Supporting information

Novel Designed Azo Substituted Semi-cyanine Fluorescent Probe for Cytochrome P450 Reductase Detection and Hypoxia Imaging in Cancer Cells

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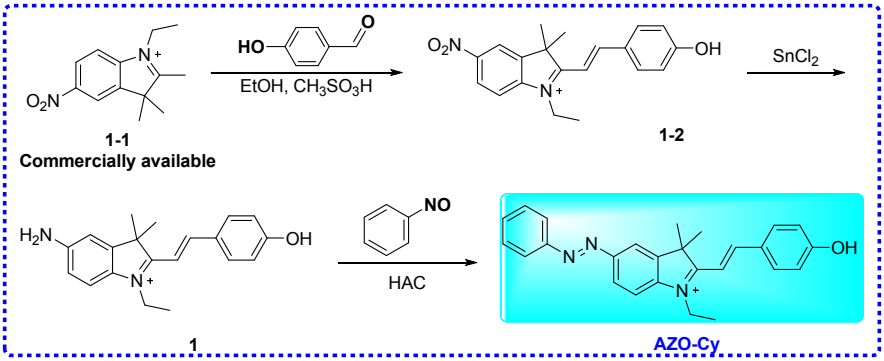
S1: The preparation of probe AZO-Cy:



Scheme 1. Synthetic schemes of **1** and probe **AZO-Cy**

Synthesis of compound 1-2

1-ethyl-2,3,3-trimethyl-5-nitroindolium iodide (600.0 mg, 2.6 mmol) and p-hydroxybenzaldehyde (377.0 mg, 3.1 mmol) were dissolved in 20 mL ethanol. Then methanesulfonic acid (272.0 mg, 2.8 mmol) was added and the mixture was stirred under nitrogen atmosphere at 80 °C for 4 h. The mixture was stirred overnight at -20 °C and filtrated to provide compound **1-4** (715.0 mg, 82%) as a bright blue solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.19 (s, 1H), 8.84 (d, J = 2.0 Hz, 1H), 8.59 (d, J = 16.0 Hz, 1H), 8.49 (dd, J = 8.8, 2.0 Hz, 1H), 8.25 (d, J = 8.8 Hz, 2H), 8.09 (d, J = 8.8 Hz, 1H), 7.52 (d, J = 16.0 Hz, 1H), 7.00 (d, J = 8.4 Hz, 2H), 4.69 (q, J = 6.8 Hz, 2H), 2.31 (s, 3H), 1.87 (s, 6H), 1.43 (t, J = 7.2 Hz, 3H), 1.23 (s, 1H).MS (ESI$^+$) calcd for $C_{20}H_{21}N_2O_3^+[M]^+$ = 337.2, Found 337.2

Synthesis of 1

Compound **1-4** (370.0 mg, 1.1 mmol) and stannous chloride dihydrate (990.0 mg, 4.4 mmol) were dissolved in 6 N aqueous hydrochloric acid (12 ml) under nitrogen atmosphere at 90 °C for 5 h. The mixture was adjusted to pH 7 with 4 N NaOH aqueous solution (12 ml). The mixture was extracted with dichloromethane. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. Then the solvent was concentrated to provide compound **1**(250.0 mg, 74%) as a bright blue solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.75 (d, J = 14.4 Hz, 1H), 7.65 (d, J = 8.4 Hz,

2H), 7.02 (d, J = 8.4 Hz, 1H), 6.71 (s, 1H), 6.56 (d, J = 8.4 Hz, 1H), 6.43 (d, J = 14.4 Hz, 1H), 6.16 (d, J = 8.8 Hz, 2H), 5.21 (s, 2H), 4.10 (q, J = 6.8, 2H), 1.59 (s, 6H), 1.24 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, MeOD-d_4) δ 178.81, 173.77, 151.55, 148.02, 143.68, 134.03, 131.20, 120.61, 120.32, 113.71, 112.32, 107.90, 100.04, 49.74, 39.23, 25.90, 11.38. MS (ESI$^+$) calcd for $C_{20}H_{23}N_2O^+$ $[M]^+$ = 307.2, Found 307.2.

S2: The preparation of PBS buffer:

8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 mL distilled water. The pH value of the solution was adjusted to 7.4 by HCl, and adding distilled water to 1L then can obtaine the PBS buffer with the concentration of 0.01 M.

Table S1.Spectroscopic data for**1** and **AZO-Cy**

Compounds	λ_{abs}(nm)	ε [$M^{-1}cm^{-1}$]	λ_{em}(nm)	Φ_f[a]
1	450	17800	---	---
AZO-Cy	450	74320	620	0.04

[a]Ref.1

Ref.

1. Lakowicz, J.R.*Principles of Fluorescence Spectroscopy*, Springer Maryland, 3rd edn., 2006, pp. 9-12

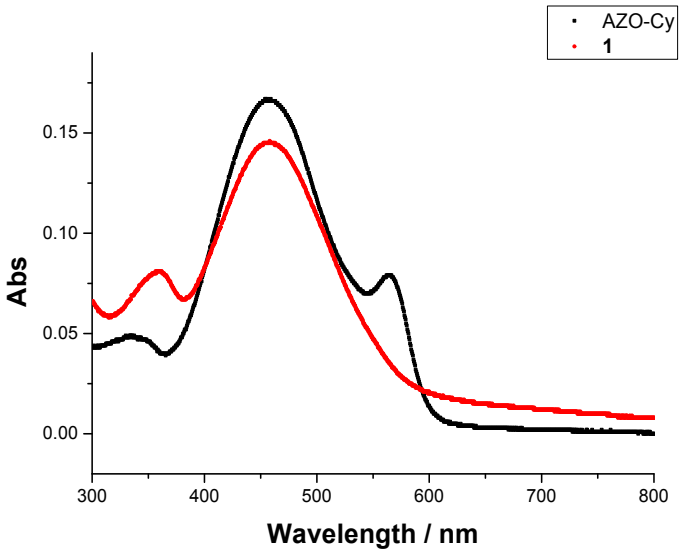


Fig. S1. UV-vis spectrum of compound **AZO-Cy** and **1**

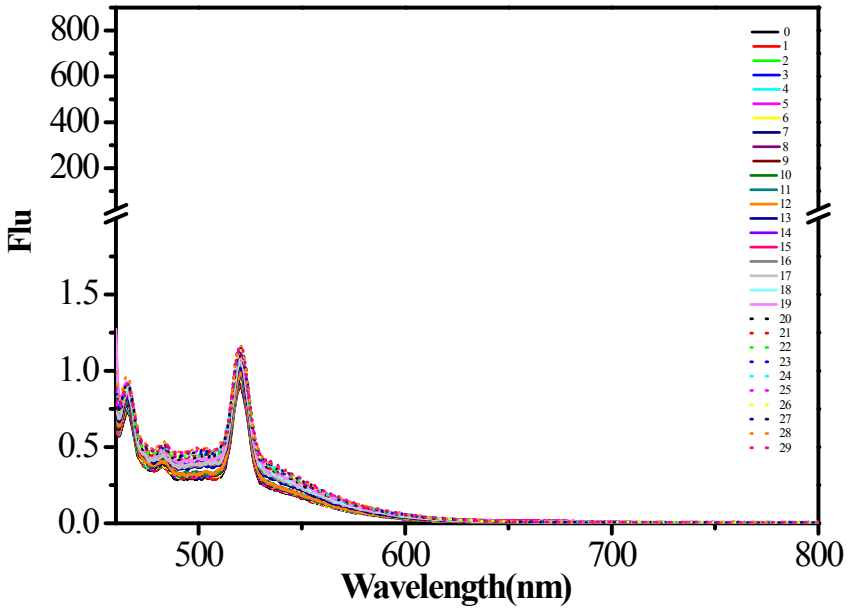


Fig. S2. Fluorescence response assays with the reductants, cations, anions and amino acids. Control: probe **AZO-Cy** (1 μM)(**0**). Fluorescence responses of **AZO-Cy** (3 μM) co-incubated with various interference (1 mM) and NADH (1 U) in PBS at 37°C with excitation wavelength at 500 nm. The reductants included VC(**1**), glutathione (**2**), cysteine (**3**), dithiothreitol (**4**), $FeCl_2$ (**5**), glucose (**6**), tyrosine (**7**), Co^{2+}(**8**),Zn^{2+}(**9**),Fe^{3+}(**10**),Ni^{2+}(**11**),Cu^{2+}(**12**),Cu^{+}(**13**), Cr^{3+}(**14**),Bi^{3+}(**15**),Ag^{+}(**16**),Mn^{2+}(**17**),Sn^{2+}(**18**),Al^{3+}(**19**),Pd^{2+}(**20**),ClO^{-}(**21**), NO_3^{-}(**22**),CH_3COO^{-}(**23**), PO_4^{3-}(**24**), HPO_4^{2-}(**25**), $P_2O_7^{4-}$(**26**), SO_3^{2-}(**27**), S^{2-}(**28**), HSO_3^{-}(**29**)

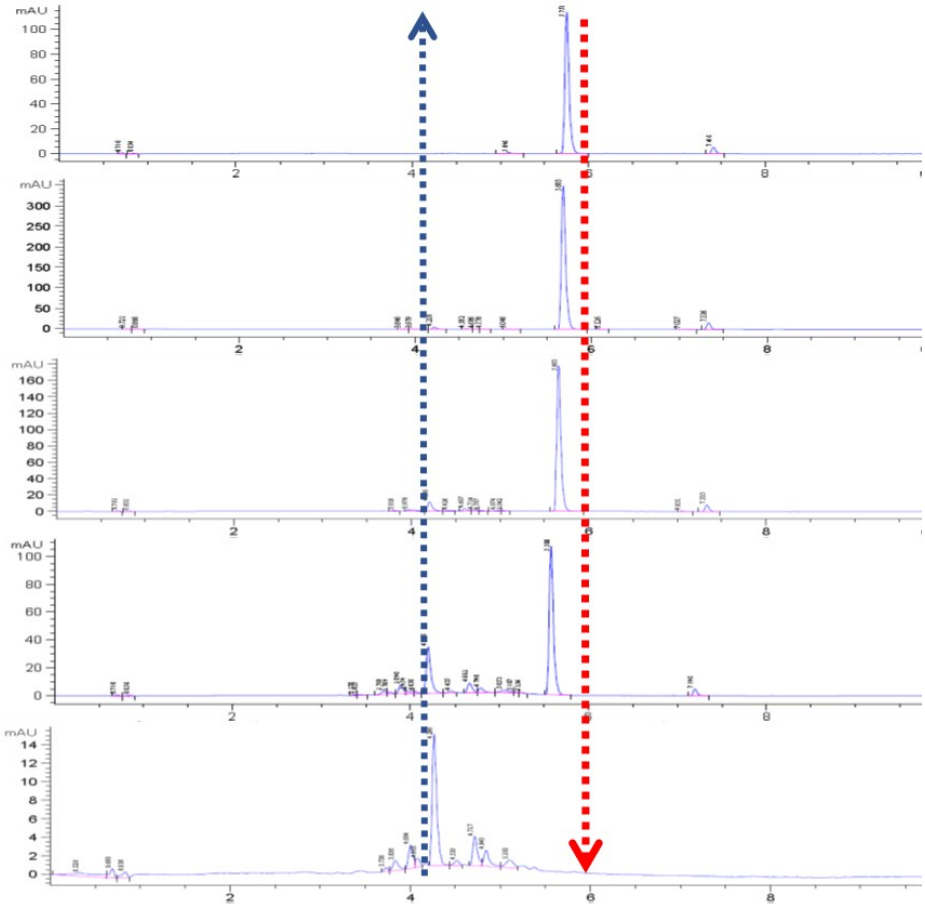


Fig. S3 HPLC analysis of metabolism of AZO-Cy reacted with cytochrome P450 reductase at different reaction time (0, 30 s, 1, 2 and 5 min). AZO-Cy (1 μM) and NADPH (0.1 mM) was treated with cytochrome P450 reductase (1 U). HPLC profiles were detected by UV at 450 nm.

LC-MS method:
chromatographic column: Agilent ZORBAX SB-Phenyl 4.6*75mm 3.5μm
Phase A: 0.025%TFA aq. solution
Phase B: 0.025%TFA CH_3CN solution
Flow: 1.5 ml/min
Column Oven: 40 ℃
Injection Volume: 3 μl

Gradient Table: Time（min） mpA（%）mpB（%）

0	95	5
8	5	95
10	5	95

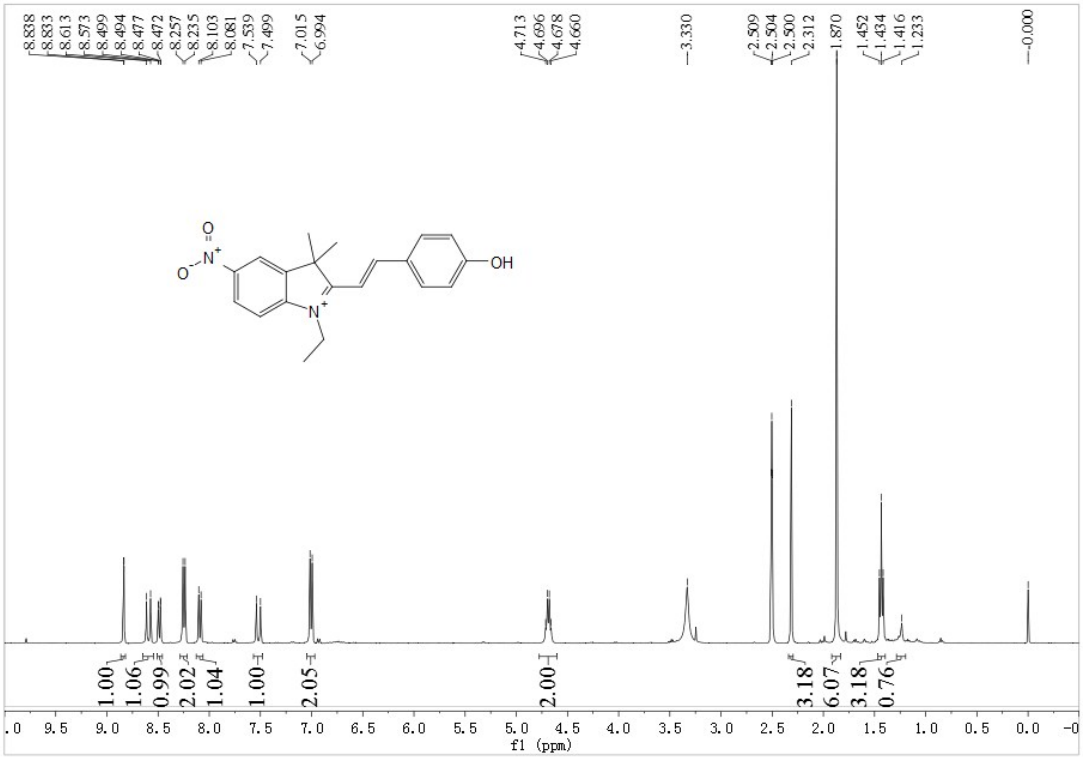
Detector: DAD (254 nm，450 nm), ELSD

MS: ESI+, frag voltage 70 V

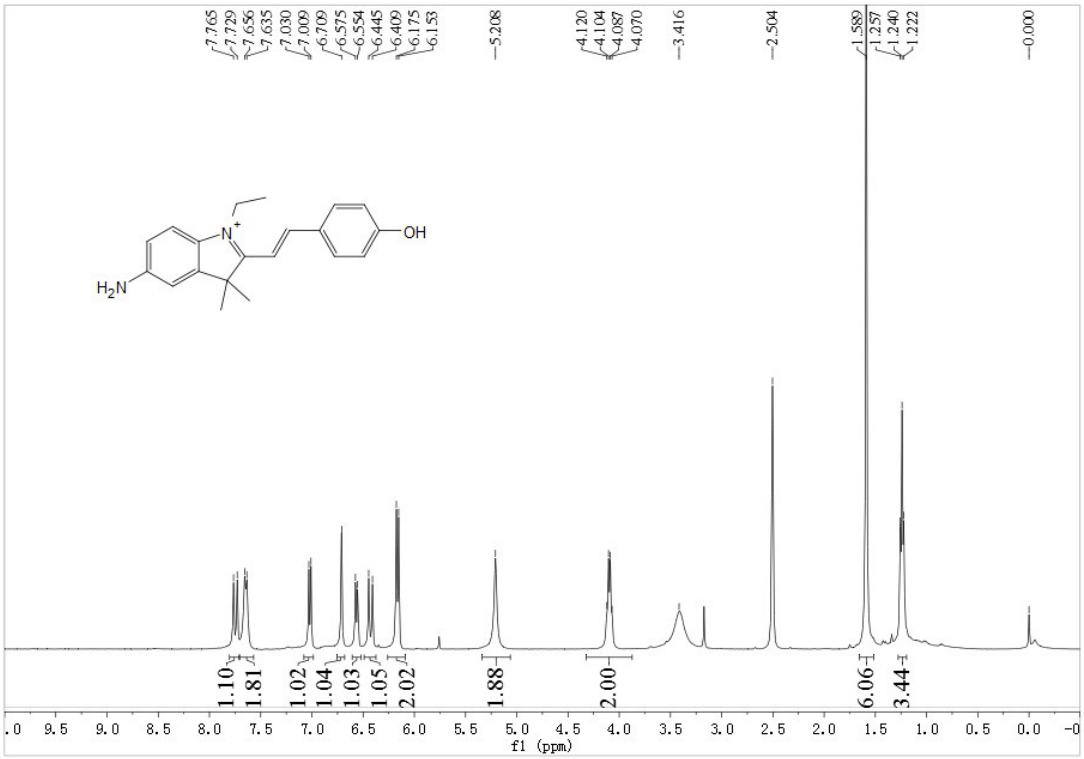
Table S2 Compared with other probes

Probe	Rate	Conditions	Ref.
AZO-DCM	reach the plateau after mixed 7.5 min	Cytochrome P450 reductase is 1 U/mL; [NADPH]= 1 mM;	Ref
MAR	About 11 min; 1. 1×10^{-3} $\mu M S^{-1}$	rat liver microsomes (226 mg/3 mL); [NADPH]= 50 μM	22
MASR	About 10 min; 2.1×10^{-3} $\mu M S^{-1}$		
BOD-Ly	reach the plateau after mixed 60 min	rat liver microsomes(100 μg mL^{-1})	26
1	None	None	28
This work	reach the plateau at about 4 min	Cytochrome P450 reductase is 1 U/mL; [NADPH]= 0.1 mM	

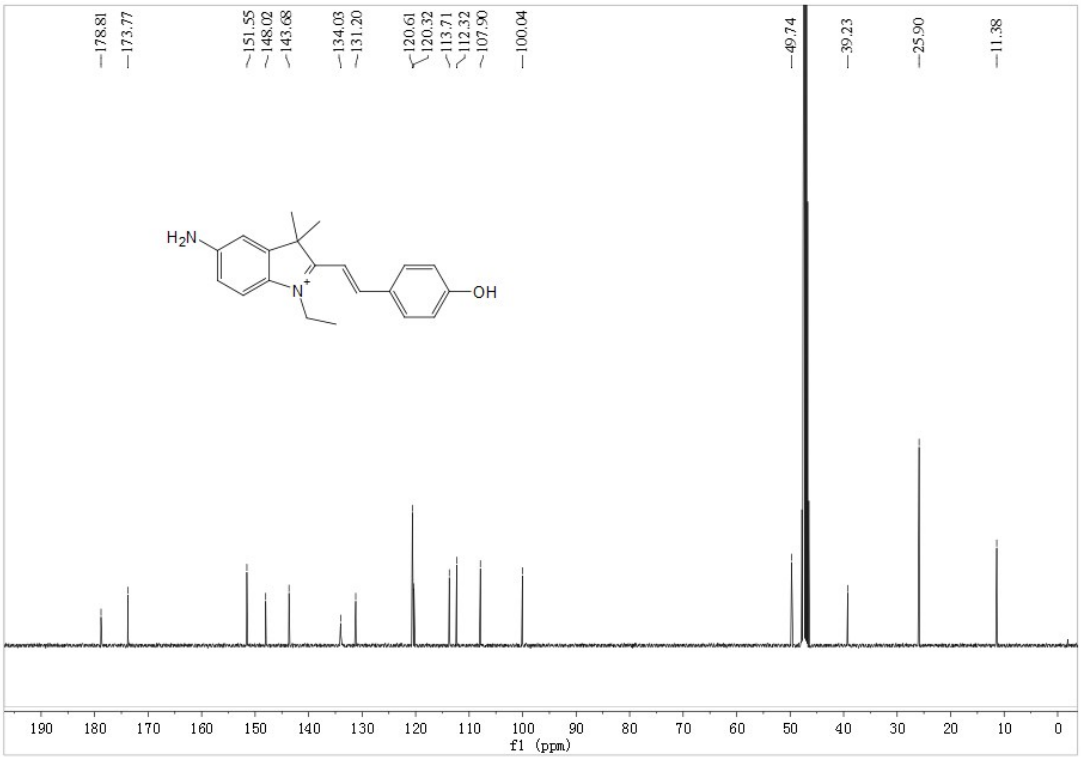
HNMR of compound 1-2



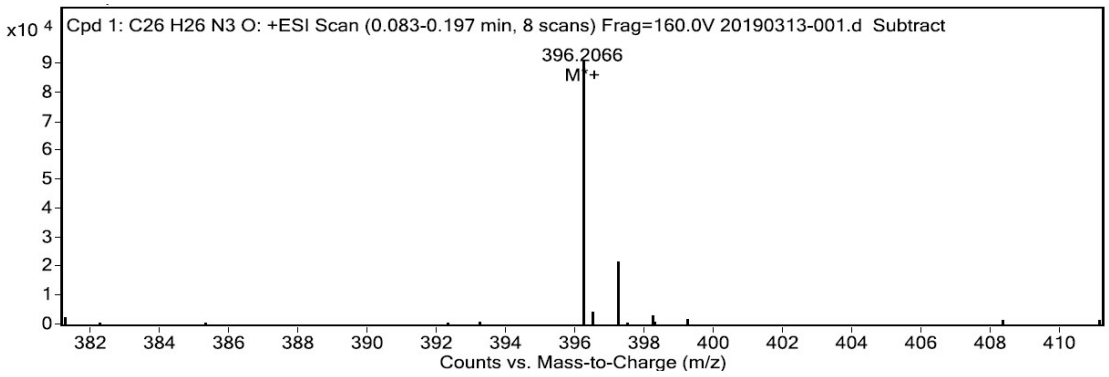
HNMR of compound 1



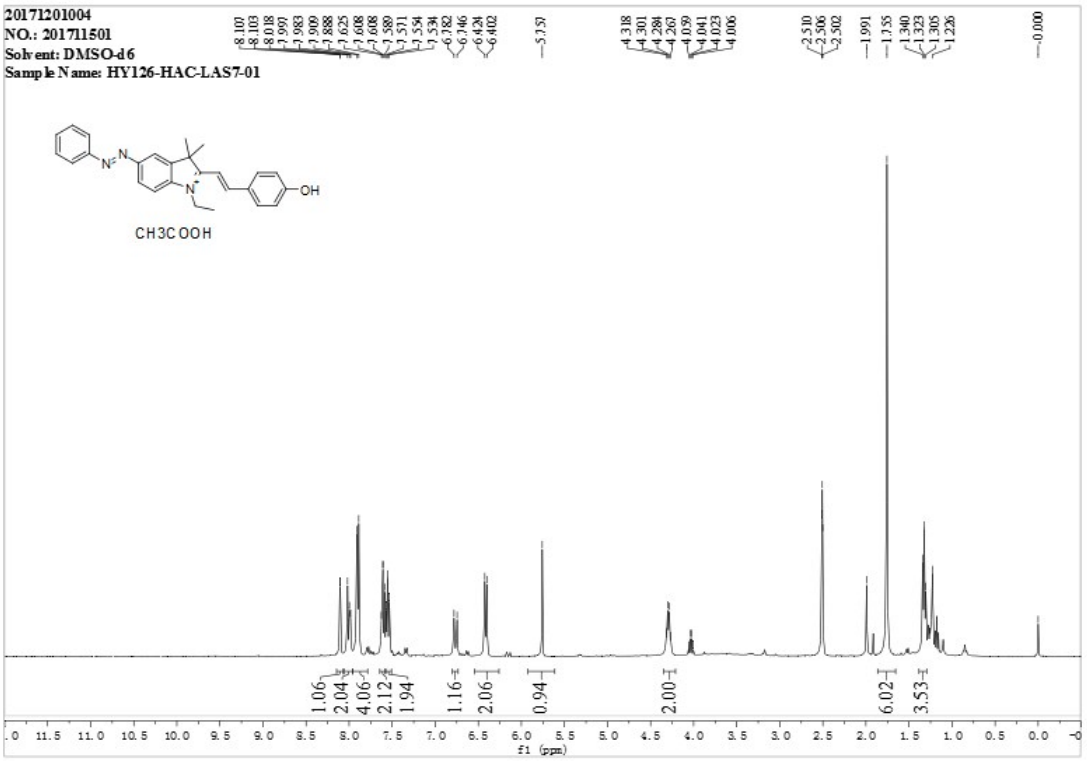
CNMR of compound 1



HRMS of AZO-Cy



HNMR of AZO-Cy



CNMR of AZO-Cy