Supporting information

Probing Serum Albumin Binding Site of Fenamates and Photochemical Protein Labeling with a Fluorescence Dye

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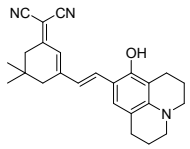
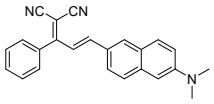
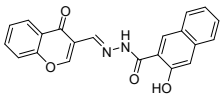
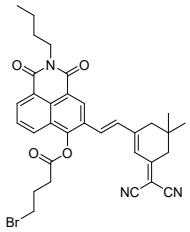
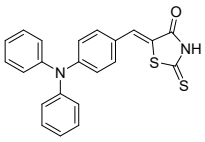
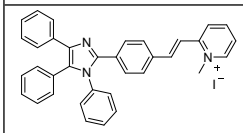
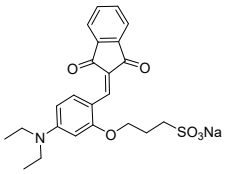
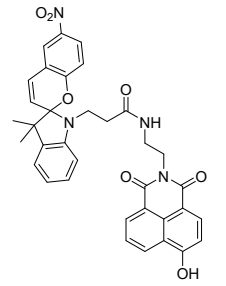
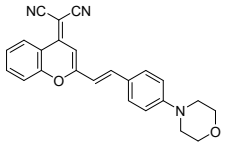
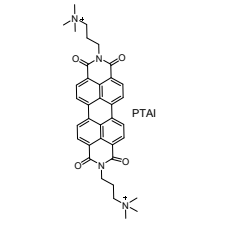
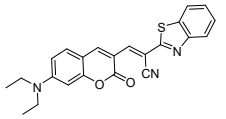
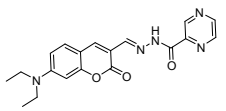
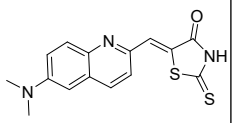
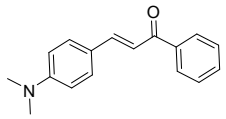
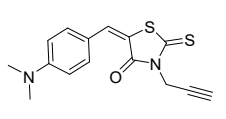
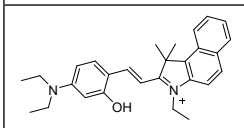
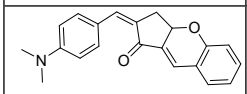
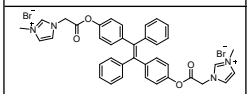
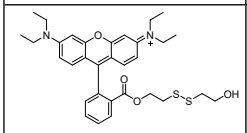
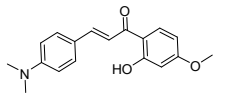
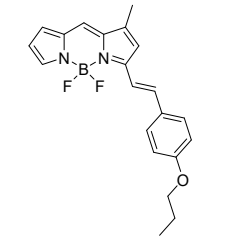
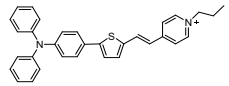
Contents

1. A table summarizing the recent HSA probes.

Table S1. Recent developments of HSA fluorescence probes

Probe structure	On/ Off	λ_{ex}, λ_{em}	Mechanism	LOD	Linear Range	Application	Ref.
 DCO2	on	590 nm 685 nm	TICT	4.64 nM	0-8 μM	HSA detection	[1]
 DNPM	on	510 nm 630 nm	ICT	0.16 μM	0-15 μM	HSA detection	[2]
 HCNH	on	370 nm 527 nm	--	10.62nM	0-4.5 μM	HSA detection	[3]
 NI-1	on	520 nm 670 nm	TICT	0.21 nM	0-0.76μM	HSA detection	[4]
 DPAR	on	485 nm 575 nm	TICT	14.87μM	0-1.36μM	HSA detection	[5]
 YS8	on	368 nm 540 nm	TICT	0.06 μM	0-1.0μM	HSA detection	[6]
 TC426	on	488 nm 550 nm	AIE	3.74 nM	0-14.8μM	HSA detection	[7]

 HSP	on	460 nm 540 nm	--	2.90 nM	0-3.0μM	HSA detection	[8]
 DCM-ML	on	480 nm 630 nm	--	3.79 nM	0-1.2μM	HSA detection	[9]
 PTAI	off	500 nm 549 nm	--	--	--	Drug (Tiagabine hydrochloride) detection	[10]
 Probe 1	on	545 nm 583 nm	TICT	0.29 nM	--	HSA detection	[11]
 NPC	on	450 nm 513 nm	ICT	8.97 nM	0-5.0μM	HSA detection	[12]
 NJUP1	on	460 nm 574 nm	TICT	18.64 nM	0-15.2μM	HSA detection	[13]
 CD1	on	400 nm 535 nm	TICT	8.65 nM	0-10.0μM	HSA detection	[14]
 RhHSA	on	436 nm 508 nm	TICT	4.55 nM	0-7.6μM	HSA detection	[15]
	on	540 nm 596 nm	TICT	6.50 nM	--	HSA detection	[16]

Probe L							
 SCD	on	490 nm 583 nm	TICT	2.28 μM	--	HSA detection	[17]
 TPE-IL	on	360 nm 460 nm	AIE	0.11 nM	0-0.15nM	HSA detection	[18]
 RHO-HS	on	550 nm 595 nm	--	0.87 μM	--	HSA detection	[19]
 4MC	on	400 nm 520 nm	AIE	16.40 nM	0-9 μM	HSA detection	[20]
 BD140	on	520 nm 585 nm	--	--	--	HSA detection, Drug-HSA interaction analysis	[21]
 TTPy	on	470 nm 650 nm	TICT	38 nM	0-15 μM	HSA detection, Drug-HSA interaction analysis	This work

Note: "--"stands for no data or not available. "On" or "Off" means the fluorescence status upon HSA binding.

2. Photophysical properties of the synthetic probes

PBS buffer (pH 7.4) was used thoroughly in the preparation of aqueous mediums unless otherwise indicated.

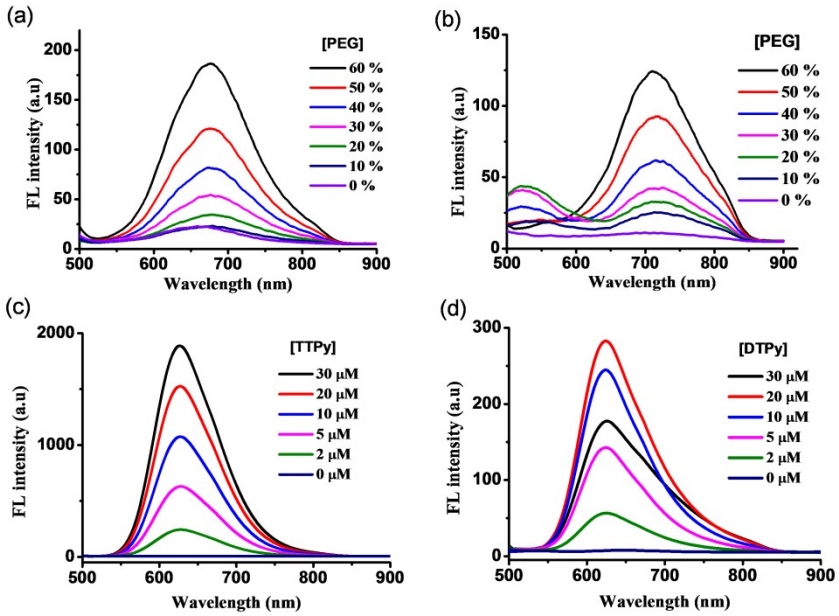


Fig. S1. (a) Fluorescence spectra of TTPy (20 μM) in PEG solutions; (b) Fluorescence spectra of DTPy (20 μM) in PEG solutions; (c) Fluorescence spectra of TTPy at different concentrations in 0.1% SDS solutions; (d) Fluorescence spectra of DTPy at different concentrations in 0.1% SDS solutions. TTPy was excited at 475 nm, DTPy was excited at 460 nm. PBS buffer (pH7.4) was used thoroughly to prepare aqueous mediums. The spectra were collected by a fluorescencespectrometer.

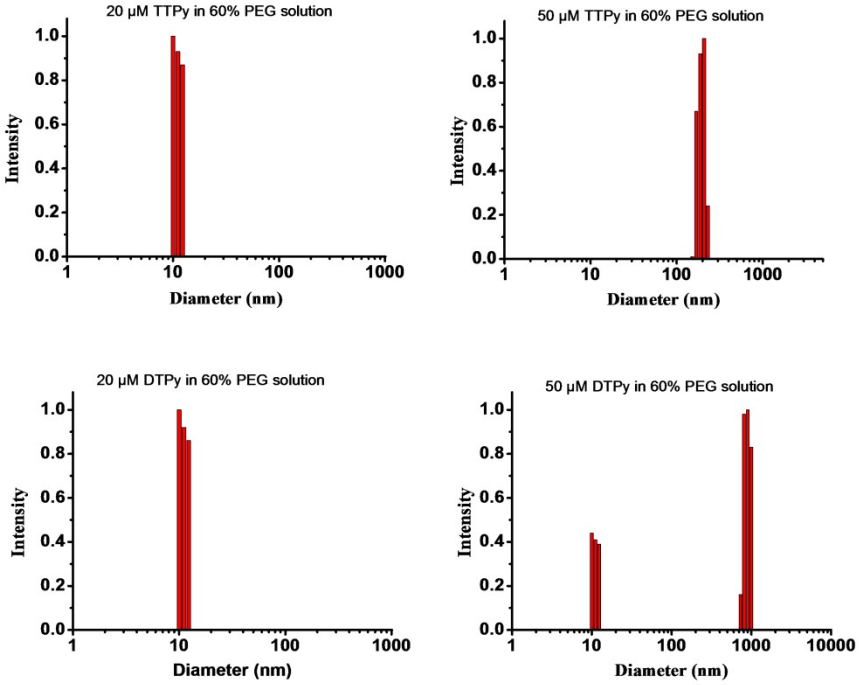


Fig.S2. DLS analysis of the aggregates formed in the TTPy and DTPy PEG solutions. Aggregates with lager size formed when increases the concentration of the probes.

3. Spectroscopic result of TTPy-HSA binding

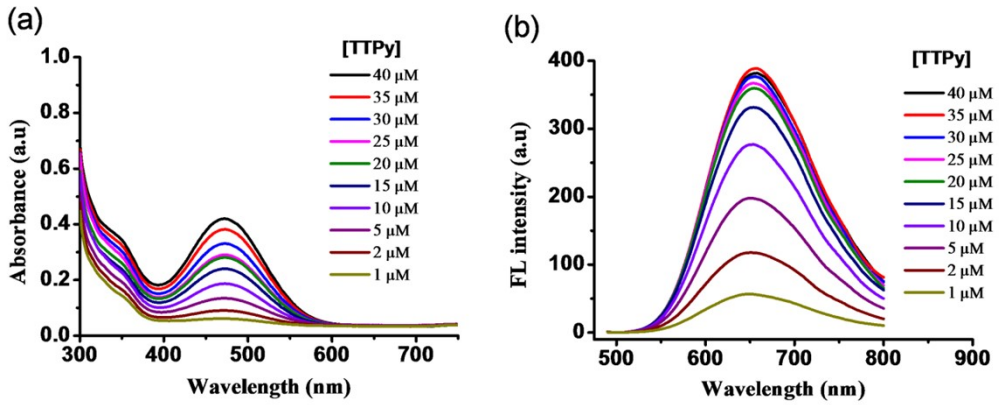


Fig. S3. The absorbance (a) and the fluorescence (b) spectra of TTPy at different concentrations in the presence of HSA (20 μM). TTPy was exited at 470 nm.

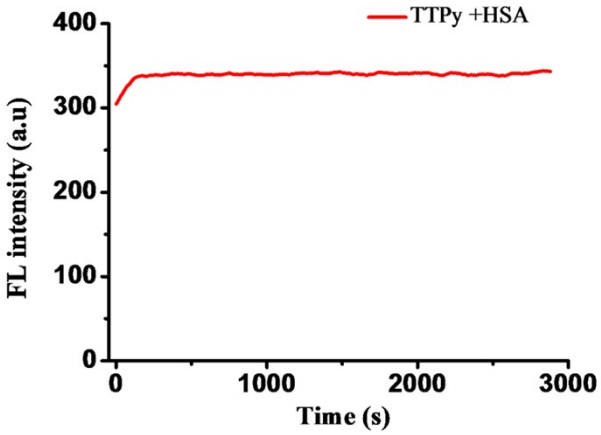


Fig. S4. The kinetic curve indicates the fluorescence intensity at 650 nm against the incubation times. The fluorescence was recorded immediately upon mixing. TTPy and HSA both at 20 μM were applied, TTPy was excited at 470 nm.

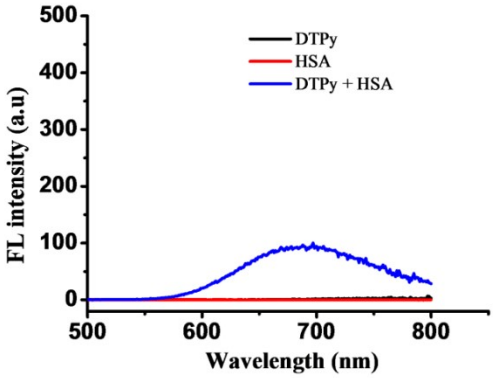


Fig. S5. The fluorescence spectra of DTPy (20 μM), HSA (20 μM) and their mixture in PBS buffer. The solutions were excited at 478 nm after 30 min incubation at rt.

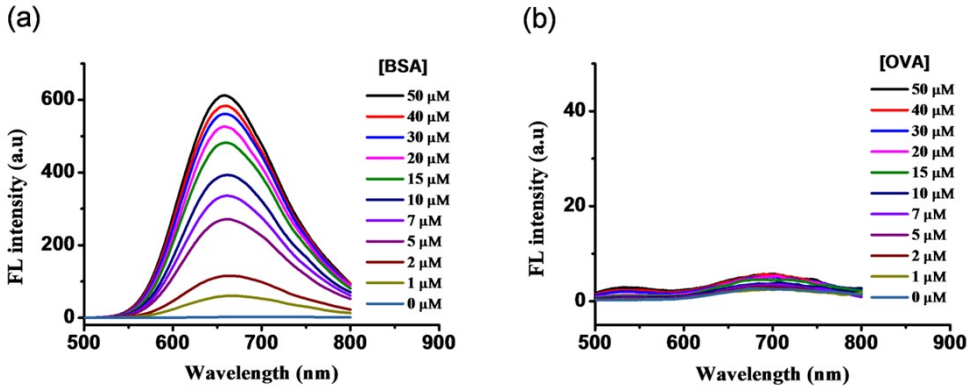


Fig. S6. The fluorescence spectra of TTPy 20 μM in the presence of BSA (a) and OVA (b) at different concentrations (0 to 50 μM). TTPy was exited at 470 nm.

4. Thermodynamic study of TTPy-HSA binding

The plate reader was set at the targeted temperature. PBS solutions of TTPy and HSA (at different concentrations), separately, in a 96-well plate were incubated for few minutes before mixing them together. The final concentration for TTPy is 10 μM, HSA (1 to 5μM). The mixtures were incubated at the targeted temperature for 30 min. The fluorescence was then recorded, TTPy was excited at 470 nm, the emission at 650 nm was collected. 3 repeats were performed for each condition.

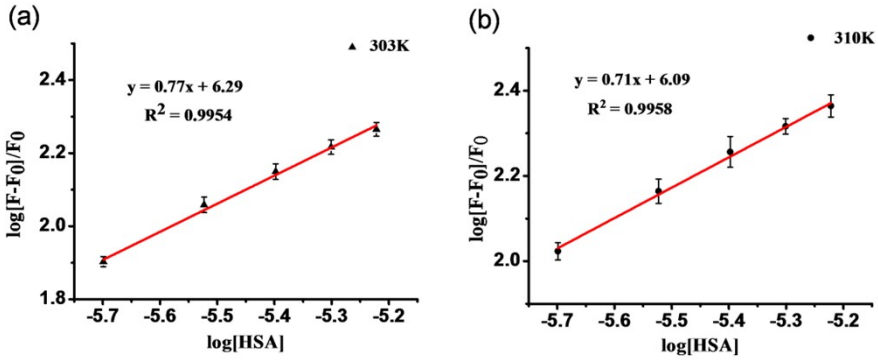


Fig. S7. The Stern-Volmer curves of TTPy-HSA system at 303 and 310 K.

The parameters calculation was performed by following the equation S1 to S3 as described in previous reports[22, 23]. F_0 stands for the intensity in the absence of HSA, F stands for the intensity of TTPy with HSA.[HSA]is the concentration of HSA.

$\log[(F-F_0)/F_0]=\log K+n\log[HSA]$ Equation S1

$\ln(K_2/K_1)=(1/T_1-1/T_2)\times(\Delta H/R)$ Equation S2

$\Delta G=\Delta H-T\times\Delta S=-RT\ln K$ Equation S3

5. Molecule docking

The crystal structure of HSA (PDB ID: 1bj5) was found from the open source protein data bank (PDB) https://www.rcsb.org/. The crystal structure was then opened with Sybyl-X2.1.1, the intrinsic

protein ligand, surrounding water molecules and ions were removed before docking. Molecular docking and calculation were conducted following the description in the article.

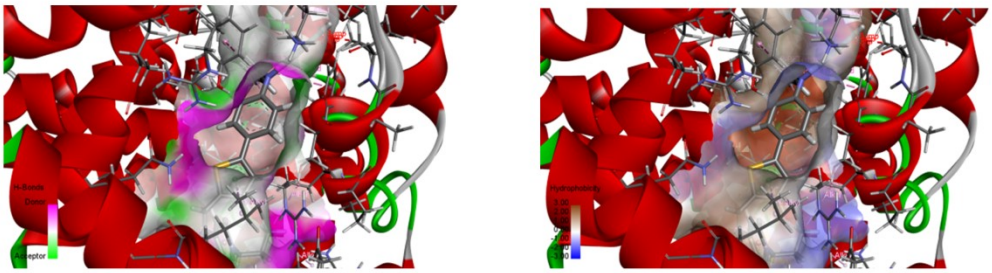


Fig. S8. The hydrogen bond surface area and hydrophobic surface area of the binding pocket obtained through molecular docking.

Table S2. T score and binding energy from Docking.

Protein	Resolution (Å)	TTPy		Mefenamic acid		Clofenamic Acid		Tolfenamic acid	
		Tscore	△G° kJ/mol	Tscore	△G° kJ/mol	Tscore	△G° kJ/mol	Tscore	△G° kJ/mol
1bj5	2.5	11.23	-64.09	7.29	-41.57	7.67	-43.77	6.74	-38.46

6. Competitive binding assay

For the competitive binding study,TTPywas firstly mixed with the testing drug in 1x PBS solution (pH 7.4) at a certain concentration. HSA was then added to the mixture to ensure the final concentrations of HSA and TTPy were both at 10 μM, the testing drug was at aimed concentrations. After 30 min incubation in a 96-well plate at room temperature, the fluorescence was then recorded by a plate reader. The emission at 650 nm upon excitation at 470 nm was collected unless otherwise indicated. Three repeats were set for each assay.

Fluorescence quenching rate was calculated using fluorescence intensity $I_{650\ nm}$, the rate was defined as the ratio of $[(I_{TTPy+HSA} - I_{TTPy+HSA+Drug}) / I_{TTPy+HSA}] \times 100\%$.

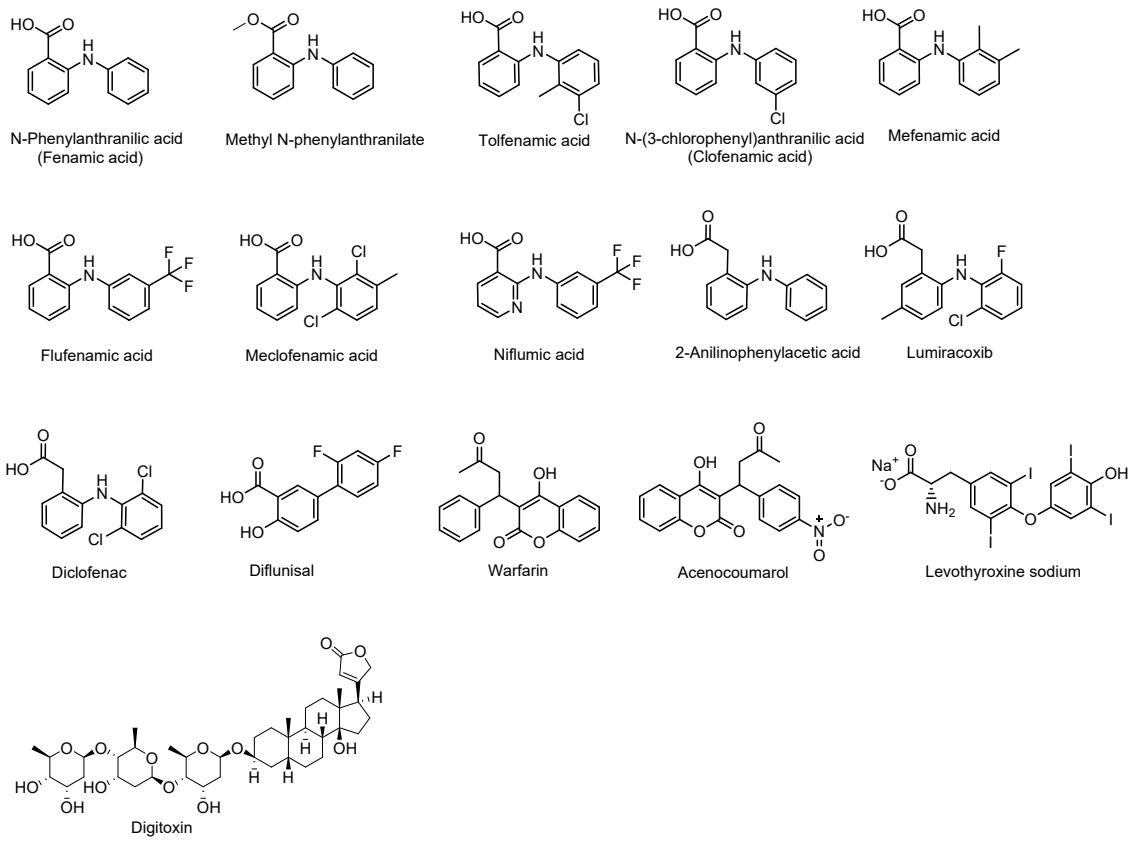


Fig. S9. Chemical structures of the drugs and compounds tested in the study.

Table S3. IC_{50} values of the tested drugs in inhibition of the fluorescence from TTPy-HSA system

No	Drugs	IC_{50} (μM)
1	Tolfenamic acid	12.14
2	Mefenamic acid	12.52
3	Flufenamic acid	36.04
4	Clofenamic acid	16.82
5	Meclofenamic acid	40.30
6	Niflumic acid	29.14
7	Diflunisal	11.48
8	Warfarin	>100
9	Acenocoumarol	>100
10	Levothyroxine	35.11
11	Digitoxin	>100

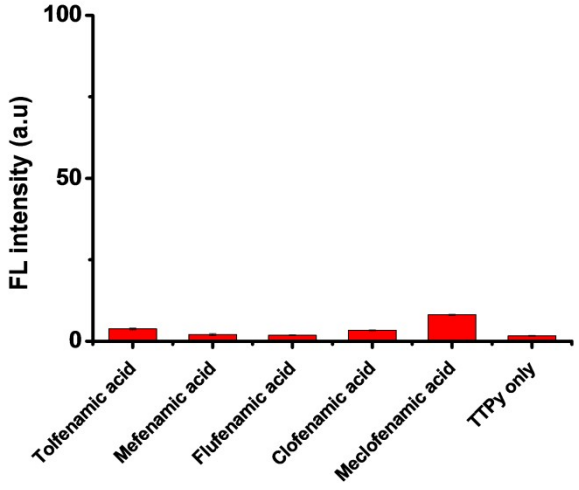


Fig. S10. Fluorescence intensity of TTPy (10 μM) solutions in the presence of 100 μM tolfenamic acid, mefenamic acid, flufenamic acid, clofenamic acid and meclofenamic acid respectively. The emission at 650 nm upon excitation at 470 nm was collected.

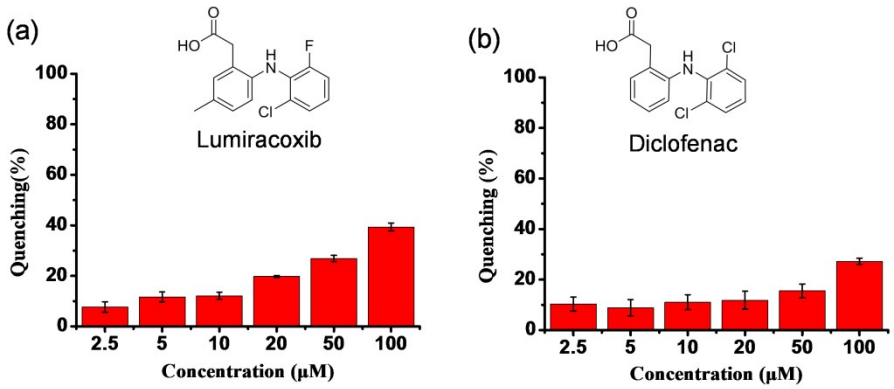


Fig. S11. (a) Fluorescence quenching rate by lumiracoxib at different concentrations; (b) Fluorescence inhibition rate by diclofenac at different concentrations. TTPy and HSA both at 10 μM were applied.

7. Photochemical protein labeling

The protein (10 μM) was incubated with TTPy (20 μM) in PBS buffer (pH 7.4) at rt for 30 min. The TTPy-protein solution was then exposed to an LED light (490 nm, 5W) for 2 min or 4 min before analysis by SDS-PAGE electrophoresis. 10%SDS-PAGE was prepared according its standard procedure. Gel electrophoresis was performed using a Bio-Rad Mini-PROTEAN® electrophoresis system. A constant voltage mode was set, 30 min at 80 V and then 100V untilthe indicator bromophenol bluerunning out of the gel.Fluorescence images were taken by Bio-Rad ChemiDoc MP Imaging System through EB channel. After the fluorescence imaging, the gels were stained again with coomassie brilliant blue.

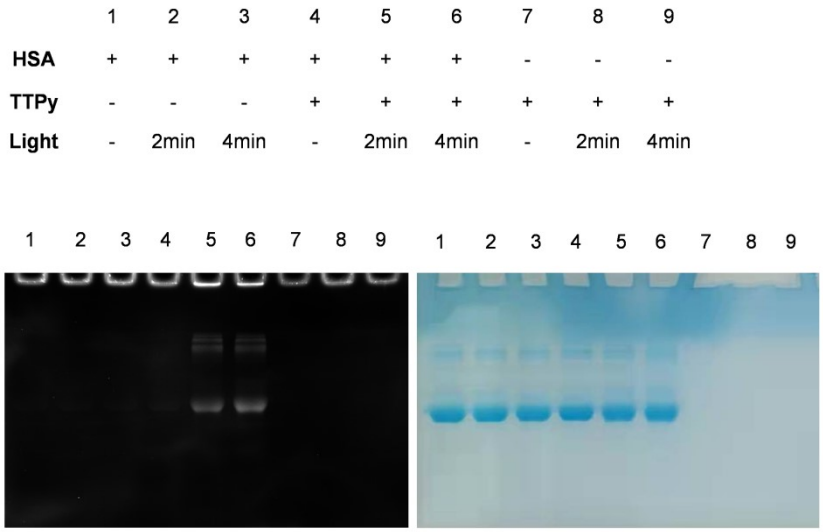


Fig. S12. Fluorescence imaging and coomassieblue staining of the PAGEgel with HSA under different treatments. HSA and TTPy were applied at 10 μM and 20 μM respectively. Irradiation was performed with a LED light (490 nm, 5W).

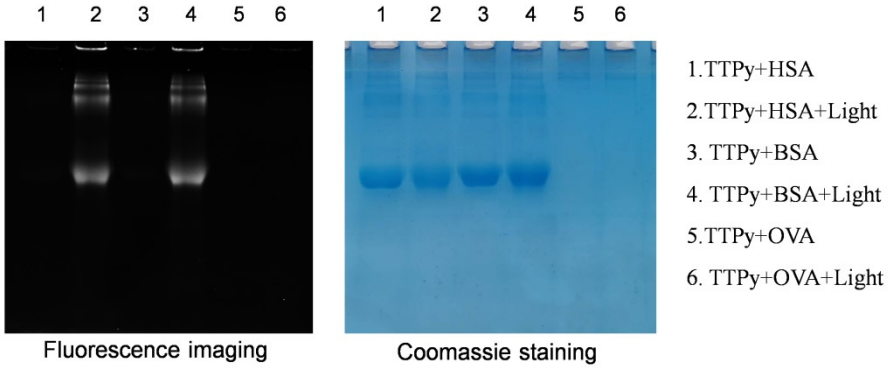


Fig. S13. Fluorescence imaging and coomassieblue staining of the PAGE gel with BSA and OVA under different treatments. Proteins and TTPy were applied at 10 μM and 20 μM respectively. Irradiation were performed with a LED light (490 nm, 5W) for 4 min.

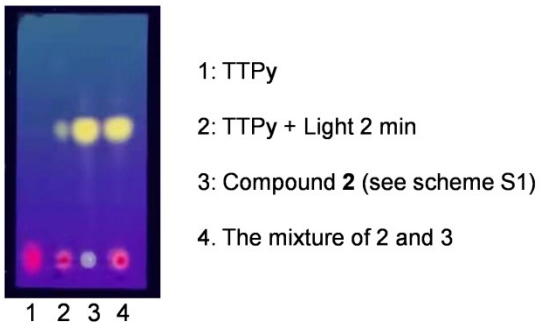


Fig. S14. Thin layer chromatography (TLC) analysis of the photochemical product of TTPy. TTPy in 0.1% SDS solution was irradiated by a LED light (490 nm, 5W) for 2 min. The eluvent is ethyl acetate/petroleum ether 1:5.

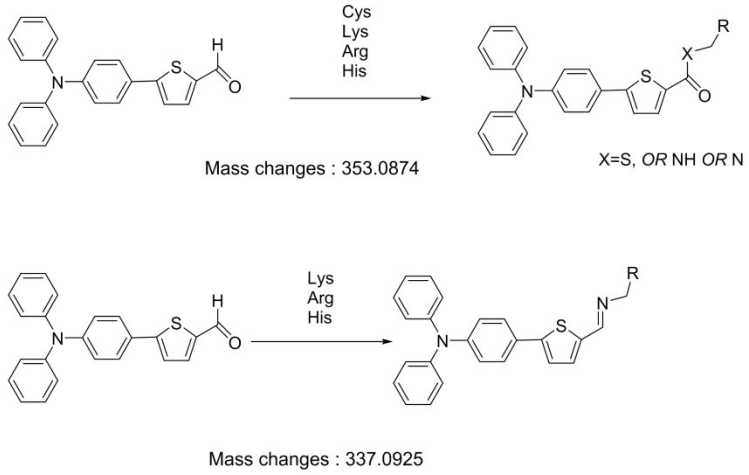


Fig. S15. The possible reaction between the photochemical product and amino acid residues.

After exposure to the LED light for 4 min, TTPy-HSA solution was digested with Chymotrypsin and Trypsin, respectively. The peptides were analyzed by a Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC-MS/MS) (conducted by Guangzhou FitGene Biotechnology Co., Ltd., China). As shown in Fig. S15, two common reactions between arylaldehyde and the potential amino acid residues with typical nucleophilic groups (such as Cys, Lys, Arg and His) have been considered. The calculated exact mass changes were used for the identification of labeling. Unfortunately, this is no reliable residue-labeling information has been found from LC-MS/MS analysis. The reaction between the aldehyde compound and amino acid residues in a protein might be complicated. More experiments are essential to reveal the exact reaction mechanism behind.

8. NMR and FTIR spectra

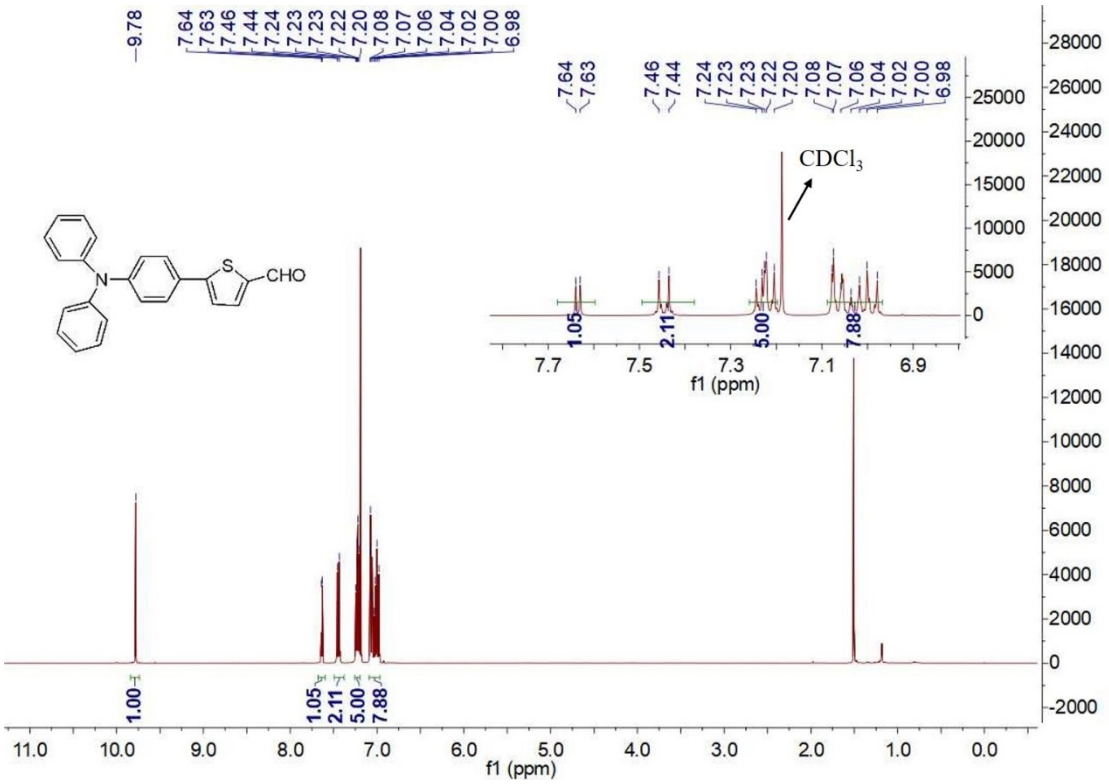


Fig.S16. [1]H NMR Spectrum of compound **2**

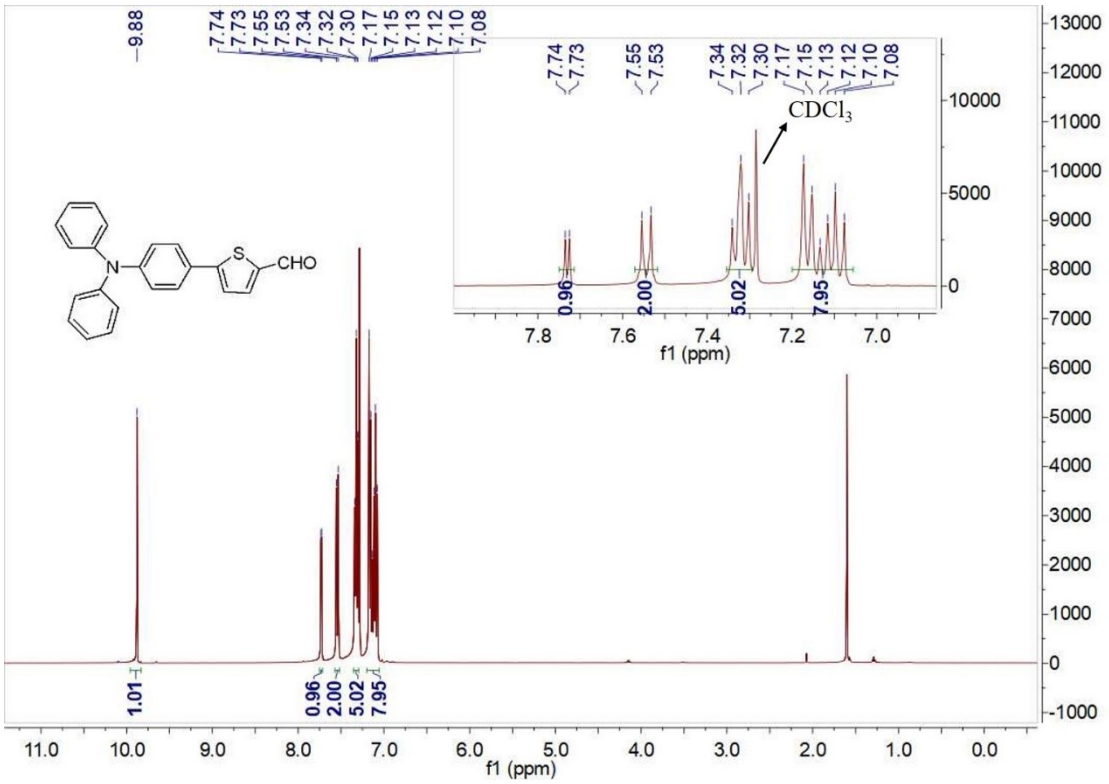


Fig. S17. [1]H NMR Spectrum of the photochemical product from **Fig. S14**

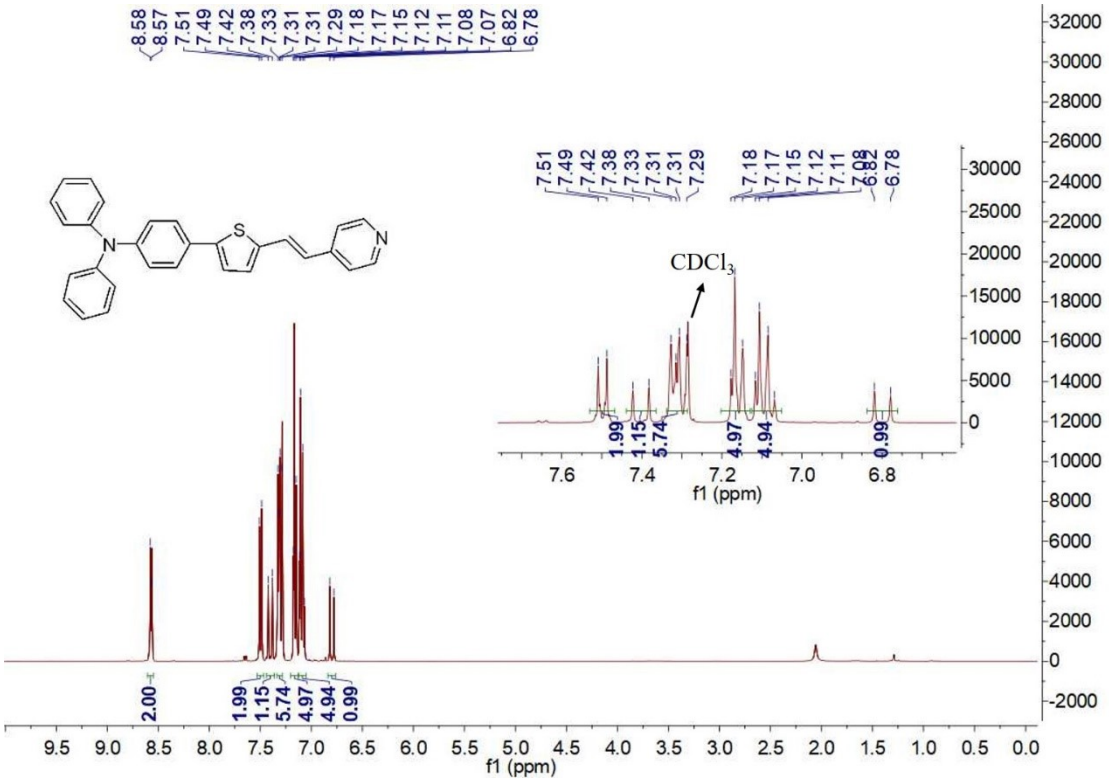


Fig.S18. [1]H NMR Spectrum of compound **3**

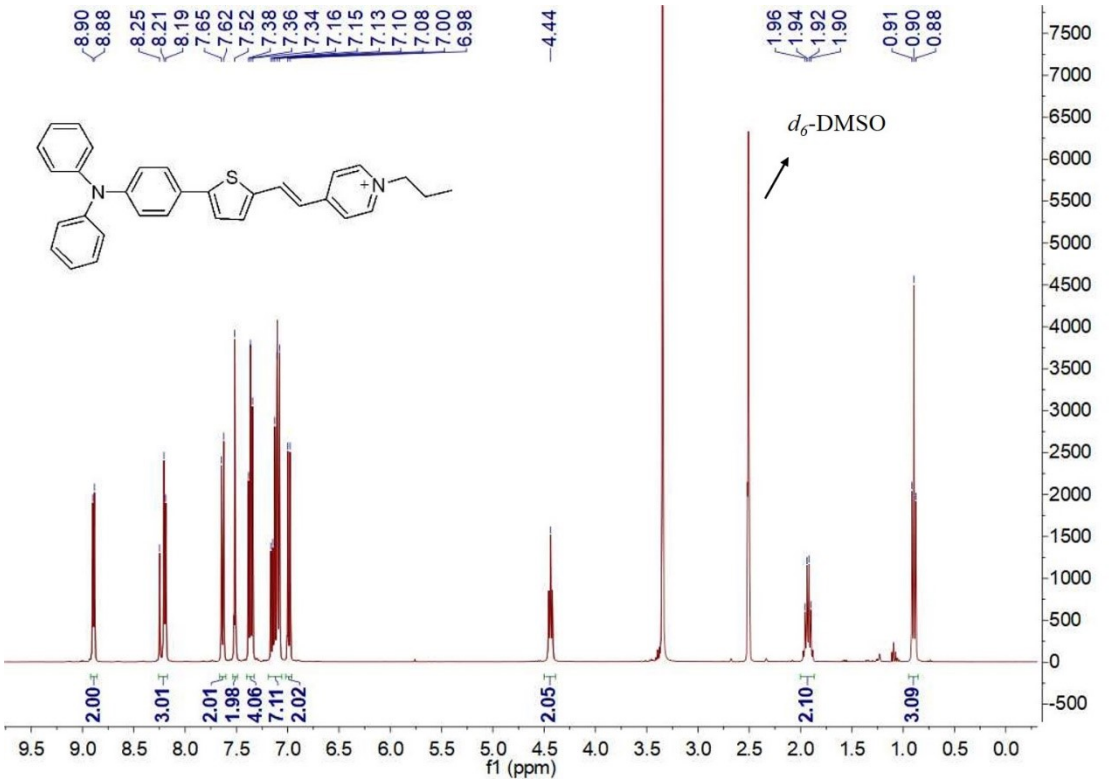


Fig.S19. [1]H NMR Spectrum of **TTPy**

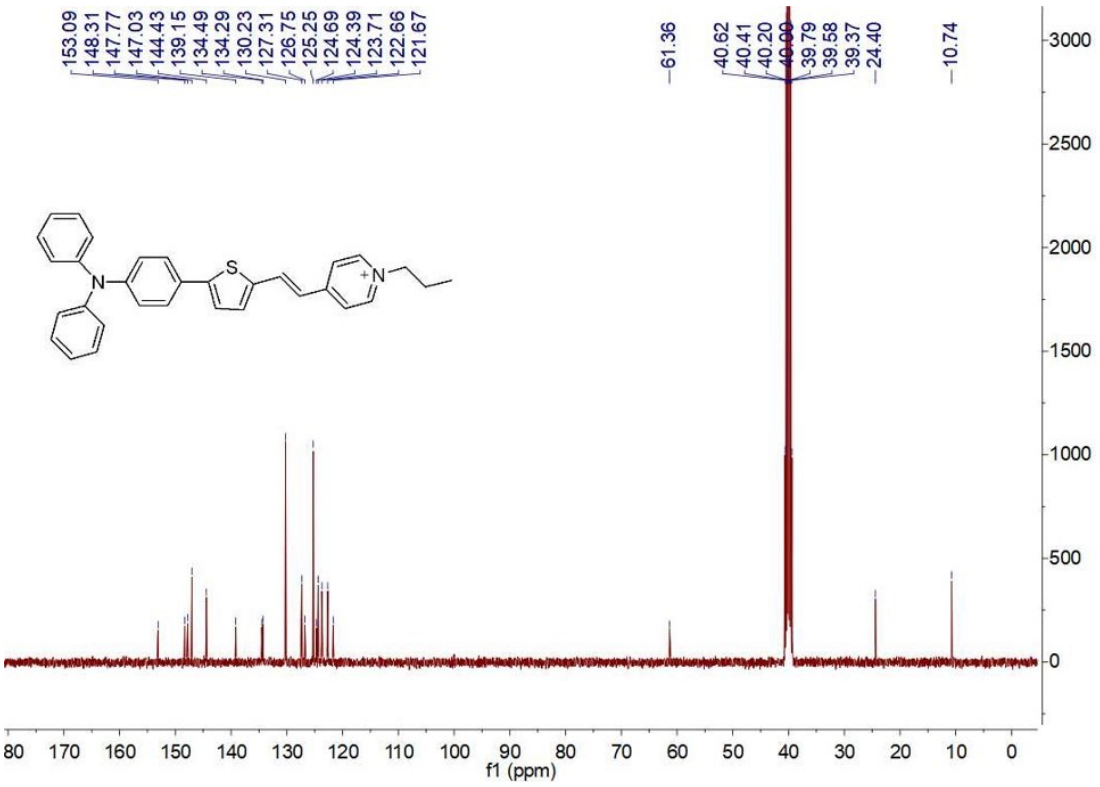


Fig.S20. ^{13}C NMR Spectrum of **TTPy**

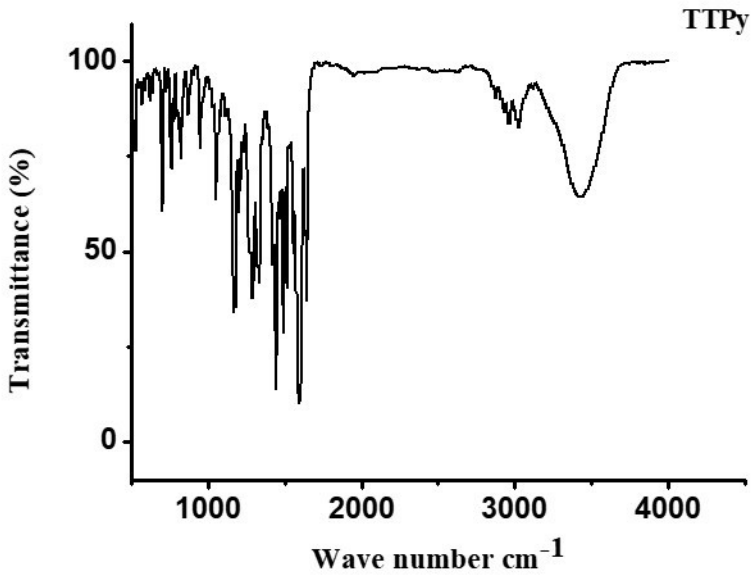


Fig. S21. FTIR Spectrum of **TTPy**

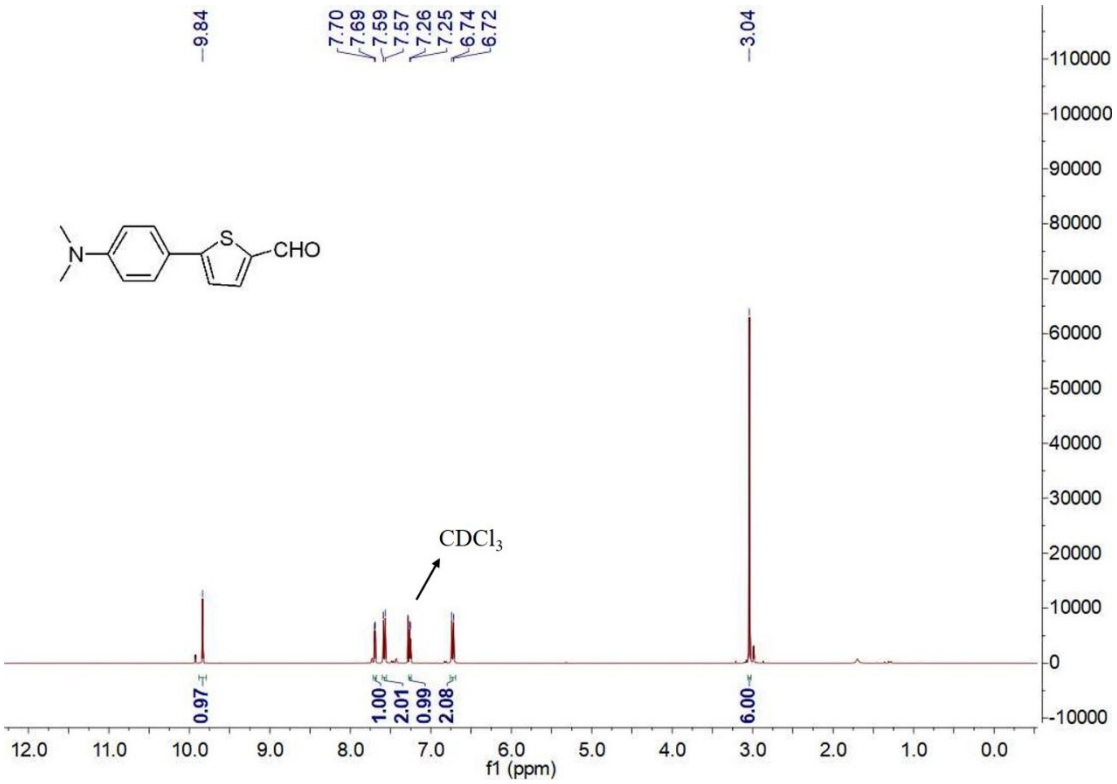


Fig.S22. ^{1}H NMR Spectrum of compound **5**

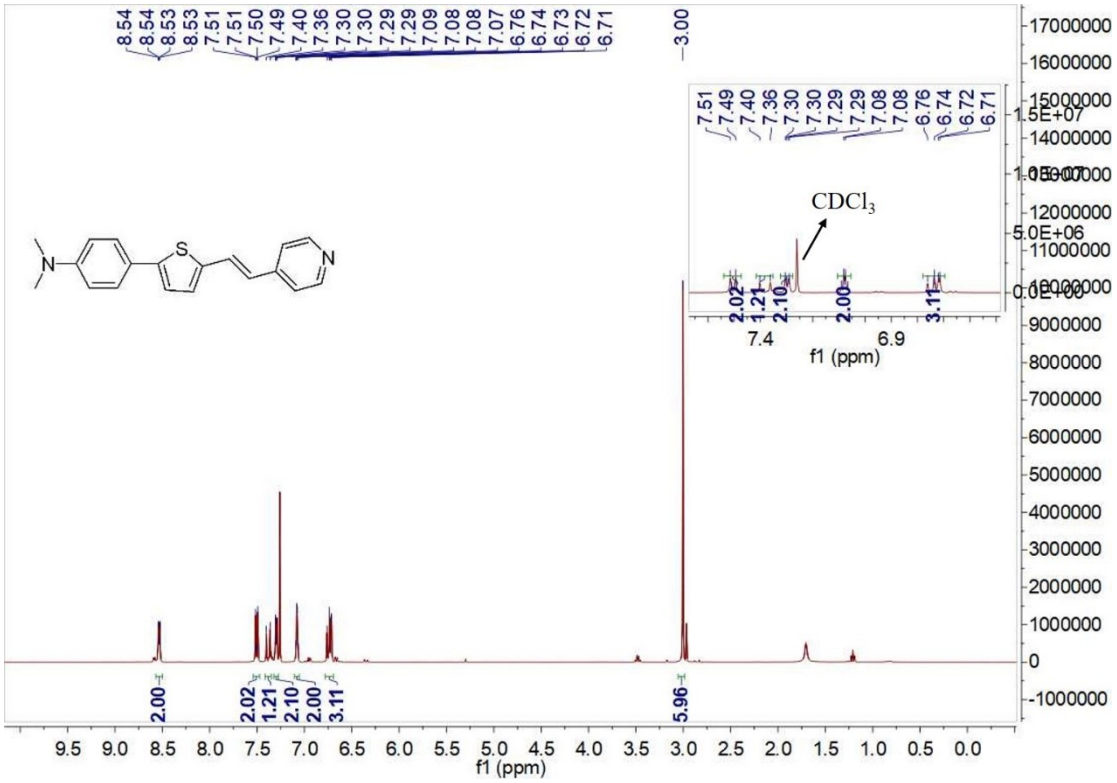


Fig.S23. ^{1}H NMR Spectrum of compound **6**

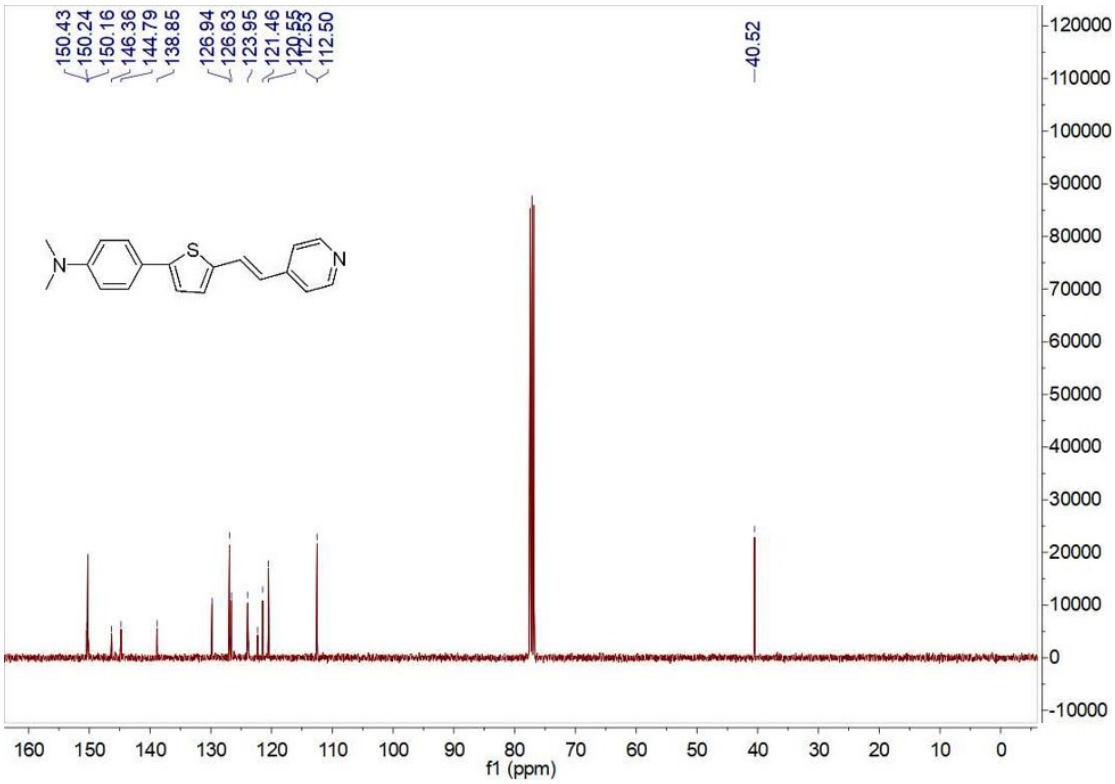


Fig. S24. ^{13}C NMR Spectrum of compound **6**

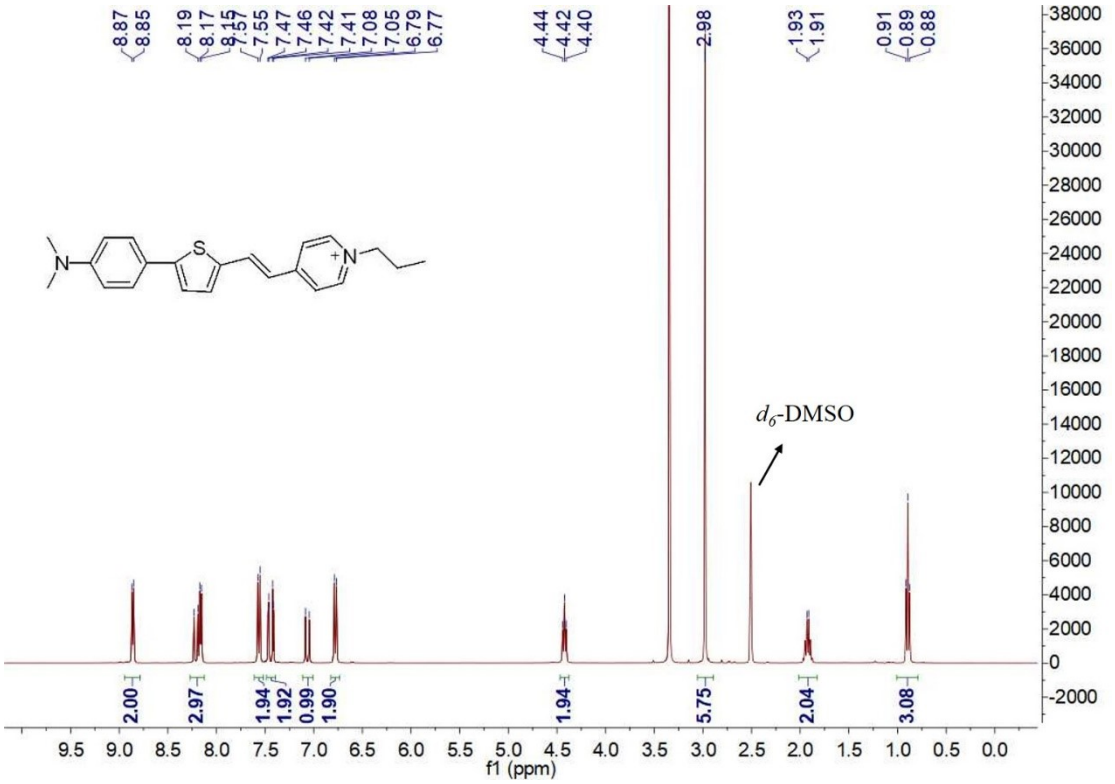


Fig.S25.^{1}H NMR Spectrum of **DTPy**

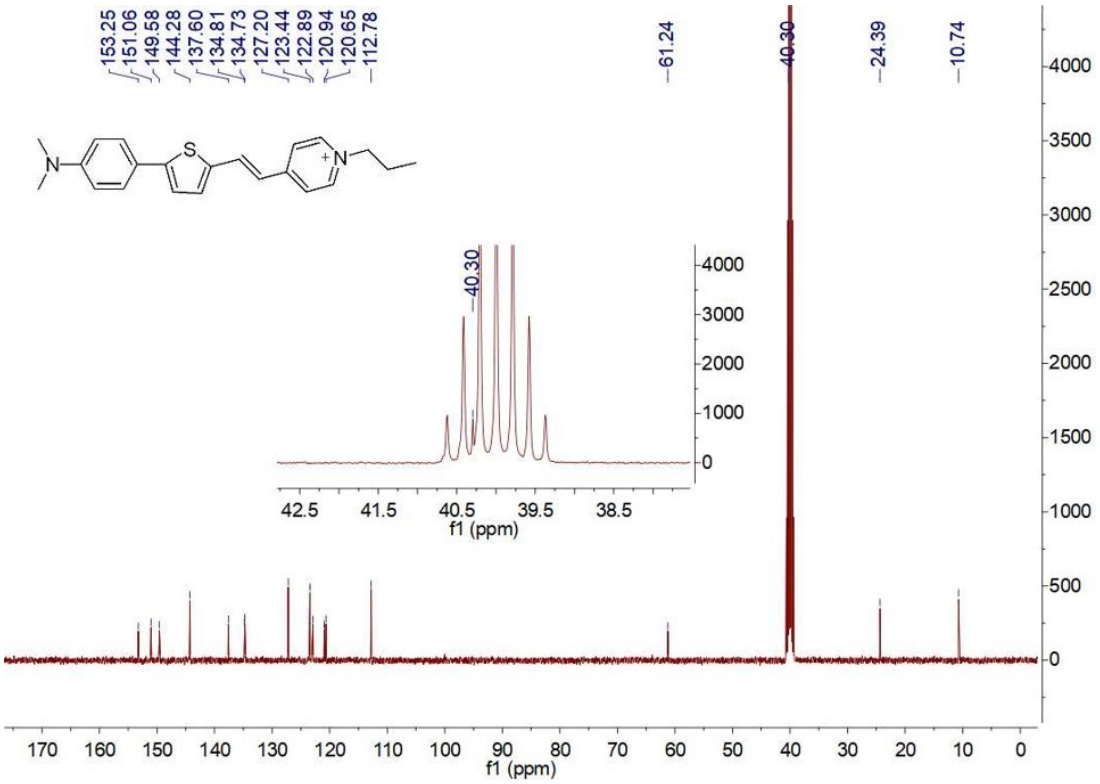


Fig.S26. ^{13}C NMR Spectrum of **DTPy**

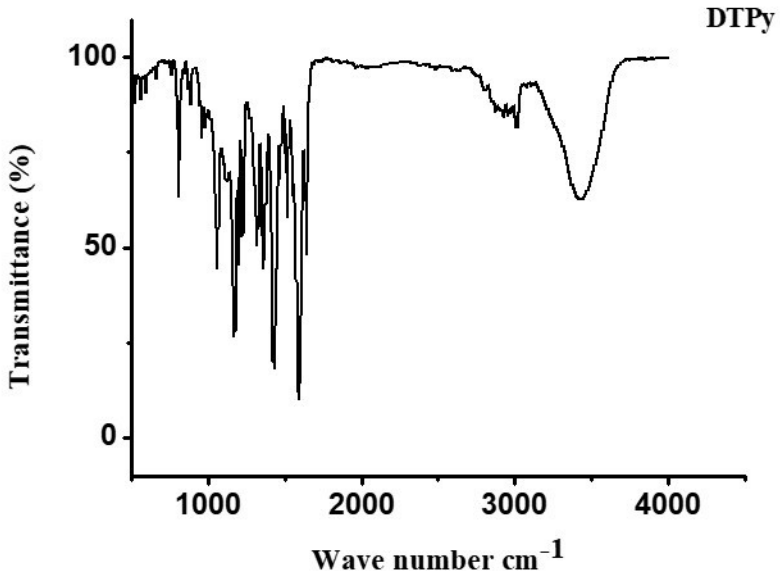


Fig. S27. FTIR Spectrum of **DTPy**

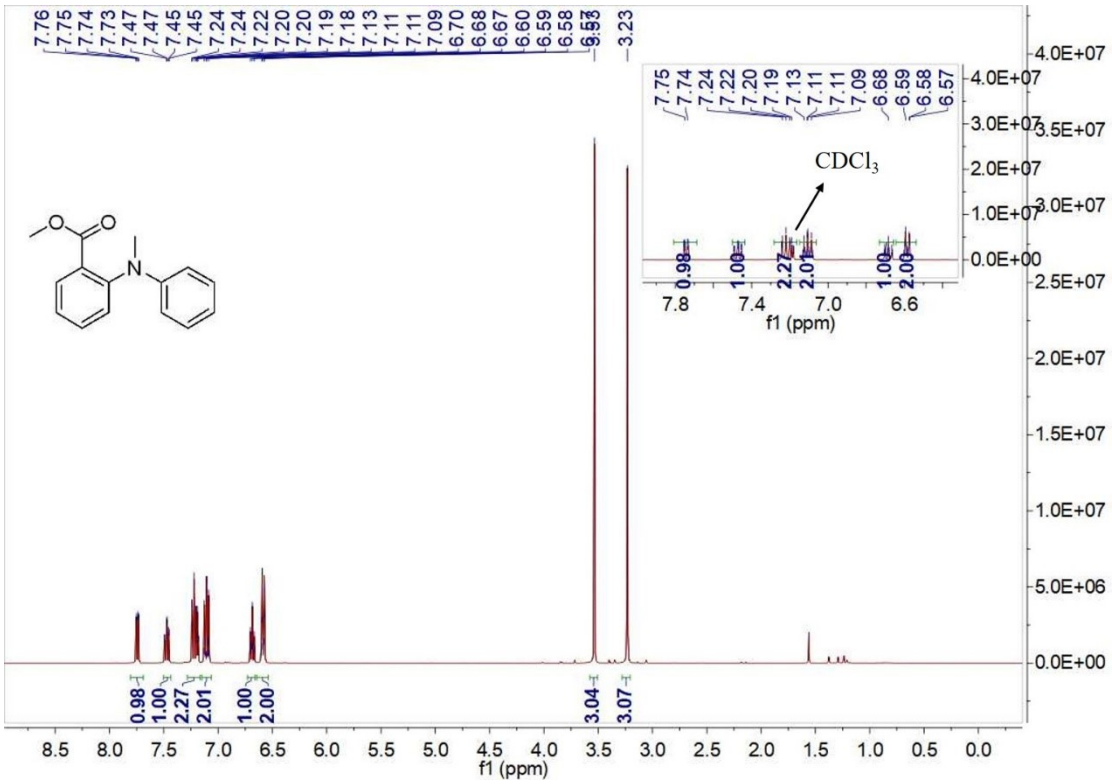


Fig.S28. [1]H NMR Spectrum of compound **7**

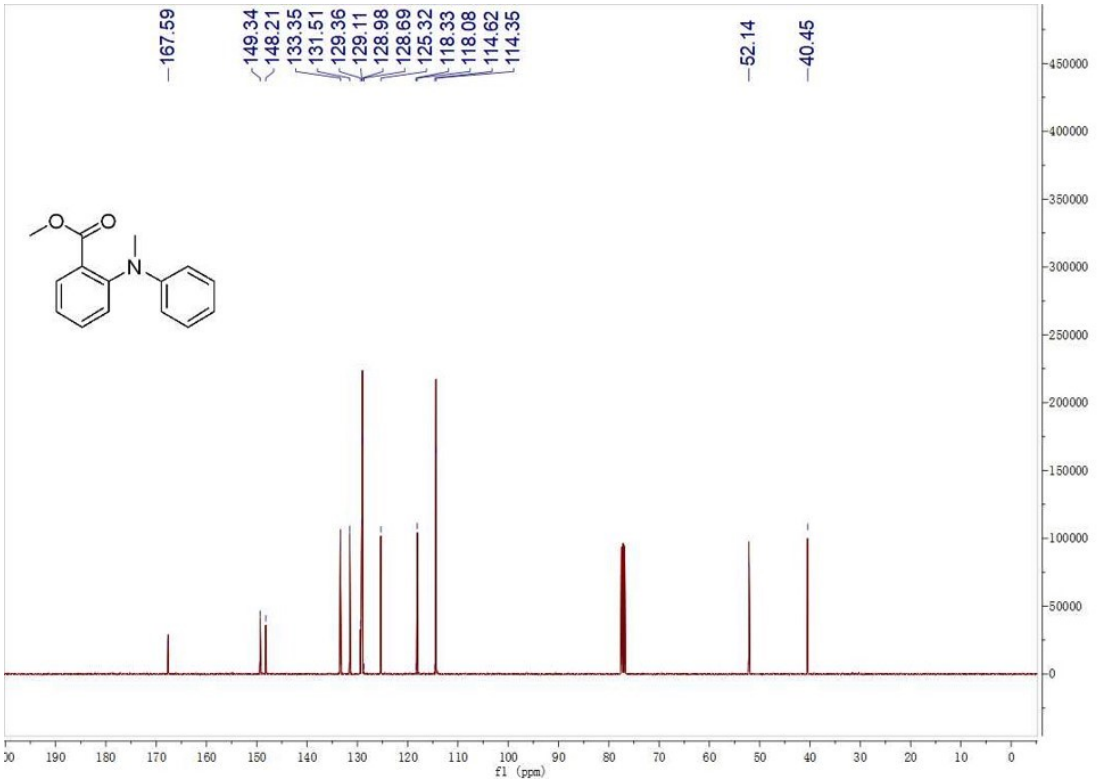


Fig. S29. [13]C NMR Spectrum of compound **7**

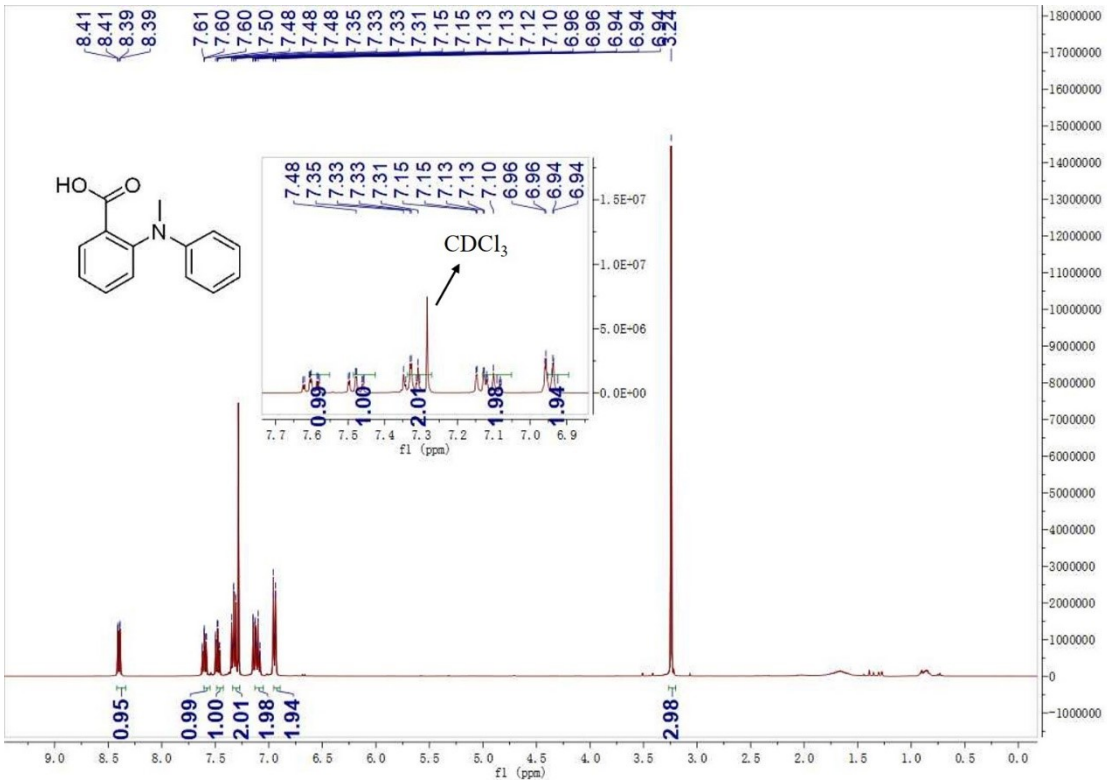


Fig.S30. [1]H NMR Spectrum of compound **8**

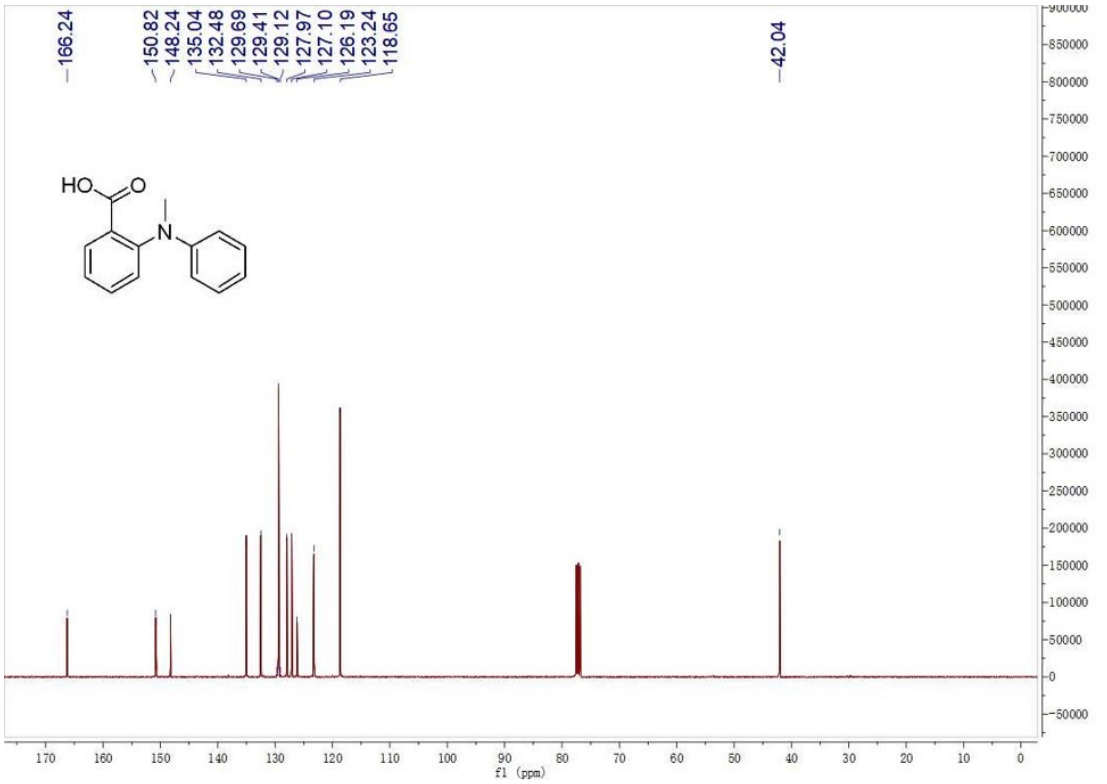


Fig.S31. [13]C NMR Spectrum of compound **8**

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