Supporting Information for

Fluorescence behavior of a unique two-photon fluorescent probe in aggregate and solution states and high sensitive detection of RNA in water solution and living systems

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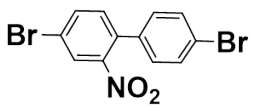
Materials

Nuclear magnetic resonance spectra (^{1}H and ^{13}C) were obtained on a Bruker Avanace 300 spectrometer. The HRMS spectra were recorded on Agilent Technologies 6510 Q-TOF LC/MS or ThermoFisher LCQ FLEET. The elemental analyses were performed on a Vario EI III instrument. The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a Cary 50 spectrophotometer using a quartz cuvette having 1 cm path length. One-photon fluorescence spectra were obtained on a HITACHI F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. Two-photon fluorescence spectra were recorded on a SpectroPro300i and the pump laser beam comes from a mode-locked Ti: sapphire laser system at the pulse duration of 200 fs, with a repetition rate of 76 MHz (Coherent Mira900-D). CD Spectrometer were recorded by Jasco J-810.

The fluorescence of DAPI was excited and collected through U-MNIBA3 and U-MWU2, respectively. The Olypus confocal microscopic image and differential interference contrast (DIC) image were taken with a 488 nm Arion laser. Fluorescence of **HVC-6** were collected with a beam splitter DM570 and BA510-540nm bandpass emission filter combination. All of TPM microscopic photos were obtained with Olympus FV 300 Laser Confocal System with a 60× water objective (N.A. = 1.25) and photomultiplier tubes and Ti: sapphire laser (Coherent) was used to excite the specimen at 800 nm. The total power provided by laser source can be maintained stable and the incident power was examined with Power Monitor (Coherent) directly. A multiphoton emission filter (FF01-750; Semrock) was used to block the IR laser. Tissues imaging using NIKON AIMP two-photon confocal microscopy.

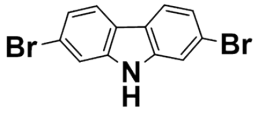
Synthesis



1

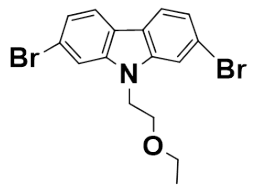
4,4'-Dibromo-2-nitrobiphenyl **(1)**: 4,4'-Dibromobiphenyl 10 g (32 mmol) was

dissolved in glacial acetic acid (120 mL), and the mixture was stirred and heated to 100 °C. Then, fuming concentrated nitric acid (95%, 40 mL) was added and the resulting mixture was allowed to react for another 30 min. After the reaction solution was cooled to room temperature, the crude product was filtered. After recrystallization from ethanol, the title compound was obtained in 90% yield. ^{1}H NMR (300 MHz, CDCl3), δ (ppm): 8.03 (d, J = 1.80 Hz, 1H), 7.76 (dd, $J1$ = 8.10 Hz, $J2$ = 1.80 Hz, 1H), 7.55–7.59 (m, 2H), 7.26-7.30 (t, J = 1.65 Hz, 1H), 7.13-7.18 (m, 2H).



2

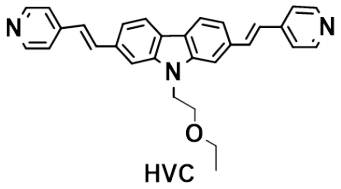
2,7-Dibromocarbazole **(2)**: 7.80 g (22 mmol) of **1** was dissolved in phosphorous acid triethyl ester (30 mL) and the mixture was heated to 150 ^{o}C under the protection of argon. The system was allowed to react for 24 h and a brown solution was obtained. The volatile solvents were then removed by vacuum distillation. The solution left was purified by column chromatography with ethyl acetate/petroleum ether (10:1, v/v) as the eluent. Finally, a white solid was obtained for **2**, in 50% yield. ^{1}H NMR (300 MHz, d6-Acetone), δ (ppm): 11.52 (s, 1H), 8.09 (d, J = 8.40 Hz, 2H), 7.75 (d, J = 1.80 Hz, 2H), 7.33 (dd, $J1$ = 1.80 Hz, $J2$ = 8.40 Hz, 2H).



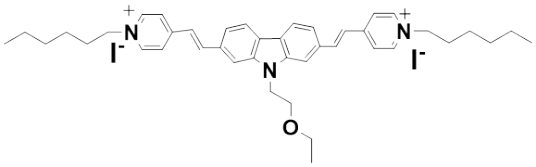
3

2,7-dibromo-9-(2-ethoxyethyl)-9H-carbazole **(3)**: 20 g KOH was added in DMF (70 mL) and the resulting solution was stirred for 30 min. 3.30 g (10 mmol) of **2** was then added and the mixture was stirred for another 40 min. Finally, 1-bromo-2-ethoxyethane (2.28 g, 15 mmol) was added dropwise and the mixture reacted for 12 h

at room temperature. White solid was found when the mixture was poured into water (500 mL). The crude residue was filtered and washed with ethanol for 3 times. A white solid was obtained for **3** after recrystallization from ethanol with a yield of 80%. ^{1}H NMR (300 MHz, DMSO-*d6*), δ (ppm): 8.12 (d, J = 8.40 Hz, 2H), 7.92 (d, J = 1.50 Hz, 2H), 4.57 (t, J = 5.10 Hz, 2H), 3.71 (t, J = 5.25 Hz, 2H), 3.35 (dd, $J1$ = 7.50 Hz, $J2$ = 6.30 Hz, 2H), 0.95 (t, J = 7.05 Hz, 3H).



9-(2-ethoxyethyl)-2,7-bis((E)-2-(pyridin-4-yl)vinyl)-9H-carbazole **(HVC)**: 3.56 g (8.0 mmol) of **3** was added into a flask containing a mixture of palladium(II) acetate (0.18 g, 0.8 mmol), tri-*o*-tolylphosphine (0.72 g, 2.4 mmol) and K_2CO_3 (8.8 g, 64.0 mmol), and to this mixture *N*-methyl-2-pyrrolidone (NMP, 40 mL) and 4-vinylpyridine (3.4 g, 32.0 mmol) was then added. The system was heated to 128 °C for 3 days under the protection of argon. A dark-red suspension was obtained. When the resulting mixture was cooled to room temperature, it was poured into H_2O (500 mL) and extracted with CH_2Cl_2. Then the organic phases were separated, the excess organic solvent was removed by vacuum distillation and a dark-red solution was obtained. The title product was obtained as a yellow solid after the residue was recrystallized from ethanol (yield: 50%). ^{1}H NMR (300 MHz, DMSO-*d6*) δ (ppm): 8.71 (d, J = 6.00 Hz, 4H), 8.17 (d, J = 8.10 Hz, 2H), 7.94 (s, 2H), 7.75 (d, J = 16.20 Hz, 2H), 7.58 (dd, $J1$ = 15.3 Hz, $J2$ = 6.90 Hz, 4H), 7.43 (s, 2H), 7.37 (s, 2H), 4.64 (t, J = 5.25 Hz, 2H), 3.82 (t, J = 5.40 Hz, 2H), 3.48 (q, J = 7.29 Hz, 2H), 0.998 (t, J = 6.90 Hz, 2H).



HVC-6

2,7-bis(1-iodododecane -4-vinylpyridium iodine)-N-ethylcarbazole **(HVC-6)**: 0.90 g (2 mmol) of HVC and excess iodine hexane were dissolved in acetone and stirred for 2 h at room temperature. Then the mixture was refluxed for another 12 h and a red residue was obtained. The residue was filtered and then washed with methanol for 3 times. The title product was obtained as a red solid after the residue was recrystallized from ethanol (Yield: 75%). IR (cm-1): 973 (υ_{trans}=C−H). ^{1}H NMR (300 MHz, DMSO-*d6*), δ (ppm): 8.97 (d, J = 6.60 Hz, 4H), 8.27–8.32 (m, 8H), 8.07 (s, 2H), 7.70 (t, J = 8.10 Hz, 2H), 4.68 (s, 2H), 4.51(t, J = 7.35 Hz, 4H), 3.85 (t, J = 5.41 Hz, 2H), 3.41 (m, 2H), 1.93 (s, 4H), 1.31 (s, 12H), 0.98 (t, J = 6.90 Hz, 3H), 0.88 (t, J = 6.30 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 153.94, 145.18, 143.00, 142.58, 134.49, 124.64, 123.83, 122.25, 120.57, 111.01, 69.00, 66.74, 60.67, 31.52, 31.42, 26.05, 22.80, 15.93, 14.76. HRMS (m/z): $[M-2I]^+/2$ calcd for $C_{42}H_{53}I_2N_3O$, found, 307.7110

General procedure for the spectral measurement

The stock solution of the probe **HVC-6** was prepared at 1 mM in DMSO. The tris buffer solutions were prepared. The solutions of various testing species were prepared from Homocysteine (Hcy), glutathione (GSH), $Zn(Ac)_2$ KBr, NH_4Cl, KCN, $CuSO_4$, $FeCl_2$, $KHPO_4$, $NaHSO_3$, KI, KNO_3, $MgCl_2$, $NaNO_3$, $NiCl_2$, K_3PO_4, Na_2SO_3, K_2SO_4, $ZnCl_2$, $NaNO_2$, KNO_3, H_2O_2, BSA, Anti-EGFR, Glucose, G-M IgG, LPS and Tubulin in the twice-distilled water. The test solution of the probe **HVC-6** (10 μM) in 5 mL buffer was prepared. For the one-photon fluorescence spectra experiments, the excitation wavelength was 488 nm, and the excitation and emission slit widths were 5 and 5 nm, respectively. For the two-photon fluorescence spectra experiments, the excitation wavelength was 800 nm.

Calculation of RNA concentration[1]

RNA concentration was calculated by bellow equation. The ultraviolet absorption intensity standed for size of the electron energy level transition probability, and abide by the lambert beer's law (1).

$$A = -\log\frac{I}{I_0} = \varepsilon c l \quad (1)$$

A is absorbancy; ε is extinction coefficient, extinction coefficient of RNA is 7700; c is molar concentration; l is length of sample pool; I_0 and I stand for The intensity of the incident light and transmission light, respectively.

Quantum yields[2]

The fluorescence quantum yields (Φ) were calculated by equation (2):

$$\Phi_s = \Phi_r\left(\frac{A_r(\lambda_r)}{A_s(\lambda_s)}\right)\left(\frac{n_s^2}{n_r^2}\right)\frac{F_s}{F_r} \quad (2)$$

In equation, s and r referred to the sample and the reference, respectively. Φ was quantum yield, F stand for the integrated emission intensity, A and n stand for the absorbance and refractive index, respectively.

Detection limit[3]

The detection limit was acquired from the RNA fluorescence titration data by previous report. According to titration experiment, the fluorescent intensity data at 550 nm were normalized between the minimum intensity and the maximum intensity. A linear regression curve was then fitted to five normalized fluorescent intensity data. To obtain the slope, the fluorescence intensity at 550 nm was plotted against the concentration of RNA. The detection limit was calculated by equation (3):

$$\text{Detection limit} = 3\sigma/k \quad (3)$$

In equation, σ is the standard deviation of the blank measurement, k is the slope of fluorescence intensity vs. RNA concentration.

Solid fluorescence imaging

For solid fluorescence imaging, first, take a small amount of solid of **HVC-6** in a petri dish, and solid powder distributed evenly in the dish. And then solid **HVC-6** was imaged with Nikon Andor wide-field fluorescence microscopy. Wide-field fluorescence images of **HVC-6** were collected between 565 and 700 nm upon excitation at 500-550 nm.

Cell culture and live cell imaging

Cancer cells (HeLa) were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and 10% bovine calf serum in a 5% CO_2 incubator at 37°C. For living cells imaging experiment of **HVC-6**, cells were incubated with 5 μM **HVC-6** in PBS (pH 7.2) for 0.5 h at 37 °C. After rinsing with PBS twice, cells were imaged immediately. **HVC-6** were dissolved in DMSO at a concentration of 1 mM and DAPI was prepared as 1 mM aqueous solution.

The one- and two-photon imaging: The living HeLa cells were incubated with 5 μM **HVC-6** in the culture medium for 30 min, and then the medium was removed and washed three times with PBS to remove the excess probe. Finally, confocal fluorescence imaging was carried out by Olympus FV 300 Laser Confocal System with a 60× water objective (N.A. = 1.25) and photomultiplier tubes and Ti: sapphire laser (Coherent) was used to excite the specimen at 800 nm. One-photon fluorescence images of **HVC-6** were collected between 565 and 700 nm upon excitation at 488 nm; Two-photon Fluorescence images of **HVC-6** were collected between 565 and 700 nm upon excitation at 800 nm.

RNase digest test of fixed cells.

Before cells digest test, cultured cells grown on glass coverslips were pretreated according to the following procedure: cells were first fixed by 4% paraformaldehyde for 30 min and then permeabilized by 0.5% Trition X-100 for 2 min at ambient temperature. DAPI was prepared as 1 mM aqueous solution. For RNase digest test,

two sets of pretreated HeLa cells were stained with 5 μM **HVC-6** for 30 min. After rinsing with PBS twice, a total 1 mL PBS (as control experiment) was added into a set of cells and 25 mg/mL DNase-Free RNase (GE) was added into the other set of cells, and then two sets of cells were incubated at 37°C in 5% CO_2 for 2 h. In addition, the RNase digest test of cells stained with 1 μM DAPI was also carried out for comparison. After rinsing with PBS twice, cells with DAPI treated were imaged with wide-field fluorescence microscopy; cells were stained by **HVC-6** were imaged using confocal fluorescence microscopy. cells with **HVC-6** and RNase treated were imaged with confocal fluorescence microscopy.

Cell viability evaluated by MTT assay

HeLa cells were seeded per well in a 96-well plate. The next day the medium was changed into a medium containing 5 μM **HVC-6**. After different incubation times (0.5, 1, 2, 6h), the medium and the excess probe were removed, and then 10 μL MTT (5 mg/mL in PBS) was added. Subsequently, the culture medium was removed, and 100 μL DMSO was added into the dishes to dissolve the formazan crystal product. The plate was shaken for 10 min, and then the absorbance at 490 nm was measured by the microplate reader. The cell viability (%) = $(OD_{490\ sample} - OD_{490\ blank})/(OD_{490\ control} - OD_{490\ blank}) \times 100\%$. $OD_{490\ sample}$ denotes the cells incubated with the probe for different incubation time, $OD_{490\ control}$ denotes the cells without the probe, $OD_{490\ blank}$ denotes the wells containing only the culture medium.

Preparation of fresh mouse liver slices and TP fluorescence imaging

Mice was bought in Shandong University College of Life Science. The slices were prepared from the liver of 14 day-old mice, and they were cut to 400 mm thickness by using a vibrating-blade microtome in 25 mM PBS (pH 7.4). The slices were incubated with 20 μM **HVC-6** in PBS buffer bubbled with 5% CO_2 for 1 h at 37 °C, and then were washed three times with PBS buffer solution, transferred to glass-bottomed dishes, and observed under a TP confocal microscope (Nikon A1MP). The fluorescence images of the slices were acquired using 800 nm excitation and

fluorescence emission windows of 500-550 nm.

Density functional theory (DFT) calculations

Density functional theory (DFT) calculations were carried out with the GAUSSIAN 09 program package. All the calculations were performed on systems in the gas phase using the Becke's three-parameter hybrid functional with the LYP correlation functional (B3LYP) and 6-31G(d, p) basis set.

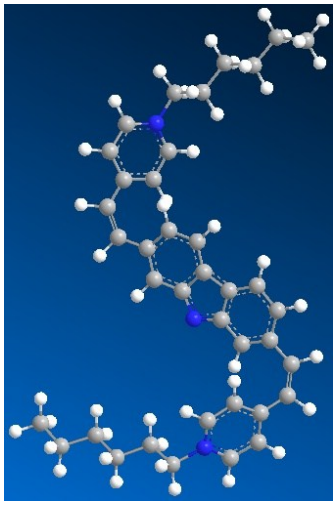


Fig. S1. DFT optimized "Helical Structures''structure of **2,7-**position carbazole derivative.

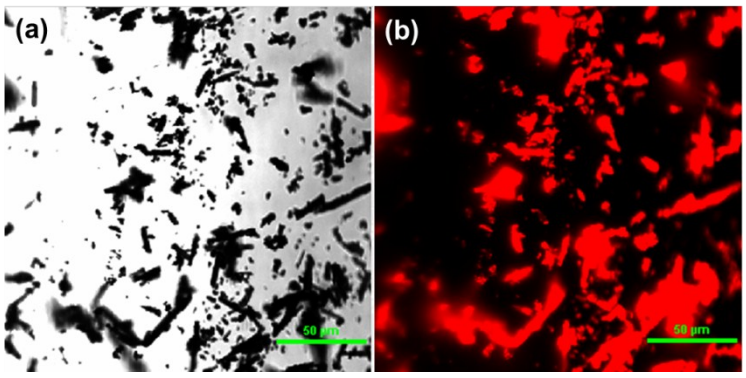


Fig. S2. (a) Solid states of the probe **HVC-6**; (b) Solid fluorescence imaging of the probe **HVC-6**. Scale bars: 50 μm. λ_{ex}: 500-550 nm; λ_{em}: >550 nm.

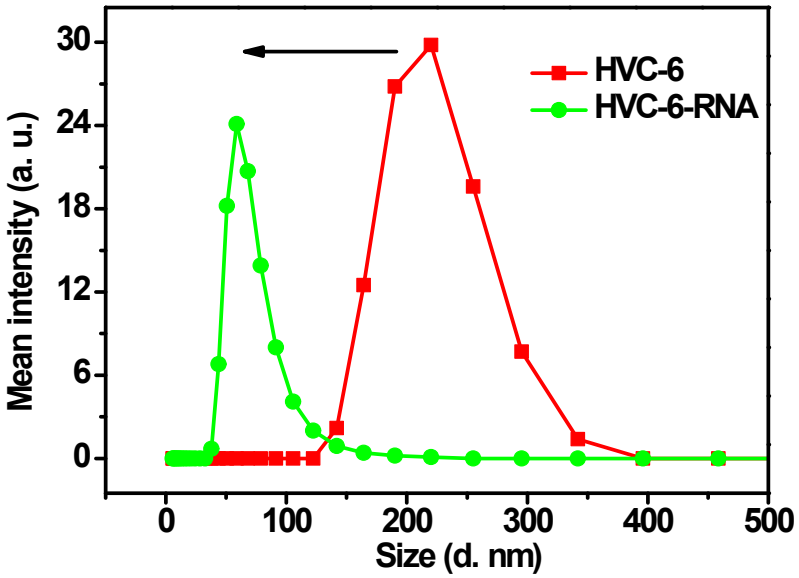


Fig. S3. Dynamic light scattering (DLS) measurements of **HVC-6** (10 μM) in the presence or absence of RNA.

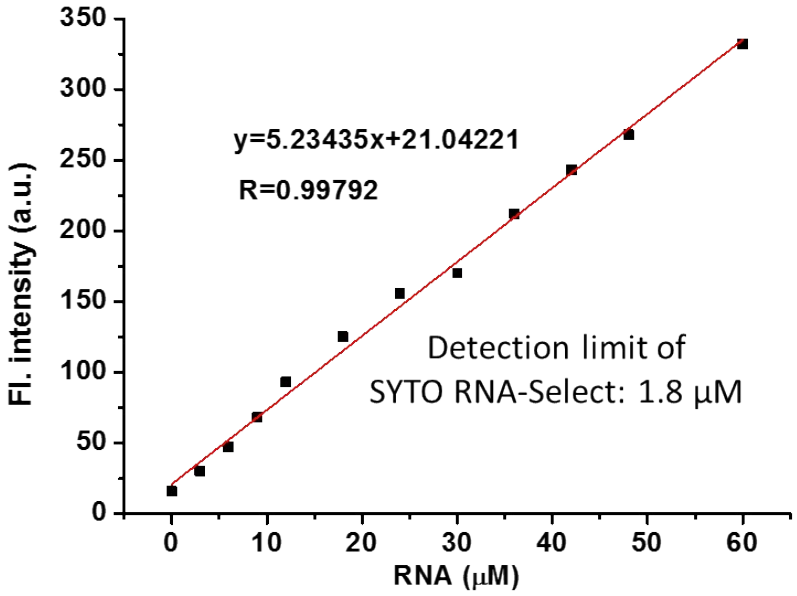


Fig. S4. Normalized response of fluorescence signal of commercially available probe SYTO RNA-Select by changing the concentration of RNA.

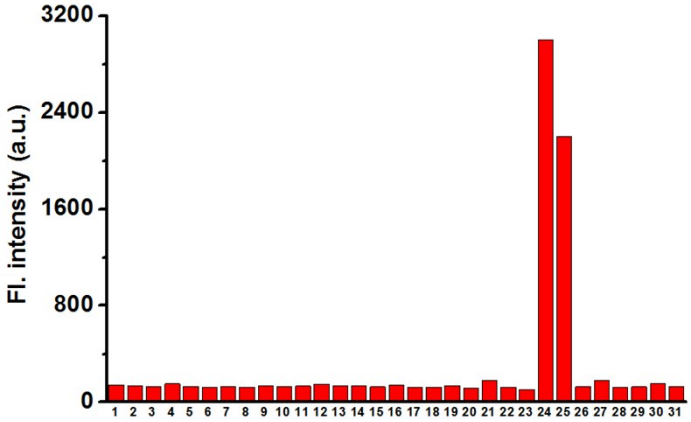


Fig. S5. Fluorescence responses of **HVC-6** (10 μM) in the presence of various relevant analytes. The concentrations of the representative analytes are: amino acids, 1.5 mM; GSH, 1.5 mM; cations and anions, 2 mM; reactive oxygen and nitrogen species, 3 mM. RNA/DNA, 5.5 mM. Legend: 1. **HVC-6**; 2, Hcy; 3, GSH; 4, Ac^-; 5, Br^-; 6, Cl^-; 7, CN^-; 8, Cu^{2+}; 9, Fe^{2+};10, HPO_4^-; 11, HSO_3^-; 12; I^-; 13, K^+; 14, Mg^{2+}; 15, Na^+; 16, Ni^{2+}; 17, PO_4^{3-}; 18, SO_3^{2-}; 19, SO_4^{2-}; 20, Zn^{2+}; 21, NO_2^-; 22, NO_3^-; 23, H_2O_2; 24, RNA; 25, DNA; 26, BSA; 27, Anti-EGFR; 28, Glucose; 29; G-M IgG; 30, LPS; 31, Tubulin. λ_{ex}: 488 nm.

Table S1. Cytotoxicity Data of **HVC-6** (5 μM) in HeLa cells[a].

Incubation time	0.5 h	1 h	2 h	6 h
Survival (%)	92±3	90±3	85±3	85±1

[a] Cell viability was quantified by the MTT assays (mean ± SD).

The RNase digest experiment: Positive control experiment was determined from a reported method.[1]

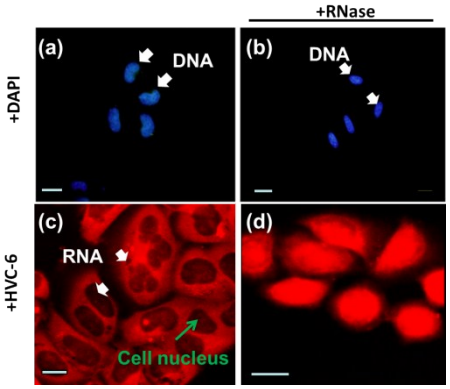


Fig. S6. The RNase digest experiment. Fluorescence imaging of HeLa cells stained with DAPI (a and b); Fluorescence imaging of HeLa cells stained with **HVC-6** before (c) or after (d) treatment with RNase (30 μg/ml) for 2 h at 37 ◦C. Conditions: 5 μM, dyes; 1 μM, DAPI; Incubation time: 30 min. λ_{ex} (DAPI) = 330-385 nm, λ_{em} (DAPI) >410 nm; λ_{ex} (**HVC-6**) = 488 nm, λ_{em} (**HVC-6**) > 565 nm; Scale bar = 20 μm.

***In site* imaging:** *In site* imaging experiment was determined from a reported method.[4] As shown in Fig. S7, the probe **HVC-6** emitted stronger fluorescent emission than commercially available probe SYTO RNA-Select under the same conditions. This experiment was carried out by the Nikon infrared spectra detector (IR SD) at the same conditions.

Imaging conditions: HV: 194; laser: 48.4; Pinhole: 0.9; λ_{ex} = 488 nm;

Other conditions see caption of Fig. S5.

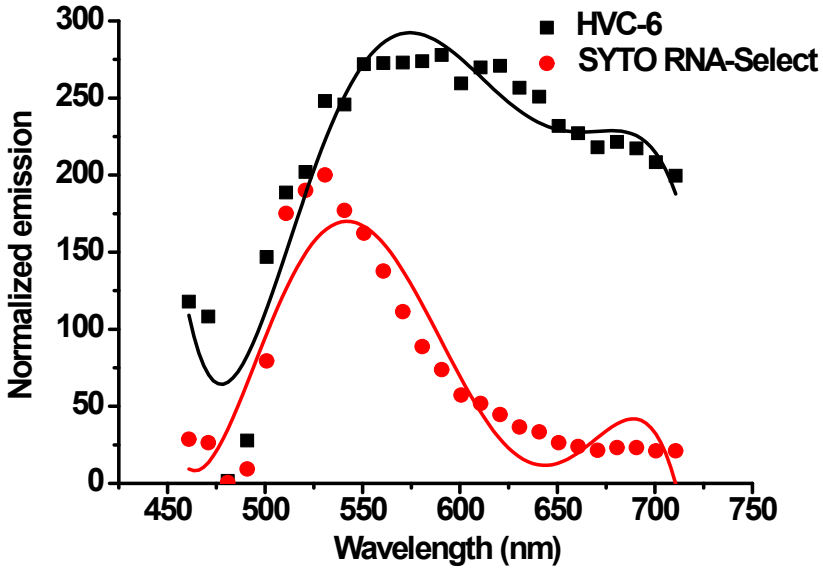


Fig. S7. Fluorescence images and spectra of the HeLa cells incubated with **HVC-6** and SYTO RNA-Select (4 μM) by the Nikon infrared spectra detector (IR SD). λ_{ex} = 488 nm.

Circular dichroism (CD) spectra: CD experiment was determined from a reported method.[5]

To further explain the mechanism of the light-up effect upon binding to RNA, the interactions between **HVC-6** and RNA were also investigated by induced circular dichroism (ICD) spectra. As shown in Fig. S8a, CD signal of RNA appeared in range of 200-300 nm, and plus and minus two signals confirm spiral chain characteristics of RNA. Due to induction of RNA, **HVC-6**, a compound without optical activity, present optically activity. Moreover, there were negative cottons effect in the electronic absorption (400-500 nm) region of **HVC-6**, implying that the electron transition easily absorbs right-circularly polarized light. Meanwhile, cotton peaks at 400 (-) and 470 (+) nm corresponded to UV absorption peaks at 400-500 nm of **HVC-6** bound with RNA (Fig. S8b). It can be assumed that the groove binding between **HVC-6** and RNA may affect the molecular conformation and bring about an asymmetric chiral center in **HVC-6**, resulting in optical activity. The results showed that **HVC-6** was capable of preferentially interact with RNA within cells.

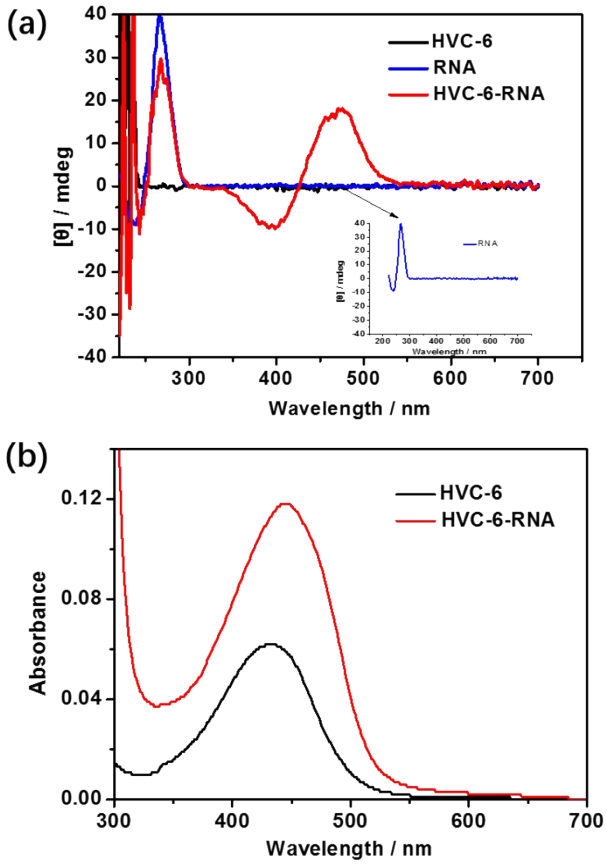


Fig. S8. (a) Circular dichroism (CD) spectra of the **HVC-6**, RNA and **HVC-6**-RNA; Inset: CD spectrum of RNA; (b) Absorbance spectra of the **HVC-6** and **HVC-6**-RNA **[HVC-6]**: 20 μM. Ratio (phosphate of RNA/dye): 15:1.

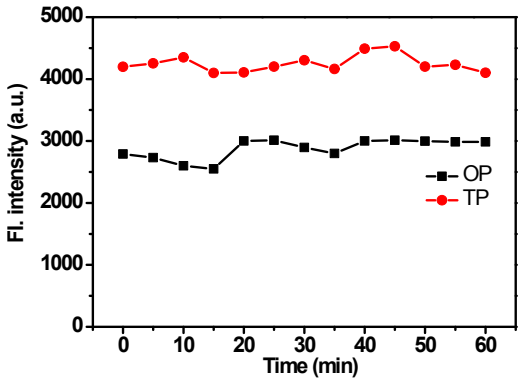


Fig. S9. One-photon (OP) and two-photon (TP) fluorescence intensities of **HVC-6** to RNA in tris buffer solution under successive irradiation for different times.
OP conditions: [**HVC-6**] = 10 μM, RNA: 500 equiv., λ_{ex} = 488 nm.
TP conditions: [**HVC-6**] = 20 μM, RNA: 800 equiv., λ_{ex} = 800 nm.

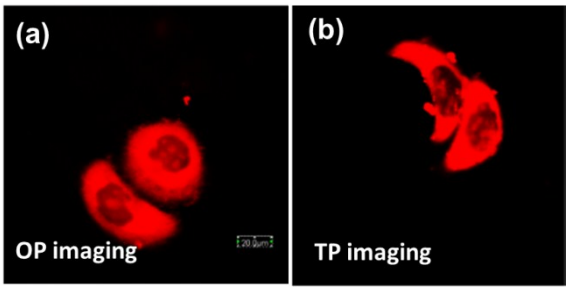


Fig. S10. One- (a) and two-photon (b) fluorescence imaging of HeLa cells stained with 5 μM **HVC-6** for 30 min, Bar = 20 mm.

Tomoscan: A two-photon microscopic imaging of **HVC-6** including 10 optical sections at different depths has been recorded using two-photon tomoscan technology (Fig. S7). These results demonstrated that RNA fluorescence came from region of certain depths.

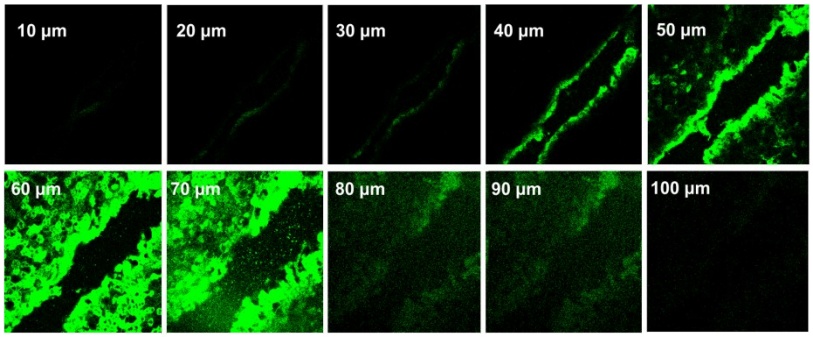


Fig. S11. TP fluorescence images (λ_{ex} = 800 nm) of the mouse liver slice incubated with 20 μM **HVC-6**.

Spectral characterization

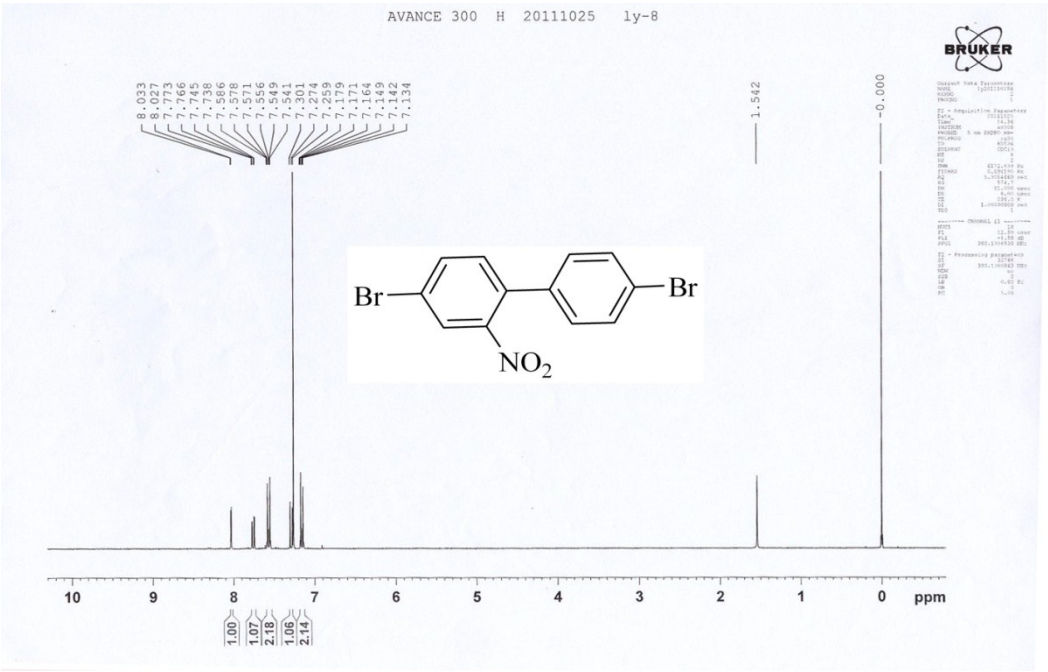


Fig. S12. [1]H NMR spectrum of the compound **2**

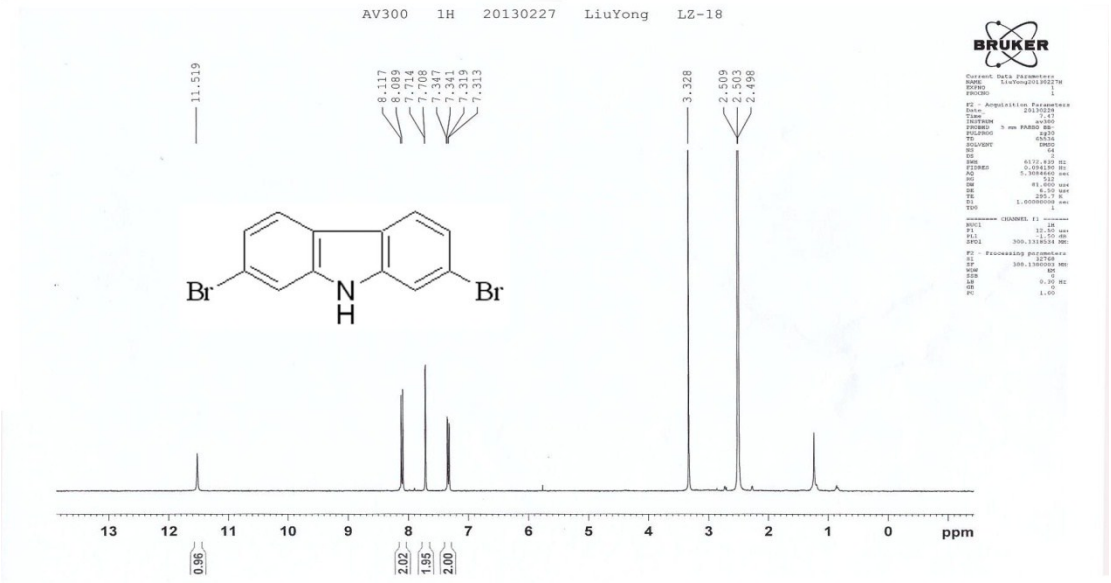


Fig. S13. [1]H NMR spectrum of the compound **2**

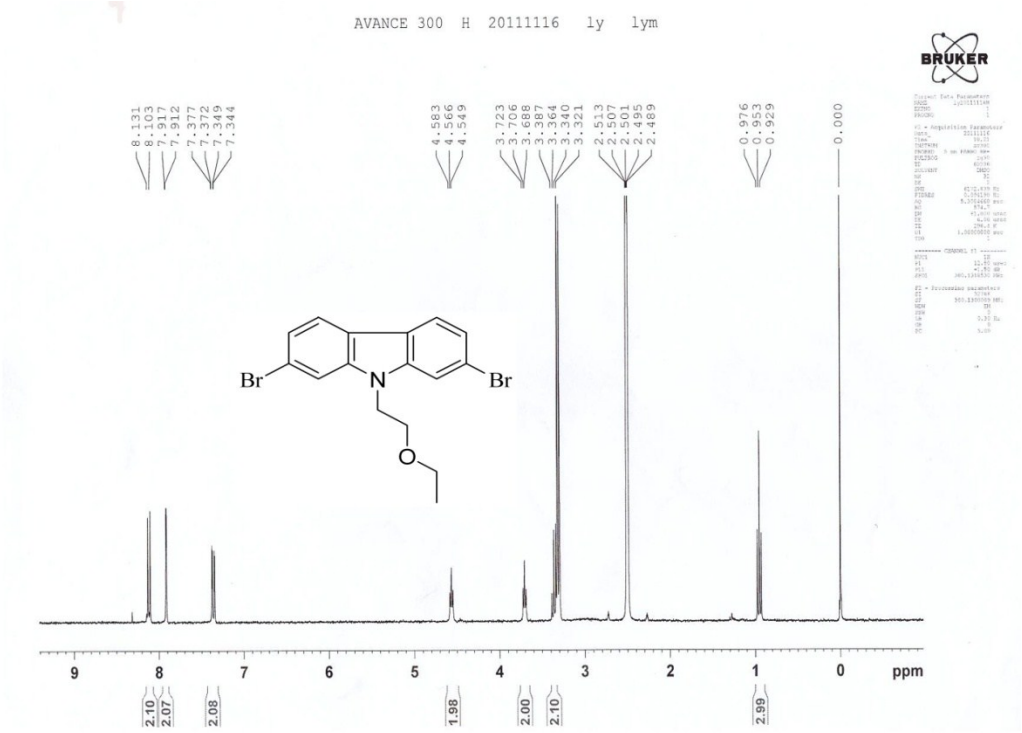


Fig. S14. [1]H NMR spectrum of the compound **3**

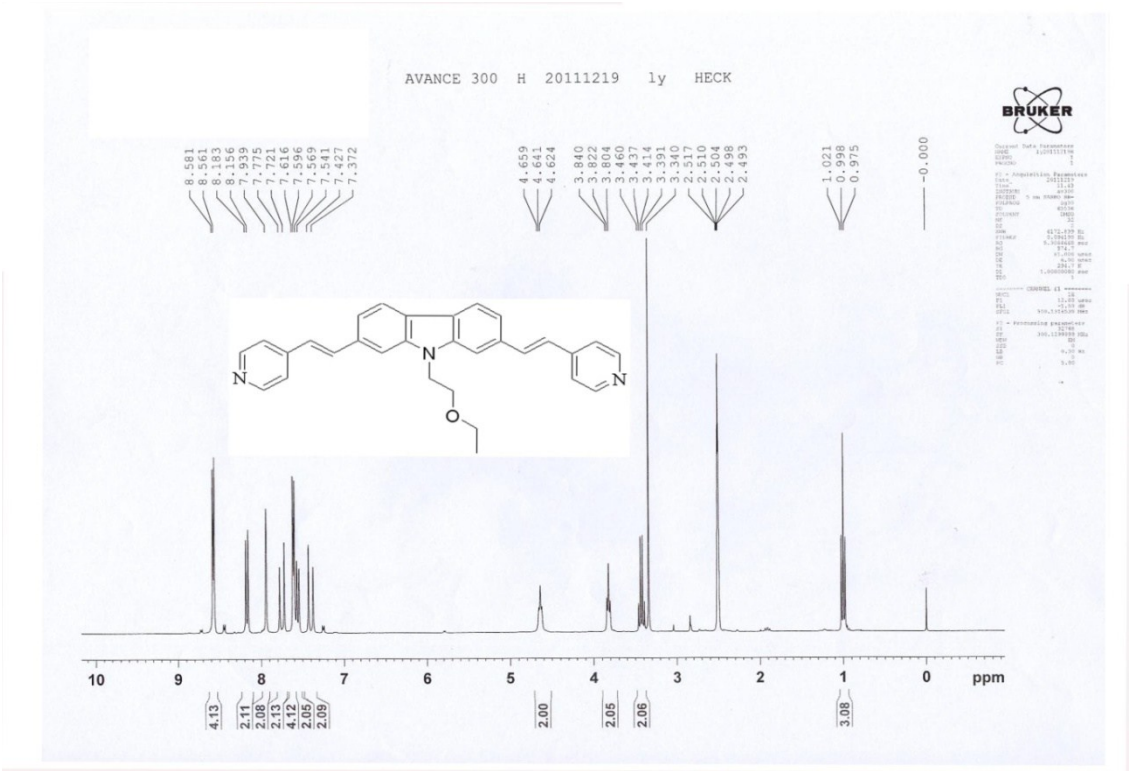


Fig. S15. [1]H NMR spectrum of the compound **HVC**

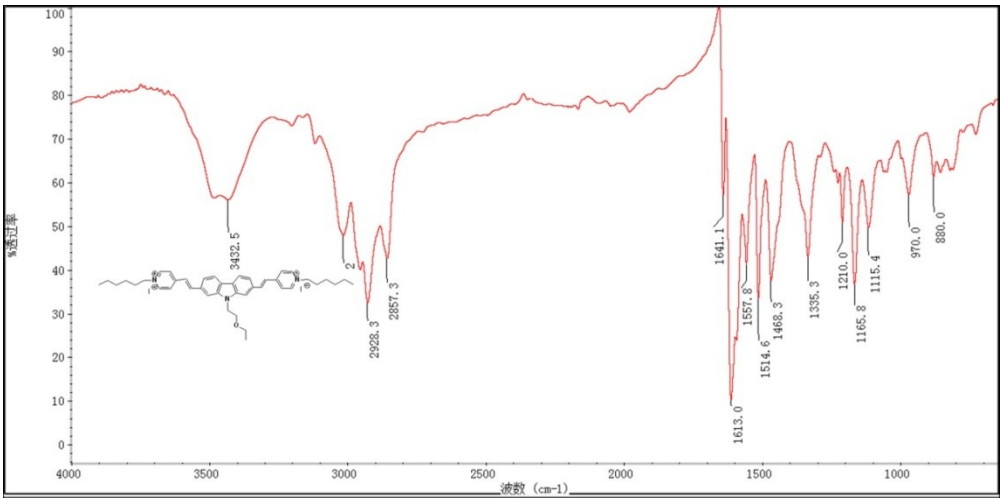


Fig. S16. FT-IR spectrum of **HVC-6**

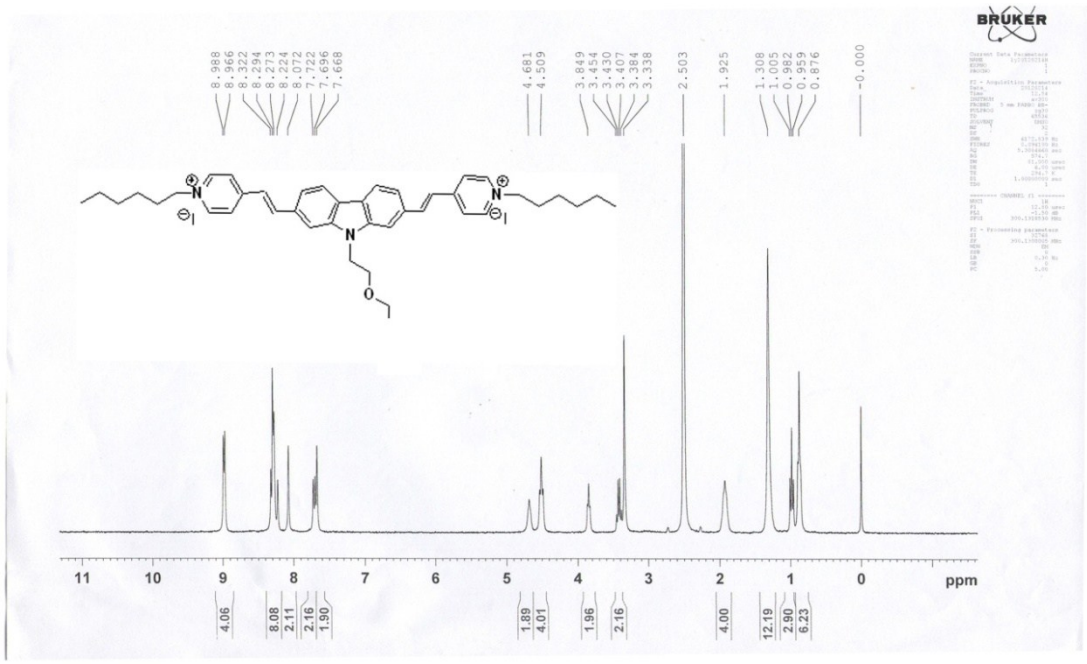


Fig. S17. [1]H NMR spectrum of the compound **HVC-6**

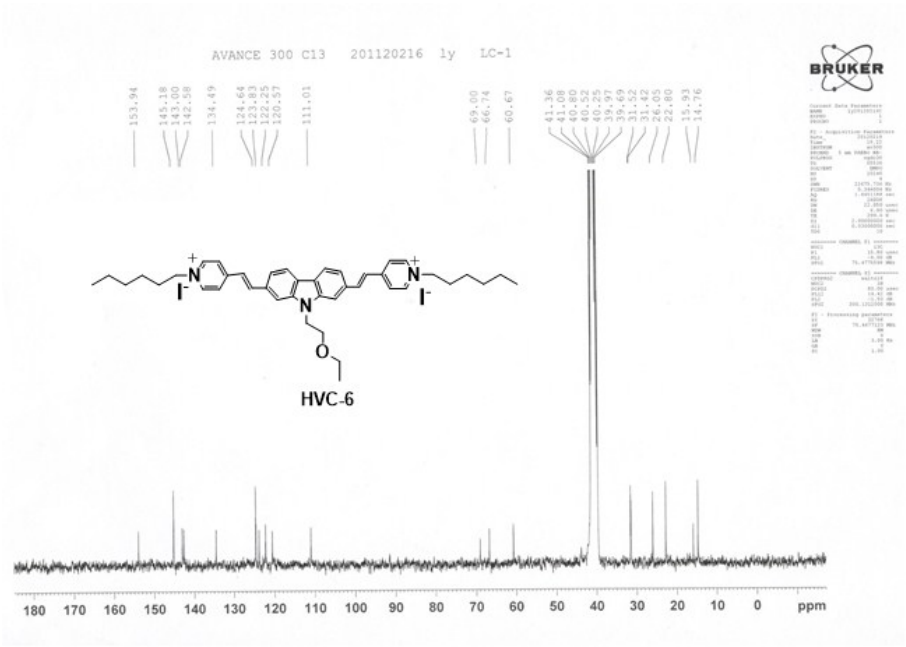


Fig. S18. ^{13}C NMR spectrum of the compound **HVC-6**

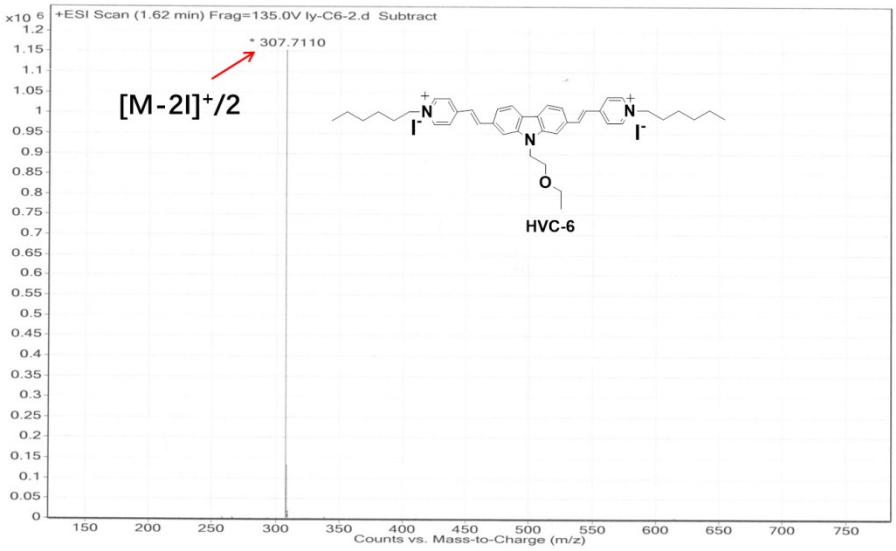


Fig. S19. HRMS spectrum of the compound **HVC-6**

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