Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information for

Rigidify styryl-pyridinium dyes to benzo[h]coumarin-based bright two-photon fluorescent probes for cellular bioimaging

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Table of contents

Materials and instruments

6-(dimethylamino)-1-hydroxy-2-naphthaldehyde was obtained through previous synthesis routes.[1] The reactants were purchased from J&K Chemical Ltd, Beijing InnoChem Science & Technology Co., Ltd, Bide Pharmatech Ltd and TCI Chemical Reagent Shanghai Co., Ltd. Hoechst 333342 was purchased from Sigma-Aldrich. Commercial probe Mito Tracker Green (MTG) was purchased from Molecular Probe. The column chromatography silica gel powder (200-300 mesh) and the thin-layer chromatography silica gel plate all from Qingdao Ocean Chemical.

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a Bruker AVANCE III spectrometer. High-resolution mass spectra (HRMS) were obtained on an Agilent Technologies 6510 Q-TOF LC/MS instrument. The UV-Vis absorption spectra were measured on a U-4100 spectrophotometer, and the fluorescence emission spectra were measured on a Horiba FluoroMax-4 fluorescence spectrophotometer. The fluorescence quantum yield was measured using a calibrated integrating sphere on Edinburgh FLS920/LP920 fluorescence spectrophotometer. Using Leica TCS SP8 for imaging.

The calculation process of yield：

Yield of (**Compound 2**) = $m_2/(n_2 \cdot M_2)$ = 840 mg/(5 mmol · 229.11 g/mol) = 73%. $n_2 = n_1 = 5$ mmol. Yield of (**Compound 3**) = $m_3/(n_3 \cdot M_3)$ = 99 mg/(0.9 mmol·316.12 g/mol) = 35%. $n_2 = n_1 = 0.9$ mmol. Yield of **BS-MN** = $m_{BS\text{-}MN}/(n_{BS\text{-}MN} \cdot M_{BS\text{-}MN})$ = 54 mg/(0.44 mmol·446.08 g/mol) = 28%. $n_{BS\text{-}MN} = n_2 = 0.44$ mmol. Yield of **BS-CN** = $m_{BS\text{-}CN}/(n_{BS\text{-}CN} \cdot M_{BS\text{-}CN})$ = 74 mg/(0.52 mmol·458.05 g/mol) = 31 %. $n_{BS\text{-}CN} = n_3 = 0.52$ mmol.

Method details about the molecular docking analysis:

Nucleic acid preparation: The initial structure of RNA was downloaded from RCSB Protein Data Bank (PDB code: 5ml7). The original ligand and water were removed by PyMOL for docking studies. Ligand (BM-CN and BM-MN) preparation: using the optimized compound structure, the partial atomic charges were obtained by restrained electrostatic potential (RESP) calculating with Gaussian 09 package at the level of HF/6-31g*. After that, the docking calculations were performed by the AutoDock 4 suite of programs, using ligand flexible docking approach that allows ligand flexibility. The Lamarckian genetic algorithm was chosen as the search protocol using the default

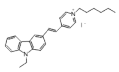
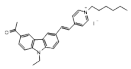
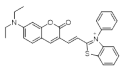
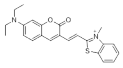
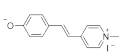
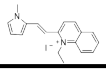
parameters except for number of GA runs (ga_run = 50) and the maximum number of energy evaluations (ga_num_evals = 2500000).

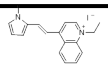
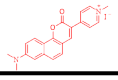
Table S1 The photophysical properties of **BS-MN** and **BS-CN**

Probes	Solvents	λ_{ab}/nm	λ_{em}/nm	Stokes shift/nm	ε^c/ 10^4 $M^{-1}\cdot cm^{-1}$	Φ_s (%)
BS-MN	Toluene	477	603	126	21800	0.84
	DCM	525	671	146	27800	7.46
	THF	480	663	183	22700	3.96
	Acetone	475	670	195	23000	3.33
	EtOH	483	668	185	23700	3.11
	MeOH	480	673	193	22900	3.03
	DMSO	476	687	211	22100	0.70
	H_2O	437	658	221	16700	0.11
BS-CN	Toluene	458	589	131	18100	0.63
	DCM	540	657	117	47700	34.48
	THF	497	647	150	20800	32.09
	Acetone	495	660	165	39800	29.61
	EtOH	509	659	150	43600	27.26
	MeOH	498	659	150	52300	25.73
	DMSO	495	664	169	36900	35.60
	H_2O	487	653	166	27200	2.65

Abbreviations: λ_{abs} = absorption maximum; λ_{em} = emission maximum; ε^c: molar extinction coefficient; Φ_s =fluorescence quantum yield in solution. Concentration: 10 μM.

Table S2 Quantum yields of cationic dyes in the literatures.

Structure	**Fluorescent probe**	**Φ_s (%)**	**Staining concentration (μM)**
	CAI[2]	9.34	2
	CAEI[2]	13.80	2
	BuBtC[3]	11.83	2
	MeBtC[3]	10.73	2
	Merocyanine[4]	5.84	5
	PQO[5]	0.30	5

	PQP[5]	0.70	5
	BS-CN	35.60	0.5

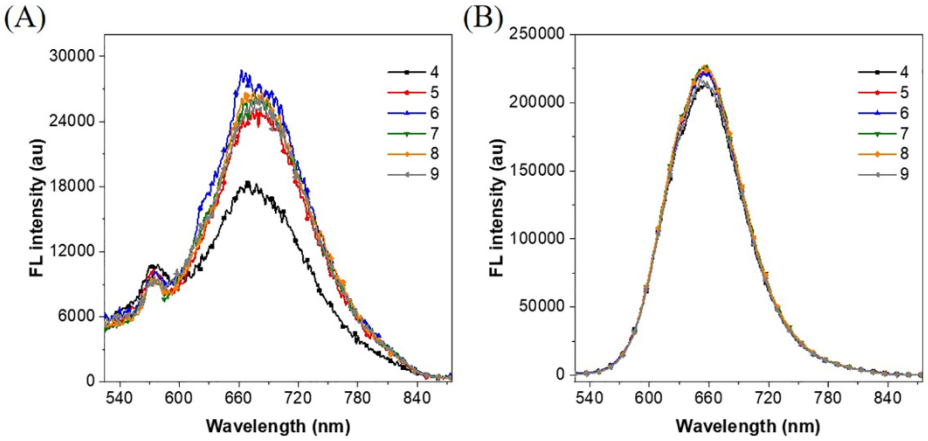


Fig. S1. Fluorescence spectra of **BS-MN** (A) and **BS-CN** (B) in buffer solutions with different pH. λ_{ex}=488 nm, 10 μM

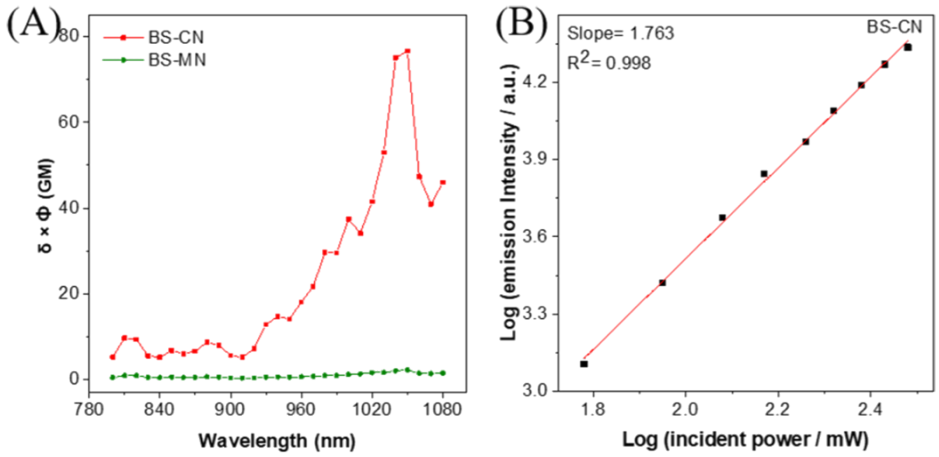


Fig. S2. Two-photon absorption cross-sections of BS-CN and BS-MN at different wavelengths in DMSO (A); Linear relationship between maximum fluorescence intensity and power (B).

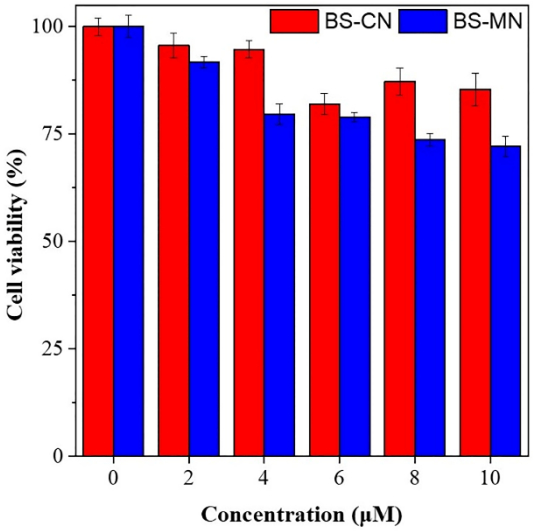


Fig. S3. Cytotoxicity of **BS-MN** and **BS-CN** at various concentrations in HeLa cells for 24 h.

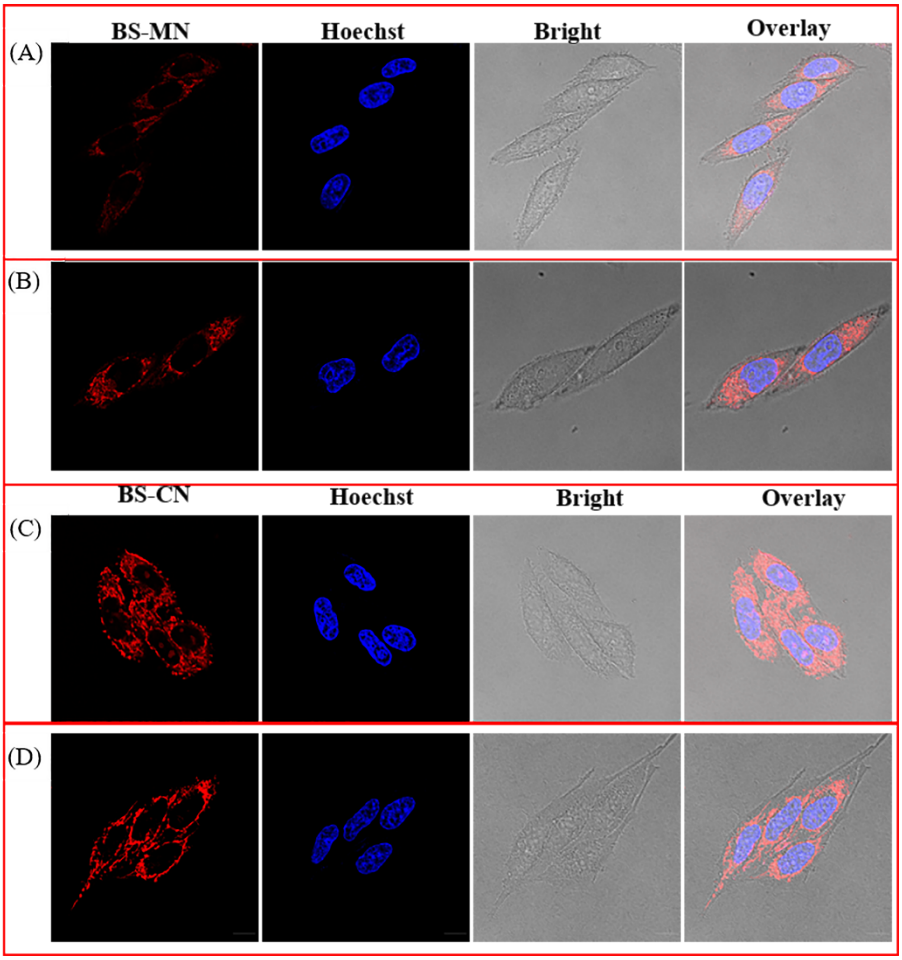


Fig. S4. Staining results of **BS-MN** (A and B) and **BS-CN** (C and D) with different concentrations in HeLa Cells. A and C: 500 nM; B and D: 1 μM BS-MN and BS-CN: λ_{ex} = 488 nm, λ_{em} = 600-700 nm, 30 min. Hoechst: 2 μM, λ_{ex} = 405 nm, λ_{em} = 420-460 nm, 10 min. Scale bar = 10 μm.

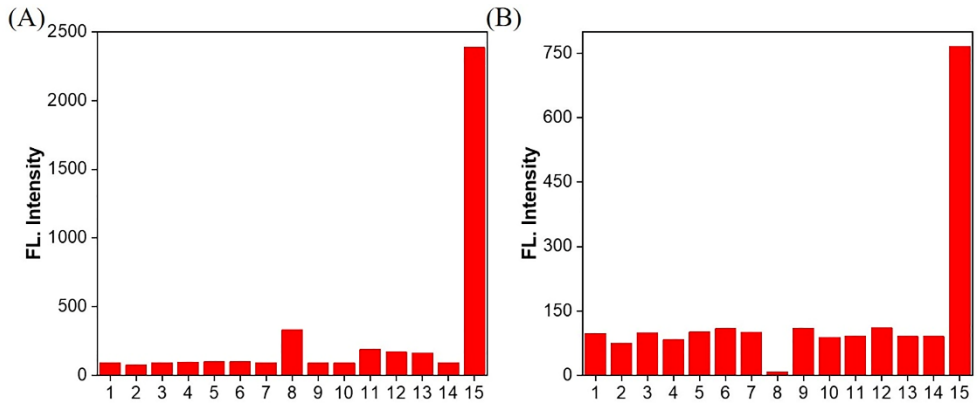


Fig. S5. The fluorescent intensity of **BS-MN** (A, 10 μM) and **BS-CN** (B, 10 μM) with 1mM of various biological reagents. 1. Ca^{2+}: $CaCl_2$, 2. Cu^{2+}: $CuSO_4$, 3. Mg^{2+}: $MgSO_4$, 4. Na^{+}: NaCl, 5. NH^{4+}: NH_4Cl, 6. K^{+}: KCl, 7. NO^{3-}: $NaNO_3$, 8. PO_3^{2-}: Na_2HPO_3, 9. SO_4^{2-}: $MgSO_4$, 10. Cl^{-}: NaCl, 11. $H_2PO_4^{-}$: NaH_2PO_4, 12. HCO_3^{-}: $NaHCO_3$, 13. HS^{-}: NaHS, 14. HSO_3^{-}: $NaHSO_3$, 15. RNA.

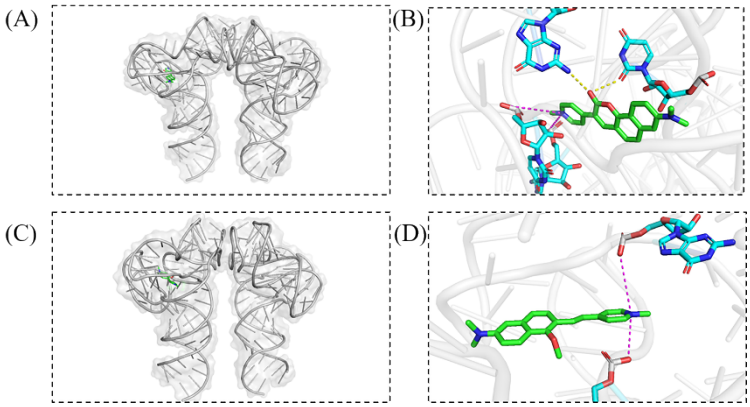


Fig. S6. The molecular docking calculations based on the structure optimized BS-CN (A and B) and BS-MN (C and D) with RNA secondary structure fragments. Yellow line: Hydrogen bond force; Purple line: electrostatic force.

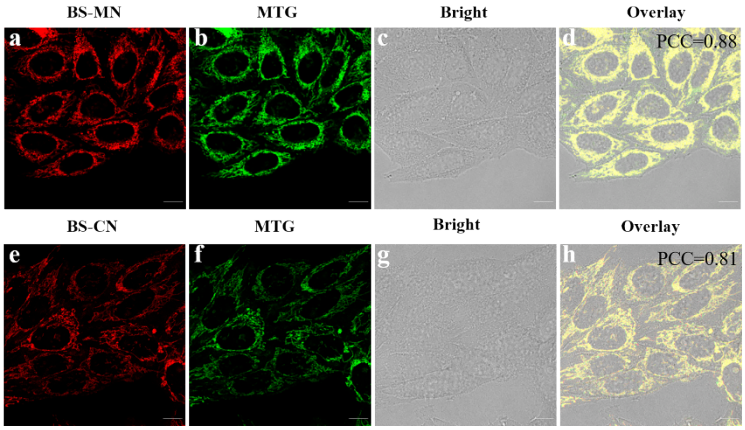
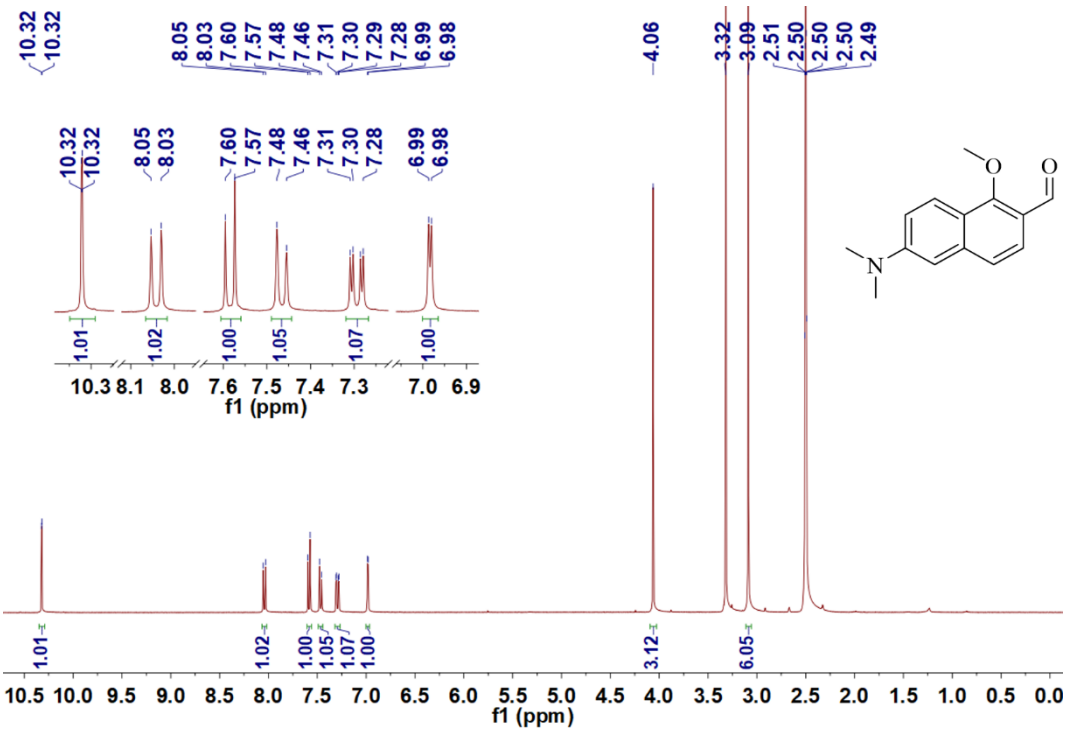


Fig. S7. Co-localization experiment with incubation time of 60 hours. BS-MN and BS-CN: λ_{ex} = 488 nm, λ_{em} = 600-700 nm; MTG: λ_{ex} = 488 nm, λ_{em} = 505-600 nm, 200 nM, 30 min. Scale bar = 10 μm.



Characterization

Fig. S8. ^{1}H NMR spectrum of **compound 2** in DMSO-d_6.

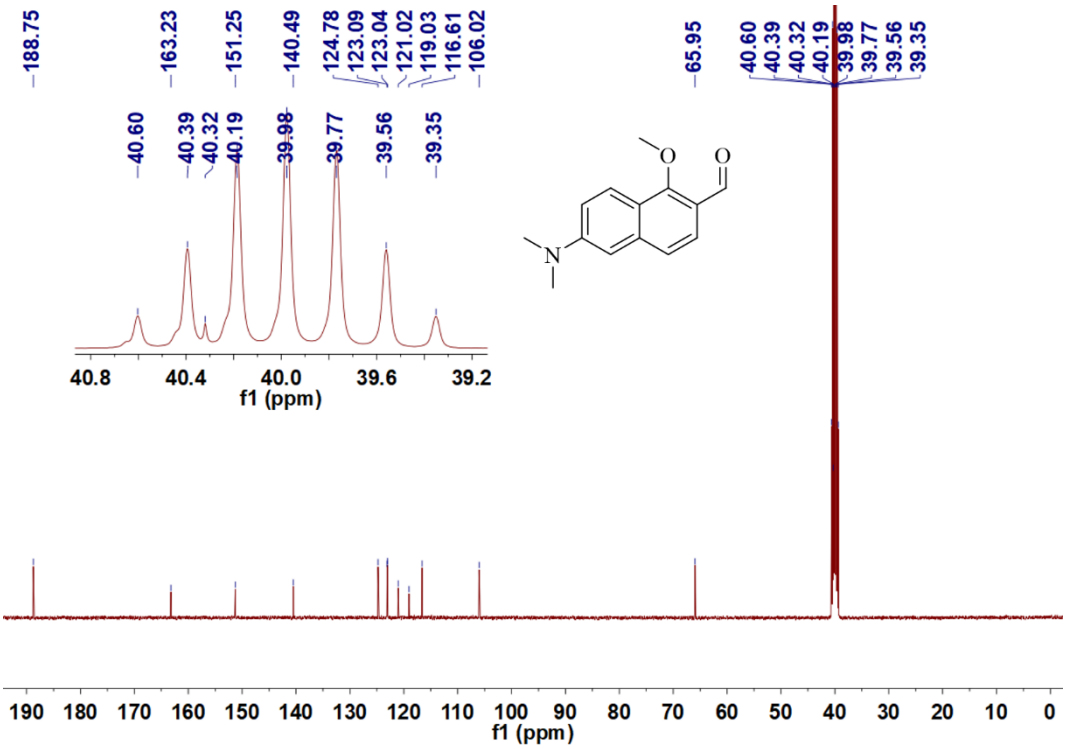


Fig. S9. ^{13}C NMR spectrum of **compound 2** in DMSO-d_6

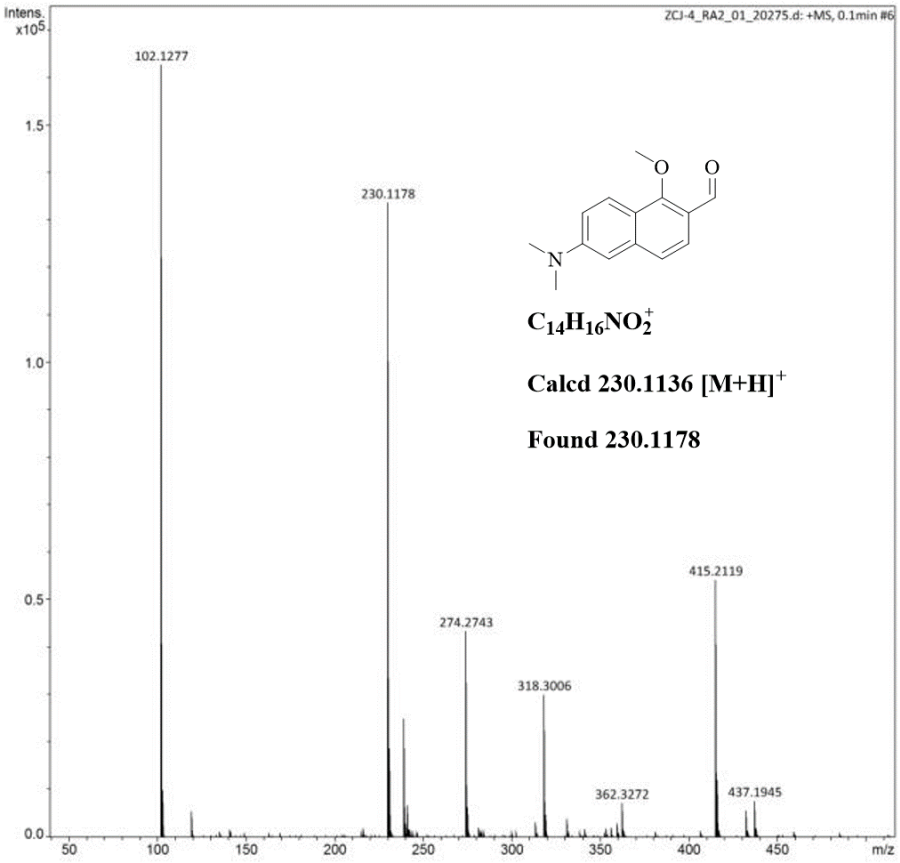


Fig. S10. HRMS of **compound 2**.

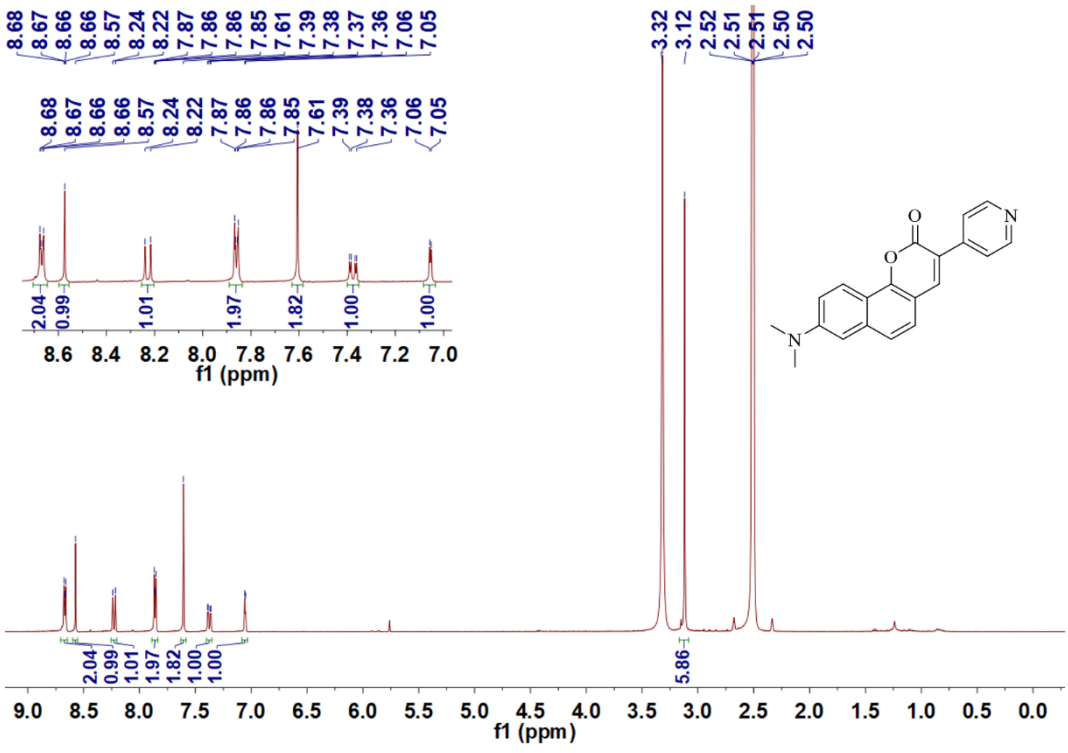


Fig. S11. ^{1}H NMR spectrum of **compound 3** in DMSO-d_6.

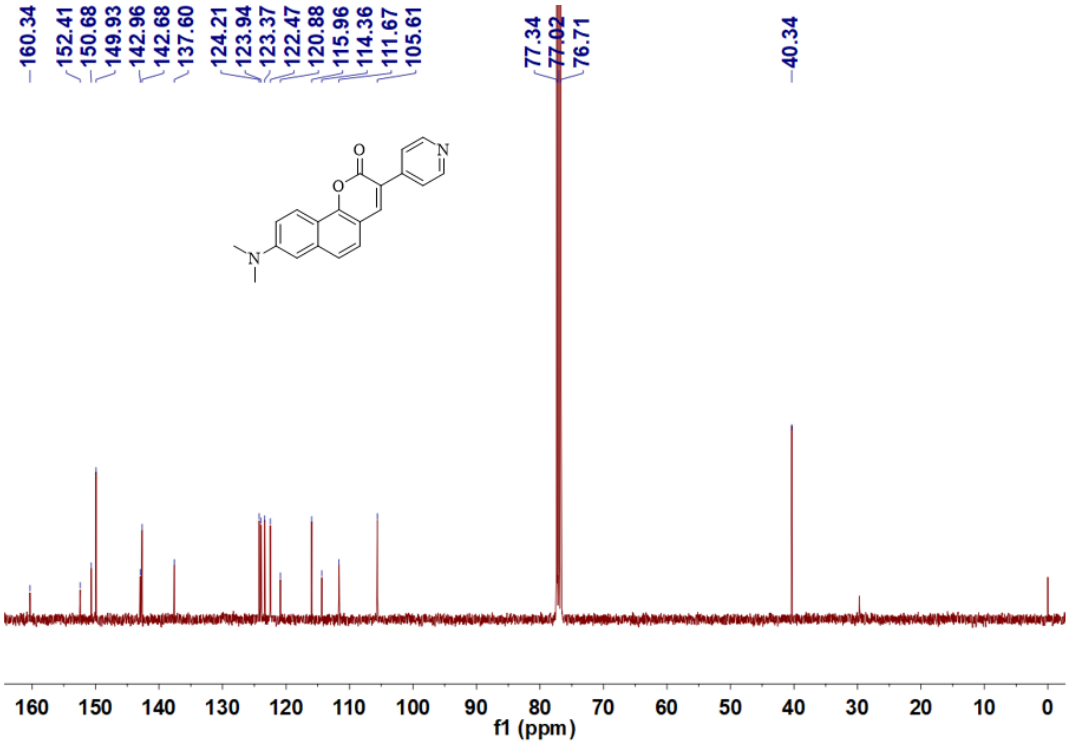


Fig. S12. ^{13}C NMR spectrum of **compound 3** in $CDCl_3$

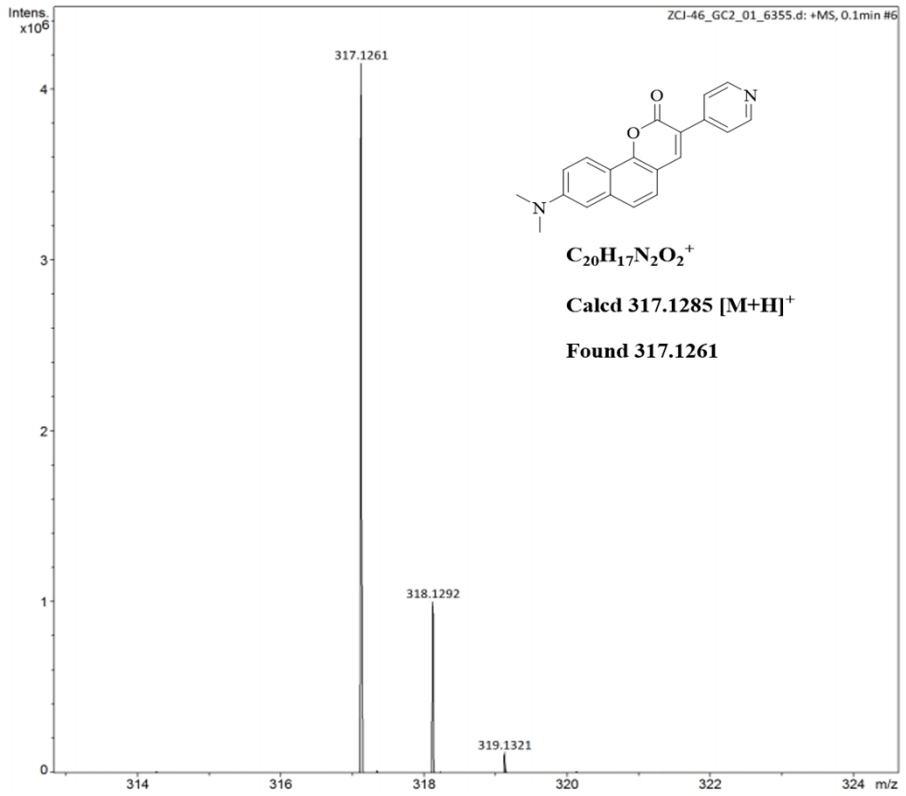


Fig. S13. HRMS of **compound 3**.

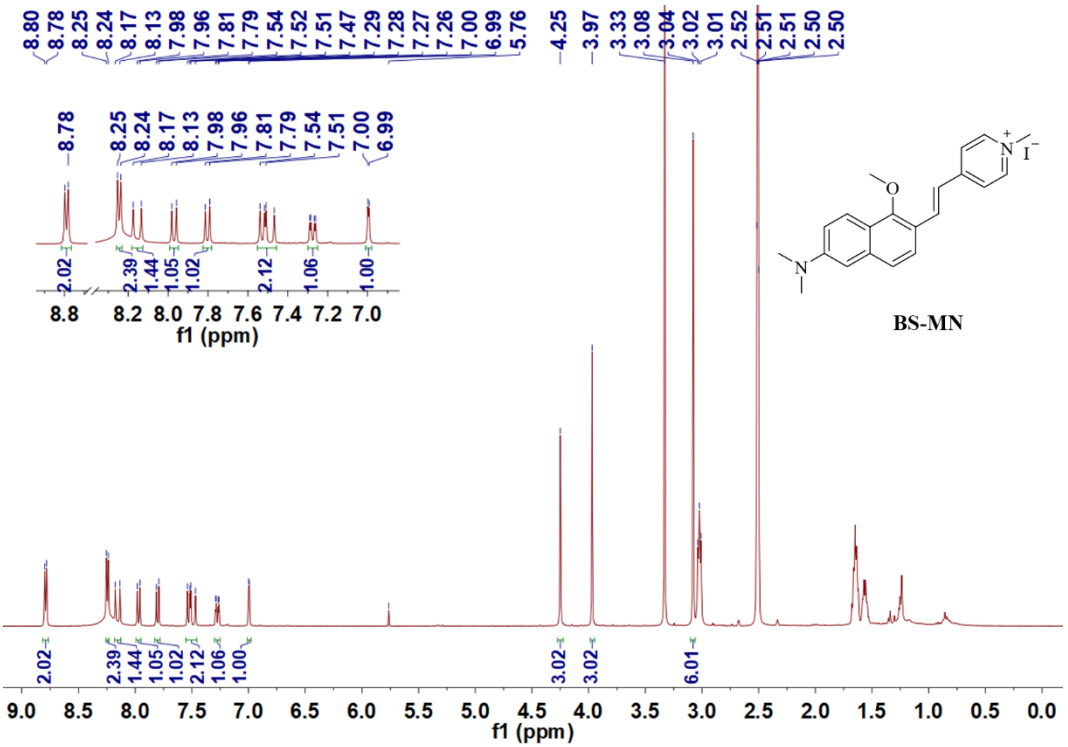


Fig. S14. ^{1}H NMR spectrum of **BS-MN** in DMSO-d_6.

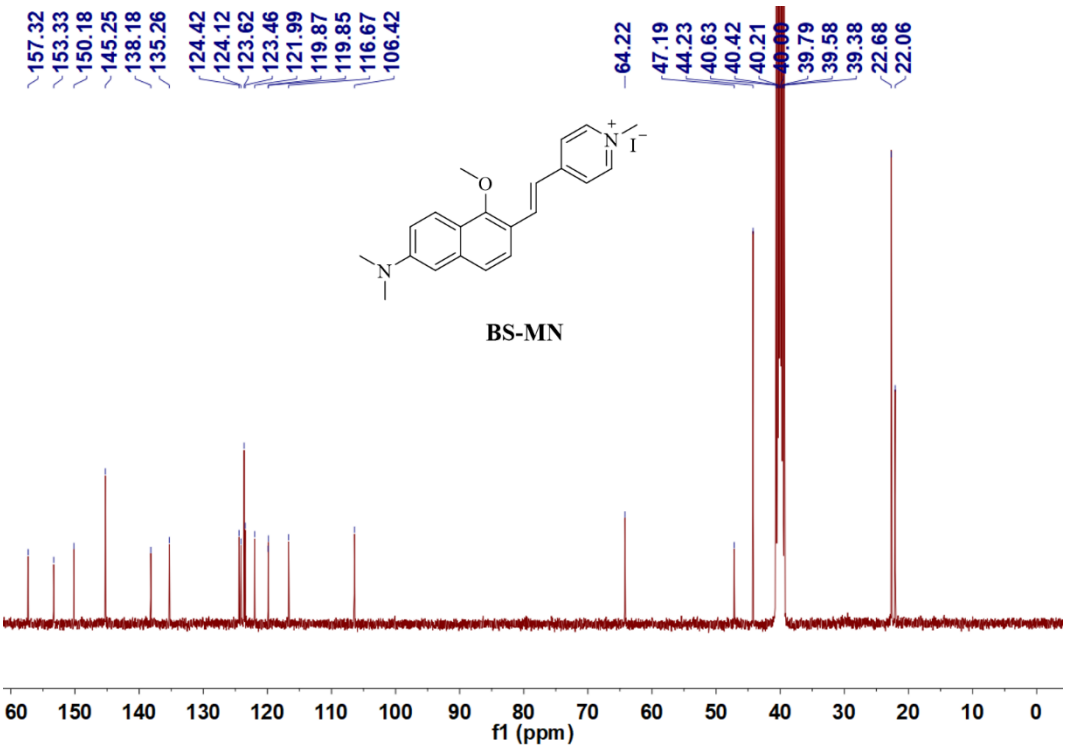


Fig. S15. ^{13}C NMR spectrum of **BS-MN** in DMSO-d_6

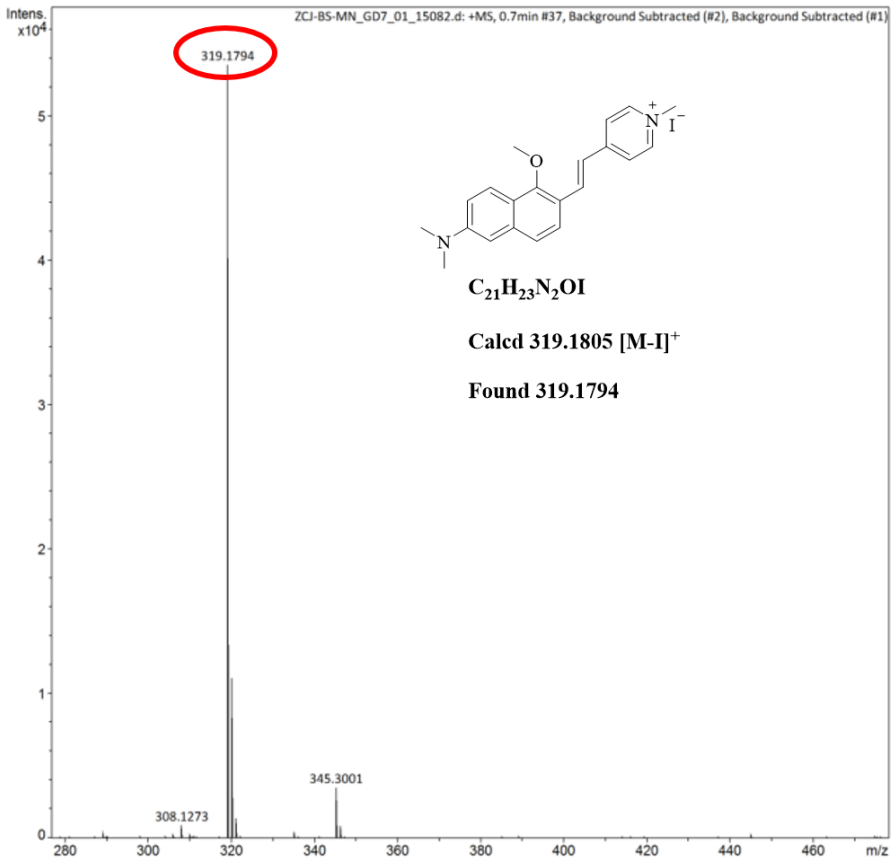


Fig. S16. HRMS of **BS-MN**.

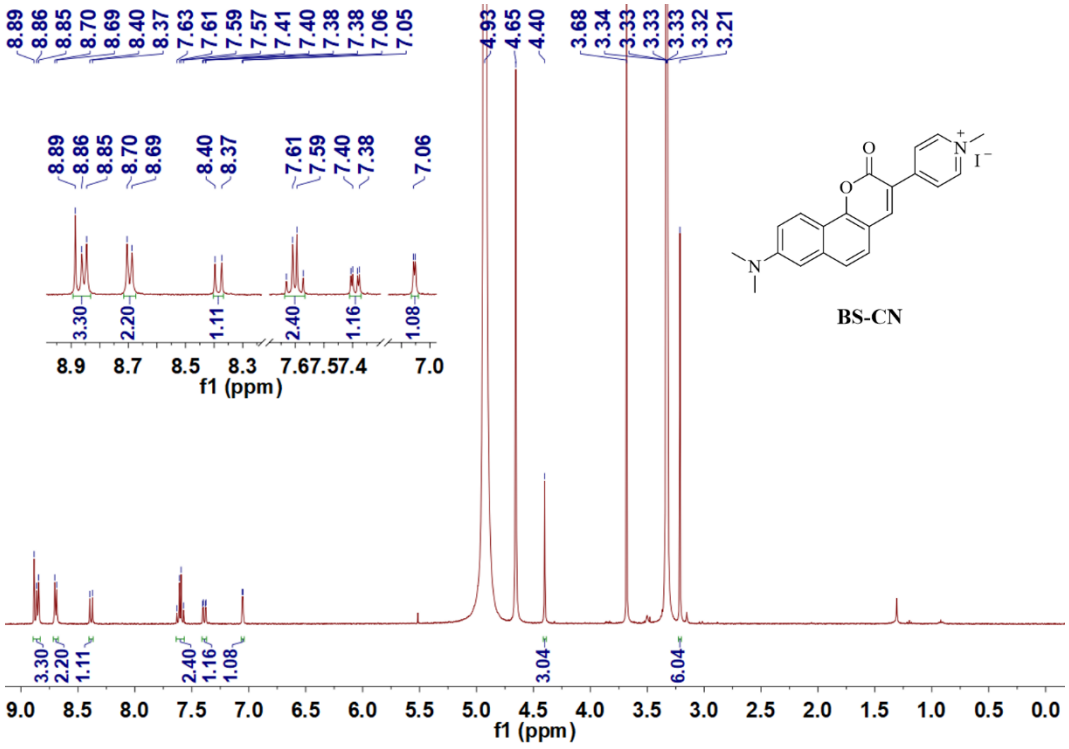


Fig. S17. ^{1}H NMR spectrum of **BS-CN** in Methanol-d_4.

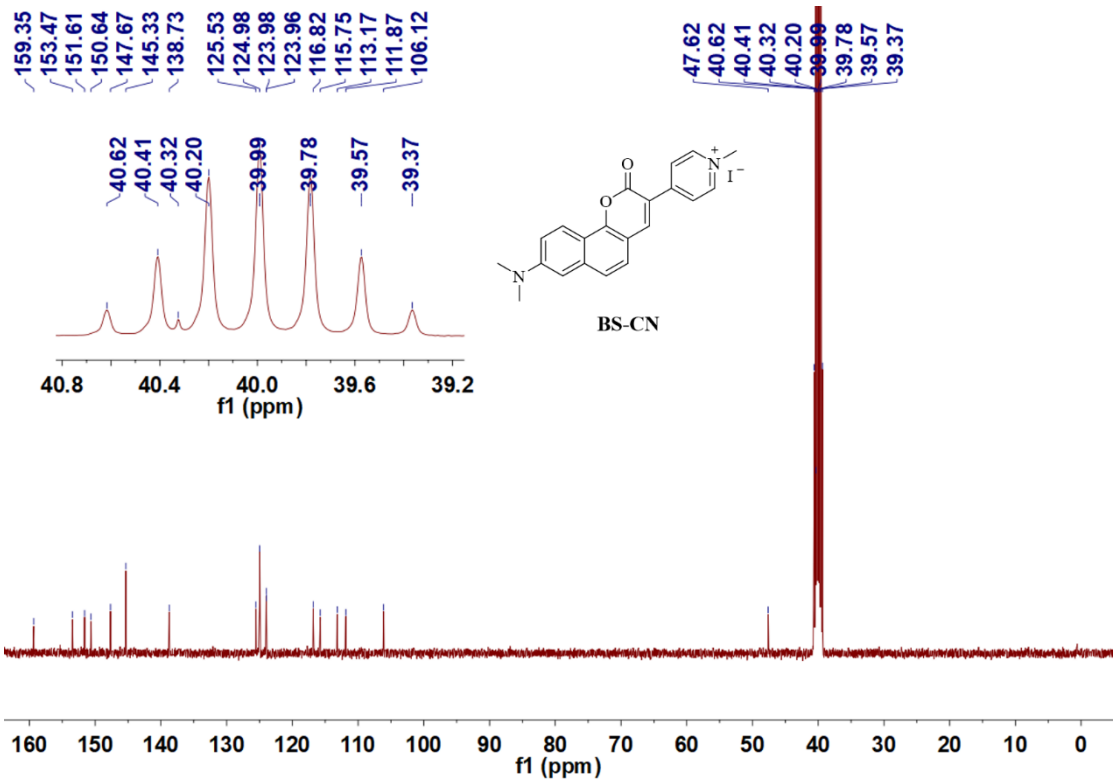


Fig. S18. ^{13}C NMR spectrum of **BS-CN** in DMSO-d_6

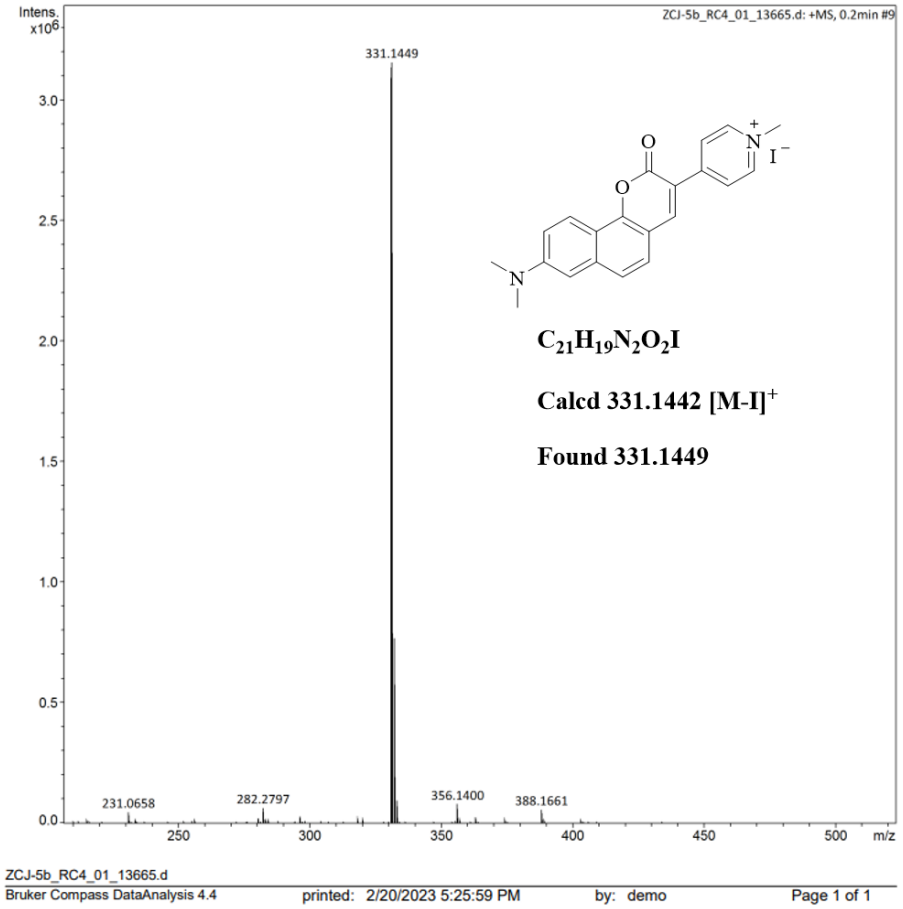


Fig. S19. HRMS of **BS-CN**

Reference

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4 W. Niu, L. Guo, Y. Li, S. Shuang, C. Dong, and M. Wong, *Anal. Chem.,* 2016, **88**, 1908−1914.

5 J. Niu, S. Yang, M. Tian, X. Yu, *Sensors and Actuators B: Chemical,* 2022, **372**, 132693.