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Electronic Supplementary Information

Biomolecular recognition at cellular level: geometrical and chemical functionality dependence of a low phototoxic cationic probe for DNA imaging

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Instruments: ^{1}H and ^{13}C NMR spectra were recorded on Jeol JNM ECX 500 MHz spectrometer in $CDCl_3$, $CDOD_3$ and DMSO-d_6. FT-IR spectra were recorded on a Carry-660 FT-IR spectrometer. HRMS-ESI spectra were recorded on Bruker Maxis Impact HD instrument. UV–vis and fluorescence spectra were recorded on Simadzu UV-2450 and Cary Eclipse spectrophotometer respectively, using 1 cm quartz cell with 5/5 slit widths. All the spectral studies were performed at 25^0C. The cell imaging experiments were performed employing Laser Scanning Confocal Microscope (version-3.0.2.0; model-IX81, Olympus) and inverted fluorescent microscope (Nikon Eclipse TS100).

Materials: Solvents and chemicals were purchased from commercial resources and used without further purification. Spectroscopic grade solvents were used for photophysical studies. Doubly ionized water used in all experiments is from Milli-Q systems. Tris-HCl (pH = 7.34, 10 X) was prepared using doubly ionized water. DNA and RNA solutions (1 mg/mL) were prepared in Tris-HCl. Stock solutions of 5 mM for each probe were prepared in DMSO and the aliquots of DNA stock solutions (0-1 mg/ml) were incubated with 10 μM solution of each probe for absorption and emission experiments.

Cell culture: HeLa cell line was used for all cell based studies and obtained from National Centre for Cell Science (NCCS), India. Cells cultured in α-MEM media (Sigma) added with 10% Fetal Bovine Serum (Sigma) and supplemented with penicillin, streptomycin and amphotericin B. Cells were maintained at 37°C in a humidified CO_2 incubator with 5% CO_2. Cells were replenished with fresh medium every alternate day.

DFT Calculations: The geometry of all the probes **P1-P5** was optimized at density functional theory (DFT) with B3LYP/6-31G (d, p) basis set [1, 2] with no symmetry constraint using Gaussian 09 suite of programmes.[3] Frequency calculation at the same level with the same basis set was performed to ensure that the geometries correspond to real minima. Gauss view software along with Chemcraft software was used for visualization purpose.[4]Mercury software was used to get ORTEP diagrams.

MTT assay: MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] assay based on a tetrazolium dye for measuring cell viability was used to assess the cytotoxicity of the probe P3.[5] For MTT assay, HeLa cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. After 24 h, cells were incubated with the probe **P3** (10 μM) for 1 h at 37°C, 5% CO_2 atmosphere. MTT reagent (5 mg/ml) was added near end point. After cell lysis with DMSO, optical density was

recorded at a wavelength of 565 nm (Tecan, M7500 pro) and cell viability was evaluated using following formula:

$$\%\ Cell\ viability = \frac{O.D.\ of\ \text{the}\ Probe\ treated\ cells}{O.D.\ of\ \text{the}\ untreated\ cells} \times 100$$

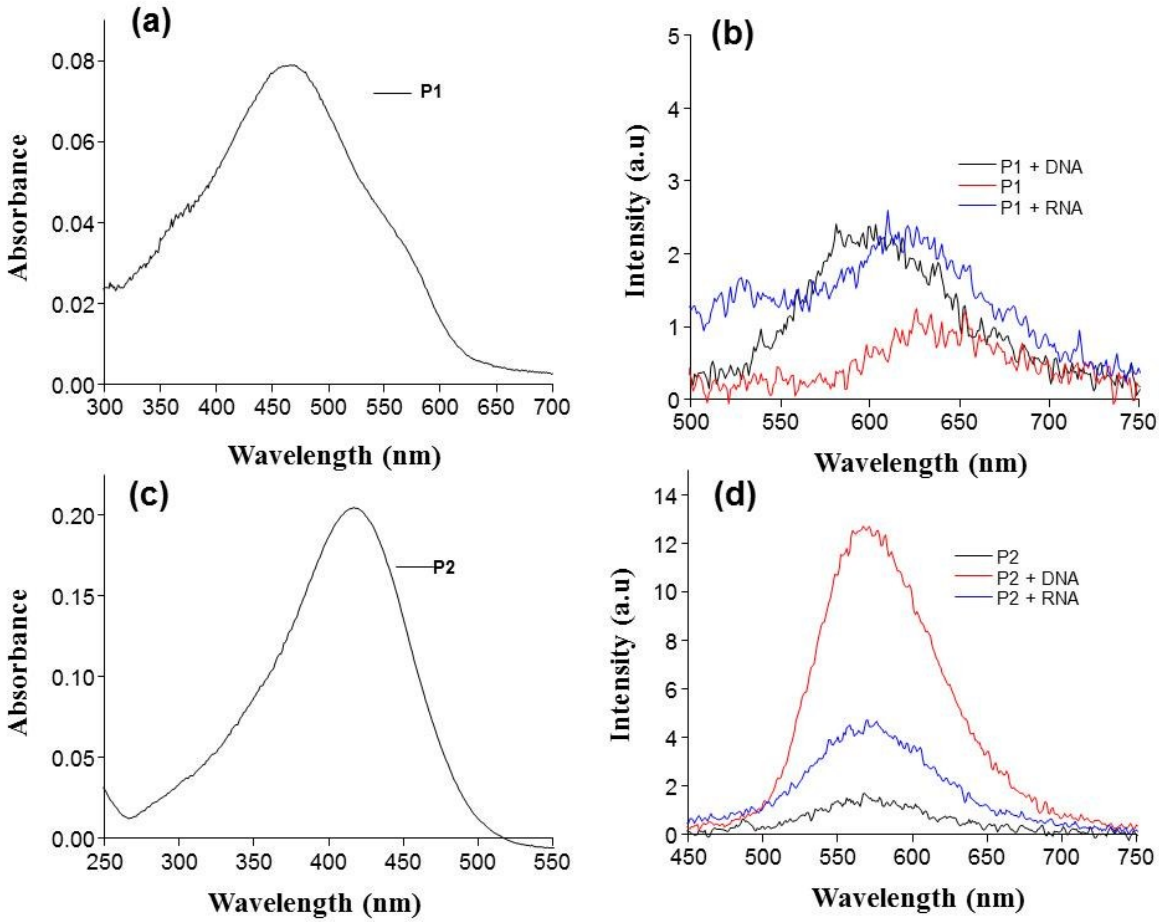
Confocal Microscopy: For confocal microscopy, 1×10^5 cells were grown on round (18 mm) glass cover slips till confluence, fixed in NBF overnight and treated with probe **P3** at a concentration of 10 μM for 2 h. Cells were then washed to remove the unbound probe and photographed for 20 min. Triton X (0.5%) and tween-20 (0.05%) were used for cell permeabilization studies. Cells stained with probe were photographed using Laser Scanning Confocal Microscope (version-3.0.2.0; model-IX81, Olympus) equipped with Fluoview FV 1000 software at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. DAPI was used as a counterstain (0.3 μM for 5 min).

Gel electrophoresis for DNA specificity: Total RNA was isolated from HeLa cells after 48 h of culture with the help of trizol reagent. 1μg of this total RNA was used to synthesize cDNA (BioRad cDNA synthesis kit) according to manufacturer's instructions. RT PCR (Reverse Transcriptase Polymerase chain reaction) for β-actin (housekeeping gene) was carried out and the PCR product was run on agarose gel for presence of the desired product. For DNA digestion experiment, DNase 1 was used at a concentration of 1X (1U) diluted in reaction buffer to a final volume of 10μl per 25μg of β-actin DNA. Equal amount of DNA was loaded per well of the agarose gel for both conditions. 10 μM of **P3** was used for detection of β-actin and gels were photographed using E- gel imager (Life Technologies) after completion of run.

Microscopy for visualizing DNA binding: Cells were grown till 60-70% confluence and stained with **P3** for duration of 2 h at 37°C and 5% CO_2 in humidified incubator. After two hours cells were washed 3 times with PBS and then incubated with DNase1 (RNase free) which was procured from Black Biotech and used at a concentration of 100 μg/ml and RNase was obtained from MagGenome and used at a final concentration of 40 μg/ml and directly added to culture media in live cells in above mentioned concentrations for 2 h after permeabilization with 0.1% triton X.[6]At end of incubation period, cells were photographed under fluorescent microscope (Nikon Eclipse TS100).

Photobelaching resistance: For photobleaching experiments, fixed cells were stained with both Syto-16 and **P3** (10 μM) for 2 h and were then continuously exposed to excitation wavelength in FITC channel (488 nm, 50 W mercury lamp, 12×10^{19} photons/pulse) for duration of 10 min for each condition using fluorescent microscope (Nikon Eclipse TS100). Fluorescence/photo bleaching was measured using Image J software. The fluorescence at 0 min was considered as 100% and fluorescence retention in each case was calculated by taking this value as reference control.

Phototoxicity assay: Cells (5×10^3cells/well) were incubated with 10 μM of probe **P3** for 1 h at 37°C. After continuous exposure of light for 5 min in FITC channel under fluorescent microscope (Nikon Eclipse TS100), the cells were incubated at 37°C for 6 h and phototoxicity was measured by MTT assay as per manufacturer's instruction.



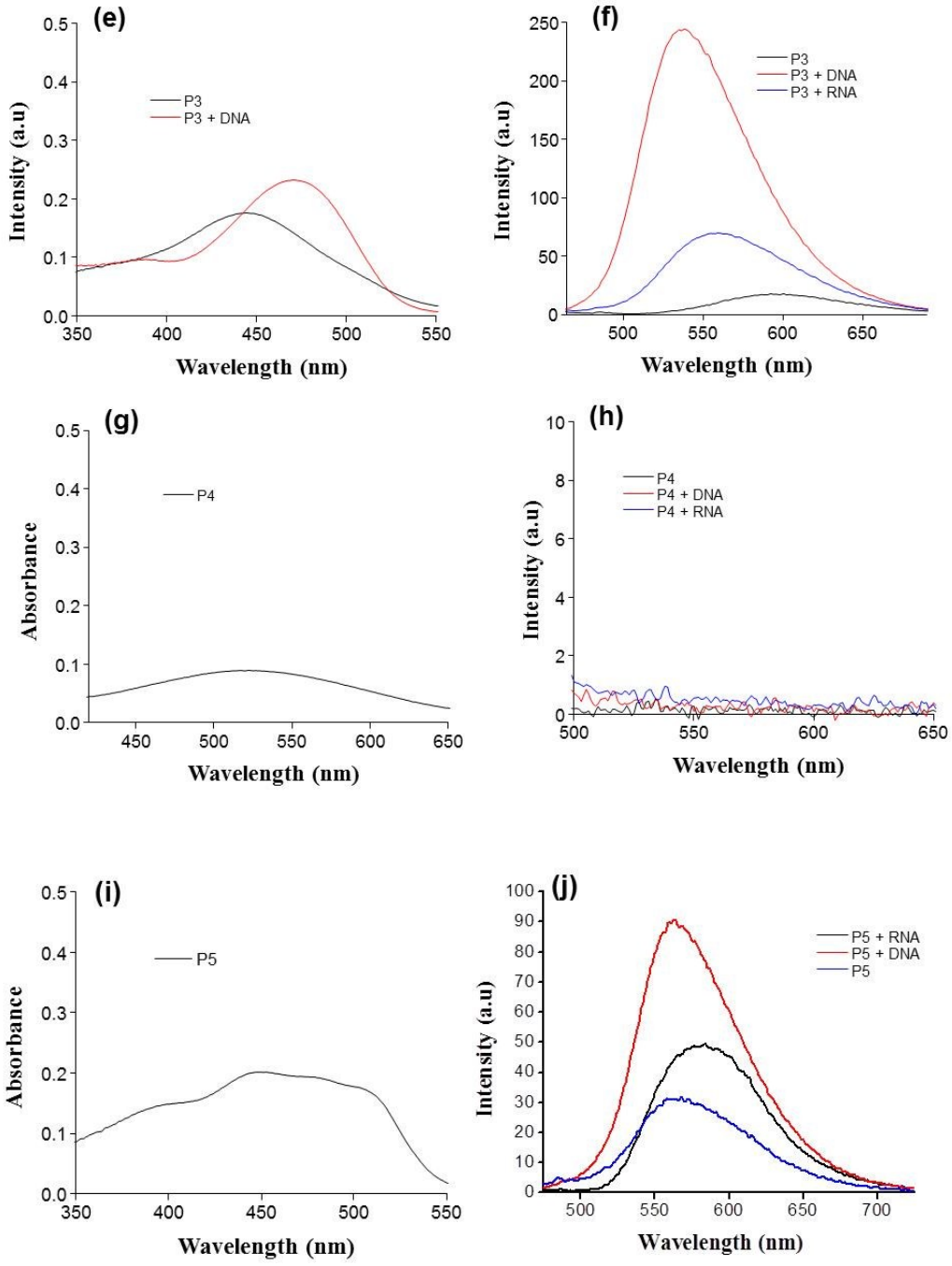


Fig. S1: Absorption and emission spectra of the probes **P1-P5**.

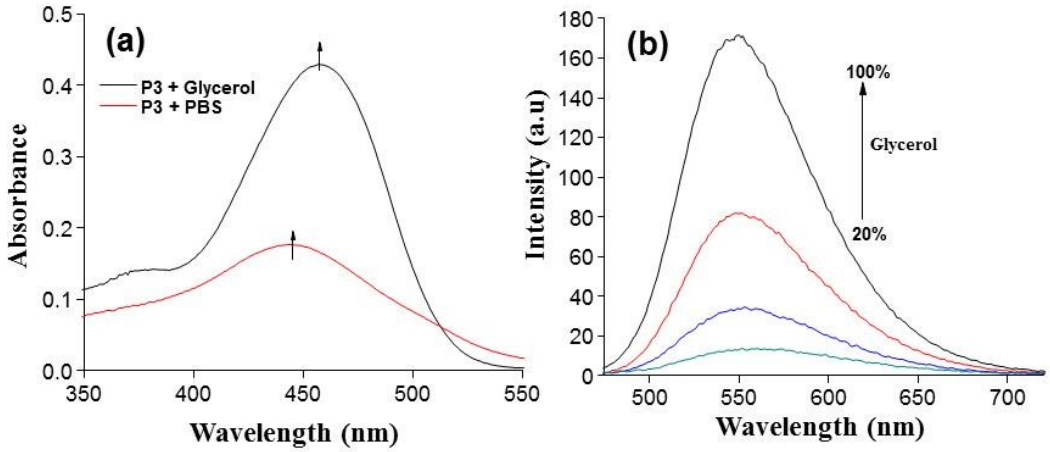


Fig. S2. a**)** Absorption profile of **P3** in PBS and glycerol, b) emission profile of **P3** in glycerol/water mixtures.

Stoichiometry and Binding constant calculation: The binding stoichiometry 1:1 between probe **P3** and DNA was evaluated as Hill coefficient (m 1) employing Hill equation.[7] Later, the corresponding binding constants was calculated employing Benesi-Hildebrand equation.[8]

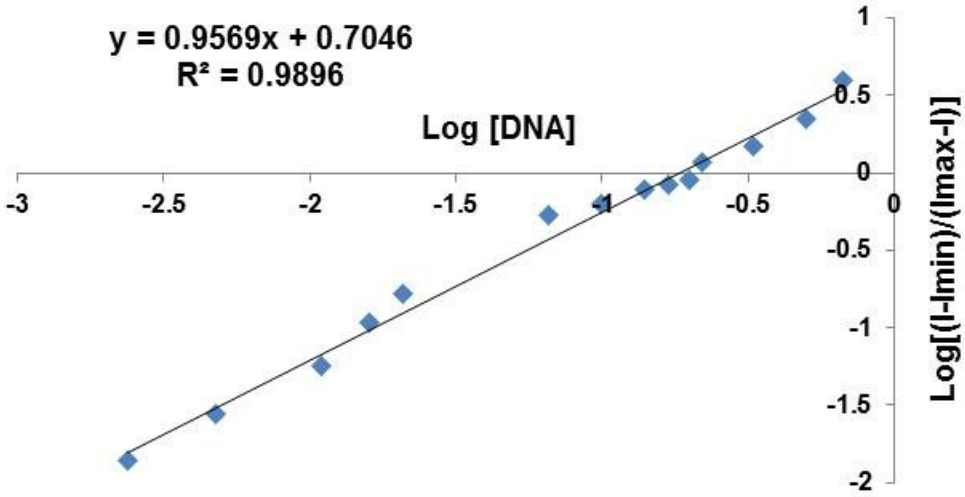


Fig. S3. Calculation of Hill coefficient, (m = 0.9569) showing 1:1 binding of DNA-Probe complex.

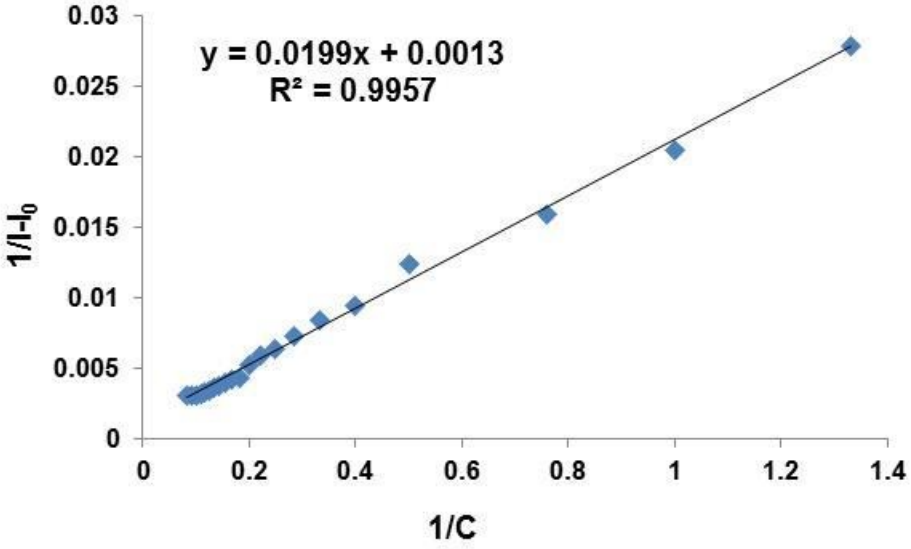


Fig. S4. Binding constant calculation of **P3.**

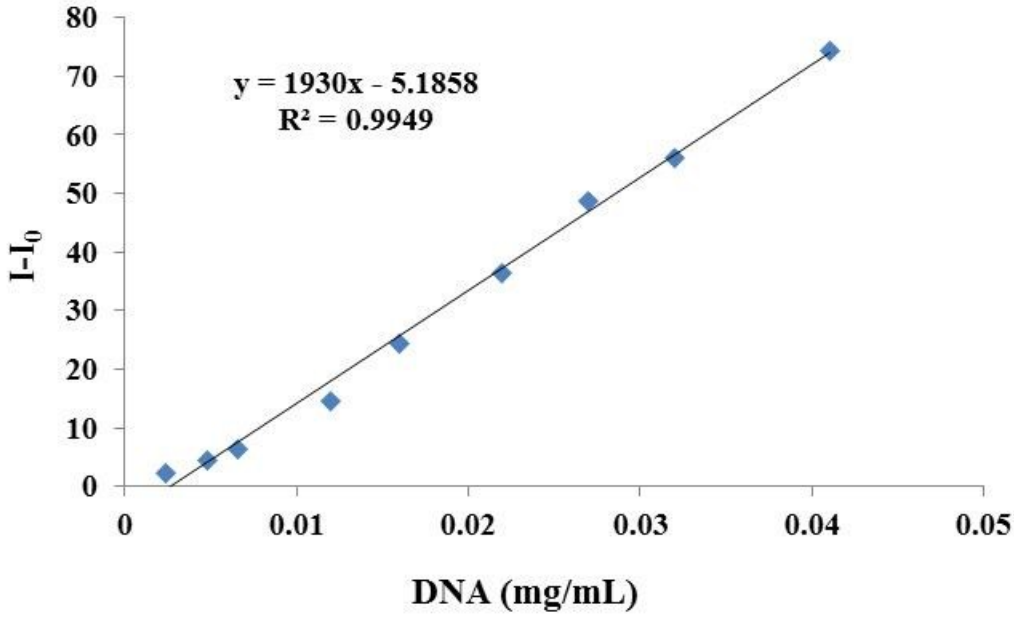


Fig. S5: Detection limit calculation of **P3.**

LOD = 0.34 μM

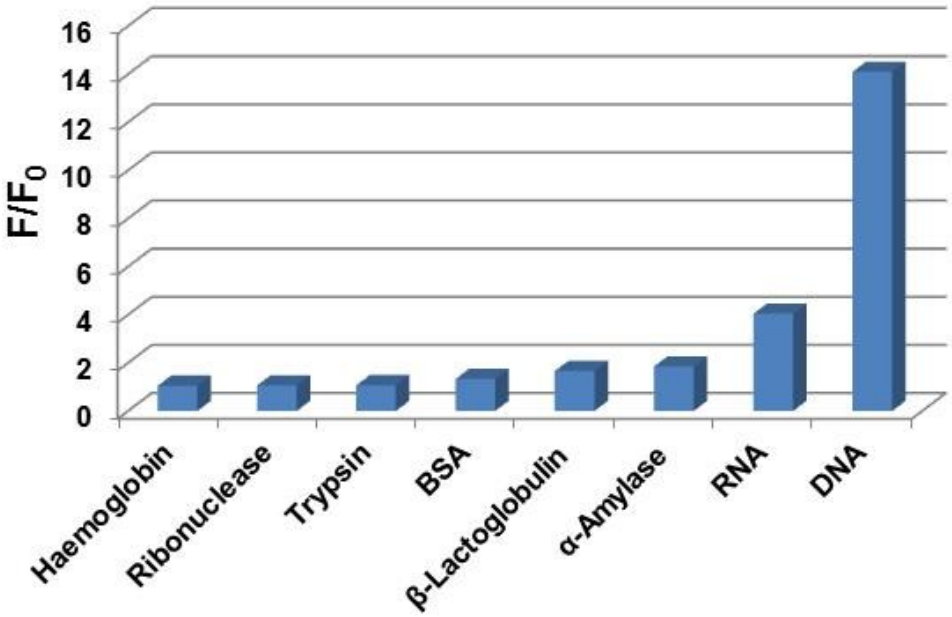


Fig. S6. Selectivity of probe **P3** toward DNA

Quantum yield calculation:

Using optically matching solutions of Fluorescein (ϕ_R = 0.95, in NaOH solution) for probe **P3**, as the standard, quantum yield was determined (on SHIMADZU UV-2450 spectrophotometer and Cary Eclipse spectro photometer, slit widths of excitation and emission are 5/5 respectively) using equation:

$$\phi_S = \phi_R \left(\frac{A_R}{A_S} \right) \left(\frac{D_S}{D_R} \right) \left[\frac{n_S}{n_R} \right]^2$$

Where ϕ_S and ϕ_R are the quantum yields of sample and the reference, A_S and A_R are the absorbance of the sample and the reference. D_S, D_R the areas of emission while n_S, n_R are the refractive indices of the sample and reference solutions respectively.

	P1	**P2**	**P3**	**P4**	**P5**
ϕ_{PBS}	0.0014	0.0019	0.0029	0.00054	0.035
ϕ_{DNA}	0.0054	0.0032	0.26	0.00084	0.044

Table S1: Calculated quantum yield of the probes

Extinction coefficient calculation: The values of extinction coefficient were evaluated for probe **P3** both in presence and absence of DNA. Absorption spectra was obtained by titrating 3ml of Tris-HCl with 0 – 10 μM concentration of probe and the value of the extinction coefficient was evaluated as the slope of the plot between absorption maxima vs concentrations. [9, 10]In the similar manner, absorption spectra of various concentration (0 – 10 μM) of probe **P3** was recorded in the presence of 1 mg/mL of DNA solution and extinction coefficient value was calculated from the slope of absorbance vs concentrations graph. The plot between absorption maxima vs concentration in absence and presence of DNA for probe **P3** are illustrated in Figure S3 respectively.

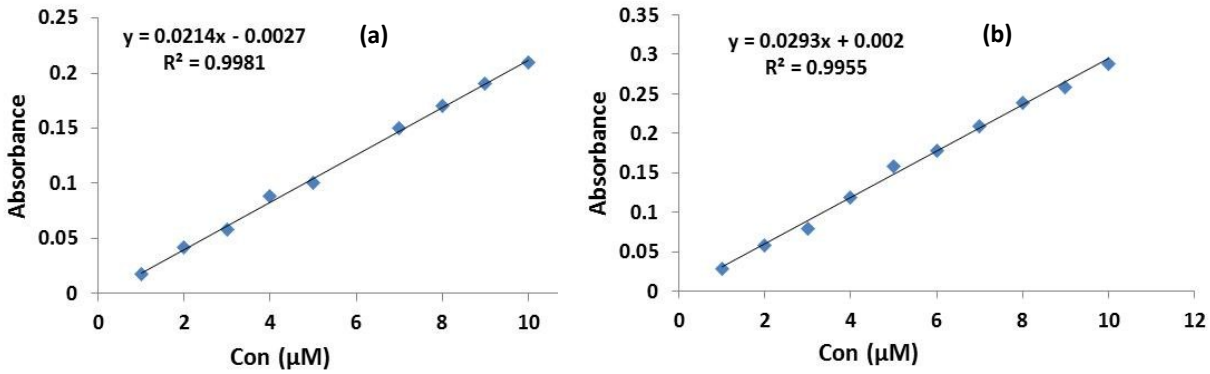


Fig. S7: Extinction coefficient calculation. (a), (b) plot between absorption maxima vs concentration (0 - 10µM) of probe **P3** in absence and presence of DNA respectively.

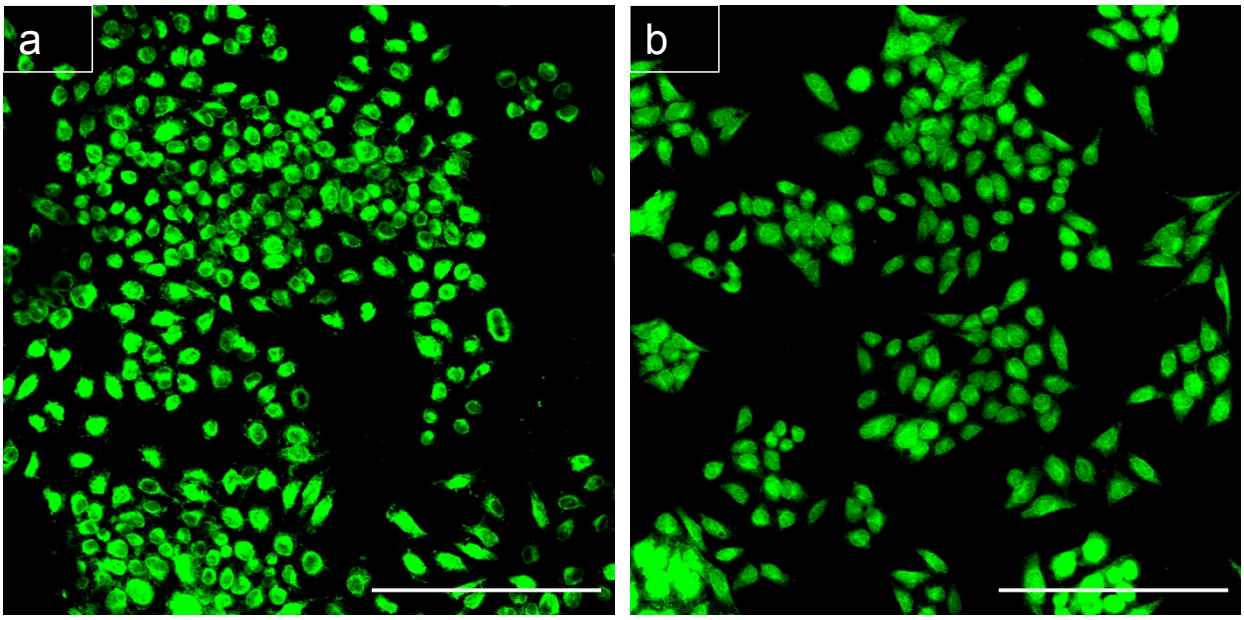


Fig. S8. Cell imaging upon incubation with 10 µM **P3. a**) Live cells, **b**) fixed cells, magnification-20x, scale bar-100um.

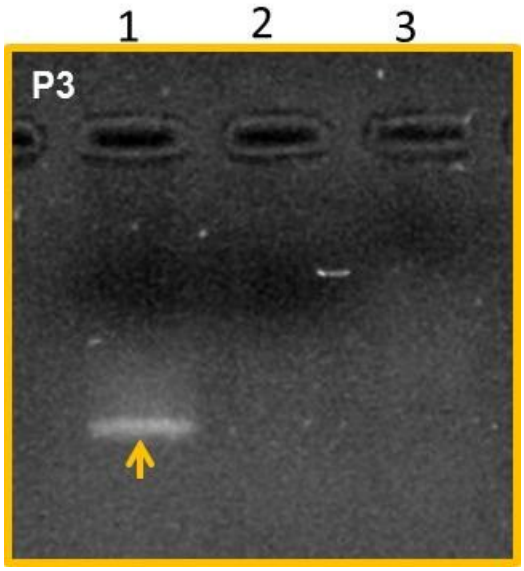


Fig. S9. Gel based experiment for DNA digestion: Lane 1 shows intact DNA labeled with the P3; Lane 2, 3 show DNA digested with DNase and labeled with P3, thus confirming the specificity of P3 for DNA.

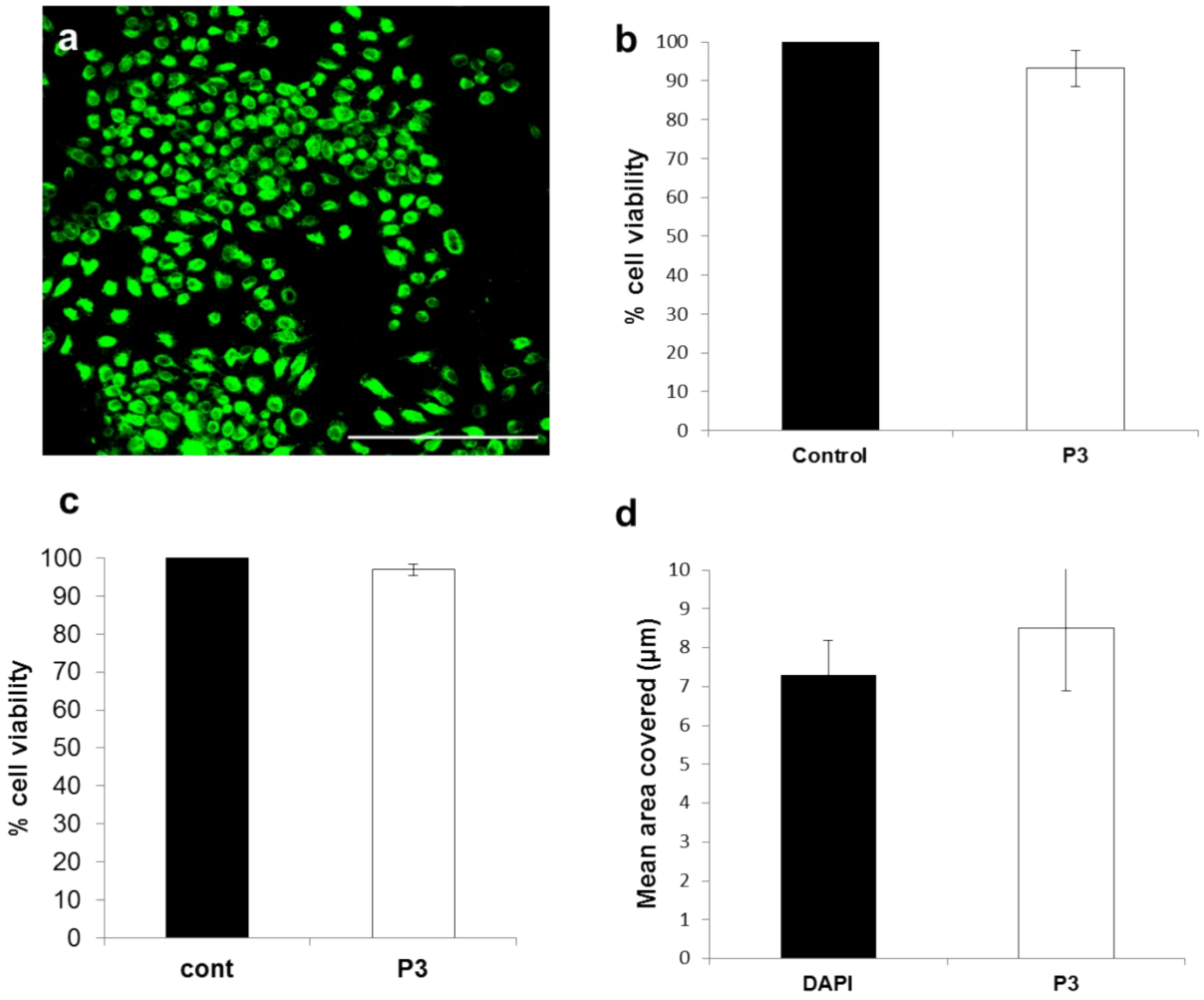


Fig. S10. **a**) Live cells, **b**) cytotoxicity assay, **c**) phototoxicity assay and **d**) cytoplasmic spillage. Mean area covered by DAPI and **P3** in cell is showing negligible spillage of **P3**. Magnification-20x, scale bar-100um.

Synthesis and Chracterization:

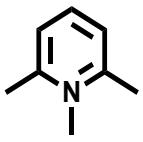
a) **Lutidinium salt (1a):** Methyl iodide (4.0 g, 3 eq) was added dropwise to a solution of 2,4,6-trimethylpyridine (1.0g, 1 eq) in dichloromethane (25 mL) at 0^0C. After stirring at room temperature overnight, the solvent was evaporated to give a white solid, which was triturated with DCM to give 1.2 g product as white crystal with yield of 52%.

b) **Compound 1b:** A mixture of N-methyl piperazine (1.0 g, 1eq) and 4-fluorobenzaldehyde (1.2 g, 1eq) in 20 mL of DMF was heated to 80^0C for overnight. The progress of the reaction was monitored by TLC. The mixture was poured on crushed ice and extracted with DCM. The crude was purified by column chromatography eluting 5% Me-OH/DCM as a brown solid (30%).

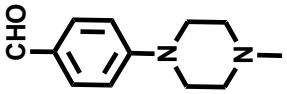
c) **Compound 1c:** A mixture of indole-3-carboxyaldehyde (0.6g, 1eq), methyl iodide (1.92 g, 3.3 eq) and cesium carbonate (2.6 g, 2 eq) was refluxed in acetonitrile (12 mL). The progress of the reaction was monitored by TLC and then solvent was evacuated on rotary evaporator. The crude was dissolved in water and extracted with ethyl acetate. Brown solid was obtained by column chromatography eluting 5% EtOAc/hexane.

d) **Compound 1d:** Phenothiazine (1eq) and Urotropine (1eq) were dissolved in DMF (1 mL/mmol). The mixture was refluxed for 6 h and then quenched with sodium carbonate. The product was extracted using DCM and washed with water 2-3 times thoroughly. The crude product was purified eluting 10% EtOAc/Hexane.

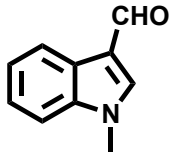
e) **General method for synthesis of P1-P5**: N-Methyl-2,4,6-trimethylpyridinium iodide (1eq) and corresponding aldehydes (3 eq) were dissolved in methanol (3 mL/mmol) into a round bottom flask, then piperidine (catalytic amount) was added. The mixture was refluxed for 6 h under the protection of N_2, and then cooled slowly. The colored solids were collected, and triturated thrice with ethyl acetate to get the pure colored powders.



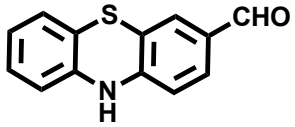
Compound 1a: Rf = 50%, White solid, Yield: 52%, M.P. - 239.5^0C, FT-IR (ν in cm^{-1}): 3015, 2965, 1421, 1264. ^{1}H NMR (500 MHz, Methanol-d_4): δ = 8.27-8.24 (m, 1H), 7.81 (d, J = 7.56Hz, 2H), 4.14 (s, 3H), 2.86 (s, 6H) ppm. ^{13}C NMR (125 MHz, Methanol-d_4): 157.7, 145.5, 128.6, 41.5, 22.2 ppm. HRMS: *m/z* calculated for $C_8H_{12}N$ [M - I] 122.097, found 122.0968.



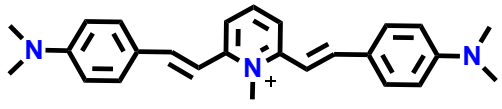
Compound 1b: Rf = 45%, Brown solid, Yield: 30%, M.P. - 61.7^{0}C, FT-IR (ν in cm^{-1}): 1665, 1580, 1365, 1210, 1165. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.78 (s, 1H), 7.75 (d, J = 8.95 Hz, 2H), 6.92 (d, J = 8.9 Hz, 2H), 3.42-3.40 (m, 4H), 2.56-2.54 (m, 4H), 2.35 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): 190.4, 154.9, 131.8, 127.0, 113.5, 54.6, 46.9, 46.0 ppm. HRMS: *m/z* calculated for $C_{12}H_{16}NO$ [M + H] $^+$205.1341, found 205.1335.



Compound 1c: Rf = 40%, Brown solid, Yield: 87%, M.P. - 71.2^{0}C, FT-IR (ν in cm^{-1}): 1670, 1598, 1385, 1227, 1173. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.96 (s, 1H), 8.30-8.29 (m, 1H), 7.64 (s, 1H), 7.35-7.31(m, 3H), 3.84 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): 184.4, 139.2, 137.8, 125.2, 123.9, 122.9, 121.9, 117.9, 109.8, 33.6 ppm. HRMS: *m/z* calculated for $C_{10}H_9NO$ [M + H] $^+$160.0762, found 160.0756.

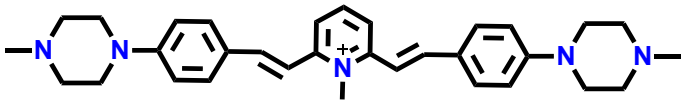


Compound 1d: Rf = 42%, Yellowish brown solid, Yield: 35%, M.P. - 186.6^{0}C, FT-IR (ν in cm^{-1}): 1668, 1505, 1469, 1199. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.71 (s, 1H), 7.47-7.43 (m, 2H), 7.01-6.97 (m, 2H), 6.94-6.84 (m, 1H), 6.56-6.52 (m, 2H), 6.16 (s, 1H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): 189.7, 146.7, 139.1, 131.5, 130.4, 128.0, 127.6, 126.8, 123.8, 118.9, 117.5, 114.9, 113.9 ppm. HRMS: *m/z* calculated for $C_{13}H_{10}NOS$ [M + H] $^+$ 228.0483, found 228.0475.

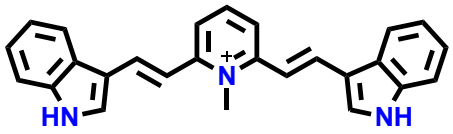


Compound P1: Rf = 81%, Violet solid, Yield: 34%, M.P. - 252.0^{0}C FT-IR (ν in cm^{-1}): 2925, 2855, 1651, 1462. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.23-8.18 (m, 2H), 8.08-8.06 (d, J = 7.6 Hz, 2H), 7.7-7.67 (m, 5H), 7.34-7.28(m, 2H), 6.79-6.76

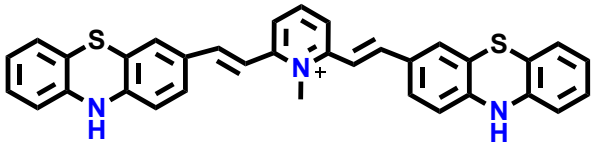
(m, 4H), 4.18 (s, 3H), 3.01 (s, 12H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): 153.6, 151.7, 142.8, 141.4, 130.5, 130.3, 122.6, 121.2, 112.8, 111.8, 111.1, 40 ppm. HRMS: *m/z* calculated for $C_{26}H_{30}N_3$ [M - I] 384.244, found 384.2434.



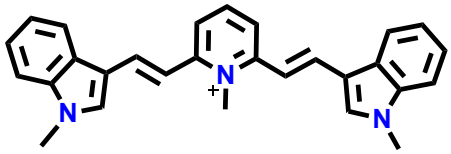
Compound P2: Rf = 42%, Red solid, Yield: 88%, M.P. - 182.0^{0}C FT-IR (ν in cm^{-1}): 2945, 2835, 1642, 1450. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.27-8.24 (m, 1H), 8.12 (d, J = 7.6 Hz, 2H), 7.71-7.66 (m, 6H), 7.40-7.37 (m, 2H), 7.02-7.0 (d, J = 8.95 Hz, 8H), 4.2 (s, 3H), 3.31-3.29 (m, 8H), 2.45-2.43 (m, 4H), 2.21 (s, 6H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): 153.6, 152.3, 142.4, 141.8, 130.2, 124.8, 121.8, 114.2, 54.37, 46.8, 45.8, 41.2 ppm. HRMS: *m/z* calculated for $C_{32}H_{40}N_5$ [M - I] 494.3284, found 494.3287.



Compound P3: Rf = 56%, Violet solid, Yield: 54%, M.P. - 227.0^{0}C FT-IR (ν in cm^{-1}): 3010, 2830, 1655, 1460. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.22-8.1 (m, 6H), 8.01 (d, J = 15.8 Hz, 2H), 7.52-7.50 (m, 3H), 7.32-7.27 (m, 2H), 7.26-7.22 (m, 4H), 4.24 (s, 3H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): 153.9, 141.2, 137.4, 136.5, 131.6, 125.1, 122.8, 121.1, 120.5, 120, 113.4, 112.6, 112.2, 40.9 ppm. HRMS: *m/z* calculated for $C_{26}H_{22}N_3$ [M - I] 376.1814, found 376.1813.



Compound P4: Rf = 61%, Violet solid, Yield: 25%, M.P. - 205.0^{0}C, FT-IR (ν in cm^{-1}): 2949, 2870, 1645, 1445. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.04 (s, 2H), 8.27-8.19 (m, 2H), 8.11-8.10 (m, 2H), 7.57-7.5 (m, 3H), 7.42-7.37 (m, 4H), 7.01-6.98 (m, 2H), 6.93-6.91 (m, 2H), 6.80-6.76 (m, 2H), 6.72-6.68 (m, 4H), 4.19 (s, 3H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): 155.2, 153.7, 153.3, 143.6, 142.9, 142.1, 141.6, 141.2, 140.3, 129.5, 128.8, 127.8, 126.2, 125.6, 122.5, 122.4, 116.9, 115.8, 114.8, 114.2, 41.4 ppm. HRMS: *m/z* calculated for $C_{34}H_{26}N_3S_2$ [M - I] 540.1568, found 540.1567.



Compound P5: Rf = 67%, Orange solid, Yield: 57%, M.P. - 265.0^{0}C FT-IR (ν in cm^{-1}): 3025, 2850, 1636, 1435. ^{1}H NMR (500 MHz, DMSO-d$_6$): δ = 8.13-8.10 (m, 7H), 8.01-7.98 (d, J = 15.8 Hz, 2H), 7.57 (m, 2H), 7.33-7.26 (m, 6H), 4.22 (s, 3H), 3.89 (s, 6H) ppm. ^{13}C NMR (125 MHz, DMSO-d$_6$): 153.8, 141.1, 137.7, 135.8, 134.7, 125.5, 122.8, 121.3, 120.5, 112.3, 110.9, 40.9, 33.2 ppm. HRMS: *m/z* calculated for $C_{28}H_{26}N_3$ [M - I] 404.2127, found 404.2127.

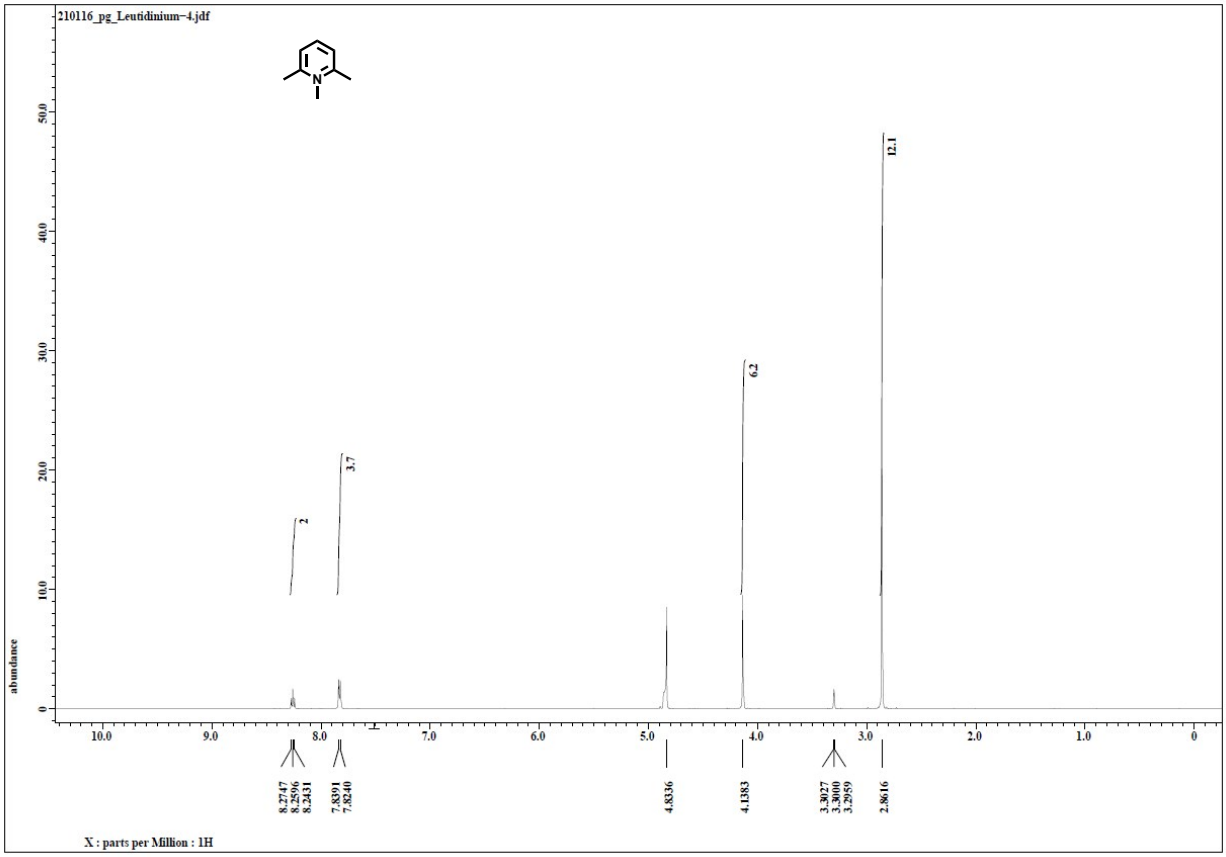


Fig. S11: ^{1}H-NMR spectra of 1a

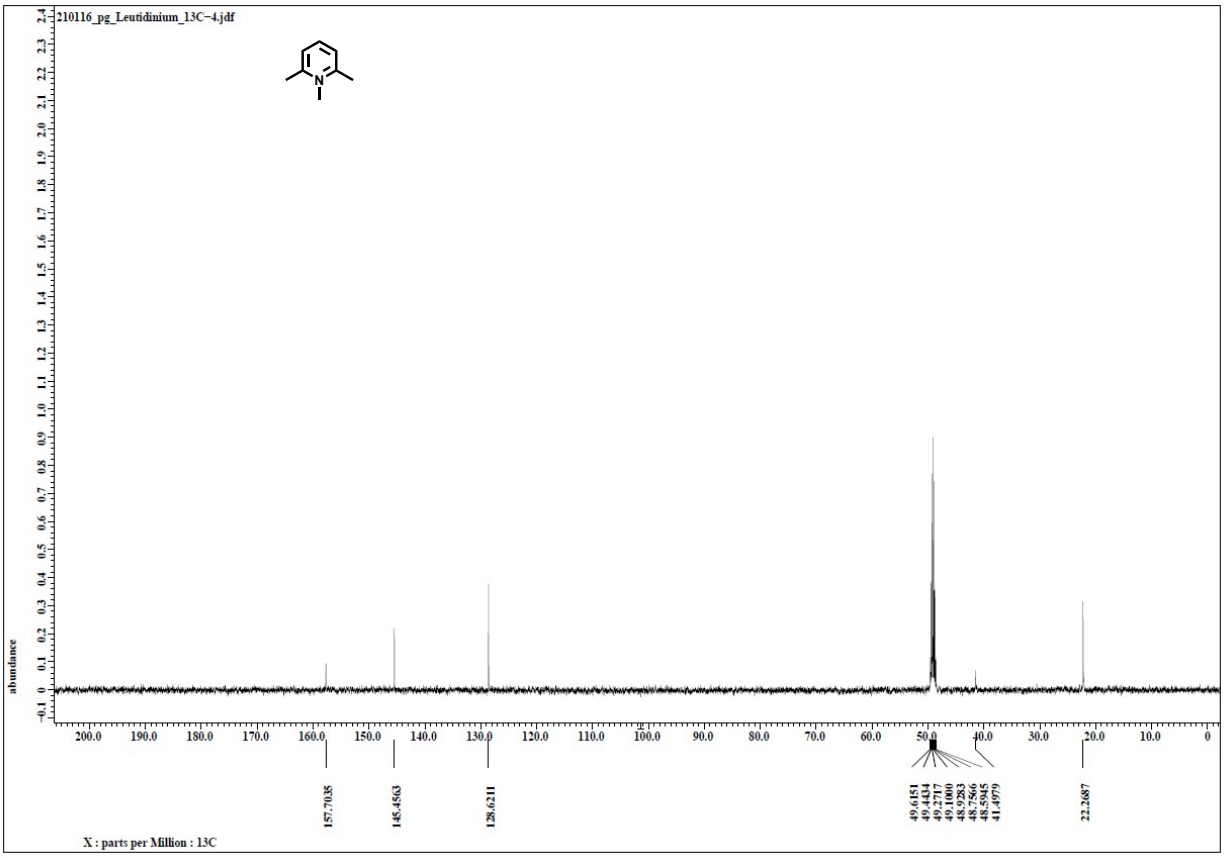


Fig. S12: ^{13}C spectra of 1a

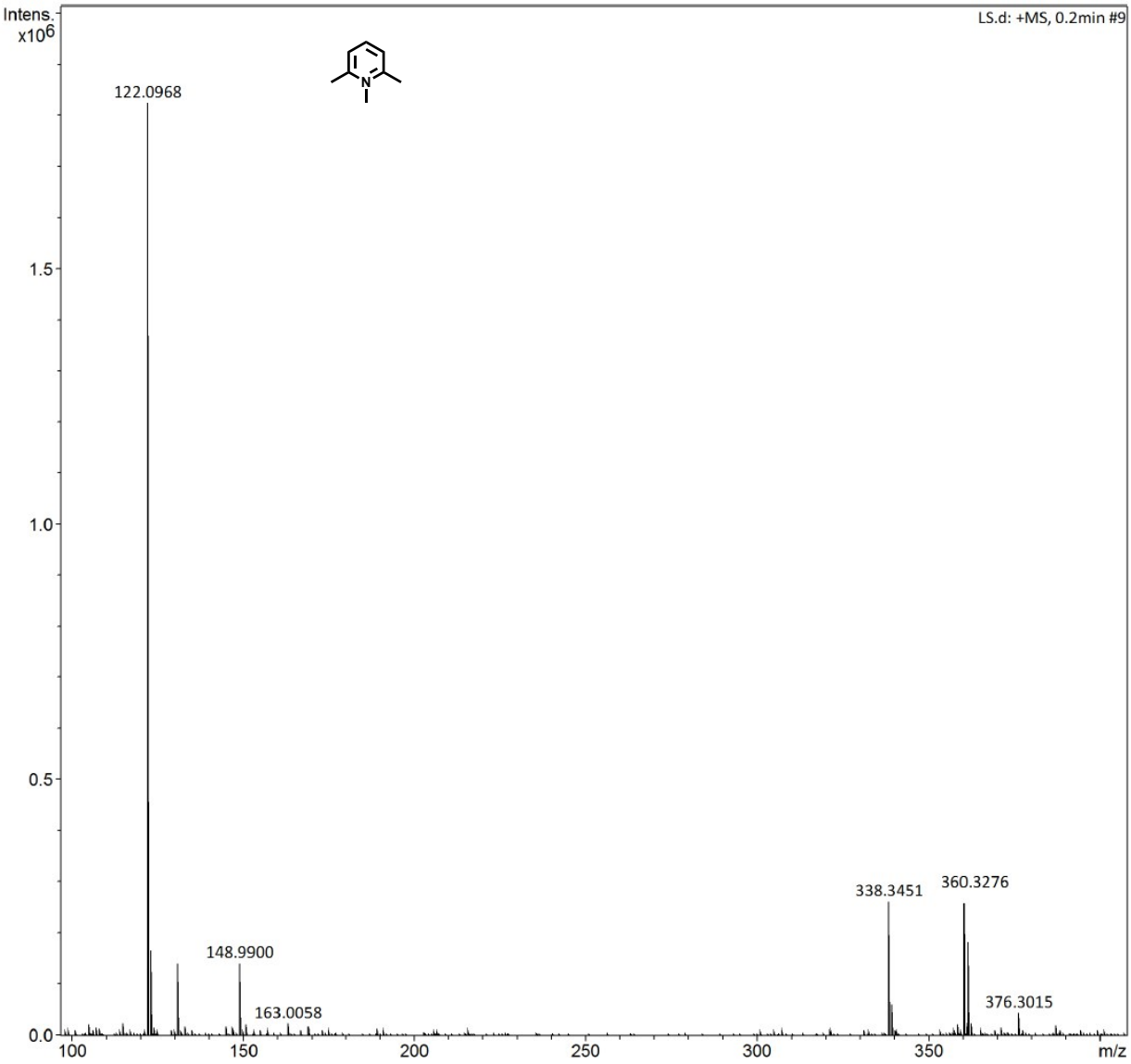


Fig. S13: HRMS spectra of 1a

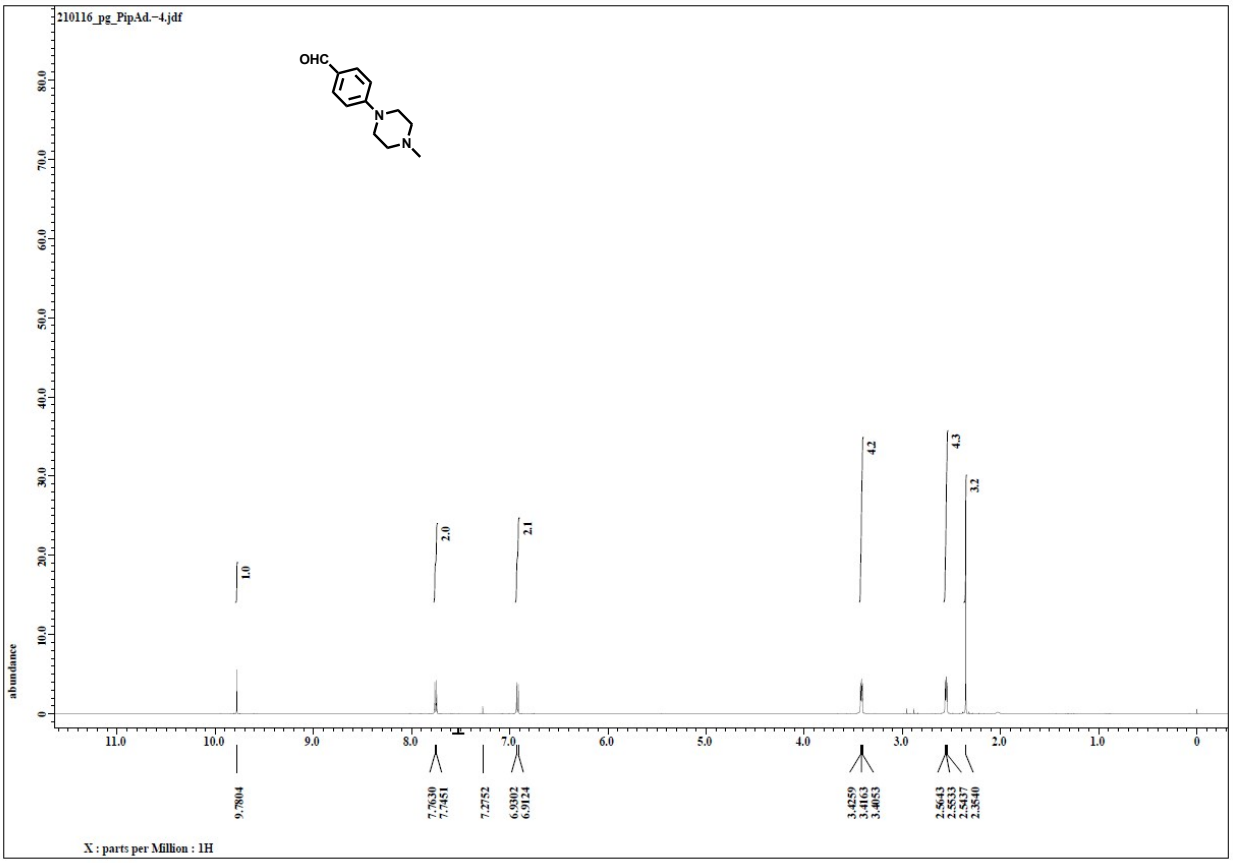


Fig. S14: ^{1}H-NMR spectra of 1b

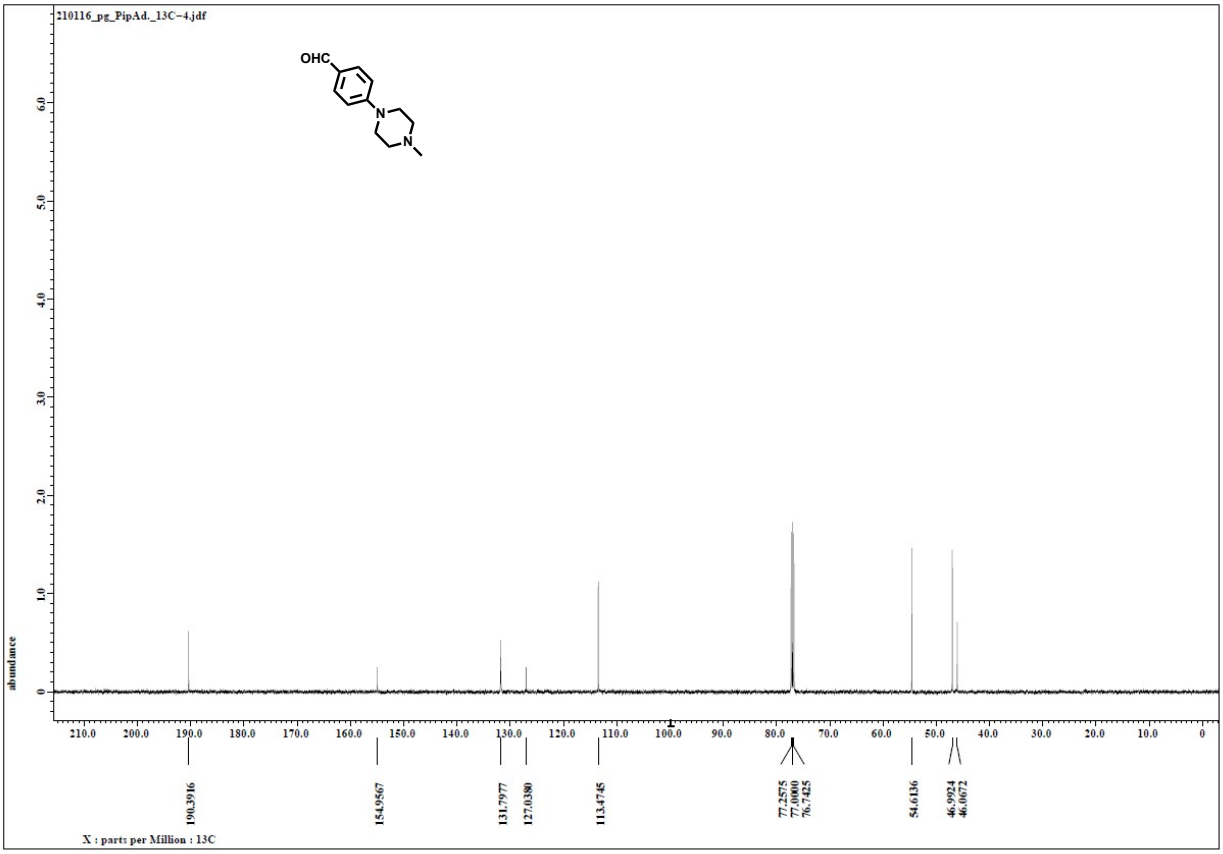


Fig. S15: ^{13}C spectra of 1b

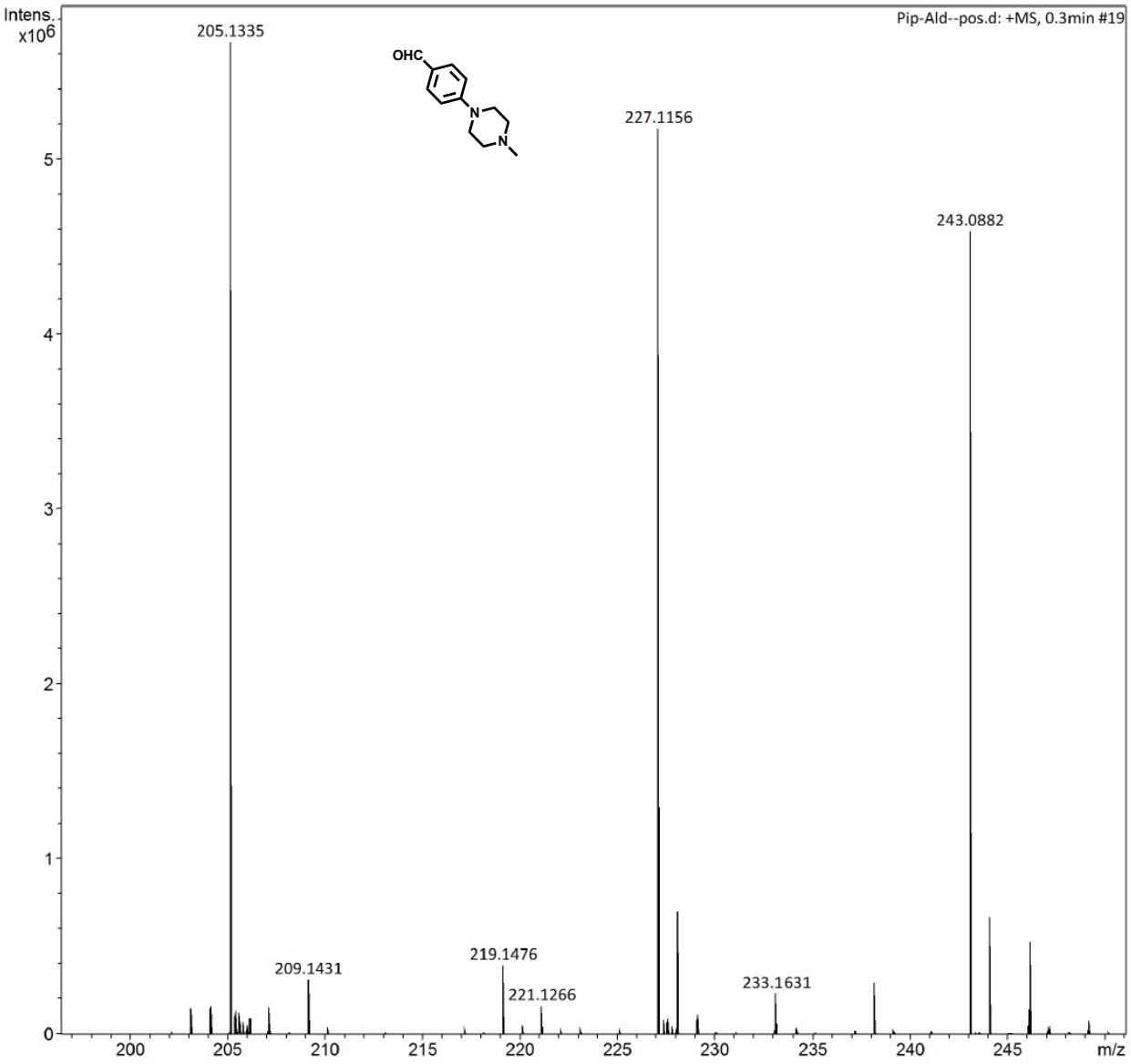


Fig. S16: HRMS spectra of 1b

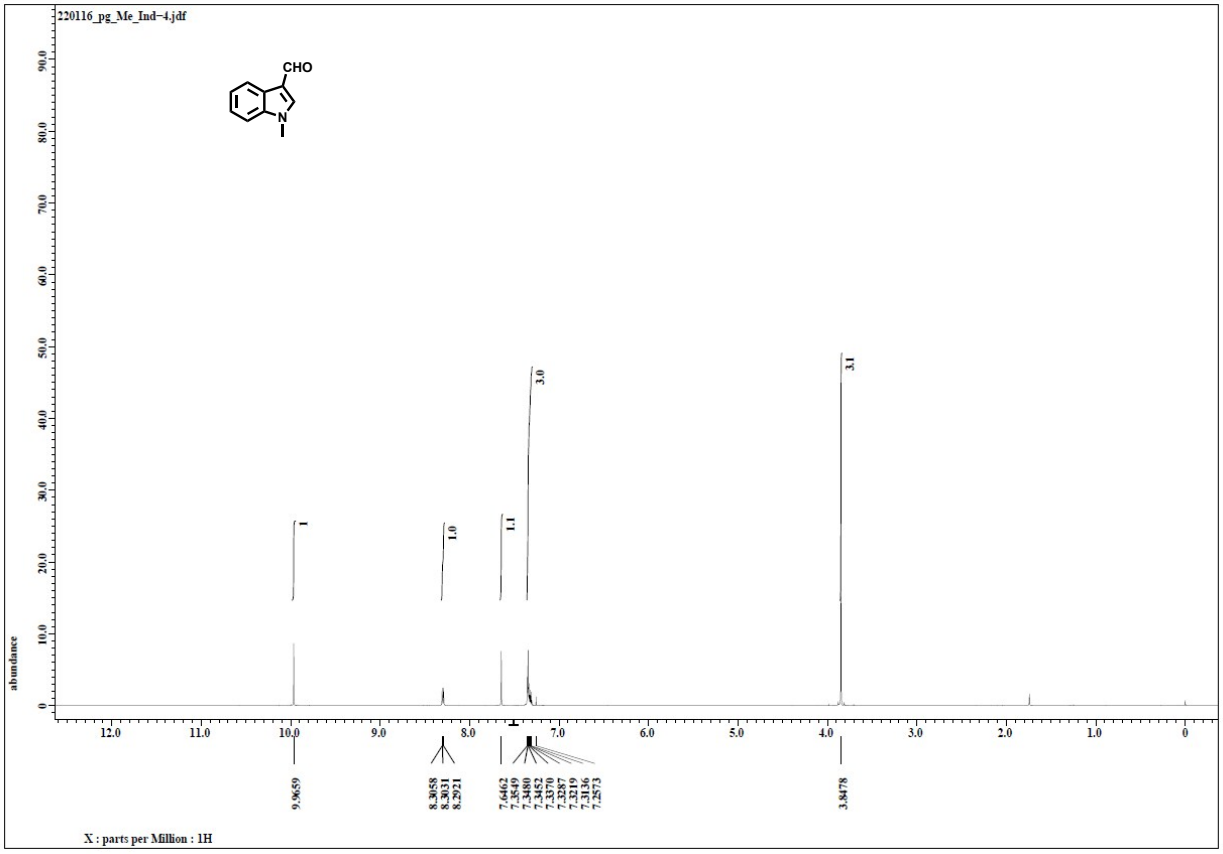


Fig. S17: ^{1}H-NMR spectra of 1c

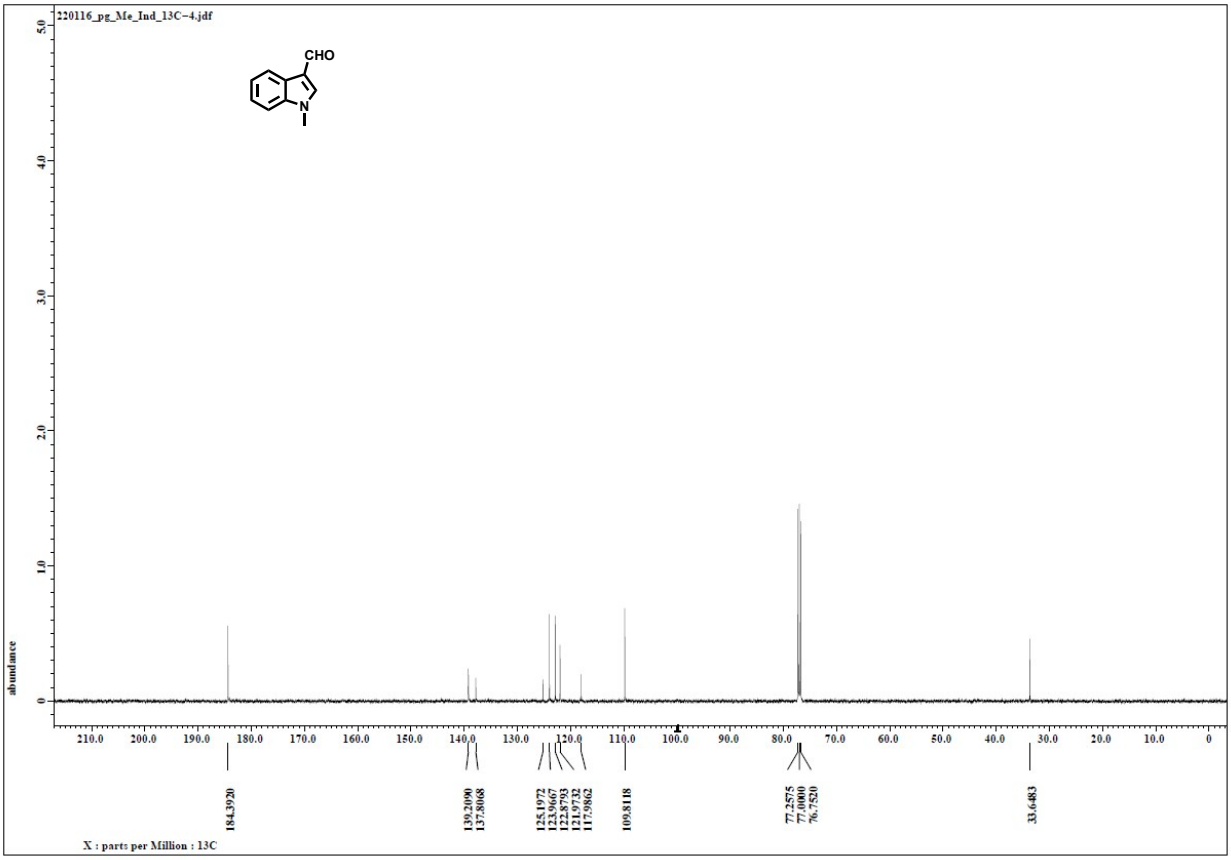


Fig. S18: ^{13}C spectra of 1c

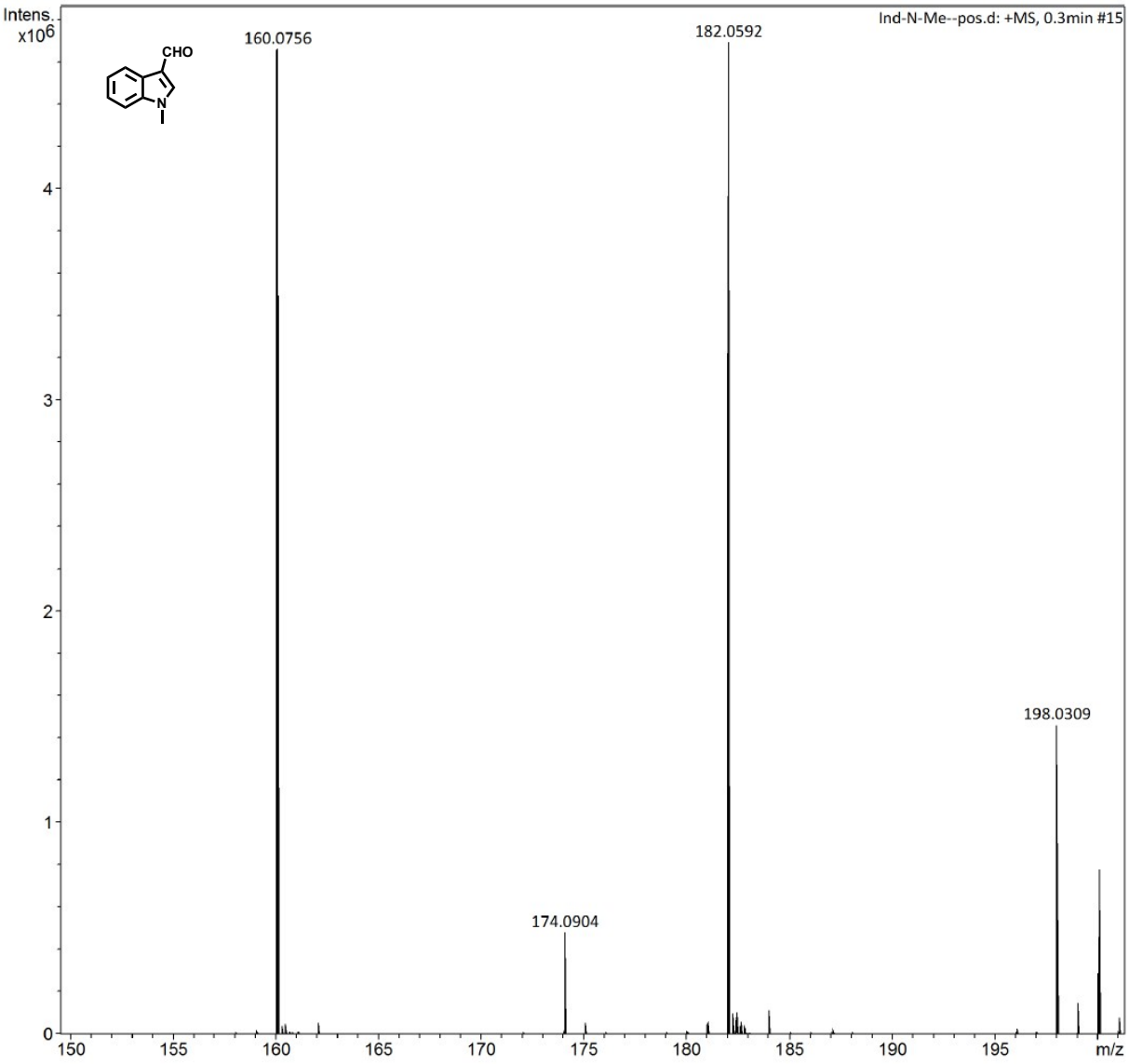


Fig. S19: HRMS spectra of 1c

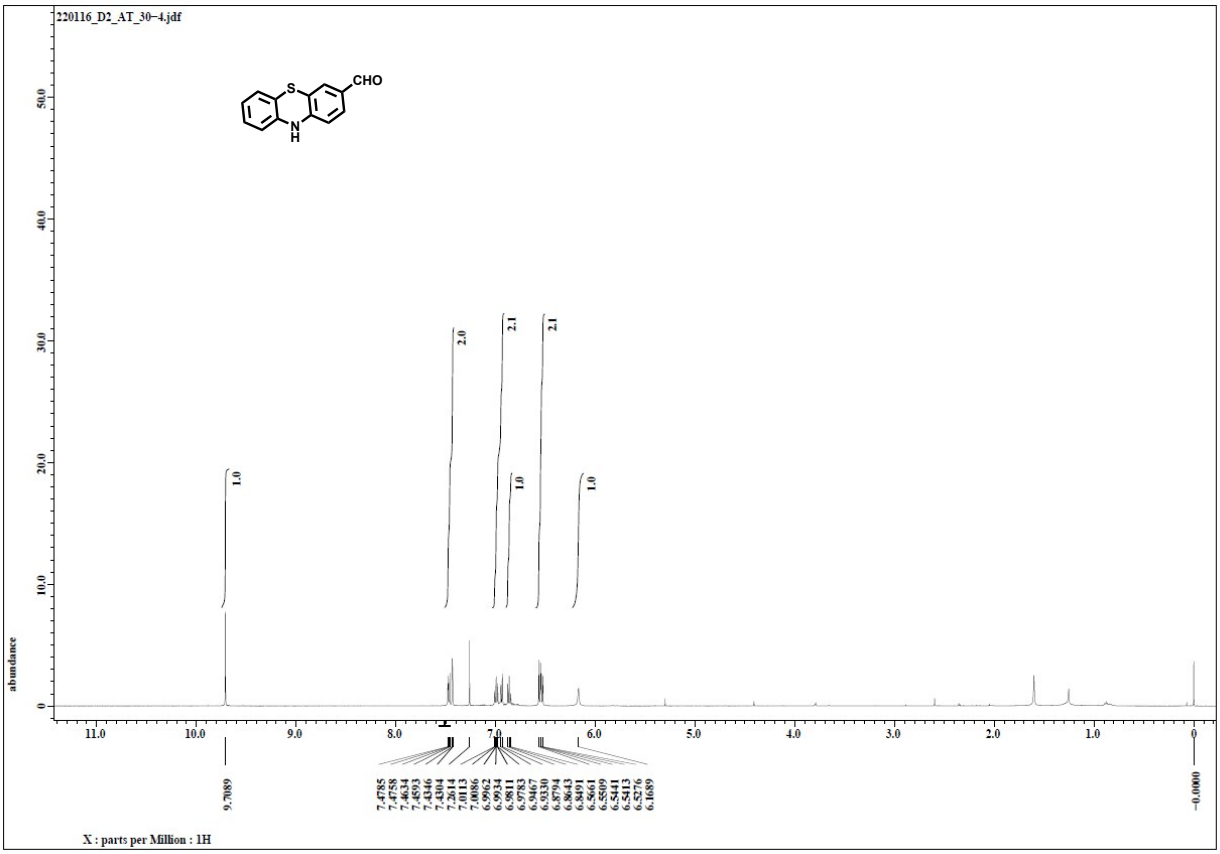


Fig. S20: ^{1}H-NMR spectra of 1d

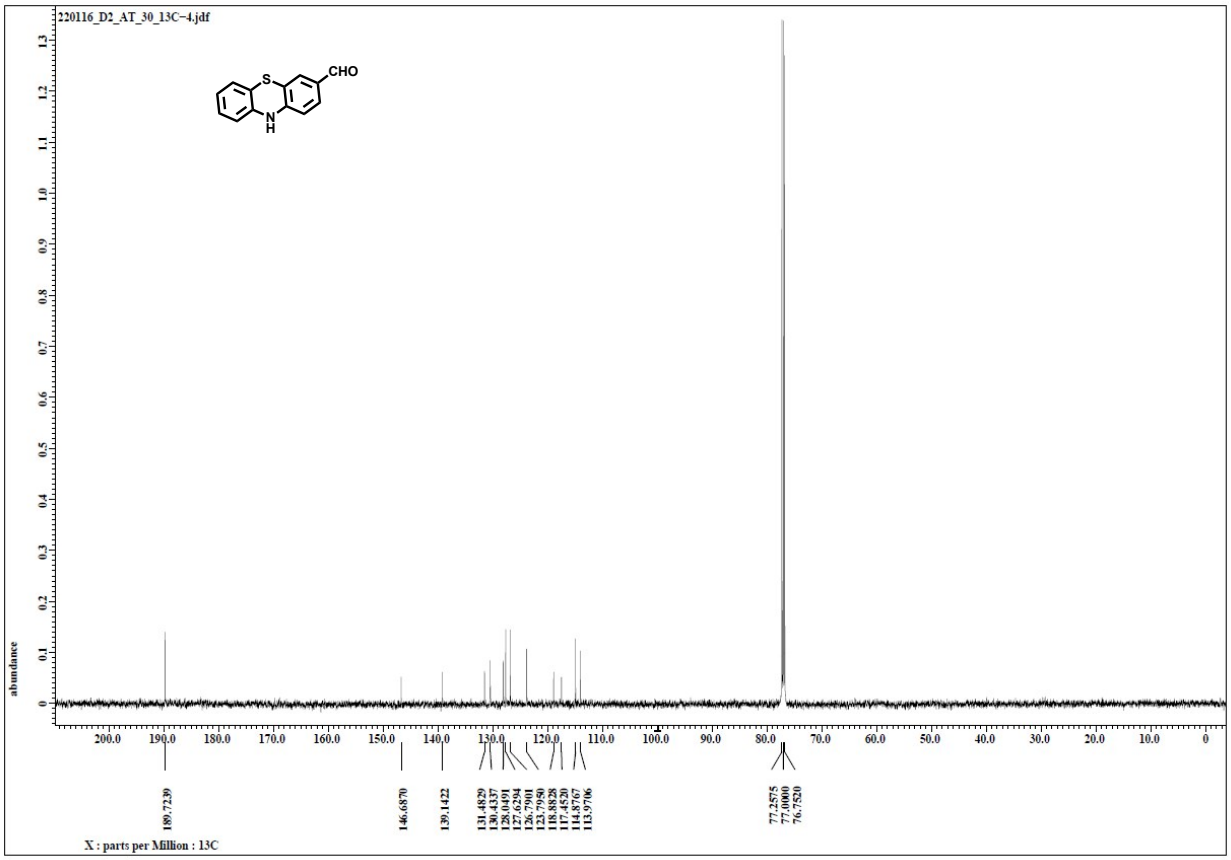


Fig. S21: ^{13}C spectra of 1d

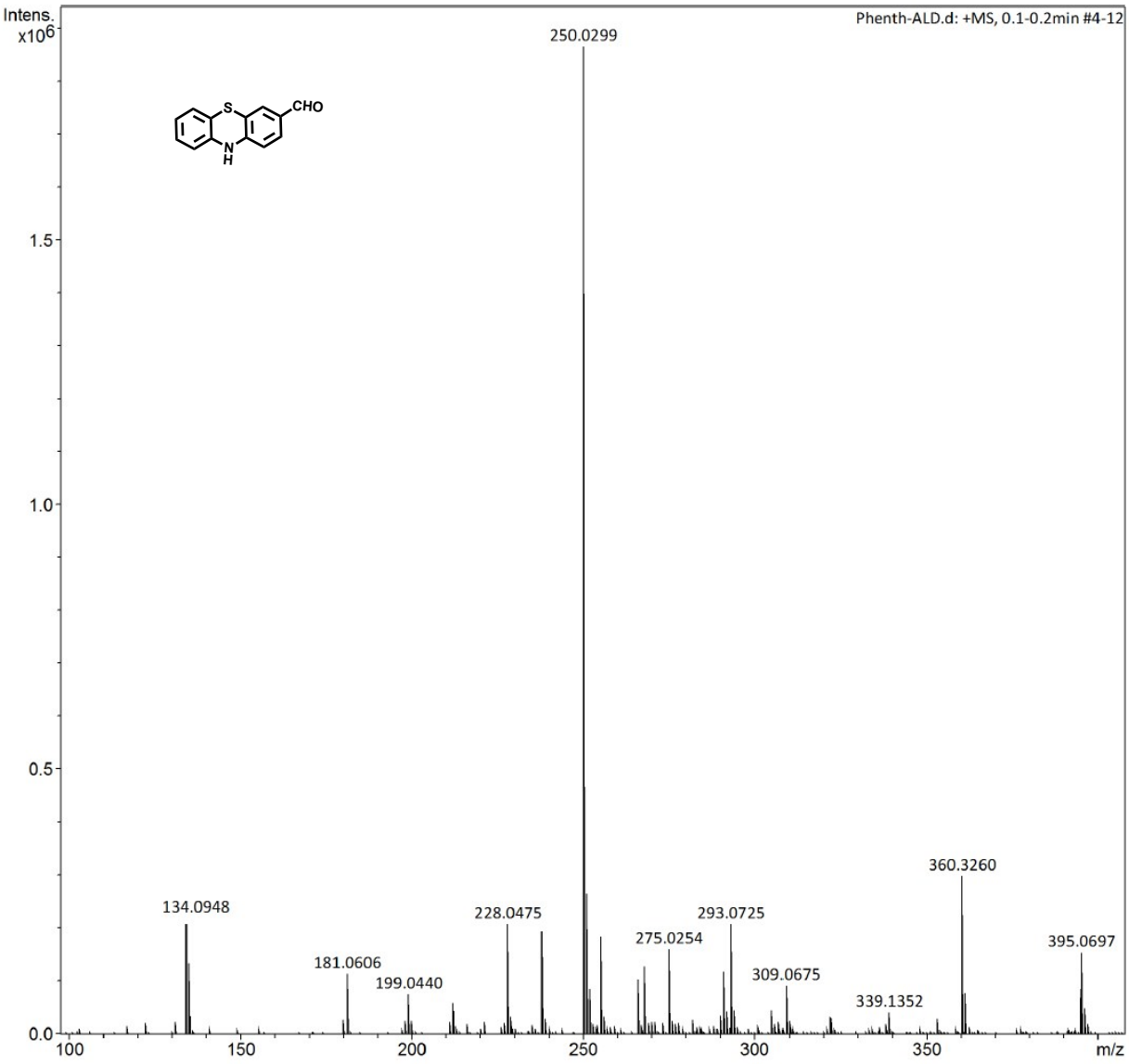


Fig. S22: HRMS spectra of 1d

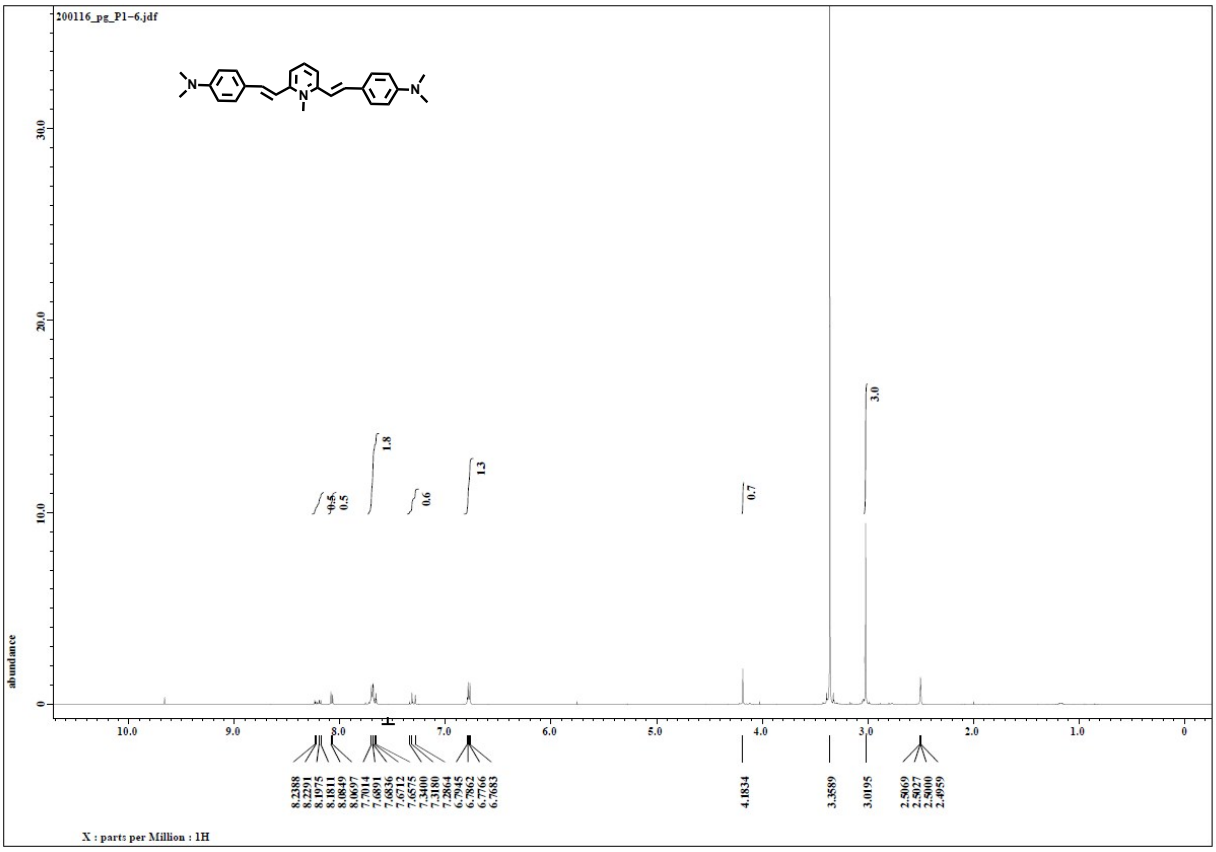


Fig. S23: ^{1}H-NMR spectra of P1

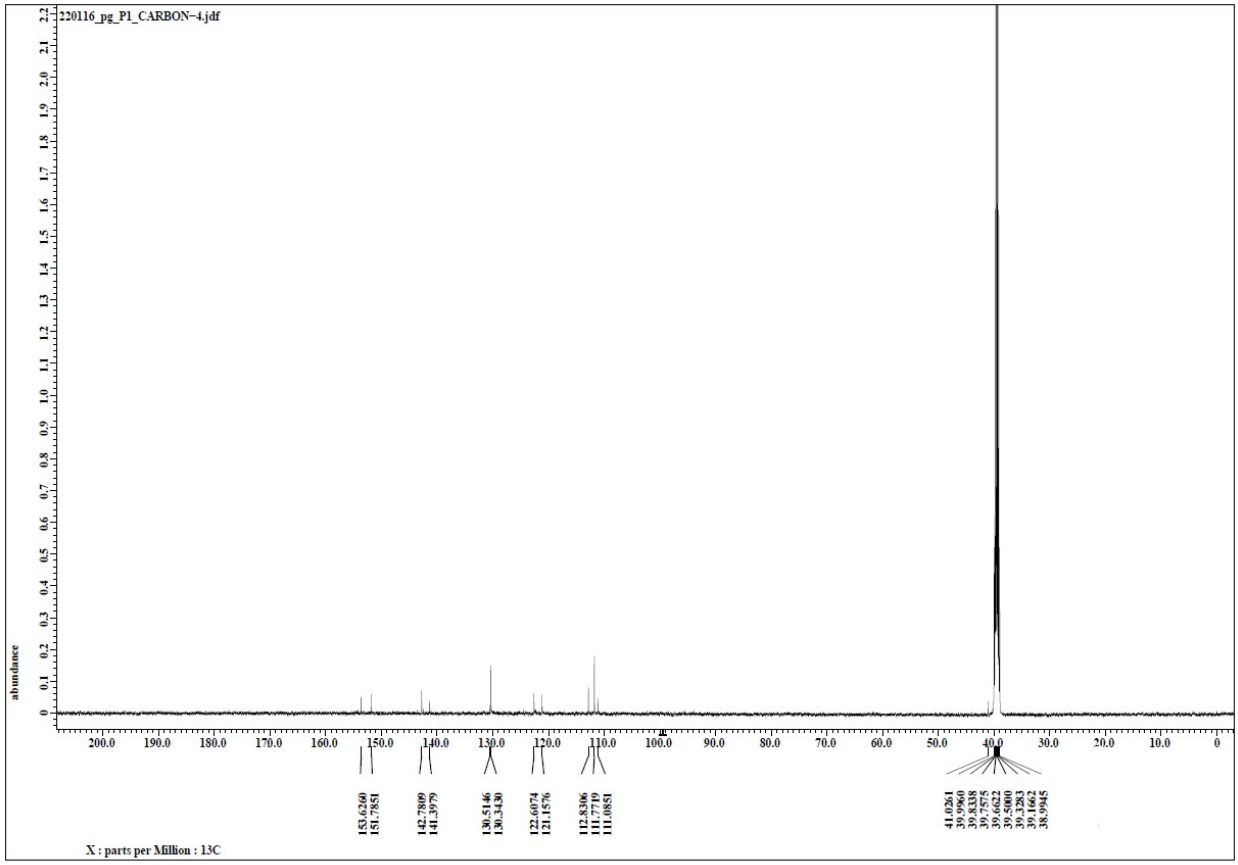


Fig. S24: ^{13}C spectra of P1

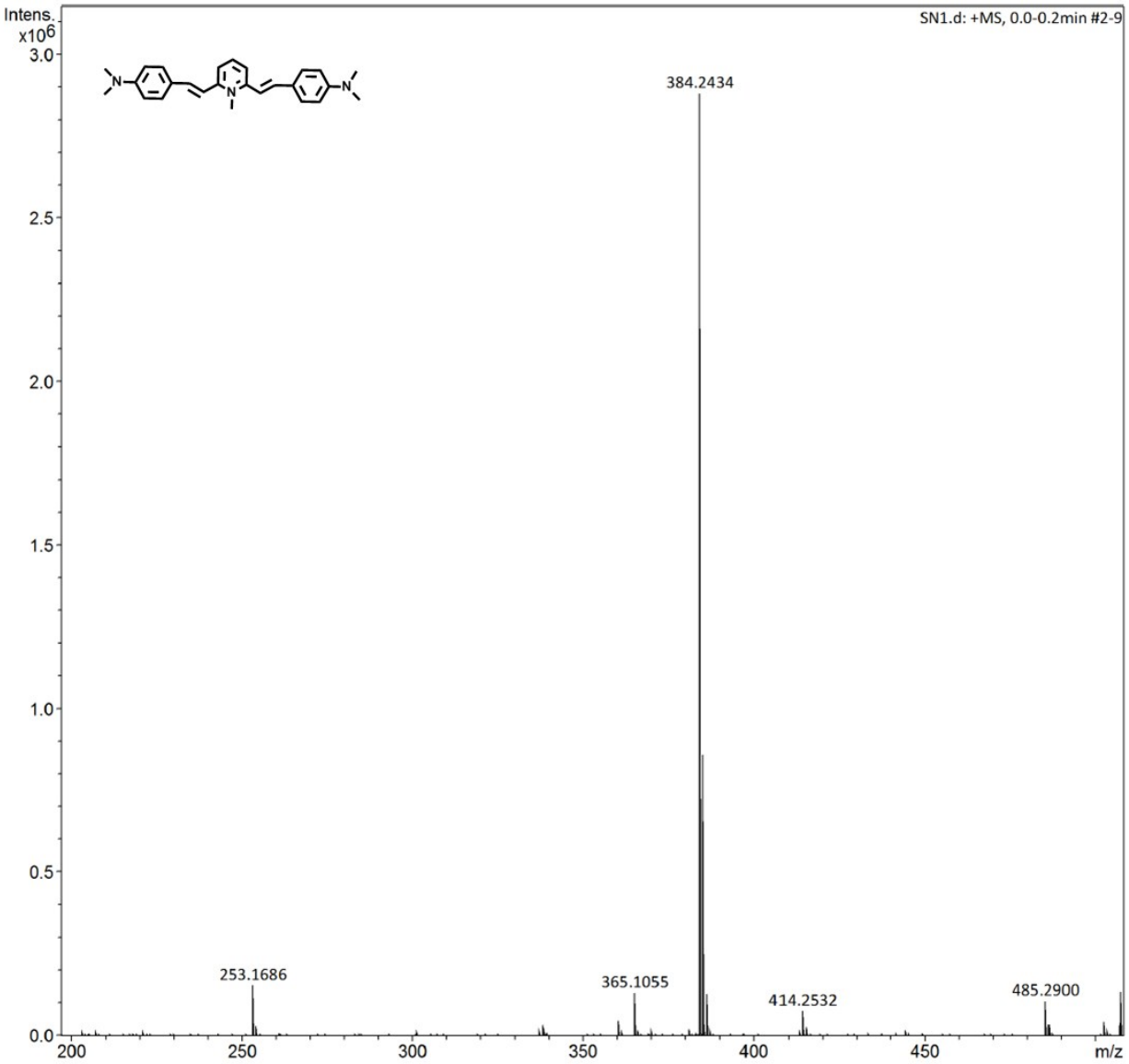


Fig. S25: HRMS spectra of P1

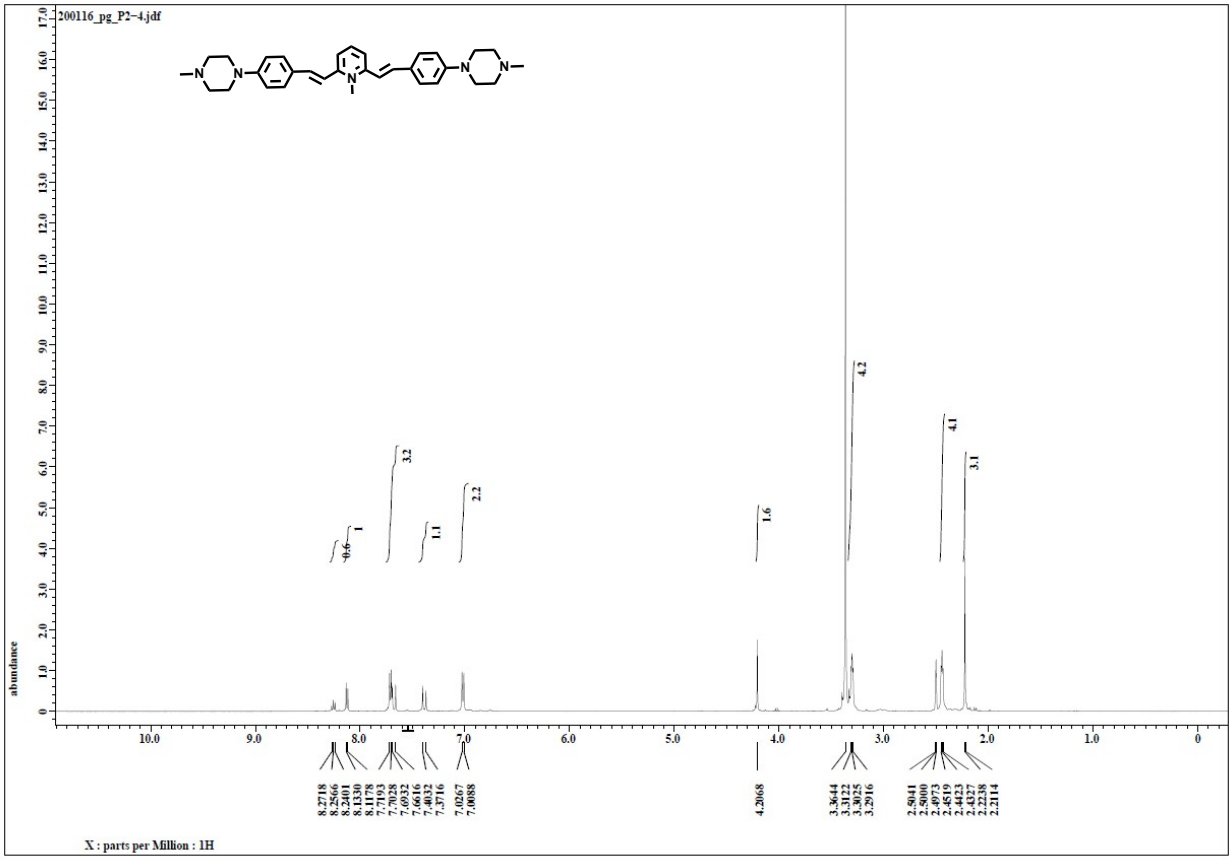


Fig. S26: ^{1}H-NMR spectra of P2

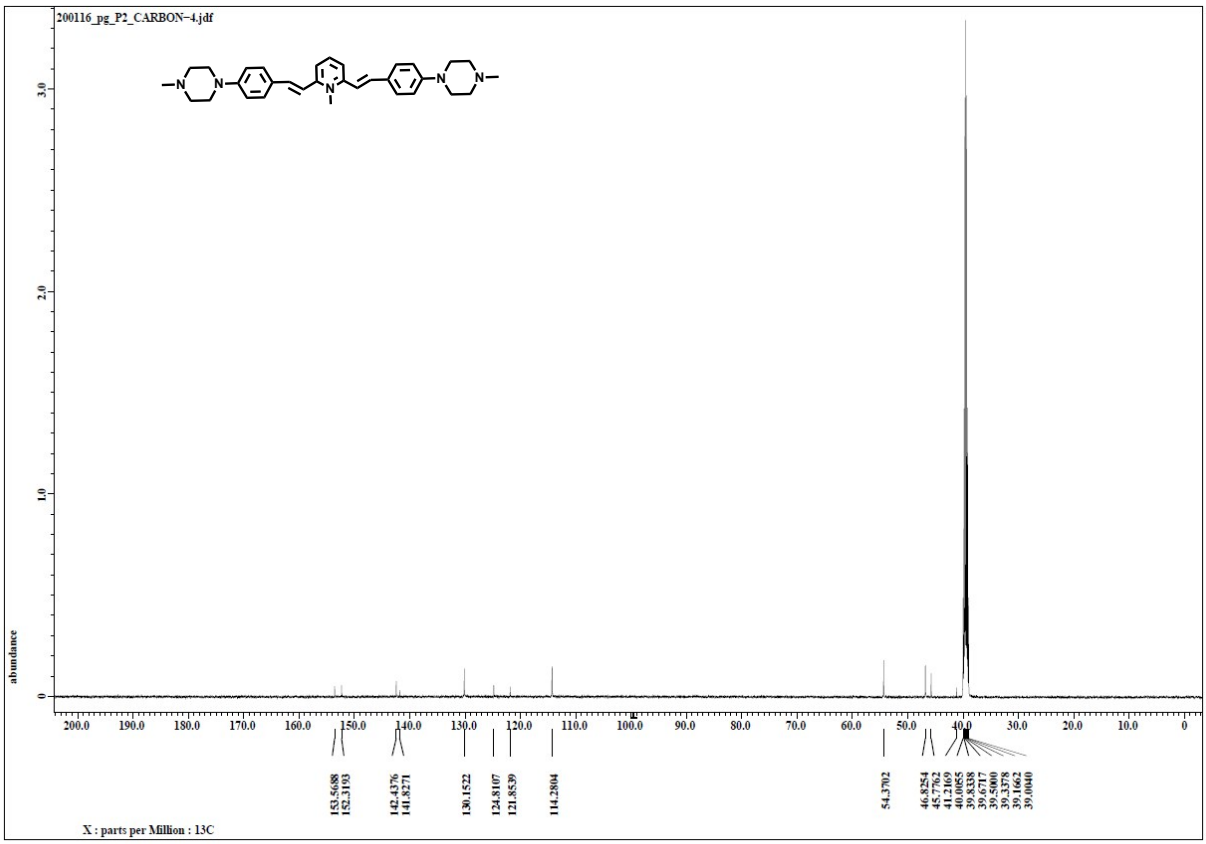


Fig. S27: ^{13}C spectra of P2

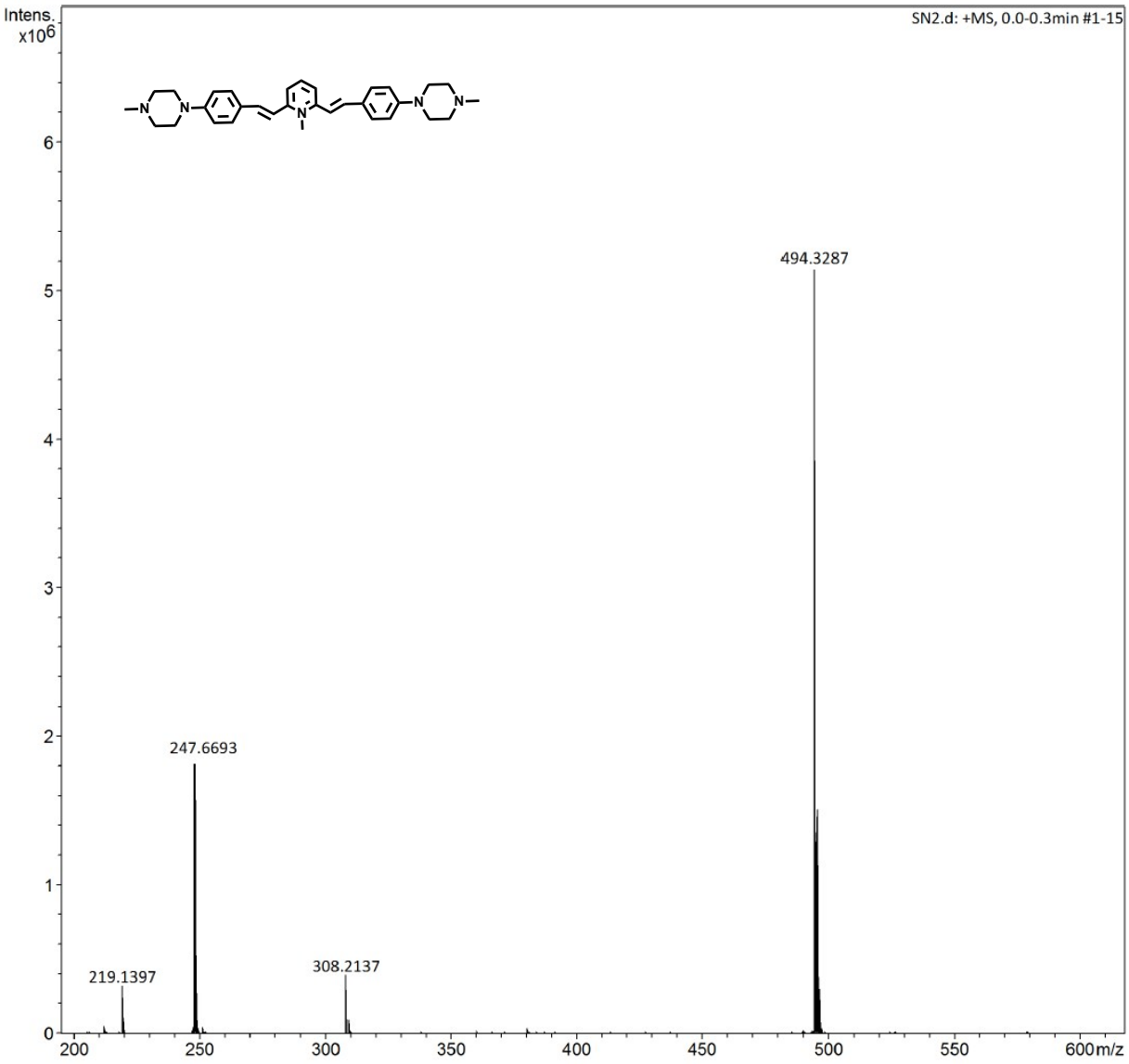


Fig. S28: HRMS spectra of P2

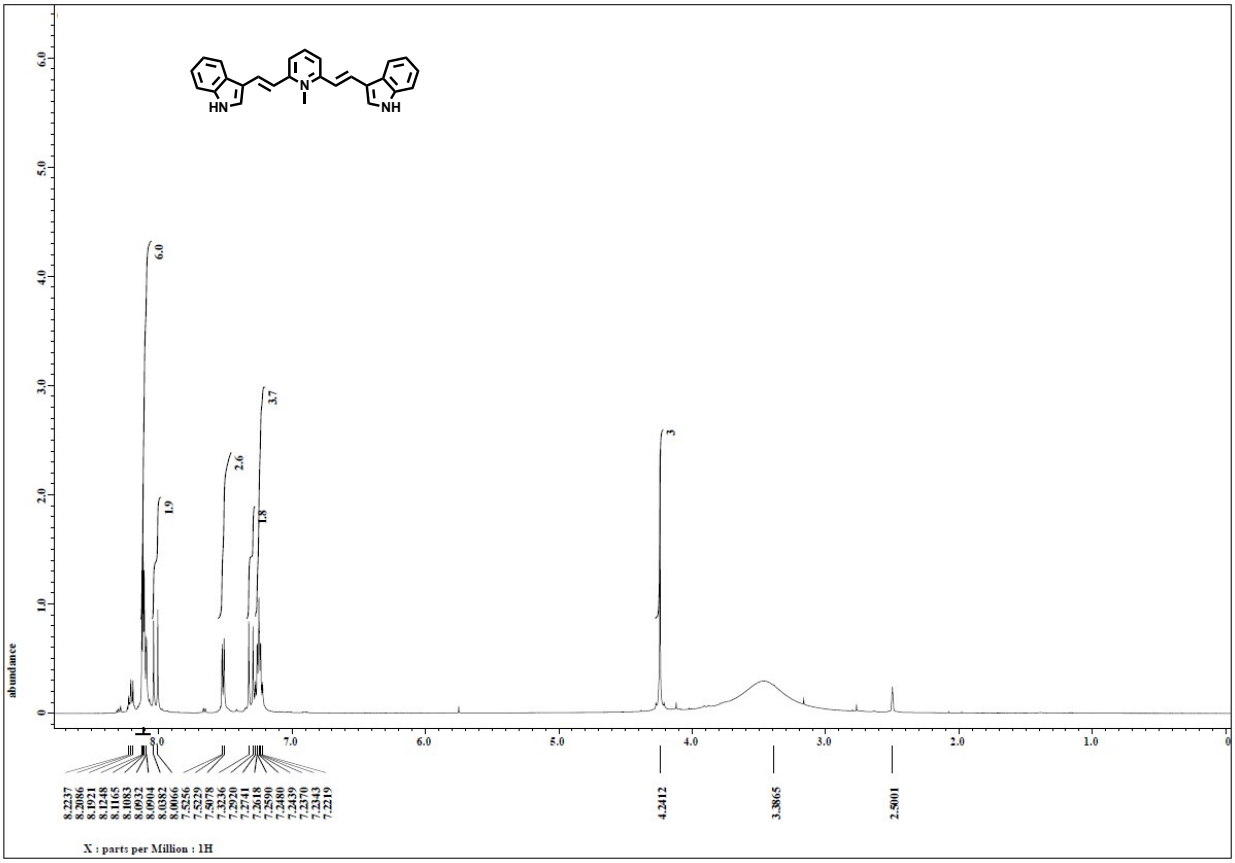


Fig. S29: ^{1}H-NMR spectra of P3

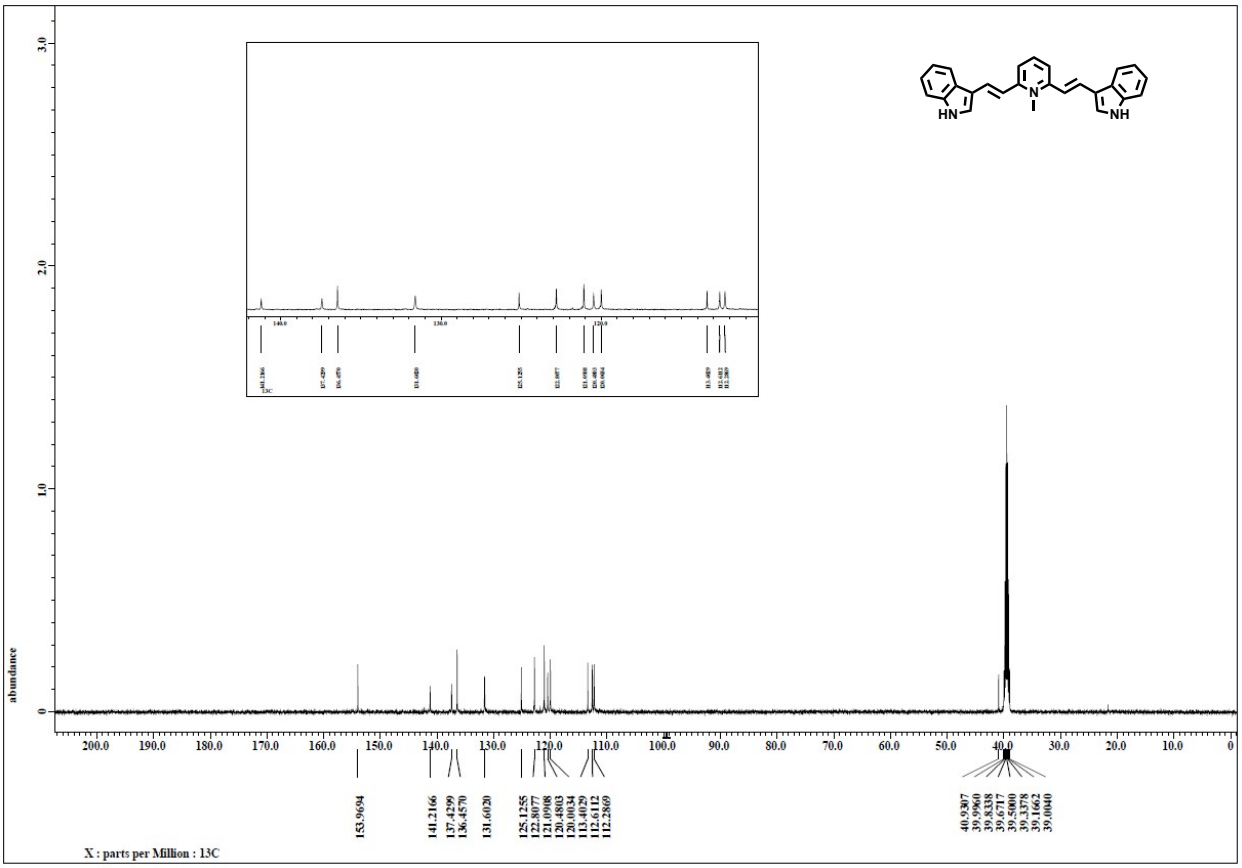


Fig. S30: ^{13}C spectra of P3

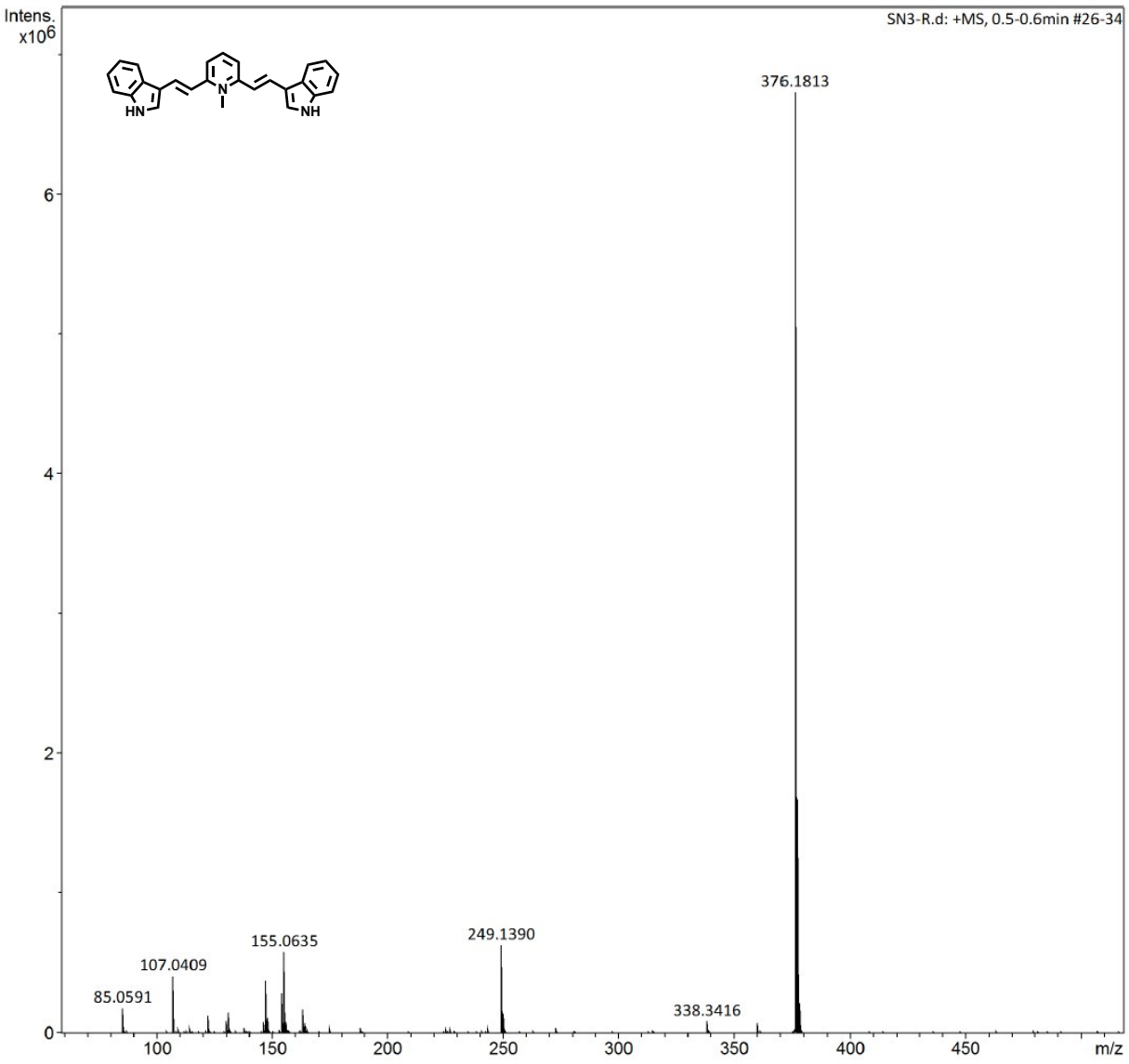


Fig. S31: HRMS spectra of P3

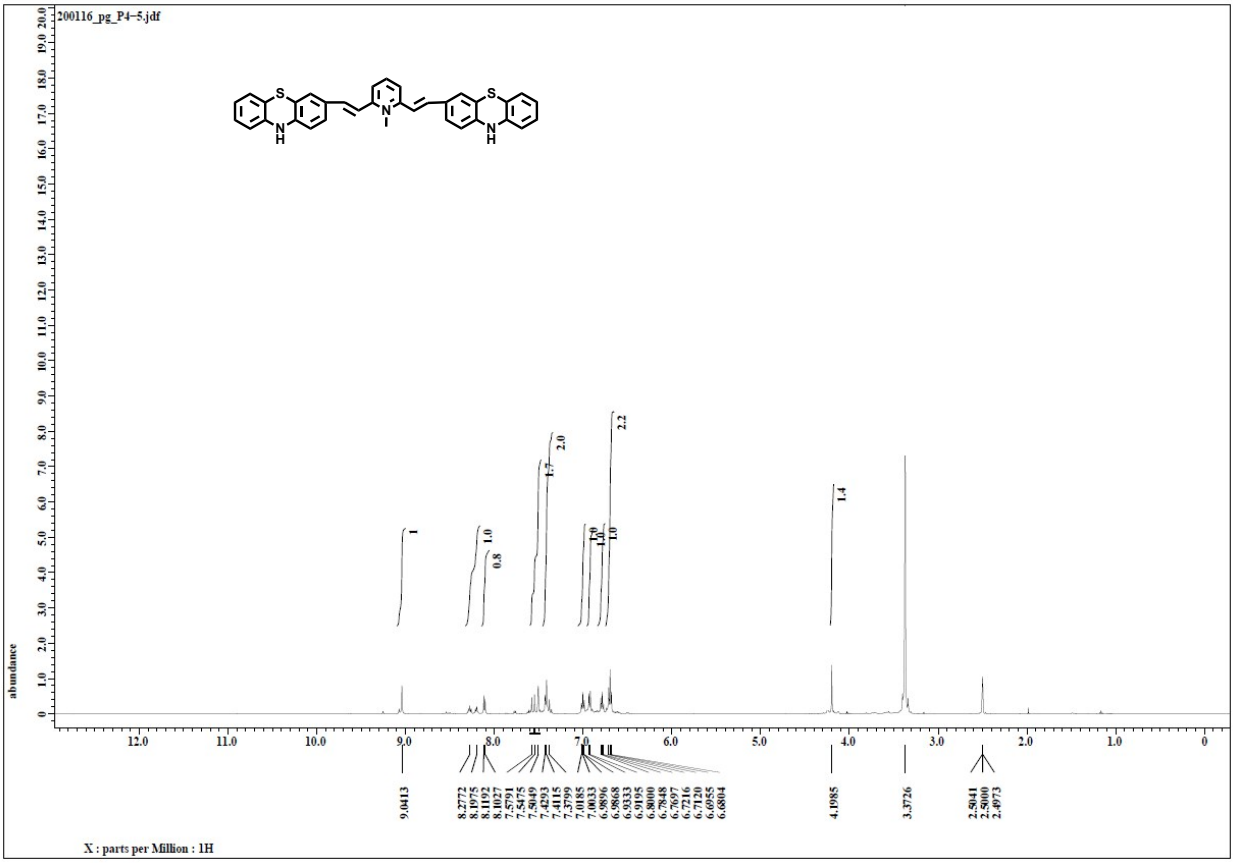


Fig. S32: ^{1}H-NMR spectra of P4

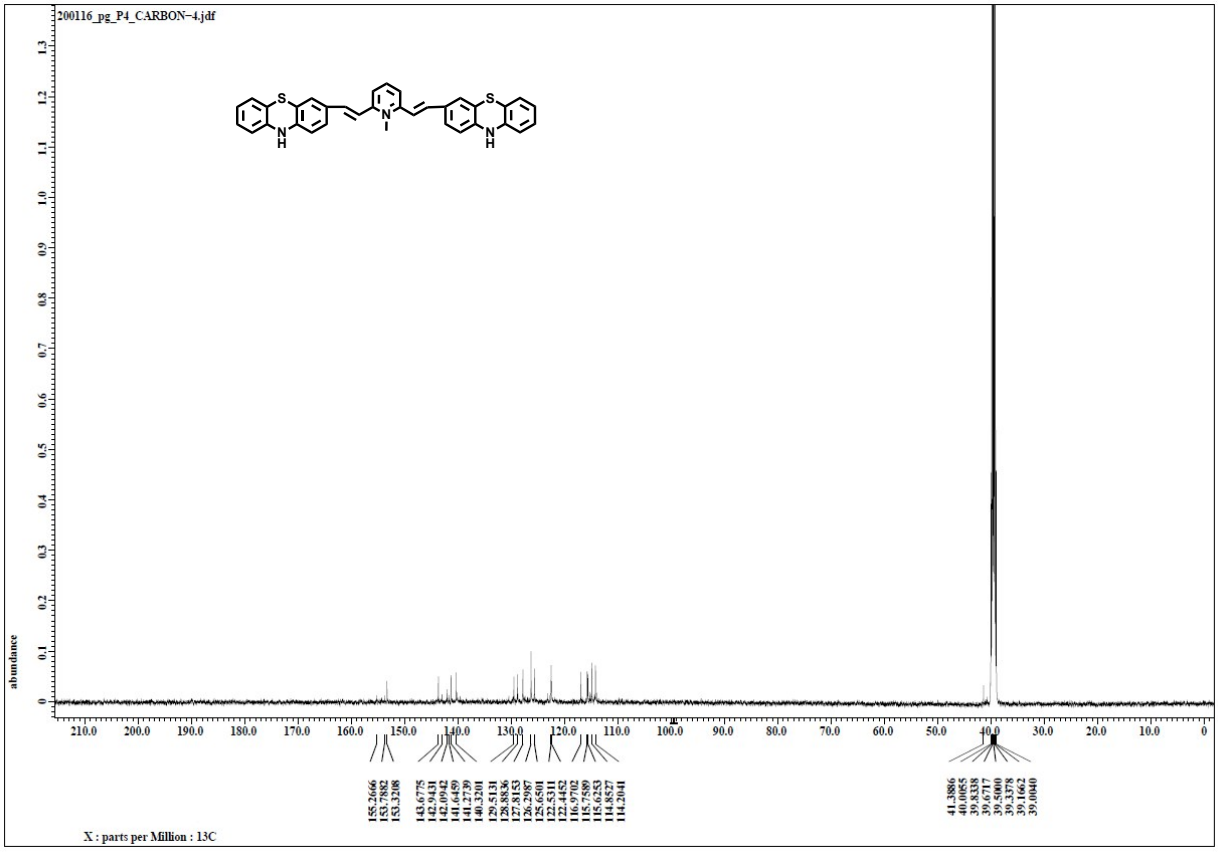


Fig. S33: ^{13}C spectra of P4

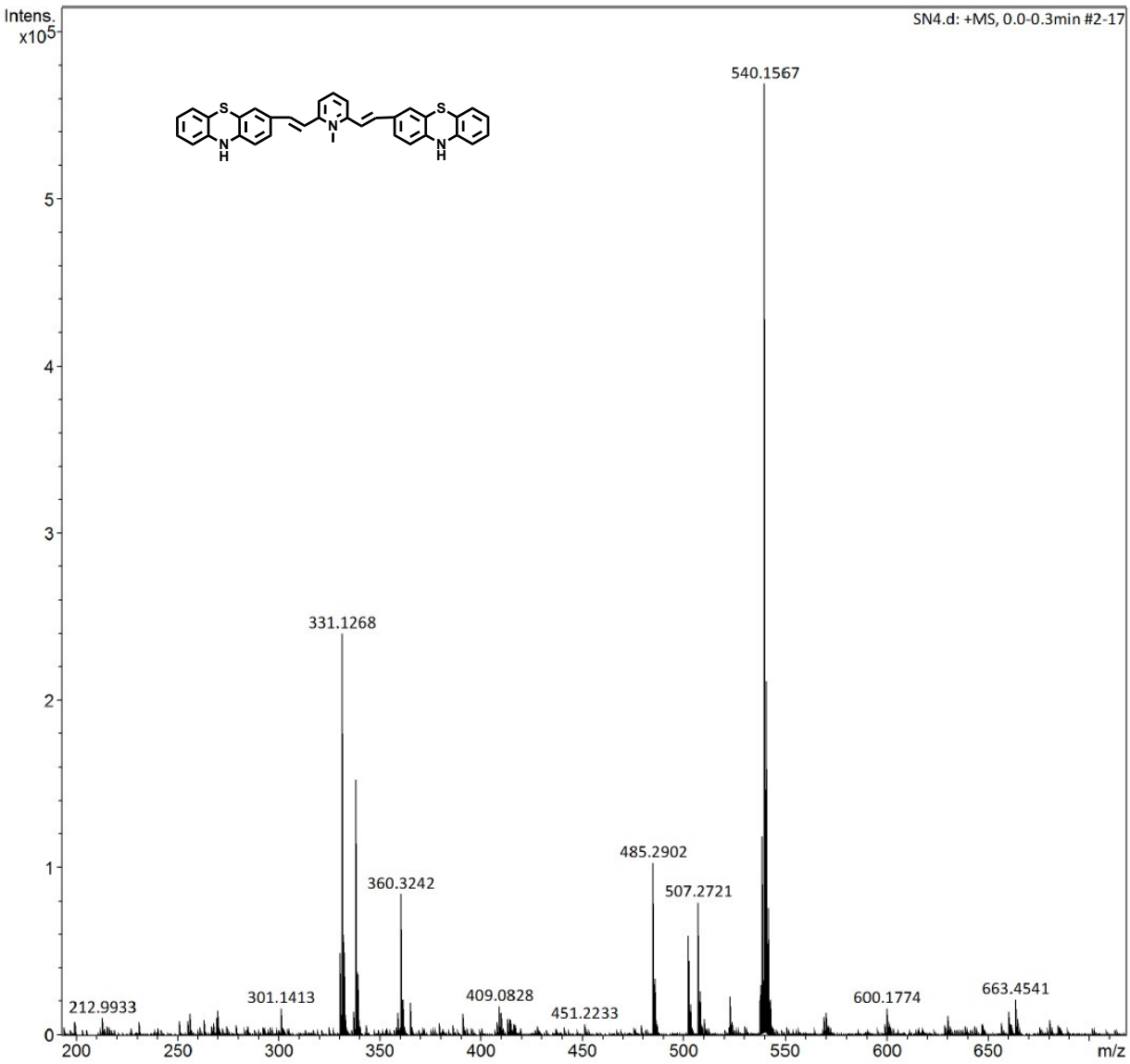


Fig. S34: HRMS spectra of P4

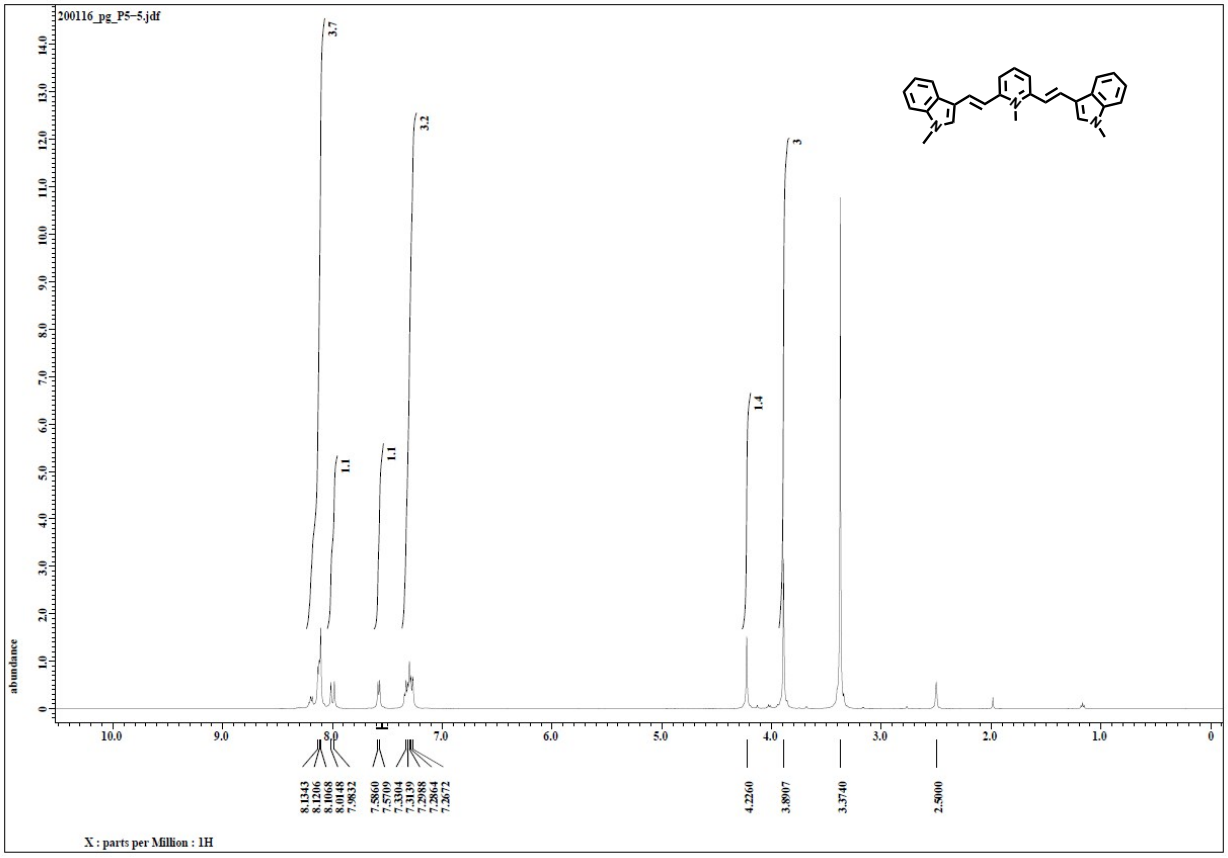


Fig. S35: ^{1}H-NMR spectra of P5

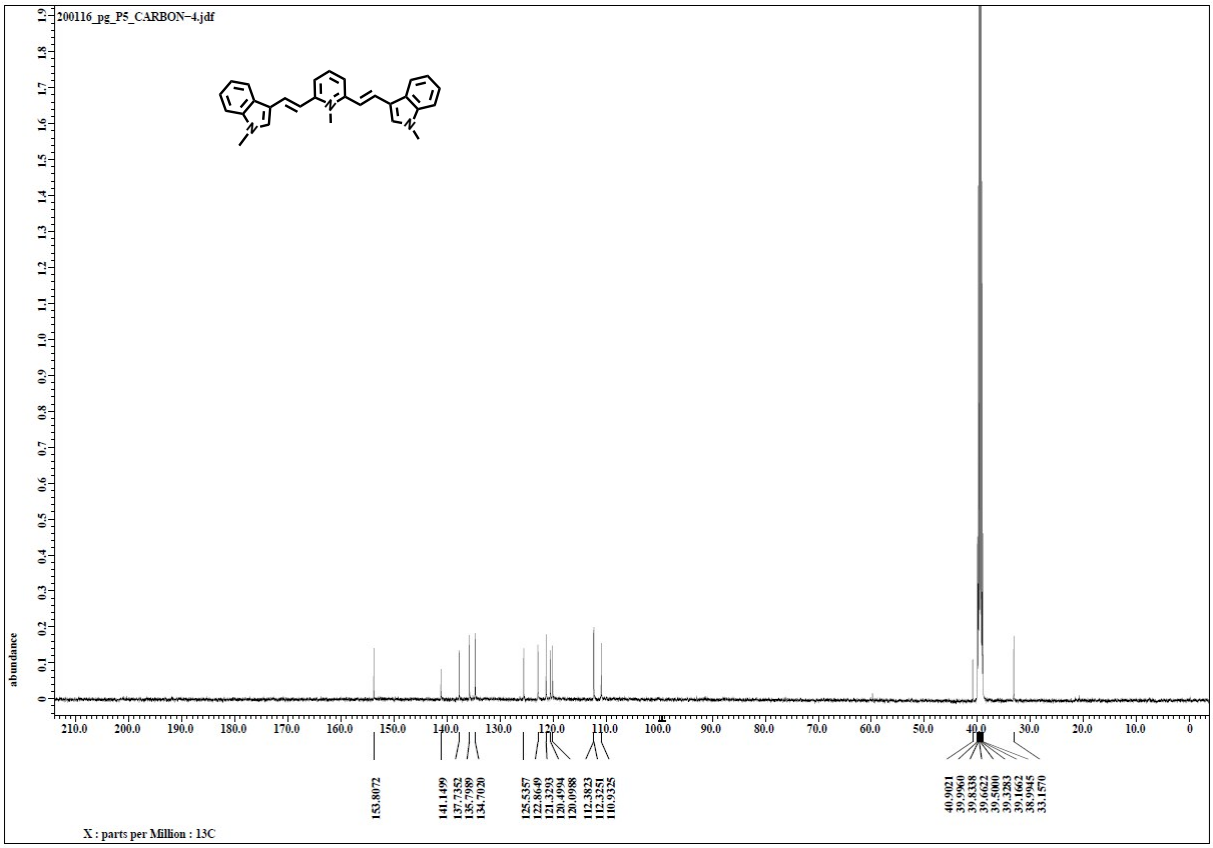


Fig. S36: ^{13}C spectra of P5

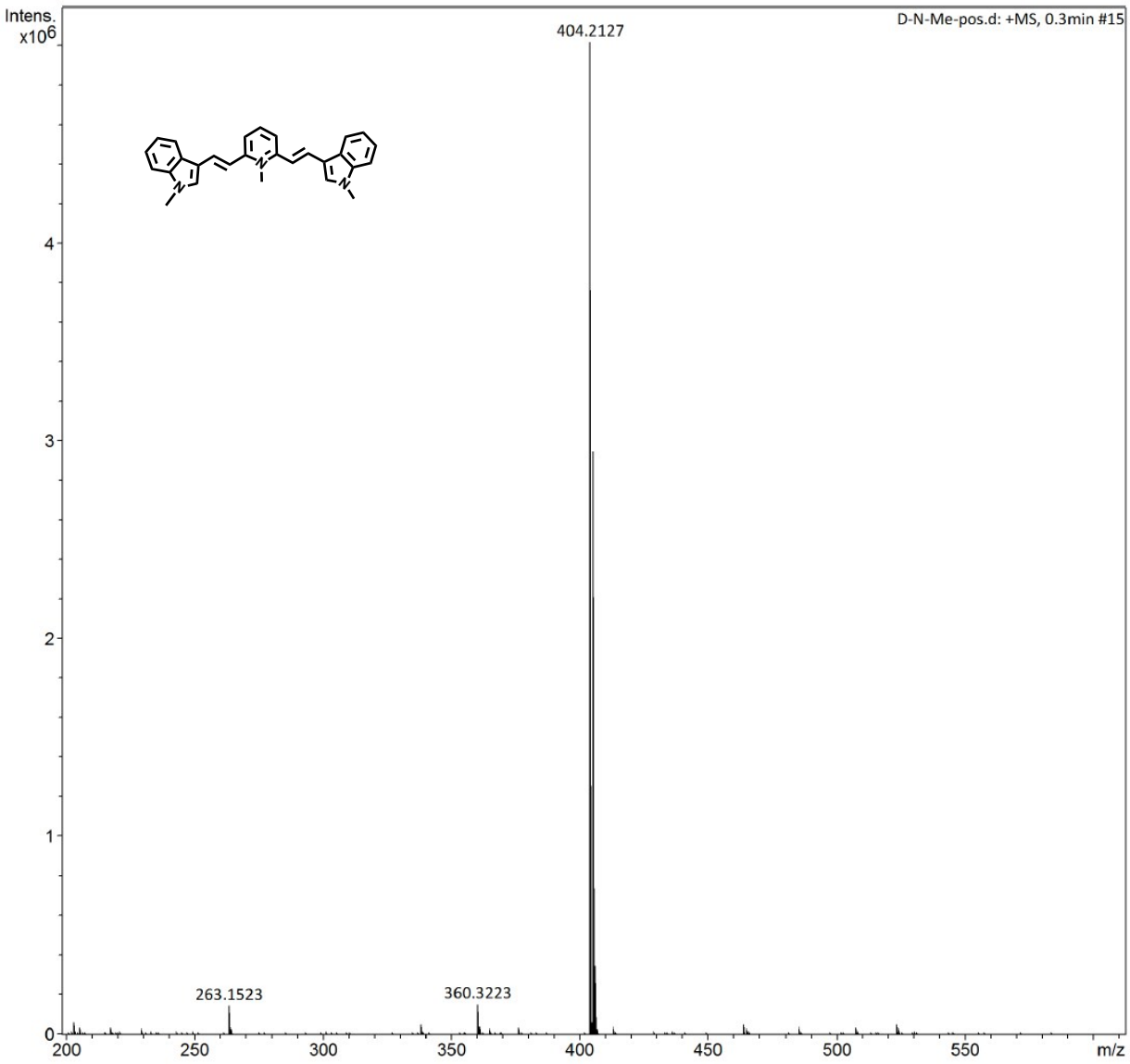


Fig. S37: HRMS spectra of P5

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