Supporting Information

TAPP-Based Fluorescent Probes for Selective Cysteine Detection: Insights into Structure-Reactivity Relationships

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1. Experimental

Materials and Methods

All reagents and solvents were purchased from commercial sources. The reaction progress was monitored by thin-layer chromatography (TLC), performed on silicagel 60. pH measurements were carried out using a Mettler-Toledo S210 pH meter. The identity of the prepared compounds was confirmed by FTIR, ^{1}H NMR, ^{13}C NMR, and HRMS (QTOF). NMR spectra were measured on Varian 500 MHz instrument at Gazi University. Chemical shifts (δ, ppm) were determined with tetramethylsilane (TMS) as the internal reference; *J* values are given in Hz. ^{1}H NMR spectra were recorded in $CDCl_3$ (δH 7.26 ppm as residual solvent peak) and ^{13}C NMR spectra in $CDCl_3$ (δC 77.16 ppm). UV-Vis spectra were measured using a T80+ double beam UV-Vis spectrophotometer from PG Instruments Ltd. Emission spectra were measured using a Hitachi F-7000 fluorescence spectrophotometer, and fluorescence measurements were carried out with a slit width of 5 nm and a PMT voltage of 950 V. All spectroscopic measurements were carried out at a concentration of 10^{-6} M to avoid aggregation and inner-filter effects.

Synthesis of 2,5-bis(4-bromophenyl)-1,4-di-p-tolyl-1,4-dihydropyrrolo[3,2-b]pyrrole (3) ($C_{32}H_{24}Br_2N_2$).

Glacial acetic acid (2 mL), toluene (2 mL), *p*-bromobenzaldehyde (4 mmol), and *p*-methylaniline (4 mmol) were placed in a 50 mL round-bottom flask equipped with a magnetic stir bar. The mixture was heated at 50 °C for 1h. After that time, $Fe(ClO_4)_3xH_2O$ (6 mol%) was added, followed by butane-2,3-dione (2 mmol). The resulting mixture was stirred at 50 °C (in an oil bath) in an open flask overnight. The oil bath was removed, and 5 mL of methanol was added to the reaction mixture. The resulting precipitate was filtered off, washed with methanol (10 mL), and dried under vacuum to afford pure product **3** as a cream solid.

Cream solid, yield: 72%. Spectral and optical properties concur with literature data.[1]

Synthesis of 4-(5-(4-bromophenyl)-1,4-di-*p*-tolyl-1,4-dihydropyrrolo[3,2-b]pyrrol-2-yl)benzonitrile (4) ($C_{33}H_{24}BrN_3$)

Compound **3** (1 mmol) and CuCN (1.2 mmol) were dissolved in 25 mL of *N*-methylpyrrolidone (NMP) solvent. The resulting mixture was heated under an argon atmosphere at 150 °C for 72 hours. After the reaction was complete, the solvent was removed under vacuum. The resulting residue was washed three times with dichloromethane (20 mL), then dried over anhydrous sodium sulfate, and the solvent was removed again. The crude product was purified by filtration using silica gel with a 1:1 (v/v) DCM/hexane eluent. Fractions with an Rf value of 0.25 were collected, and the target derivative **4** was obtained by removing the solvent.[2]

Yellow solid, yield: 61%. FTIR (cm^{-1}): 3117, 3034, 2921, 2852, 2220, 1598, 1511; ^{1}H NMR (500 MHz, $CDCl_3$) δ; 7.46 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.34 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.26 (d, *J* = 8.5Hz, 2H, Ar-H), 7.20 (t, *J* = 8.4 Hz, 4H, Ar-H), 7.16–7.13 (m, 4H, Ar-H), 7.07 (d, *J* = 8.6 Hz, 2H, Ar-H), 6.46 (s, 1H, pyrrole-H), 6.33 (s, 1H, pyrrole-H), 2.40 (s, 3H, CH_3), 2.39 (s, 3H, CH_3) ppm.

Synthesis of 4-(5-(4'-formyl-[1,1'-biphenyl]-4-yl)-1,4-di-*p*-tolyl-1,4-dihydropyrrolo[3,2-b]pyrrol-2-yl)benzonitrile (5) ($C_{40}H_{29}ON_3$).

In an inert atmosphere, 4-(5-(4-bromophenyl)-1,4-di-p-tolyl-1,4-dihydropyrrolo[3,2-b]pyrrol-2-yl)benzonitrile (4) (0.5 mmol) was dissolved in 10:5 mL of THF:H_2O. After that, potassium carbonate (6 mmol) and (4-formylphenyl)boronic acid (1.2 mmol) were added to this solution, respectively. The reaction mixture was stirred for 10 min, the palladium catalyst was added, and the mixture was heated under a reflux condenser for 24 h. After the reaction was complete, it was cooled, then poured into ice-cold water (50 mL), and the mixture was extracted twice with DCM (25 mL). The organic phases were collected, and the solvent was removed. The obtained derivative **5** was purified by column chromatography on silica gel using a DCM/hexane (6/4) solvent system.[3]

Yellow solid, , yield: 85%, m.p.: 290 °C. FTIR (cm^{-1}): 3033, 2920, 2852, 2725, 2115, 1695, 1595, 1511; ^{1}H NMR (500 MHz, $CDCl_3$) δ; 9.97 (s, 1H, CHO), 7.85 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.67 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.45 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.39 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.25 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.19 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.14 (s, 6H, Ar-H), 7.10 (d, *J* = 8.4 Hz, 2H, Ar-H), 6.41 (s, 1H, pyrrole-H), 6.35 (s, 1H, pyrrole-H), 2.34 (s, 3H, CH_3), 2.32 (s, 3H, CH_3) ppm.^{13}C NMR (126 MHz, $CDCl_3$) δ 190.8, 145.5, 137.0, 136.2 (x2C), 136.0, 135.6, 135.3, 135.0, 134.1, 132.9, 132.59, 132.56, 131.3, 130.9, 129.3, 129.1, 128.9, 127.5, 126.6, 126.2, 126.1, 124.21, 124.19, 118.2, 107.6, 95.1, 93.5, 20.1 ppm. HRMS (APCI) (*m/z*): $[M]^+$: 567.23106 (Calc.567.23267) (–2.84 ppm)

Synthesis of 2-((4'-(5-(4-cyanophenyl)-1,4-di-*p*-tolyl-1,4-dihydropyrrolo[3,2-b]pyrrol-2-yl)-[1,1'-biphenyl]-4-yl)methylene)malononitrile (6) ($C_{49}H_{33}N_5$).

In a 10 mL flask under an argon atmosphere, Et_3N (0.1 mL) was added to a solution of malononitrile (2 mmol) and the derivative of **5** (1 mmol) in $CHCl_3$. The reaction mixture was stirred overnight at 80°C and treated with brine after the starting material had been consumed (monitored by TLC). The organic phase was dried over Na_2SO_4, and the crude product was purified by chromatography column [silica gel, DCM: hexane (7:3)] and recrystallization from methanol.

Orange-brown solid, yield: 53%, m.p.: 202 °C. FTIR (cm^{-1}): 3117, 3030, 2920, 2851, 2221, 1597, 1577, 1511; ^{1}H NMR (500 MHz, $CDCl_3$) δ; 7.96 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.75 (d, *J* = 8.5 Hz, 2H, Ar-H) 7,75(s, 1H, -C=CH), 7.53 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.47 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.33 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.27 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.21 (s, 6H, Ar-H), 7.17 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.48 (s, 1H, pyrrole-H), 6.44 (pyrrole-H), 2.41 (s, 3H, -CH_3), 2.40 (s, 3H, -CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.0, 145.7, 136.9, 136.1, 136.0, 135.4, 135.4, 135.3, 135.2, 135.1, 133.2, 133.1 132.6, 131.6, 130.9, 130.4, 129.1, 129.0, 128.7, 127.5, 126.7, 126.5, 126.0, 124.23, 124.19, 118.2, 113.0, 111.9, 107.7, 95.1, 93.7, 20.1 ppm. HRMS (APCI) (*m/z*): $[M+H]^+$: 616.24957; (Calc.616.25194) (3.85 ppm).

Synthesis of ethyl 2-cyano-3-(4'-(5-(4-cyanophenyl)-1,4-di-*p*-tolyl-1,4-dihydropyrrolo[3,2-b]pyrrol-2-yl)-[1,1'-biphenyl]-4-yl)acrylate (7) ($C_{45}H_{35}O_2N_4$).

In a 10 mL flask under an argon atmosphere, Et_3N (0.1 mL) was added to a solution of ethyl cyanoacetate (2 mmol) and derivative of **5** (1 mmol) in $CHCl_3$. The reaction mixture was stirred overnight at 80°C and treated with brine after the starting material had been consumed (monitored by TLC). The organic phase was dried over Na_2SO_4, and the crude product was purified by chromatography column [silica gel, DCM: hexane (7:3)] and recrystallization from methanol.

Red solid, yield: 64%, m.p.: 250 °C. FTIR (cm^{-1}): 3115, 3028, 2919, 2843, 2219, 1727, 1589; ^1H NMR (500 MHz, $CDCl_3$) δ; 8.24 (s, 1H, -C=CH), 8.03 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.70 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.51 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.44 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.30 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.24 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.19 (s, 6H, Ar-H), 7.15 (d, *J* = 8.3 Hz, 2H, Ar-H), 6.47 (s, 1H, pyrrole-H), 6.41 (s, 1H, pyrrole-H), 4.37 (q, *J* = 7.1 Hz, 2H, $-CH_2$-OCO), 2.39 (s, 3H, $-CH_3$), 2.38 (s, 2H, $-CH_3$), 1.39 (t, *J* = 7.1 Hz, 2H, $-CH_3$) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.7, 154.4, 145.4, 138.0, 137.2, 137.0, 136.9, 136.7, 136.3, 136.1, 134.0, 133.7, 133.6, 132.4, 132.0, 131.8, 130.3, 130.1, 130.0, 128.5, 127.7, 127.3, 126.9, 125.3, 125.2, 119.3, 115.8, 108.6, 102.2, 96.2, 94.6, 62.7, 20.1, 14.2 ppm. HRMS (APCI) (*m/z*): $[M+H]^+$: 663.27545; (Calc. 663.27648) (–1.55 ppm).

2. Support Figures (FTIR, NMR, HRMS spectra)

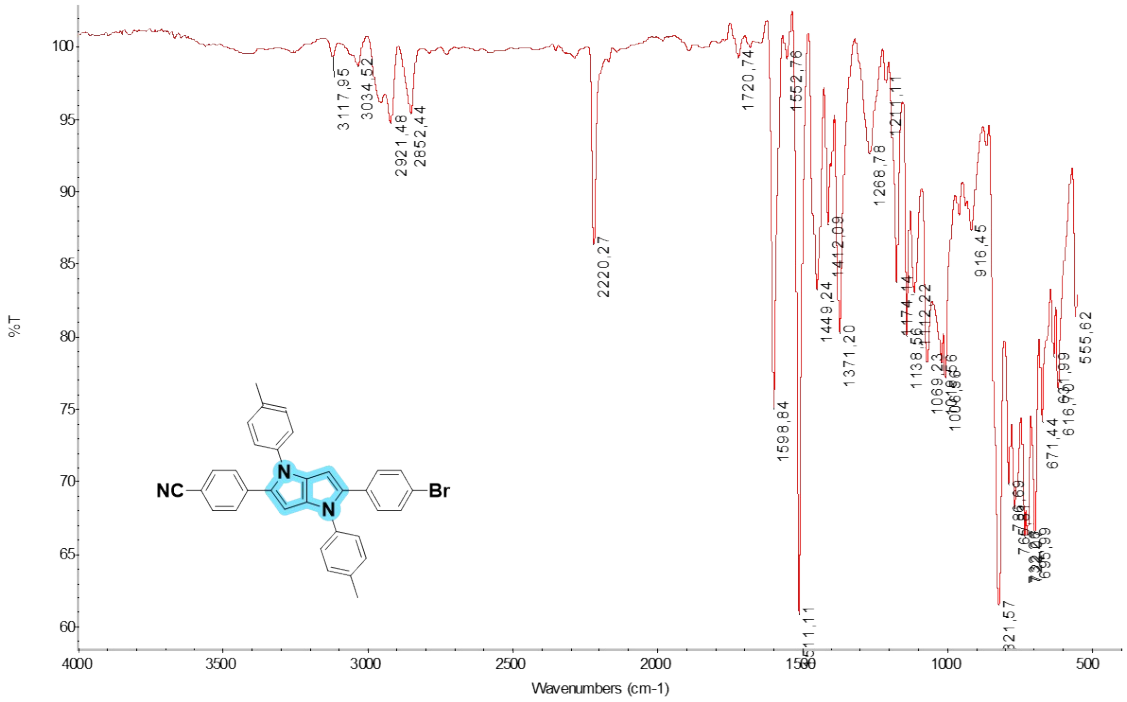

Figure SI 1. FTIR spectrum of **4**

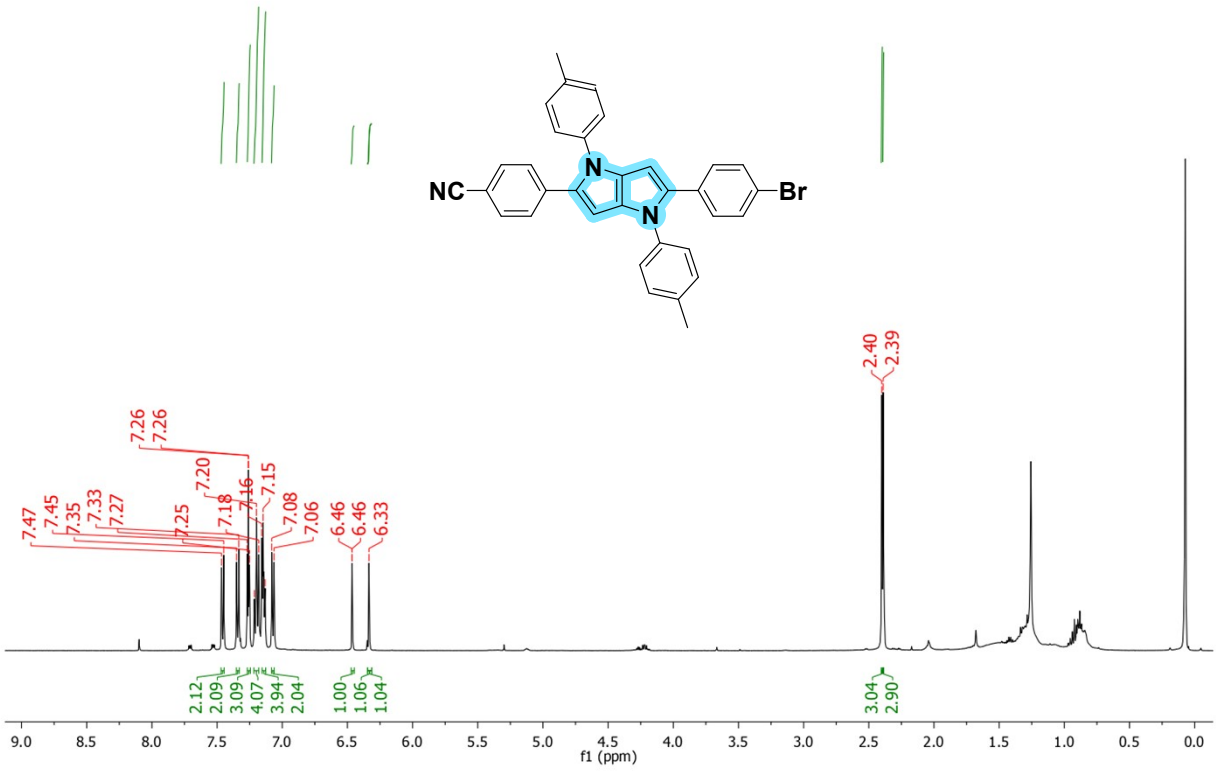

Figure SI 2. ^{1}H NMR spectrum of **4** ($CDCl_3$, 500 MHz)

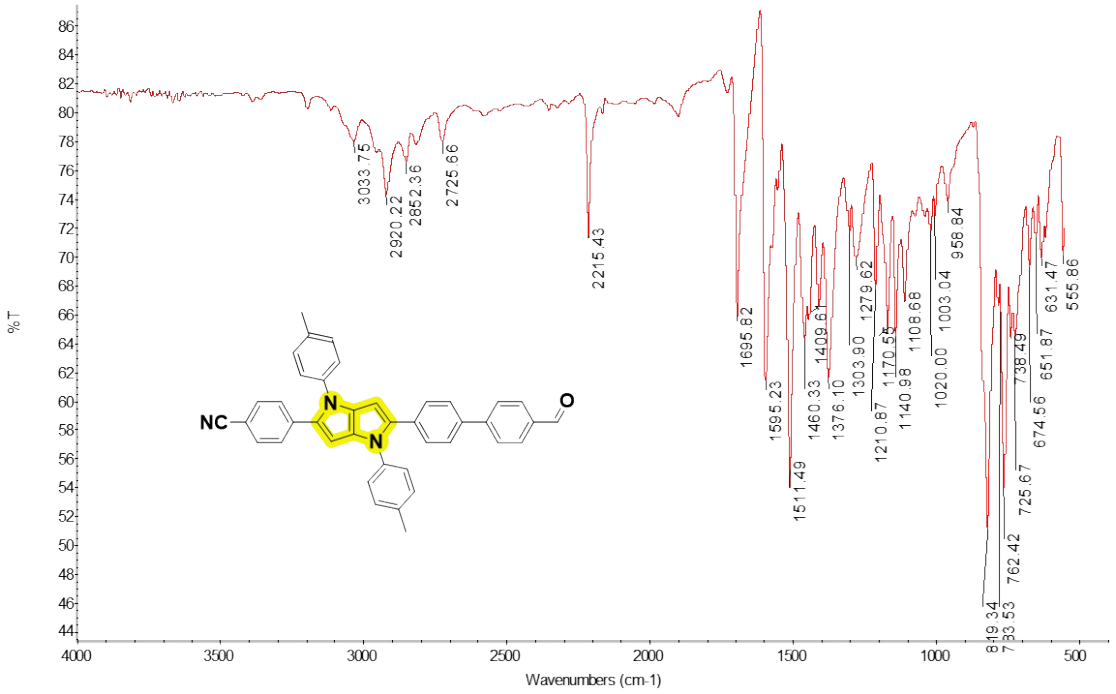

Figure SI 3. FTIR spectrum of **5**

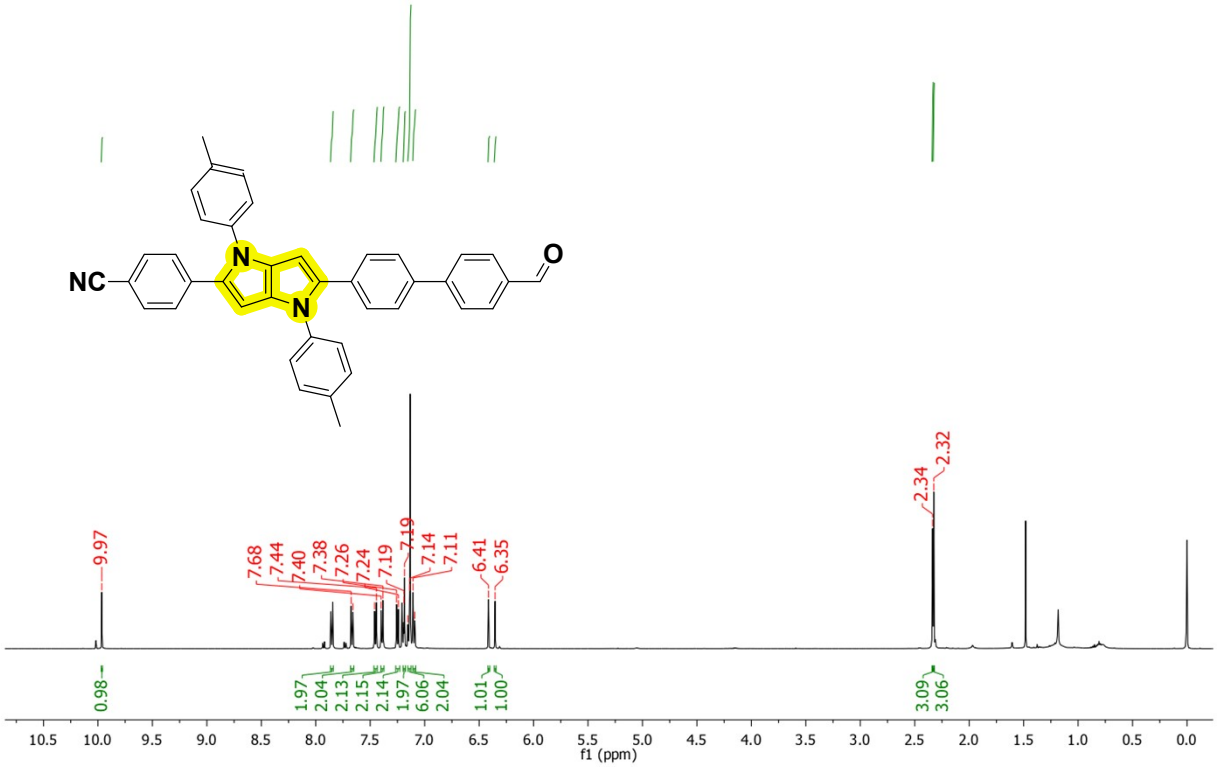

Figure SI 4. ^{1}H NMR spectrum of **5** ($CDCl_3$, 500 MHz)

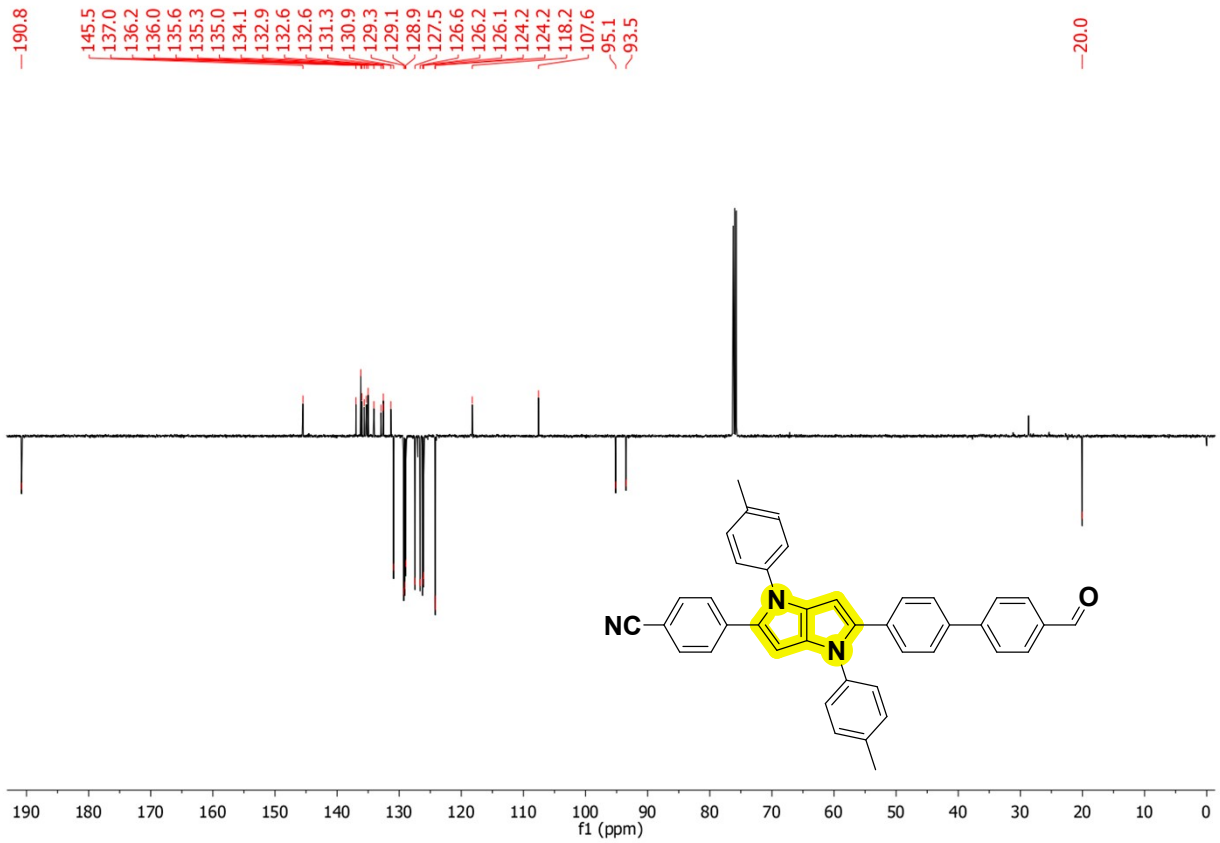

Figure SI 5. ^{13}C NMR spectrum of **5** ($CDCl_3$, 126 MHz)

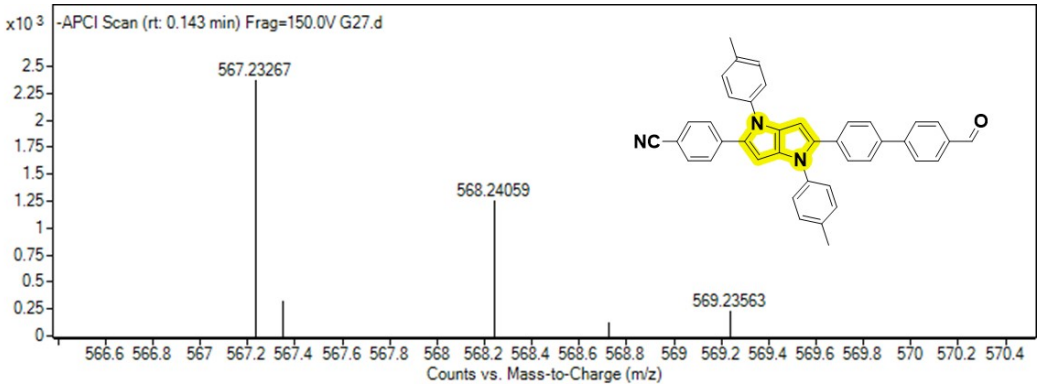

Figure SI 6. HRMS spectrum of **5**

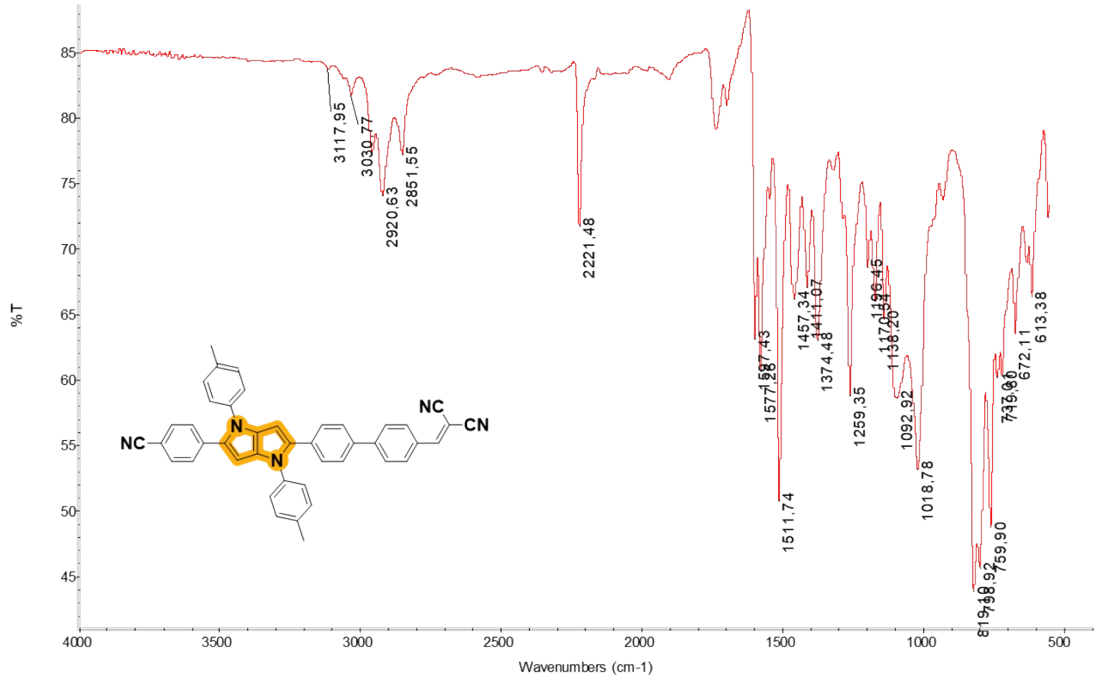

Figure SI 7. FTIR spectrum of **6**

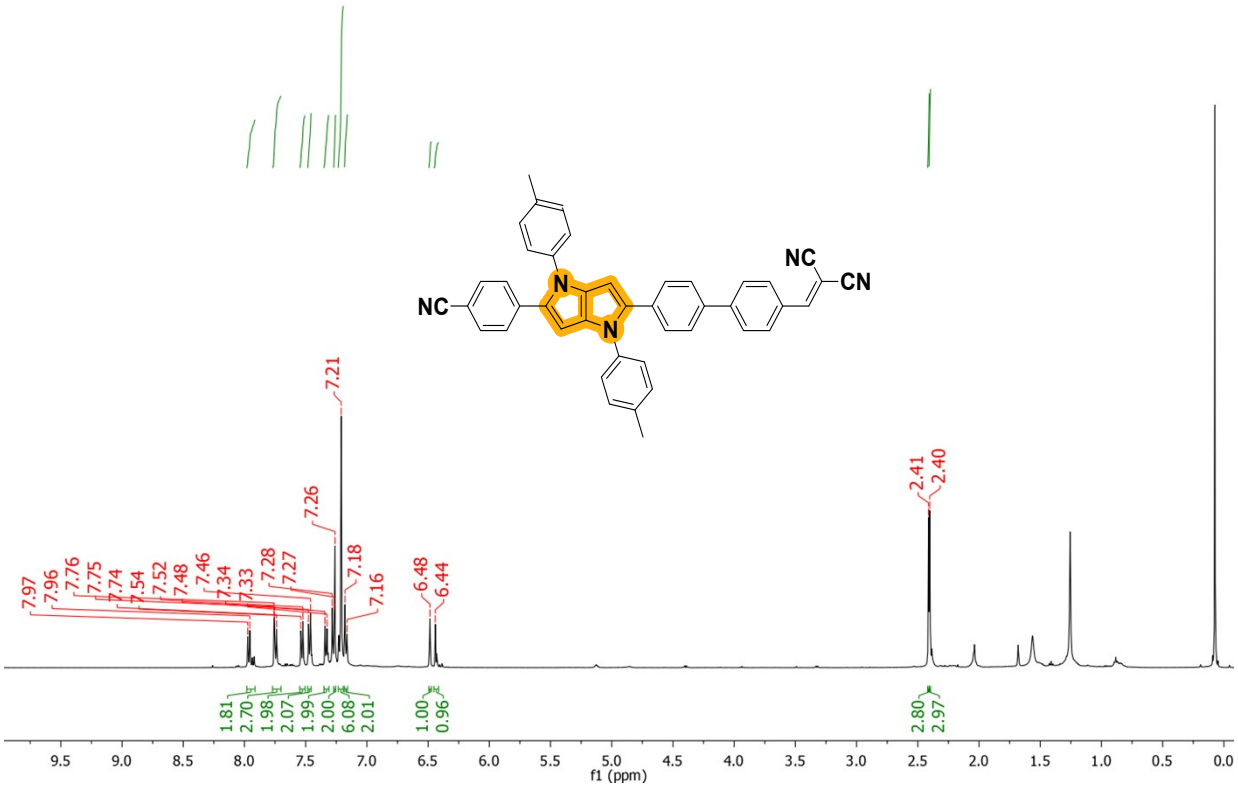

Figure SI 8. ^{1}H NMR spectrum of **6** ($CDCl_3$, 500 MHz)

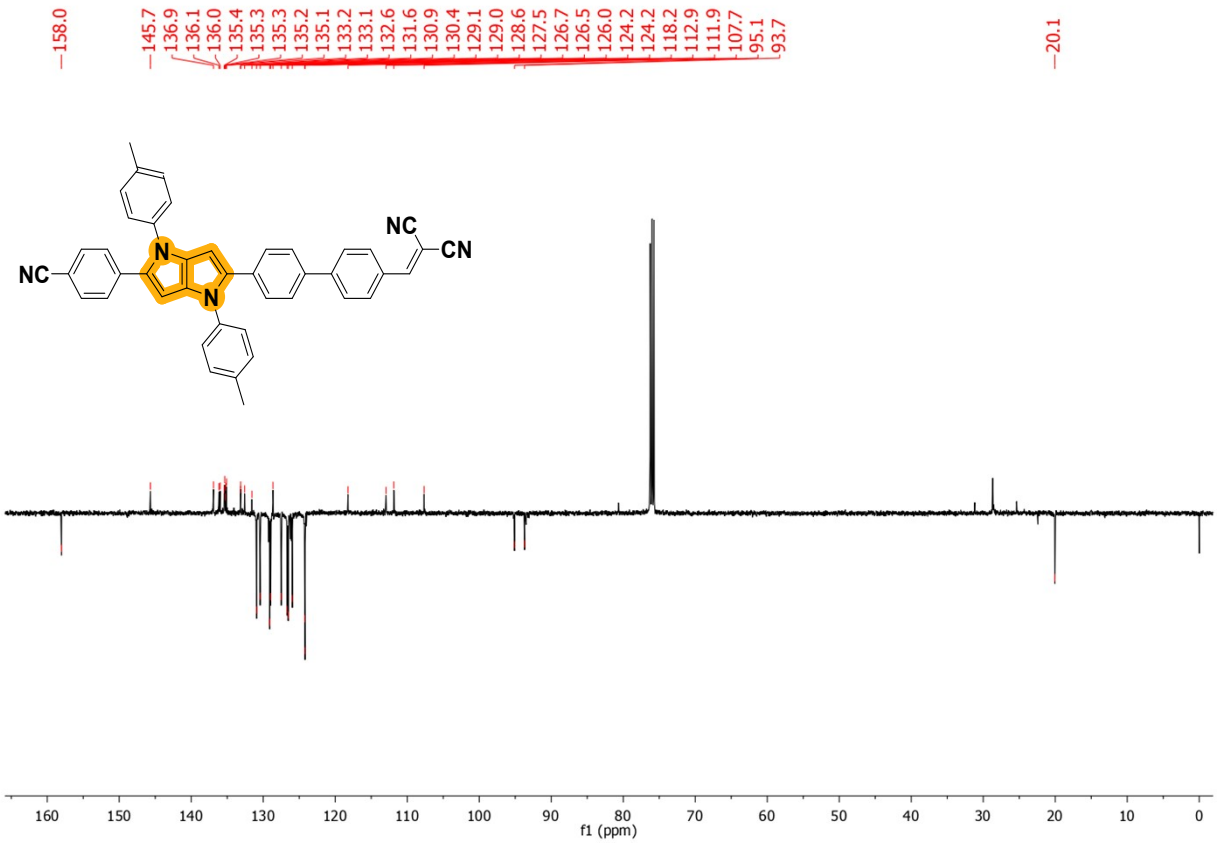

Figure SI 9. ^{13}C NMR spectrum of **5** ($CDCl_3$, 126 MHz)

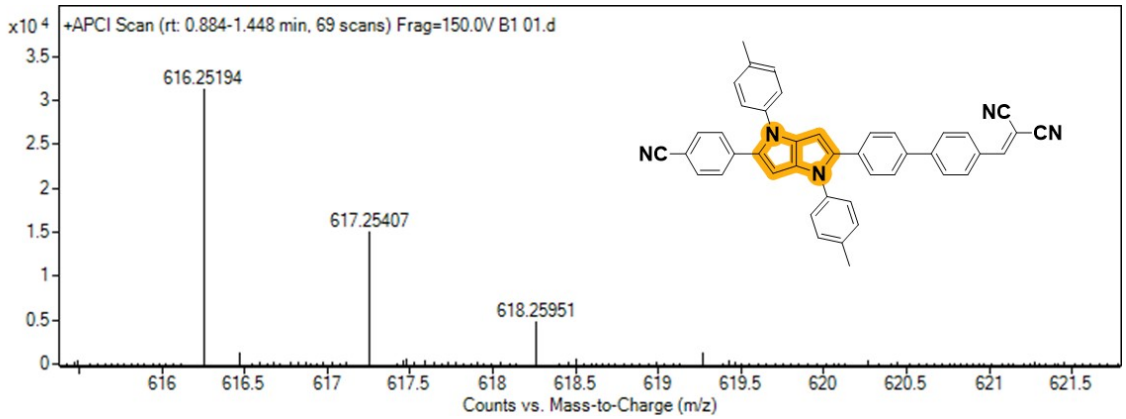

Figure SI 10 HRMS spectrum of **6**

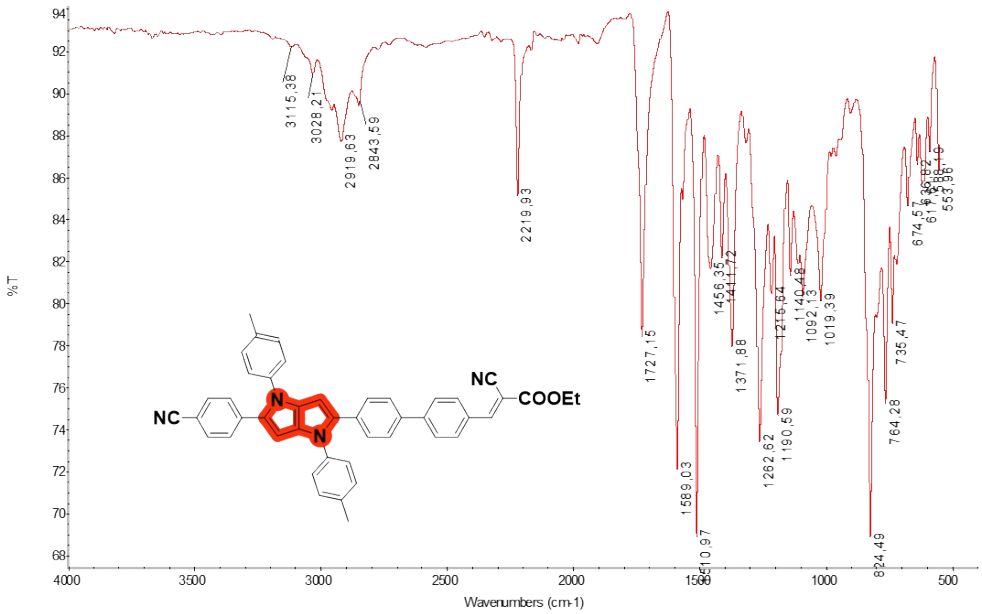

Figure SI 11. FTIR spectrum of **7**

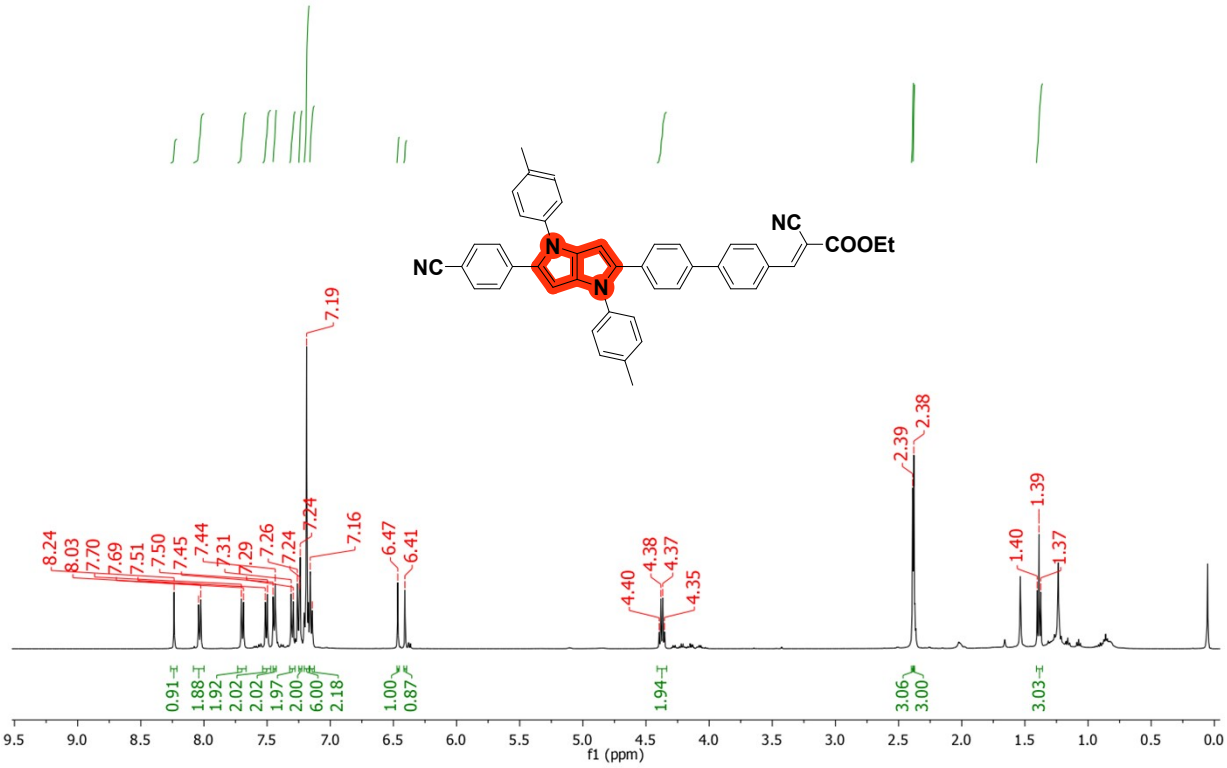

Figure SI 12. ^{1}H NMR spectrum of **7** ($CDCl_3$, 500 MHz)

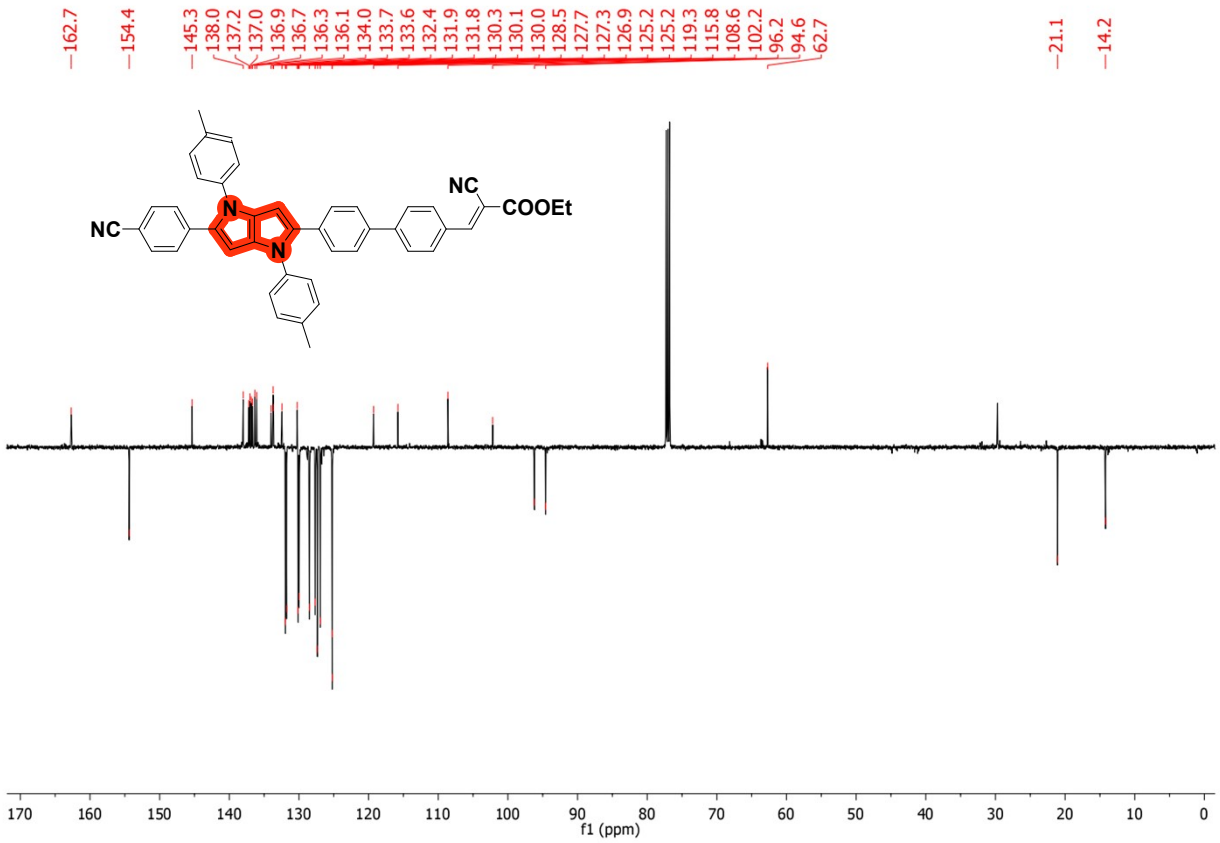

Figure SI 13. ^{13}C NMR spectrum of **7** ($CDCl_3$, 126 MHz)

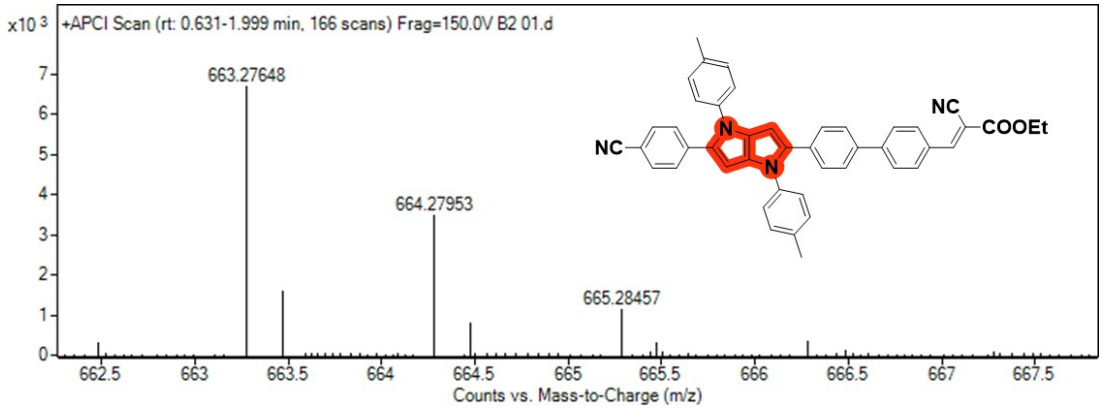

Figure SI 14 HRMS spectrum of **7**

3. Photophysical Properties

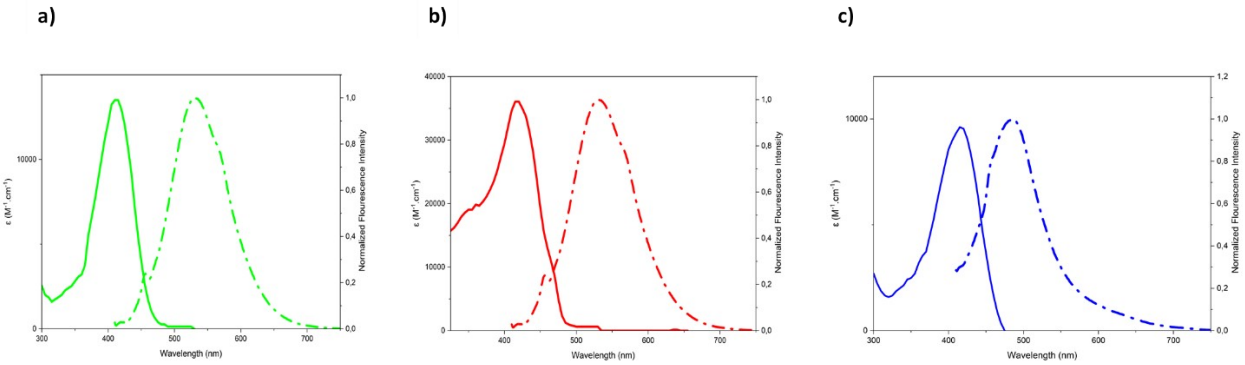

Figure SI 15. Molar extinction spectrum (solid line) and normalized fluorescence emission spectrum (short-dotted line, excitation at 400 nm of **5**) **a)** in THF, **b)** in DCM, and **c)** in DMSO

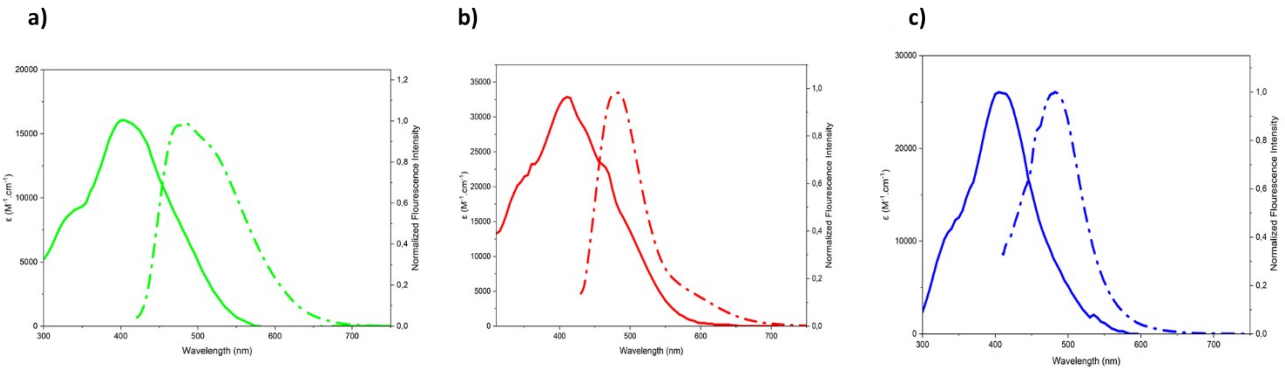

Figure SI 16. Molar extinction spectrum (solid line) and normalized fluorescence emission spectrum (short-dotted line, excitation at 400 nm of **6**) **a)** in THF, **b)** in DCM, and **c)** in DMSO

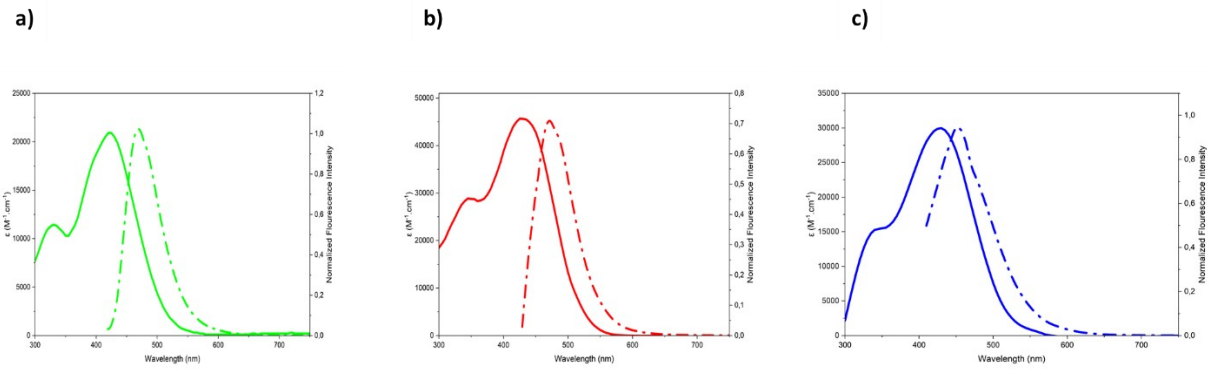

Figure SI 17. Molar extinction spectrum (solid line) and normalized fluorescence emission spectrum (short-dotted line, excitation at 400 nm of **7**) **a)** in THF, **b)** in DCM, and **c)** in DMSO

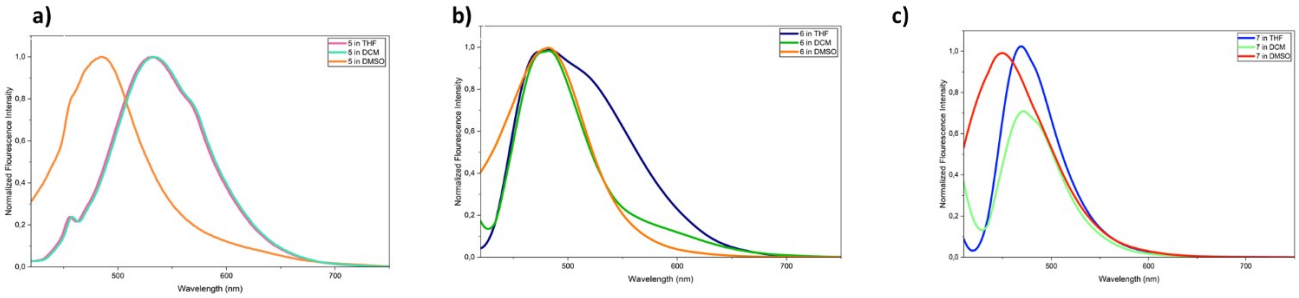

Figure SI 18. Normalized fluorescence emission spectrum in various solvents, **a)** for compound **5**; **b)** for compound **6** and **c)** for compound **7**

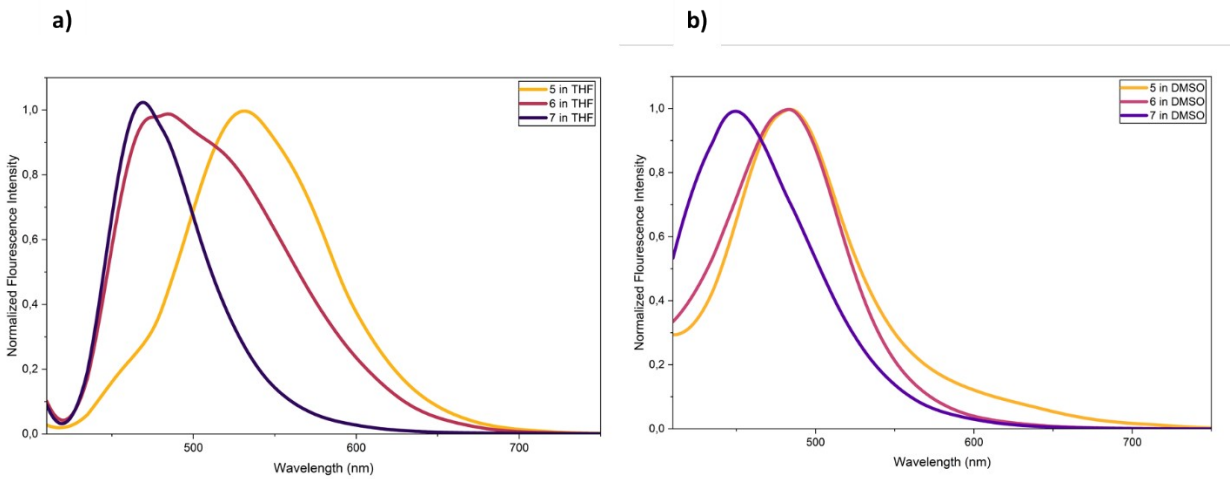

Figure SI 19. **a)** Normalized fluorescence emission spectrum of compounds **5-7** in THF **b)** in DMSO

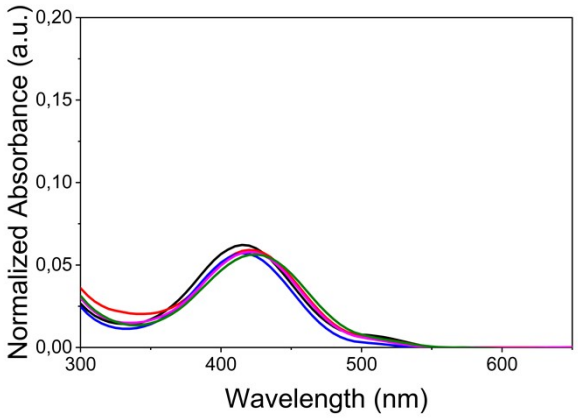

Figure SI 20. UV-vis spectra of compound **5** (0.1 μM) upon gradual addition of Cys (0-50 μM) in PBS buffer (pH 7.4).

4. Comparison with Reported Fluorescent Probes for Cysteine Detection

Table S1. Summary of turn-on fluorescent probes developed for Cys detection with corresponding analytical performance parameters

Probe Type	Response Time	LOD	Reference
NIR probe with methacryloyl group	N/A	0.46 μM	[4]
NIR probe based on Xanthene hybrid tetrahydro-acridine salt	6 min	0.50 μM	[5]
Lipid droplet-targeting probe	<8 min	39.0 nm	[6]
BODIPY-based probe	10 min	11.2 nm	[7]
Reversible pyrene-based probe	50 s	0.47 μM	[8]
TAPP-based π-conjugated aldehyde systems	**<50 s**	**0.37 μM**	**This work**

5. Spectroscopic data of response of 5 toward Cys

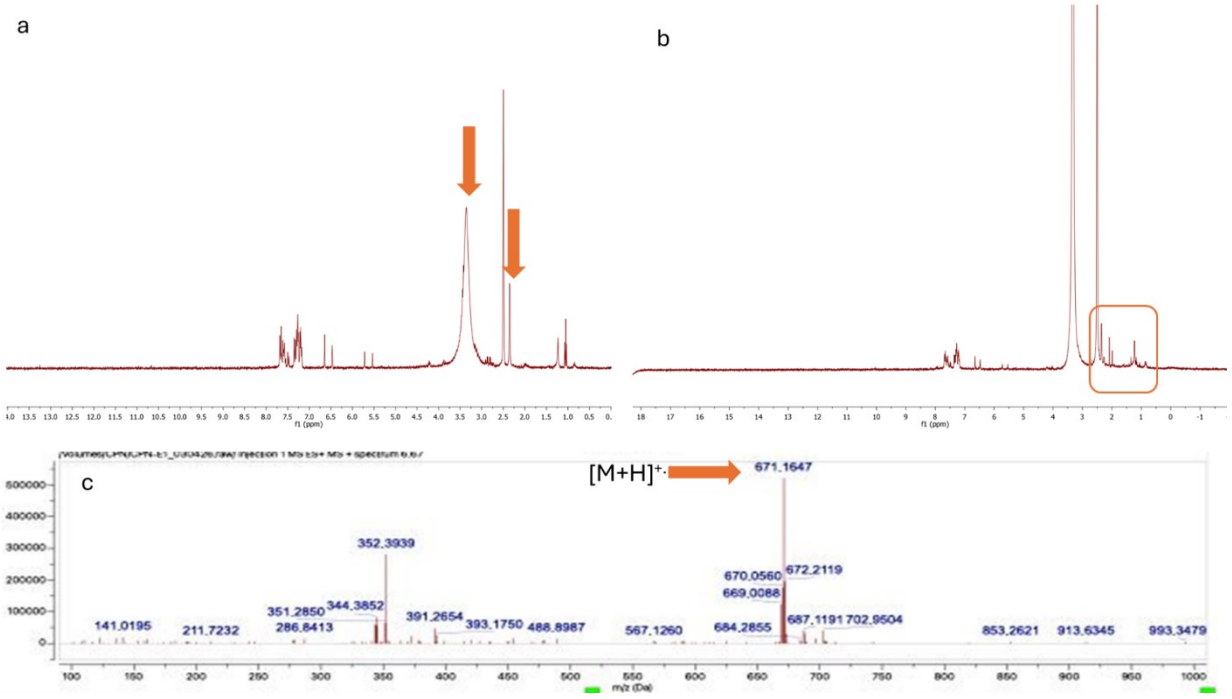

Figure SI 21. Spectroscopic characterization of the reaction between compound **5** and cysteine. (a) ^{1}H NMR spectrum recorded in $CDCl_3$, displaying new aliphatic signals in the 2.5-4.0 ppm region, consistent with the formation of a new product. (b) ^{1}H NMR spectrum recorded in DMSO-d_6 showing the disappearance of the aldehyde proton signal upon reaction with cysteine. (c) HRMS spectrum of the reaction mixture showing a peak at m/z 671.1647 corresponding to the protonated cysteine adduct ($[M+H]^+$).

6. Computational Studies

All computational studies were performed using ORCA 6.1. DFT calculations were performed to optimize the molecules using the B3LYP/6-31G(d) basis set.

Table S2. Cartesian coordinates (Å) of optimized stationary points for compound 5. The first column represents the atomic number of each atom.

6	-3.53941	-1.46137	-4.56096	6	3.216458	-4.96103	-6.40103
6	-2.12874	-1.46007	-5.10342	6	2.898926	-6.31151	-6.44202
6	-1.79529	-0.73209	-6.25346	6	1.642149	-6.72692	-6.91875
6	-0.50376	-0.75862	-6.77948	6	1.303593	-8.11782	-6.95305
6	-1.11539	-2.20537	-4.48332	7	1.02831	-9.24881	-6.98009
6	0.184024	-2.22689	-4.98619	6	0.721878	-5.75908	-7.36003
6	0.494268	-1.50811	-6.14662	6	1.050484	-4.41022	-7.32543
7	1.819852	-1.48994	-6.6703	1	-4.06636	-0.5342	-4.81089
6	2.54903	-0.32617	-6.8523	1	-3.54964	-1.57375	-3.47123
6	3.865426	-0.67463	-7.15394	1	-4.1254	-2.2918	-4.97801
7	4.602708	0.490725	-7.29808	1	-2.55918	-0.14099	-6.75369
6	6.0226	0.50843	-7.41759	1	-0.26452	-0.2041	-7.68236
6	6.81665	1.232155	-6.5205	1	-1.3421	-2.76873	-3.58088
6	8.20401	1.212656	-6.65044	1	0.960487	-2.79036	-4.47893
6	8.83601	0.460912	-7.65145	1	6.346742	1.80552	-5.72814
6	10.34304	0.41476	-7.75742	1	8.808119	1.785805	-5.9506
6	8.025503	-0.26516	-8.5344	1	10.79742	1.356189	-7.42972
6	6.635049	-0.24077	-8.42875	1	10.66691	0.222668	-8.78608
6	3.731448	1.571627	-7.0579	1	10.76204	-0.38378	-7.12989
6	4.101287	2.980913	-7.21862	1	8.48672	-0.85026	-9.32699
6	3.513618	3.952266	-6.38456	1	6.018859	-0.78983	-9.13497
6	3.794073	5.304425	-6.54394	1	2.835	3.634142	-5.59806
6	4.681845	5.752611	-7.53887	1	3.303091	6.026676	-5.89745
6	4.987392	7.192956	-7.70438	1	4.932065	7.641021	-5.59153
6	5.067772	8.048536	-6.58914	1	5.420388	10.04815	-5.87493
6	5.3534	9.400983	-6.74759	1	5.910981	11.94907	-7.23184
6	5.569326	9.937567	-8.0241	1	5.651756	9.522731	-10.1289
6	5.873529	11.37472	-8.18527	1	5.119805	7.10833	-9.86057
8	6.073558	11.92797	-9.25114	1	5.973087	5.089521	-9.1389
6	5.492268	9.093741	-9.14392	1	5.450225	2.712356	-8.88833
6	5.205474	7.745319	-8.98486	1	1.581738	1.693255	-6.60582
6	5.26901	4.781986	-8.37066	1	4.842688	-2.69061	-7.36367
6	4.986898	3.428853	-8.21828	1	4.177985	-4.64987	-6.00393
6	2.457447	1.081856	-6.76904	1	3.611952	-7.05245	-6.09377
6	3.982694	-2.08116	-7.12589	1	-0.24498	-6.0744	-7.74008
6	2.707949	-2.57285	-6.83631	1	0.33595	-3.68297	-7.69437
0	2.304564	-3.97846	-6.84328				

Table S3. calculated Gibbs free energy, zero-point vibrational energies (ZPVE), and lowest vibrational frequencies for compound 5.

ZPVE (Hartree)	Gibbs Free Energy (Hartree)	Lowest Freq. (cm^{-1})

0.57891546 -1779.54913533 11.87

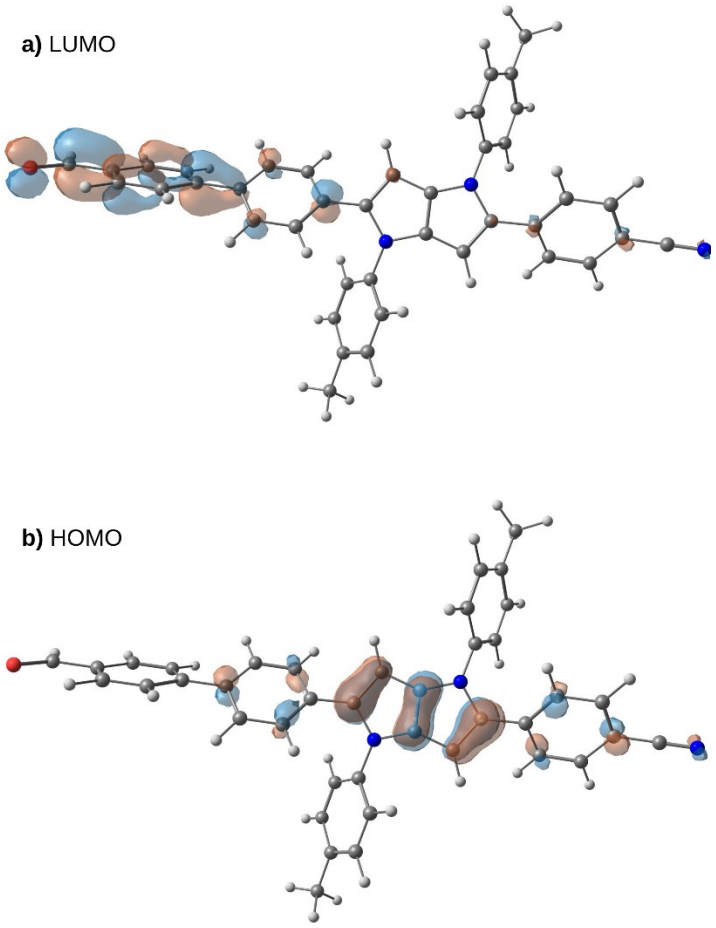

Figure SI 22. Optimized geometry and frontier molecular orbitals of compound 5 at the B3LYP/6-31G(d) level.

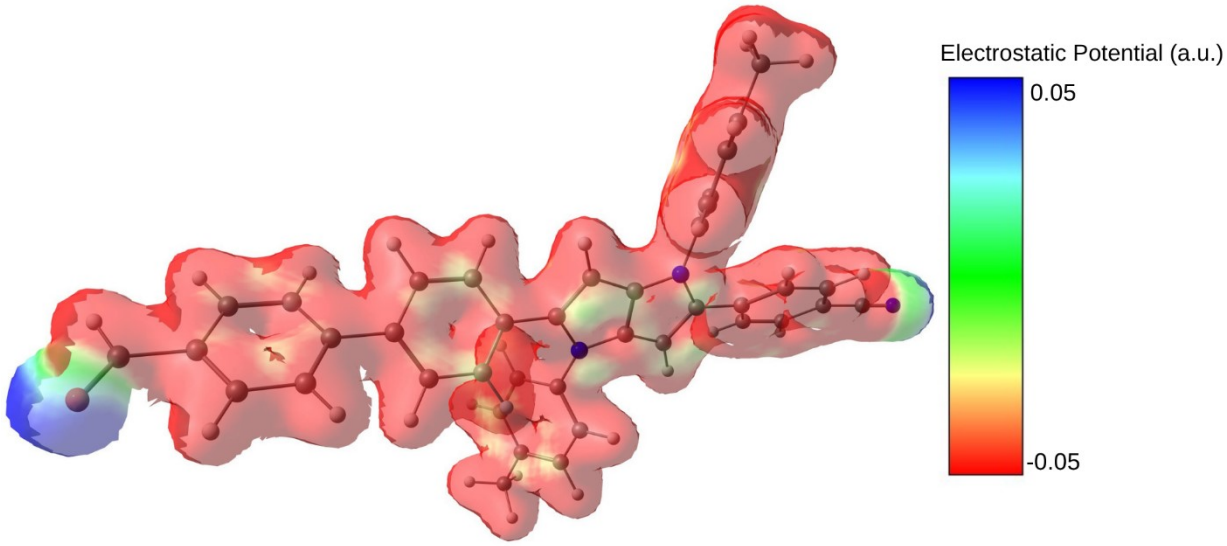

Figure SI 23. Molecular electrostatic potential (MEP) map of Probe 5 calculated at the B3LYP/6-31G(d). The potential colors range from -0.05 a.u. (red, nucleophilic regions) to +0.05 a.u. (blue, electrophilic regions).

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