Supporting Information

Selective Tracking of Ovarian-Cancer-Specific γ-Glutamyltranspeptidase by a Ratiometric Two-Photon Fluorescent Probe

Ben Shi,[a,#] Zhenyu Zhang,[a,#] Qingqing Jin,[b,#] Zhiqiang Wang,[c] Jie Tang,[a] Ge Xu,[a] Tianli Zhu,[a] Xueqing Gong,[c] Xinjing Tang,*[,b] Chunchang Zhao*[,a]

[a] Key Laboratory for Advanced Materials, Institute of Fine Chemicals, School of Chemistry and Molecular Engineering, East China University of Science &Technology, Shanghai ,200237, China.

[b] State Key Laboratory of Natural and Biomimetic Drugs , School of Pharmaceutical Sciences, Peking University, Beijing , 100191 , China

[c] Key Laboratory for Advanced Materials， Institute of Fine Chemicals, Centre for Computational Chemistry and Research Institute of Industrial Catalysis, School of Chemistry and Molecular Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, PR China

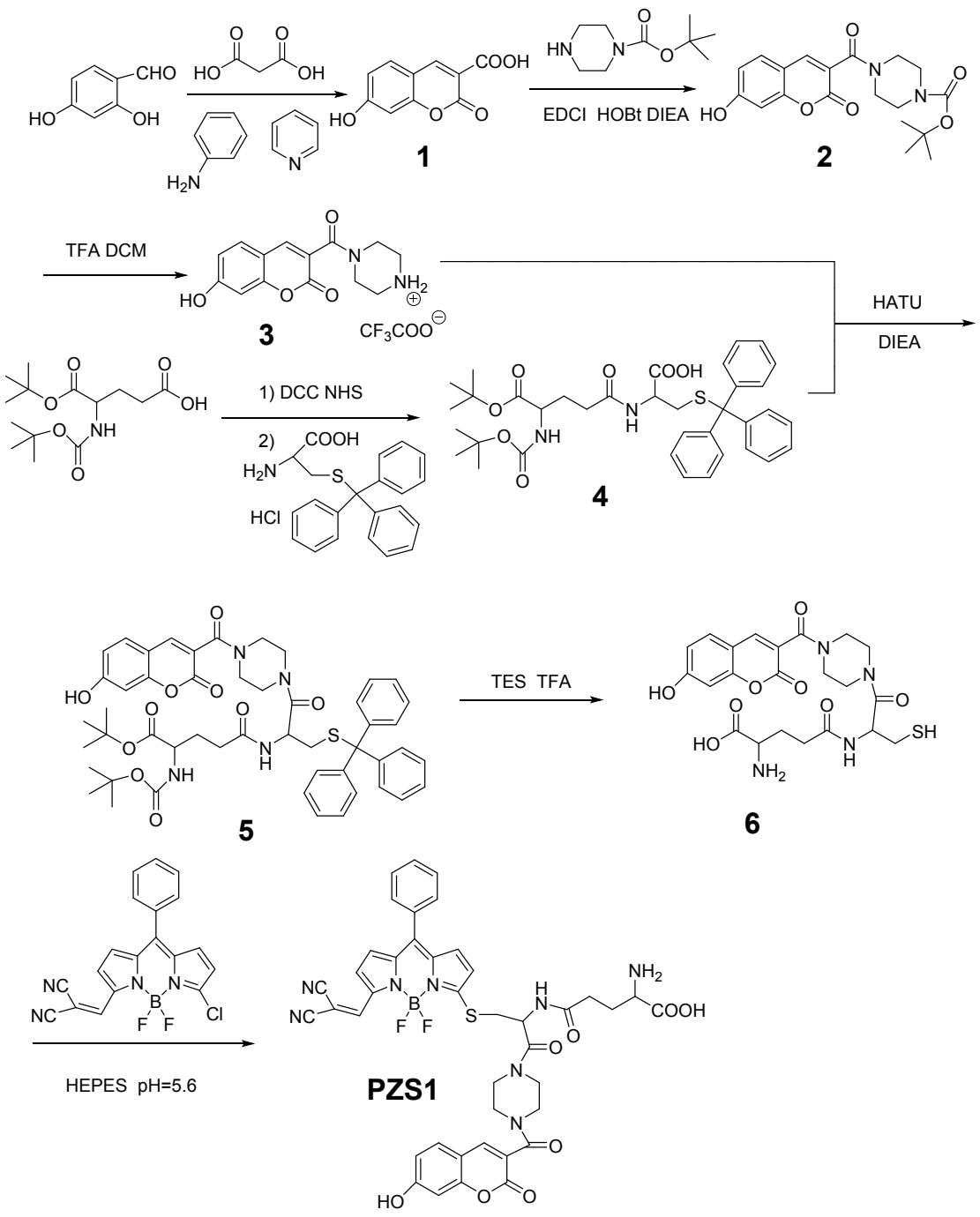
[#] These authors contributed equally.

1. Reagents

Chemicals for synthesis were purchased from commercial suppliers and used without further purification. γ-Glutamyltranspeptidase (GGT) from equine kidney was obtained from Sigma. GGsTOP, GGT inhibitor, was purchased from Wako (Japan). Other biochemical including glucoamylase, aprotinin, Collagen hydrolase, phosphatase, lipase and human plasma were purchased from Sigma-Aldrich.

2. Cells culture and imaging. OVCAR5 cells and human umbilical vein endothelial cells (HUVECs) were cultured under growth conditions (37 °C, a humidified atmosphere of 5/95 CO2/air) in RPMI (Roswell Park Memorial Institute) 1640 medium that is supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were seeded in a glass bottom dish and allowed to adhere for 24h. For imaging of HUVEC cells or OVCAR5 cells, these cells were incubated with PZS1 (10 μM) in culture medium at 37 °C for 15min, 30 min, 45 min and 1h, respectively. Then fluorescence images were captured in two channels (blue channel = 400-500 nm, red channel = 600−650 nm) using two-photon microscopy (TPM) with TP excitation at 700 nm. For assay the inhibitory effect of GGsTOP, ovarian cancer cells were pretreated with GGsTOP for 30 minutes then loaded with PZS1 (10 μM) for another 30 minutes in culture medium at 37 °C. Then fluorescence images were captured in two channels (blue channel = 400-500 nm, red channel = 600-650 nm) using two-photon microscopy (TPM) with TP excitation at 700 nm.

3. Synthesis.



Chemical compound **1**，**2**，**3** were synthesized according to previous literature[1] [2]

Synthesis of 1. 2,4-dihydroxybenzaldehyde (6.74g, 48.8 mmol) and malonic acid (10.16 g , 97.7 mmol) were dissolved in 44 mL of anhydrous pyridine in a 100 mL round flask. 4 mL aniline was added dropwisly. The mixture was stirred overnight under argon at room temperature. The precipitate was transferred to 70 mL ethanol and stirred for 1 h then filtered to afford yellow solid which was washed with 0.1 M chlorhydric acid, water and ethylic ether, then the yellow **1**

was obtained (6.40g, yield, 63%).

Synthesis of 2. A mixture of solution of **1** (1.2 g, 6.6 mmol), N-Boc-piperazine (1.8 g, 8.6 mmol), EDCI•HCl (1.2 g, 8.6 mmol), HOBt-H2O (1.6 g, 8.6 mmol), and DIEA (3.4mL, 19.8 mmol) in dry DMF (10.0 mL) was stirred for 24 h at room temperature. After dilution with H_2O, the mixture was extracted with AcOEt, and the organic layer was washed with brine followed by dried over anhydrous Na_2SO_4. The solvent was removed by evaporation, and the residue was purified by column chromatography (SiO_2, DCM:EA= 10/1 →4/1) to give a faint yellow solid **2** (1.5 g, yield, 60%).

Synthesis of 3. To a solution of **2** (3.0 g, 7.9 mmol) in dry CH_2Cl_2 (20 mL) was added TFA (20 mL). The mixture was stirred for 10 min at room temperature. The solvent was evaporated, and then CH_3OH added to the residue, followed by filtration to give **3** (1.94 g,yield, 63%) as a colorless powder.

Synthesis of 4. To a solution of 5-(tert-butoxy)-4-((tert-butoxycarbonyl)amino)-5-oxopentanoic acid (1.12 g, 3.70 mmol) in anhydrous DCM (20 mL) was added N-hydroxysuccinimide (0.85 g, 7.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.42 g, 7.4 mmol). The resulted mixture was stirred at room temperature for 16 h under a nitrogen atmosphere. After removing the solvent, the white residue was added to anhydrous DMF (50 mL) containing S-Trityl-L-cysteine (2.71 g, 7.4 mmol) and triethylamine (0.74 g, 7.4mmol). The mixture was stirred at room temperature for 10 h under argon. Then the solvent was removed, and the residue was redissolved in acetone (15 mL) and then poured into 1M HCl (100 mL). Light yellow solid precipitated, which was filtered off, washed with H_2O (50 mL) and dried under the high vacuum. **4** was thus obtained as a white spumescence solid (1.87g, yield, 79%). ^{1}H NMR (400

MHz, DMSO) δ 12.73 (s, 1H), 8.22-8.20 (d, 1H), 7.32-7.25 (m, 15H), 7.15-7.13(d, 1H), 4.16-4.11 (q, 1H), 3.80-3.76 (q, 1H), 2.46 (s, 1H), 2.38-2.34 (dd, 1H), 2.20-2.16 (t, 2H), 1.91-1.83 (m, 1H), 1.75-1.66 (m, 1H), 1.38 (s, 18H). ^{13}C NMR (100 MHz, DMSO) δ 172.7, 171.4, 171.3, 156.1, 146.9, 144.3, 129.5, 128.0, 126.9, 82.5, 80.6, 67.0, 60.4, 53.4, 51.8, 51.7, 33.1, 29.4, 28.3, 27.0, 21.0,14.2. HRMS (m/z): Calcd for $C_{36}H_{44}N_2O_7S_1Na$ $[M+Na]^+$: 671.2760, found: 671.2761.

Synthesis of 5. 3 (1.37 g, 5.0 mmol), **4** (3.24 g, 5.0 mmol), HATU (2.28 g, 6.0 mmol), and DIEA (3.47 ml, 20.0 mmol) were dissolved in anhydrous DMF (20ml), and the resulted mixture was stirred at room temperature for 10 h. After the reaction finished, the solution were removed under high vacuum. The residue was washed with water for three times and extracted with EA, the organic lay was purified by column chromatography (SiO_2, DCM:CH_3OH = 100/1→50/1) to give a white solid **5** (3.5 g, yield, 77%). ^{1}H NMR (400 MHz, DMSO) δ 10.79 (s, 1H), 8.32-8.29 (d, 1H), 8.09 (s, 1H), 7.61-7.59 (t, 1H), 7.34-7,21 (m, 15H), 7.14-7.08 (q, 1H), 6.85-6.83 (d, 1H), 6.78-6.76 (d, 1H), 4.53-4.43 (dd, 1H), 3.79-3.71 (m, 1H), 3.60-3.40(m, 8H), 2.45-2.33 (m, 2H), 2.15-2.11 (t, 2H) 1.89-1.78 (m, 1H), 1.72-1.62(m, 1H), 1.38-1.36 (d, 18H). ^{13}C NMR (100 MHz, DMSO) δ 171.5, 170.8, 168.3, 163.6, 162.2, 158.0, 155.7, 155.4, 151.1, 144.3, 130.5, 129.1, 128.0, 126.7, 120.7, 119.4, 113.6, 110.8, 101.9, 80.2, 78.0, 66.2, 33.5, 31.3, 28.1, 27.6, 26.6. HRMS (m/z): Calcd for $C_{50}H_{56}N_4O_{10}S$ $[M+Na]^+$: 927.3615, found: 927.3607.

Synthesis of 6. The solution of compound **5** (904 mg, 1 mmol) was dissolved in TFA (3.7 ml, 50 mmol) was stirred for 10 h. To the solution was then added TES (0.24 ml, 1.5 mmol). The mixture was stirred for another 5 h, and the solvent was removed. Ether (10 ml) was used to wash the residue to get a white solid **6** (498 mg, yield, 98%). ^{1}H NMR (400 MHz, D_2O) δ 8.02 (s, 1H), 7.50-7.48 (d, 1H), 6.84-6.81 (dd, 1H), 6.75-6.74 (d, 1H), 3.76-3.47 (m, 10H), 2.89-2.68 (m, 2H), 2.40-

2.28 (m, 2H), 2.06-1.98 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 173.8, 173.0, 172.8, 170.4, 166.0, 161.6, 155.6, 149.9, 145.7, 130.9, 117.5, 114.3, 111.5, 102.5, 66.0, 53.3, 43.1, 30.8, 29.9, 29.1, 28.3, 25.9, 14.0. HRMS (m/z): Calcd for $C_{22}H_{27}N_4O_8S$ $[M+H]^+$: 507.1550, found: 507.1541.

Synthesis of PZS1. To a solution of **6** (200 mg, 0.4 mmol) in HEPES (50 ml, pH=5.8) was added a solution of dicyano-BODIPY-Cl (151 mg, 0.4 mmol) in acetonitrile (10 ml). The mixture was stirred overnight, and the acetonitrile was removed. The residue was washed with water (100 ml), DCM (100 ml), acetone (20 ml) to give the final product (83 mg, yield, 24%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.14 (s, 1H), 8.01-7.99 (d, 1H), 7.63-7,49 (m, 7H), 7.33-7.25 (m, 2H), 6.84-6.80 (m, 1H), 6.77-6.72 (m, 2H), 5.26-5.15 (m, 1H), 3.82-3.61 (m, 9H), 3.50-3.46 (m, 2H), 2.4-2.50 (m, 2H), 2.21-2.11(m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 174.7, 172.1, 170.4, 167.5, 162.9, 152.6, 148.7, 143.6, 138.0, 135.2, 132.3, 129.0, 122.3, 118.9, 115.2, 111.8, 103.4, 64.9, 55.8, 44.1, 30.8, 28.6, 28.1, 22.6, 16.3. HRMS (m/z): Calcd for $C_{41}H_{36}BN_8O_8F_2S$ $[M+H]^+$: 849.2438, found: 849.2433.

4. Calculation methods

The Gaussian 09 program package (Frisch, M. J.; et al. Gaussian 09, Revision A.1; Gaussian, Wallingford, 2009) was used for spin-unrestricted density functional theory (DFT) calculations. All chemical structures were optimized without symmetry constrains at the B3LYP level (Becke, A. D. *Phys. Rev. A*， 1988, **38**, 3098.) with the 6-311G basis sets for all atoms. During calculations, the water solvation effect was also taken into account by using SMD model (A. V. Marenich, C. J. Cramer, D. G. Truhlar. *J. Phys. Chem. B.*, 2009, **113**, 6378.).

5. The FRET efficiency of PZS1 using receptor photobleaching.

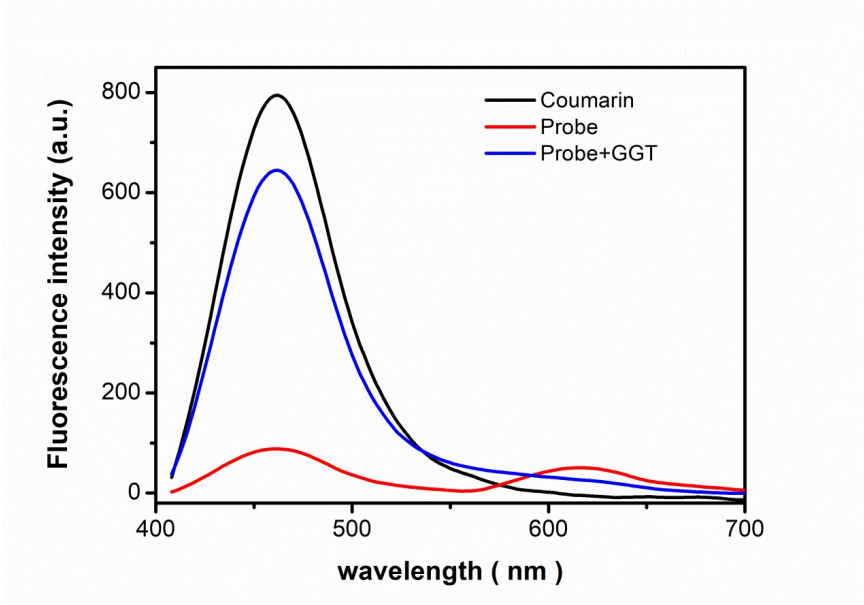


Figure S1. Calculation FRET efficiency. According to the way of receptor photobleaching to calculate the FRET efficiency: $E=1-IF_n / IF_0$, the efficiency of PZS1 is 88.8%, after reaction with GGT, the number is changed to 19.1%.

6. The fluorescence color change of PZS1 treated with GGT.

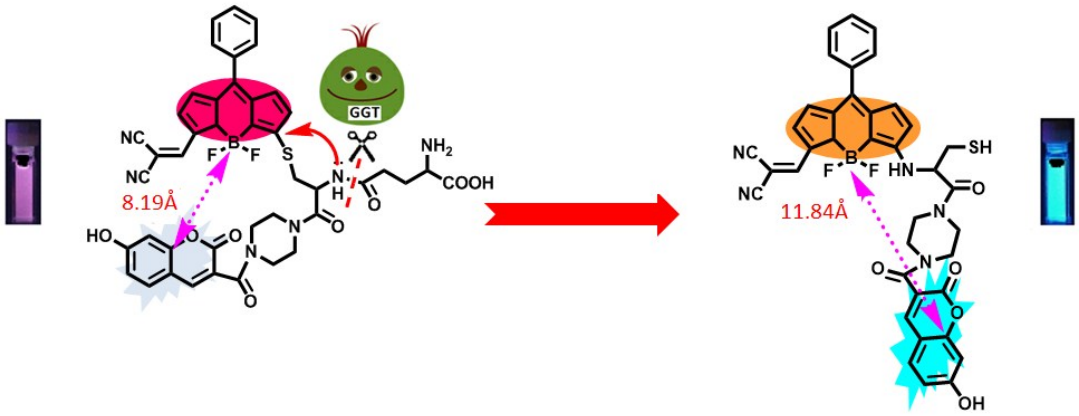


Figure S2. The fluorescence color changes from pink to bluish green upon addition of GGT.

7. Fluorescence changes of PZS1 with GGT and the inhibitor GGsTOP.

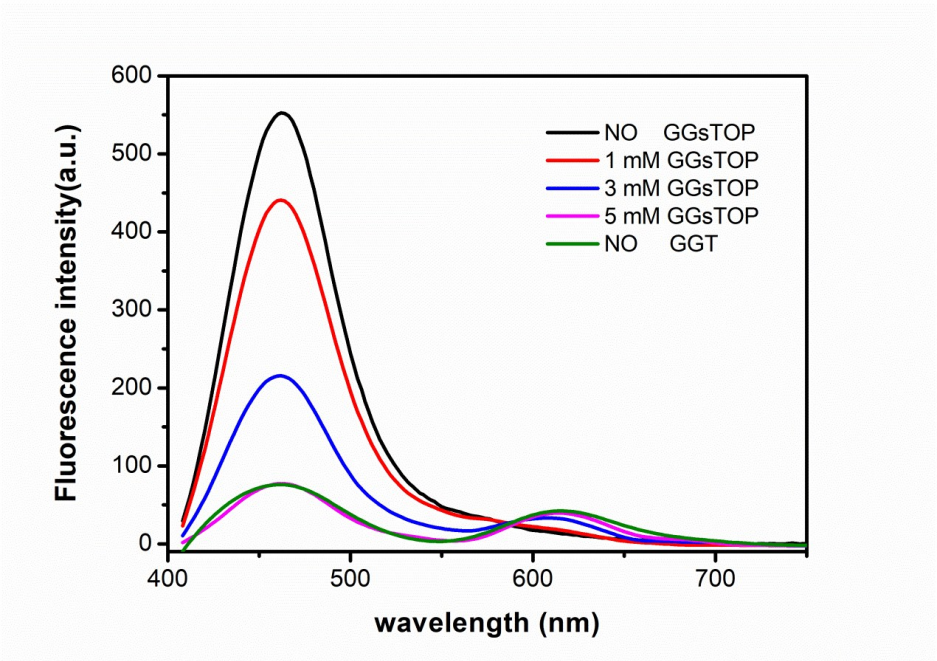
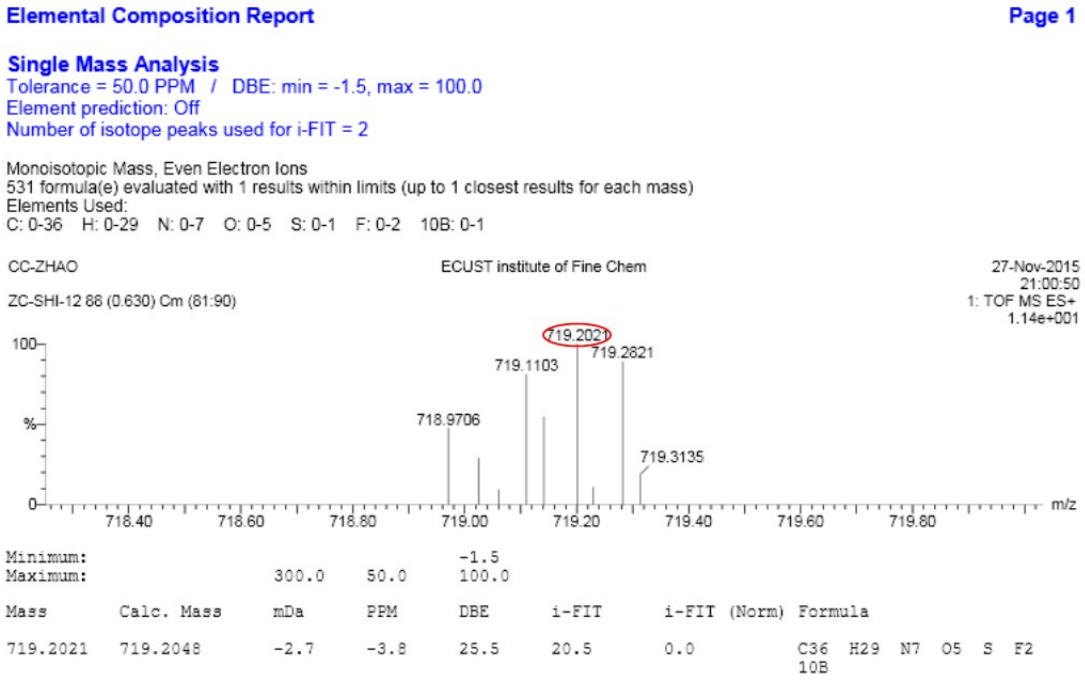


Figure S3**.** The ratiometric fluorescence changes of PZS1 (10 μM) in the presence of GGT (600 U/L) pretreated with various concentrations of GGsTOP.

8. HRMS spectra of the mixtures of probes and GGT.



Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
531 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-36 H: 0-29 N: 0-7 O: 0-5 S: 0-1 F: 0-2 10B: 0-1

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Maximum:		300.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
719.2021	719.2048	-2.7	-3.8	25.5	20.5	0.0	C36 H29 N7 O5 S F2 10B

Figure S4. HRMS analysis of PZS1 treated with GGT.

9. The titration experiments of probe with GGT.

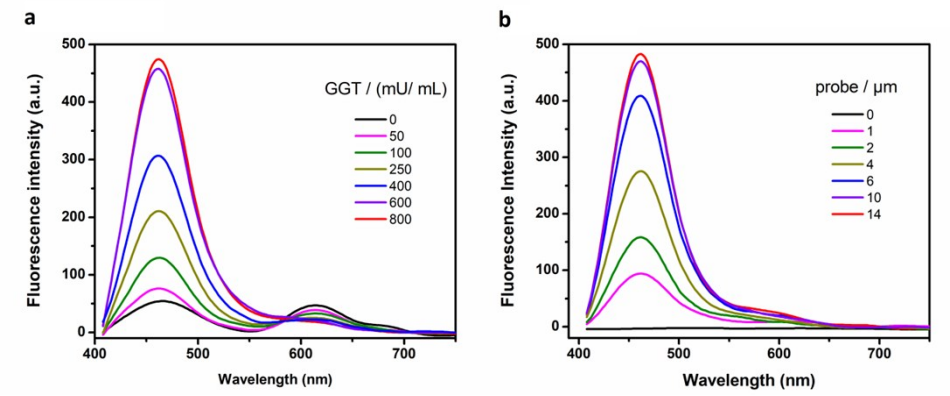


Figure S5. (a) The emission spectra of PZS1 (10 μM) in the presence of different amount of GGT; (b) the emission spectra of different concentrations of PZS1 in the presence of GGT (600 U/L). Data are recorded 110 min after addition of analytes.

10. Determination of K_m and V_{max}.

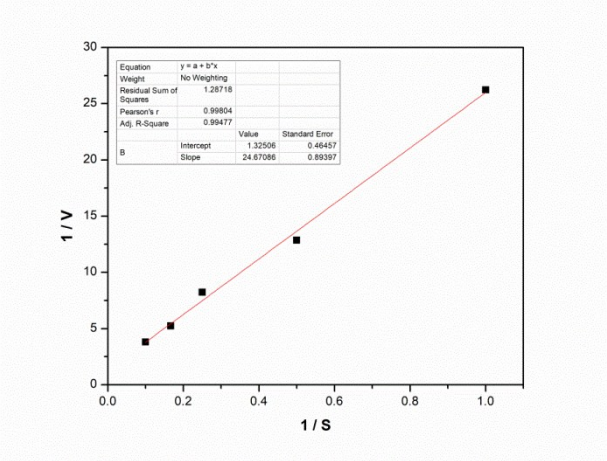


Figure S6. Plots of 1/V as function of 1/S. Equation (1) and (2) can be obtained according to Michaelis-Menten equation, where V represents the velocity and [S] is the substrate concentration, K_m is the Michaelis constant. From the linear plot, the slope and the intercept can be determined, then K_m, V_{max} were calculated to be 18.62 μM and 0.75 μM • min^{-1}.

$$V = V_{max} \times [S]/(K_m + [S]) \quad (1)$$

$$1/V = (K_m/V_{max}) \times (1/[S]) + 1/V_{max} \quad (2)$$

11. Two-photon microscopy of PZS1 for differentiating ovarian cancer cells.

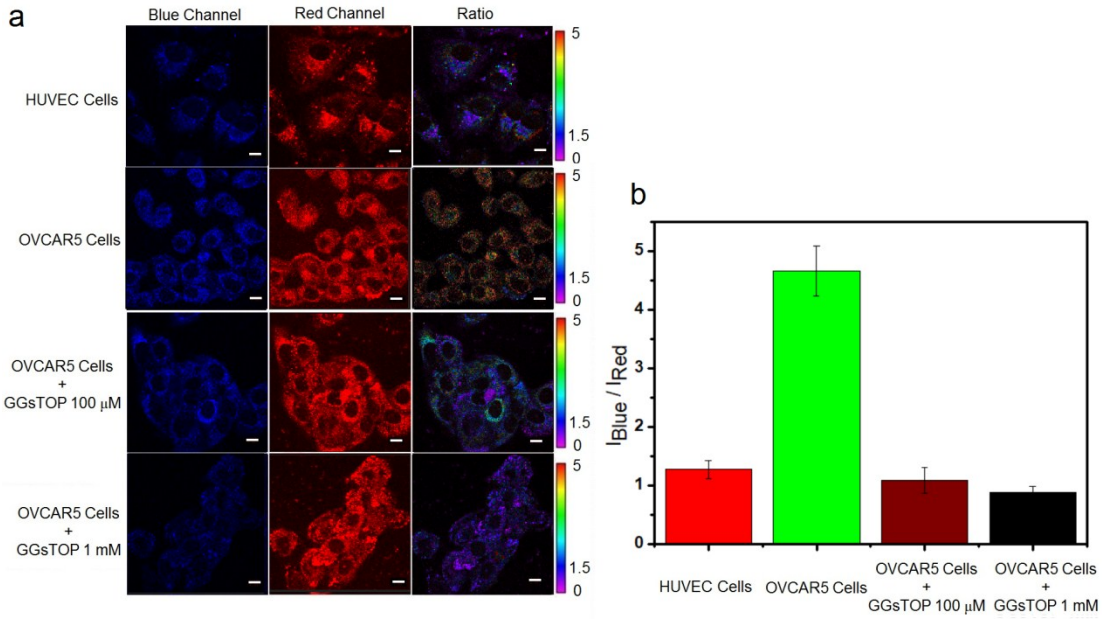


Figure S7. (a) Differentiation of ovarian cancer cells from normal cells via living-cell tracking of GGT activity with PZS1 (10 μM). (b) The average emission ratio (I_{blue}/I_{red}) in fluorescence images obtained in Fig. 4 and Figure S7a.

12. Time-dependent two-photon microscopy of PZS1 in ovarian cancer cells.

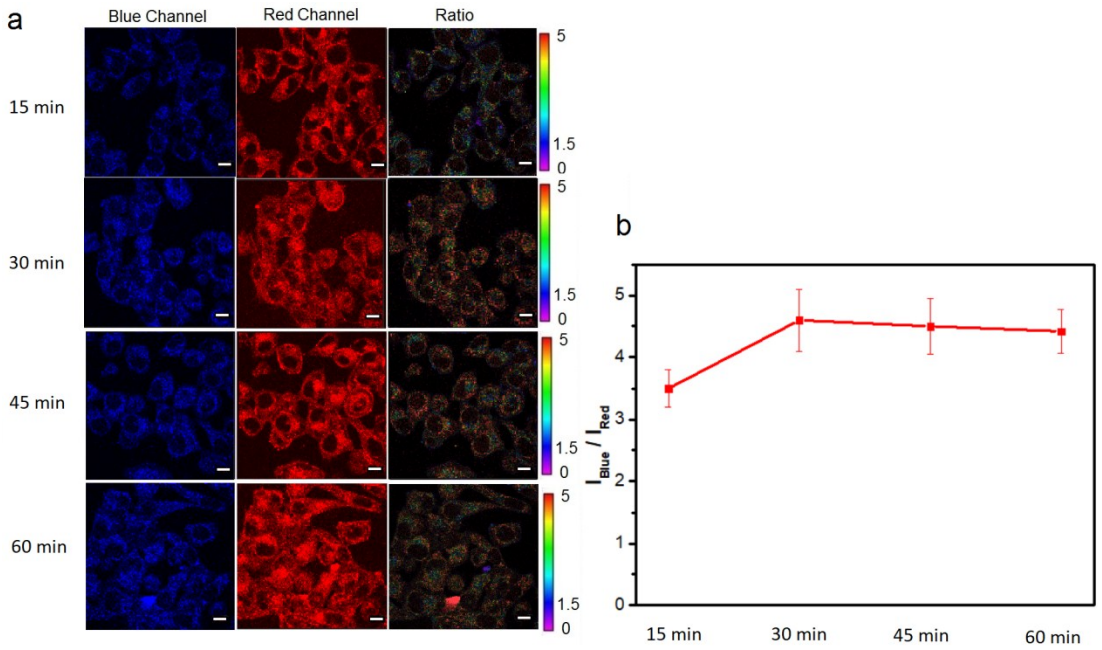
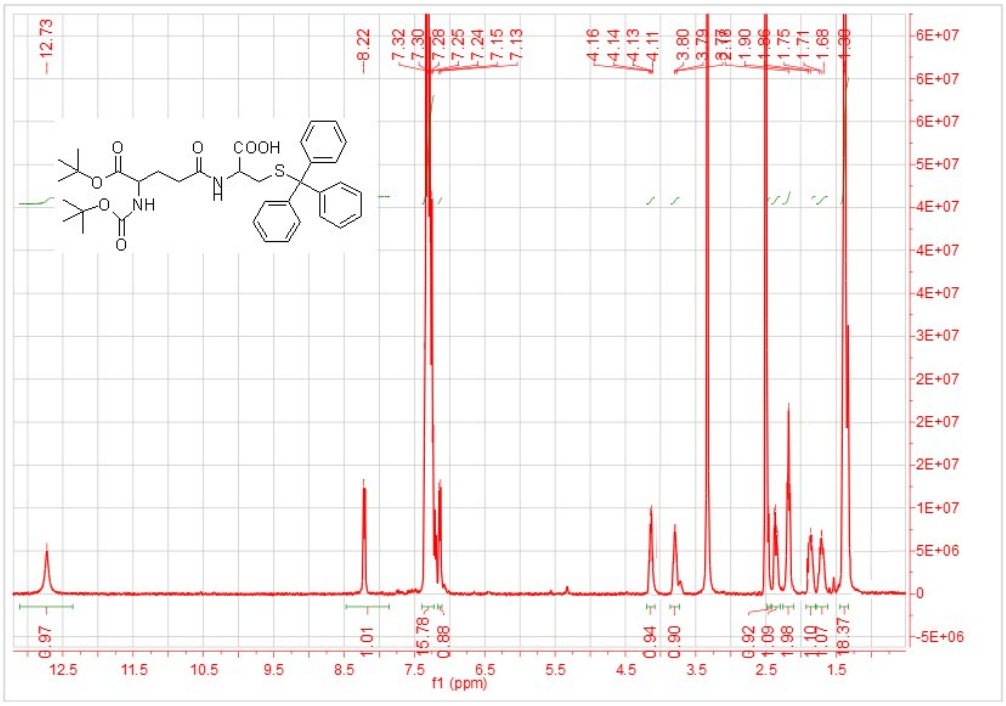
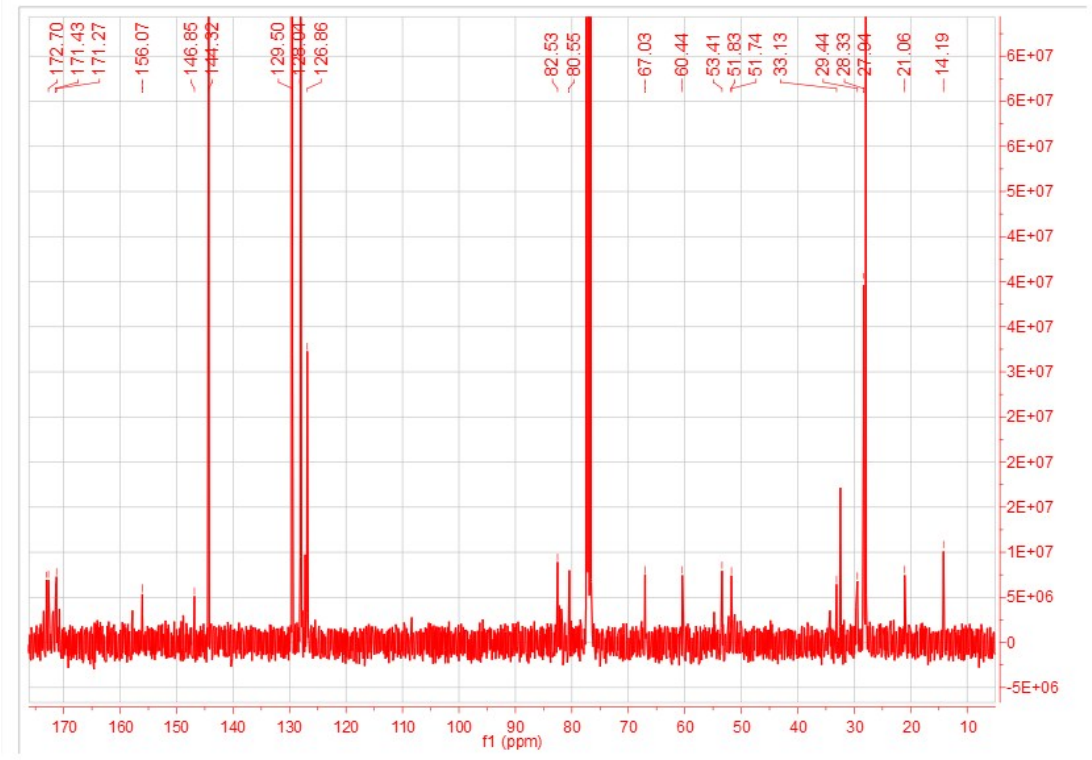


Figure S8. (a) Time-dependent confocal imaging of OVCAR5 cells via tracking of GGT activity with PZS1 (10 μM). (b) The average emission ratio (I_{blue}/I_{red}) in fluorescence images.

13. The ^{1}H-NMR, ^{13}C-NMR and HRMS spectra.





Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
3638 formula(e) evaluated with 232 results within limits (up to 1 closest results for each mass)
Elements Used:
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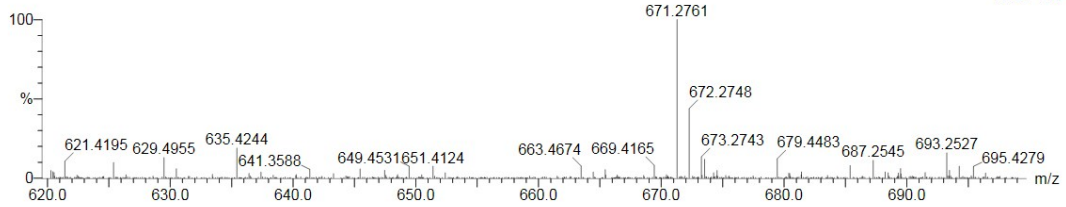

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Maximum:		300.0	50.0	100.0			
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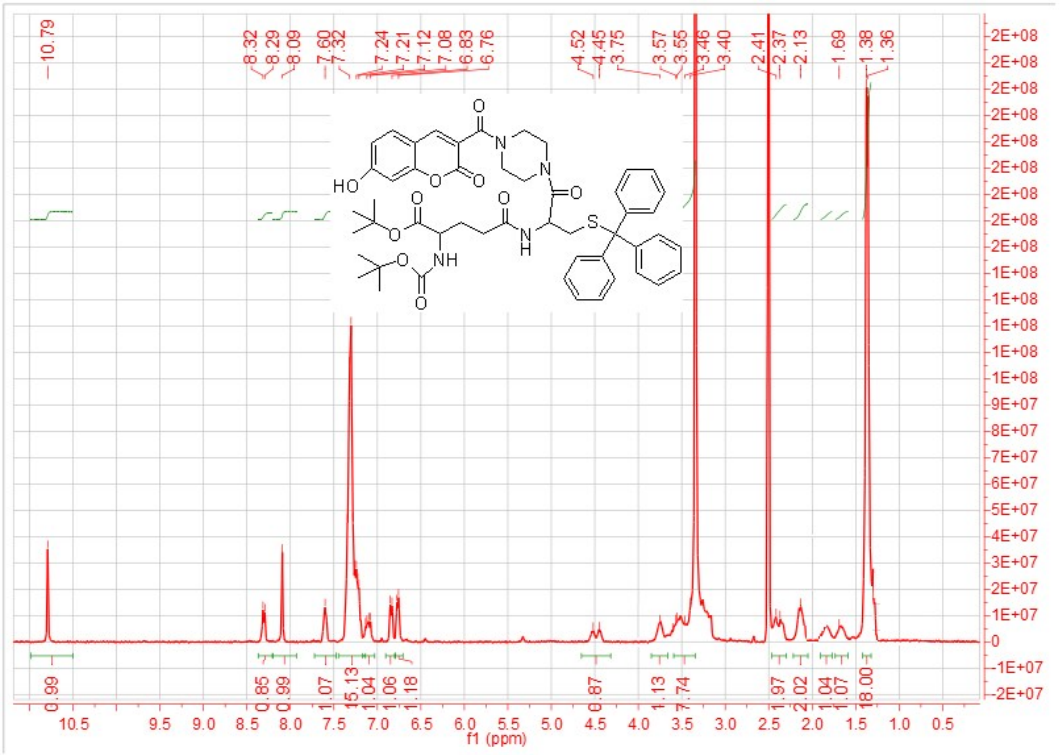
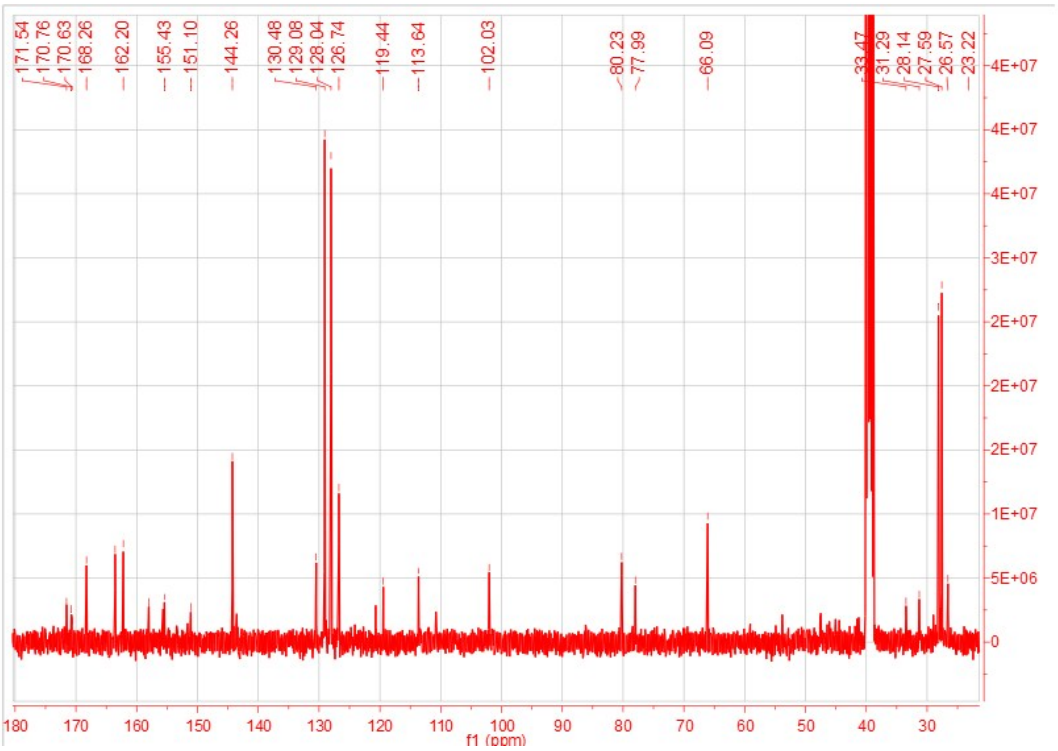
Figure S9. Characterizations for compound **4**.





Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
201 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
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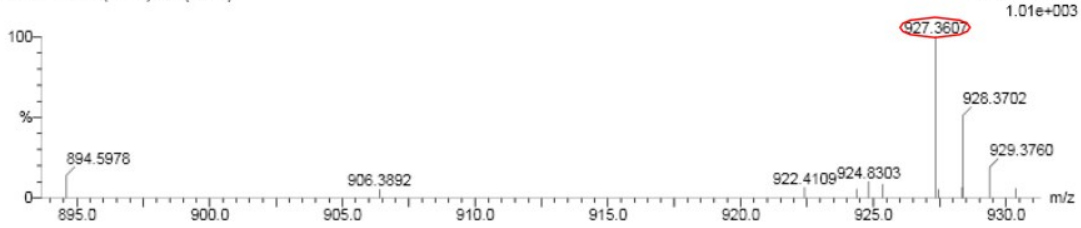

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Maximum:		300.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
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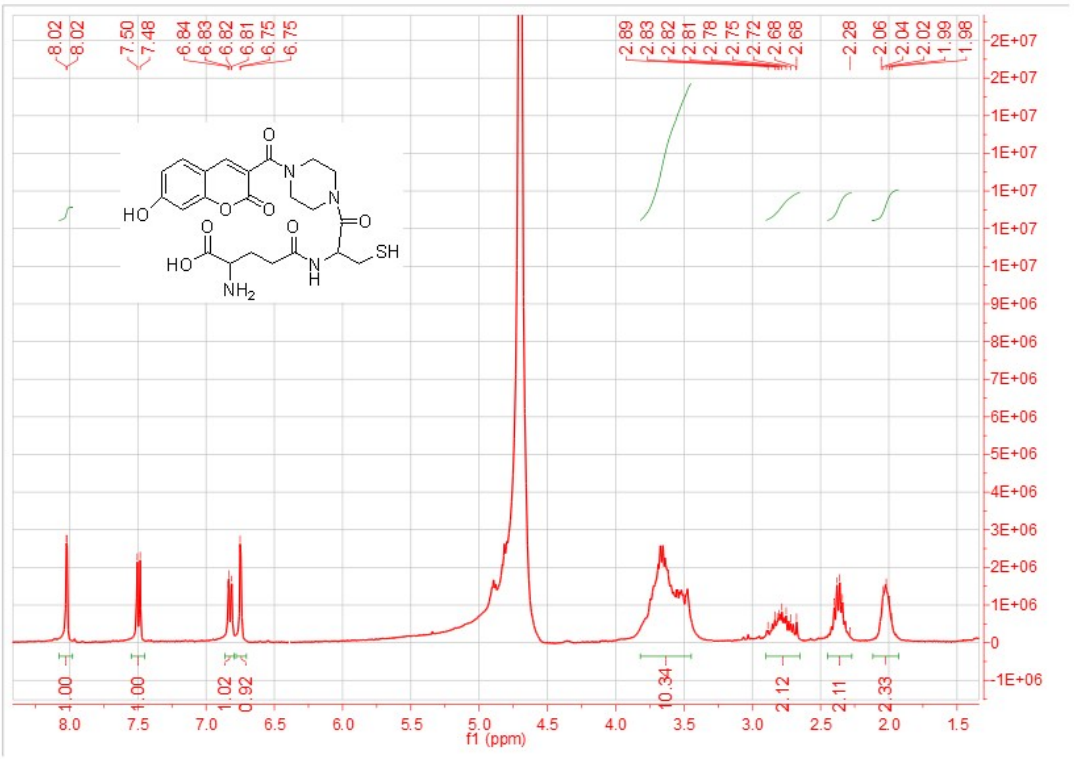
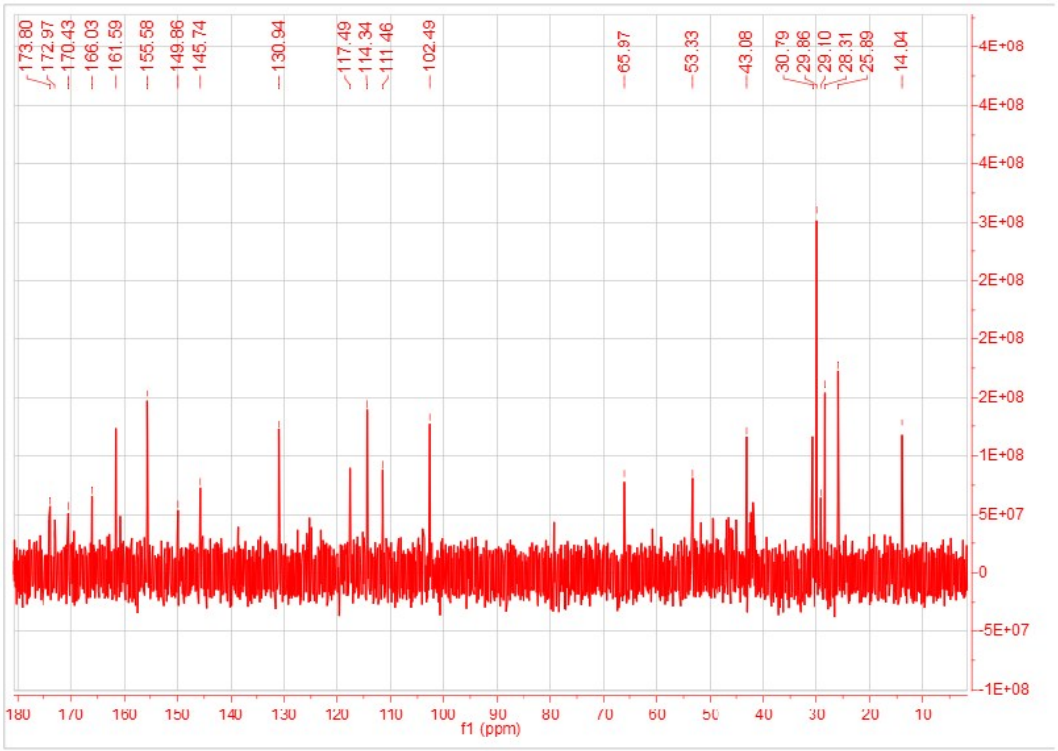
Figure S10. Characterizations for compound **5**.


8.02
8.02
7.50
7.48
6.84
6.83
6.82
6.81
6.75
6.75
2.89
2.83
2.82
2.81
2.78
2.75
2.72
2.68
2.68
2.28
2.06
2.04
2.02
1.99
1.98
HO
O
N
N
O
O
O
SH
HO
O
O
N
H
NH2
1.00
1.00
1.02
0.92
10.34
2.12
2.11
2.33
f1 (ppm)


173.80
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170.43
166.03
161.59
155.58
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145.74
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117.49
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102.49
65.97
53.33
43.08
30.79
29.86
29.10
28.31
25.89
14.04
f1 (ppm)

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
88 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
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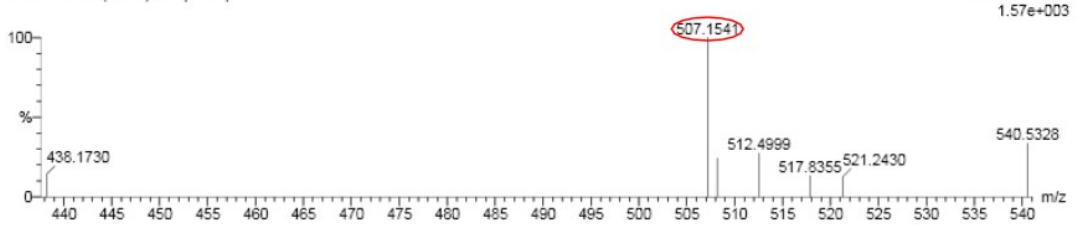
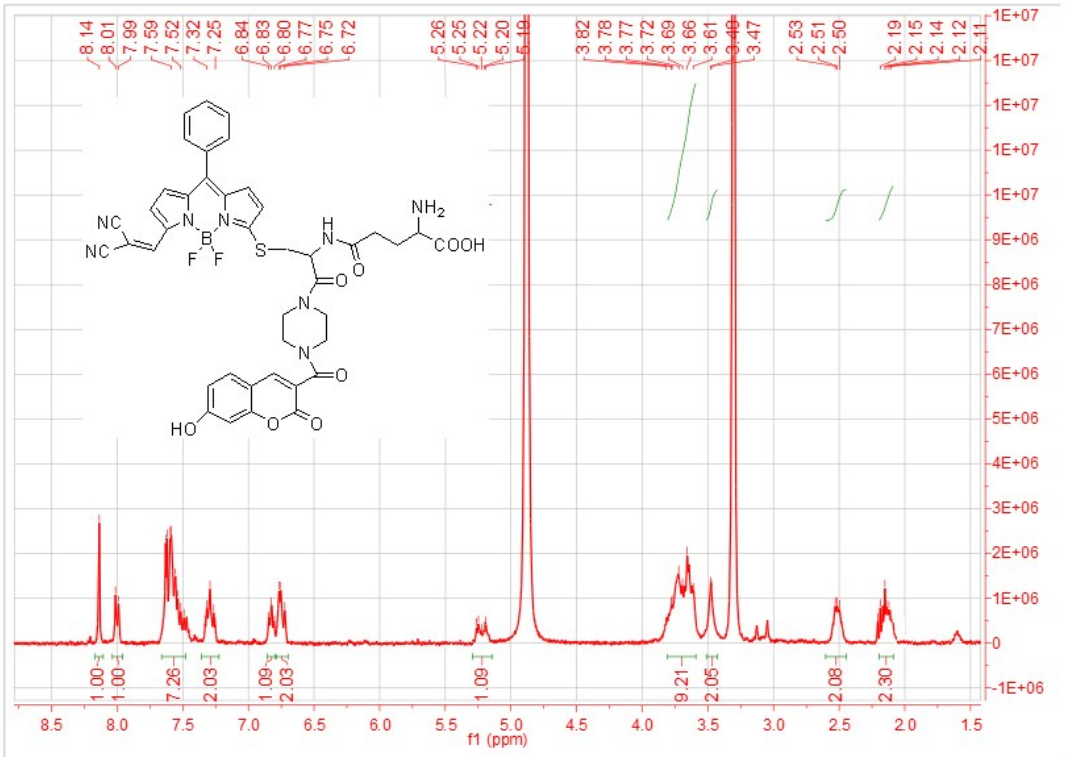
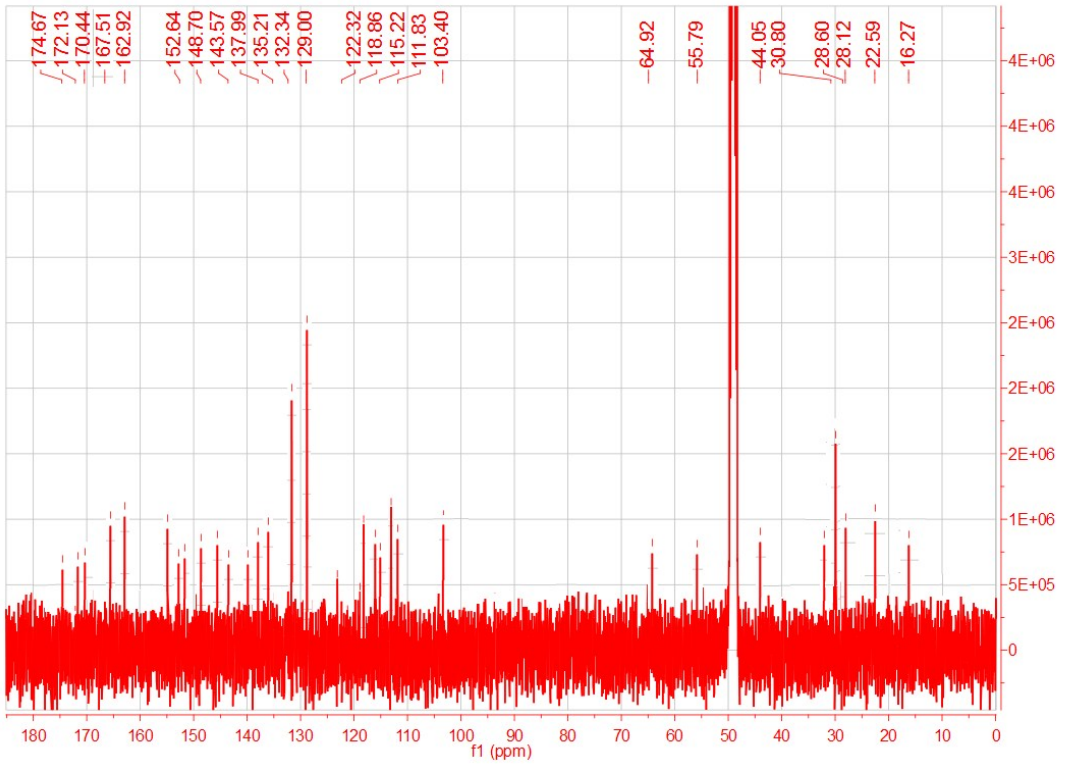


Minimum:				-1.5			
Maximum:		300.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
507.1541	507.1550	-0.9	-1.8	11.5	8.2	0.0	C22 H27 N4 O8 S

Figure S11. Characterizations for compound **6**.





Elemental Composition Report **Page 1**

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
920 formula(e) evaluated with 1 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-41 H: 0-56 B: 0-1 N: 0-8 O: 0-8 F: 0-2 S: 0-1

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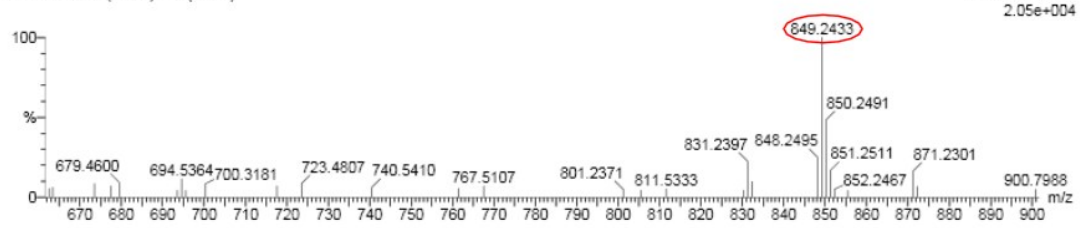
ZC-SHI-07 65 (0.615) Cm (63:91) 1: TOF MS ES+ 2.05e+004



Minimum:				-1.5			
Maximum:		300.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
849.2433	849.2438	-0.5	-0.6	27.5	26.8	0.0	C41 H36 B N8 O8 F2 S

Figure S12. Characterizations for compound **PZS1**.

References:

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