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*Supporting Information*

# A Highly Selective Space-folded Photo-induced Electron Transfer Fluorescent Probe for Carbonic Anhydrase Isozymes IX and Its Applications for Biological Imaging

*Shenyi Zhang, Chunmei Yang, Weiqiang Lu, Jin Huang, Weiping Zhu, Honglin Li, Yufang Xu*, Xuhong Qian**

*Shanghai Key Laboratory of Chemical Biology, State Key Laboratory of Bioreactor Engineering, and School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China*

*E-mail: yfxu@ecust.edu.cn, xhqian@ecust.edu.cn*

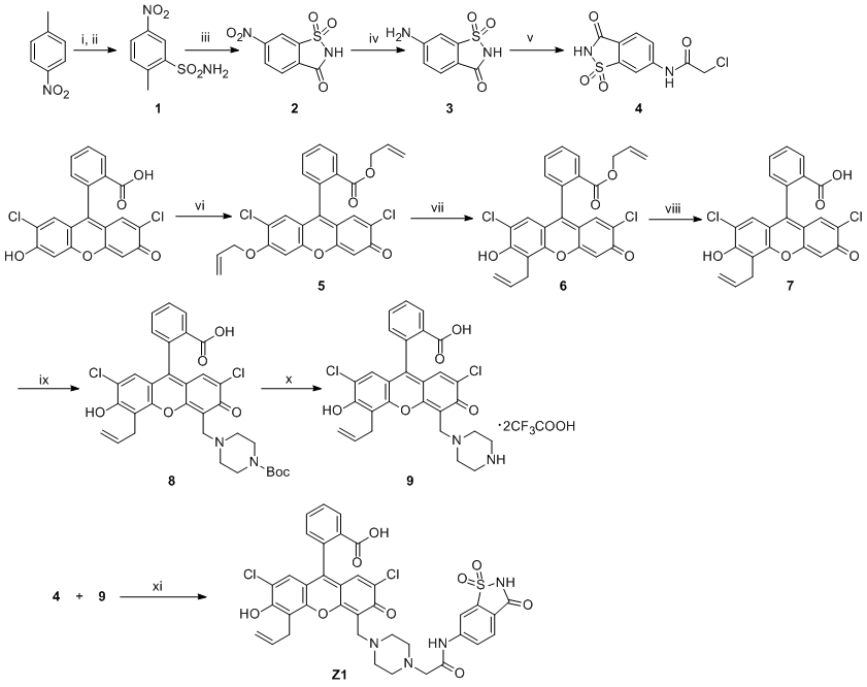
**Contents:**

## 1. Materials and general techniques

Acetonitrile ($CH_3CN$) was distilled from phosphorus pentoxide ($P_2O_5$) under anhydrous conditions. N, N-dimethylformamide (DMF) was distilled from calcium hydride ($CaH_2$) under anhydrous conditions. Other solvents were of analytic grade. All reactions were carried out under a helium atmosphere with analytic grade solvents, unless noted. Mass spectra were measured on a HP 1100 LC-MS spectrometer. Double distilled water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 10 mM Tris-HCl buffer (pH = 7.1, containing 0.1 mM $ZnCl_2$ and < 1 % DMSO) at 37 ºC unless special instructions. Fluorescence spectra were determined on a VARIAN CARY Eclipse Fluorescence spectrophotometer. Absorption spectra were determined on a VARIAN CARY 100 Bio UV-Visible spectrophotometer. $^1$H NMR and $^{13}$C NMR were measured on a BrukerAV-400 spectrometer with chemical shifts reported in ppm (in $CD_3OD$, DMSO or $CDCl_3$; TMS as internal standard). All pH measurements were made with a Sartorius basic pH-Meter PB-10. All the cell images were collected on Leica DMRIB Fluorescence Microscopy. All $K_i$ values were measured on an SX20 stopped-flow instrument (British Applied Photophysics Company).

All reactions were monitored by thin-layer chromatography (TLC) using UV-light (254 nm) and Flu-light (365 nm). Silica gel (300 - 400 mesh) was used for column chromatography.

## 2. Synthesis of probe Z1




**Scheme S1** Synthesis of **Z1**. i) chlorosulfonic acid, 60 °C, 48 h; ii) $NH_4OH$, 50 °C, 2 h; iii) $CrO_3$, $H_2SO_4$, r.t., 24 h; iv) Pd/C, MeOH, r.t., 4 h; v) chloracetyl chloride, $NaHCO_3$, $H_2O$, r.t., 2 h; vi) bromopropylene, $NaHCO_3$, DMF, r.t., 3.5 h; vii) $Ph_2O$, 200 °C, 2 h; viii) lithium hydroxide, 1, 4-dioxane, $H_2O$, 100 °C, 2.5 h; ix) N-Boc-piperazine, paraformaldehyde, MeCN, 80 °C, 4.5 h; x) trifluoroacetic acid, r.t., 5 h; xi) $Et_3N$, DMF, r.t., 12 h.

**2-Methyl-5-nitrobenzensulfonamide (1)[1].**

P-nitrotoluene (22.0 g, 160 mmol) was added to a solution of chlorosulfuric acid (53.0 ml, 802 mmol) kept at 60 °C for 48 h with calcium chloride tube. The reaction mixture was cooled to room tempeture, poured onto ice and extracted with ether (1 L). The organic layer was washed with aqueous sodium chloride, mixed with concentrated aqueous ammonia (300 mL), and heated to evaporate ether completely with stirring. The aqueous layer was then filtered and crude product was recrystallized from water to afford primrose yellow needles (mp 182-183 °C) in 35 % yield.

**6-Nitrosaccharin (2)[1].**

Concentrated sulfuric acid (84.0 mL) was added gradually to water (67.0 mL) contained chromium trioxide (9.0 g, 900 mmol). Then **1** (4.3 g, 20 mmol) was added to the resulting solution batchwisely and the mixture was stirred at room temperature for 24 h. The reaction mixture was poured onto ice, then filtrated with a glass filter to obtain crude product. Purification by dissolution in 10 % aqueous sodium hydrogencarbonate, filtration, and reprecipitation of the filtrate with 5 % aqueous hydrochloric acid provided a white powder (mp 209-210 °C) in 40 % yield.

$^1$H NMR (400 MHz, DMSO-$d_6$, 20 °C): δ 8.45 (dd, $J$ = 1.6, 2.0 Hz, 1 H), 8.42 (s, 1 H), 7.85 (d, $J$ = 8 Hz, 1 H), EI MS (*m/e*) 228 ($M^+$, 100).

**6-Aminosaccharin (3).**

To a solution of **2** (5.7 g, 20 mmol) in methnol (50 mL), 10 % Pd/C (570 mg) was added slowly in icebath. The black mixture was stirring for 4 h with a hydrogen balloon, and then filtered through diatomite filter lay to make filtrate clear. The filtrate was evaporated to dryness to afford a slightly yellow powder (mp 263-264 °C) with no more purification in 80 % yield.

$^1$H NMR (400 MHz, DMSO-$d_6$, 20 °C): δ 7.24 (s, 1 H), 7.22 (s, 1 H), 6.69 (s, 2 H), 6.66 (d, $J$ = 2.0 Hz, 1 H), 6.64 (d, $J$ = 1.6, 1 H). EI MS (*m/e*) 198 ($M^+$, 100).

**6-(2-Chloroacety)-aminosaccharin (4).**

To a solution of **3** (1.0 g, 5 mmol), sodium hydrogencarbonate (1.3 g, 15 mmol) in water (30 mL), 2-chloroacetyl chloride (1.2 g, 10 mmol) was added dropwise in icebath stirring at room tempeture for 2 h. Whereafter, 2 M hydrochloric acid was added to the reaction mixture to effect precipitation. The precipitate was filtered to afford a slightly yellow powder (mp > 300 °C) in 80 % yield.

$^1$H NMR (400 MHz, DMSO-$d_6$, 20 °C): δ 10.85 (s, 1 H), 8.07 (s, 1 H), 7.71 (d, $J$ = 8.0 Hz, 1 H), 7.67 (dd, $J$ = 4.0, 8.4 Hz, 1 H), 4.33 (s, 2 H). EI MS (*m/e*) 274 ($M^+$, 36) and 198 (100).

**Allyl 2-(6-(allyloxy)-2,7-dichloro-3-oxo-3H-xanthen-9-yl)benzoate (5)[2].**

To a solution of 2', 7'-dicholoroflurescein (1.0 g, 2.5 mmol), potassium carbonate (2.1 g, 15 mmol) in DMF (15 mL), allyl bromide (0.9 mL, 10 mmol) was added dropwise in ice cooling, and the reaction mixture was stirred at room temperature for 3.5 h. The mixture was poured to water (400 mL), and extracted with acetylacetic ester (100 mL).

The organic layer were dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by column chromatography (silica gel: 100 mL, eluent: 33.3 % EtOAc in Petroleum ether) to afford a bright orange solid in 85 % yield.

$^1$H NMR (400 MHz, $CDCl_3$, 20 $^o$C): δ 8.29 (d, *J* = 7.6 Hz, 1 H), 7.79 (t, *J* = 7.2, 6.8 Hz, 1 H), 7.73 (d, *J* = 7.2 Hz, 1 H), 7.36 (t, *J* = 7.6, 9.6 Hz, 1 H), 6.97 - 6.82 (m, 3 H), 6.69 (t, *J* = 7.6, 9.2 Hz, 1 H), 6.11 - 6.04 (m, 1 H), 5.66 - 5.58 (m, 1 H), 5.49 (dd, *J* = 7.2, 7.2 Hz, 1 H), 5.38 (d, *J* = 10.4 Hz, 2 H), 4.72 (dd, *J* = 4.4, 4.8 Hz, 2 H), 4.48 (s, 2 H). $^{13}$C NMR (100 MHz, $CDCl_3$, 20 $^o$C): δ 178.67, 164.76, 158.59, 153.25, 150.48, 149.30, 133.79, 132.87, 131.48, 131.34, 130.86, 130.59, 130.10, 129.08, 129.03, 126.74, 119.49, 118.88, 118.14, 115.94, 111.97, 111.09, 109.93, 101.16, 70.32, 66.14. EI MS (*m/e*) 480 ($M^+$, 100).

**Allyl 2-(5-allyl-2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoate (6)**[2].

A solution of **5** (240 mg, 0.5 mmol) in diphenyl ether (2.0 mL) was heated at 200 $^o$C in an oil bath and stirred for 2 h at same temperature. Subsequently, the reaction mixture cooled to room temperature and transferred directly to a silica gel column. The column chromatography performed with 33.3 % EtOAc in Petroleum ether to afford **6** as a bright orange solid in 65 % yield.

$^1$H NMR (400 MHz, $CDCl_3$, 20 $^o$C): δ 8.29 (d, *J* = 7.6 Hz, 1 H), 7.79 (t, *J* = 7.6 Hz, 6.4 Hz, 1 H), 7.72 (t, *J* = 7.6 Hz, 7.6 Hz, 1 H), 7.37 (t, *J* = 7.6 Hz, 10.8 Hz, 1 H), 7.13 (s, 1 H), 6.91 - 6.83 (m, 3 H), 6.69 (d, *J* = 10.0 Hz, 1 H), 6.12 - 6.03 (m, 1 H), 5.68 - 5.58 (m, 1 H), 5.49 (dd, *J* = 5.6 Hz, 5.6 Hz, 1 H), 5.39 (dd, *J* = 5.2 Hz, 5.2 Hz, 1 H), 5.16 (s, 1 H), 5.12 (d, *J* = 6.0 Hz, 1 H), 4.74 (d, *J* = 5.2 Hz, 1 H), 4.71 (d, *J* = 5.6 Hz, 1 H), 4.49 (t, *J* = 5.6 Hz, 3.2 Hz, 2 H). $^{13}$C NMR (100 MHz, $CDCl_3$, 20 $^o$C): δ 178.82, 164.77, 158.50, 154.35, 153.22, 150.14, 149.26, 133.83, 132.86, 131.49, 131.35, 130.85, 130.59, 130.07, 129.18, 129.06, 126.65, 119.52, 119.34, 118.89, 118.17, 115.92, 109.82, 101.28, 70.30, 66.15, 29.71. EI MS (*m/e*) 480 ($M^+$, 100).

**2-(5-allyl-2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (7)**[2].

A solution of **6** (96 mg, 0.2 mmol), lithium hydrate (5.3 mg, 0.3 mmol), 1, 4-dioxane (2.0 mL) and $H_2O$ (0.8 mL) was refluxed for 2.5 h. The reaction mixture was cooled to room temperature, and removed in vacuo. 1 N hdrochloric acid was added to the residue, then the residue aqueous was extracted with EtOAc (20 mL). The combined organic layers were washed with saturated brine (20 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by column chromatography (silica gel: 150 mL, eluent: 66.7 % EtOAc in Petroleum ether) to afford a bright orange solid in 90 % yield.

$^1$H NMR (400 MHz, $CDCl_3$, 20 $^o$C): δ 8.06 (d, *J* = 7.6 Hz, 1 H), 7.75 - 7.66 (m, 2 H), 7.19 (d, *J* = 7.2 Hz, 1 H), 6.98 (s, 1 H), 6.72 (s, 1 H), 6.62 (s, 1 H), 6.07 - 5.98 (m, 1 H), 5.17 (d, *J* = 17.2 Hz, 1 H), 3.69 (d, *J* = 6 Hz, 1 H), 3.50 (s, 1 H), 2.98 (s, 2 H). EI MS (*m/e*) 441 (M+1, 100).

**2-(5-allyl-4-((4-(tert-butoxycarbonyl)piperazin-1-yl)methyl)-2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (8).**

A solution of N-Boc-piperazine (127 mg, 0.68 mmol) and paraformaldehyde (41 mg, 1.36 mmol) was refluxed for 0.5 h in MeCN. The reaction mixture was cooled to room temperature, and **7** (100 mg, 0.2 mmol) was added. Subsequently, the mixture was refluxed again for 4.5 h, then cooled to room temperature, and removed in vacuo. The crude product was purified by column chromatography (silica gel: 150 mL, eluent: 16.7 % EtOAc in DCM) to afford a red solid in 76 % yield.

$^{1}$H NMR (400 MHz, $CDCl_3$, 20 $^{o}$C): δ 8.08 (d, *J* = 7.2 Hz, 1 H), 7.69 - 7.78 (m, 2 H), 7.22 (d, *J* = 7.2 Hz, 1 H), 6.67 (s, 1 H), 6.63 (s, 1 H), 6.08 - 5.98 (m, 1 H), 5.16 - 5.10 (m, 2 H), 4.13 (dd, *J* = 14.8 Hz, 15.2Hz, 2 H), 3.70 - 3.68 (m, 1 H), 3.81 - 3.35 (br, 4 H), 2.82 - 2.52 (br, 4 H), 2.33 (d, *J* = 3.6 Hz, 1 H), 2.28 (s, 1 H), 1.15 (s, 9 H). $^{13}$C NMR (100 MHz, $CDCl_3$, 20 $^{o}$C): δ 168.70, 156.00, 154.42, 151.56, 150.84, 148.14, 135.36, 134.53, 130.32, 129.56, 128.91, 127.56, 126.87, 126.02, 125.78, 125.52, 125.33, 123.93, 117.14, 115.88, 115.16, 112.41, 110.37, 108.64, 82.59, 80.38, 54.57, 52.63, 28.38.

**2-(5-allyl-2,7-dichloro-6-hydroxy-3-oxo-4-(piperazin-1-ylmethyl)-3H-xanthen-9-yl)benzoic acid 2,2,2-trifluoroacetate (9).**

A solution of **8** and trifluoroacetic acid (0.5 mL) was stirred for 5 h at room temperature. Subsequently, the reaction mixture was removed directly in vacuo to afford crude solid without purification.

**2-(5-allyl-2,7-dichloro-4-((4-(2-((1,1-dioxido-3-oxo-2,3-dihydrobenzo[d]isothiazol-6-yl)amino)-2-oxoethyl)piperazin-1-yl)methyl)-6-hydroxy-3-oxo-3H-xanthen-9-yl) benzoic acid (Z1).**

To a solution of **9** (20 mg, 0.04 mmol), triethylamine (33 mg, 0.3 mmol) stirring in anhydrous DMF for 0.5 h, **4** (51 mg, 0.2 mmol) was added at room temperature. The reaction mixture was stirred for 12 h in dark, and then removed in vacuo. The crude product was purified by column chromatography (silica gel: 150 mL, eluent: 16.7 % MeOH in DCM) to afford a red solid in 20 % yield.

$^{1}$H NMR (400 MHz, $CD_3OD$, 20 $^{o}$C): δ 8.27 (s, 1 H), 8.14 (d, *J* = 7.2 Hz, 1 H), 7.81 - 7.92 (m, 1 H), 7.73 (d, *J* = 8.0 Hz, 1 H), 7.70 - 7.63 (m, 2 H), 7.24 (d, *J* = 7.2 Hz, 1 H), 7.14 (d, *J* = 9.6 Hz, 2 H), 6.13 - 6.03 (m, 1 H), 5.12 (d, *J* = 17.2 Hz, 1 H), 5.04 (d, *J* = 9.6 Hz, 1 H), 4.55 (s, 2 H), 3.74 (d, *J* = 5.2 Hz, 2 H), 3.63 - 3.59 (m, 1 H), 3.56 - 3.50 (m , 1 H), 3.42 (s, 4 H), 3.38 (s, 2 H), 2.93 (s, 3 H). $^{13}$C NMR (100 MHz, $CD_3OD$, 20 $^{o}$C): δ 169.43, 169.01, 168.93, 168.85, 163.224, 154.04, 147.69, 145.30, 141.97, 135.99, 134.02, 131.25, 129.76, 129.72, 129.53, 129.22, 128.86, 128.79, 128.35, 127.12, 123.59, 123.17, 114.11, 113.80, 110.61, 110.24, 104.53, 62.96, 60.50, 51.79, 50.19, 27.45. HRMS (ESI): [M - $H^+$] calcd for $C_{37}H_{29}N_4O_9SCl_2^-$, 775.1038; found, 775.1035.

## 3. Absorption and emission spectra of free probes Z1

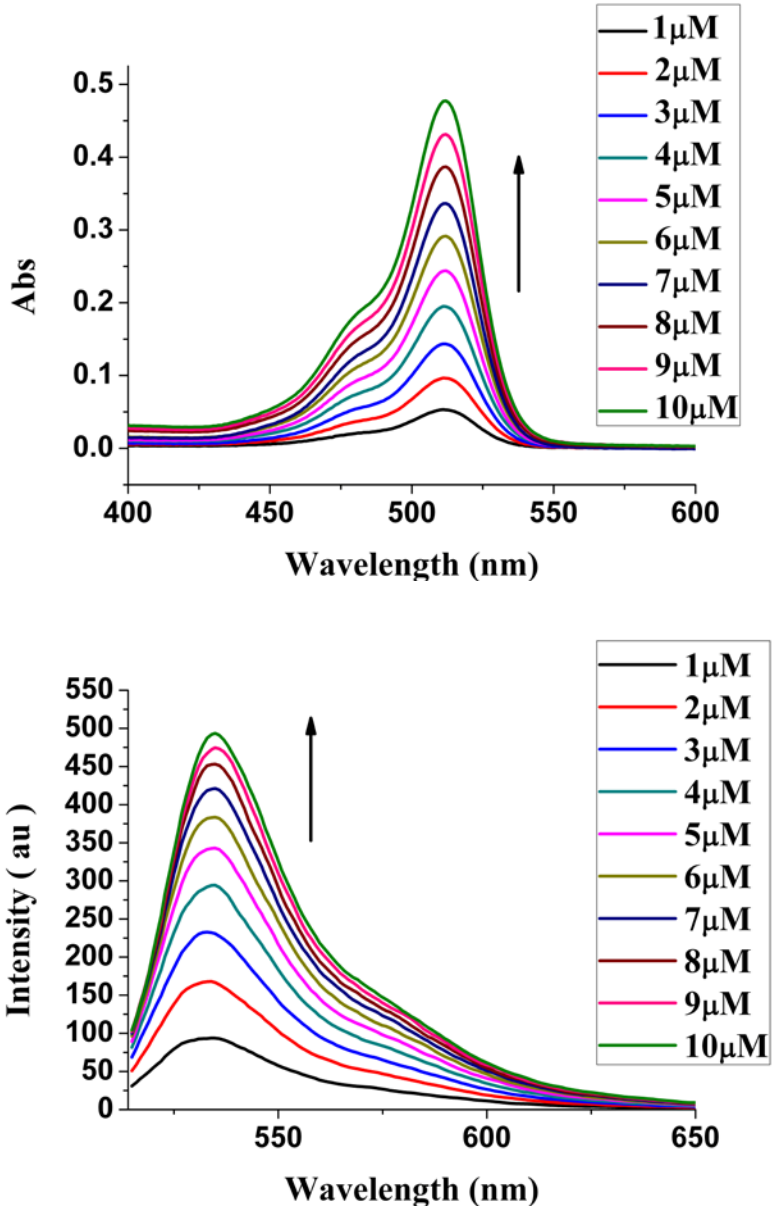



**Fig S1** The UV absorption spectra and fluorescent emission spectra of **Z1** in enzyme buffer (10 mM Tris-HCl, pH = 7.1, with 0.1 mM $ZnCl_2$ and < 1 % DMSO). The excitation wavelength was 511 nm.

## 4. Expression and purification of carbonic anhydrase IX

The catalytic domain of hCA IX in pFastBac vector was subcloned into pGEX-4T-1 vector and then expressed in *Escherichia coli* BL21 bacterial strain.[3] The bacterial cells were resuspended in the lysis buffer (10 mM Tris-HCl, 150 mM NaCl and 0.2 % Triton X-100, pH 7.5). The bacterial cells were sonicated. The obtained lysate was centrifuged for 30 min at 10,000 rpm, at 4 $^{o}$C and the supernatant was then applied to a prepacked Glutathione Sepharose 4B column. The mixture of the supernatant and resin were shaked gently and combind for about four hours, extensively washed with phosphate-buffered saline, pH 7.4, and the fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. [4]

## 5. CA inhibition assay.

A stopped-flow instrument has been used for measuring the $CO_2$ hydration reaction catalyzed by CA IX, by following the change of the maximum absorbance of a pH indicator. Phenol red was used (at a concentration of 0.2 mM ) as the indicator, working at the absorbance maximum of 557 nm, 10 mM Hepes, 0.1 M $Na_2SO_4$ (pH 7.5) as assay buffer, following the CAIX-catalyzed $CO_2$ hydration reaction for 5 s. Saturated $CO_2$ solutions in water at 25 °C were used as substrate.[5-7] Stock solutions of inhibitors (50 mM) were prepared in DMSO and diluted into different concentrations. Inhibitors and enzyme solutions were preincubated together for 15 min at 25 °C prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results.

## 6. Fluorescent emission spectra of Z1 combined with CA I, CA II and GST protein.

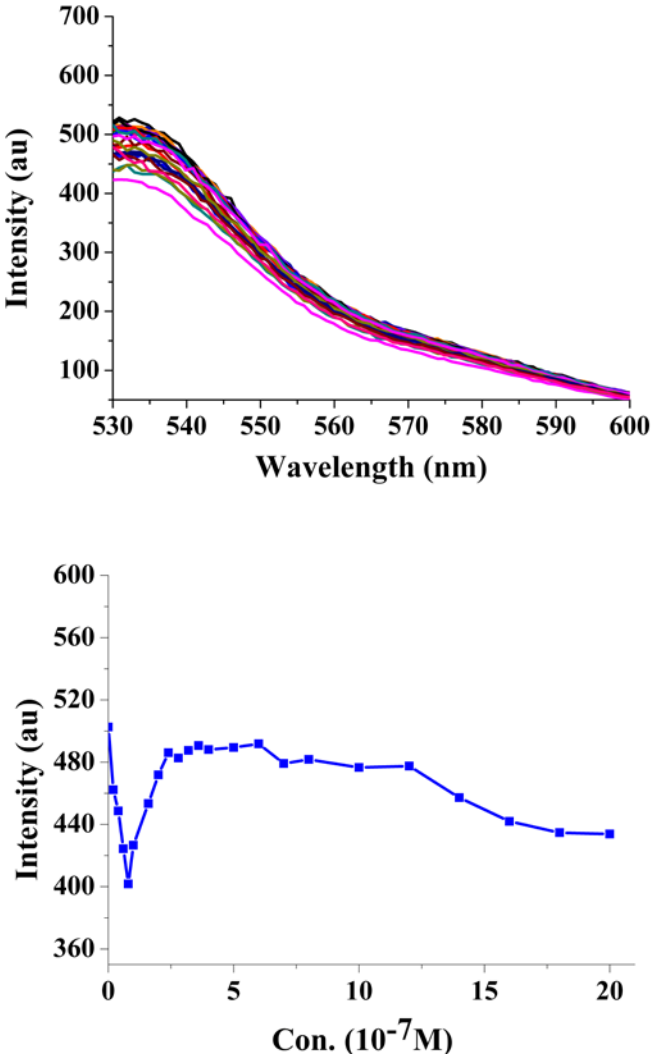



**Fig S2** The direct fluorescent response of **Z1** to CA I (above) and the corresponding emission intensity at 534nm (below). Fluorescent responses of **Z1** (0.1 μM) with 0-2 μM CA I in enzyme buffer (10 mM Tris-HCl, pH = 7.1, contained 0.1 mM $ZnCl_2$ and < 1 % DMSO) at 37 °C. Fluorescent excitation was provided at 511 nm.

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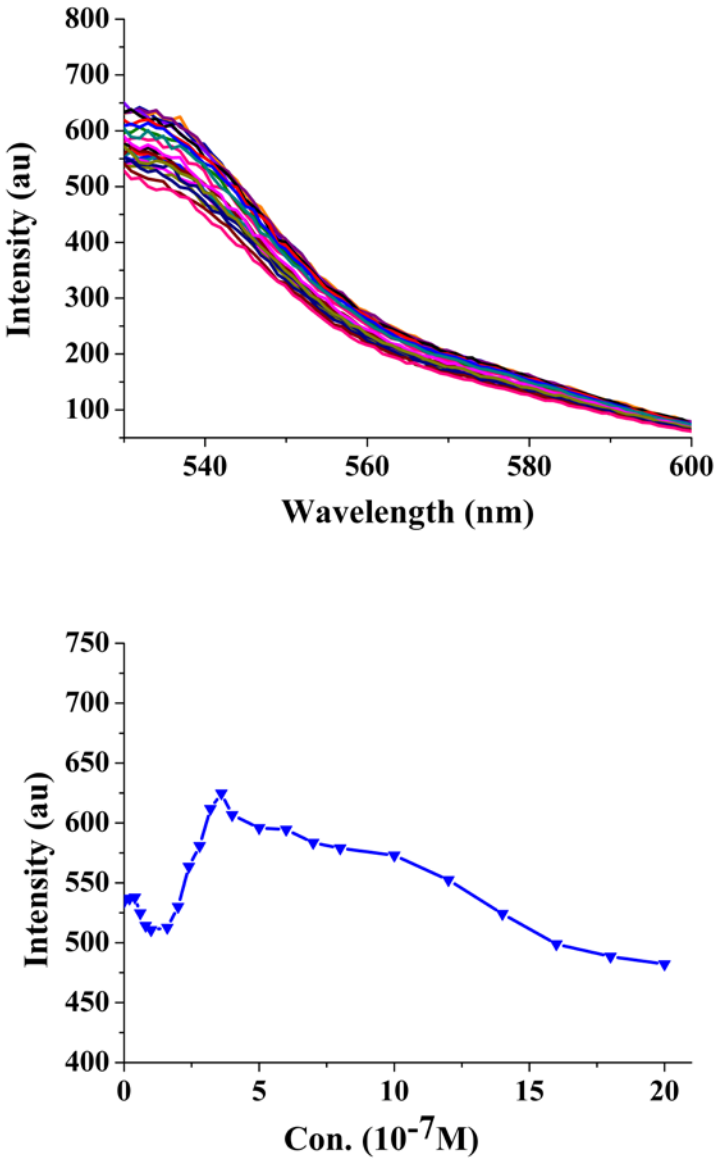


**Fig S3** The direct fluorescent response of **Z1** to CA II (above) and the corresponding emission intensity at 534nm (below). Fluorescent responses of **Z1** (0.1 μM) with 0-2 μM CA II in enzyme buffer (10 mM Tris-HCl, pH = 7.1, contained 0.1 mM $ZnCl_2$ and < 1 % DMSO) at 37 $^{o}$C. Fluorescent excitation was provided at 511 nm.

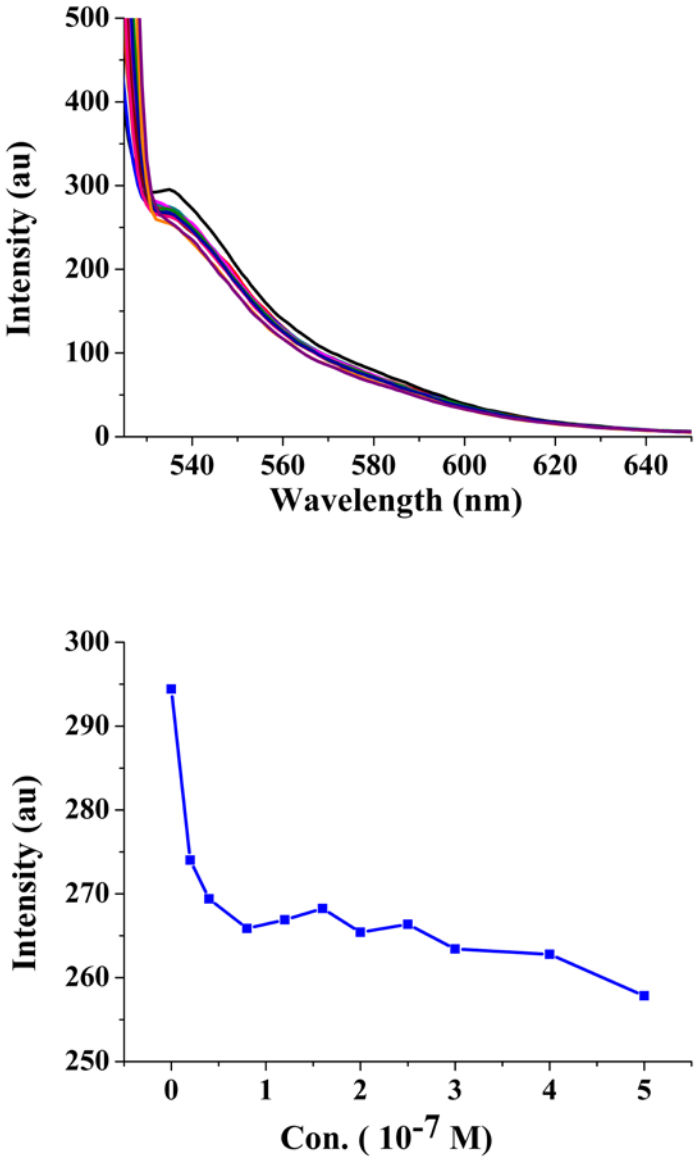


**Fig S4** The direct fluorescent response of **Z1** to GST protein (above) and the corresponding emission intensity at 534nm (below). Fluorescent responses of **Z1** (0.1 μM) with 0-0.5 μM GST in enzyme buffer (10 mM Tris-HCl, pH = 7.1, contained 0.1 mM $ZnCl_2$ and < 1 % DMSO) at 37 $^{o}$C. Fluorescent excitation was provided at 511 nm.

## 6. Fluorescent polarization spectra of Z1 with CA IX in buffer

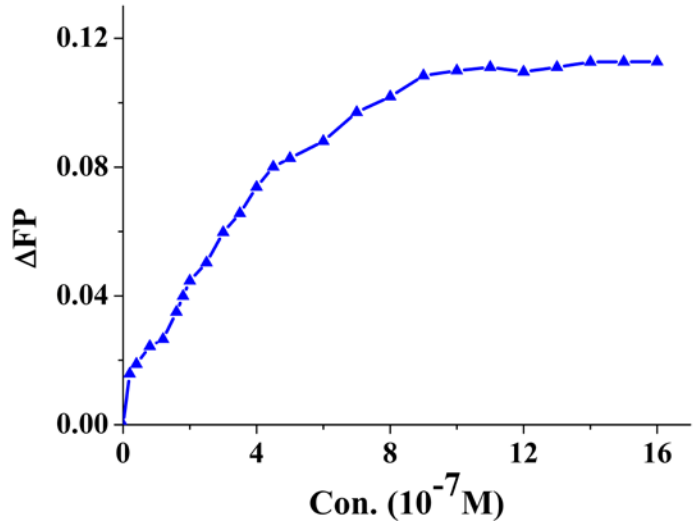


**Fig S5** Fluorescent polarization spectra of **Z1** (0.1 μM) with 0-1.6 μM CA IX in enzyme buffer (10 mM Tris-HCl, pH = 7.1, contained 0.1 mM $ZnCl_2$ and < 1 % DMSO) at 37 °C.

## 7. The relationship between CA IX concentration and fluorescent intensity

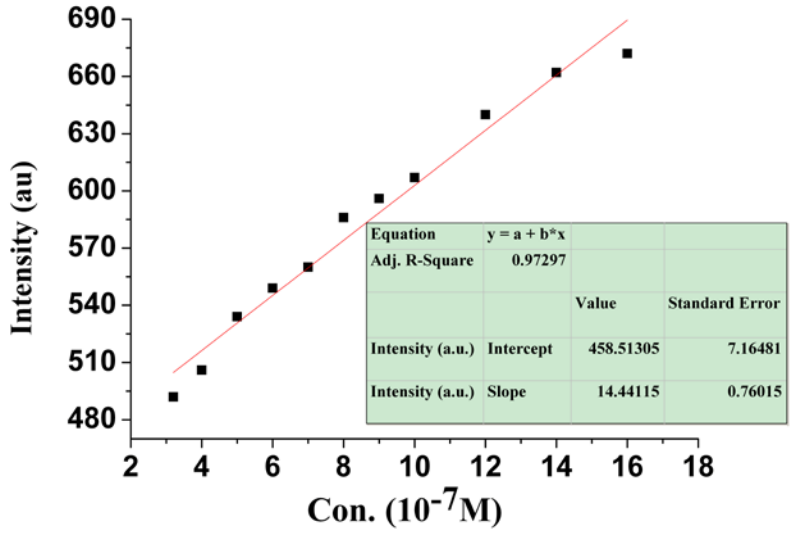


**Fig S6** the relationship between CA IX concentration and fluorescent intensity at 573 nm in enzyme buffer (10 mM Tris-HCl, pH = 7.1, contained 0.1 mM $ZnCl_2$ and < 1 % DMSO) at 37 °C.

## 8. Cell culture and imaging

SiHa cells were seeded in 24-well plates with a density of $5\times10^4$ cell $ml^{-1}$ in 1 ml Dulbecco's Modified Eagle Medium (DMEM, Gibco/Invitrogen) containing 10 % Fetal Bovine Serum and incubated in hypoxia (95 % $N_2$ and 5 % $CO_2$) or normoxia (5 % $CO_2$) for 48 h, then the cells were incubated with 10 μM probe **Z1** in DMEM without serum for

different time. Subsequently, the media was removed and the cells were washed twice with phosphate buffered solution (PBS). Fluorescence imaging was performed with Leica DMRIB Fluorescence Microscopy. We set the same exposure time, 0.02 s for bright field and 0.5 s for fluorescent field.

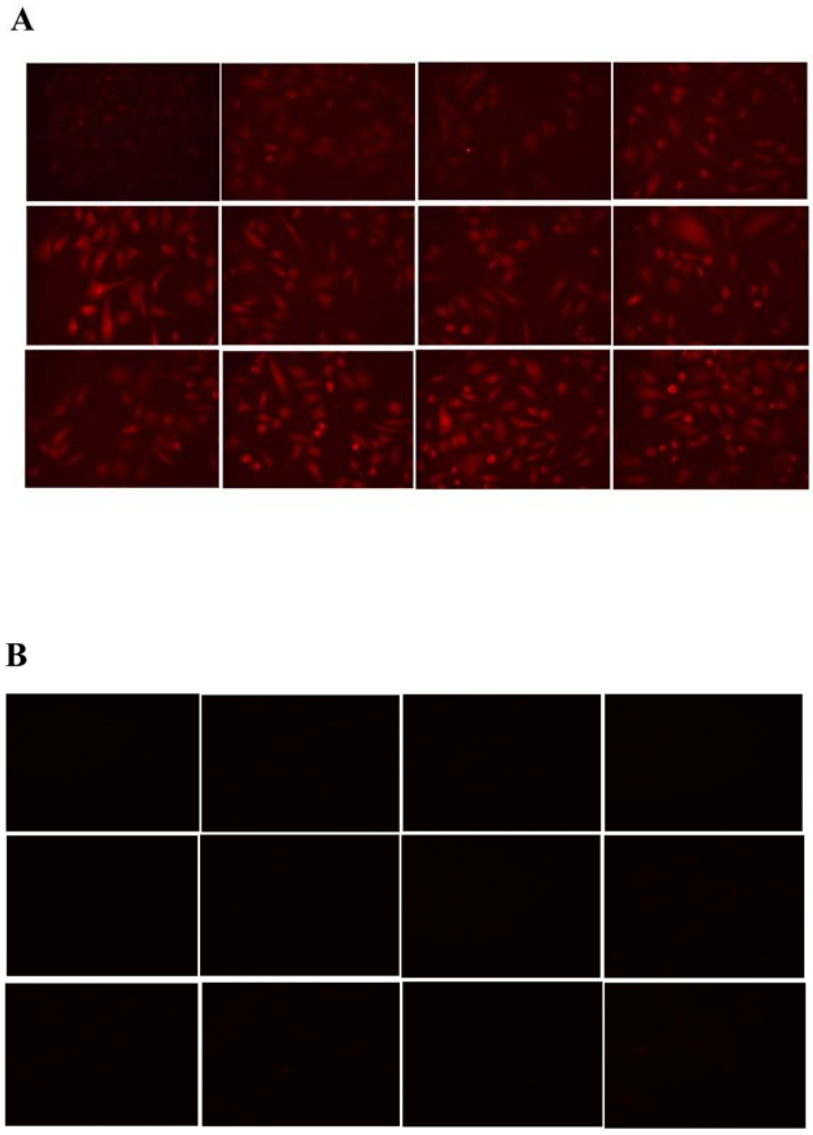


**Fig S7** The fluorescent image of **Z1** (10 μM) incubation in hypoxia (A) and normoxia (B) SiHa cells at 1 min, 2 min, 4 min, 6 min, 8 min, 10 min, 12 min, 14 min, 16 min, 18 min, 20 min and 22 min, respectively.

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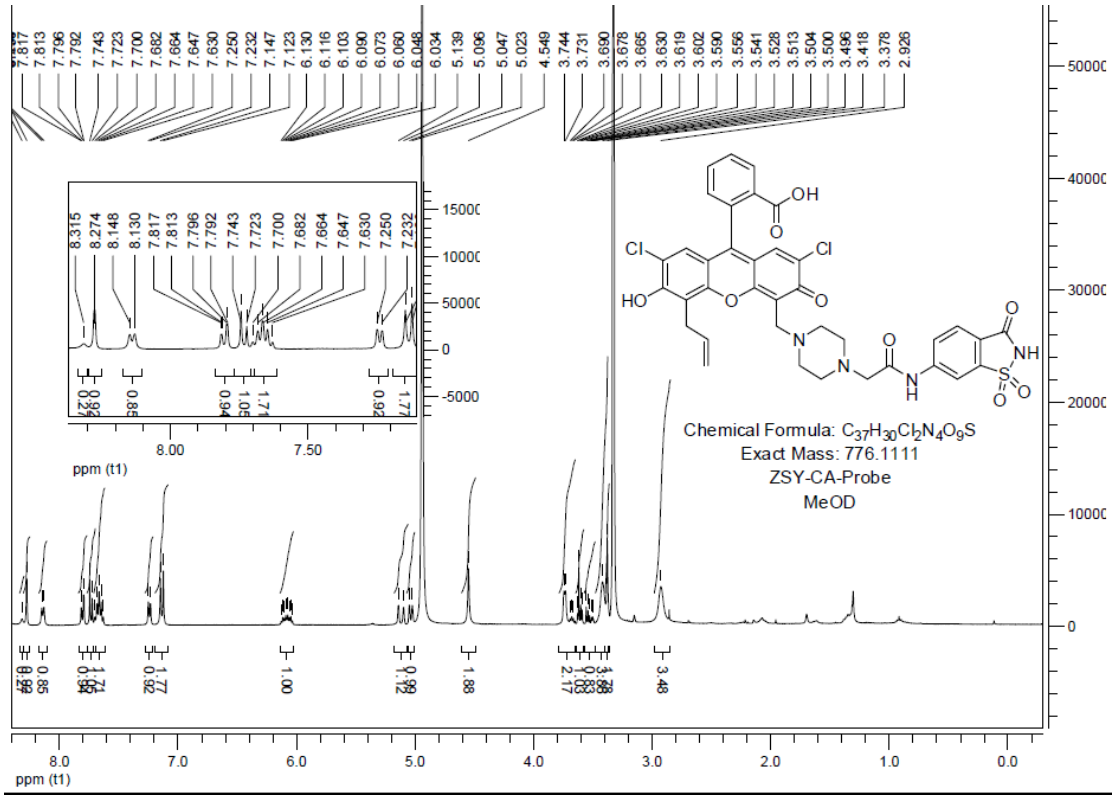
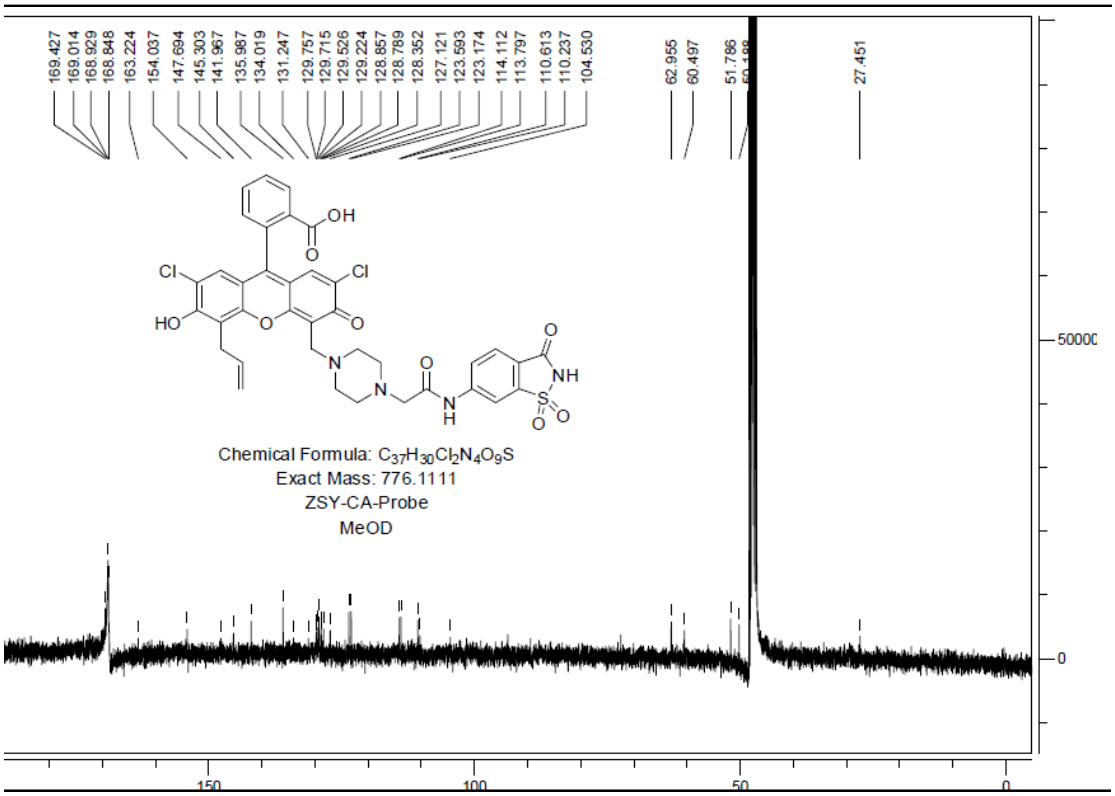
## 10. The characterization data of probe



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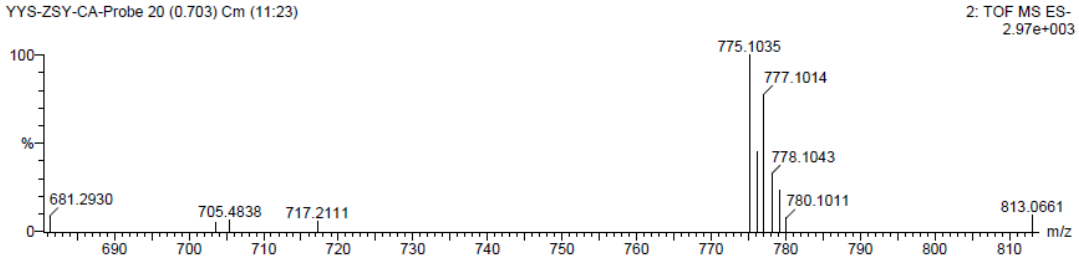
Monoisotopic Mass, Even Electron Ions
769 formula(e) evaluated with 6 results within limits (up to 1 best isotopic matches for each mass)
Elements Used:
C: 0-40 H: 0-30 N: 0-6 O: 0-9 S: 0-2 Cl: 0-3
YYS
YYS-ZSY-CA-Probe 20 (0.703) Cm (11:23)




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Minimum: | | | | -1.5 | | | |
| Maximum: | | 3.0 | 50.0 | 100.0 | | | |
| Mass | Calc. Mass | mDa | PPM | DBE | i-FIT | i-FIT (Norm) | Formula |
| 775.1035 | 775.1032 | 0.3 | 0.4 | 24.5 | 9.0 | 0.0 | C37 H29 N4 O9 S Cl2 |

## 11. Reference

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