Electronic Supplementary Information

A responsive fluorescent probe for detecting and imaging pyruvate kinase M2 in live cells

Ya Zhang,‡[a] Huishan Li,‡[a] Hailing Mai,[a] Dong Luo,[a] Xinpei Ji,[a] Zhengting Liu,[a] Shiyong Peng,[a] Xuetao Xu,[a] Yinghui Zhang,[a] Rongfeng Lan*[b] and Hongguang Li*[a, c]

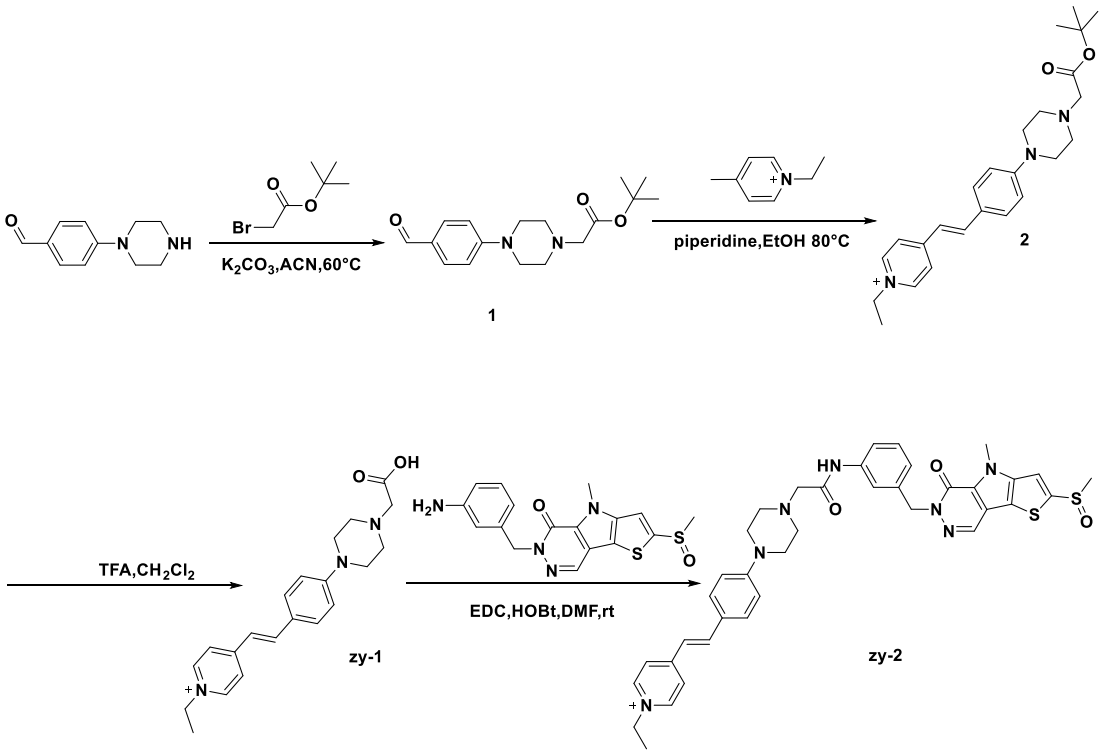
[a] *Guangdong Provincial Key Laboratory of Large Animal Models for Biomedicine, School of Biotechnology and Health Science, Wuyi University, Jiangmen 529020, China.*
[b] *College of Basic medical sciences, Shenzhen University Health Science Center, Shenzhen 518060, China.*
[c] *South China Institute of Large Animal Models for Biomedicine, Wuyi University, Jiangmen 529020, China.*

Table of Contents

1. Synthesis of Compounds

General information about the synthesis

Each step of the reaction was monitored by thin-layer chromatography (TLC), on silica gel plates (0.2 mm, 60F-254) using UV light as a visualizing method. Flash column chromatography was carried out on 100-200 mesh silica gels. Analytical and preparative HPLC was performed on a Waters 2545/2767/QDa HPLC. High-resolution mass spectrometry (HRMS) was measured on an AB Sciex X550R Q-TOF spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) spectrometer. The ^{1}H NMR chemical shifts were based on tetramethylsilane, TMS (δ = 0.00). The following abbreviations are used to interpret the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, br = broad. TEPP-46 (CAS No. 1221186-53-3) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. All the reagents were obtained commercially with high quality and used without further purification.



Scheme S1. Synthetic routes of **zy-1** and **zy-2.**

Synthesis of tert-butyl 2-(4-(4-formylphenyl) piperazin-1-yl)acetate (1)

Tert-butyl bromoacetate (61.6 mg, 0.32 mmol) was added into the solution of 4-piperazine-1-benzaldehyde (50 mg, 0.26 mmol) in anhydrous acetonitrile (3 mL), followed by potassium carbonate (181.6 mg 1.31 mmol). The resulting mixture was stirred at 60^{o}C for 6h. The solid was removed by filtration. The filtrate was concentrated, and the residue was purified by silica gel flash column chromatography (DCM : MeOH = 200 : 1) to give a white solid as compound **1** (66.92 mg, 0.22 mmol, yield: 82.3%). ^{1}H NMR (500 MHz, Chloroform-d) δ(ppm): 9.73 (s, 1H), 7.71 (d, *J* = 10 Hz, 2H), 6.88 (d, *J* = 5 Hz, 2H), 3.42 (t, *J* = 5Hz, 4H), 3.15 (s, 2H), 2.69 (t, *J* = 5Hz,4H), 1.44 (s, 9H). ^{13}C NMR (125 MHz, Chloroform-d) δ(ppm): 190.46, 169.34, 154.97, 131.86, 127.07, 113.55, 81.41, 59.77, 52.44, 46.98, 28.13. HRMS (+ESI) m/z calcd. for $C_{17}H_{25}N_2O_3$ $[M + H]^+$ 305.1865, found 305.1858.

Synthesis of (E)-4-(4-(4-(carboxymethyl) piperazin-1-yl) styryl)-1-ethylpyridin -1-ium (zy-1)

1-ethyl-4-methylpyridin-1-ium (15 mg, 0.12 mmol) was added into the solution of **1** (30 mg,0.098 mmol) in anhydrous acetonitrile (3 mL), followed by piperidine (10 μL). The reaction mixture was stirred at 80 ^{o}C for 6h. The resulting solution was concentrated, and the residue was purified by silica gel flash column chromatography (DCM: MeOH = 50 : 1) to give a red solid as the intermediate compound **2**. To the solution of compound **2** (19 mg, 0.0465 mmol) in CH_2Cl_2 (1 mL), trifluoroacetic acid (0.5 mL, 6.53 mmol) was added dropwise. The mixture was stirred at room temperature for 12 h. The reaction solvents were removed by rotary evaporation. The residue was dissolved in MeOH and purified by HPLC to give a yellow solid as the product **zy-1** (10.57mg, 0.03mmol, yield: 64.7%).^{1}H NMR (500 MHz, DMSO-d^{6}) δ (ppm): 8.83 (d, *J* = 5 Hz, 2H), 8.41 (s, 1H), 8.10 (d, *J* = 5 Hz, 2H), 7.92 (d, *J* = 15 Hz, 1H), 7.60 (d, *J* = 5 Hz, 2H), 7.24 (d, *J* = 15 Hz, 1H), 7.01 (d, *J* = 10 Hz, 2H), 4.46 (q, *J* = 5 Hz, 2H), 3.31(t, *J* = 5 Hz, 4H), 3.03 (s,

2H), 2.63 (t, *J* = 5 Hz, 2H), 1.49 (t, *J* = 5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d^6) δ(ppm): 172.27, 165.71, 153.95, 152.81, 143.95, 141.93, 130.40, 125.01, 123.25, 118.94, 114.79, 55.27, 52.34, 47.16, 16.63. HRMS (+ESI) m/z calcd. for $C_{21}H_{26}N_3O_2$ $[M]^+$ 352.2020, found 352.2010.

Synthesis of (E)-1-ethyl-4-(4-(4-(2-((3-((4-methyl-2-(methylsulfinyl)-5-oxo-4,5-dihydro-6H-thieno[2',3':4,5]pyrrolo[2,3-d]pyridazin-6-yl)methyl) phenyl)amino)-2-oxoethyl)piperazin-1-yl)styryl)pyridin-1-ium (zy-2)

TEPP-46 (7.5 mg, 0.02 mmol) was added into the mixture of N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) (4.6 mg, 0.024 mmol), HOBt (3.7 mg, 0.024 mmol) and **zy-1** (10.6 mg, 0.03 mmol) in dimethylformamide (10 mL). The reaction mixture was stirred at room temperature for 4 h. The solvents were removed by rotary evaporation. The residue was dissolved in MeOH and purified by HPLC to give an orange-red solid as the product **zy-2** (8.87mg, 0.0126mmol, yield: 62.8%). ^{1}H NMR (500 MHz, Methanol-d4) δ (ppm): 8.68 (d, *J* = 10 Hz, 2H), 8.47 (d, *J* = 5 Hz, 1H), 8.23 (s, 1H), 8.05 (d, *J* = 5 Hz, 1H), 7.86 (d, *J* = 5 Hz, 1H), 7.83 (s, 1H), 7.63-7.58 (m, 3H), 7.32 (t, *J* = 5 Hz, 1H), 7.19 (m, 1H), 7.07 (t, *J* = 5 Hz, 1H), 6.98 (d, *J* = 10 Hz, 1H), 6.76-6.72 (m, 1H), 5.44 (q, *J* = 15 Hz, 2H), 5.33 (s, 1H), 4.53 (q, *J* = 5 Hz, 2H), 4.29 (d, *J* = 5 Hz, 3H), 3.41 (s, 3H), 3.08 (d, *J* = 5 Hz, 3H), 2.79 (t, *J* = 5 Hz, 3H), 2.03 (s, 1H), 1.64 (t, *J* = 5 Hz, 3H). ^{13}C NMR (125 MHz, Methanol-d^4) δ(ppm): 152.71, 151.29, 142.95, 129.79, 125.41, 122.96, 114.53, 55.42, 52.64, 48.56, 47.35, 43.01, 39.00, 33.26, 15.23. ^{13}C NMR (125 MHz, Methanol-d^4) δ(ppm):155.36, 152.71, 151.29, 145.50, 142.95, 142.08, 138.15, 132.51, 129.79, 128.81, 125.41, 123.81, 122.96, 119.15, 118.12, 117.08, 114.65, 113.32, 55.42, 53.76, 52.64, 48.57,46.73, 43.01, 33.26, 15.23. HRMS (+ESI) m/z calcd. for $C_{38}H_{40}N_7O_3S_2$ $[M]^+$ 706.2629, found 706.2636; calcd. for $C_{38}H_{40}N_7O_3S_2$ $[M + H]^{2+}$ 353.6354, found 353.6347.

2. Measurement of Photophysical Properties

UV-Visible absorption spectra in the spectral range 200 to 1100 nm were recorded by a HITACHI Ultraviolet visible spectrophotometer (UV-3900). Fluorescence spectra were recorded by an Edinburgh Instruments FLS1000 transient steady-state fluorescence spectrometer. The solvents for photophysical measurements were spectroscopically pure. The fluorescent quantum yields of **zy-1** and **zy-2**, were measured by the comparative method [S1] using rhodamine B (Φ = 97 %) in ethanol as a reference. Quantum yields of **zy-1** and **zy-2** were evaluated by the following equation [S2]:

$$\Phi_S = (\frac{G_s}{G_R})(\frac{n_s}{n_R})^2 \Phi_R$$

Where the subscripts R and S denote the reference and sample, respectively, Φ is the quantum yield, G is the slope of the integrated emission intensity versus absorbance in the plot, and n is the refractive index of the solvent. The correction curves were obtained by comparing the experimentally recorded spectrum of standard rhodamine B with the published data.

Stability testing of zy-1 and zy-2: The stability of **zy-1** and **zy-2** was evaluated in PBS buffer at 37 ºC. Emission spectra were recorded for 10 μM of **zy-1** or **zy-2** in PBS buffer at t = 0 h and after incubation at 37 ºC for 1, 4, 8, 12, 16 and 24 h. The temperature of the solutions was cooled down to room temperature before the spectra were determined.

Emission spectra in different solvents: Stock solutions of 1 mM **zy-1** and **zy-2** were prepared in DMSO. Then, the stock solutions of **zy-1** or **zy-2** in DMSO were added to dioxane, tetrahydrofuran (THF), N, N-dimethylformamide (DMF), acetonitrile (MeCN), methanol (MeOH), water (H_2O) or H_2O/Glycerol mixed solvents, respectively, to obtain the final 10 μM dye concentrations. In all measurements, the excitation wavelength was 419 nm, the excitation slit width was 2 nm, and the emission slit widths was 2 nm.

pH-dependent emission: The emission of **zy-1** and **zy-2** in PBS buffer (pH from 3.0 to 11.0) was determined under excitation at 419 nm at room

temperature. The pKa values were calculated by the Henderson-Hosslbalch equation [S3]:

$$pH = pKa + \log(\frac{[A]}{[HA]})$$

Fluorescence titration of zy-1 and zy-2 with PKM2: The PKM2 protein used for fluorescence titration was purchased from Guangzhou Pubo instrument Co., Ltd. (Item No. RPA588Hu02-200). The probe (**zy-1** or **zy-2**) was dissolved in ultra-pure water and prepared as a 2 mM stock solution. PKM2 protein was diluted at different concentrations and incubated with **zy-2** (1 μM) for 10 min at 37 ^{o}C. Fluorescence emission spectra were recorded at 450-750nm at an excitation wavelength of 419 nm. The width of the excitation slit was 2 nm and the width of the emission slit was 2 nm.

Limit of detection (LOD) test. LOD = 3σ/S, where σ is the standard deviation of the 11 blank signals and S is the slope of the linear regression curve of the fluorescence intensity of **zy-2** versus the concentration of PKM2.

Binding selectivity test

To evaluate the selectivity of the **zy-2** probe for PKM2, the probe (10 μM) was incubated with pyruvate kinase M2 (PKM2) (17.94 μM), bovine serum albumin (BSA) (17.94 μM), glutathione (GSH)(17.94 μM), cellulose (17.94 μM), human serum albumin (HSA) (17.94 μM), pepsin (PEP)(17.94 μM), Lysozyme (LZ) (17.94 μM) and acetylcholinesterase (AchE) (17.94 μM) for 10 min at 37 ^{o}C. Fluorescence emission spectra were recorded at 450-750 nm with an excitation wavelength of 419 nm. The excitation slit width was 2 nm and the emission slit width was 2 nm.

3. Molecular Docking.

Molecular docking was performed on the Autodock vina developed by Oleg Trott et al.[S4] The tetrameric structure of PKM2 (PDB: 3GQY) was accessed from the Protein Database Bank (www.rcsb.org). After removing the ligands and substrates in PyMOL

software, the PKM2 tetramer molecule was opened in MGL Tools 1.5.7, polar hydrogens were added and the grid box of the docking region was set, including the number of points and centers in the X, Y and Z dimensions. The protein molecule was saved a .pdbqt file. Similarly, **zy-2** as well as TEPP46 were opened in MGL Tools 1.5.7 as ligands, selected the torsions and saved as .pdbqt files. Docking calculations were performed in CD-DOS and the results were displayed in PyMOL.

4. Western Blotting

Proteins extracted from HeLa or HK2 cells were completely denatured in 1xSDS-PAGE sample loading buffer after heating at 100 ºC for 10 minutes. Proteins were then separated by 10% SDS-PAGE and transferred to nitrocellulose membranes and blocked with 5% skim milk for 1 h. Proteins were detected separately with the primary antibodies shown. Antibody specific for PKM1 (#7067) was provided by Cell Signaling Technology, Inc. and the anti-PKM2 antibody (#15822-1-AP) was provided by Proteintech Group, Inc. After incubation with anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling Technology, Inc.), the protein band signal was visualized with Pierce ECL Western Blotting Substrate (#32106, Thermo PIERCE) and imaged with the Tanon 6100C system.

5. Pyruvate Kinase Activity

Pyruvate kinase activity was measured with a fluorescence pyruvate kinase-lactate dehydrogenase coupled assay [S6]. All compounds were tested in kinetic mode by coupling the generation of pyruvate by pyruvate kinase to NADH consumption by lactate dehydrogenase. 1 μL of the test sample (TEPP46, **zy-2** or DMSO, final concentration was 1 μM) was added to the solution of PKM2 in 59 μL working buffer (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$), and then incubated at 37 ºC for 15 min. And then, 40 μL of substrate mixture (final concentration, 0.5 mM PEP, 4.0 mM ADP, 0.12 mM NADH and 1 unit LDH) was added to the wells, and the plates were placed in a microplate reader, and then the fluorescence intensity of

NADH was measured at 0, 0.5, 1, 2 and 5 min, respectively. The excitation and emission wavelength was 340 and 465 nm respectively.

6. Cell Culture and Cytotoxicity

Cell culture: HeLa or HK-2 cells were cultured in DMEM medium and supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 ^{o}C in 5% CO_2 incubator.

MTT cell cytotoxicity assays: Cytotoxicity of **zy-1** and **zy-2** were detected by MTT method. HeLa cells were incubated with different concentrations of **zy-1** and **zy-2** probes (0-55 μM) for 24 h. Cells were then incubated with thiazolyl blue solution for 4 h. Then, the thiazolyl blue solution was thoroughly dissolved by dimethyl sulfoxide (DMSO), and the absorbance of the solutions was measured using a multifunctional enzyme reader (490 nm). Quadruplicates were performed.

7. Live Cell Imaging

Live cell imaging of **zy-1** and **zy-2** was performed on a confocal laser scanning microscope, Leica TCS SP8. HeLa and HK-2 cells were seeded in 35-mm confocal dishes overnight and then incubated with **zy-1** or **zy-2** (10 μM) for 4 h. The cells were washed 3 times with PBS before imaging. Image analysis was performed using Image J software.

siRNA interference of PKM2 in HeLa cells

HeLa cells were transfected with siRNA (si156) as described by Goldberg, et al. [S5] The sequences were 5'- CCAUAAUCGUCCUCACCAATT-3' (sense) and 5'-UUGGUGAGGACGAUUAUGGTT-3' (anti-sense). SiRNAs were provided by Shanghai GenePharma Co., Ltd and dissolved in DEPC water. For cell transfection in 6-well plates, siRNA (final concentration 10nM) was transfected into HeLa cells using Rfect siRNA Transfection Reagent (#11011, Biogen & Bio-trans) in Gibco Opti-MEM medium. After 48 h, cells were incubated with **zy-2** (10 μM) for 4 h and

then subjected to microscopic imaging or Western blotting assay.

Time-dependent imaging: HeLa cells were seeded overnight on 35-mm confocal dishes. Cells were then incubated with **zy-2** (10 μM) for different incubation times (0.5, 1, 2, 3, 4, 5, and 6 h), and washed 3 times by PBS prior to imaging.

Concentration-dependent imaging: HeLa cells were seeded on 35-mm confocal dishes overnight and then incubated with **zy-2** (0.5, 2.5, 5, 10, 20 and 40 μM) for 4 h, and the cells were washed by PBS 3 times before imaging.

TEPP46 competition imaging: HeLa cells were seeded on 35-mm confocal overnight and then treated with **TEPP46** (0, 10, 30 or 50 μM) for 0.5 h at 37 ^{o}C. Cells were then incubated with **zy-2** (10 μM) at 37 ^{o}C for 2 h. Cells were washed 3 times with PBS prior to imaging.

8. Co-localization imaging

HeLa cells were seeded on a 35-mm confocal dish and allowed to adhere for 24 h. Cells were washed three times with PBS (pH=7.4) and then incubated with **zy-2** (10 μM) for 4 hr at 37 ^{o}C. Cells were subsequently fixed with 4% formaldehyde in PBS for 30 min at 4 °C, and permeabilized with 0.5% Triton X-100 in PBS for 15 min. Cells were then blocked with 5% BSA, 10% normal goat serum, and 0.1% Triton X-100 in PBS at room temperature for 30 min. Subsequently, cells were treated with anti-PKM2 antibody at 4 ^{o}C overnight, respectively, and washed three times with PBST. After incubation with Alexa Fluor 488 anti-Rabbit IgG (diluted 1:100) and subsequent washing, the cells were finally imaged. For fluorescence imaging, a Leica/TCS SP8 laser confocal fluorescence microscope with a 60x objective lens was used. The probe **zy-2** was excited at 405 nm and emission spectra were collected at 600-620 nm. Alexa Fluor 488 anti-rabbit IgG were excited at 488 nm, and emission spectra were collected at 509-529 nm. Fluorescence imaging was performed under the same experimental conditions in all groups. Image analysis was performed using Image J software.

9. Statistical Analysis

All data represent at least three independent experiments and are expressed as mean ± S.D. Statistical analyses were performed using Origin 2019b (64Bit) statistical software and plotted in GraphPad Prism 8.0.

10. Supplementary Figures and Tables

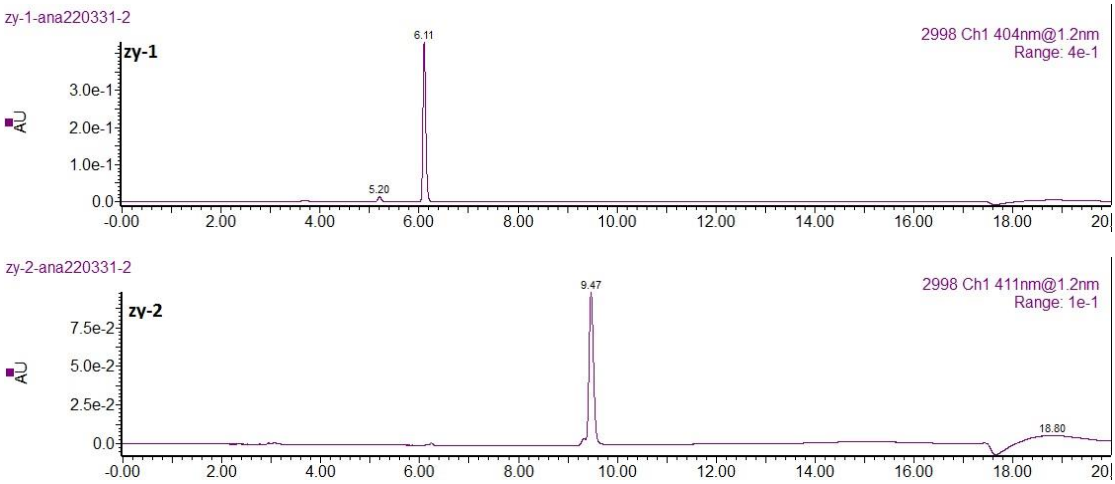


Fig. S1 HPLC traces of **zy-1** and **zy-2**. HPLC analysis was performed on a Waters 2545 LC system with a 2998 DAD detector. The column used was a SunFire® C18, 5 μm, 250 x 4.6 mm analytical column. The LC elution profiles were shown in Table S1.

Table S1. Solvent gradients used for characterization of **zy-1** and **zy-2** by HPLC.

Time/min	A (0.05% HCOOH in water)/%	B (Methanol)/%
0	90	10
10	40	60
13	0	100
15	90	10
20	90	10

Flow rate = 1 mL/min

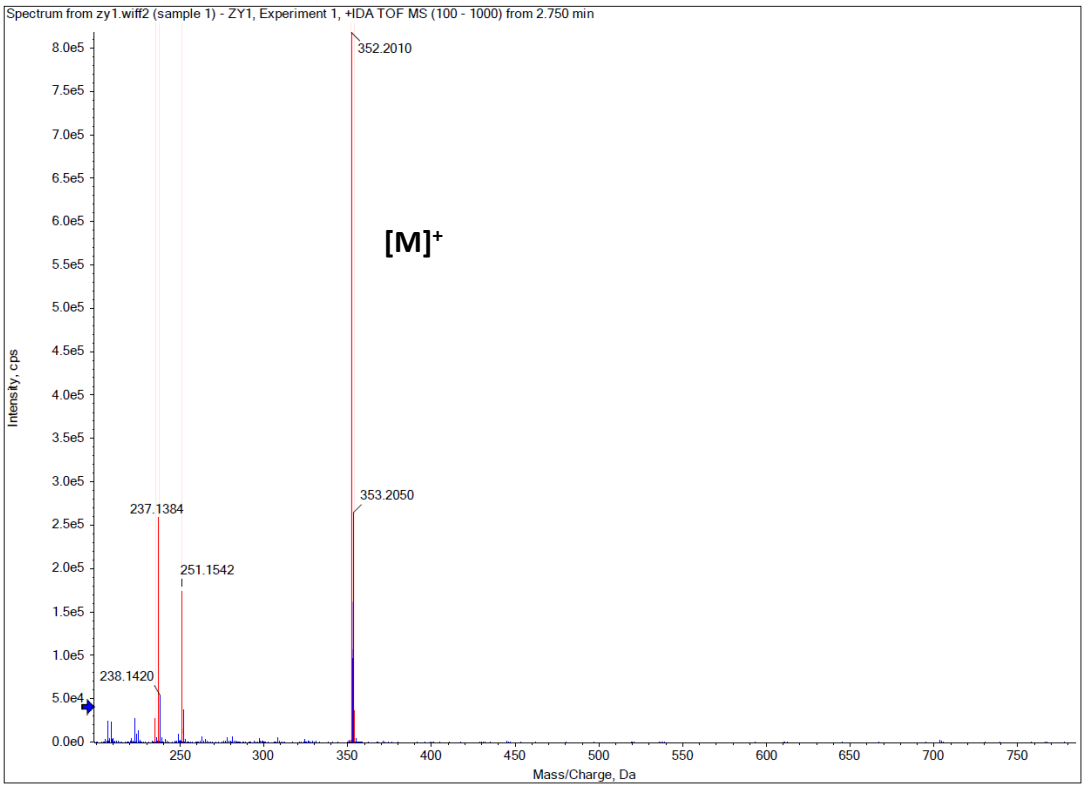


Fig. S2 HRMS (+ESI) spectrum of **zy-1**. (m/z calcd. for $C_{21}H_{26}N_3O_2$ $[M]^+$ 352.2020, found 352.2010.)

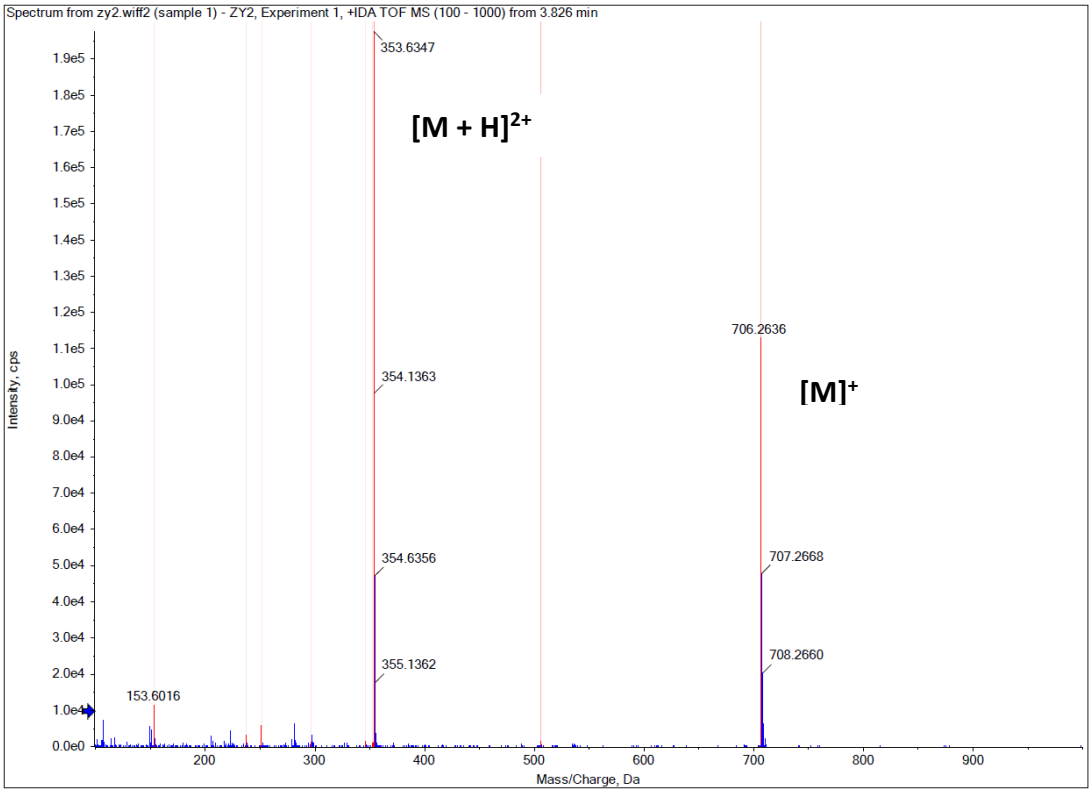


Fig. S3 HRMS (+ESI) spectrum of **zy-2**. (m/z calcd. for $C_{38}H_{40}N_7O_3S_2$ $[M]^+$ 706.2629, found 706.2636; calcd. for $C_{38}H_{40}N_7O_3S_2$ $[M + H]^{2+}$ 353.6354, found 353.6347.)

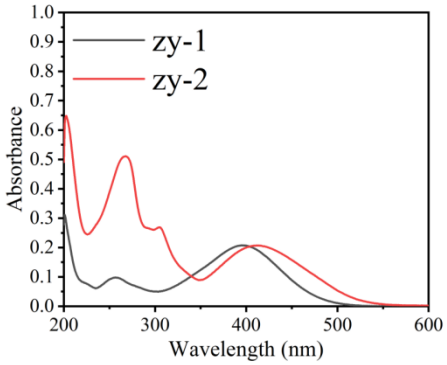


Fig. S4 Absorption spectra of **zy-1** and **zy-2** (10 μM) in PBS.

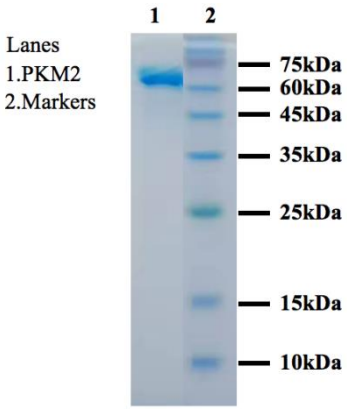


Fig. S5 Purified PKM2 (purchased) in protein a gel stained with coomassie blue.

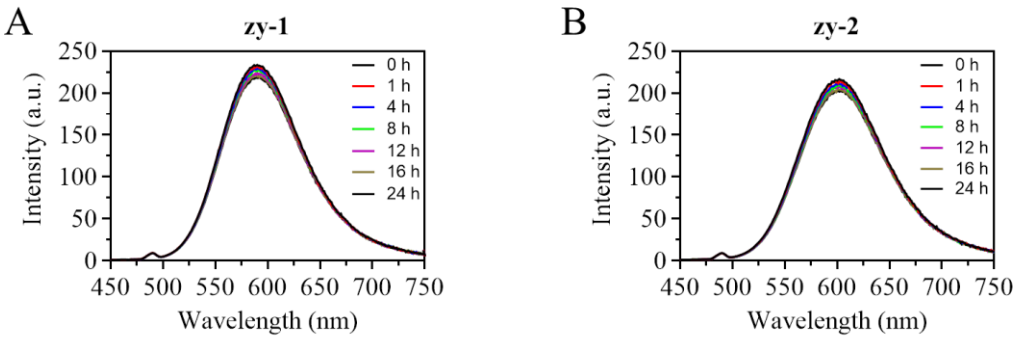


Fig. S6 Stability test of **zy-1** and **zy-2**. Emission spectra of **zy-1** and **zy-2** in PBS buffer at t = 0 h and after incubation at 37 °C for 1, 4, 8, 12, 16 and 24 h (concentration = 10 μM, λ_{ex} = 419 nm).

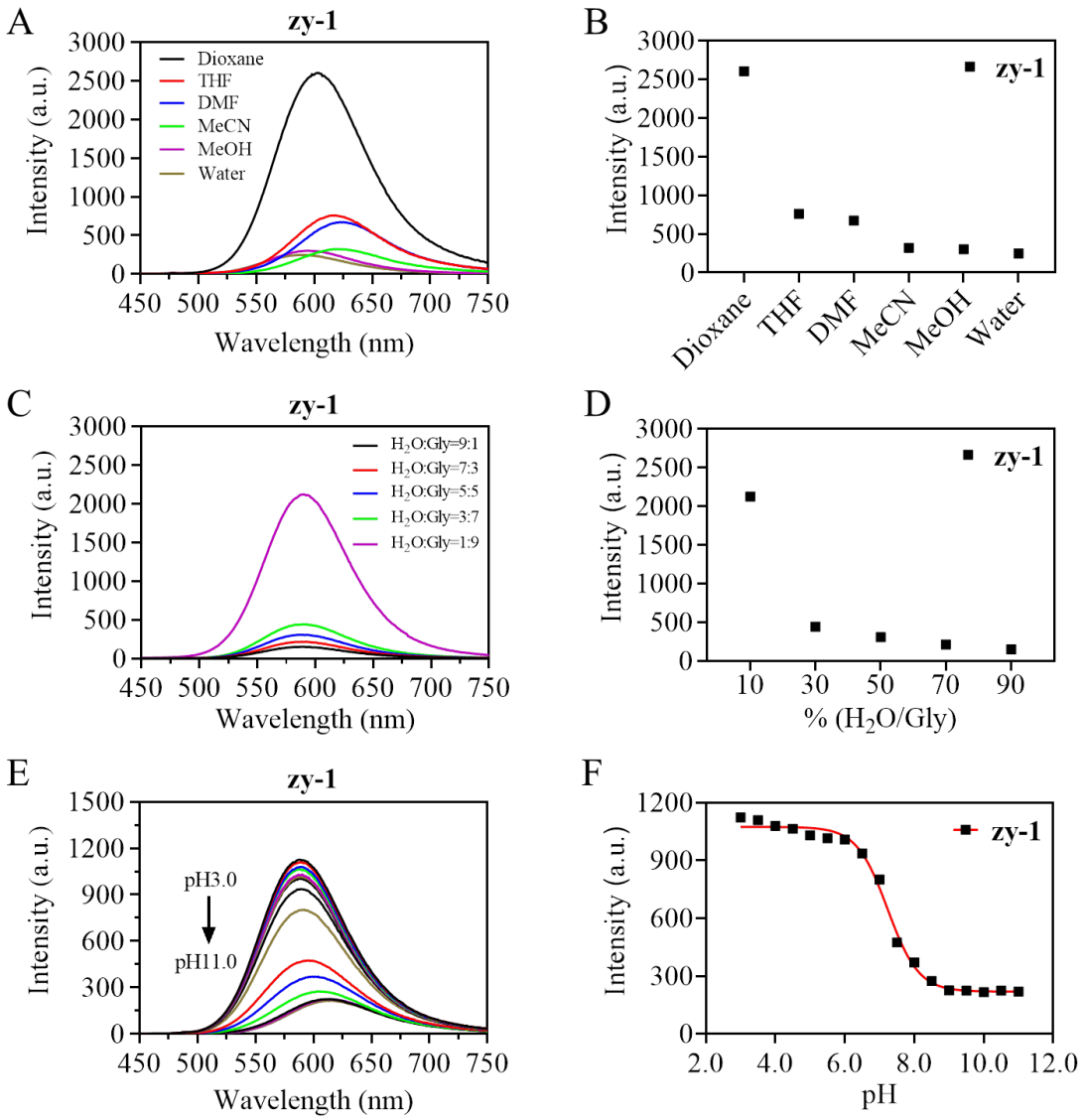


Fig. S7 Polarity-, viscosity- and pH-dependent fluorescence emission of **zy-2**. Emission spectra of **zy-1** (10 μM) in different solvents (A), in the H_2O/Glycerol system (C), and in various pH buffers (E) with λ_{ex}=419 nm, λ_{ex}=450-750 nm. (B, D and F) Scatter plots of the most intense fluorescence emitted by **zy-1** in A, C and E respectively.

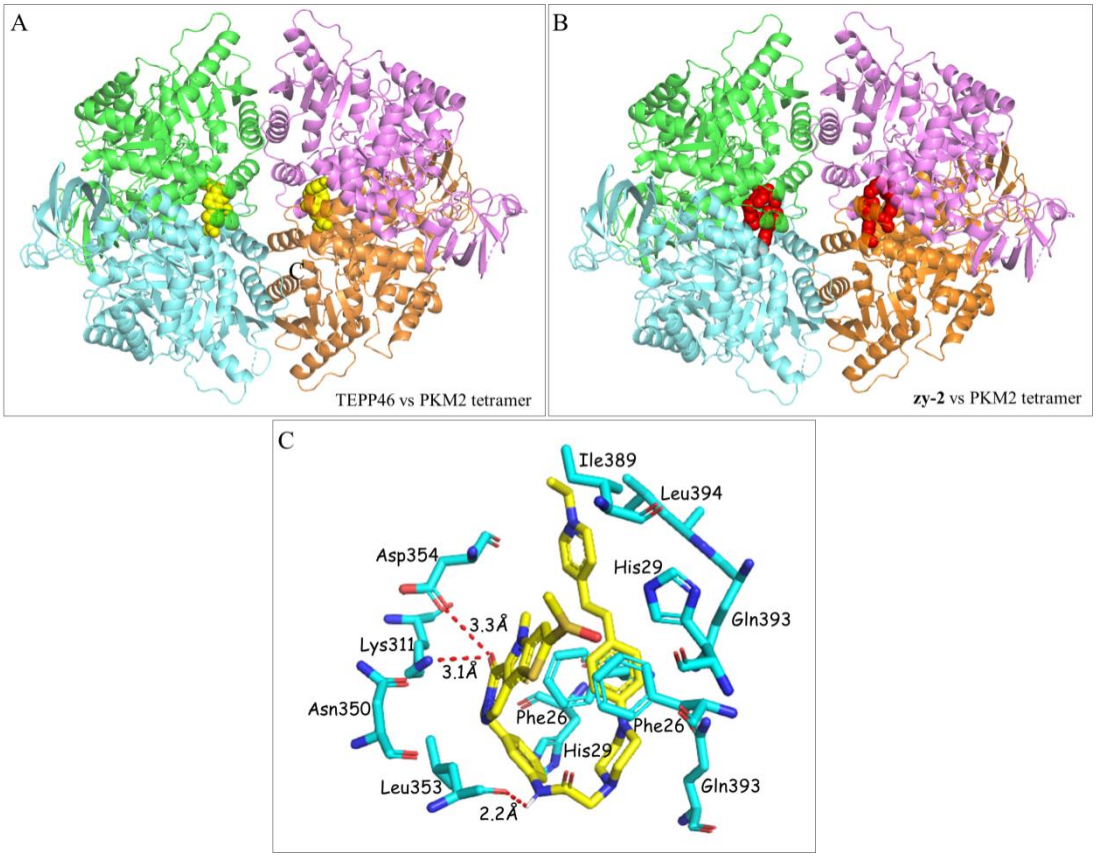


Fig. S8 Molecular docking of **zy-2** to PKM2. (A) TEPPE46 (yellow) and (B) **zy-2** (red) are docked in the TEPP46-binding pocket in the PKM2 tetramer, respectively. The protein structures are represented in a cartoon model, while the ligand molecules are represented in a sphere model. (C) Molecular docking models show that **zy-2** has a hairpin-like structure and that the TEPP46 molecule forms hydrogen bonds with amino acid residues such as Asp354, Lys311, and Leu353 in a polar environment. Meanwhile, the fluorophore of **zy-2** is oriented toward the hydrophobic environment, such as the two Phe26 and Gln393, which are derived from the two monomers of PKM2, and Ile389, Leu394, His29, respectively.

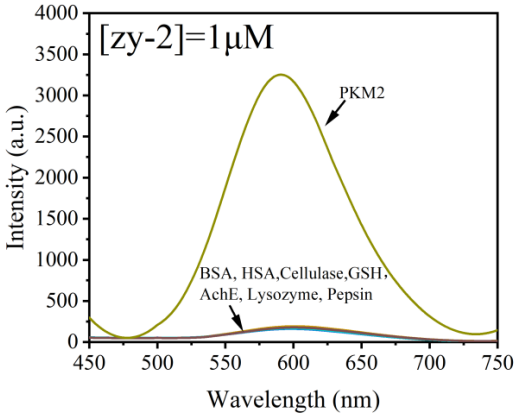


Fig. S9 The emission spectra of **zy-2** for PKM2 (17.94 μM) or biologically relevant substances. (BSA, HSA, Cellulase, GSH, AchE, Lysozyme, Pepsin.)

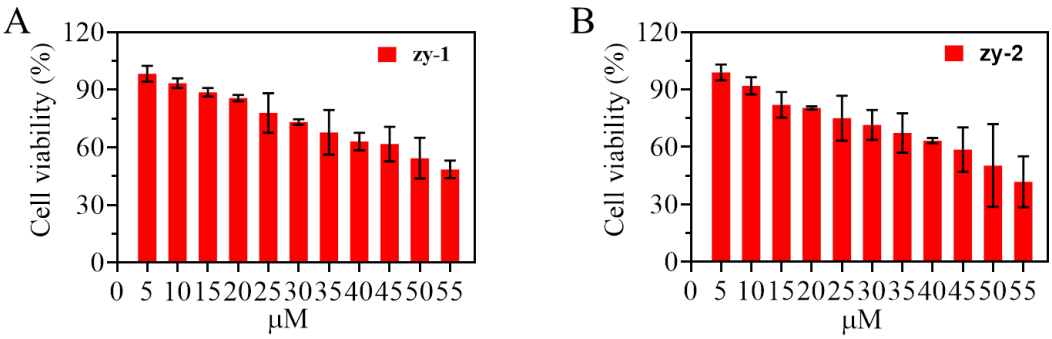


Fig. S10 MTT Cytotoxicity of **zy-1** and **zy-2** in HeLa cells.

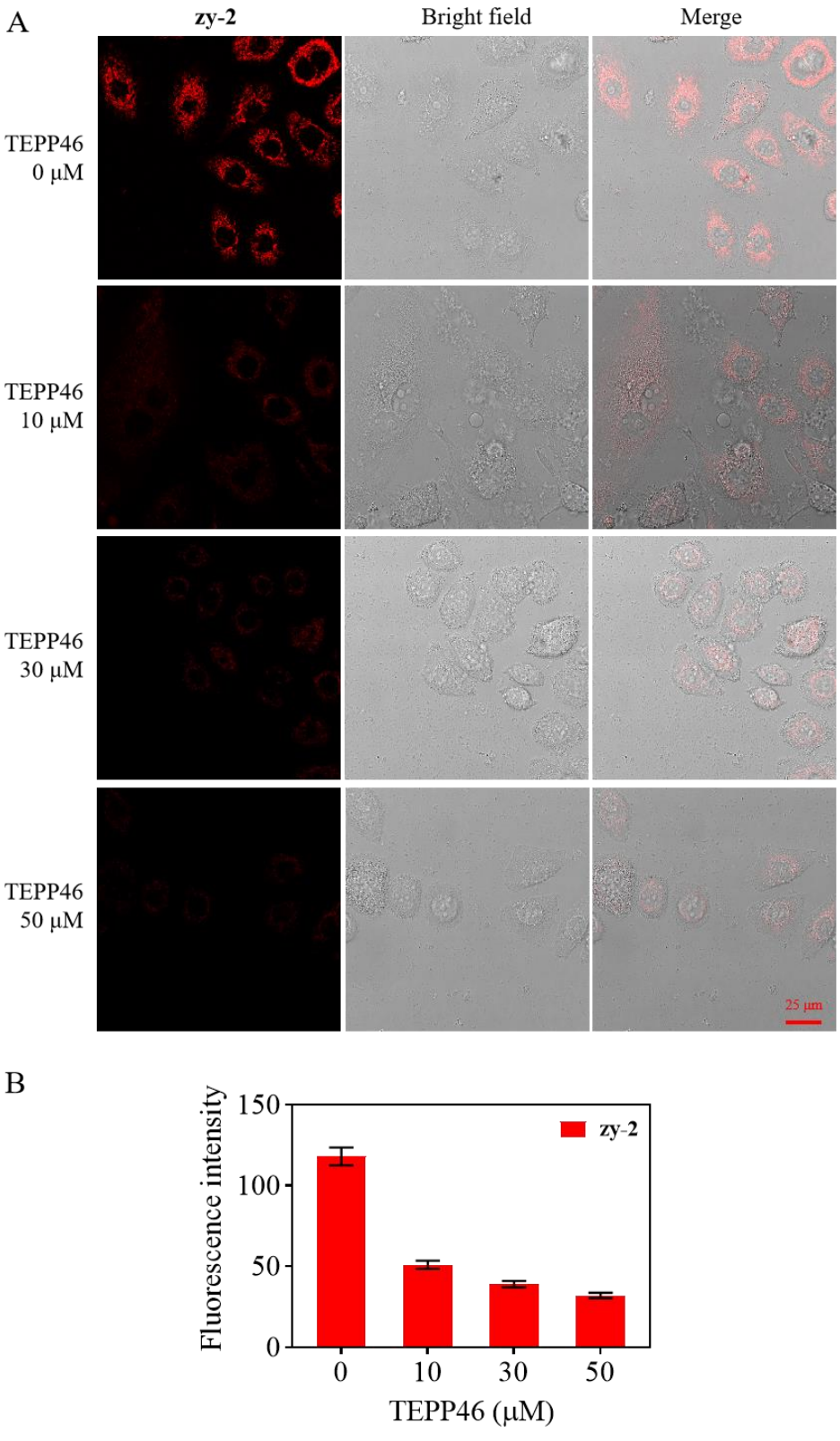


Fig. S11 Imaging of PKM2 in HeLa cells by **zy-2** in the presence of TEPP46 competition. (A) Imaging of PKM2 by **zy-2** in HeLa cells that were pretreated with 0, 10, 30, or 50 μM TEPP46, λ_{ex} = 405 nm, λ_{em} = 600 nm-620 nm. (B) Corresponding fluorescence intensity of **zy-2** in A.

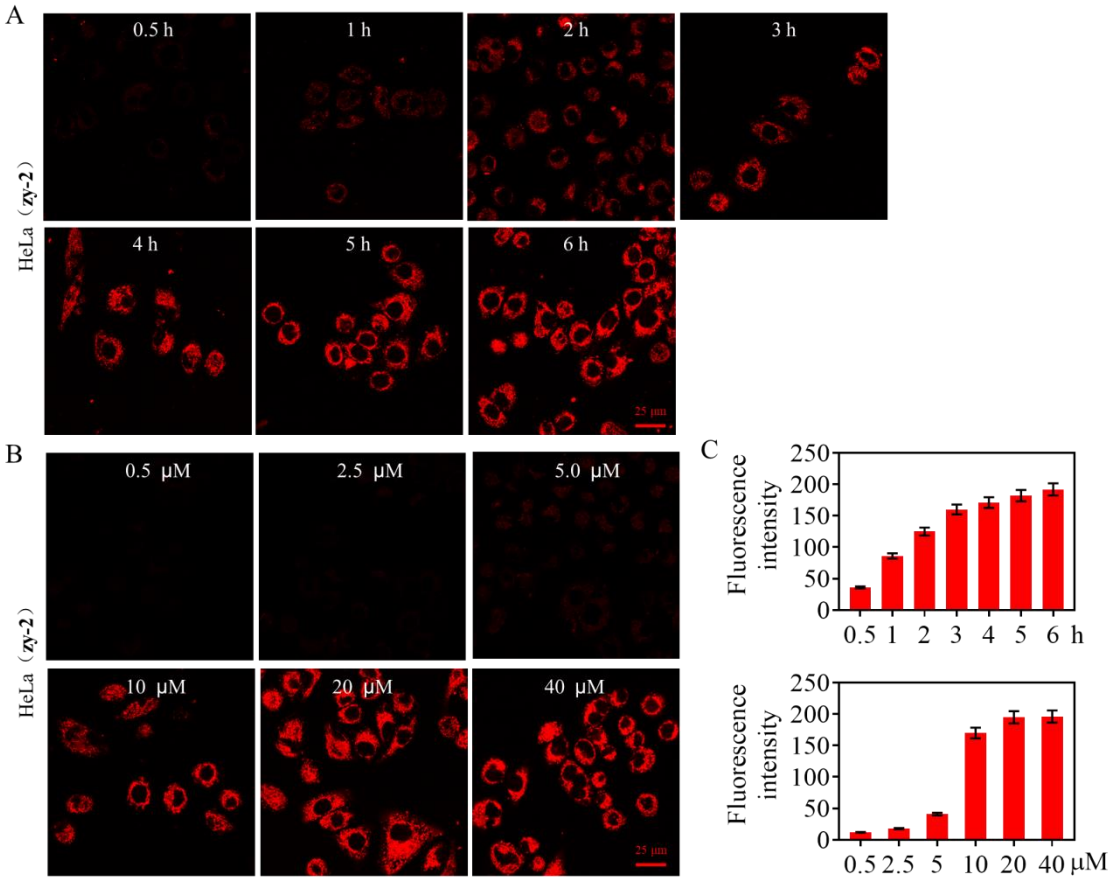


Fig. S12 Time- or concentration- dependent imaging of **zy-2** in HeLa cells. (A) Rapid responsive emission (0.5-6 h) of **zy-2** (10 μM) to PKM2 in HeLa cells. (B) Concentration-dependent emission of **zy-2** to PKM2. In A-B, λ_{ex} = 405 nm, λ_{em} = 600 nm-620 nm. (C) Fluorescence intensity of **zy-2** in (A-B) in a time- or concentration-dependent manner.

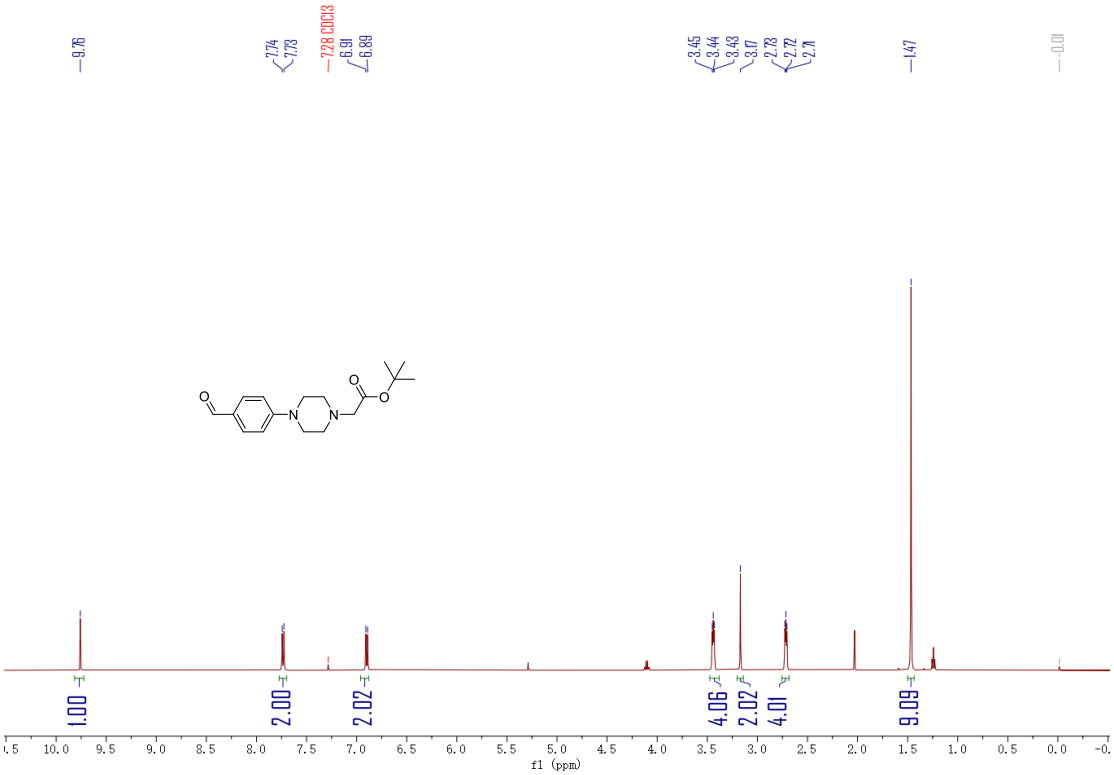


Fig. S13 ^{1}H NMR spectrum of **Compound 1**. (500 MHz, $CDCl_3$)

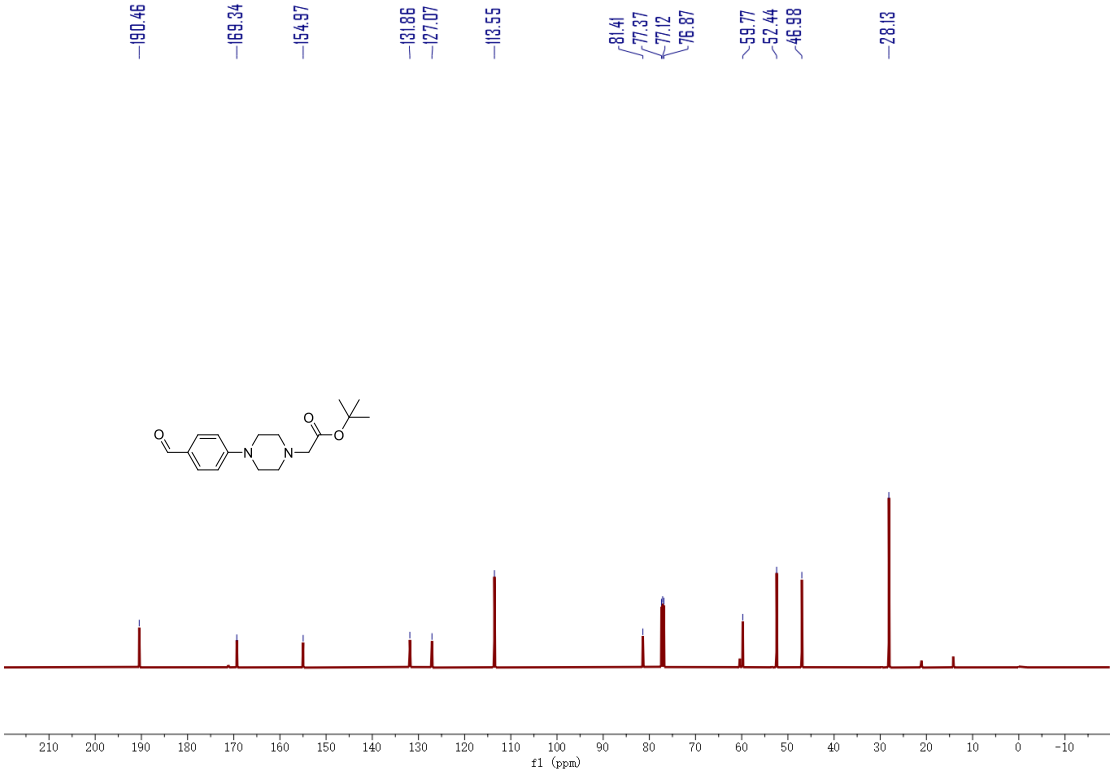


Fig. S14 ^{13}C NMR spectrum of **Compound 1**. (125 MHz, $CDCl_3$)

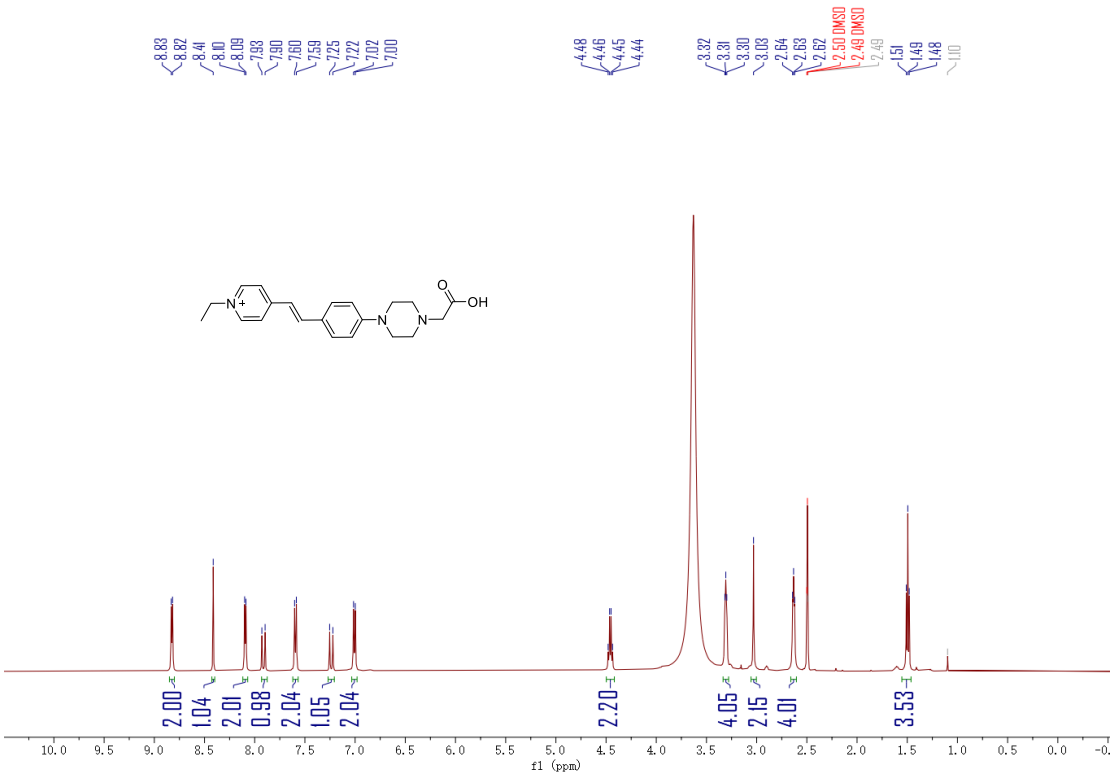


Fig. S15 [1]H NMR spectrum of **zy-1**. (500 MHz, DMSO-d[6])

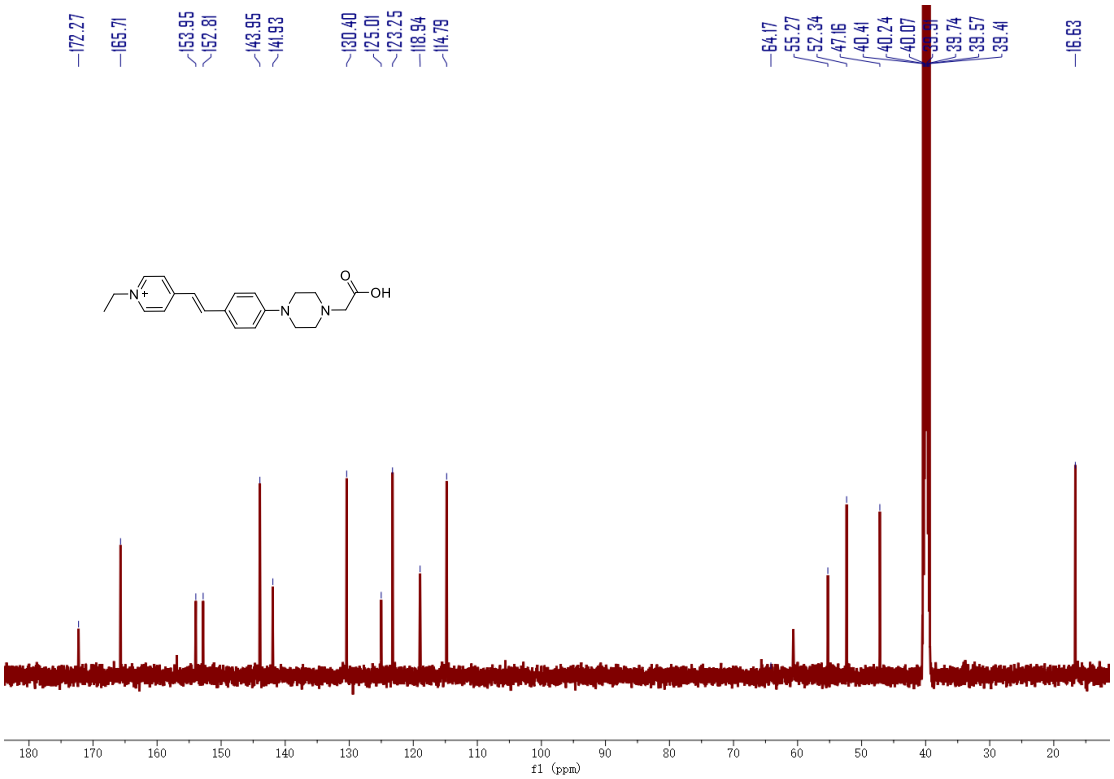


Fig. S16 [13]C NMR spectrum of **zy-1**. (125 MHz, DMSO-d[6])

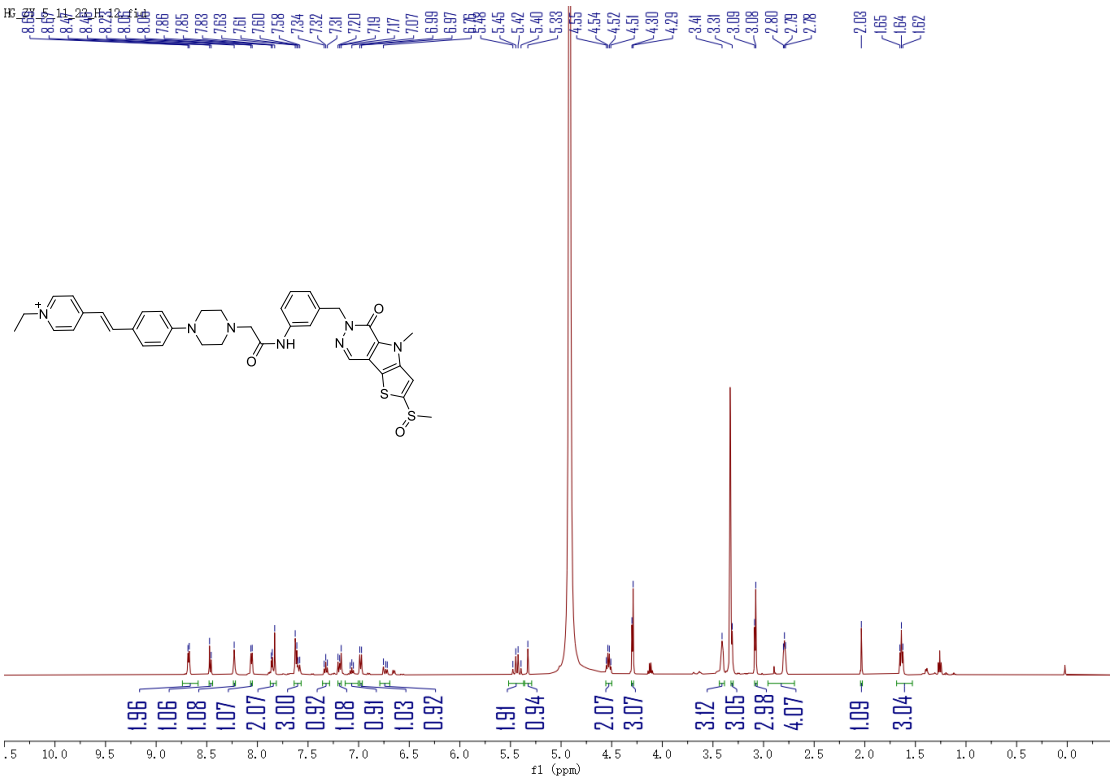


Fig. S17 ^{1}H NMR spectrum of **zy-2**. (500 MHz, methanol-d^{4})

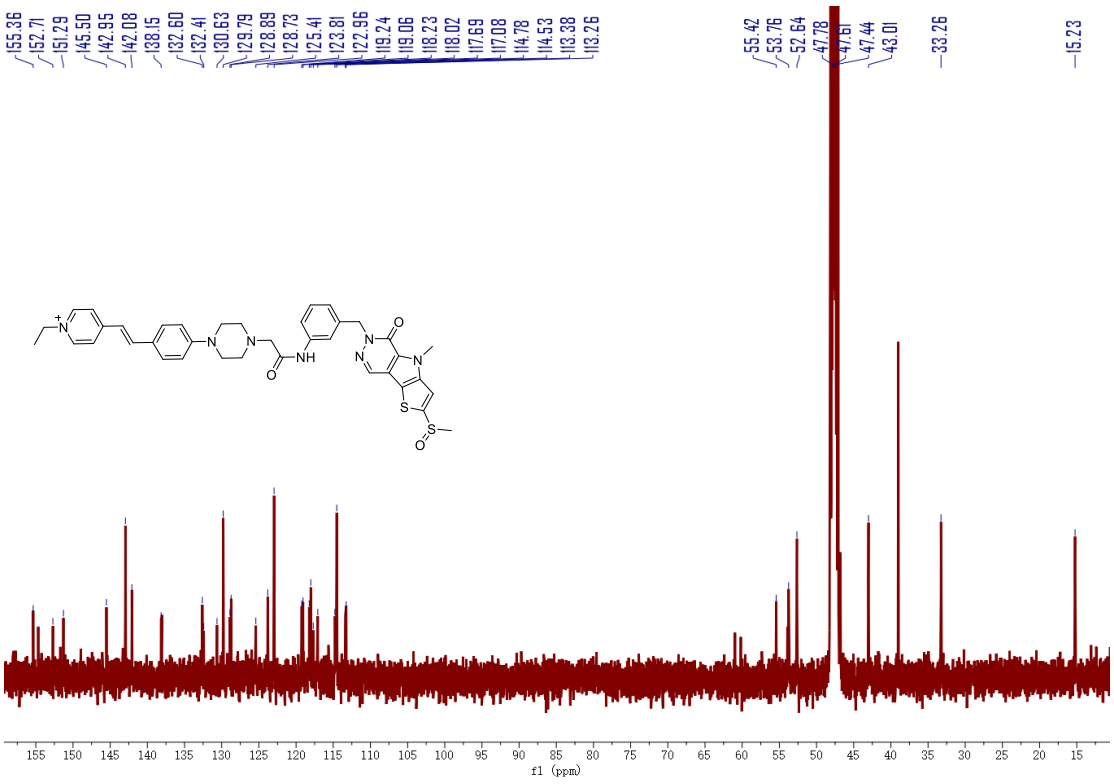


Fig. S18 ^{13}C NMR spectrum of **zy-2.** (125 MHz, methanol-d^{4})

11. References

[S1] A. T. R. Williams, S. A. Winfield and J. N. Miller, *Analyst*, 1983, **108**, 1067-1071.

[S2] A. M. Brouwer, *Pure Appl Chem*, 2011, **83**, 2213-2228.

[S3] O. Finikova, A. Galkin, V. Rozhkov, M. Cordero, C. Hägerhäll, S. Vinogradov, *J Am Chem Soc,* 2003, **125**, 4882-4893.

[S4] O. Trott and A. J. Olson, *J. Comput Chem*, 2010, **31**, 455-461.

[S5] M. S. Goldberg, P. A. Sharp, *J Exp Med*, 2012, **209**, 217-224.

[S6] M. G. Vander Heiden, H. R. Christofk, E. Schuman, A. O. Subtelny, H. Sharfi, E. E. Harlow, J. Xian and L. C. Cantley, *Biochem Pharmacol*, 2010, **79**, 1118-1124.