Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Optimising Molecular Rotors to AIE Fluorophores for Enhanced Mitochondria Uptake and Retention

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1. Materials and Methods

Materials

1-Bromohexane, 6-bromohexanoic acid, 4-bromophenyl acetonitrile, ethyl bromoacetate, iodomethane, 1,3-propanesultone, 4-pyridylacetonitrile hydrochloride, tricyclohexylphosphine tetrafluoroborate, tris(dibenzylideneacetone)dipalladium(0) were obtained from Sigma Aldrich. 4-pyridylboronic acid was obtained from Boron Molecular. Potassium hydroxide and potassium phosphate tribasic were obtained from Chem-supply. 4-Dimethylaminobenzaldehyde, potassium hexafluorophosphate, were obtained from AK Scientific. Anhydrous solvents were dried by molecular sieves (4Å). Hexanes refer to the fraction of boiling point range 40-60^{o}C. Flash chromatography was carried out on silica gel (Merck Kieselgel 60 (230 – 400 mesh)) under pressure of nitrogen. For biological experiments, materials were obtained from Sigma Aldrich or Thermo Scientific. MitoTracker^{TM} Deep Red were obtained from Thermofisher Scientific.

Synthesis Characterisation

^{1}H (400MHz or 500MHz) & ^{13}C NMR (100MHz, 125MHz) spectra were acquired on Agilent MR400 or Agilent DD2 instrument. The chemical shift data for each signal are given as δ. High-resolution mass spectra were acquired using a Thermo Scientific Q Exactive Plus Orbitrap LC-MS/MS instrument.

X-ray Diffraction Experiments

Single crystals of **ASC** and **ASCP** for crystal structure determination were grown by using DMF and diethylether as a co-solvent. X-ray diffraction intensity data for **ASCP** and **ASC** were collected with a Rigaku Synergy Diffractometer using either Cu- Kα or Mo- Kα radiation with the temperature during data collection maintained at 100.0(1)K using an Oxford Cryosystems cooling device. The structures were solved by direct methods and difference Fourier synthesis.[1] Thermal ellipsoid plots were generated using the program Mercury integrated within the WINGX suite of programs.[2-3] Experimental electrostatic potential data for **ASC** acquired from measured X-ray crystallography data through LS refinement of scale, xyz and Ui,j against high order reflections (d < 0.8Å). Charge density parameters were refined using CGLS against the full dataset (with appropriate constraints and restraints). Charge density parameters were then deconstrained and refined against the full dataset. Scale, xyz and Ui,j parameters were then LS refined against the full dataset until convergence. Visualisation of electrostatic potential were done using MoPro software.[4-6] For solid state powder diffraction experiments, upon synthetic characterisation, **ASC** and **ASCP**, were subjected to grinding and were measured as slurries in oil at 100K on a Rigaku Synergy Single Crystal Diffractometer using the Gadolfi method for powders incorporated into the Crysalis-Pro software.

Photophysical Characterisation

Absorbance and fluorescence spectra were obtained on a Cary 300 UV-Vis spectrometer and Cary Eclipse fluorimeter (Agilent Technologies Inc., Santa Clara, CA, USA), respectively. Data were plotted using Origin 2018 (OriginLab Corp., Northampton, MA, USA). Absolute photoluminescence quantum yield measurements were acquired by using an integrating sphere (F3018, Horiba Jobin Yvon) on a Jobin Yvon FluorologOR-3 fluorimeter. All spectra for the absolute quantum yield measurements were corrected for the light source noise, wavelength sensitivity and the transmittance of filters. 488 nm excitation wavelength was used for all measurements. 10μM dye concentration was used for solution state quantum yield measurements. 2 mM dye concentration in 2 wt% PMMA acetone solutions were spin-coated on glass substrate (600 rpm, 1min) for dye-PMMA film fabrication. For solid state fluorescence spectra measurements upon synthetic characterisation, **ASC** and **ASCP** were subjected

to grinding and the crystalline samples were sandwiched between two quartz slides. Measurements were carried out on a Jobin Yvon FluorologOR-3 fluorometer. 488 nm was used as excitation wavelength for all measurement.

Dye Aggregation Characterisation

Stock solution (1 mM, in DMSO) of ASC or ASCP was added to the co-solvent system with desired volume ratio. After dilution, the mixtures were then thoroughly mixed and immediately transferred to a 1cm quartz cuvette. Particle size distributions were measured in triplicate on Zetasizer Nano ZEN3600 (Malvern Instruments Ltd, Malvern, WR14 1XZ, United Kingdom). 10μM dye concentration was used for all measurements.

DFT Calculations

DFT calculations were carried out using B3LYP/ 6-31g* with Gaussian16, using the University of Melbourne HPC system, Spartan, for all calculations. Visualisation of structures were done using Avogadro 1.20[7] and GaussView 5.0. No negative frequencies for optimised structures were observed.

Cell Culture

A549 cells (Human Lung Carcinoma Epithelial Cells) were kindly provide by Prof Weisan Chen, La Trobe University. The cells were cultured in DMEM (Life Technologies, Catalog Number: 11965118) supplemented with 10% fetal bovine serum (Corning, Australia origin, Catolog Number: 35-076-CV) at 37°C in 5% CO_2 air with humidification.

Cell Viability Assay

2×10^4 cells were plated onto 96-well plates 24hr prior to dye application. CLARIOstar monochromator plate reader (BMG Labtech) in fluorescence intensity mode with excitation at 560/15nm and emission at 590/20nm was used to assess cell viability using Alamar Blue assay (Thermo Fisher Scientific, Catalog number: DAL1025).

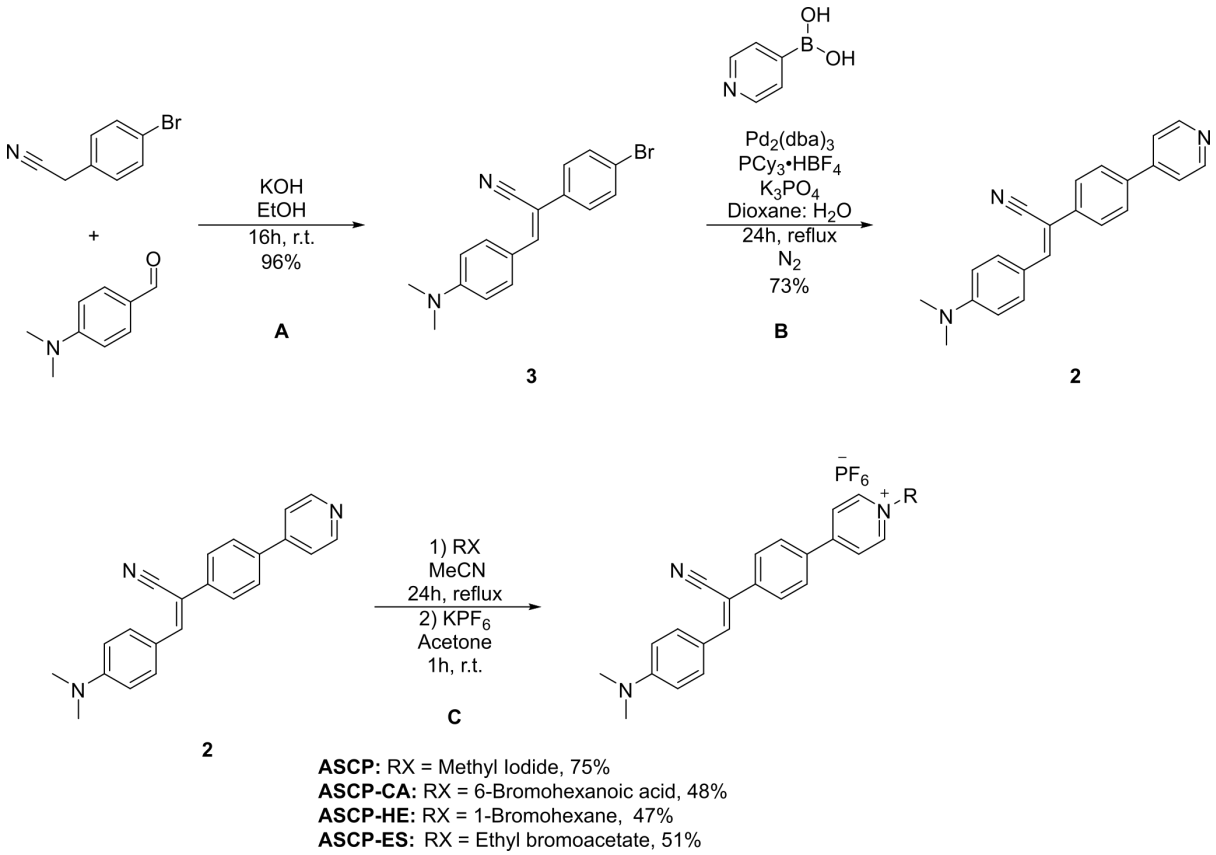
Cell Staining

All dyes were dissolved in DMSO as 5 mM, **ASCP-SO** was dissolved in DMSO as 2 mM stock. Stock solution of dyes were kept at -20 °C in the dark. A549 cells (1.5×10^4) were plated either on an ibidi μ-Slide 8 Well, ibiTreat (ibidi, Catalog number: 80826-90) for fixed cell imaging or 15 mm glass bottom culture dishes (Nest, Catalog number: 801002) for live cell imaging. Plated cells were treated with freshly diluted dye (5μM) for 30min at 37°C and subsequently washed. For co-staining experiments, cells were further incubated in a **MITO DR** solution (500nM) for 20min at 37°C and subsequently washed. For fixed cell imaging, cells were fixed on plate with 4% (w/v) paraformaldehyde (PFA) in PBS for 15min at room temperature. For live cell imaging, cells were imaged at 37°C and 5% CO_2 air.

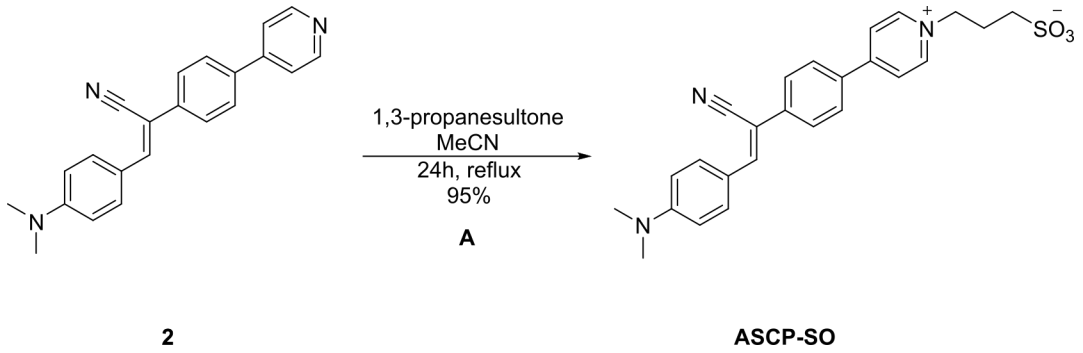
Confocal Laser Scanning Microscopy

After staining, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15min at room temperature. For live cell imaging, cells were imaged at 37 °C and 5% CO_2 air. Images were acquired on a Zeiss LSM 780 microscope using a 63× objective lens. For image acquisition, the pixel frame size was set at 1024×1024 or 512×512 and the pixel dwell time was 25.4 μs or 50.4 μs. For **ASCP** and derivatives (excitation: 488 nm; emission: 615 – 650 nm), **ASC** (excitation: 488 nm; emission 520 – 560 nm) and Mitotracker™ Deep Red (excitation: 633 nm; emission 660 – 700nm).

2. Synthesis Procedures of Compounds:



Scheme S1 Synthetic scheme for probes **ASCP**. **A** Knoevenagel Condensation, **B** Palladium catalysed Suzuki cross-coupling, **C** Alkylation and anion conversion of halide to PF_6^-.



Scheme S2 Synthetic scheme for **ASCP-SO**. **A** Alkylation using 1,3-propanesultone to make **ASCP-SO**.

(Z)-2-(4-bromophenyl)-3-(4-(dimethylamino)phenyl)acrylonitrile (3): 4-Bromophenylacetonitrile (8.77 g, 45.0 mmol) and 4-dimethylaminobenzaldehyde (7.45 g, 50.0 mmol) was added with potassium hydroxide (3.40 g, 55.0 mmol) in 100 mL of ethanol and stirred at room temperature for 16 h. Upon completion of reaction, the yellow precipitates were filtered, washed with ethanol and dried under reduced pressure to yield the product as a yellow coloured solid in 96% yield (14.1 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 9.0 Hz, 2H), 7.50 (q, *J* = 8.8 Hz, 4H), 7.37 (s, 1H), 6.71 (d, *J* = 8.9 Hz, 2H), 3.06 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.79, 142.79, 134.58, 131.93, 131.40, 126.87, 121.79,

121.23, 119.08, 111.56, 103.13, 40.00. HRMS (ESI+): *m*/*z*. 327.04925 [$C_{17}H_{16}N_2Br$ $(M+H)^+$, calcd 327.04914].

(Z)-3-(4-(dimethylamino)phenyl)-2-(4-(pyridin-4-yl)phenyl)acrylonitrile (2): 3 (8.15 g, 25 mmol) was added to 4-pyridineboronic acid (3.38 g, 27.5 mmol), $Pd_2(dba)_3$ (220 mg, 0.25 mmol), $P(Cy)_3{\cdot}HBF_4$ (221 mg, 0.60 mmol), K_3PO_4 (13.3 g, 62.5 mmol) and placed under N_2. An N_2 sparged solution of dioxane (70 mL) and H_2O (34 mL) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion, the reaction mixture was cooled and extracted with DCM (3 x) and washed with water (3 x). The organic layer was then dried over magnesium sulfate, filtered and concentrated. The crude product was then purified by column chromatography (99:1 to 9:1 DCM: EtOAc) to obtain the pure product as an orange solid in 73% yield (5.92 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, *J* = 6.0 Hz, 2H), 7.89 (d, *J* = 8.9 Hz, 2H), 7.76 – 7.68 (m, 4H), 7.53 (d, *J* = 6.0 Hz, 2H), 7.48 (s, 1H), 6.74 (d, *J* = 8.9 Hz, 2H), 3.08 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.86, 150.36, 147.31, 142.96, 137.38, 136.42, 131.52, 127.40, 126.02, 121.32, 119.24, 111.61, 103.44, 40.04. HRMS (ESI+): *m*/*z*. 327.16637 [$C_{22}H_{20}N_3$ $(M+H)^+$, calcd 326.16517].

(ASCP): Compound **2** (200 mg, 0.614 mmol) was added to MeCN (10 mL). Iodomethane (131 mg, 0.912 mmol) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature, precipitated with Et_2O, filtered and washed with Et_2O. The precipitate was then redissolved in acetone (20 mL) and saturated KPF_6 aqueous solution (20 mL) and stirred for 1 h. Acetone was then removed and the precipitate was filtered and washed with water to obtain the pure product as a red solid in 75% yield (224 mg). ^{1}H NMR (400 MHz, DMSO) δ 9.00 (d, *J* = 6.7 Hz, 2H), 8.54 (d, *J* = 6.7 Hz, 2H), 8.19 (d, *J* = 8.6 Hz, 2H), 8.06 (s, 1H), 7.96 – 7.92 (m, 4H), 6.85 (d, *J* = 9.0 Hz, 2H), 4.32 (s, 1H), 3.06 (s, 3H), 2.50 (s, 6H). ^{13}C NMR (100 MHz, DMSO) δ 153.59, 152.59, 145.99, 145.07, 139.02, 132.66, 132.19, 131.06, 129.37, 129.18, 127.49, 126.24, 124.61, 124.14, 121.84, 120.96, 119.48, 112.09, 101.00, 56.84, 47.46, 40.04. HRMS (ESI+): *m*/*z*. 340.18026 [$C_{23}H_{22}N$ $(M-PF_6)^+$, calcd 340.18082].

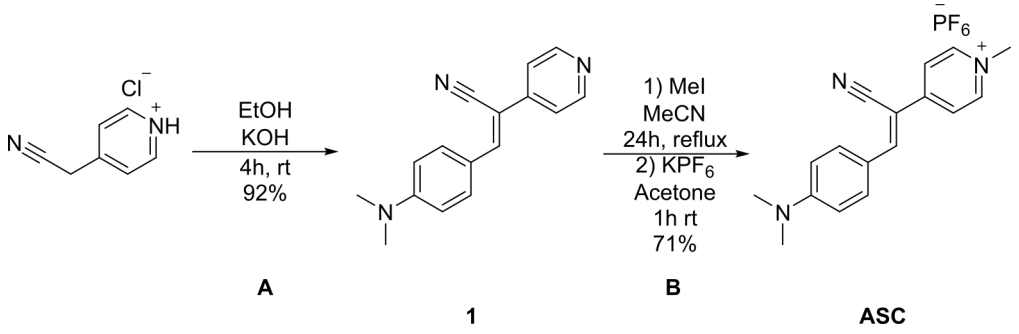
(ASCP-CA): Compound **2** (100 mg, 0.307 mmol) was added to MeCN (5 mL). 6-bromohexanoic acid (599 mg, 3.07 mmol) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature, precipitated with Et_2O, filtered and washed with Et_2O. The precipitate was then redissolved in acetone (10 mL) and saturated KPF_6 aqueous solution (10 mL) and stirred for 1 h. Acetone was then removed and the precipitate was filtered and washed with water to obtain the pure product as a red solid in 48% yield (92.3 mg). ^{1}H NMR (400 MHz, DMSO) δ 12.02 (s, 1H), 9.08 (d, *J* = 6.6 Hz, 2H), 8.55 (d, *J* = 6.6 Hz, 2H), 8.19 (d, *J* = 8.5 Hz, 2H), 8.04 (s, 1H), 7.94 – 7.89 (m, 4H), 6.83 (d, *J* = 8.9 Hz, 2H), 4.55 (t, *J* = 7.3 Hz, 2H), 3.04 (s, 6H), 2.22 (t, *J* = 7.2 Hz, 2H), 2.01 – 1.86 (m, 2H), 1.65 – 1.48 (m, 2H), 1.34 – 1.29 (m, 2H). ^{13}C NMR (100 MHz, DMSO) δ 174.75, 153.96, 152.60, 150.73, 145.14, 145.09, 139.06, 132.66, 132.20, 131.81, 129.27, 127.84, 126.22, 126.06, 124.53, 120.96, 119.47, 112.09, 100.99, 60.11, 33.75, 30.80, 25.39, 24.24. HRMS (ESI+): *m*/*z*. 440.23345 [$C_{28}H_{30}N_3O_2$ $(M-KPF_6)^+$, calcd 440.23325].

(ASCP-HE): Compound **2** (100 mg, 0.307 mmol) was added to MeCN (5 mL). 1-bromohexane (507 mg, 3.07 mmol) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature, precipitated with Et_2O, filtered and washed with Et_2O. The precipitate was then redissolved in acetone (10 mL) and saturated KPF_6 aqueous solution (10 mL) and stirred for 1 h. Acetone was then removed and the precipitate was filtered and washed with water to obtain the pure product as a red solid in 47% yield (80.1 mg). ^{1}H NMR (400 MHz, CD_3CN) δ 8.66 (d, *J* = 6.6 Hz, 2H), 8.29 (d, *J* = 6.6 Hz, 2H), 8.02 (d, *J* = 8.5 Hz, 2H), 7.97 – 7.91 (m, 4H), 7.81 (s, 1H), 6.85 (d, *J* = 8.9 Hz, 2H), 4.50 (t, *J* = 7.5 Hz, 1H), 3.09 (s, 6H), 2.01 – 2.00 (m, 2H), 1.45 – 1.29 (m, 6H), 0.93 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CD_3CN) δ 155.21, 152.55, 144.65, 144.18, 139.59, 132.55, 131.79, 128.72, 126.16, 124.69, 120.86, 118.91, 111.65, 101.63, 60.93, 39.27, 30.75, 25.26, 22.08, 13.20. HRMS (ESI+): *m*/*z*. 410.25917 [$C_{28}H_{32}N_3$ $(M-PF_6)^+$, calcd 410.25907].

(ASCP-ES): Compound **2** (100 mg, 0.307 mmol) was added to MeCN (5 mL). ethyl bromoacetate (512 mg, 3.07 mmol) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature, precipitated with Et_2O, filtered and washed with Et_2O. The precipitate was then redissolved in acetone (10 mL) and saturated KPF_6 aqueous solution (10 mL) and stirred for 1 h. Acetone was then removed and the precipitate was filtered and washed with water to obtain the pure product as a red solid in 51% yield (86.8 mg). ^{1}H NMR (400 MHz, DMSO) δ 9.02 (d, *J* = 6.7 Hz, 2H), 8.64 (d, *J* = 6.7 Hz, 2H), 8.21 (d, *J* = 8.5 Hz, 2H), 8.06 (s, 1H), 7.94 (d, *J* = 8.5 Hz, 4H), 6.84 (d, *J* = 9.0 Hz, 2H), 5.60 (s, 2H), 4.25 (q, *J* = 7.1 Hz, 2H), 3.04 (s, 6H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 166.98, 155.00, 152.63, 146.54, 145.24, 139.44, 132.44, 132.25, 129.46, 126.26, 124.21, 120.95, 119.46, 112.10, 100.91, 62.78, 59.89, 40.04, 14.42. HRMS (ESI+): *m*/*z*. 412.20206 [$C_{26}H_{26}N_3O_2$ $(M\text{-}PF_6)^+$, calcd 412.20195].

(ASCP-SO): Compound **2** (100 mg, 0.307 mmol) was added to MeCN (5 mL). 1,3-Propanesultone (375 mg, 3.07 mmol) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature, precipitated with Et_2O, filtered and washed with Et_2O to obtain the pure product as a red solid in 95% yield (131 mg). ^{1}H NMR (500 MHz, DMSO) δ 9.10 (d, *J* = 6.7 Hz, 2H), 8.55 (d, *J* = 6.7 Hz, 2H), 8.19 (d, *J* = 8.6 Hz, 2H), 8.06 (s, 1H), 7.94 (dd, *J* = 10.9, 8.8 Hz, 4H), 6.85 (d, *J* = 9.0 Hz, 2H), 4.71 (t, *J* = 6.7 Hz, 2H), 3.05 (s, 6H), 2.46 (t, *J* = 7.1 Hz, 2H), 2.31 – 2.19 (m, 2H). ^{13}C NMR (125 MHz, DMSO) δ 153.96, 152.59, 145.36, 145.08, 138.98, 132.81, 132.20, 132.02, 129.29, 128.62, 126.21, 126.18, 124.56, 122.80, 121.01, 119.49, 112.10, 101.05, 59.15, 47.43, 27.75. HRMS (ESI+): *m*/*z*. 448.16856 [$C_{25}H_{26}N_3O_3S$ $(M+H)^+$, calcd 448.16894].

(Z)-3-(4-(dimethylamino)phenyl)-2-(pyridin-4-yl)acrylonitrile (1): 4-pyridylacetonitrile hydrochloride (2.00 g, 12.9 mmol) and 4-dimethylaminobenzaldehyde (2.12 g, 14.2 mmol) was added with potassium hydroxide (2.39 g, 42.6 mmol) in 30 mL of ethanol and stirred at room temperature for 4 h. Upon completion of the reaction, 30mL of water was added to the reaction mixture. The yellow precipitates were filtered, washed with water and dried under reduced pressure to yield the product as an orange coloured solid in 92% yield (2.95 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (dd, *J* = 4.9, 1.3 Hz, 1H), 7.91 (d, *J* = 9.0 Hz, 1H), 7.58 (s, 1H), 7.51 (dd, *J* = 4.8, 1.4 Hz, 1H), 6.73 (d, *J* = 9.0 Hz, 1H), 3.09 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.37, 150.29, 144.95, 143.06, 132.21, 120.58, 119.29, 118.49, 111.56, 101.10, 40.02. HRMS (ESI+): *m*/*z*. 250.13342 [$C_{16}H_{16}N_3$ $(M+H)^+$, calcd 250.13387].

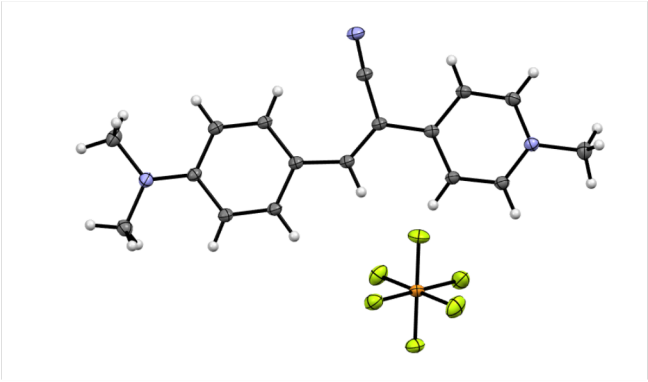


Scheme S3 Synthetic scheme for **ASC**. **A** Knoevenagel condensation. **B** Alkylation and anion conversion of halide to PF_6.

(ASC): Compound **1** (200 mg, 0.803 mmol) was added to MeCN (10 mL). Iodomethane (171 mg, 1.21 mmol) was then added to the reaction mixture, stirred and refluxed for 24 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature, precipitated with Et_2O, filtered and washed with Et_2O. The precipitate was then redissolved in acetone (20 mL) and saturated KPF_6 aqueous solution (20 mL) and stirred for 1 h. Acetone was then removed and the precipitate was filtered

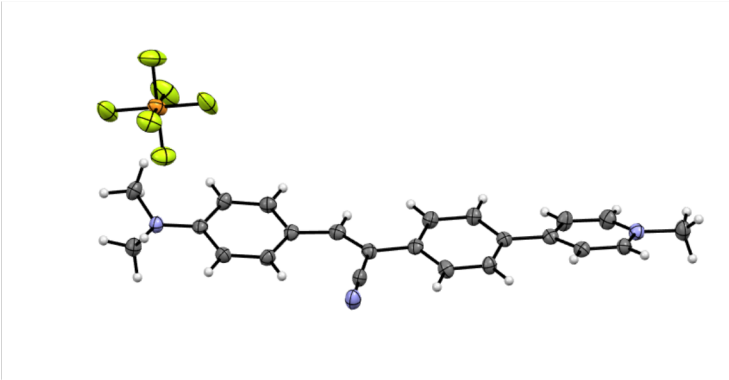
and washed with water to obtain the pure product as a dark blue solid in 71% yield (234 mg). ^{1}H NMR (400 MHz, DMSO) δ 8.80 (d, *J* = 6.9 Hz, 2H), 8.44 (s, 1H), 8.17 (d, *J* = 7.0 Hz, 2H), 8.04 (d, *J* = 9.1 Hz, 2H), 6.91 (d, *J* = 9.1 Hz, 2H), 4.22 (s, 3H), 3.11 (s, 6H). ^{13}C NMR (100 MHz, DMSO) δ 154.32, 151.05, 145.30, 134.44, 121.39, 119.95, 118.41, 112.52, 95.17, 47.08. HRMS (ESI+): *m*/*z*. 264.14933 [$C_{17}H_{18}N_3$ $(M\text{-}PF_6)^+$, calcd 264.14952].

A



ASC

B



ASCP

Figure S1 Ortep X-ray diffraction crystal structures obtained of molecules **ASC** and **ASCP**.

Table S1 Crystal data and structure refinement for **ASC**.

Identification code	shelx	
Empirical formula	C17 H18 F6 N3 P	
Formula weight	409.31	
Temperature	100.2(6) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P 21/c	
Unit cell dimensions	a = 6.35200(10) Å	a= 90°.
	b = 8.22740(10) Å	b= 91.2980(10)°.
	c = 33.0348(6) Å	g = 90°.
Volume	1725.97(5) $Å^3$	
Z	4	
Density (calculated)	1.575 Mg/m^3	
Absorption coefficient	0.228 mm^{-1}	
F(000)	840	
Crystal size	0.450 x 0.433 x 0.134 mm^3	
Theta range for data collection	3.956 to 51.315°.	
Index ranges	-13<=h<=11, -16<=k<=18, -72<=l<=71	
Reflections collected	91442	
Independent reflections	18826 [R(int) = 0.0528]	
Completeness to theta = 25.242°	99.5 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.00000 and 0.59562	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	18826 / 0 / 247	
Goodness-of-fit on F^2	1.086	
Final R indices [I>2sigma(I)]	R1 = 0.0459, wR2 = 0.1353	
R indices (all data)	R1 = 0.0736, wR2 = 0.1504	
Extinction coefficient	n/a	
Largest diff. peak and hole	0.760 and -0.844 $e.Å^{-3}$	

Table S2 Crystal data and structure refinement for **ASCP**.

Identification code	shelx	
Empirical formula	C23 H22 F6 N3 P	
Formula weight	485.40	
Temperature	100.0(1) K	
Wavelength	1.54184 Å	
Crystal system	Monoclinic	
Space group	C 2/c	
Unit cell dimensions	a = 30.1428(6) Å	a= 90°.
	b = 6.19510(10) Å	b= 93.329(2)°.
	c = 23.4009(4) Å	g = 90°.
Volume	4362.46(13) $Å^3$	
Z	8	
Density (calculated)	1.478 Mg/m^3	
Absorption coefficient	1.736 mm^{-1}	
F(000)	2000	
Crystal size	0.2 x 0.1 x 0.03 mm^3	
Theta range for data collection	2.937 to 77.178°.	
Index ranges	-37<=h<=38, -7<=k<=7, -29<=l<=20	
Reflections collected	27533	
Independent reflections	4559 [R(int) = 0.0537]	
Completeness to theta = 67.684°	99.9 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	1.00000 and 0.65361	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	4559 / 0 / 304	
Goodness-of-fit on F^2	1.116	
Final R indices [I>2sigma(I)]	R1 = 0.1012, wR2 = 0.2844	
R indices (all data)	R1 = 0.1046, wR2 = 0.2856	
Extinction coefficient	0.00012(4)	
Largest diff. peak and hole	1.286 and -0.626 $e.Å^{-3}$	

3. Photophysical Studies, X-ray Crystallography & DFT Calculations

Table S3 Summary of absorbance maxima for all compounds measured in different solvent.

	Absorbance Maxima, $\lambda_{abs\ max}$(nm)					
	ASC	**ASCP**	**ASCP-CA**	**ASCP-HE**	**ASCP-ES**	**ASCP-SO**
PBS	506	436	436	438	445	441
DMSO	506	459	457	459	466	457
MeCN	504	448	446	449	456	443
EtOH	509	456	455	458	463	452
Acetone	505	450	449	452	458	443
^{i}PrOH	511	458	454	460	467	451
THF	507	454	454	457	462	427
Chloroform	530	474	468	472	478	477
Toluene	516	411	455	461	465	424

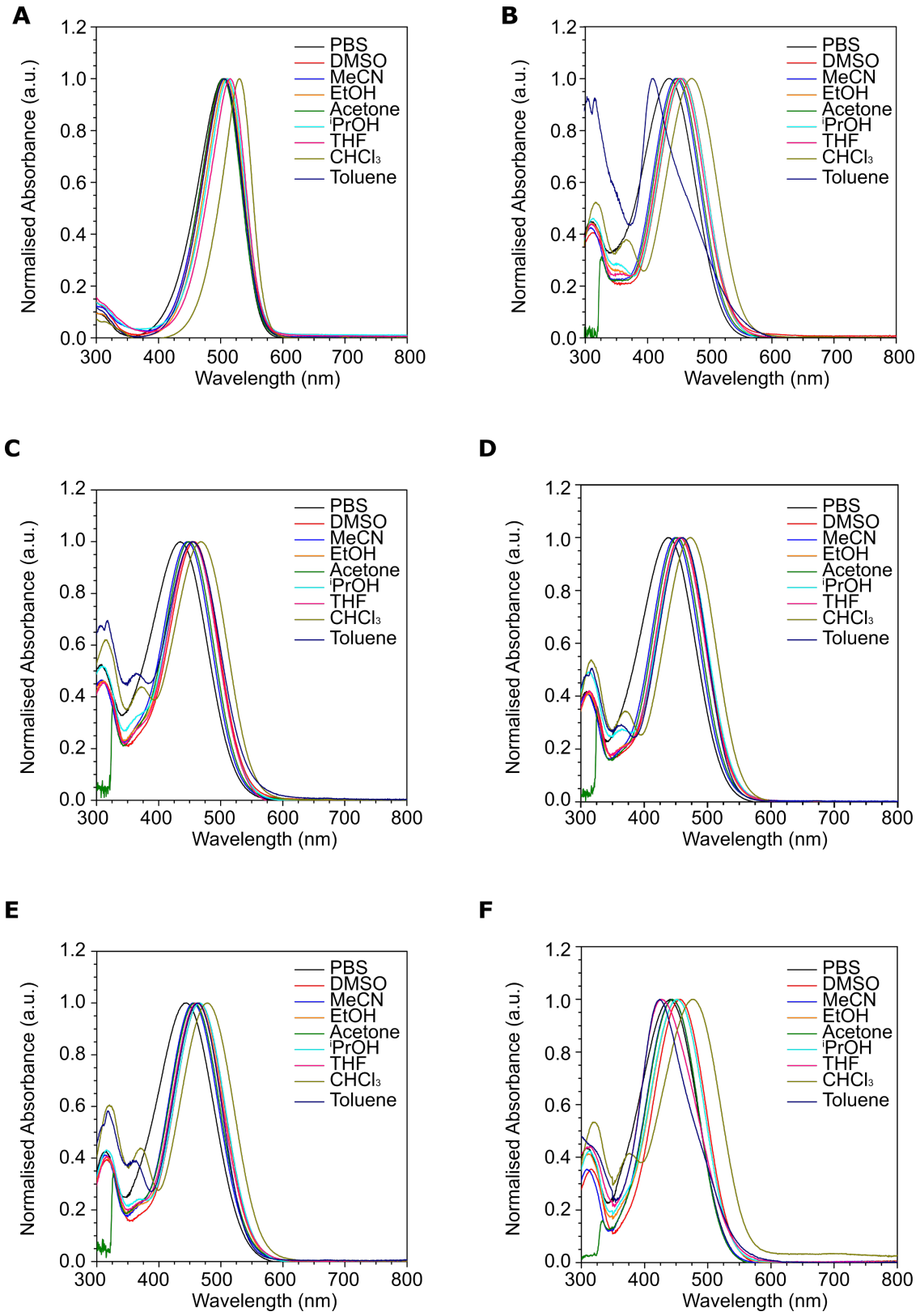


Figure S2 Absorbance spectra for **ASC** and ASCP derivatives in different solvents. (A) **ASC**, (B) **ASCP**, (C) **ASCP-CA**, (D**) ASCP-HE**, **(**E) **ASCP-ES**, (F**) ASCP-SO**. PBS was Na_2HPO_4 at 20 mM concentration. 10 μM dye concentration was used for all measurements.

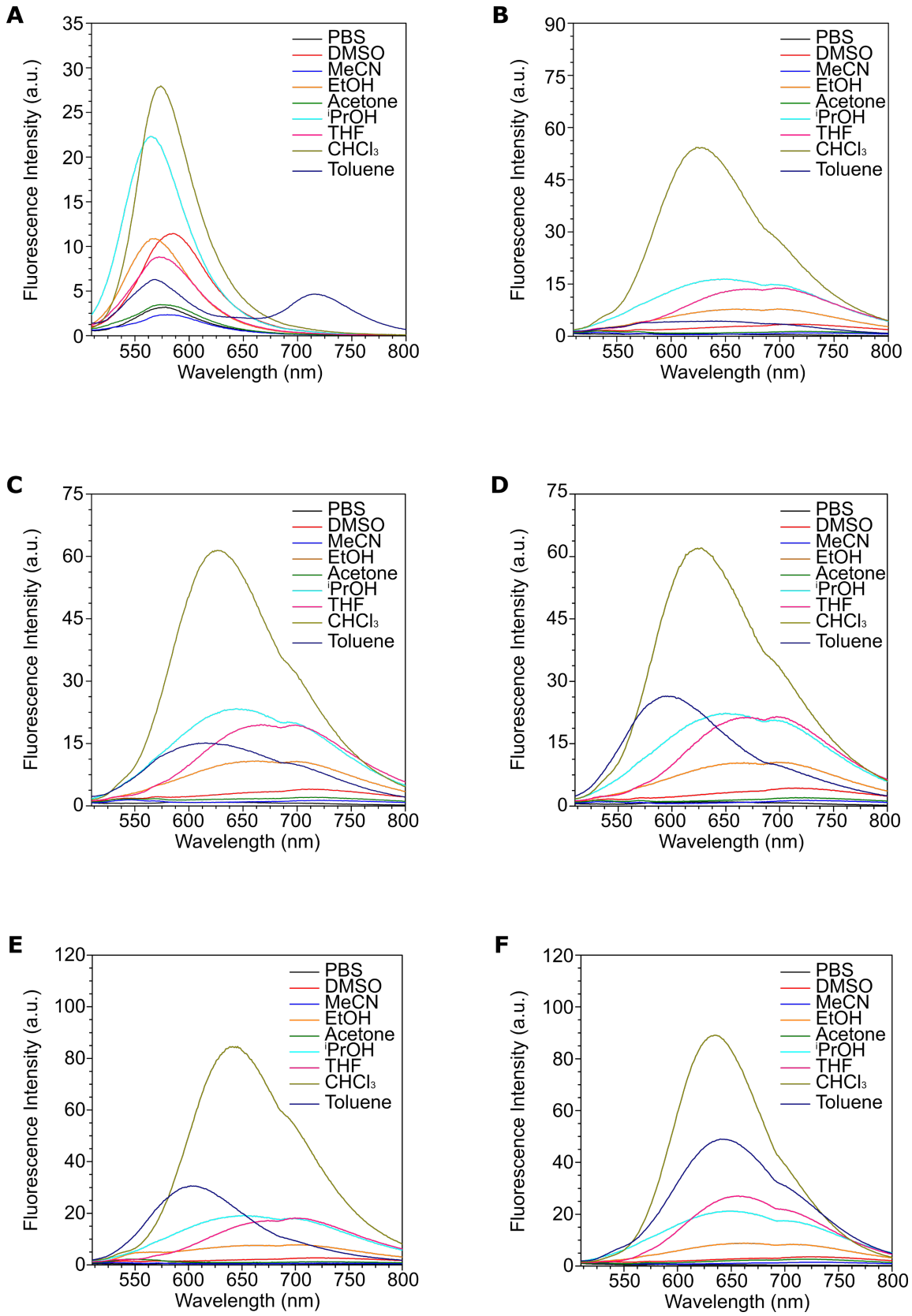


Figure S3 Fluorescence emission spectra for **ASC** and ASCP derivatives in different solvents. (A) **ASC**, (B) **ASCP**, (C) **ASCP-CA**, (D) **ASCP-HE**, (E) **ASCP-ES**, (F) **ASCP-SO**. PBS was Na_2HPO_4 at 20mM concentration. 10 μM dye concentration and 488nm excitation was used for all measurements.

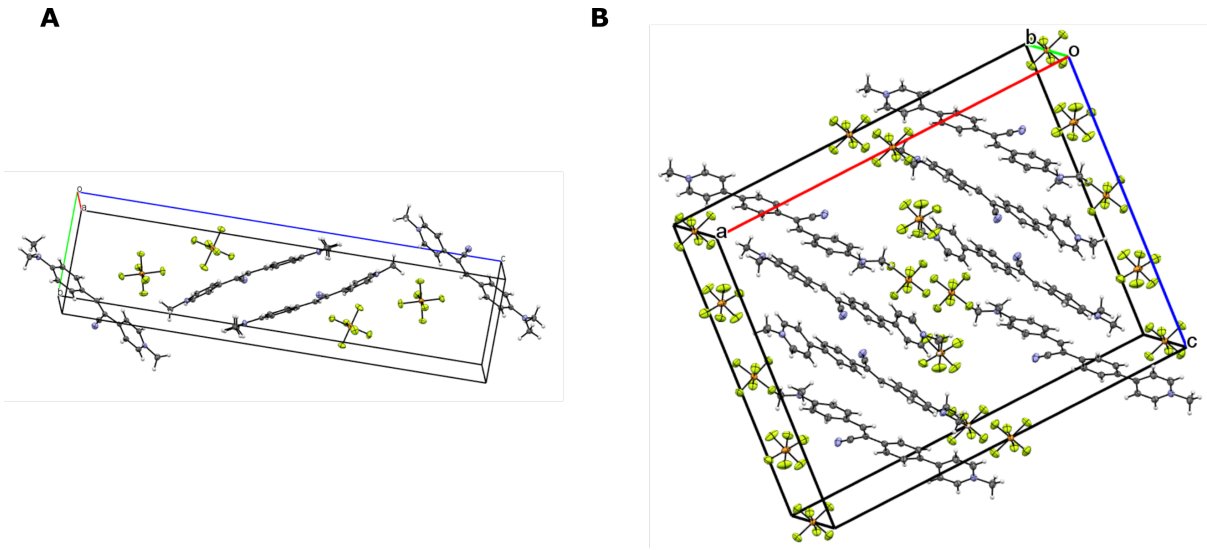


Figure S4 Crystal packing from x-ray crystallography data (|τ|°) between phenyl rings and central double bond for (A) **ASC** and (B) **ASCP**.

Table S4 Summary and comparison of photophysical characteristic of dyes measured in ^{i}PrOH.

	Absorbance Maxima, λ_{absmax}**(nm)**	**Emission Maxima,** $\lambda_{em\ max}$**(nm)**	**Stokes Shift,** $\lambda_{em\ max}$ **(nm) –** $\lambda_{abs\ max}$**(nm)**
ASC	511	564	53
ASCP	458	649	191
ASCP-CA	454	644	190
ASCP-HE	460	650	190
ASCP-ES	467	652	185
ASCP-SO	451	646	195

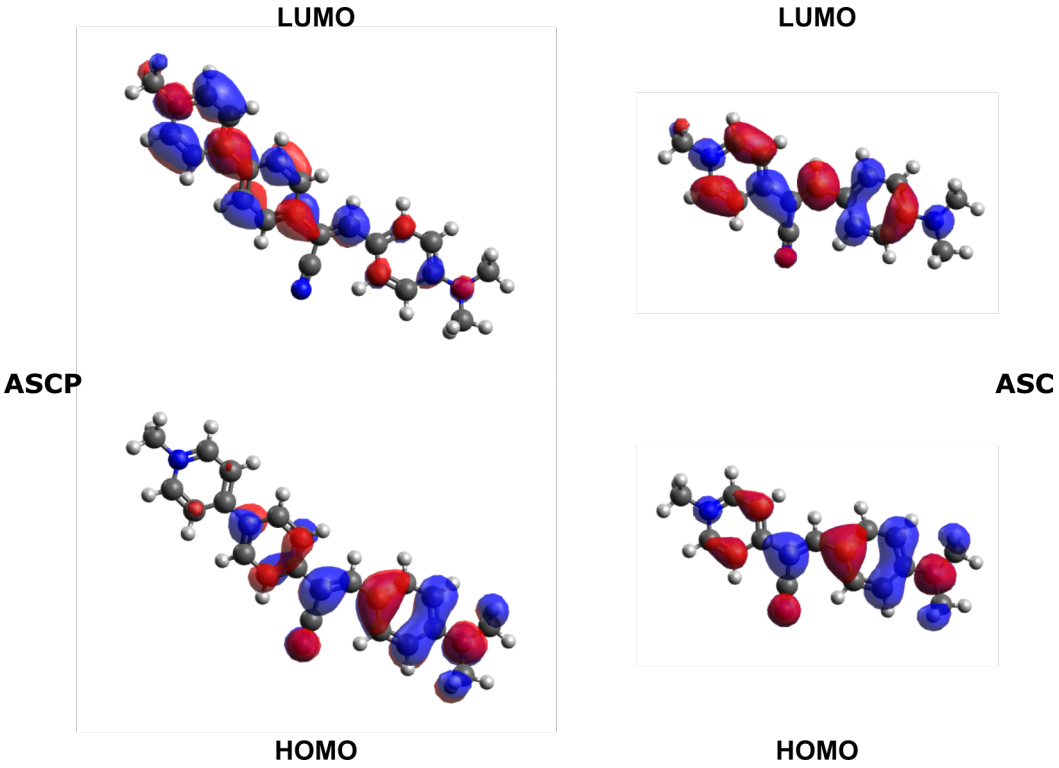


Figure S5 DFT calculations of HOMO and LUMO orbitals for **ASCP** and **ASC**. DFT calculations were carried out at the B3LYP/6-31g* level with IEFPCM model in water. No negative frequencies for optimised structures were observed.

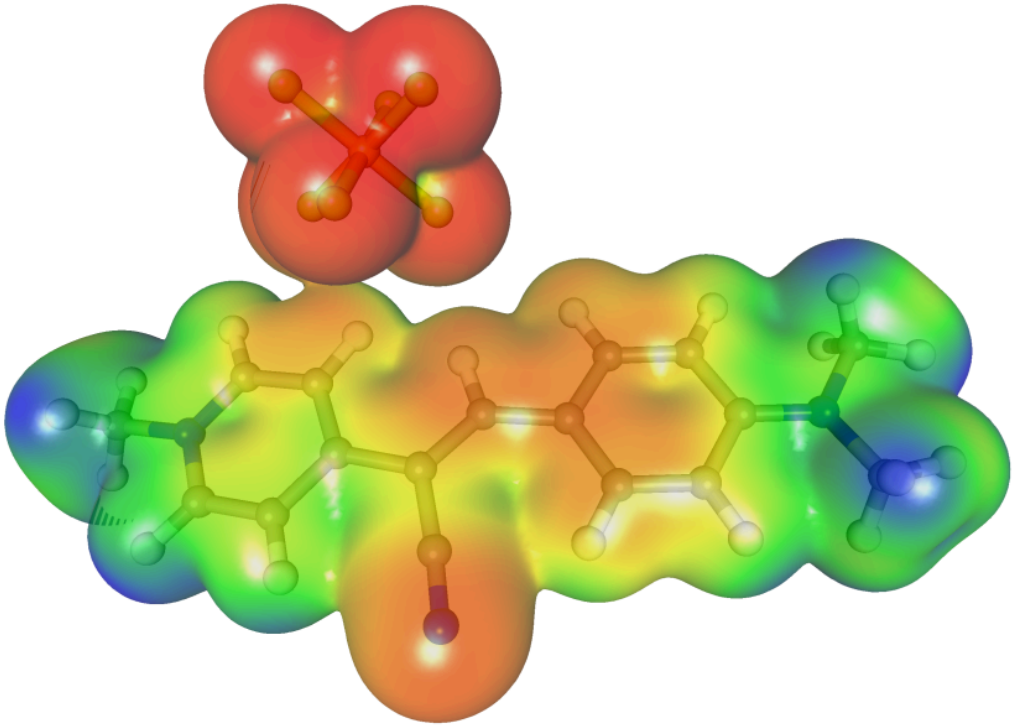


Figure S6 Heat map representation of electrostatic potential data for **ASC** based on acquired X-ray crystallography data. Red: negative electrostatic potential, Blue: positive electrostatic potential.

Table S5 Absolute photoluminescent quantum yield (PLQY) of dyes in PMMA matrix (2% weight ratio).

	Photoluminescent Quantum Yield (PLQY)			
	1	**2**	**3**	**Mean**
ASC	6.8%	6.8%	6.8%	7% (6.8%)
ASCP	18.4%	18.4%	18.7%	19% (18.5%)
ASCP-CA	23.2%	23.3%	23.3%	23% (23.2%)
ASCP-HE	22.4%	22.4%	22.4%	22% (22.4%)
ASCP-ES	20.7%	20.7%	20.8%	21% (20.7%)

Note that due to the poor solubility of **ASCP-SO** in acetone, its PLQY was not measured. Summary of triplicate measurements to obtain absolute photoluminescent quantum yield (PLQY) of dyes in PMMA matrix. Note that due to the poor solubility of **ASCP-SO** in acetone, its PLQY was not measured.

Table S6 Summary of triplicate measurements for absolute photoluminescent quantum yield (PLQY)

	DMSO	$CHCl_3$	Glycerol (95%)	Crystalline	PMMA
ASC	0.3%	0.5%	5.5%	6.3%	6.8%
ASCP	0.5%	4.3%	5.3%	6.7%	18.5%

Note crystalline **ASC** only has excimer emission (Refer to Figure S10). For measurement in chloroform ($CHCl_3$), 99% chloroform condition was used. For glycerol (95%), water was used as the co-solvent.

Table S7 Dynamic light scattering measurements in different dye aggregate forming solvent mixtures.

	ASC Particle Diameter (nm)	SD
$CHCl_3$ (80%)	224.2	304.5
Toluene (80%)	409.9	337.8

	ASCP Particle Diameter (nm)	SD
$CHCl_3$ (99%)	154.1	35.7
Toluene (99%)	166.5	47.9

The remaining solution was made up with DMSO. 10μM dye concentration was used. Note for **ASC**, higher percentage volume of $CHCl_3$ or toluene resulted in aggregate sizes too large for the machine to measure.

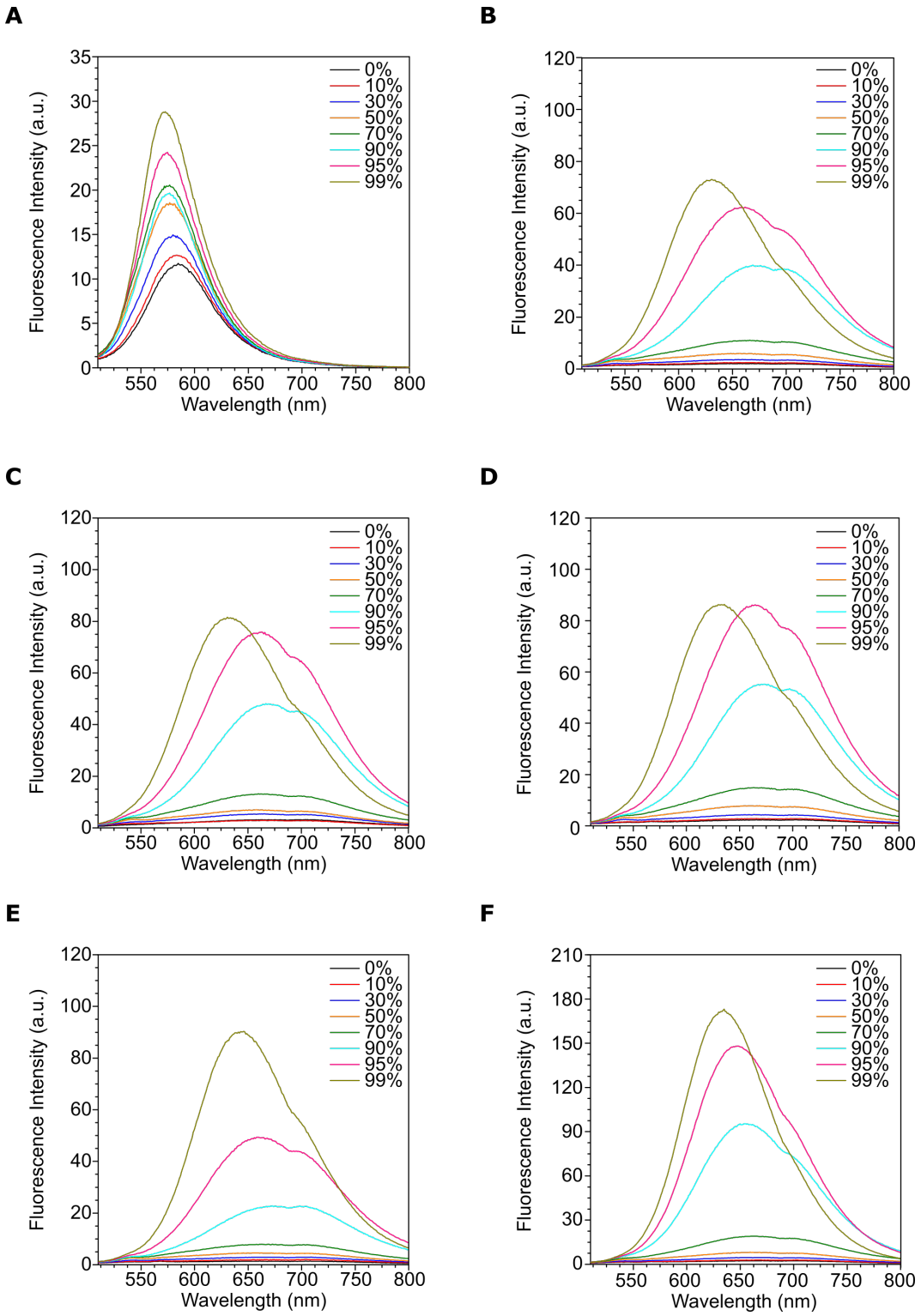


Figure S7 Measured fluorescence emission spectra for dye self-aggregation experiments. (A) **ASC**, (B) **ASCP**, (C) **ASCP-CA**, (D**) ASCP-HE**, **(**E) **ASCP-ES**, (F**) ASCP-SO**. Dyes were measured in DMSO and chloroform ($CHCl_3$) mixtures in increasing fraction of $CHCl_3$ from 0, 10, 30, 50, 70, 90, 95, 99%. 488nm excitation was used for all measurements. 10 μM dye concentration and 488nm excitation was used for all measurements.

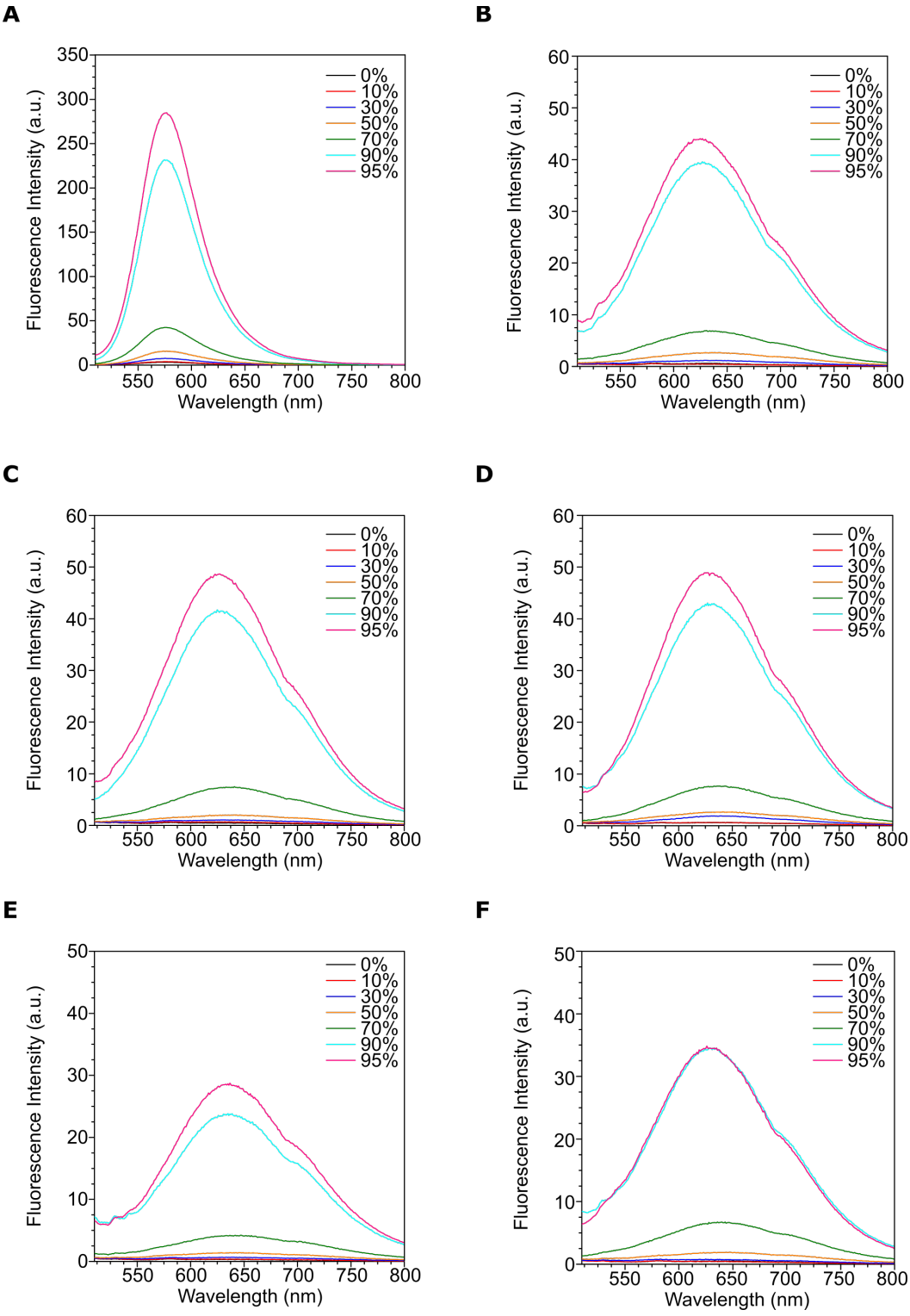
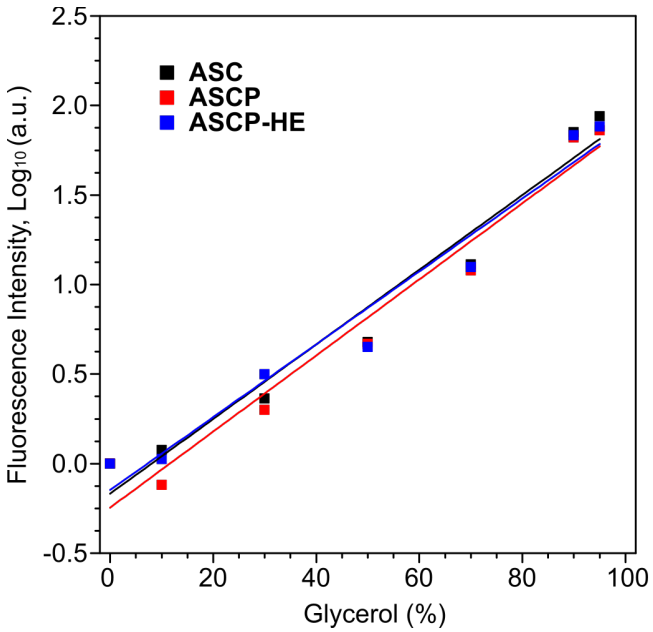


Figure S8 Measured fluorescence emission spectra for dye viscosity experiments. (A) **ASC**, (B) **ASCP**, (C) **ASCP-CA**, (D**) ASCP-HE**, **(**E) **ASCP-ES**, (F**) ASCP-SO**. Dyes were measured in water and glycerol mixtures in increasing fraction of glycerol from 0, 10, 30, 50, 70, 90, 95%. 10 μM dye concentration and 488nm excitation was used for all measurements.



Equation	y = a + bx	
ASC		
Adj. R-Square	0.955	
	Value	**Standard Error**
Intercept (a)	-0.167	0.111
Slope (b)	0.021	0.002
ASCP		
Adj. R-Square	0.953	
	Value	**Standard Error**
Intercept (a)	-0.246	0.116
Slope (b)	0.021	0.002
ASCP-HE		
Adj. R-Square	0.956	
	Value	**Standard Error**
Intercept (a)	-0.147	0.108
Slope (b)	0.020	0.002

Figure S9 Linear fit plot of fluorescence intensity for dye viscosity experiments. Data extracted from Figure 3c.

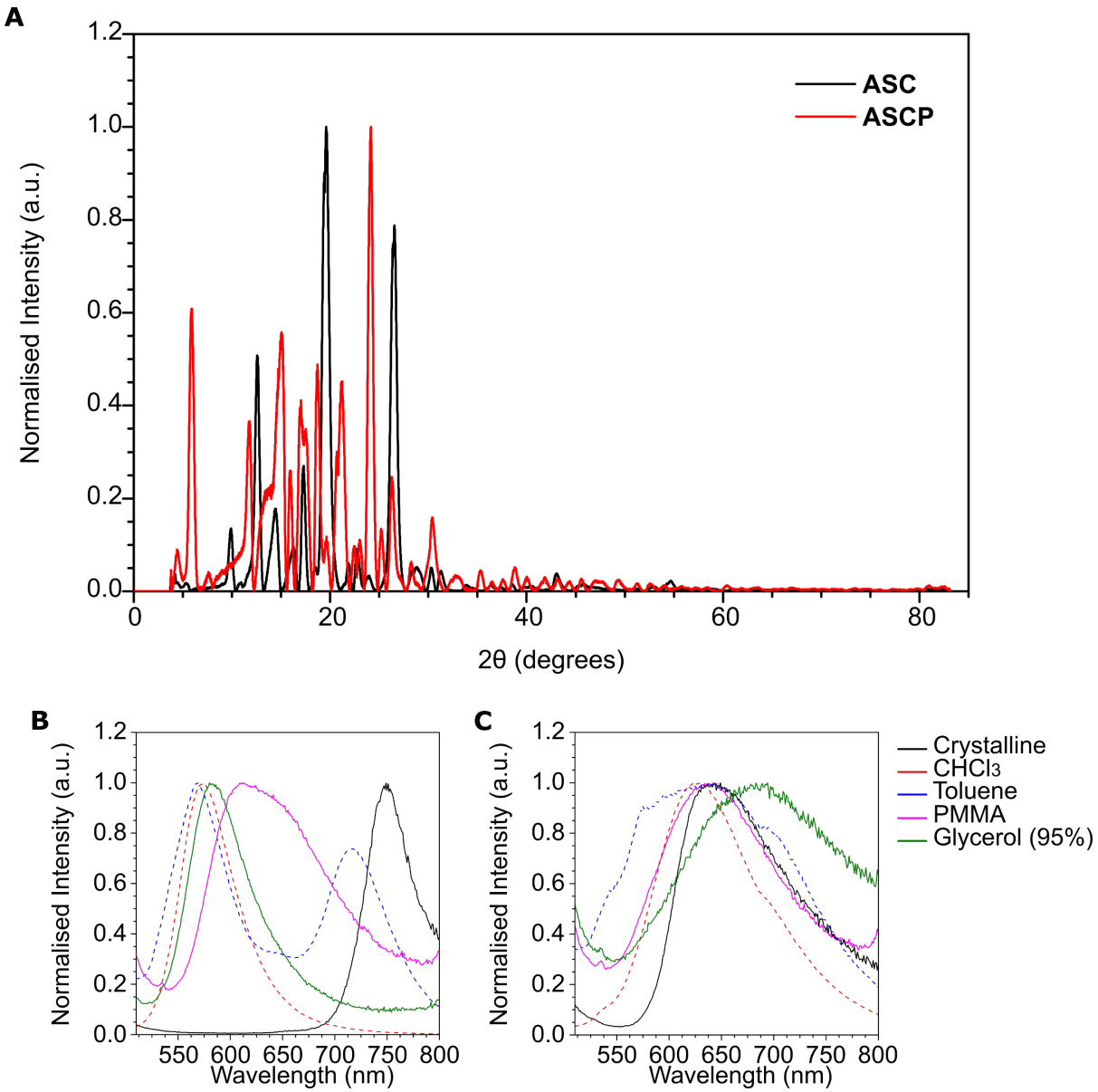


Figure S10 Solid state experiments. (A) X-ray powder diffraction experiments confirm crystalline nature of **ASC** and **ASCP** used for solid state fluorescence emission measurements. (B) Comparison of solution and crystalline fluorescence emission spectra for **ASC**. (C) Comparison of solution and crystalline fluorescence emission spectra for **ASCP**.

4. Cell Viability and Confocal Laser Scanning Microscopy

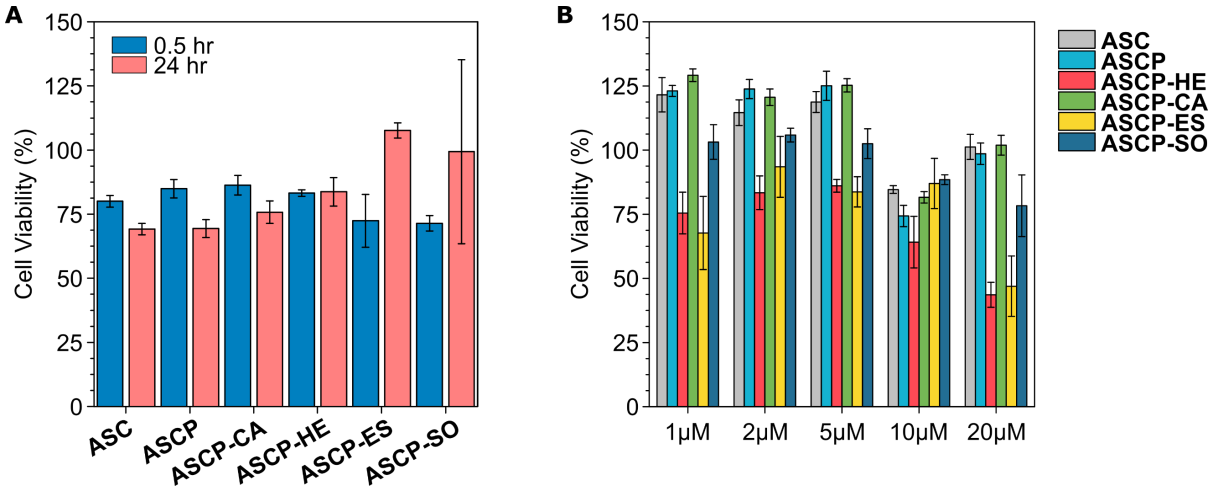


Figure S11 Cell viability experiments. (A) Time dependent cytotoxicity assay for dyes in A549 cells after treatment with 5 μM dye. (B) Concentration dependent cytotoxicity assay for dyes in A549 cells after treatment with respective dye concentrations for 0.5 hr. alamarBlue™ cell viability assay was recorded by a plate reader. n = 3 biological replicates; mean ± s.d.

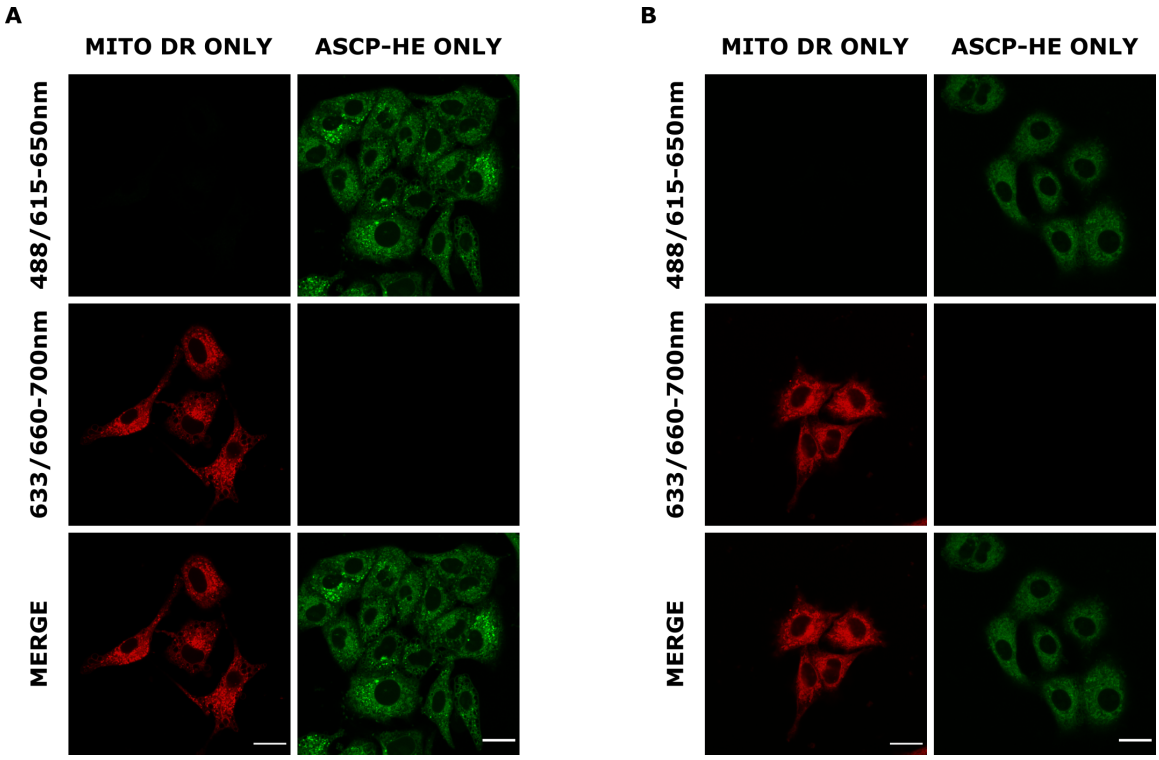


Figure S12. Control images for dye crosstalk in A549 cells. (A) Minimal crosstalk between dyes for the settings used for CLSM colocalization experiments for fixed cells. (B) Minimal crosstalk between dyes for the settings used for CLSM colocalization experiments for live cells. 500nM of Mitotracker™ Deep Red (**MITO DR**) counterstain was used to visualise mitochondria. 5μM of **ASCP-HE** was used. Scale bar, 20μm.

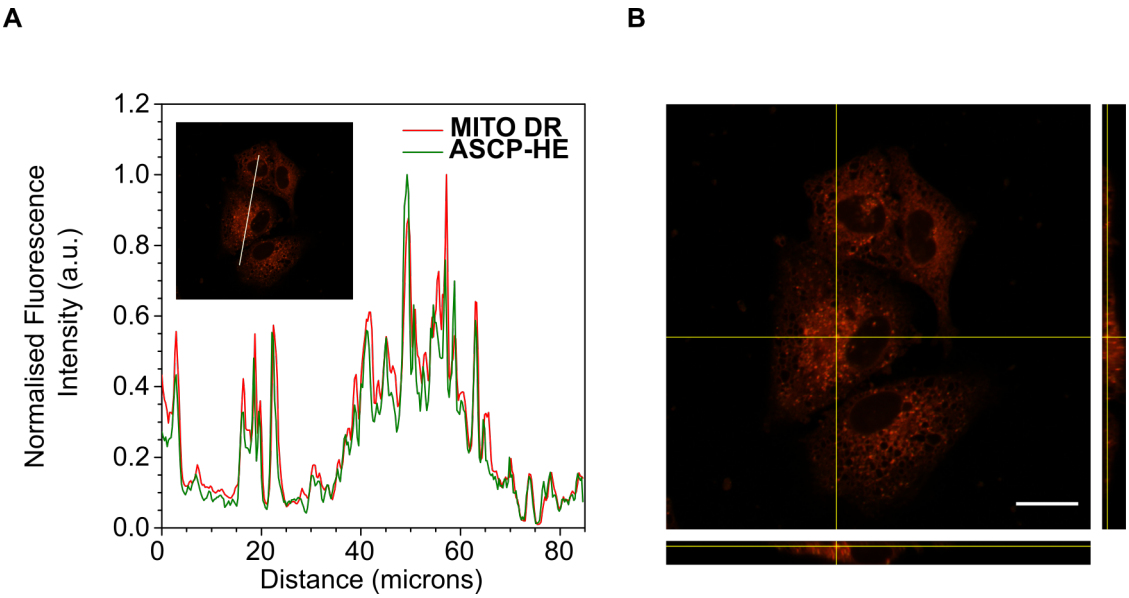


Figure S13 Z-stack image for confirmation of colocalization of **ASCP-HE** and **MITO DR**. (A) Normalised fluorescence intensity kymograph at single Z-plane for A549 cells. Fluorescence intensity measured along white line. (B) Orthoslice representation for Z-stack image of A549 cells confirming localisation of **ASCP-HE** into A549 cells. 500nM Mitotracker^{TM} Deep Red counterstain was used to visualise mitochondria. 5μM of **ASCP-HE** was used. Scale bar, 20 μm.

5. ^{1}H and ^{13}C NMR Spectra for compounds

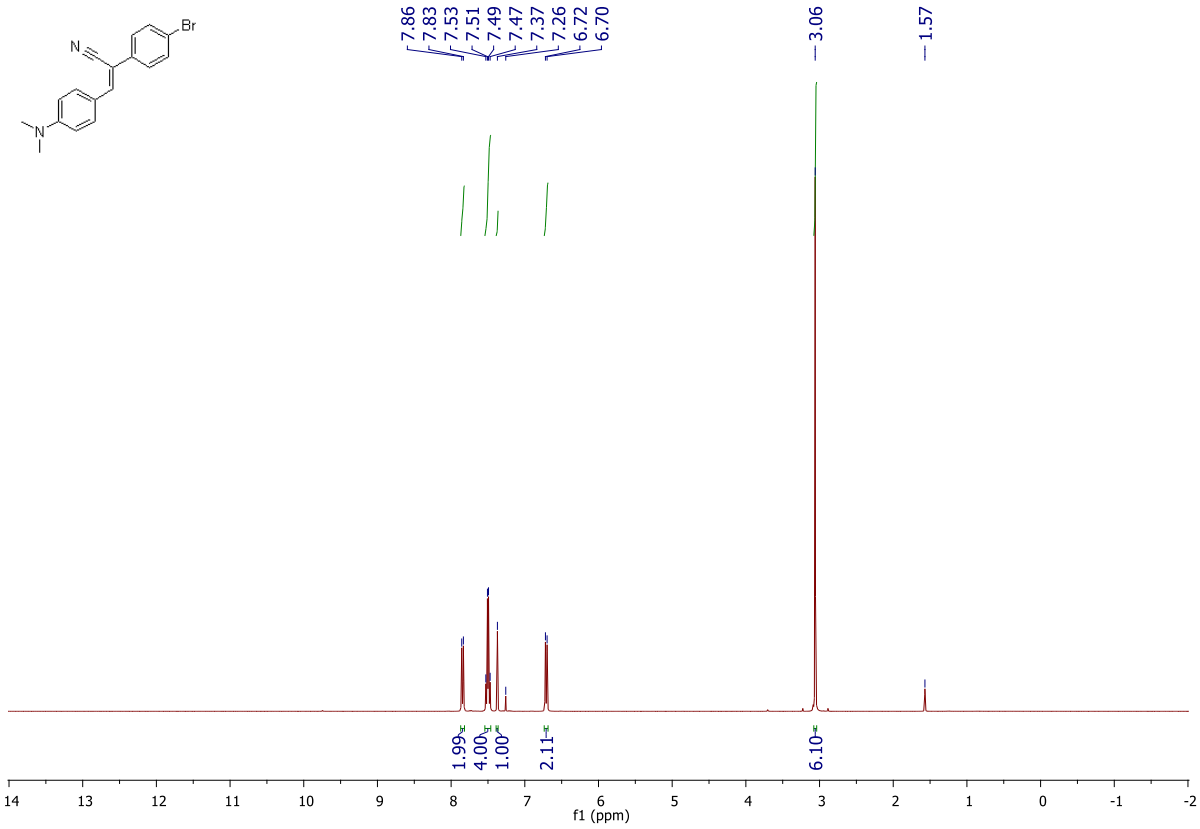


Figure S14 ^{1}H NMR of **3** in $CDCl_3$

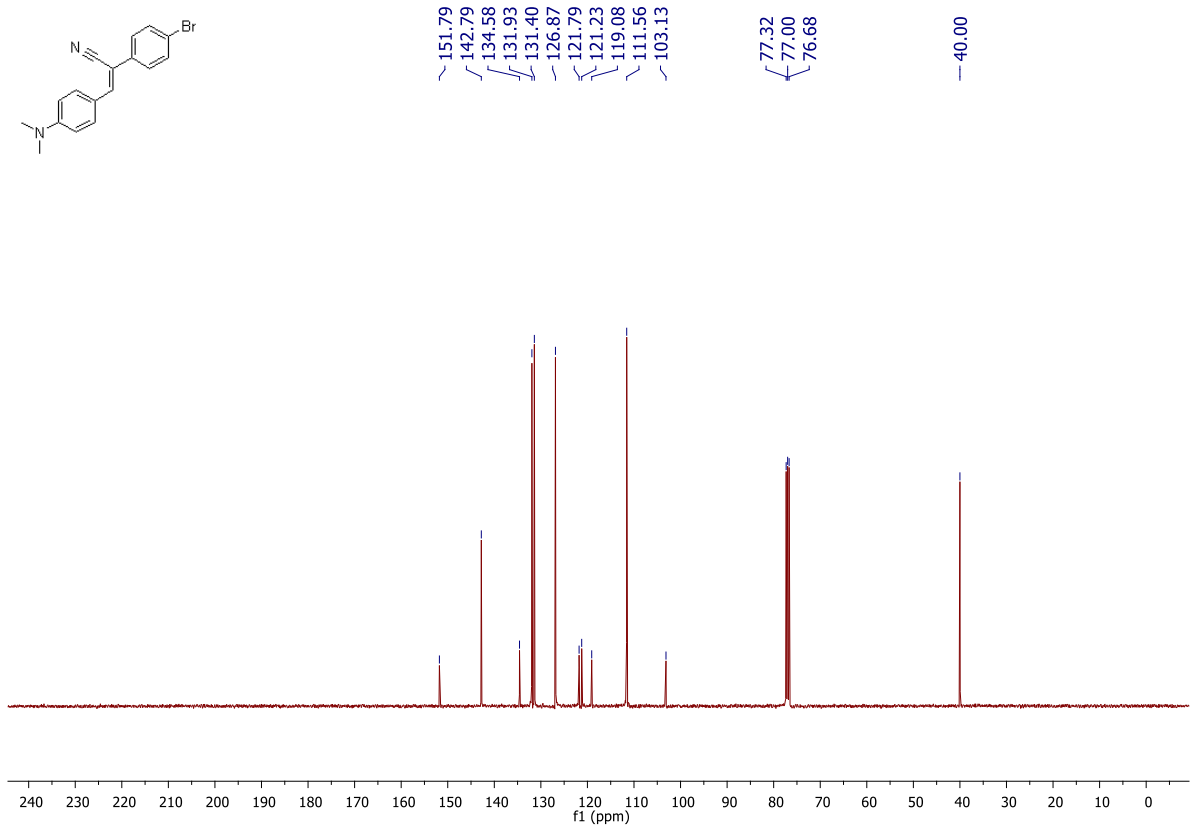


Figure S15 ^{13}C NMR of **3** in $CDCl_3$

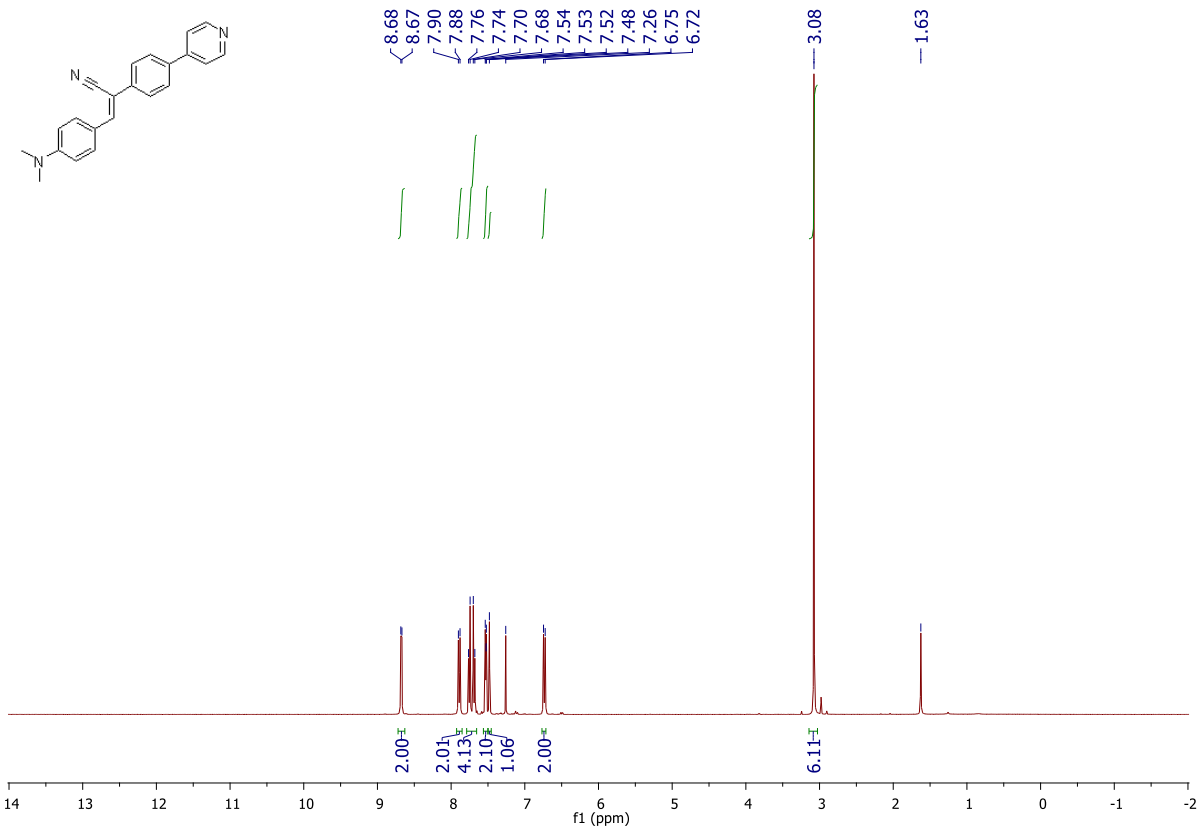


Figure S16 ^{1}H NMR of **2** in $CDCl_3$

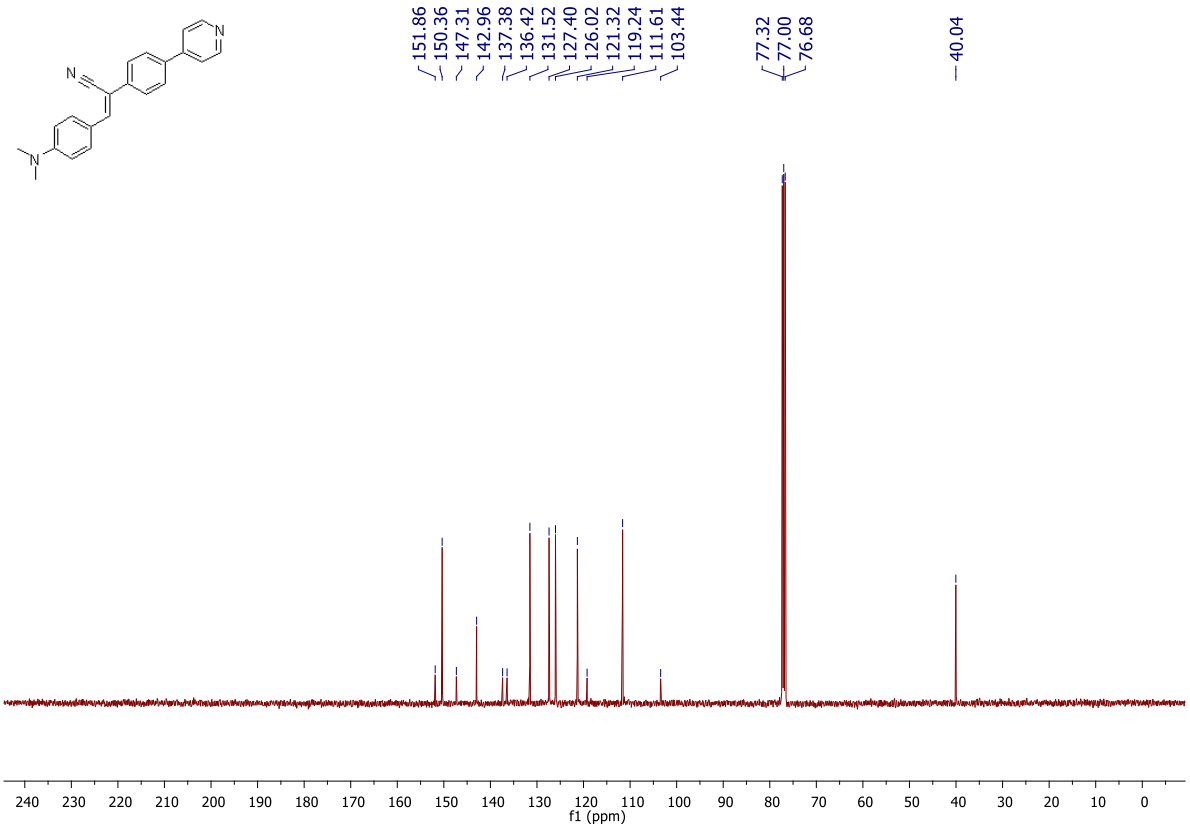


Figure S17 ^{13}C NMR of **2** in $CDCl_3$

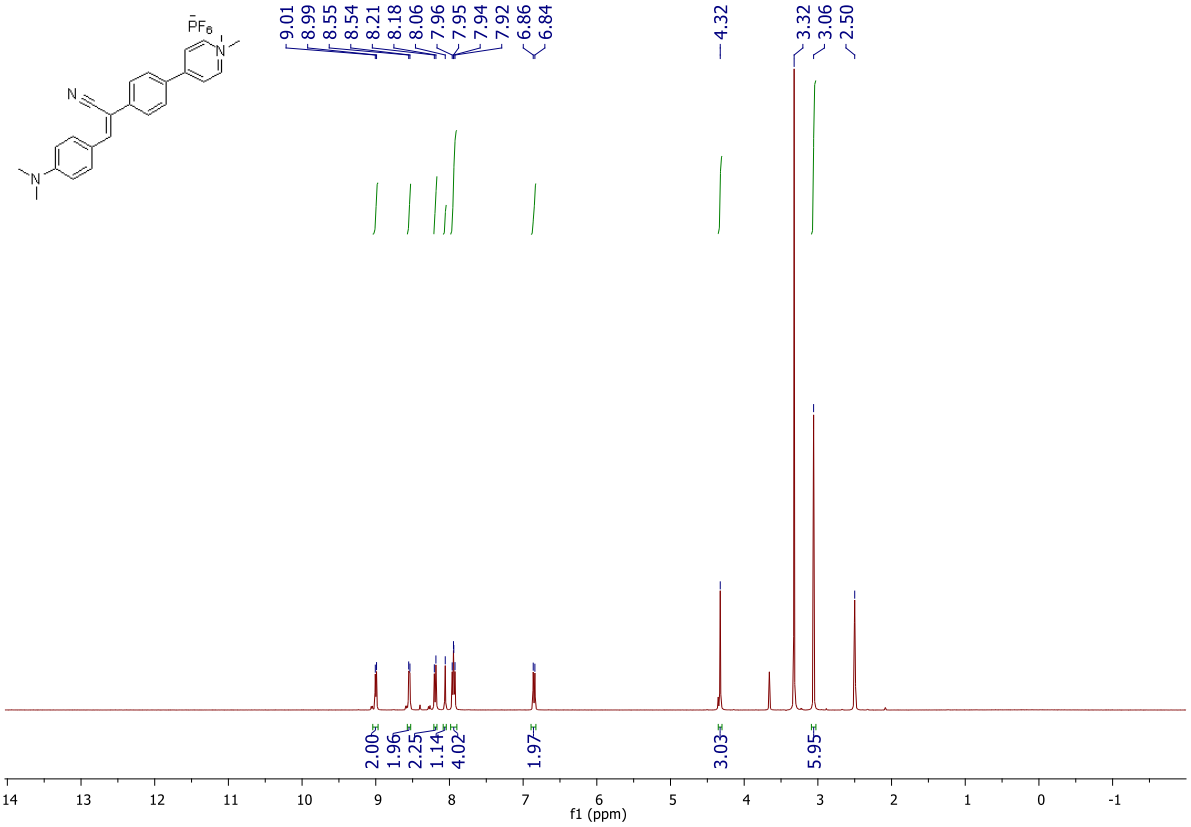


Figure S18 ^{1}H NMR of **ASCP** in DMSO.

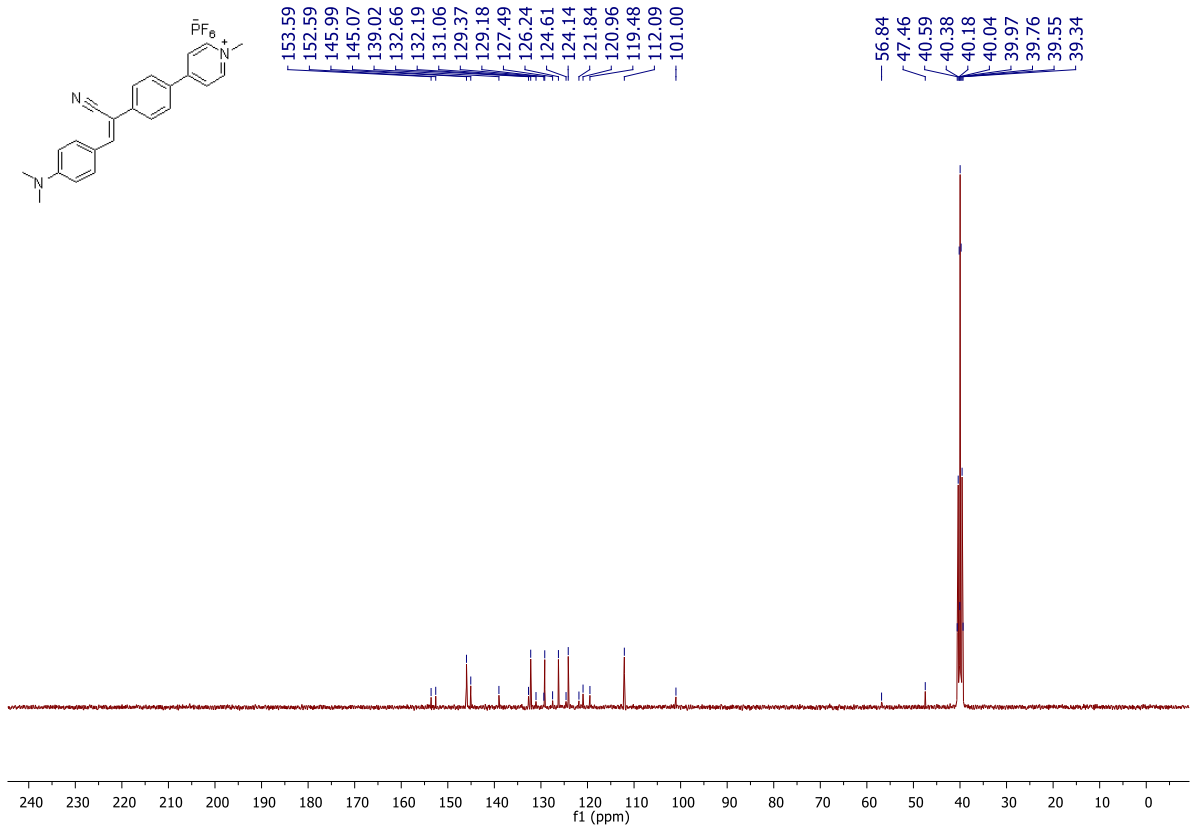


Figure S19 ^{13}C NMR of **ASCP** in DMSO.

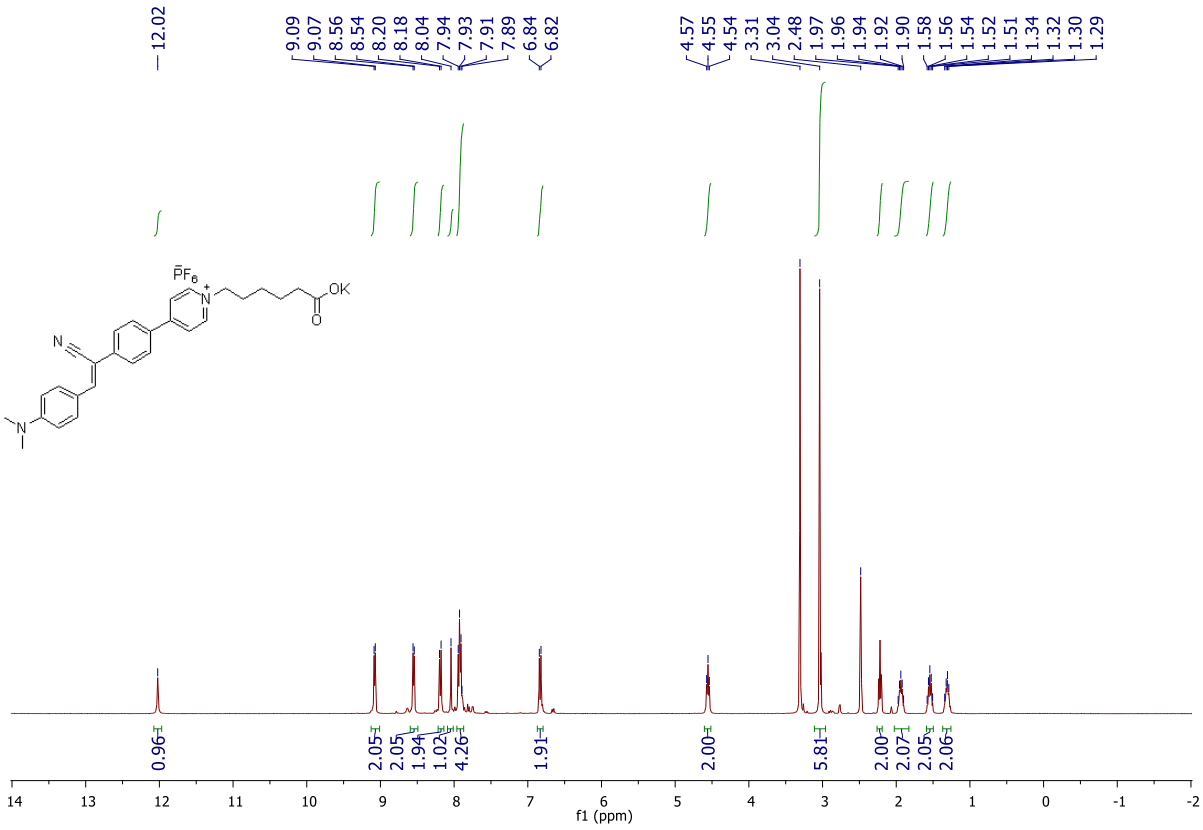


Figure S20 ^{1}H NMR of **ASCP-CA** in DMSO.

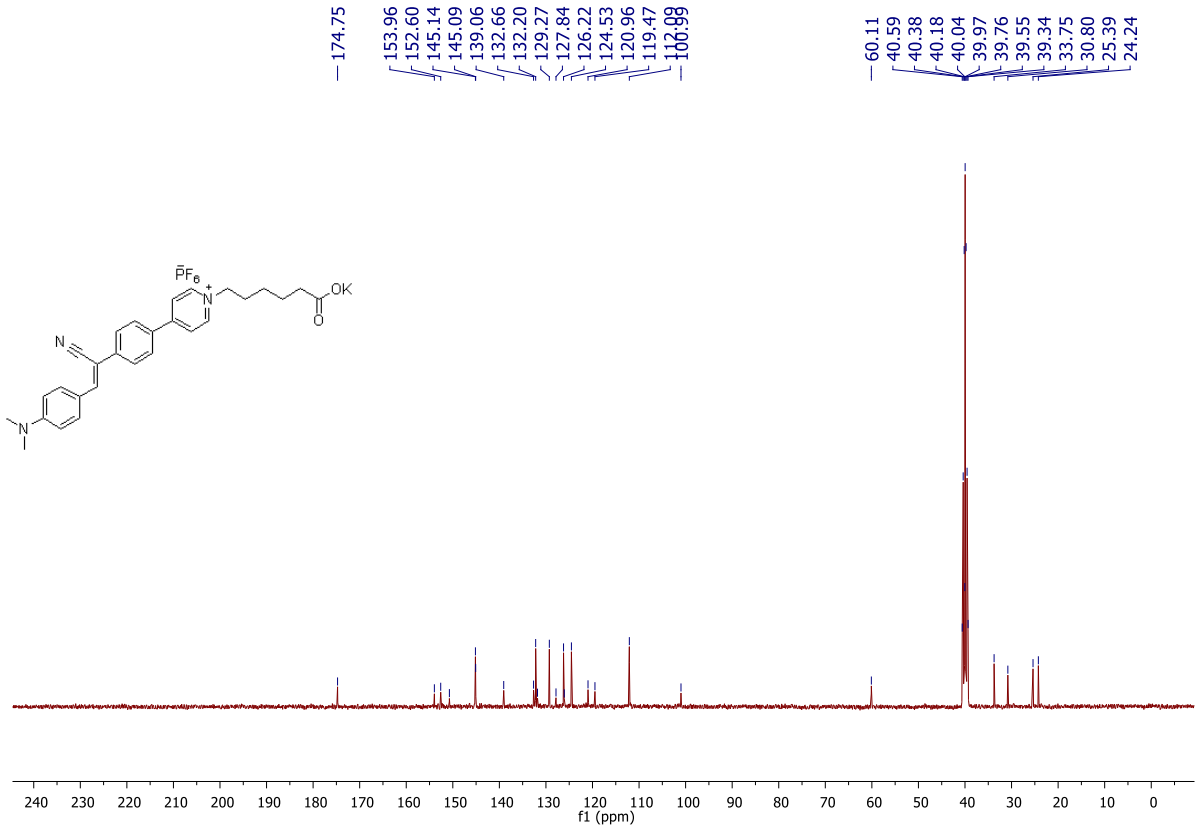


Figure S21 ^{13}C NMR of **ASCP-CA** in DMSO.

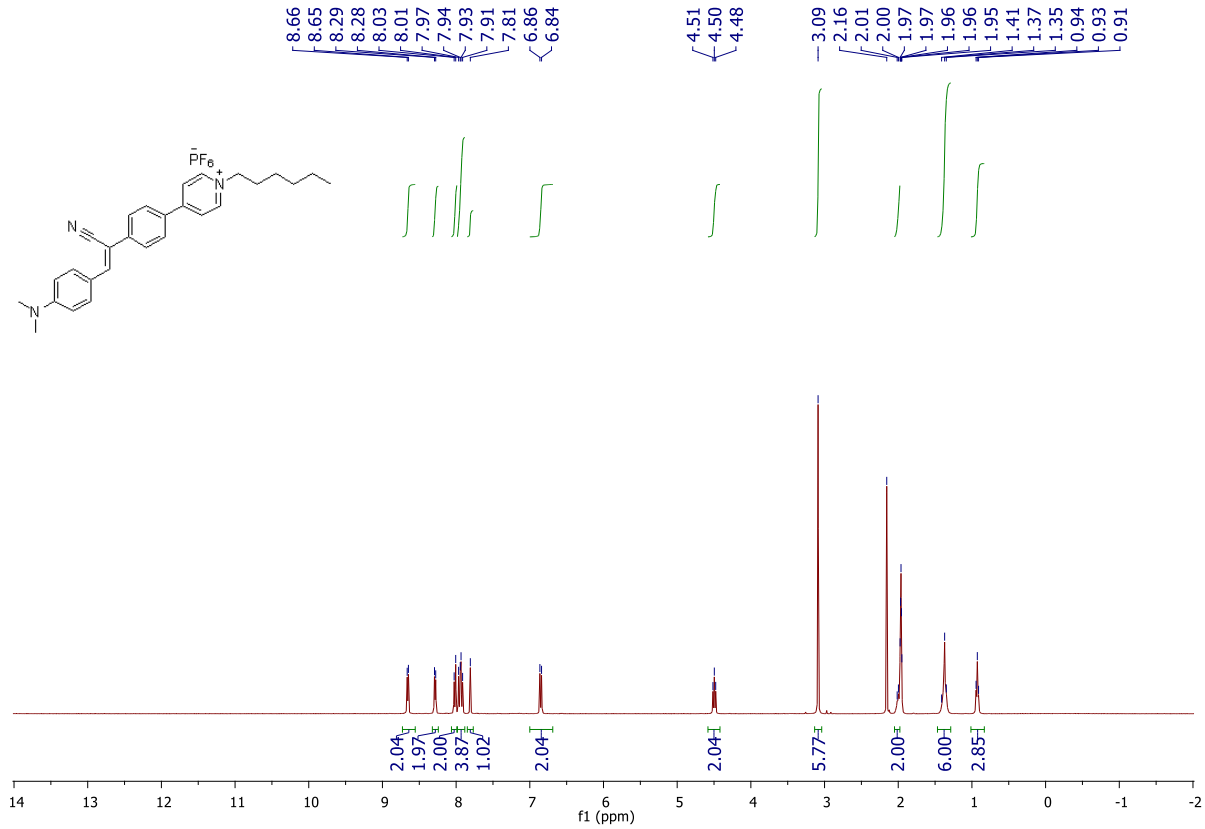


Figure S22 ^{1}H NMR of **ASCP-HE** in CD_3CN.

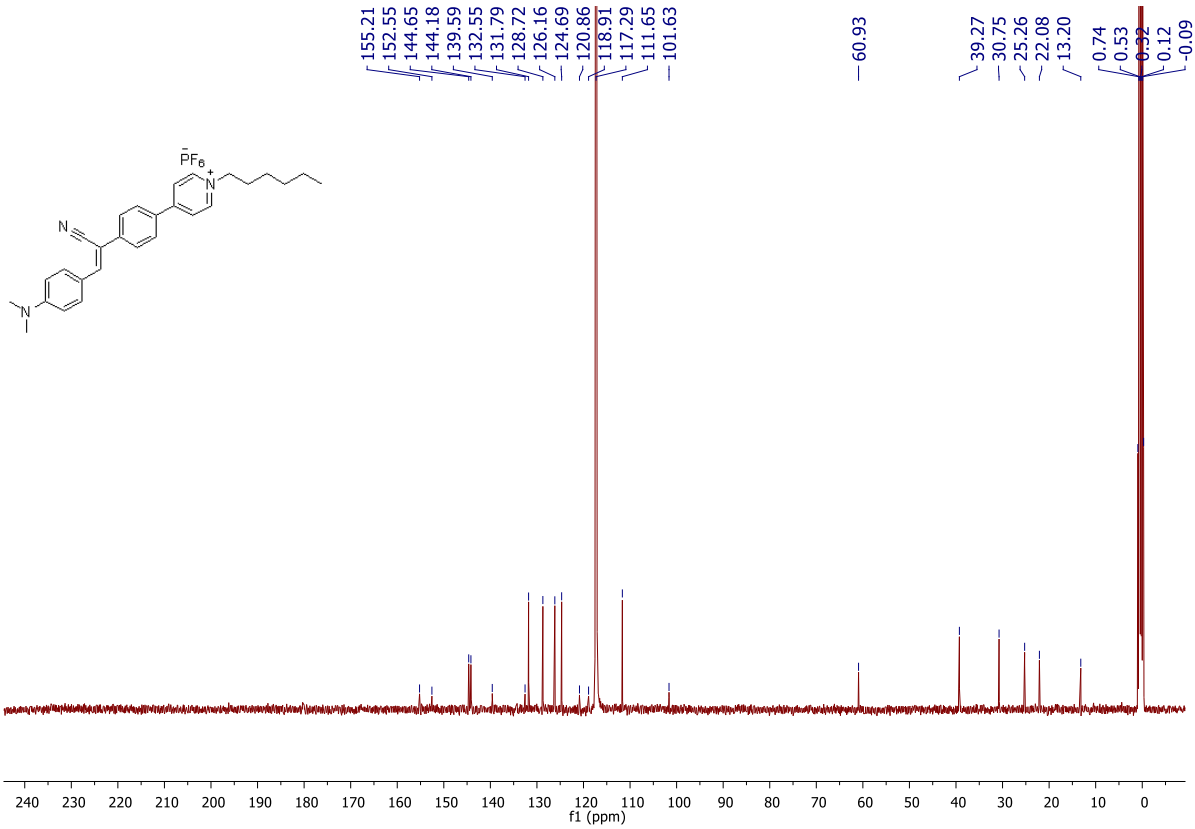


Figure S23 ^{13}C NMR of **ASCP-HE** in CD_3CN.

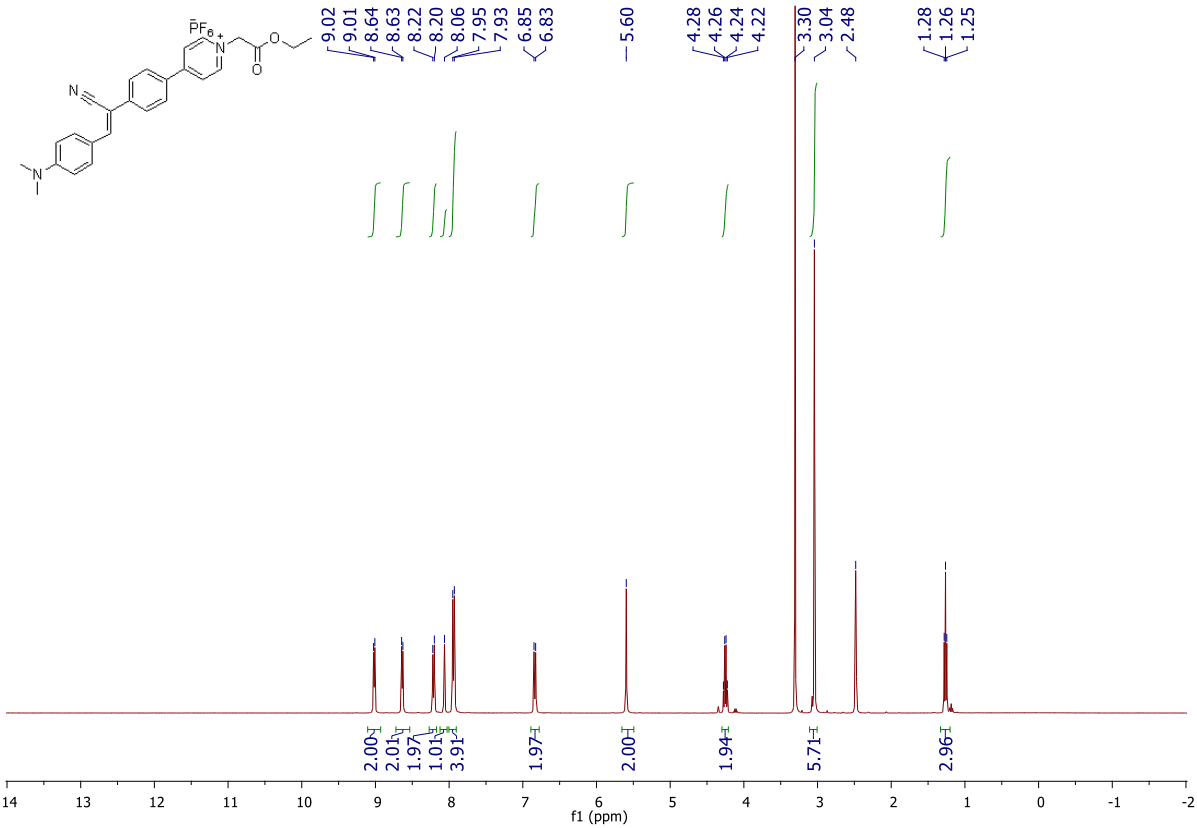


Figure S24 ^{1}H NMR of **ASCP-ES** in DMSO.

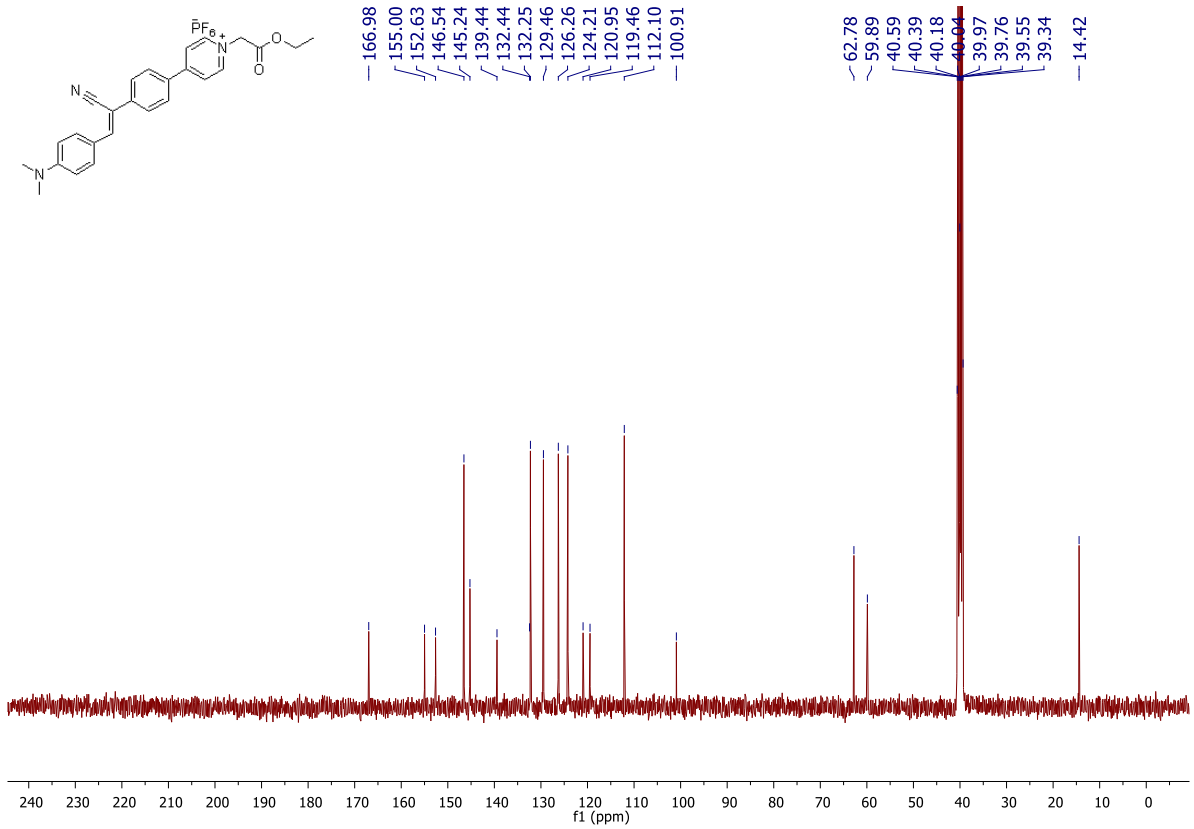


Figure S25 ^{13}C NMR of **ASCP-ES** in DMSO.

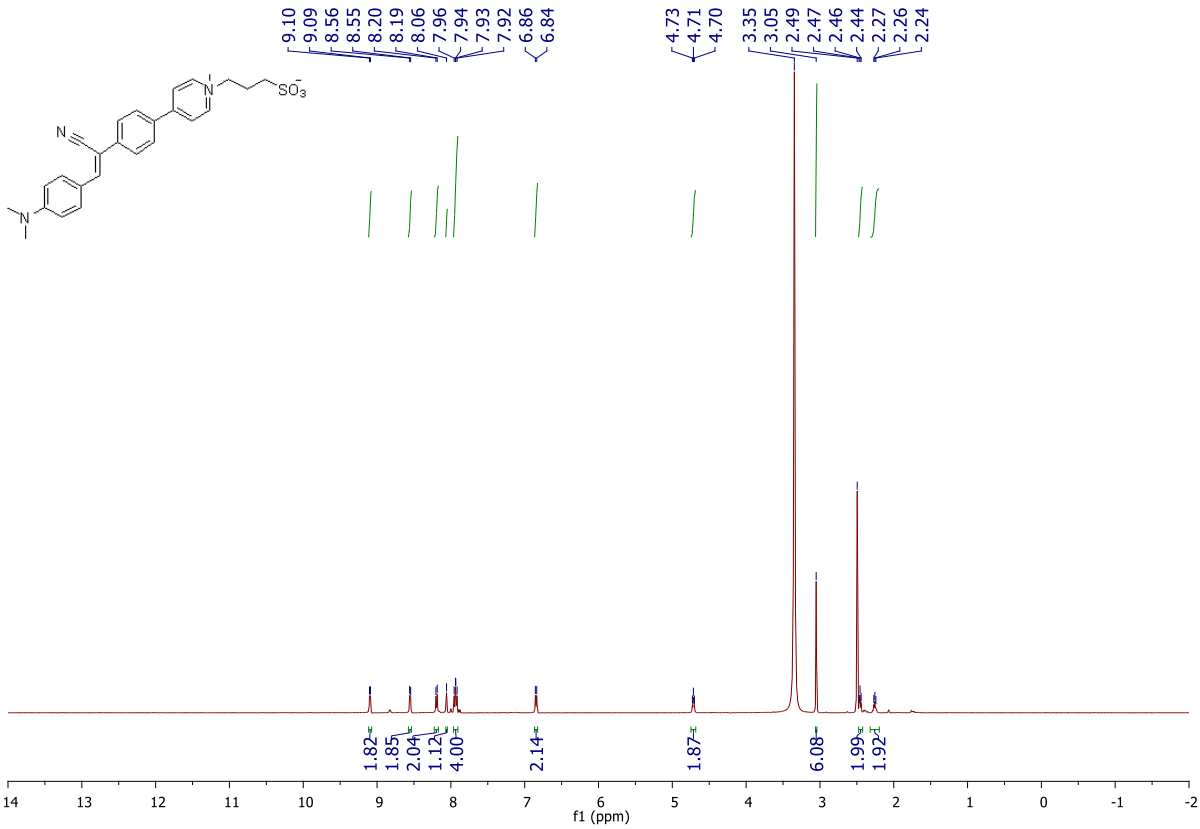


Figure S26 ^{1}H NMR of **ASCP-SO** in DMSO.

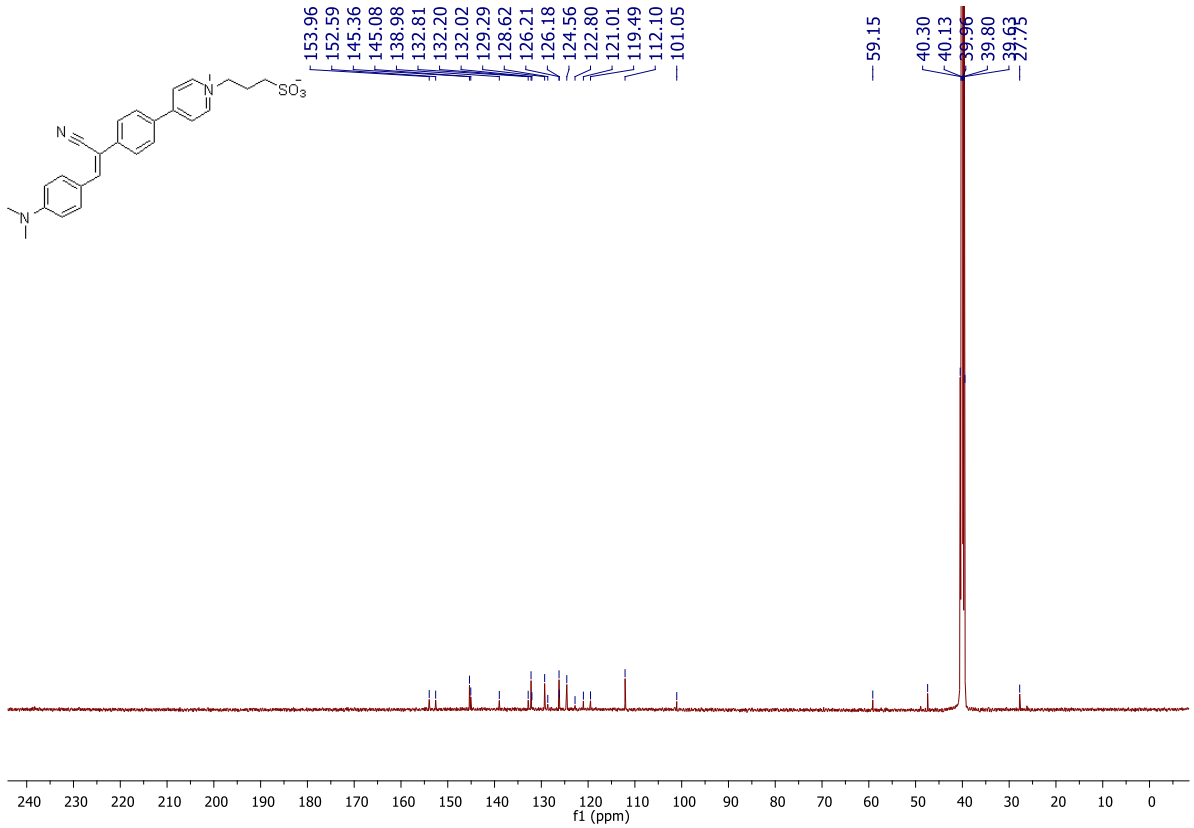


Figure S27 ^{13}C NMR of **ASCP-SO** in DMSO.

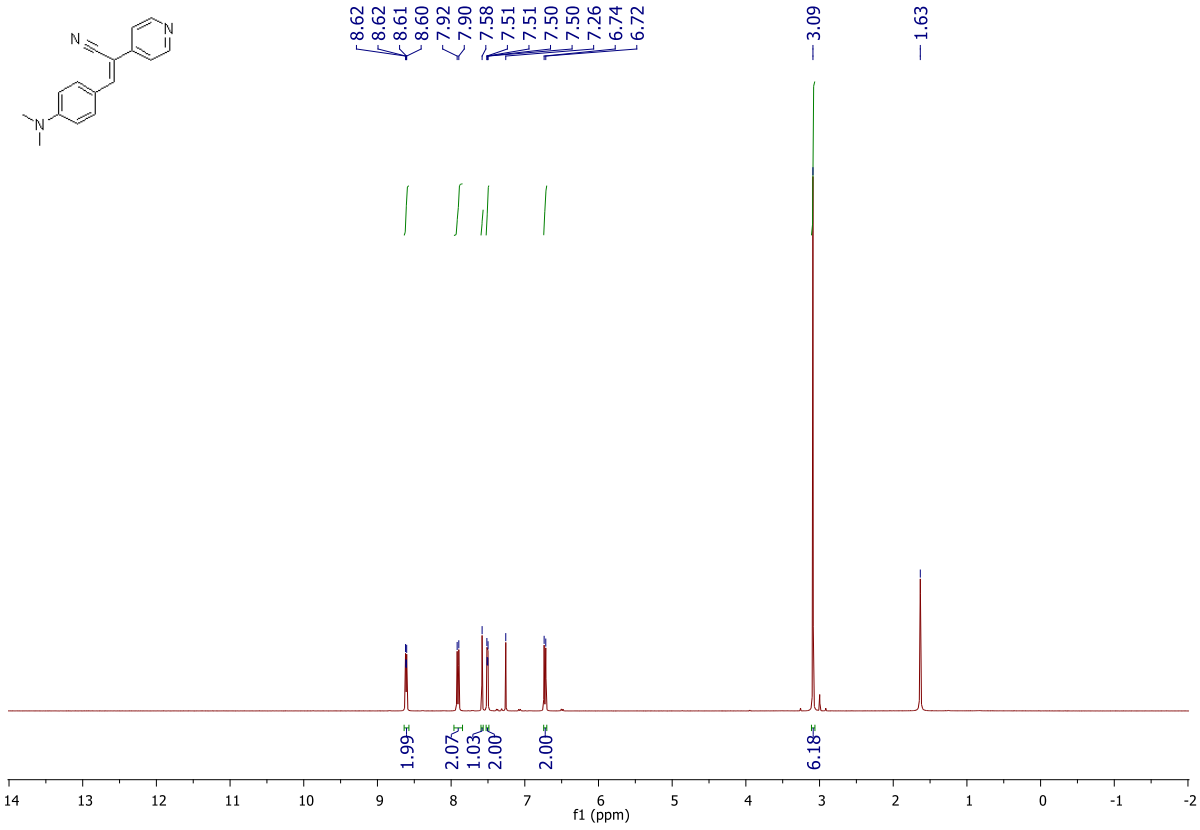


Figure S28 ^{1}H NMR of **1** in $CDCl_3$.

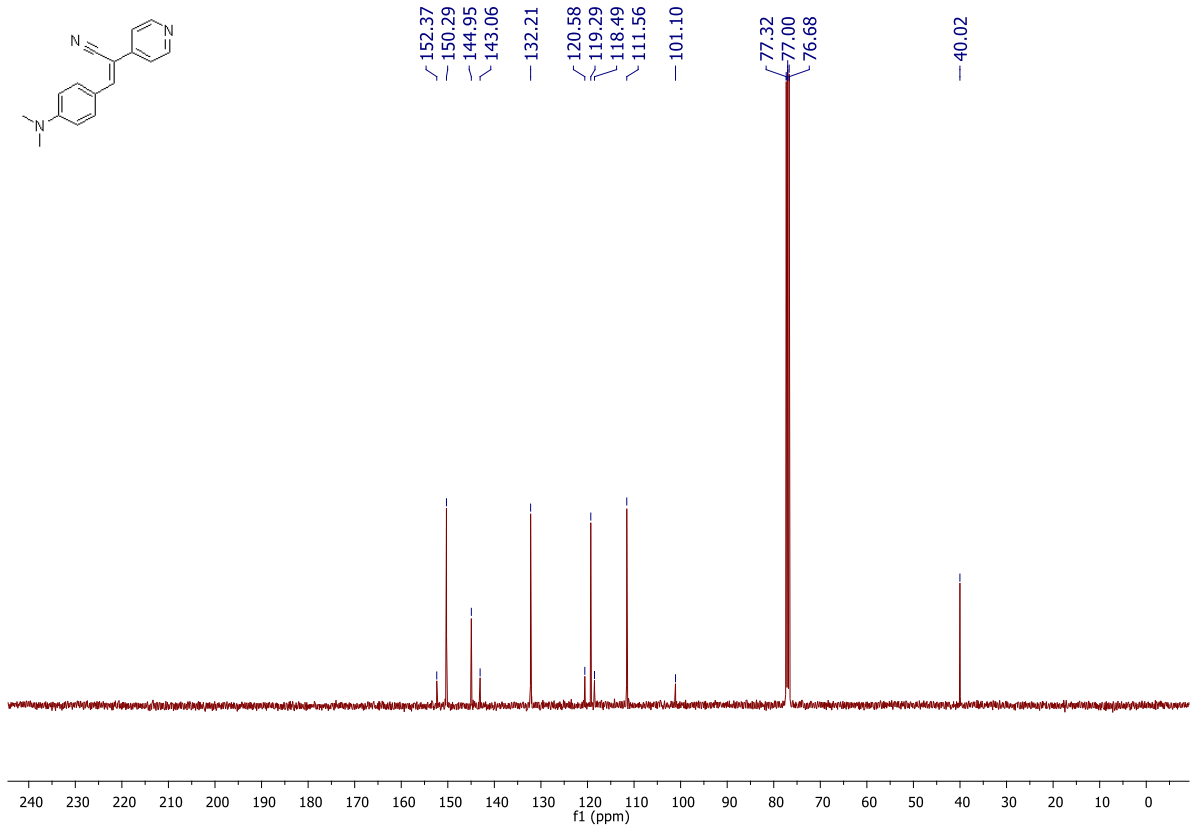


Figure S29 ^{13}C NMR of **1** in $CDCl_3$.

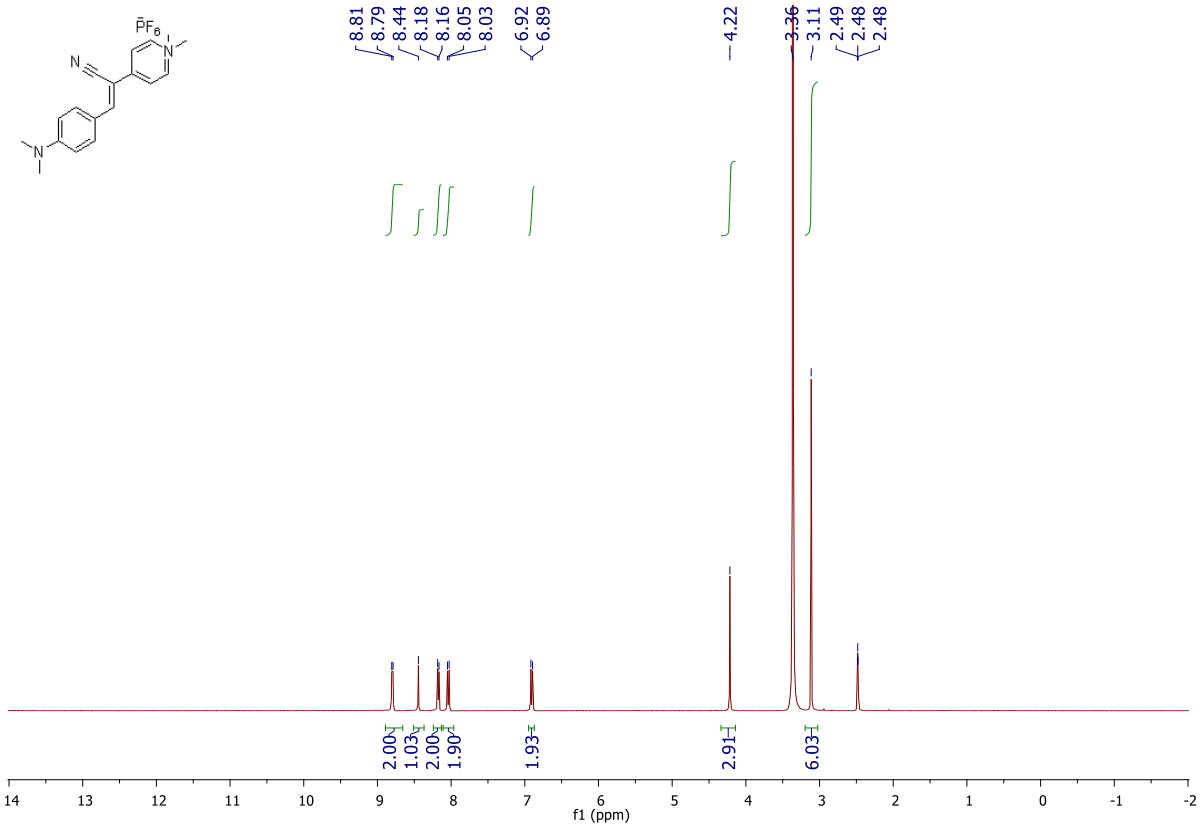


Figure S30 ^{1}H NMR of **ASC** in DMSO.

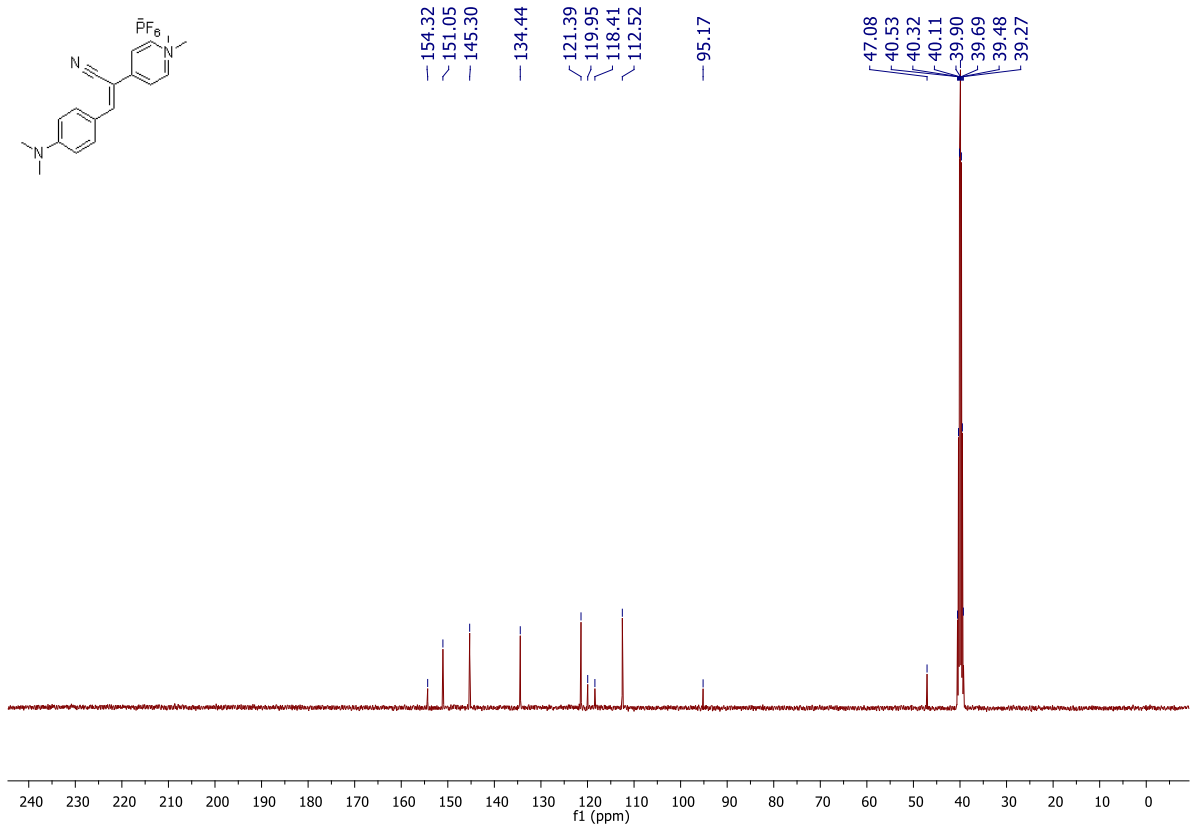


Figure S31 ^{13}C NMR of **ASC** in DMSO.

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