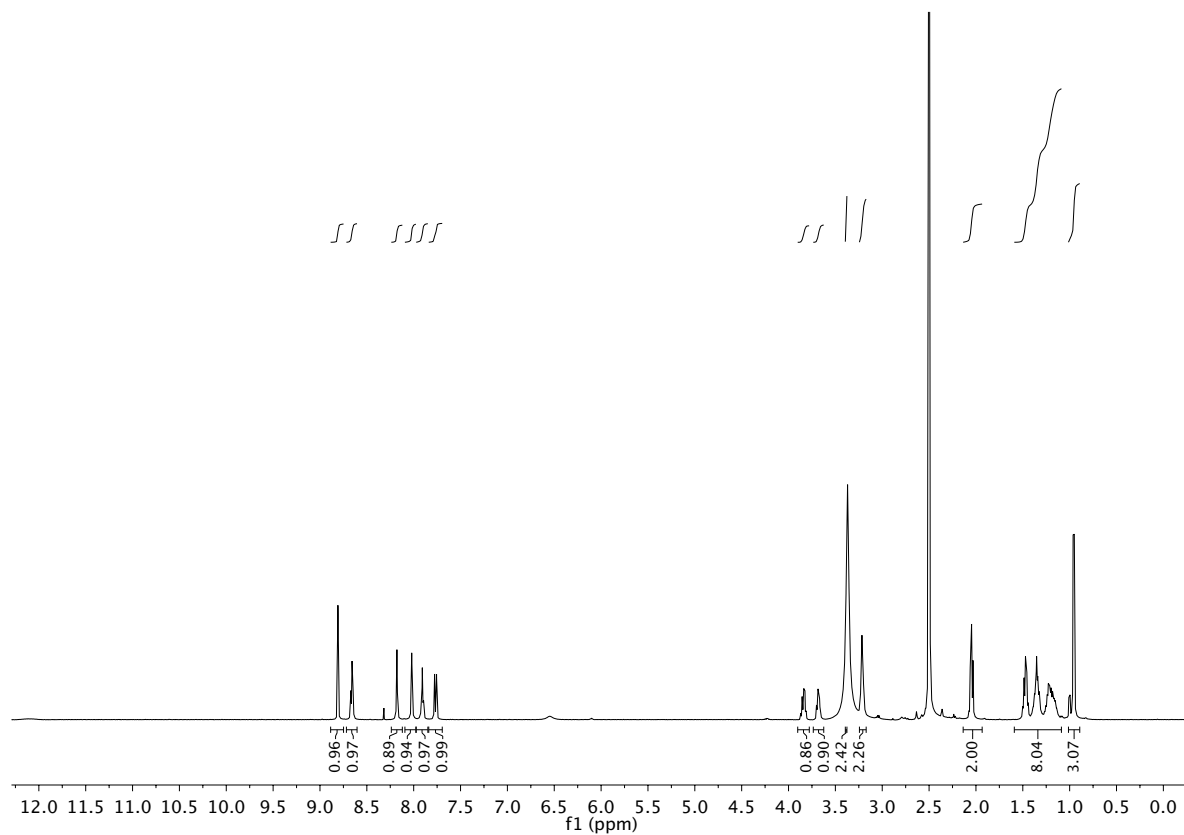
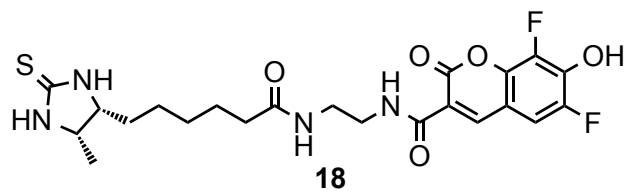


Figure S33. ¹H NMR (500 MHz, DMSO-*d*₆) spectrum of **18**.

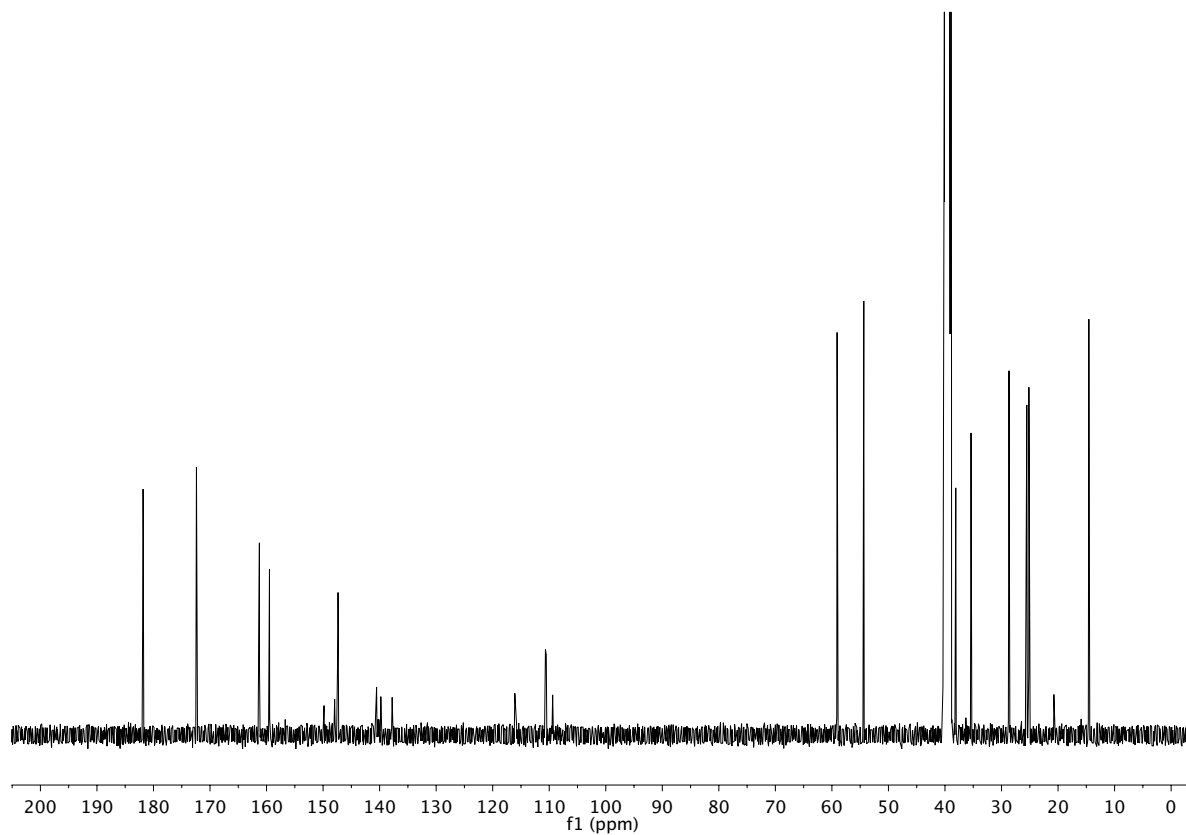
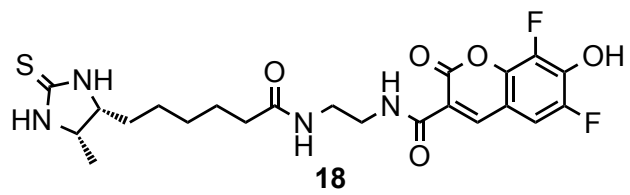


Figure S34. ¹³C NMR (126 MHz, DMSO-*d*₆) spectrum of **18**.

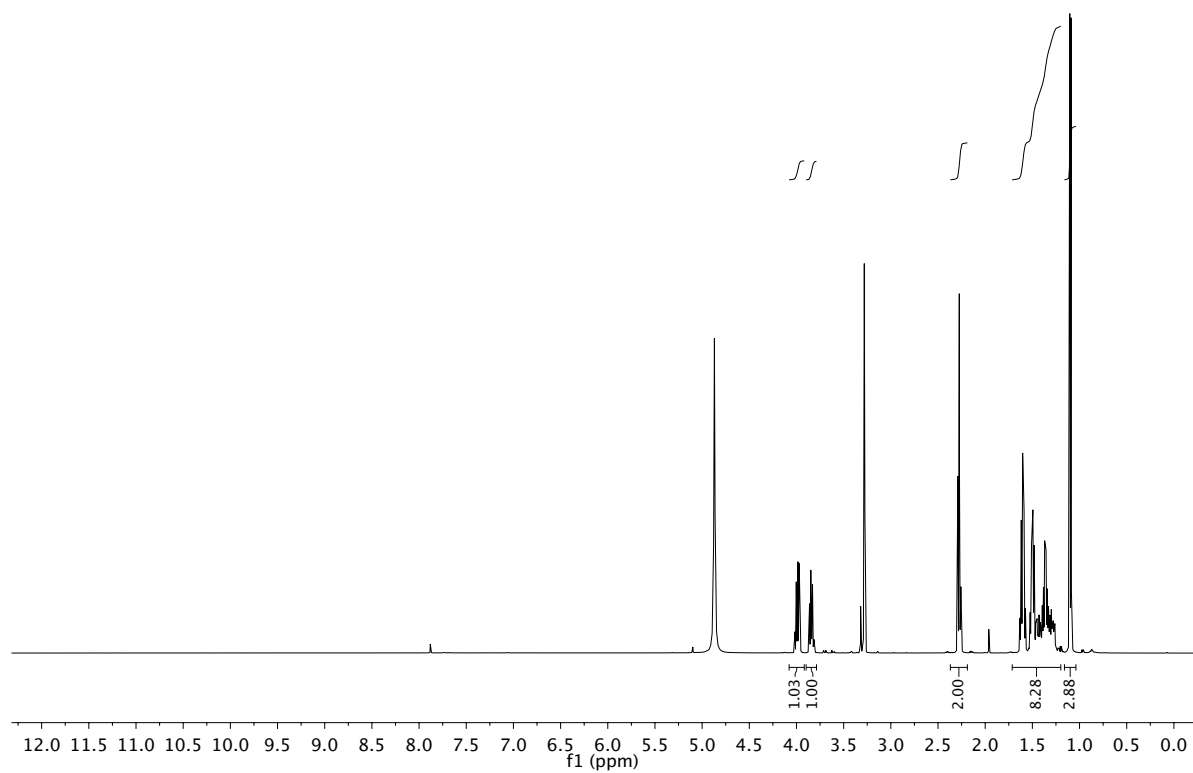
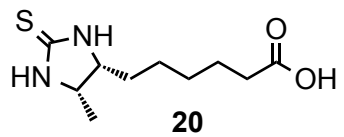


Figure S35. ^1H NMR (500 MHz, CD_3OD) spectrum of **20**.

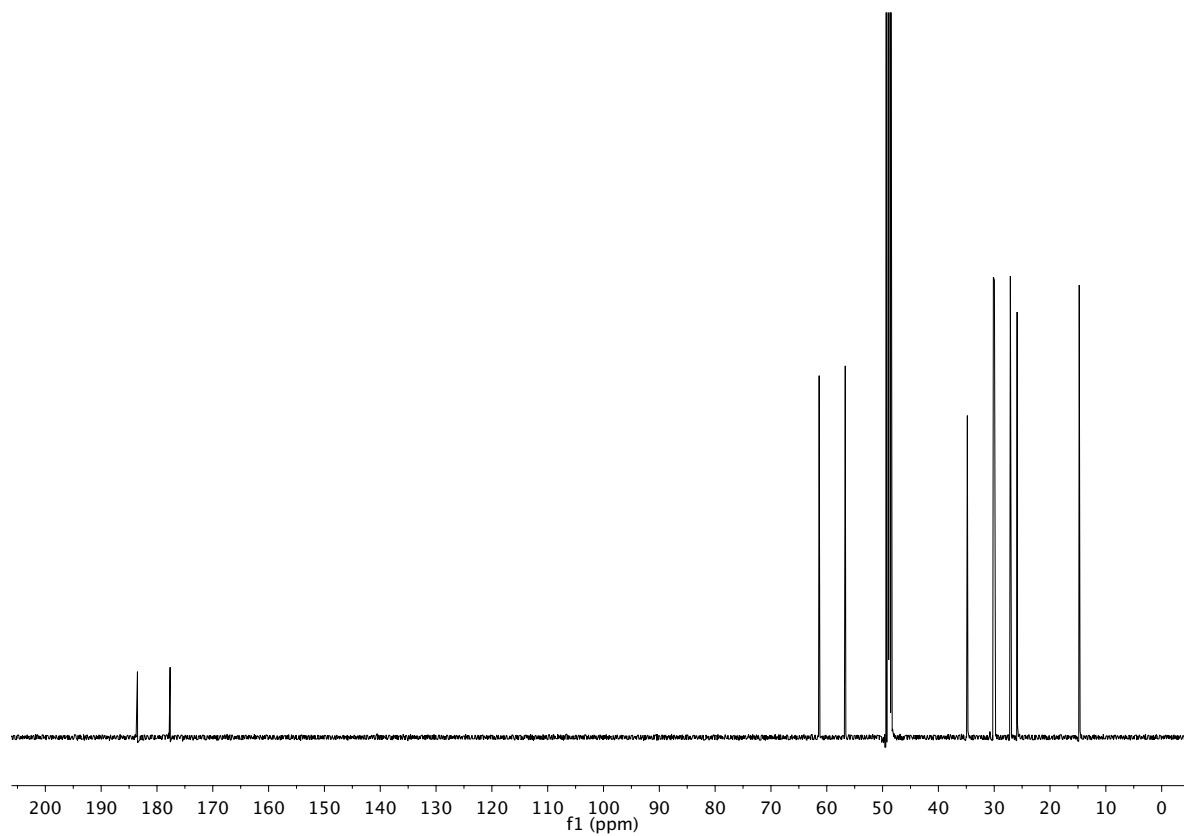
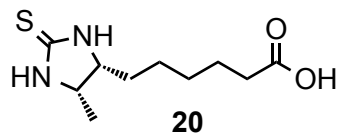


Figure S36. ^{13}C NMR (126 MHz, CD_3OD) spectrum of **20**.

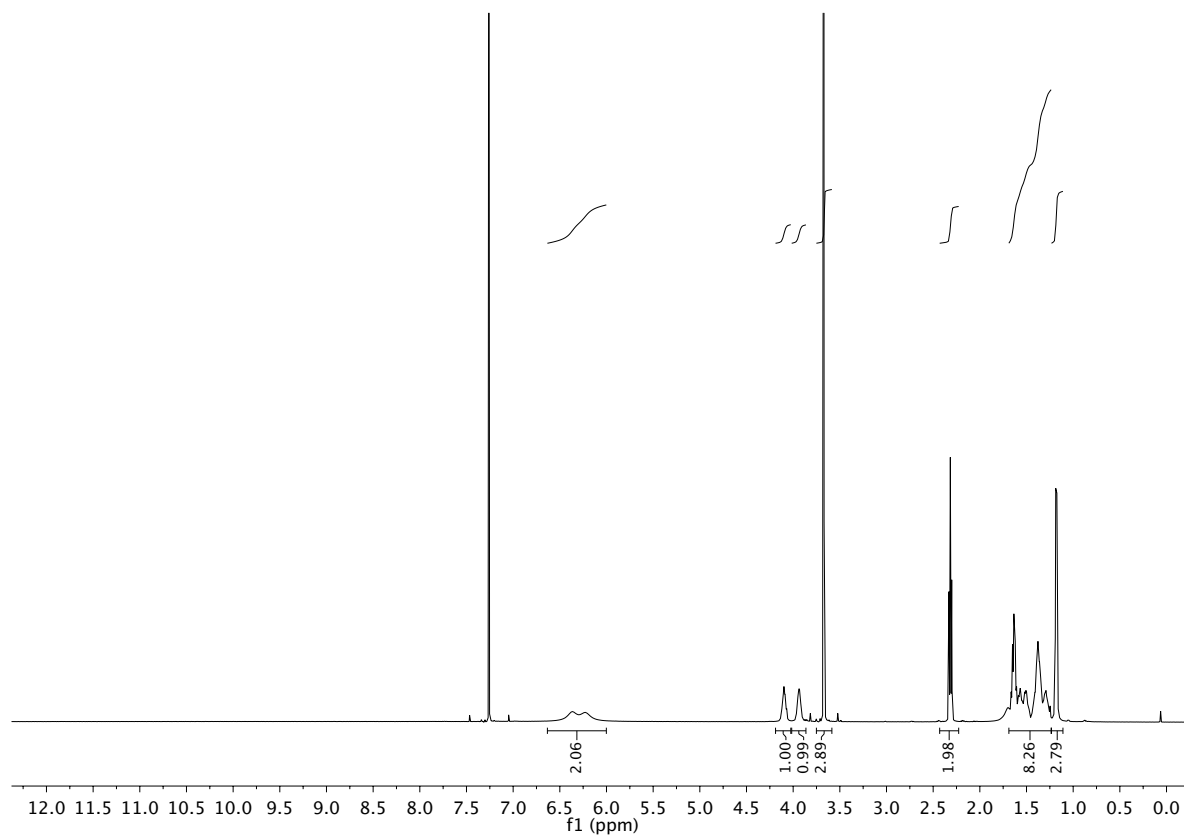
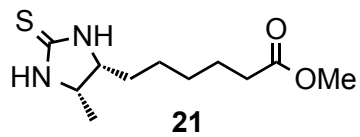


Figure S37. ^1H NMR (500 MHz, CDCl_3) spectrum of **21**.

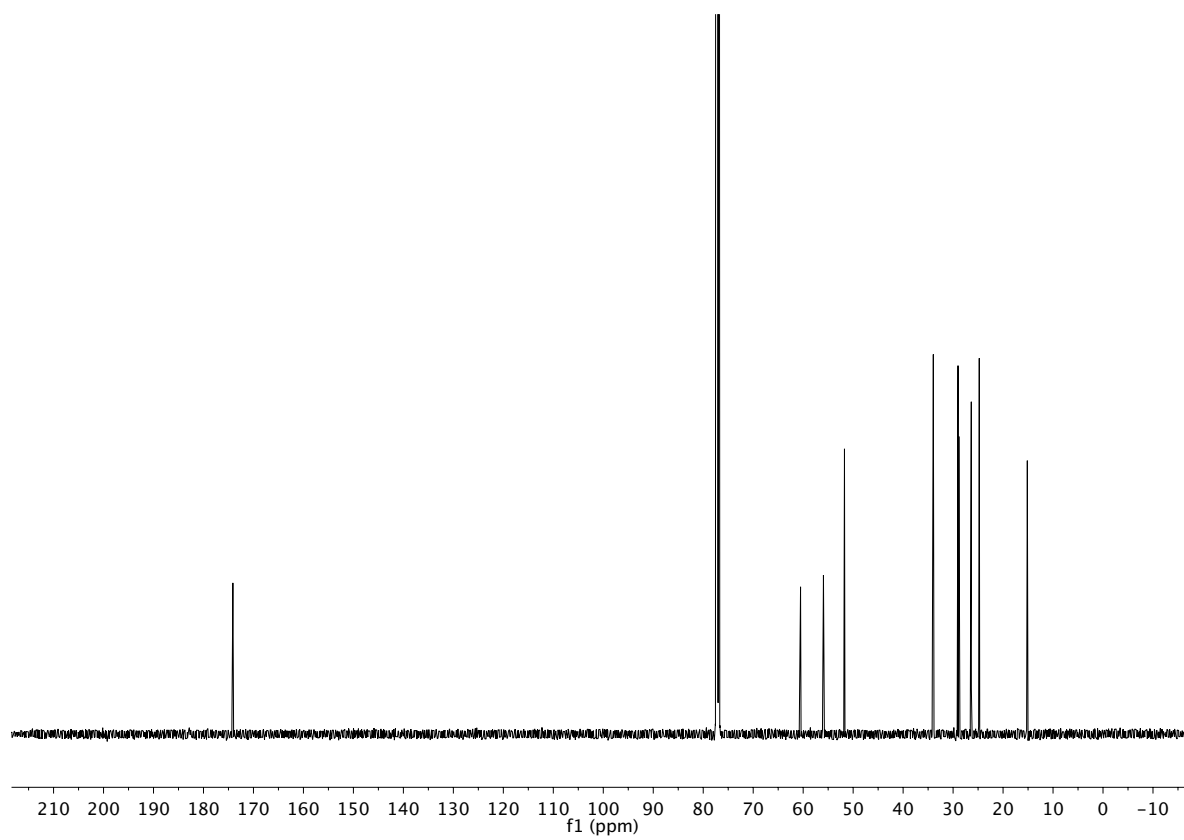
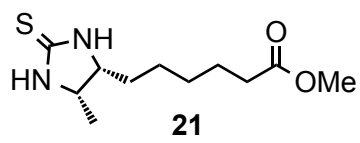


Figure S38. ^{13}C NMR (126 MHz, CDCl_3) spectrum of **21**.

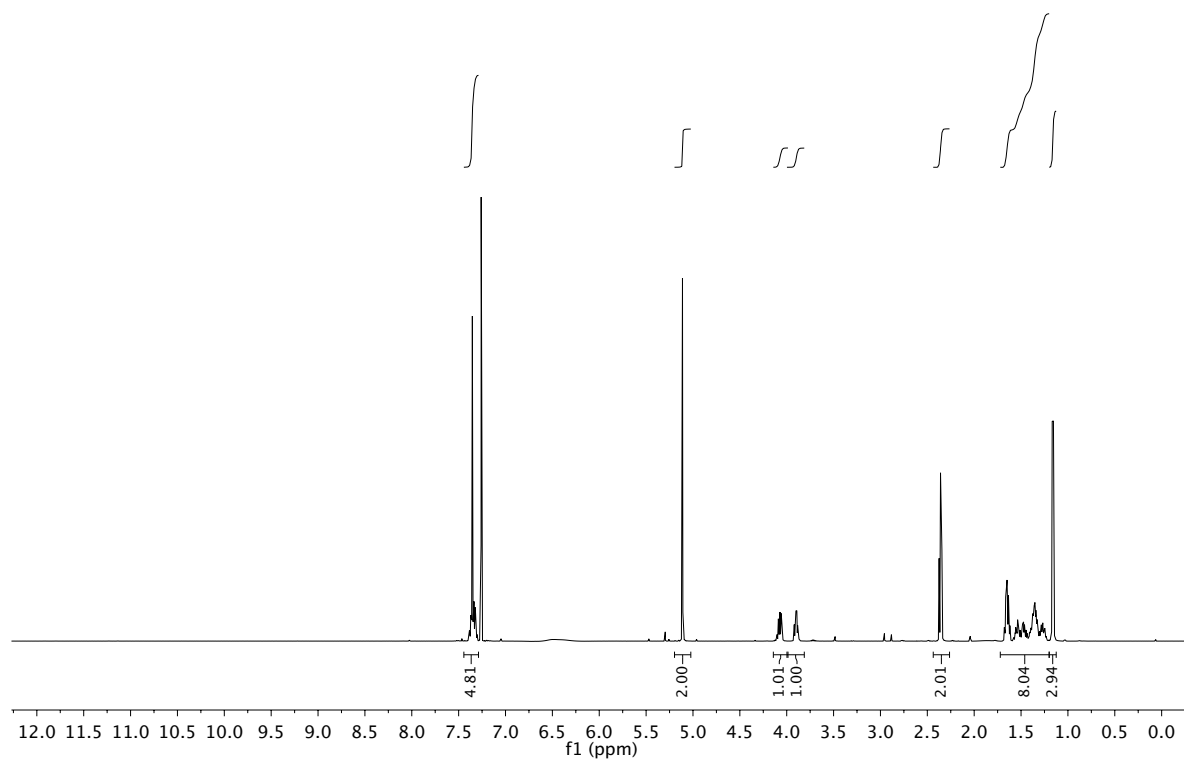
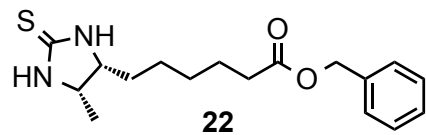


Figure S39. ¹H NMR (500 MHz, CDCl₃) spectrum of **22**.

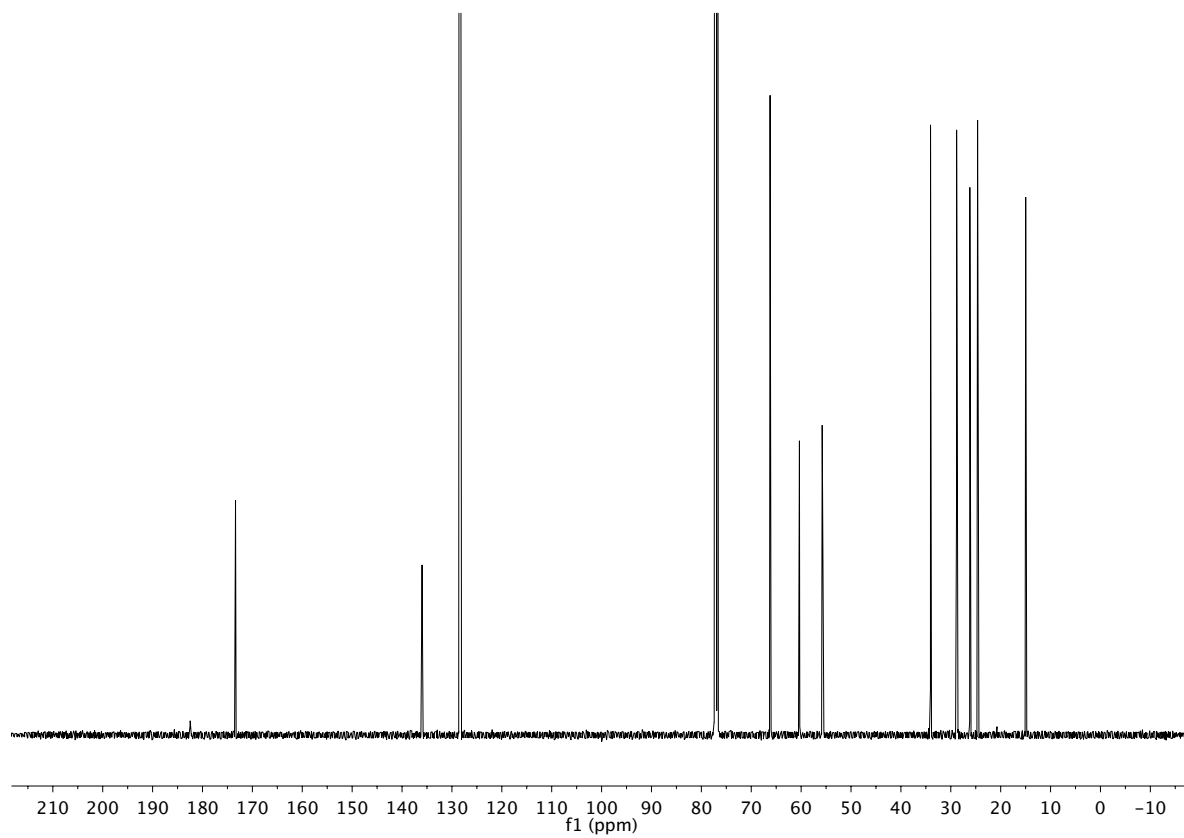
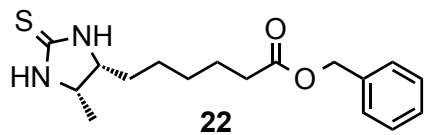


Figure S40. ^{13}C NMR (126 MHz, CDCl_3) spectrum of **22**.

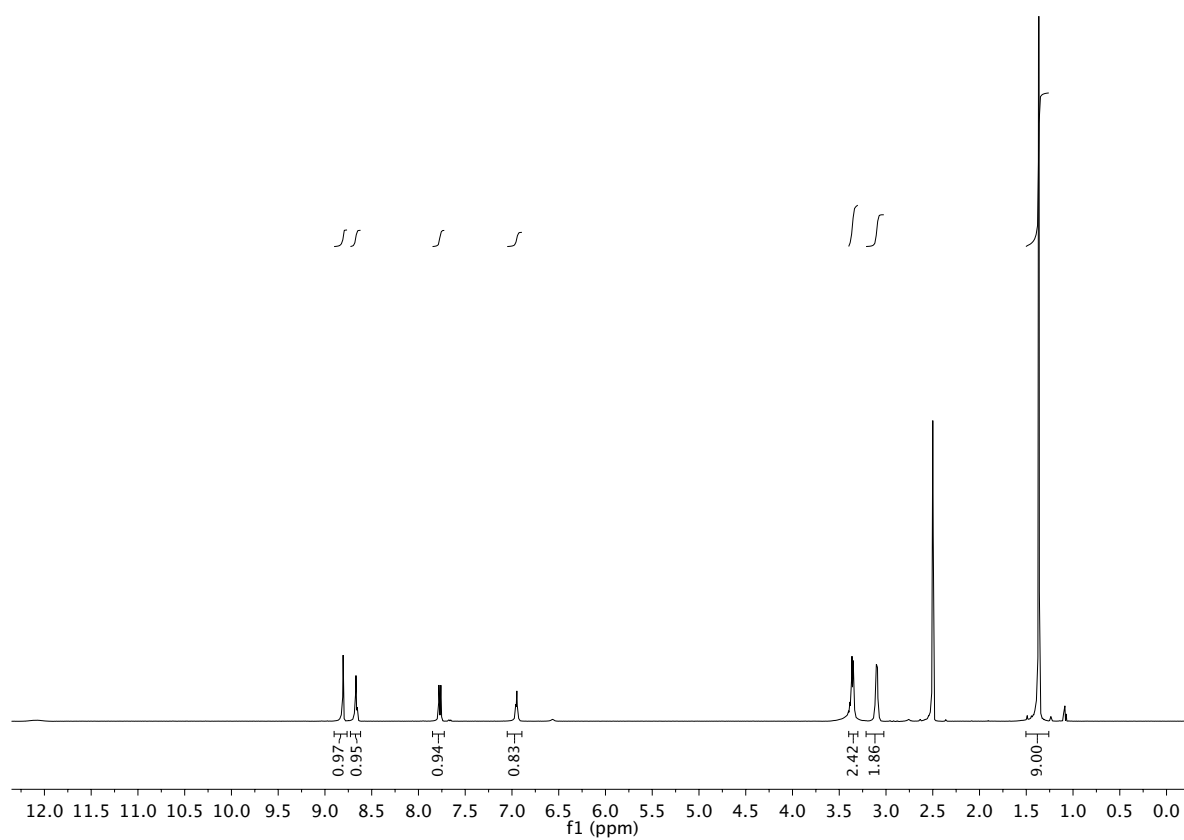
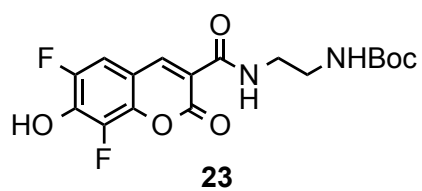


Figure S41. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **23**.

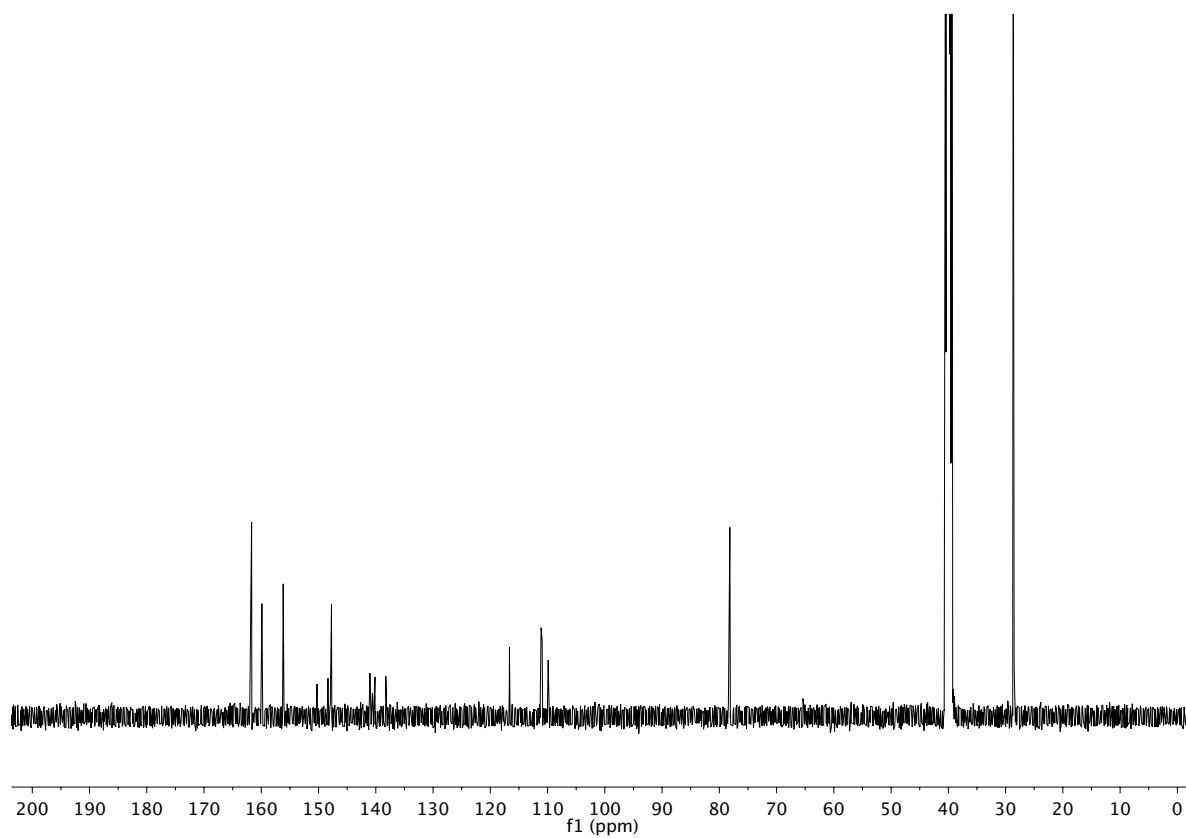
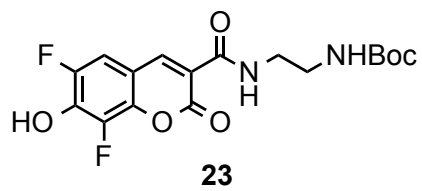


Figure S42. ^{13}C NMR (126 MHz, DMSO- d_6) spectrum of **23**.

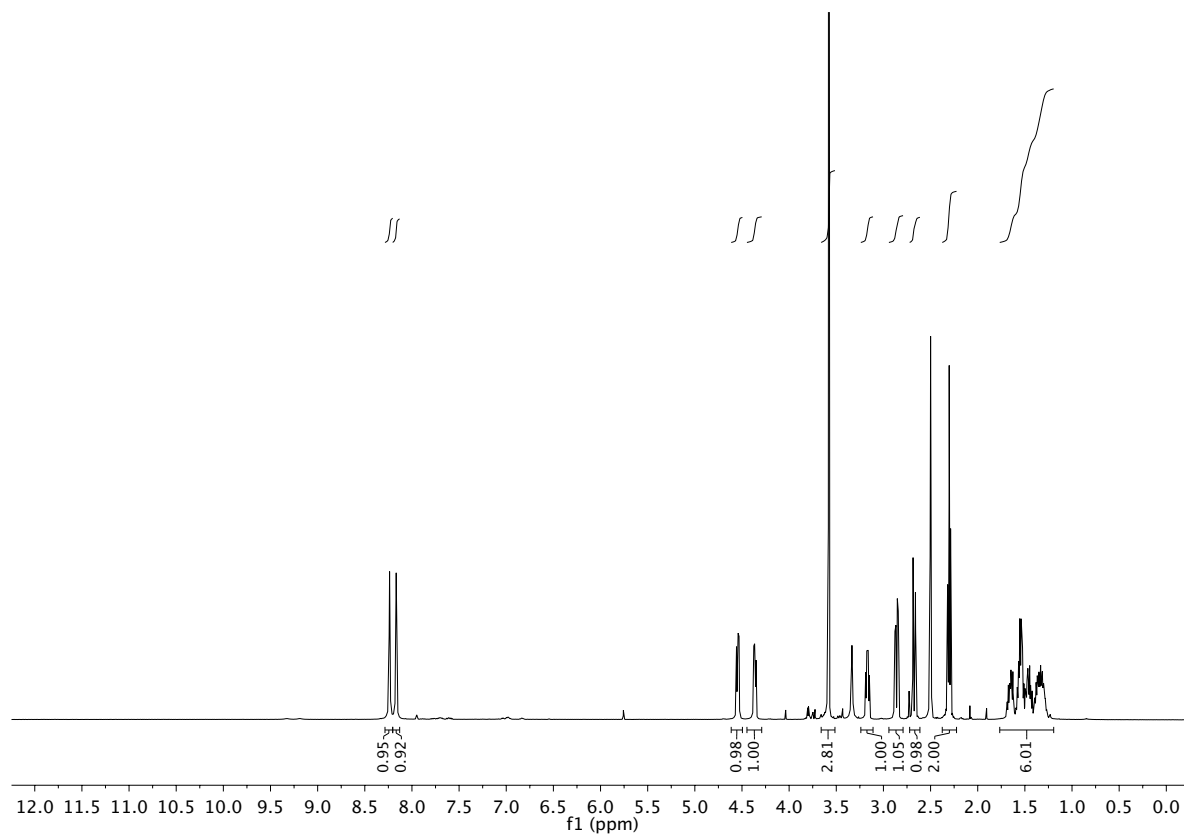
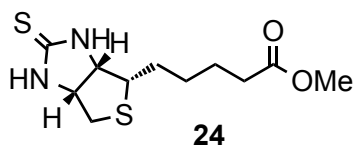


Figure S43. ¹H NMR (500 MHz, DMSO-*d*₆) spectrum of **24**.

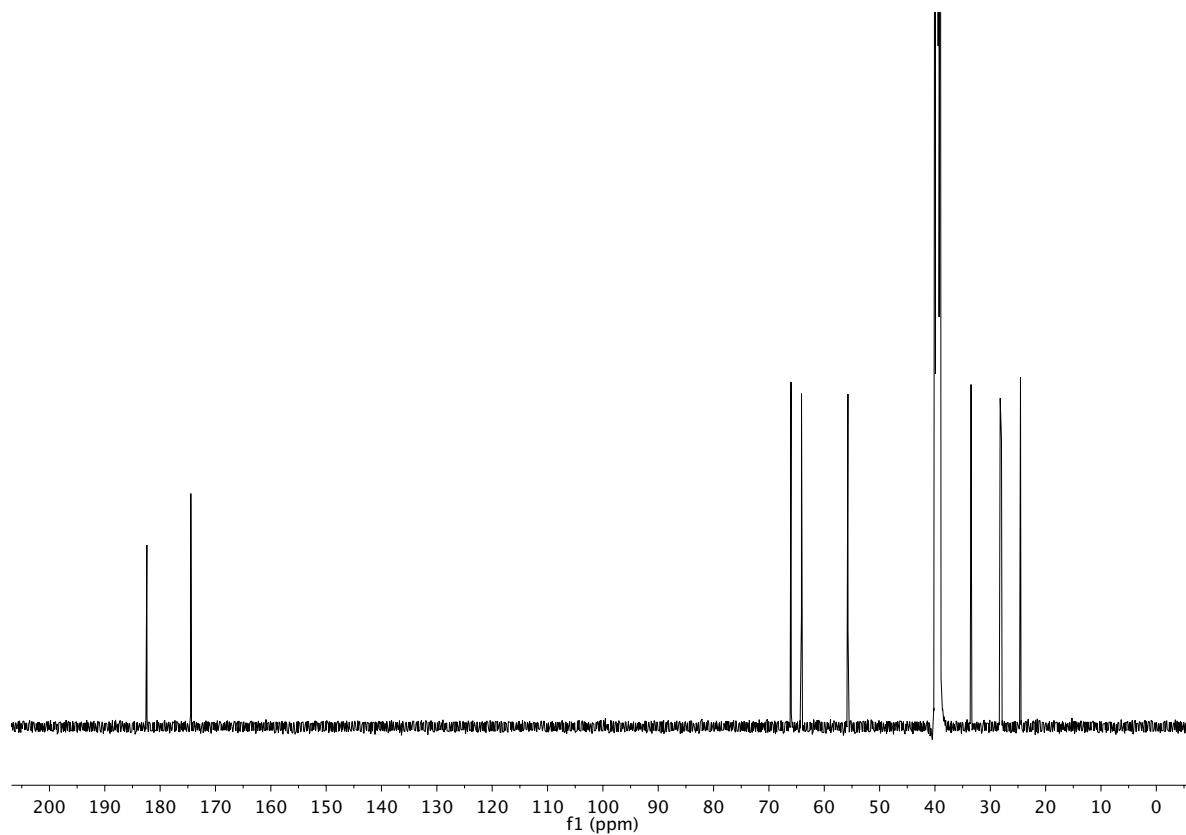
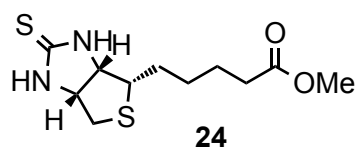


Figure S44. ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) spectrum of **24**.

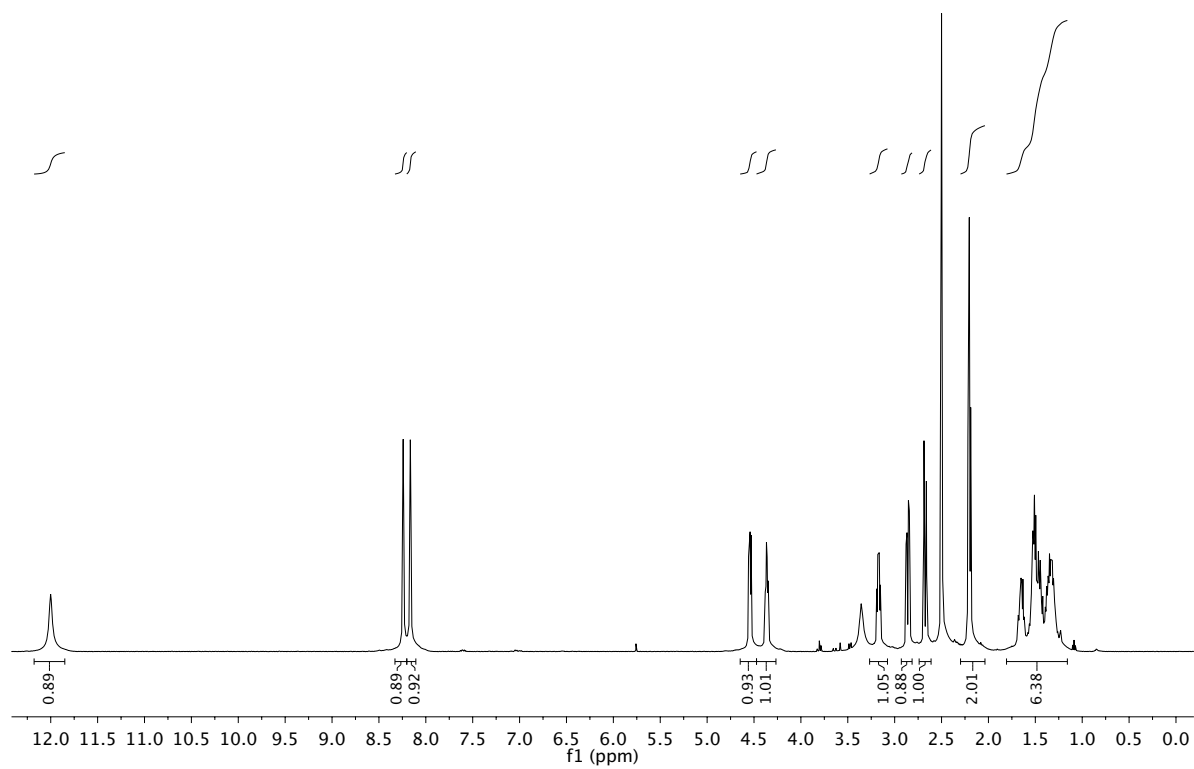
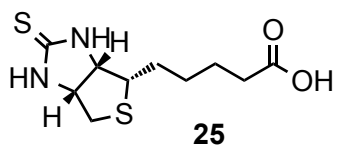


Figure S45. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) spectrum of **25**.

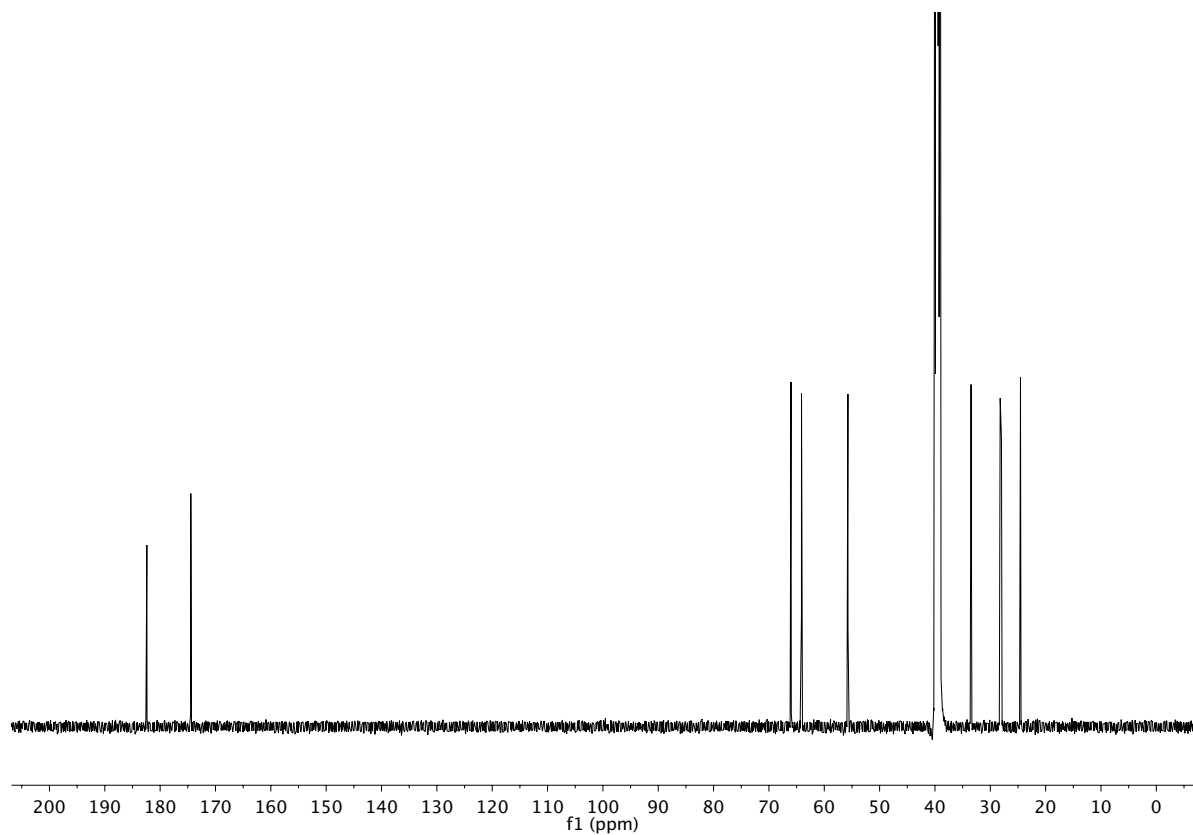
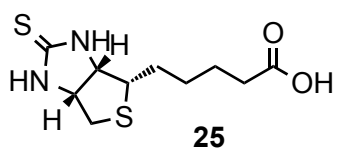


Figure S46. ¹³C NMR (126 MHz, DMSO-*d*₆) spectrum of **25**.

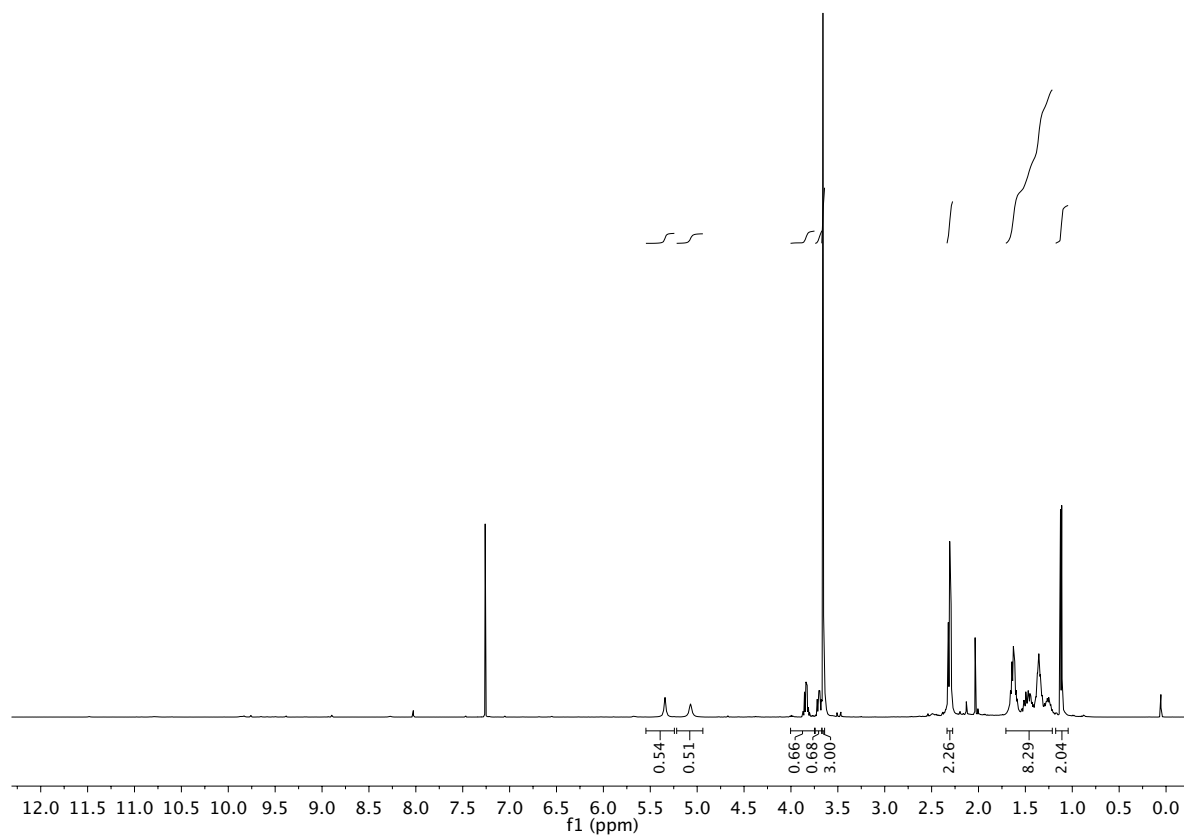
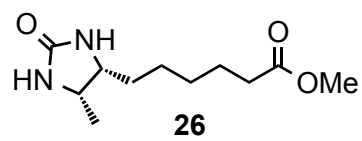


Figure S47. ^1H NMR (500 MHz, CDCl_3) spectrum of **26**.

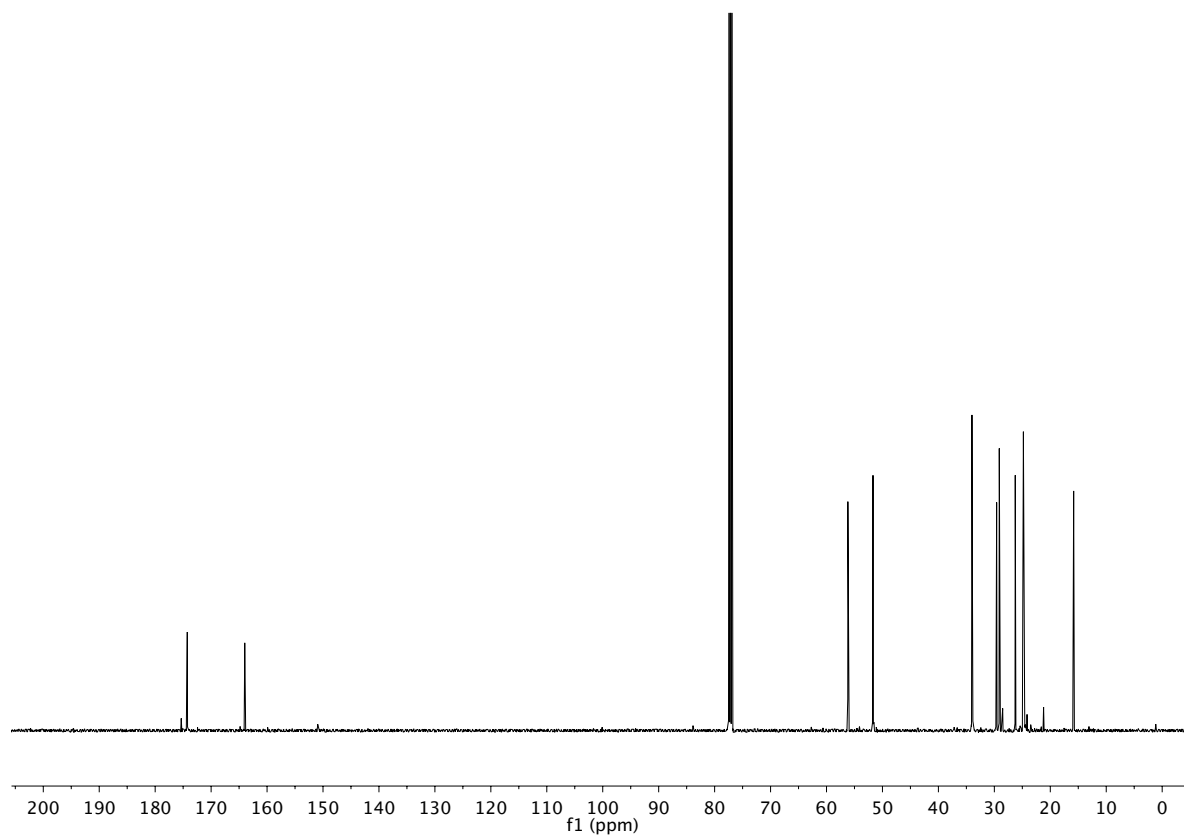
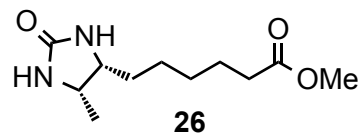


Figure S48. ¹³C NMR (126 MHz, CDCl₃) spectrum of **26**.

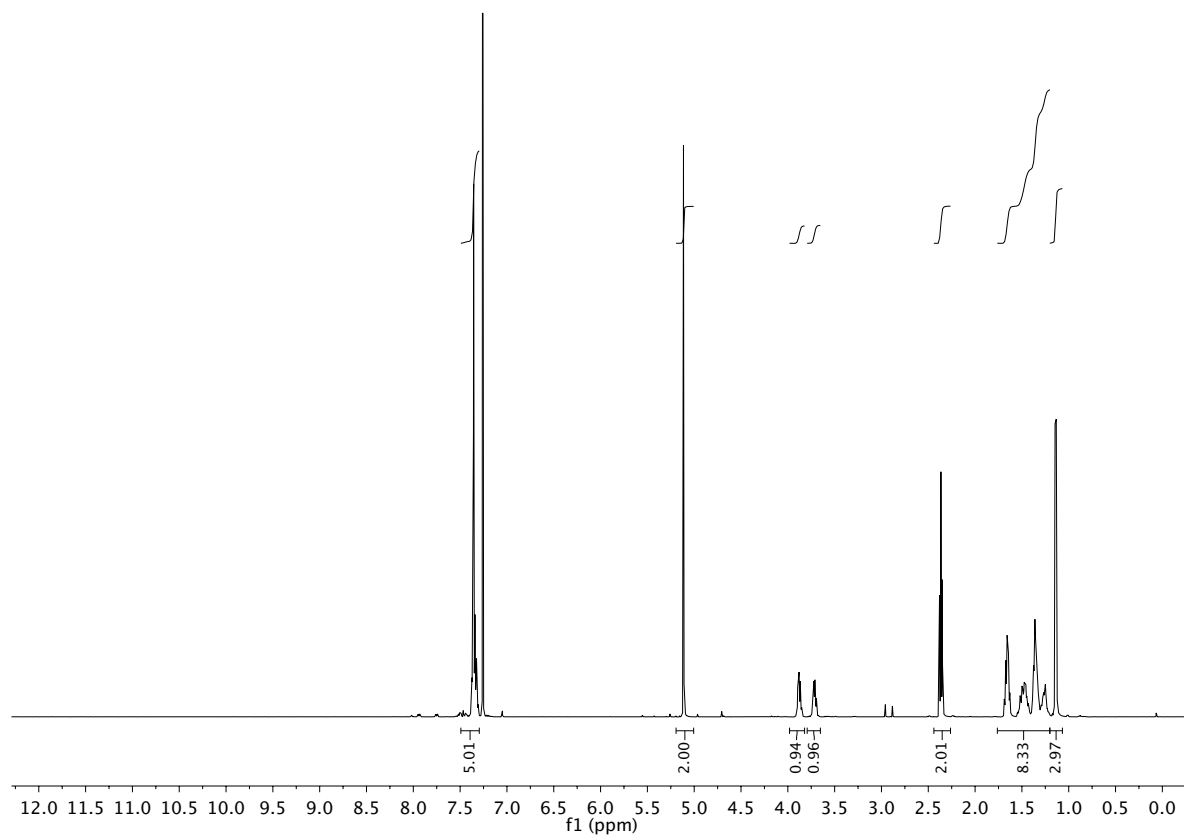
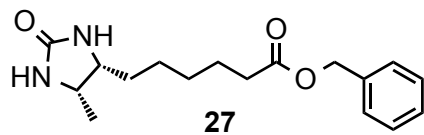


Figure S49. ¹H NMR (500 MHz, CDCl₃) spectrum of **27**.

