*Supporting Information for*

# A biotin-guided fluorescent probe for dual-mode imaging of viscosity in cancerous cells and tumor tissue

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## Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments; Mass spectrometric analyses were measured on a Finnigan MAT 95 XP spectrometer; High resolution mass spectrometric (HRMS) analyses were measured on an Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an AVANCE III 400 MHz Digital NMR Spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a Shimadzu UV-2700 power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer with a 1 cm standard quartz cell; The fluorescence imaging of cells was performed with a Nikon A1MP confocal microscope; The fluorescence lifetime imaging were measured by LSM880 NLO confocal laser scanning microscope (Carl Zeiss Co., Germany) with a time-correlated single photon counting (TCSPC) unit (Simple-Tau-152NX, Becker & Hickl, Germany); The SPCImage software was used for TCSPC Imaging (FLIM) data, and fluorescence decay analysis. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

## Viscosity determination and spectral measurement

The solvents were obtained by mixing methanol-glycerol systems in different proportions. Measurements were carried out with NDJ-8S rotational viscometer, and each viscosity value was recorded. The solutions of **Biotin-V** of different viscosity were prepared by adding the stock solution (1.0 mM) to solvent mixture (methanol-glycerol solvent systems) to obtain the final concentration of the dye (10.0 μM). These solutions were sonicated for 5 minutes to eliminate air bubbles. After standing for 1 hour at a constant temperature, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer. For measure the response to viscosity, all the measurements were made according to this procedure.

## Fluorescence lifetime detection

Variable viscosity was prepared by adjusting the volume ratio of glycerol to MeOH. The fluorescence decay curves of the probe **Biotin-V** at different viscosity were measured with the excitation wavelength at 550 nm and emission at 608 nm, and the fluorescence lifetime was calculated. An excellent straight fitting was obtained and the quantitative relationship between the fluorescence lifetime of **Biotin-V** and the viscosity of the solution is described by Förster-Hoffmann equation.

$$\text{Log } \tau = C + x \log \eta$$

Where $\tau$ is the fluorescence lifetime; $\eta$ stands the viscosity of solution; C is a concentration- and temperature-dependent constant and x is a sensor- and temperature-dependent constant.

## Culture and preparation of living cells for imaging experiment

Different types of cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% $CO_2$ and 95% air at 37 °C. Before the experiments, seed the cells in 35-mm glass-bottomed dishes at a density of $2\times10^5$ cells per dish in 2 mL of culture medium and incubate them inside an incubator containing 5% $CO_2$ and 95% air at 37 °C. Incubate the cells for 24 h. Cells will attach to the glass surface during this time.

## Cytotoxicity assay

*In vitro* cytotoxicity was measured using the colorimetric methyl thiazolyl tetrazolium (MTT) assay on HeLa cells. Cells were seeded into the 96-well tissue culture plate in the presence of 100 μL Dulbecco's modifed eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% $CO_2$ atmosphere for overnight and then incubated for 24 h in the presence of **Biotin-V** at different concentrations (0, 1, 5, 10, 20, 30 μM). Then cells were washed with PBS buffer and 100 μL supplemented DMEM medium was added. Subsequently, 10 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. Violet formazan was dissolved in 100 μL sodium dodecyl sulfate solution in

the water-DMF mixture. Absorbance of the solution was measured at 570 nm using a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without **Biotin-V**.

## Imaging of viscosity in HeLa cells with stimulated by nystatin and monensin

Before the experiments, the well-prepared cells were washed with PBS (pH=7.4) buffer three times. We use nystatin stimulated HeLa cells. Firstly, HeLa cells were incubated with probe **Biotin-V** (5 μM) (containing 0.1 % DMSO as a cosolvent) for 30 min at 37 ˚C. Wash cells twice with 1mL PBS at room temperature, and then add 1 mL PBS and observe under a confocal microscopy. Secondly, HeLa cells were coincubated with nystatin and monensin (10 μM) for 30 min at 37 ˚C and then washed with PBS twice, and the cells were incubated with **Biotin-V** (5 μM) for 20 min at 37 ˚C, and then washed with PBS three times, and the fluorescence intensity images and FLIM images were acquired through a Nikon A1MP confocal microscopy with a cooled CCD camera and LSM880 NLO confocal laser scanning microscope (Carl Zeiss Co., Germany) with a time-correlated single photon counting (TCSPC) module respectively.

## Preparation of tumor-bearing mice models and organs for imaging experiment

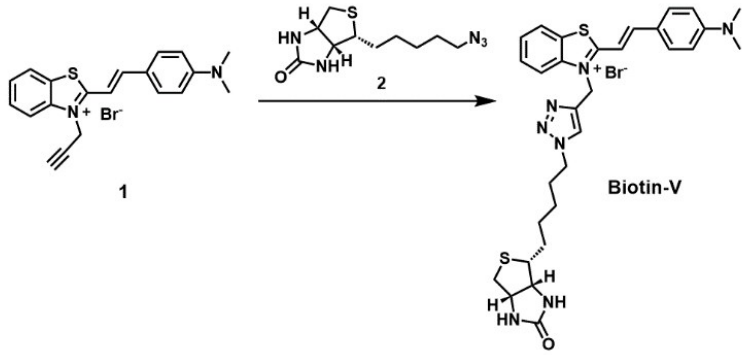
Five-week old female mice were purchased from School of Pharmaceutical Sciences, Shandong University, and the studies were approved by the Animal Ethical Experimentation Committee of Shandong University. All animals were kindly kept during experiment according to the requirements of the National Act on the use of experimental animals (China).

4T1 cells were grafted into the mice to produce tumor models and on day 14 post-injection, a tumor was obtained. Then the tumor-bearing mice was utilized for *in vivo* imaging. Before in vivo imaging, the mice were anesthetized by 4% chloral hydrate aqueous solution (150 μL). and their abdominal fur were removed by an electric shaver. **Biotin-V** (50 μL, 1mM) was then injected into the abdominal position of the tumor-grafted mice by hypodermic injection for 30

min. The mouse was then imaged by using an *in vivo* imaging system with an excitation filter of 580 nm and an emission filter of 620 nm.

The organs were loaded with 10 μM **Biotin-V** for 30 min. Then all organs were imaged under IVIS Lumina XR system using a 580 nm excitation and a 620 nm emission filter. The statistical analysis was performed from three separate biological replicates. A region of interest (ROI) was created around in each image and the mean fluorescence intensity was measured by an *in vivo* imaging system software and averaged across the three fields imaged. The probe loaded organs was sectioned by slicer, and the fluorescence lifetime imaging of liver and tumor tissue slices was studied by FLIM system.

## Synthesis




**Scheme S1**. Synthesis of Viscosity fluorescent probe of **Biotin-V**.

***Synthesis of compound Biotin-V***

Compound **1** (100 mg, 0.25 mmol, 1.0 eq) and biotin azide **2**（64 mg，0.25 mmol，1.0 eq）were dissolved in anhydrous DCM (10 mL) under a nitrogen atmosphere. To this solution, tetrakis(acetonitrile)copper(I) hexafluorophosphate (19 mg, 0.05 mmol, 20 mol%) and DIPEA (25 μL, 0.14 mmol, 0.57 eq.) were added and the flask was covered with aluminium foil and stirred at room temperature for 24 h. Then the reaction solution was washed with a saturated solution of EDTA in 17% $NH_3/H_2O$ (10 mL), and the aqueous layer was extracted with DCM (3 × 15 mL). The combined organic layers were dried over $Na_2SO_4$, filtered and concentrated *in vacuo* to give crude product. The resulting residue was purified by column chromatography

on silica gel (MeOH / $CH_2Cl_2$ = 1: 10, v/v) to afford the compound **Biotin-V** as a gray solid (105 mg, yield: 64%). $^1$H-NMR (400 MHz, $CDCl_3$) 8.41 (s, 1H), 8.31 (d, *J* = 8.0 Hz, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 8.14 (d, *J* = 15.1 Hz, 1H), 8.02 – 7.84 (m, 3H), 7.77 (t, *J* = 7.6 Hz, 1H), 7.67 (t, *J* = 7.7 Hz, 1H), 6.87 (d, *J* = 8.7 Hz, 2H), 6.40 (d, *J* = 7.9 Hz, 2H), 6.19 (s, 2H), 4.58 (s, 1H), 4.32 (dt, *J* = 18.3, 6.6 Hz, 3H), 4.08 (s, 1H), 3.13 (s, 6H), 3.02 (q, *J* = 6.7 Hz, 1H), 2.79 (dd, *J* = 12.4, 5.1 Hz, 1H), 1.84 – 1.67 (m, 2H), 1.53 (d, *J* = 10.3 Hz, 1H), 1.45 – 1.07 (m, 8H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.0, 163.2, 154.0, 151.3, 141.4, 140.3, 133.6, 129.3, 127.8, 124.9, 124.4, 122.0, 116.6, 112.4, 106.6, 61.4, 59.7, 55.8, 50.0, 49.0, 43.4, 30.6, 29.9, 29.5, 28.6, 28.4, 26.3, 17.7. HRMS (ESI) m/z calcd for $C_{30}H_{36}N_7OS_2^+$ [$M^+$]: 574.2423; Found 574.2415.

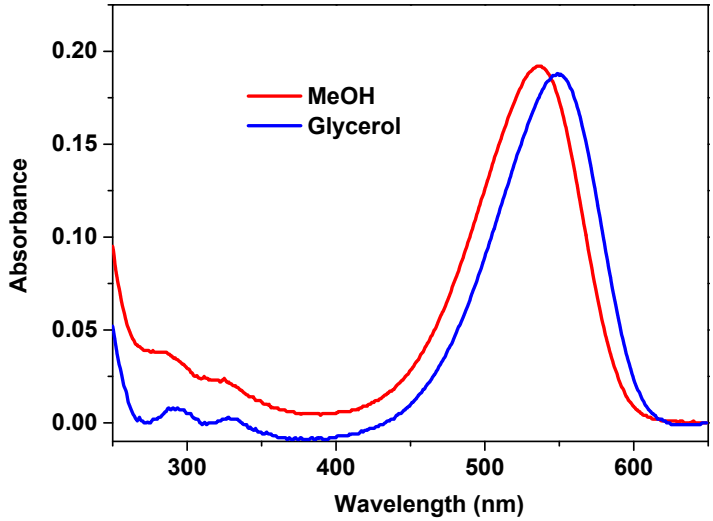



**Figure S1** Absorption spectra of the probe of **Biotin-V** (10 μM) in MeOH (red line) and in Glycerol (blue line).

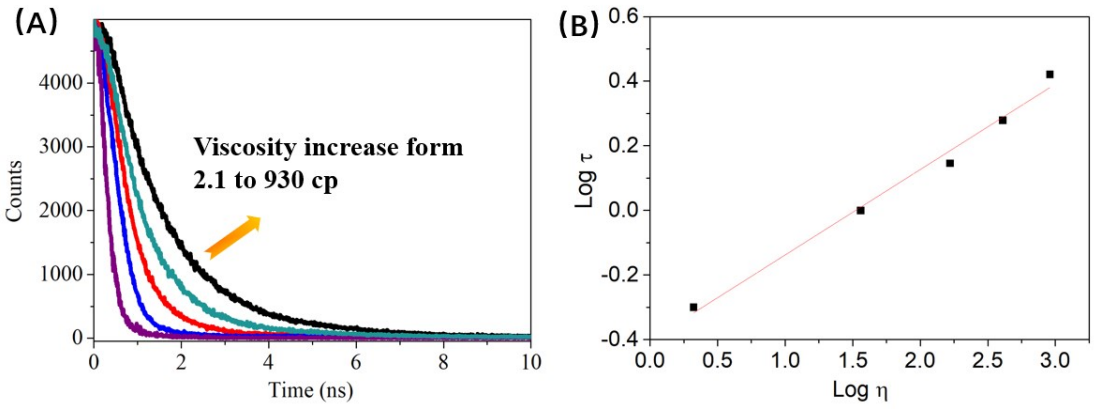


**Figure S2** (A) Fluorescence decays and (B) the linear relationship of lifetimes with different viscosity of the fluorescent dye **Biotin-V**.

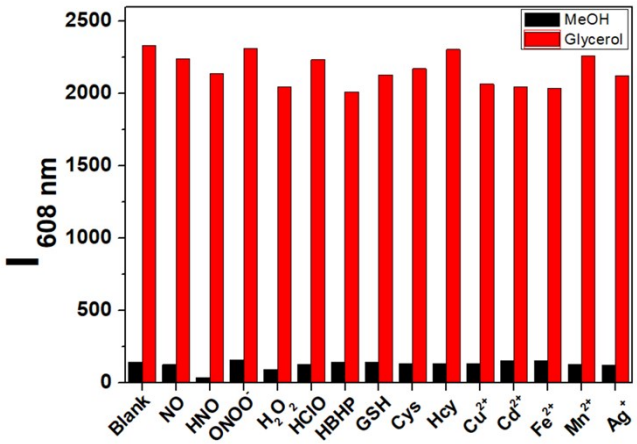



**Figure S3** (A) Fluorescence intensities of the probe **Biotin-V** (10 μM) treated with various species (Blank; NO (50 μM); HNO (50 μM); ONOO- (50 μM); $H_2O_2$ (50 μM); HClO (50 μM); TBHP (50 μM); GSH (5 mM); Cys (100 μM); Hcy (100 μM); $Cu^{2+}$ (50 μM); $Cd^{2+}$ (50 μM) ; $Fe^{2+}$ (50 μM); $Mn^{2+}$ (50 μM); $Ag^{+}$(50 μM) in methanol and glycerol solution.

To verify that the probe's response to viscosity was caused by the TICT mechanism, the structure of the viscosity response part of the probe **Biotin-V** in ground state and excited state were optimized by DFT with B3LYP/6-31G(d) using Gaussian 09. As shown in Fig. S4A, benzthiophene and diethylaminobenzene stayed almost in a planar conformation in the ground state, displaying large conjugated system. However, in the excited state, benzthiophene and diethylaminobenzene were in a perpendicular state to each other (Fig. S4B), meaning that the twisted internal charge transfer process takes placed in the viscosity response part in the excited state. The electron density in the HOMO and LUMO were localized on the entire molecule in the ground state, while in the excited state, the HOMO centered at the benzthiophene moiety

and the LUMO centered at diethylaminobenzene core, indicating that a strong charge separation exists in the excited state, which was a relatively obvious feature of the TICT molecule. With the increase of solvent polarity, the emission band shows a bathochromic shift along with the decrease of fluorescence intensity (Fig. S5), which also the feature of the TICT molecule.

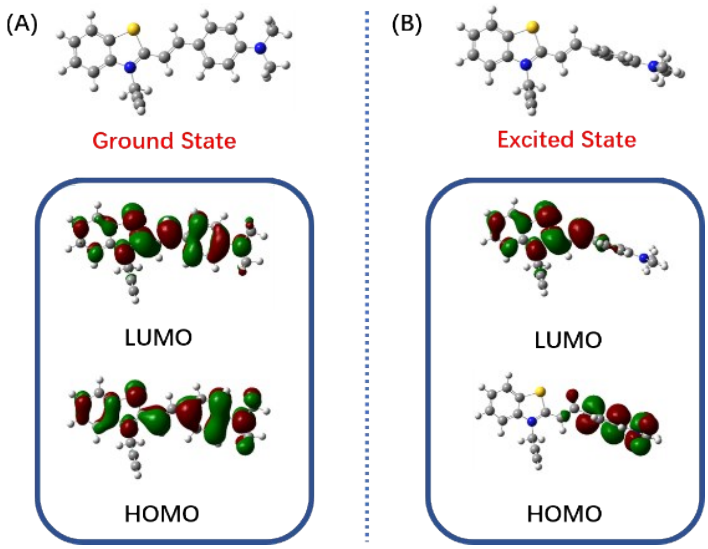



**Figure S4** DFT/TDDFT optimized structure the viscosity response part of the probe **Biotin-V** in ground state (A) and excited state (B). The vertical excitation related calculations are based on the optimized ground state geometry ($S_0$ state) and excited state ($S_1$ state), at the B3LYP/6-31G (d) level using Gaussian 09W.

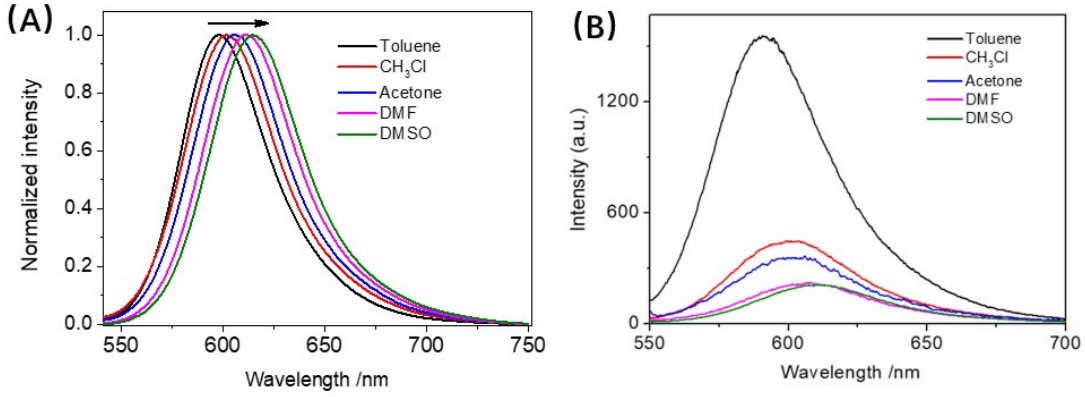



**Figure S**5 (A) Normalized fluorescence spectra and (B) fluorescence spectra of **Biotin-V**

(10 μM) in different polarity of solvents (toluene, chloroform, acetone, DMF and DMSO).

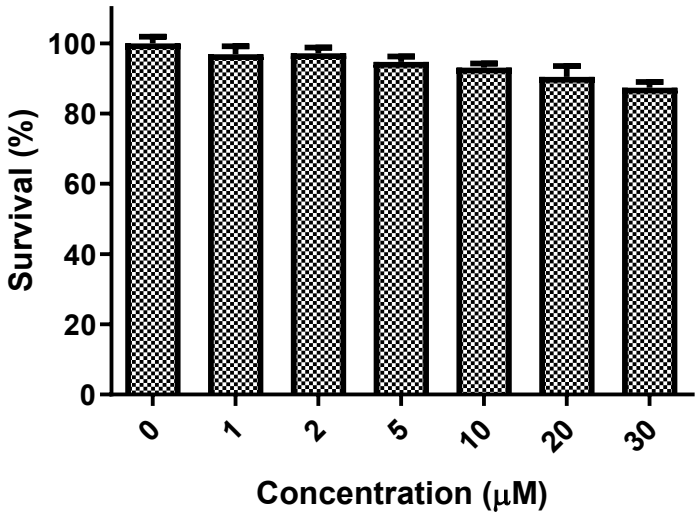



**Figure S6** Cytotoxicity assays of **Biotin-V** at different concentrations for HeLa cells

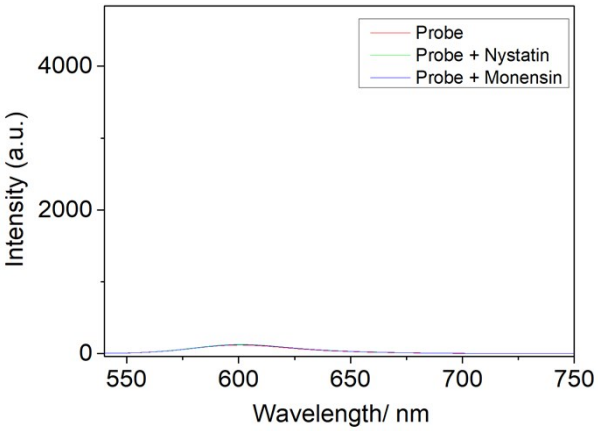



**Figure S7** Fluorescence spectra of probe **Biotin-V** (10 μM) in the absence and presence 10 μM of nystatin or 10 μM of monensin in MeOH for 30 min.

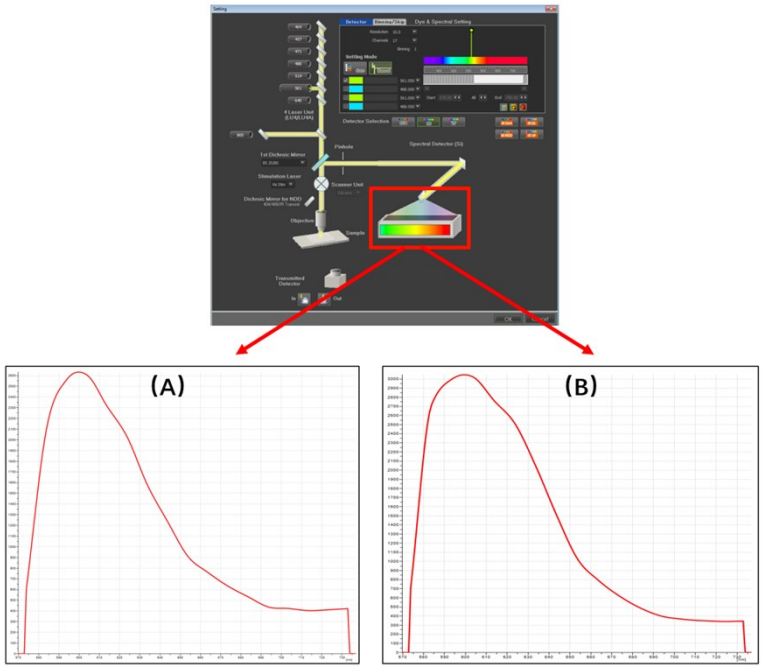



**Figure S8** Fluorescence emission spectrum of **Biotin-V** (5 μM) in HeLa cells (A) and in HeLa cells pretreated with monensin (10 μM) (B) using full spectrum detector of the confocal microscopy.

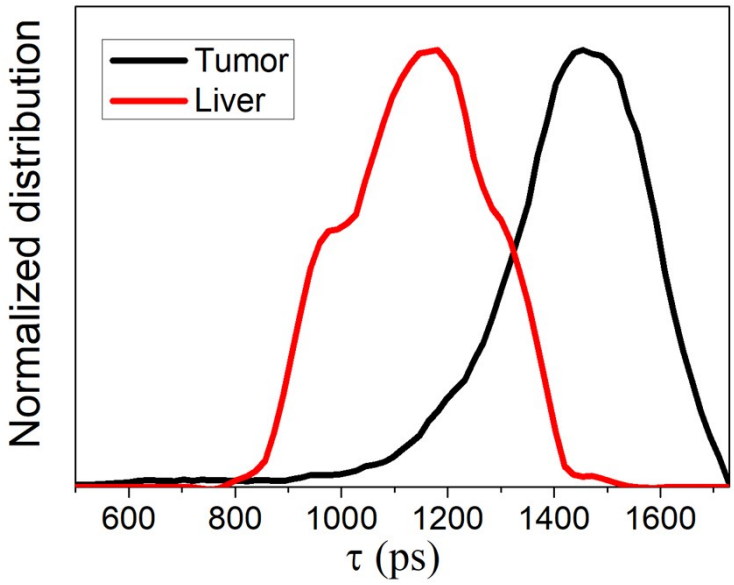



**Figure S9** The histograms of average lifetime (τ) of the probe **Biotin-V** (5 μM) in tumor and liver.

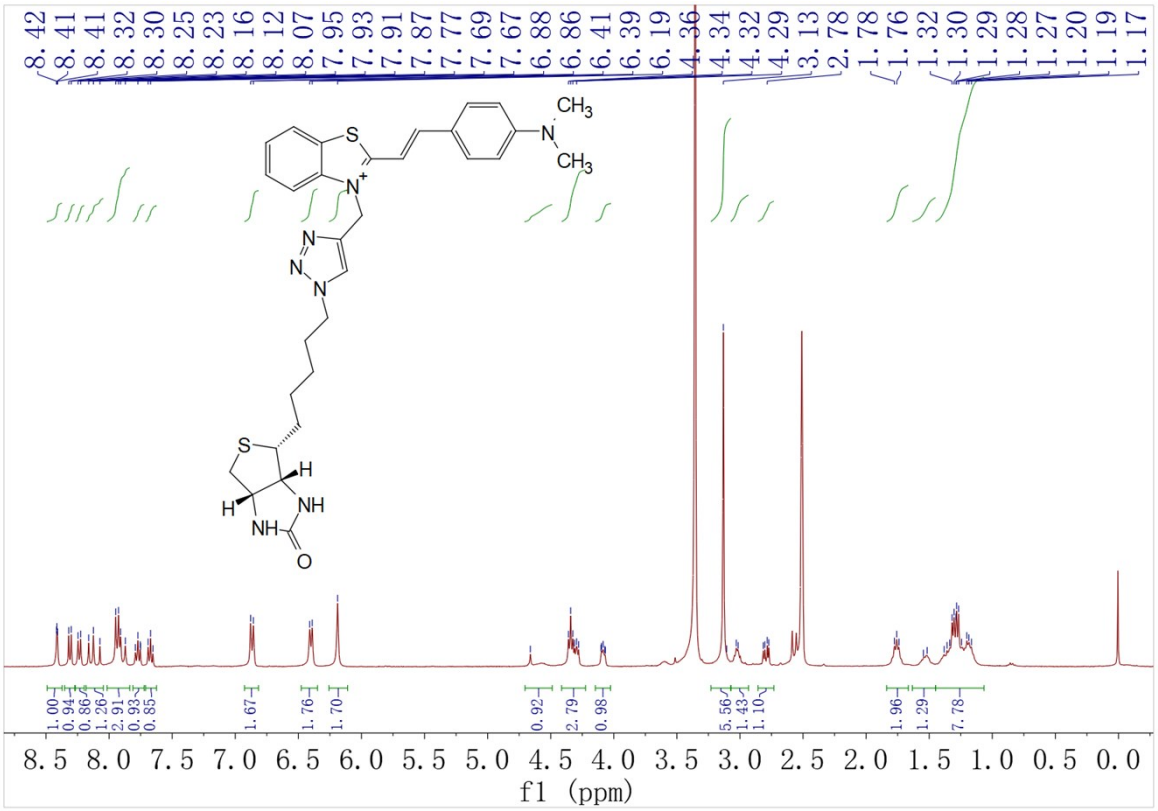



**Figure S10** [1]H-NMR (DMSO-$d_6$) spectrum of **Biotin-V**.

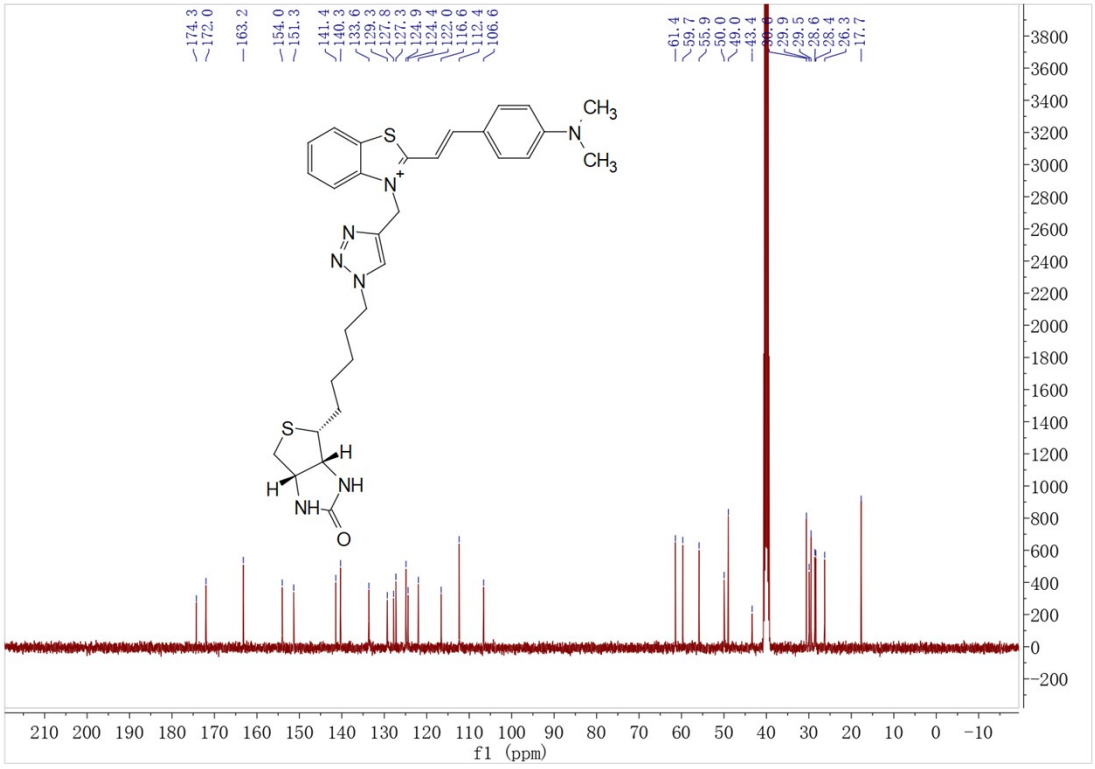



**Figure S11** [1]C-NMR (DMSO-$d_6$) spectrum of **Biotin-V**.

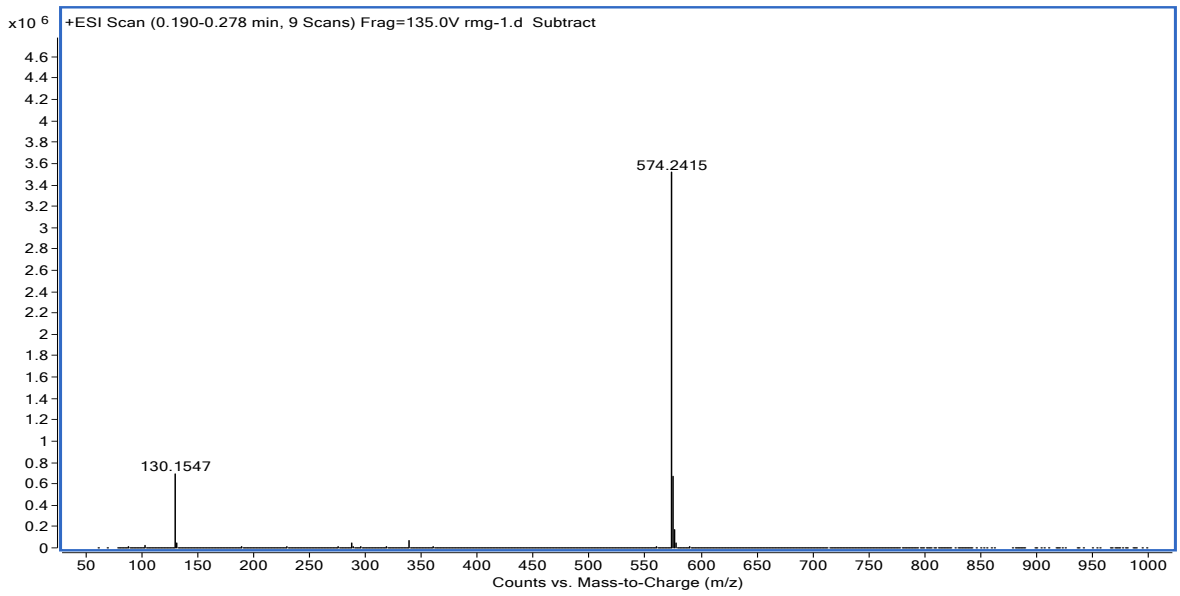



**Figure S12** HRMS (ESI) spectrum of **Biotin-V.**