Supporting Information for

TPZ, a bright centrosymmetric two photon scaffold for bioimaging

Zuhai Lei^{a,†}, Ping Yue^{b,†}, Xueli Wang^c, Xinran Li^a, Yi Li^a, Haihong He^a, Xiao Luo^a, Xiangming Meng^{b,*}, Jinquan Chen^{c,*}, Xuhong Qian^{a,*}, Youjun Yang^{a,*}

a. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, China 200237

b. Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai, China 200237

c. Department of Chemistry, Anhui University, Hefei, China

d. State Key Laboratory of Precision Spectroscopy, East China Normal University, 3663 North Zhongshan Road, Shanghai 200062, China

| Page | Content | | |
|----------|---|--|--|
| S2-S8 | General methods, experimental synthesis and characterizations. | | |
| S9-S33 | The ¹ H-NMR, ¹³ C-NMR, ¹⁹ F-NMR and HRMS of all compounds. | | |
| S34 | Fig S33 The X-ray single crystal of compound 7. | | |
| S35 | The excited kinetics of TZP2 . | | |
| Table S1 | | | |

General Methods

The THF for reaction was dried over a Na/benzophenone still. CH₂Cl₂ was driver over CaH₂. Solvents for chromatography were of reagent grade. All chemicals of analytical grades were purchased and used without further treatment. The ¹H-NMR and ¹³C-NMR spectra were acquired with a Bruker AV-400 spectrometer. Chemicals shifts were referenced to the residue solvent peaks and listed in unit of ppm. ESI-HRMS and EI-HRMS spectra were acquired on a TOF mass spectrometer and a Micromass GCT spectrometer respectively.

Experimental

Spectroscopic methods. UV-Vis absorption spectra were acquired over a SHIMADZU UV-2600 spectrophotometer. Fluorescence spectra were collected on a PTI-QM4 steady-state fluorimeter, equipped with a 75 W Xeon arc lamp and a model 810 type PMT. Voltage of the PMT was set to 950 V. The excitation/emission slits were set to 2 nm. All spectra were collected with a 1-cm quartz cuvette (3.4 mL) and uncorrected.

The extinction coefficients were calculated with the Beer-Lambert law with absorption spectra of dilute solutions of each compound (O.D. < 0.05). The absolute fluorescence quantum yield of **TPZ1** was measured as 0.5 in DMSO using a Hamamatsu C9920-01 integrating sphere system. Fluorescence quantum yield of **TPZ2** was calculated following the literature procedures with **TPZ1** as the reference.

Measurement of two-photon absorption cross-section (\delta). Two-photon excitation fluorescence (TREF) spectra were collected using a femtosecond laser pulse and Ti:sapphire system (680-1080 nm, 80 MHz, 140 fs, Chameleon II) as the light source. All measurements were at room temperature without purging oxygen.

Two-photon absorption cross-sections were calculated with a two-photon induced fluorescence technique. The two- photon absorption cross sections (δ) are determined by comparing their TPEF to that of fluorescein in different solvents (equation 1):

$$\delta = \delta_{ref} \frac{F \Phi_{ref} c_{ref} n_{ref}}{F_{ref} \Phi c n}$$
(1)

The subscript ref. stands for the reference molecule. δ is the TPA cross-section value, *c* is the concentration of the solution, *n* is the refractive index of the solution (because of the low concentrations of the solutions, the refractive indices of the solutions were replaced with those of the solvents), *F* is the TREF integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The δ_{ref} value of reference was taken from the literature.¹

Cytotoxicity assays. The MTT assay was performed to test the cytotoxic effect of the dye in cells. Mcf-7 cells were passed and plated to *ca.* 70% confluence in 96-well plates 24 h before treatment. Prior to **TPZ1-2** treatment, DMEM (Dulbecco,s Modified Eagle Medium) with 10% FCS (Fetal Calf Serum) was removed and replaced with fresh DMEM, and aliquots of **TPZ1-2** stock solutions (1 mM DMSO) were added to obtain final concentrations of 0, 5, 10, 15, 20, and 25 μ M respectively. The treated cells were incubated for 24 h at 37 °C under 5% CO₂. Subsequently, cells were treated with 5 mg mL⁻¹ MTT (40 μ L per well) and incubated for an additional 4 h (37 °C, 5% CO₂). Then the cells were dissolved in DMSO (150 μ L per well), and the absorbance at 570 nm was recorded. The cell viability (%) was calculated according to the following equation:

Cell viability % = OD_{570} (sample)/ OD_{570} (control) * 100 (2)

Where OD_{570} (sample) represents the optical density of the wells treated with various concentrations of **TPZ1-2** and OD_{570} (control) represents that of the wells treated with DMEM containing 10% FCS. The percent of cell survival values is relative to untreated control cells.²



Cell culture. For two-photon bio-imaging, Mcf-7 cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 μ g mL⁻¹) and streptomycin (100 μ g mL⁻¹) at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air. Cells were cultured and stained with **TPZ1-2** (10 μ M) within 30 min and washed with PBS buffer. Cell imaging was carried out on a confocal microscope (Zeiss LSM 710 Meta NLO). Two-photon fluorescence microscopy images of labeled cells were obtained by exciting the probe with a mode-locked titanium-sapphire laser source set at 850 or 920 nm wavelength for **TPZ1-2**, respectively.

Preparation of fresh mouse liver slices and two-photon fluorescence imaging. For two-photon bio-imaging, slices were prepared from the liver of a 7-day-old mouse. Slices were cut to 180 μ m thickness by using a vibrating-blade microtome in 10 mM PBS buffer (pH = 7.4). Slices were incubated with **TPZ1-2** (10 μ M) in PBS buffer bubbled with 95% air and 5% CO₂ for 1 h at 37 °C. Then, slices were washed three times with PBS buffer, and transferred to glass-bottomed dishes. Mouse liver slice imaging was carried out on a confocal microscope (Zeiss LSM 710 Meta NLO). Two-photon fluorescence microscopy images of fresh mouse liver slices were obtained by exciting the probe with a mode-locked titanium-sapphire laser source set at 850 or 920 nm wavelength for **TPZ1-2**, respectively.

Preparation of zebrafish and two-photon fluorescence imaging. For two-photon bioimaging *in vivo*, 5-day-old zebrafishes were prepared. Zebrafishes were fed with **TPZ1-2** (10 μ M) in PBS buffer at 28 °C for 1 h. All the fishes were terminally anaesthetized using MS222, and images were carried out on a confocal microscope (Zeiss LSM 710 Meta NLO). Twophoton fluorescence microscopy images of fresh mouse liver slices were obtained by exciting the probe with a mode-locked titanium-sapphire laser source set at 850 or 920 nm wavelength for **TPZ1-2**, respectively.

Animals. All procedures involving animals were approved by and conformed to the guidelines of the Anhui University Animal Care Committee, School of life science. We have taken great efforts to reduce the number of animals used in these studies and also taken effort to reduce animal suffering from pain and discomfort.

Synthetic scheme, procedures and characterizations.



2-bromo-4-fluorobenzaldehyde (100 g, 493 mmol), glycol (61.2 g, 986 mmol) and catalytic mount of p-toluenesulfonic acid were dissolved in 300 mL toluene, then heated to reflux with a Dean-Stark apparatus. After the reaction was completed, the toluene was removed and treated with saturated sodium bicarbonate solution then extracted with DCM (30 mL x 3). The organic solvent was removed by reduce pressure, then the residue was purified by reduced pressure (100 Pa, 85°C) distillation to give the oil product. ¹H-NMR (400 MHz, CDCl₃) δ 7.56-7.52 (m, 1H), 7.29-7.26 (m, 1H), 7.04-6.98 (m, `H), 6.00 (s, 1H), 4.07-3.96 (m, 4H); ¹⁹F-NMR (376 MHz, CDCl₃) δ -110.40 (t, *J* = 11.28 Hz); ¹³C-NMR (101 MHz, CDCl₃) δ 163.8, 161.4, 132.9, 129.3, 129.2, 123.1, 123.0, 120.2, 119.9, 114.6, 114.4, 102.0, 65.4; HRMS (EI) *m/z* Calcd for C₉H₈BrFO₂ [M]⁺, 247.9671; Found, 347.9676.



8.2 mL n-BuLi (2.5 M, 1 eq.) was added to the 30 mL anhydrous THF solution of compound 3-8 at -78 °C and kept reacting for 15 minutes then 2 mL DMF (21.2 mmol, 1.1eq.) was added with a syringe. Warmed to room temperature and extracted with DCM after treated with saturated ammonium chloride followed by column chromatography (petroleum ether / ethyl acetate 100 / 10, v/v) to give the oil product (3.2 g, 82%); ¹H-NMR (400 MHz, CDCl₃) δ 10.40 (d, *J* = 2.44 Hz, 1H), 7.71 (q, *J* = 8.56 Hz, 1H), 7.62 (dd, *J* = 8.80 Hz, 2.72Hz, 1H), 7.31-7.26 (m, 1H), 6.30 (s, 1H), 4.15-4.09 (m, 4H); ¹⁹F-NMR (376 MHz, CDCl₃) δ -111.10; ¹³C-NMR (101 MHz, CDCl₃) δ 190.1, 163.5, 135.5, 129.5, 120.4, 120.0, 115.8, 100.9, 65.3; HRMS (EI) *m/z*, Calcd for C₁₀H₉FO₃ [M]⁺, 196.0536, Found, 196.0537.



compound **3** (3 g, 14.41 mmol, 1 eq.) and 1, 4- cyclohexanedione monoethylene acetal **4** (1.13 g, 7.2 mmol, 0.5 eq) were dissolved in 10 mL ethanol then 40% NaOH solution was slowly added until the solid precipitated. Kept reacting for half hour then filtered and collected the residue and recrystallized to give the product in 92% yield. Yellow solid, 3.2 g. ¹H-NMR (400 MHz, CDCl₃) δ 8.06 (s, 2H), 7.60 (t, *J* = 8.40 Hz, 2H), 7.06 (td, *J* = 8.30 Hz, 2.04 Hz, 2H), 6.93 (td, *J* = 9.32 Hz, 1.84 Hz, 2H), 5.84 (s, 2H), 4.16-4.12 (m, 4H), 4.02-3.99 (m, 4H), 3.87 (s, 4H), 2.98 (s, 4H); ¹⁹F-NMR (376 MHz,

CDCl₃) δ -112.13 (t, *J* = 15.04 Hz); ¹³C-NMR (101 MHz, CDCl₃) δ 187.3, 162.7 (*J* = 252 Hz), 137.2, 137.1, 136.5, 134.6, 132.4, 132.3, 129.1, 129.0, 115.8, 115.6, 115.2, 115.0, 106.6, 101.4, 65.4, 64.7, 37.1. HRMS (ESI) *m/z*, Calcd for C₂₈H₂₆F₂O₇ [M+H]⁺, 513.1719, Found, 513.1724.



7.8 mL n-BuLi (2.5 M) was added to the 20 mL THF solution of diphenyl ether (3.4 g, 5.1 mmol) at -0 °C and kept reacting for 1 hour then the mixture was transferred to 30 mL THF solution of compound **5** (5 g, 9.8 mmol) and reacted for 1 hour further. Then treated with saturated ammonium chloride and extracted with DCM. Next, DCM was concentrated and 2 mL methanesul-fonic acid was added then kept reacting for half hour. After the reaction was finished, the methanesulfonic acid was neutralized by saturated sodium bicarbonate followed by column chromatography (petroleum ether / ethyl acetate 100 / 5, v/v) to give the yellow solid product in 32% yield, 1.5 g. ¹H-NMR (400 MHz, CDCl₃) δ 9.05 (s, 2H), 8.05 (t, *J* = 7.18 Hz, 2H), 7.50 (s, 2H), 7.32-7.18 (m, 8H), 6.78 (t, *J* = 7.46 Hz, 2H), 6.60 (d, *J* = 7.84 Hz, 2H); ¹⁹F-NMR (376 MHz, CDCl₃) δ -109.17—109.24 (m); ¹³C-NMR (101 MHz, CDCl₃) δ 183.9, 162.3 (*J* = 250 Hz), 149.2, 147.9, 137.3, 137.2, 132.4, 132.3, 131.0, 130.9, 130.9, 128.9, 128.7, 128.5, 128.2, 127.6, 127.6, 123.8, 117.9, 117.7, 111.0, 110.8, 46.1. HRMS (ESI) *m/z* Calcd for C₃₄H₁₉F₂O₂ [M+H]⁺, 497.1357, Found, 497.1352.



Compound **7** (500 mg, 1.01 mmol) and 1 g sodium methoxide were dissolved in 100 mL methanol. To the solution 10 mL DMSO was added. Then the mixture was heated to 80 °C for 24 h. After the reaction was completed, methanol was removed and the concentrated solution was poured into 500 mL ice-water. Filtrated and collected the residue which was the desired product. 430 mg, yield 82%, yellow solid.¹H-NMR (400 MHz, CDCl₃) δ 8.98 (s, 2H), 7.94 (d, *J* = 7.8 Hz, 2H), 7.41 (s, 2H), 7.30 (dd, *J* = 7.8 Hz, 2.4 H, 2H), 7.19 (t, *J* = 8.2 Hz, 2H), 7.12 (dd, *J* = 8.0 Hz, 2.4 Hz, 2H), 6.88 (d, *J* = 2.4 Hz, 2H), 6.78 (t, *J* = 8.4 Hz, 2H), 6.65 (d, *J* = 7.8 Hz, 2H), 3.82 (s, 6H); ¹³C-NMR (101 MHz, CDCl₃) δ 184.0, 159.8, 149.2, 147.8, 137.9, 131.2, 131.2, 130.1, 129.1, 128.4, 127.9, 127.3, 126.5, 123.7, 120.4, 116.6, 105.11, 7.35, 77.0, 76.7, 55.3, 46.1. HRMS (ESI) *m/z* Calcd for C₃₆H₂₄O₄ [M+Na]⁺, 543.1572; Found, 543.1571.



To the solution of 500 mg starting material **8** in 200 mL 1,2-dichloromethane, 1 mL boron tribromide was added at r.t. and the reaction was kept reacting for 3h before 30 mL water was added. Then extracted with DCM, dried with anhydrous sodium sulfate and followed by chromatography on silica gel (PE/DCM/EA 100/20/10, v/v,) to get the product. Yellow solid, 400 mg, yield 85%. ¹H-NMR (400 MHz, DMSO) δ 10.28 (s, 2H), 8.87 (s, 2H), 8.08 (d, *J* = 9.04 Hz, 2H), 7.35(d, *J* = 7.56 Hz, 2H), 7.21 (d, *J* = 10.24Hz, 2H), 6.89 (s, 2H), 7.13 (dd, *J* = 8.92 Hz, 2.16Hz, 2H), 6.89 (d, *J* = 1.76 Hz, 2H), 6.82 (t, *J* = 7.08 Hz, 2H); 6.57 (dd, *J* = 7.88 Hz, 1.56Hz, 2H); ¹³C-NMR (101 MHz, DMSO) δ 182.47, 158.28, 148.50, 147.48, 137.69, 131.72, 130.71, 128.94, 128.67, 128.20, 128.15, 126.06, 125.33, 123.86, 120.37, 116.73, 107.91, 45.46.



To the solution of 200 mg starting material **9** in 100 mL anhydrous dichloromethane at ice-bath, 2.5 eq. triflic anhydride was added. Then 1 mL pyridine was slowly added and kept reacting for 1h before 100 mL water was introduced. Then extracted with DCM, dried with anhydrous sodium sulfate and followed by chromatography on silica gel (PE/DCM/EA 100/20/1, v/v,) to get the product. White solid, 290 mg, yield 95%. ¹H-NMR (400 MHz, CDCl₃) δ 9.11 (s, 2H), 8.15 (d, *J* = 9.04 Hz, 2H), 7.30 (s, 2H), 7.58 (s, 2H), 7.38 (d, *J* = 9.08 Hz, 2H), 7.34 (d, *J* = 8.20 Hz, 2H), 7.22 (d, *J* = 7.40 Hz, 2H), 6.81 (t, *J* = 7.34 Hz, 2H), 6.58 (d, *J* = 7.80 Hz, 2H); ¹⁹F-NMR (376 MHz, CDCl₃) δ -72.83. ¹³C-NMR (101 MHz, CDCl₃) δ 183.6, 149.2, 148.9, 148.5, 136.3, 132.7, 132.1, 130.8, 130.5, 129.0, 128.9, 128.6, 127.8, 124.1, 120.9, 120.3, 119.3, 117.3, 46.3. HRMS (ESI)⁺ *m/z*, Calcd for C₃₆H₁₈F₆O₈S₂ (M+H)⁺: 757.0426, found: 757.0424.



Compound **10** (100 mg, 132.16 μ mol), palladium acetate (1.2 mg, 6.5 μ mol), cesium carbonate (86 mg, 264.3 μ mol) and trimethylamine (100 mg, 1.32 mmol) were dissolved in 30 mL anhydrous 1,4-dioxane then heat to 100 °C for 8 h under argon protection. After the reaction was completed, cooled to r.t. and then 30 mL water was added and extracted with DCM followed by chromatography on silica gel

to get the product (PE/EA, 20:1). Yellow solid, 64 mg, yield 80%. ¹H NMR (400 MHz, CDCl₃) δ 8.85 (s, 2H), 7.84 (d, *J* = 9.2 Hz, 2H), 7.27 - 7.25 (m, 2H), 7.16 - 7.12 (m, 4H), 7.03 (dd, *J*₁ = 9.2 Hz, *J*₂ = 2.5 Hz, 2H), 6.77 - 6.70 (m, 4H), 6.52 (d, *J* = 2.3 Hz, 2H), 3.38 (q, *J* = 7.1 Hz, 8H), 1.14 (t, *J* = 7.1 Hz, 12H); ¹³C NMR (101 MHz, CDCl₃) δ 183.6, 149.1, 148.0, 147.6, 138.5, 131.4, 131.1, 129.8, 128.6, 128.4, 127.5, 124.9, 124.4, 123.6, 116.3, 103.7, 46.1, 44.4, 12.8; HRMS (ES⁺) Calcd. for [M+H]⁺, 603.3012; Found, 603.3009.



The compound **7** (100mg, 201.41 µmol), dimethylamine (2 mL, 2.01 mmol, 1 M THF solution) and sodium carbonate (43mg, 402 µmol) were dissolved in 10 mL DMSO at r.t.. Kept reacting and monitored with TLC. After the reaction was finished, poured it into 300 mL water and kept standing for half hour then filtered and the residue was further purified with chromatography to get the product. Eluent PE/DCM/EA 100/25/2, v/v, yellow solid, 69 mg, yield 65%. ¹H-NMR (400 MHz, CDCl₃) δ 9.03 (s, 1H), 8.90 (s, 1H), 8.03 (t, *J* = 7.26 Hz, 1H), 7.88 (d, *J* = 9.20 Hz, 1H), 7.46 (s, 1H), 7.28 (d, *J* = 8.24 Hz, 2H), 7.24 (d, *J* = 8.24 Hz, 2H), 7.21 (s, 1H), 7.17 (td, *J* = 7.68 Hz, 1.32Hz, 2H), 7.12 (dd, *J* = 9.20 Hz, 2.4 Hz 1H), 6.77 (td, *J* = 7.04 Hz, 0.88 Hz, 2H), 6.65 (dd, *J* = 7.88 Hz, 1.16 Hz, 2H), 6.57 (d, *J* = 1.96 Hz, 1H), 3.02 (s, 6H); ¹⁹F-NMR (376 MHz, CDCl₃) δ -110.06- -110.13 (m); ¹³C NMR (101 MHz, CDCl₃) δ 183.7, 150.3, 149.2, 148.4, 147.5, 138.5, 132.3, 132.2, 131.1, 130.9, 130.8, 129.1, 128.9, 128.7, 128.5, 128.1, 127.9, 124.9, 124.8, 123.7, 117.6, 117.3, 116.9, 116.7, 110.9, 110.7, 104.7, 46.1, 40.3. HRMS (ESI)⁺, *m/z*, Calcd for C₃₆H₂₄FNO₂ (M+H)⁺: 522.1869, found: 522.1871.

Supporting References.

C. Xu and W. W. Webb, J. Opt. Soc. Am. B. 1996, 13, 481.
W. Lin, B. Mohands, C. P. Fontaine and R. A. Colvin, *BioMetals*, 2007, 20, 891.





Fig S3 The ¹⁹F-NMR of compound 2 in CDCl_{3.}



Fig S4 The HR-MS of compound 2.









Fig S7 The ¹⁹F-NMR of compound 3 in CDCl_{3.}



Fig S8 The HR-MS of compound 3.









Fig S11 The ¹⁹F-NMR of compound 5 in CDCl_{3.}



Fig S12 The HR-MS of compound 5.









Fig S15 The ¹⁹F-NMR of compound 7 in CDCl_{3.}





S21



Fig S18 The ¹³C-NMR of compound 8 in CDCl_{3.}



Fig S19 The HR-MS of compound 8.

















Fig S25 The HR-MS of compound 10.







Fig S28 The HR-MS of compound TPZ 2.









Fig S31 The ¹⁹F-NMR of compound TPZ1 in CDCl_{3.}





Fig S33 The X-ray single crystal of compound 7.



Figure S34. The transient absorption spectra of **TPZ2** in DMSO.



Figure S35. (a) femtosecond transient absorption kinetics, (b) TCSPC and (c) excited state relaxation mechanism for **TPZ2**.

| 波长 | Tau0 (ps) | Tau1 (ps) | Tau2(ps) | Tau3(ps) |
|--------|---------------|--------------|-------------|--------------|
| 514 nm | 1.8478±0.0497 | 13.247±0.625 | 2343 | 2659 |
| 720 nm | 2.2434±0.224 | 12.748±1.040 | 2343.3±55.2 | |

Table S1. The global fitting results of the excited state kinetics of **TPZ2**.