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Electronic Supplementary Information (ESI)

Novel pyrene-based two-photon active fluorescent dye efficiently excited and emitting in the 'tissue optical window (650–1100 nm)'

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1. Instrumentals

NMR and high resolution mass spectrometry

All the ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz JEOL LMN-EX400 instrument with tetramethylsilane (TMS) as the internal standard. HRMS spectra (FAB) were obtained by JEOL JMS-700 mass spectrometer.

Absorption, fluorescence spectroscopy, and absolute fluorescence quantum yield

The UV-Vis spectra were recorded with a JASCO U-670 UV-Vis Spectrophotometer. Fluorescence spectra were obtained using an absolute photoluminescence quantum yield measurement system (C9920-02G, Hamamatsu Photonics K. K.).

All photophysical measurements performed in solutions were carried out using dilute solutions (1 μM) 1 cm path length quarz cells at room temperature (298 K). In addition, all samples solutions were deaerated by bubbling with argon gas for 15 min before the measurements.

TP absorption cross section

Two-photon absorption (TPA) spectra were measured using open-aperture Z-scan measurements.[1] A femtosecond pulsed beam from an optical parametric amplifier (OPA-800C, Spectra-Physics, Santa Clara, CA) pumped by a beam from a regenerative amplifier (Spitfire, Spectra-Physics) was used as the light source. The typical pulse duration was 150–200 fs and the repetition rate was 1 kHz. The incident beam was focused by a plano-convex lens (f = 150 nm). The sample solution was scanned along the incident beam axis. The average peak power was varied from 0.01 to 0.4 mW, corresponding to one-axis peak powers of 6 to 240 GW/cm^2. The measurements revealed that the two-photon absorbance (q_0) is proportional to the incident power (and hence I_0). This is a reliable indication that the transmittance changes observed from the Z-scan measurements were purely due to TPA and not to any other nonlinear optical process.

Single photon fluorescence microscopy

Single photon fluorescence images were taken on a fluorescence microscope Eclipse 50i (Nikon Instruments Inc.) equipped with digital camera DS-Ril (Nikon Instruments Inc.). Red emission of **PY** was collected by with G-2A (excitation filter 510-560 nm, dichroic mirror 565 nm, barrier filter 590 nm). Green emission of **FLW** was collected by V-2A (excitation filter 380-420 nm, dichroic mirror 430 nm, barrier filter 450 nm).

Two-photon fluorescence microscopy

Two-photon fluorescence microscopy (TPFM) was carried out using a Mira Ti:sapphire femtosecond laser (Coherent, Santa Clara, CA) as the light source. A galvano scanner (C10516, Hamamatsu Photonics K. K.) was used as the laser scanning unit. Cells were placed in a 35 mm glass-base dish. **PY** was excited by a 950 nm femtosecond laser through a water immersion objective (UApo 340 40X, OLYMPUS, Tokyo, Japan). The sample was put on a power stage and scanned along the optical axis. A photomultiplier tube (R928, Hamamatsu Photonics K. K.) was used for signal detection.

Computational methodology

The equilibrium structures of the compounds investigated in this work were fully optimized by using the RB3LYP method with 6-31+G** basis set, The Analytical frequencies were obtained to ensure that a local energy minimum has been located. Then, the singlet- and triplet-spin excited states for the minima have been calculated by time-dependent density functional theory (TD-DFT). All calculations were performed by using the Gaussian 09 program package.[2]

2. Materials

Chemicals

Unless otherwise noted, all reagents and chemicals were used without further purification. *n*-butyllithium, 1-bromobutane, titanium tetrachloride, dichloromethy methylether and 4-methylpyridine were obtained from TCI (Tokyo, Japan). Spectrograde DMSO and water, and piperidine were prepared from Wako Pure Chem. (Tokyo, Japan). Iodomethane was purchased from Nacalai Tasque (Kyoto, Japan).

1,6-dibromopyrene 1,6-dibutylpyrene (**1**), and 1,4-dimethylpyridin-1-ium iodide were synthesized from parent pyrene according to previous reports.[3-5]

Sythetic details

Synthesis of 3,8-dibutylpyrene-1,6-dicarbaldehyde (2)

To a stirred solution of 1,6-dibutyllpyrene (0.33 g, 1.06 mmol) and dichloromethyl methyl ether (0.50 mL, 5.30 mmol) in 5 mL of dry CH_2Cl_2 was added at 0 °C a solution of titanium tetrachloride (0.6 mL, 5.30 mmol) in 2 mL of dry CH_2Cl_2. This mixture was stirred for 24 h at room temperature. The reaction mixture was poured into a large amount of ice-water and extracted with three times with chloroform. The combined organic layers were washed with $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel (chloroform:hexane = 3:1) and subsequently recrystallized with MeOH to afford objective compound as a yellow solid (0.16 g, 41 %).

^{1}H NMR (400 MHz, $CDCl_3$): δ 10.8 (s, 2H), 9.52 (d, J = 9.6 Hz, 2H), 8.47 (d, J = 9.6 Hz, 2H), 8.35 (s, 2H), 3.43 (t, J = 7.9 Hz, 4H), 1.89 (tt, J = 7.9 Hz, 7.4 Hz, 4H), 1.56 (tq, J = 7.4 Hz, 7.3 Hz, 4H), 1.05 (t, J = 7.3 Hz, 6H) ppm.; ^{13}C NMR (100 MHz, $CDCl_3$): δ 193.4, 139.1, 133.0, 129.6, 128.4, 126.1, 125.8, 125.6, 34.3, 33.8, 23.3, 14.4 ppm.; M S (FAB) Calcd for $C_{26}H_{26}O_2$: 370.1933, Found: 371 ($[M+H]^+$)

Synthesis of 4,4'-((1*E*,1'*E*)-(3,8-dibutylpyrene-1,6-diyl)bis(ethene-2,1-diyl))bis(1-methylpyridin-1-ium) iodide (PY)

To a solution of 3,8-dibutylpyrene-1,6-dicarbaldehyde (0.10 g, 0.27 mmol) and 1,4-dimethylpyridin-1-ium iodide (0.16 g, 0.67 mmol) in 10 mL of $CHCl_3$ and 30 mL of MeOH was added a catalyc amount of piperidine (5 drops), then the mixture was refluxed for 12 h. The solvents were removed in vacuo and the crude residue was washed with hot MeOH and $CHCl_3$ two times to give objective compounds as a red solid. (0.13g. 59 %)

^{1}H NMR (400 MHz, DMSO): δ 9.06 (d, *J* = 7.9 Hz, 2H), 8.97 (d, *J* = 9.5 Hz, 2H), 8.90 (d, *J* = 6.1 Hz, 4H), 8.54 (s, 2H), 8.48 (d, *J* = 6.1 Hz, 4H), 8.42 (d, *J* = 9.5 Hz, 2H), 7.85 (d, *J* = 7.9 Hz, 2H), 4.29 (s, 6H) 3.46-3.34 (t, *J* = 7.5 Hz, 4H), 1.89 (tt, *J* = 7.5 Hz, 7.5 Hz, 4H), 1.51 (tq, *J* = 7.5 Hz, 7.3 Hz, 4H), 1.05 (t, *J* = 7.3 Hz, 6H) ppm.; ^{13}C NMR (100 MHz, DMSO): δ 153.3, 145.9, 138.9, 137.4, 130.7, 130.3, 129.0, 126.9, 126.6, 126.1, 125.2, 124.8, 124.5, 47.8, 34.7, 33.6, 23.2, 14.8.; HRMS (FAB) Calcd for $C_{40}H_{42}N_2^{2+}$: 550.3348, Found: 550.3351 ([M]$^+$)

3. Water solubility of PY.

Small amount of dye was dissolved in DMSO to prepare the stock solutions (2.6×10^{-3} M and 2.6×10^{-4} M). The solution was added to a measuring flask containing 5.0 mL of spectrograde water by using a micro syringe. In all cases, the concentration of DMSO in water was kept less than to be 1 %. The plots of fluorescence intensity against the total amount of the dye were linear at low dye content and showed downward curvature as more dye was added (**Fig. S1**). We estimated the water solubility to about 2.0 µM, by taking the maximum value of the plot on the straight line at low concentration.

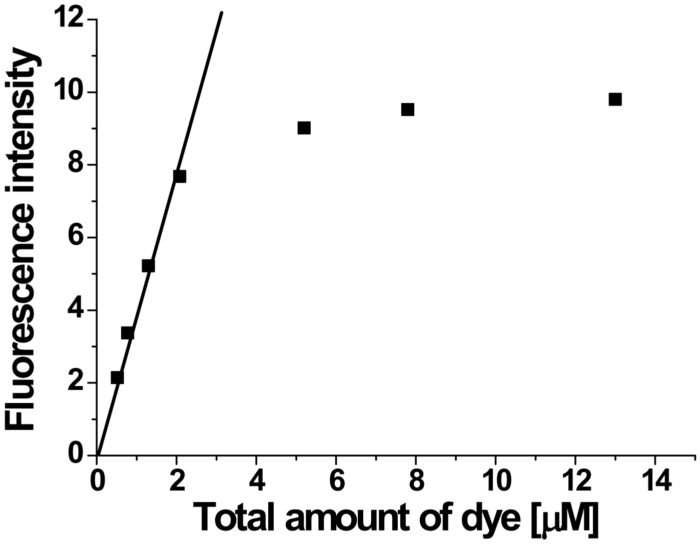

Fig S1. Concentration dependency of the fluorescence intensity of **PY** in water (λ_{ex} = 500 nm).

4. DFT calculation

Using DFT calculation, we investigated at which positions in pyrene and anthracene substitution of an electron acceptor group would efficiently affect the absorption spectra. We adopted the cyano group as an acceptor group instead of the vinyl group-linked pyridinium moieties used experimentally to simplify the calculation.

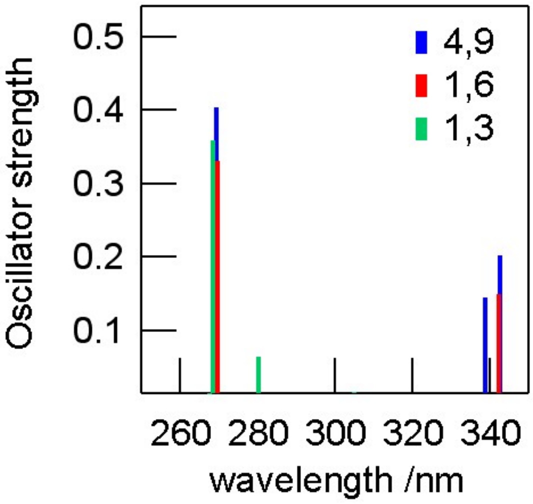

Fig S2. Simulated absorption spectra of 1,3-, 1,6-, and 4,9-dicyanopyrene.

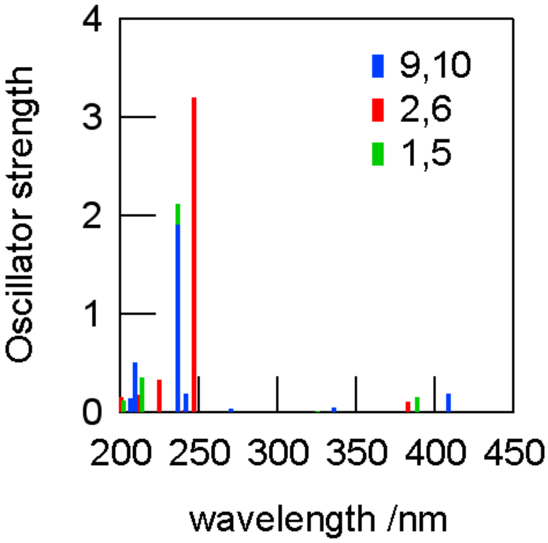

Fig S3. Simulated absorption spectra of 1,5-, 2,6-, and 9,10-dicyanoanthracene.

Table S1. Atom coordinates and absolute energies of 1,3-, 1,6-, and 4,9-dicyanopyrene in theoretical calculations.

1,3-dicyanopyrene (ground): E(RB3LYP) = -800.295612872 A.U.

Center number	Atomic number	Coordinate (Angstroms)		
		X	Y	Z
1	6	-0.018487	-0.000345	-0.010568
2	6	-0.017076	-0.000208	1.387812
3	6	1.203854	-0.000058	2.114284
4	6	2.43183	-0.000049	1.383464
5	6	2.432614	-0.000189	-0.045529
6	6	1.184294	-0.000337	-0.72386
7	6	1.244097	0.000085	3.547564
8	6	3.673515	0.000101	2.089882
9	6	3.686761	0.000242	3.517375
10	6	2.433647	0.000228	4.216138
11	6	4.920437	0.000388	4.19174
12	1	4.929437	0.000495	5.278223
13	6	6.121583	0.000397	3.482635
14	6	6.117409	0.00026	2.0878
15	6	4.907369	0.000111	1.371889
16	6	4.867752	-0.000032	-0.062334
17	6	3.685237	-0.000176	-0.743273
18	1	3.677482	-0.000282	-1.828376
19	1	5.807632	-0.000024	-0.607486
20	1	0.307371	0.000078	4.095333
21	1	-0.960735	-0.000459	-0.54663
22	1	2.445264	0.000336	5.302614
23	1	7.065196	0.000511	4.019474
24	1	7.055958	0.000268	1.540396
25	6	-1.26963	-0.000222	2.082868
26	7	-2.286104	-0.000233	2.650943
27	6	1.141778	-0.00048	-2.155706
28	7	1.110712	-0.000596	-3.319736

1,6-dicyanopyrene (ground): E(RB3LYP) = -800.296877178 A.U.

Center number	Atomic number	Coordinate (Angstroms)

		X	Y	Z
1	6	-0.022541	-3.531854	0
2	6	-1.222176	-2.833189	0
3	6	-1.238187	-1.426443	0
4	6	0.000051	-0.714493	0
5	6	1.238187	-1.425811	0
6	6	1.202906	-2.843793	0
7	6	-2.46404	-0.680262	0
8	6	-0.000051	0.714493	0
9	6	-1.238187	1.425811	0
10	6	-2.467123	0.683413	0
11	6	-1.202906	2.843793	0
12	6	0.022541	3.531854	0
13	6	1.222176	2.833189	0
14	6	1.238187	1.426443	0
15	6	2.46404	0.680262	0
16	6	2.467123	-0.683413	0
17	1	3.405833	-1.228237	0
18	1	3.402786	1.226909	0
19	1	-3.402786	-1.226909	0
20	1	-0.019673	-4.616585	0
21	1	-2.162784	-3.37599	0
22	1	-3.405833	1.228237	0
23	1	0.019673	4.616585	0
24	1	2.162784	3.37599	0
25	6	-2.423124	3.594317	0
26	7	-3.41368	4.206877	0
27	6	2.423124	-3.594317	0
28	7	3.41368	-4.206877	0

4,9-dicyanopyrene (ground): E(RB3LYP) = -800.295115588 A.U.

Center number	Atomic number	Coordinate (Angstroms)		
		X	Y	Z
1	6	0.022587	-3.530278	0
2	6	1.228008	-2.832495	0
3	6	1.238358	-1.426669	0

4	6	-0.000847	-0.714072	0
5	6	-1.228008	-1.443059	0
6	6	-1.19418	-2.846539	0
7	6	2.461885	-0.683965	0
8	6	0.000847	0.714072	0
9	6	1.228008	1.443059	0
10	6	2.468084	0.687936	0
11	6	1.19418	2.846539	0
12	1	2.126941	3.401774	0
13	6	-0.022587	3.530278	0
14	6	-1.228008	2.832495	0
15	6	-1.238358	1.426669	0
16	6	-2.461885	0.683965	0
17	6	-2.468084	-0.687936	0
18	1	-3.402701	1.225747	0
19	1	3.402701	-1.225747	0
20	1	0.02619	-4.615753	0
21	1	2.171825	-3.370316	0
22	1	-2.126941	-3.401774	0
23	1	-0.02619	4.615753	0
24	1	-2.171825	3.370316	0
25	6	3.719201	1.387746	0
26	7	4.729897	1.965437	0
27	6	-3.719201	-1.387746	0
28	7	-4.729897	-1.965437	0

Table S2. Atom coordinates and absolute energies of 1,5-, 2,6-, and 9,10-dicyanoanthracene in theoretical calculations.

1,5-dicyanoanthracene (ground): E(RB3LYP) =		-724.053253220		A.U.
Center number	Atomic number	Coordinate (Angstroms)		
		X	Y	Z
1	6	0.007988	-0.001266	-0.028384
2	6	0.009101	0.003823	1.344004
3	6	1.233954	0.003029	2.08113
4	6	2.48669	-0.003236	1.365005

5	6	2.440722	-0.00846	-0.078703
6	6	1.232454	-0.007462	-0.747758
7	6	1.243547	0.008206	3.483735
8	6	3.68476	-0.00401	2.086509
9	6	3.694353	0.001166	3.489114
10	6	2.441616	0.007432	4.205239
11	6	2.487584	0.012656	5.648947
12	6	3.695852	0.011658	6.318002
13	6	4.920319	0.005461	5.598627
14	6	4.919205	0.000373	4.22624
15	1	0.297168	0.01291	4.016793
16	1	-0.92844	-0.000603	-0.576662
17	1	-0.927024	0.008564	1.895212
18	1	1.222637	-0.011459	-1.832635
19	1	4.631139	-0.008714	1.553451
20	1	3.70567	0.015654	7.402879
21	1	5.856746	0.004798	6.146906
22	1	5.855331	-0.004369	3.675032
23	6	3.66467	-0.014752	-0.824165
24	7	4.665924	-0.019842	-1.418668
25	6	1.263636	0.018947	6.394409
26	7	0.262382	0.024037	6.988912

2,6-dicyanoanthracene (ground): E(RB3LYP) = -724.052512885 A.U.

Center number	Atomic number	Coordinate (Angstroms)		
		X	Y	Z
1	6	-0.018689	3.728361	0
2	6	-0.926016	2.688802	0
3	6	-0.480319	1.334489	0
4	6	0.940325	1.067248	0
5	6	1.847861	2.174812	0
6	6	1.392977	3.464528	0
7	6	-1.383377	0.260496	0
8	6	1.383377	-0.260496	0
9	6	0.480319	-1.334489	0
10	6	-0.940325	-1.067248	0

11	6	-1.847861	-2.174812	0
12	1	-2.91542	-1.974164	0
13	6	-1.392977	-3.464528	0
14	6	0.018689	-3.728361	0
15	6	0.926016	-2.688802	0
16	1	-1.991453	2.896861	0
17	1	2.91542	1.974164	0
18	1	2.084551	4.299911	0
19	1	-2.084551	-4.299911	0
20	1	1.991453	-2.896861	0
21	1	2.451154	-0.464735	0
22	1	-2.451154	0.464735	0
23	6	0.480319	-5.086622	0
24	7	0.843353	-6.192602	0
25	6	-0.480319	5.086622	0
26	7	-0.843353	6.192602	0

9,10-dicyanoanthracene (ground): E(RB3LYP) = -724.050945626 A.U.

Center number	Atomic number	Coordinate (Angstroms)		
		X	Y	Z
1	6	-3.673395	0.711275	0
2	6	-2.491438	1.407635	0
3	6	-1.239253	0.719981	0
4	6	-1.23909	-0.720251	0
5	6	-2.491115	-1.408194	0
6	6	-3.673233	-0.712101	0
7	6	-0.000155	1.406481	0
8	6	0.000155	-1.406481	0
9	6	1.239253	-0.719981	0
10	6	1.23909	0.720251	0
11	6	2.491115	1.408194	0
12	1	2.492488	2.493221	0
13	6	3.673233	0.712101	0
14	6	3.673395	-0.711275	0
15	6	2.491438	-1.407635	0
16	1	-4.617065	1.24791	0

17	1	-2.493099	2.492661	0
18	1	-2.492488	-2.493221	0
19	1	-4.61678	-1.24895	0
20	1	4.61678	1.24895	0
21	1	4.617065	-1.24791	0
22	1	2.493099	-2.492661	0
23	6	0.000339	-2.838105	0
24	7	0.000339	-4.002757	0
25	6	-0.000339	2.838105	0
26	7	-0.000339	4.002757	0

5. Confirmation of selective staining of PY into mitochondria in Hek293 cells.

Cell lines, culture conditions and treatment

Hek293 cells were grown in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich Japan), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Japan) at 37 °C in a humidified atmosphere containing 5% CO_2. Hek293 cell were treated with medium containing 500 nM of **PY** for 16 hours. For imaging, Hek293 cells were washed several times with phenol-red-free medium Opti-MEM (Invitrogen) supplemented with 10% (v/v) FBS.

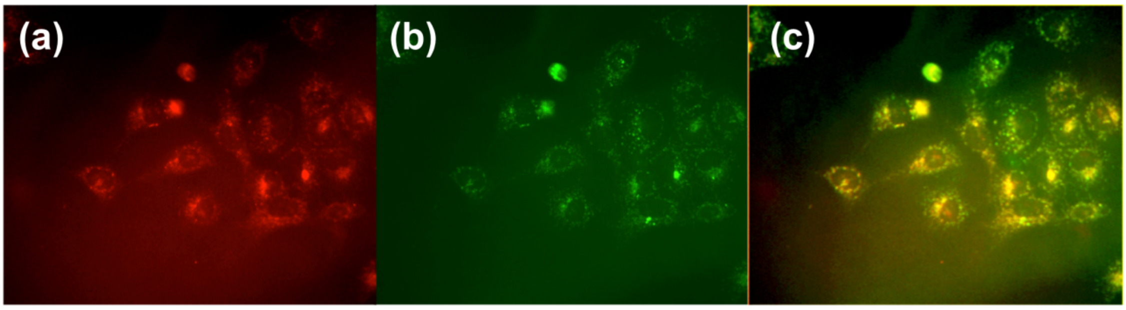

Fig. S4 One-photon fluorescence microscope images of Hek293 cells. (a) shows cells stained with **PY**. (b) shows cells stained with **FLW**. (c) is a marged image of (a) and (b).

6. ^{1}H and ^{13}C NMR spectra

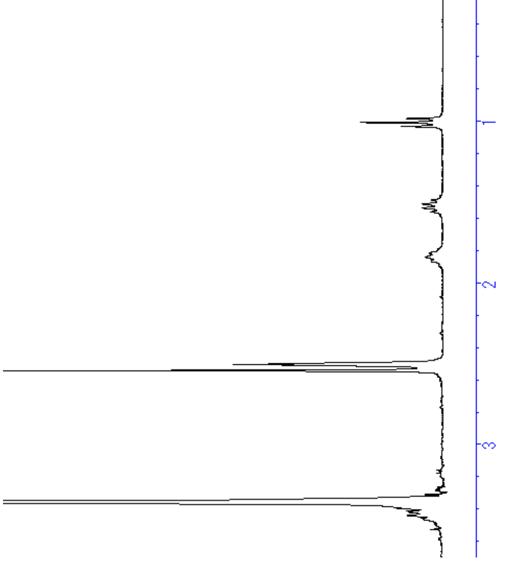

Fig. S5 ^{1}H NMR spectrum of **PY** (D

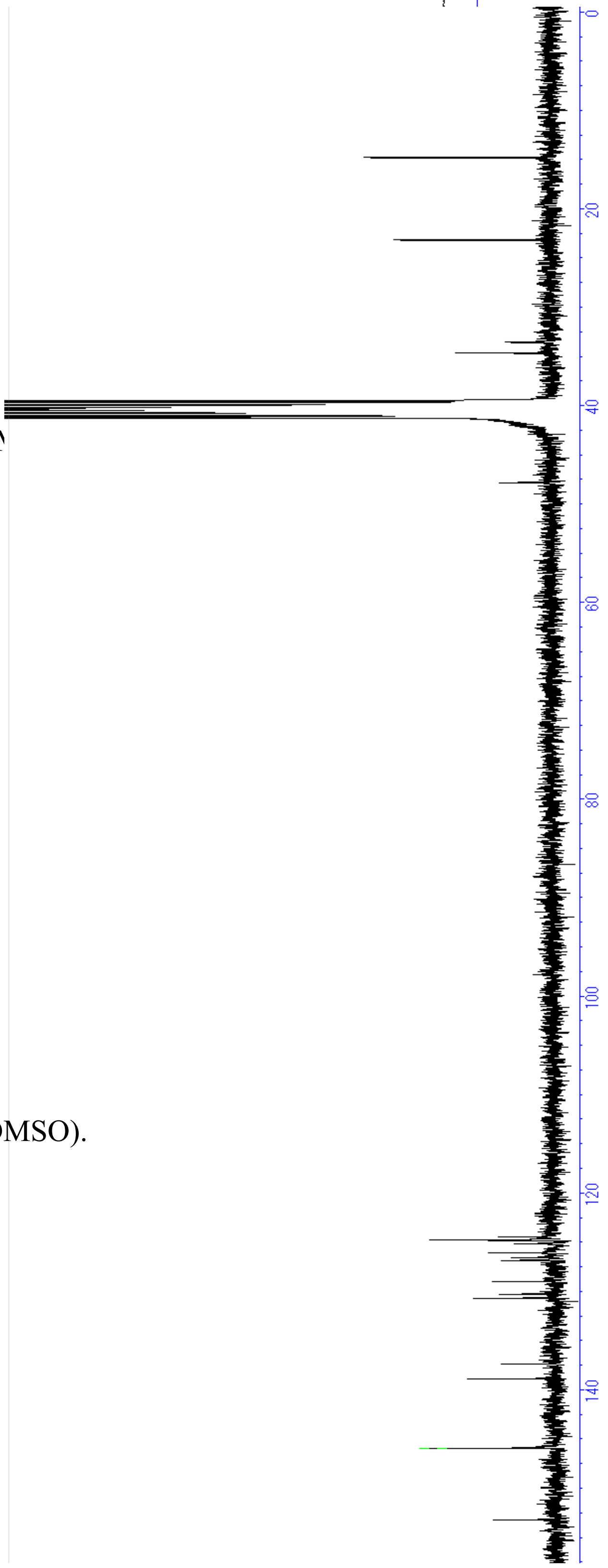

Fig. S6 ^{13}C NMR spectrum of **PY** (DMSO).

7. References.

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