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A ratiometric fluorescent sensor for rapid detection of the pyroglutamate aminopeptidase-1 in tumor mouse

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Experiment Section

Measurements of photophysical properties

Steady-state UV-vis absorption and fluorescence spectroscopy. UV–vis absorption spectra were recorded on a Varian UV-Cary5000 spectrophotometer, and for the corrected steady-state emission spectra, a Hitachi F-7000 spectrofluorometer was employed (Scan speed: 2400 nm/min, Delay: 0.0 s, EX Slit: 5.0 nm, EM Slit: 5.0 nm, PMT, Voltage: 700 V, Response: 2.0 s). Freshly prepared samples in 1-cm quartz cells were used to perform all UV–vis absorption and emission measurements. For the determination of the fluorescence quantum yields (Φ_f of probe **DP-1**, only dilute solutions with an absorbance below 0.1 at the excitation wavelength was used). Fluorescein[1] (λ_{abs} = 490 nm, λ_{em} = 535 nm, Φ_f = 0.89) and 4-Dicyanomethyl-2-methyl-P-ditolidine styrene-4h-pyran[2] (λ_{abs} = 467 nm, λ_{em} = 618 nm, Φ_f = 0.435) were used as the standard of fluorescence quantum yield. The Φ_f values reported in this work are the averages of multiple (generally 3), fully independent measurements. In all cases, correction for the solvent refractive index was applied.

The limit of detection calculation. The detection limit (DL) for **DP-1** with PGP-1 was calculated by the linear function in Figure 1c and the following equation:

$$DL = (3\sigma / k) \times 10^{-6}$$

Where σ is the standard derivation of fluorescence intensity of **DP-1** blank solutions; k is the slope of the linear calibration curve in Figure 1c; the concentration of **DP-1** is10 μM.

The specific test experiments. For metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}) were 2 mM, for reactive species (S^{2-}, SO_3^{2-}, H_2O_2, $ONOO^-$) were 100 μM, for amino acids (Glu, Asp, Gly, Pro, Ile, Tyr, Ser, Lys, Ala, Arg, Met, Phe, Cys, Hcy and GSH) were 1mM, for enzymes and proteins were 2 μg/mL.

K^+, Na^+, Ca^{2+}, Mg^{2+}: A 10 mM stock solution of these ions was firstly prepared in deionized water.

Glu, Asp, Gly, Pro, Ile, Tyr, Ser, Lys, Ala, Arg, Met, Phe, Cys, Hcy and GSH: A 10 mM stock solution of amino acids were firstly prepared in deionized water.

S^{2-}, SO_3^{2-}: A 10 mM stock solution of $Na_2S \cdot 9H_2O$ and Na_2SO_3 were firstly prepared in deionized water.

H_2O_2: H_2O_2 solution was provided by diluting 30 % H_2O_2.

$ONOO^-$: At 0℃, H_2O_2 solution (0.7 M) was added to the aqueous $NaNO_2$ solution (0.6 M), followed by the addition of HCl solution (0.6 M), and NaOH solution (3 M) was added within 1-2 seconds. MnO_2 was added to remove excess H_2O_2, and stirring was continued for 15 minutes at 0℃. The mixture was then filtered with a 0.4-micron filter. The concentration of $ONOO^-$ was determined by UV analysis with the extinction coefficient at 302 nm (ε=1670 M-1 cm -1). Aliquots of the solution were stored at -20℃ for use.[3, 4]

Cytotoxicity Tests. The cytotoxic activity experiment of **DP-1**, against HepG2 cells and RAW264.7 cells were tested according to standard 3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-etrazolium, inner salt (MTS) assay procedures. HepG2 cells and RAW264.7 cells were seeded in 96-well assay plates at a density of 10^4 cells per well (100 μL total volume/well) for 24 h. The prepared **DP-1** (5 μM, 10 μM, 20 μM, 50 μM and 100 μM) were added in the serum-free medium

and incubated with the cells for 24 h after that. The optical absorbance of the cells was detected at 490 nm through a microplate reader (German Berthold Mithras 2LB943). The control experiment was finished by detecting the growth culture medium without **DP-1** (0 μM). The cell viability was estimated according to the following equation: cell viability (%) = mean of absorbance value of treatment group/mean absorbance value of control × 100%.

Cell Culture and Confocal Microscopy Imaging. To obtain the cell permeability of **DP-1**, HepG2 cells and RAW264.7 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) subjoined with 10% FBS (fetal bovine serum) in 1.0 mL of culture medium for different periods of time (0-24 h or 0-16 h) with various concentrations of FIA (0-0.25 %, v/v) or LPS (0-0.5 μg/mL). For inhibitor experiments, cells were treated according to the above method and then incubated with iodoacetamide (60 nM) in Petri dishes for 1h. Then, cells were incubated with **DP-1** (5 μM) for 20 min at 37 °C. Each experiment included at least three biological replicates, and at least 3 images from different randomly selected fields of view were captured from each experimental condition. Confocal fluorescence images of HepG2 cells and RAW264.7 cells were carried out on an Olympus FV1000-IX81 laser confocal microscope at an excitation wavelength of 488 nm and emission channels of 490-580 nm (green) and 580-700 nm (red).

Fluorescence Imaging in Inflammatory Model Mice. All mice related experiments were performed in accordance with the National Institutes of Health guidelines on the use of animals in research and were approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University. Five- to six-week-old female BALB/c nude mice with a body weight of approximately 16 g were purchased from Chongqing Tengxin Biotechnology Co., LtD. Mice were divided into two groups: one experimental group (mice were treated with 40 μg/kg LPS in 100 μL solution before 24 h and then co-incubated with **DP-1**) and a control group (treated with **DP-1**). The mice were anesthetized with isoflurane (RWD life Science, Shenzhen, China) and

were maintained in a stereotaxic frame (RWD Life Science, Shenzhen, China). All mice were tail vein injected with 100 μL **DP-1**, and then the fluorescence images of each mouse were measured (λ_{ex}: 500 nm; λ_{em}: 620 nm) at the different times within 120 min. After body fluorescence images acquiring, the major organs were collected and examined using an *in vivo* optical imaging system (IVIS Spectrum, PerkinElmer). In all animal experiments, signals were collected from a defined ROI and total flux intensities (photons/s) analyzed using Living Image Software 4.5.5.

Fluorescence Imaging in Tumor Mice Model. All mice related experiments were performed in accordance with the National Institutes of Health guidelines on the use of animals in research and were approved by the Laboratory Animal Welfare and Ethics Committee of the Army Medical University. Briefly, the HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine solution (FBS) and 1% streptomycin-penicillin using a 37 °C, 5% CO_2 humidified chamber. When the cells reached ~80% confluence, the cells were collected and approximate 10^7 cells in 50 μL PBS were mixed with 50 μL Matrigel (Corning, NY, USA), and the mixture was injected into the back of five- to six-week-old female nude mice. When the tumor volume >200 mm^3, about 50 μL the probe (finally is 50 μM) was intratumorally injected into the tumor and the fluorescence images were measured (λ_{ex}: 500 nm; λ_{em}: 620 nm) at the different times within 90 min using an *in vivo* optical imaging system (IVIS Spectrum, PerkinElmer). In all animal experiments, signals were collected from a defined ROI and total flux intensities (photons/s) analyzed using Living Image Software 4.5.5.

Table S1. Spectroscopic/photophysical data of **DP-1** in the absence and addition of PGP-1.

Compounds	λ_{abs} / nm	λ_{ex} / nm	λ_{em} / nm	Φ_f
DP-1	422	500	564	0.008 ±0.0005
DP-1+PGP-1	525	500	616	0.027 ±0.003

Table S2. Comparison of the proposed method with other methods for PGP-1 detection.

Linear range	Detection Limit	React time	ratiometric	Tumor model	Reference
0.05–1.75 μg/mL	5.6 ng/ mL	30 min	no	no	[5]
0.01–0.25 μg/mL	0.18 ng/ mL	26 min	no	no	[6]
0-0.28 μg/mL	**0. 25 ng/mL**	**10 min**	**yes**	**yes**	**This work**

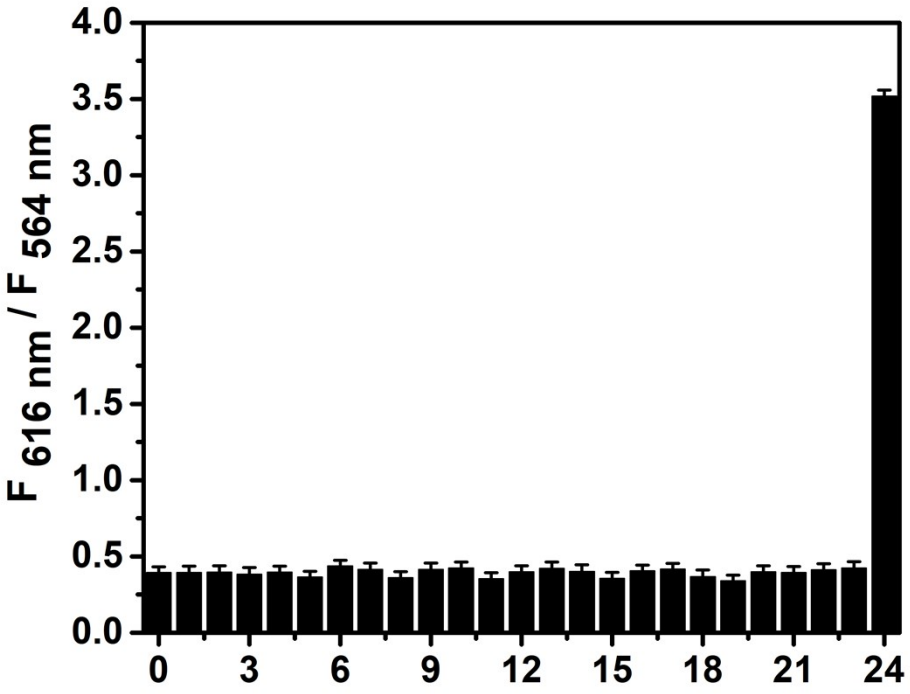
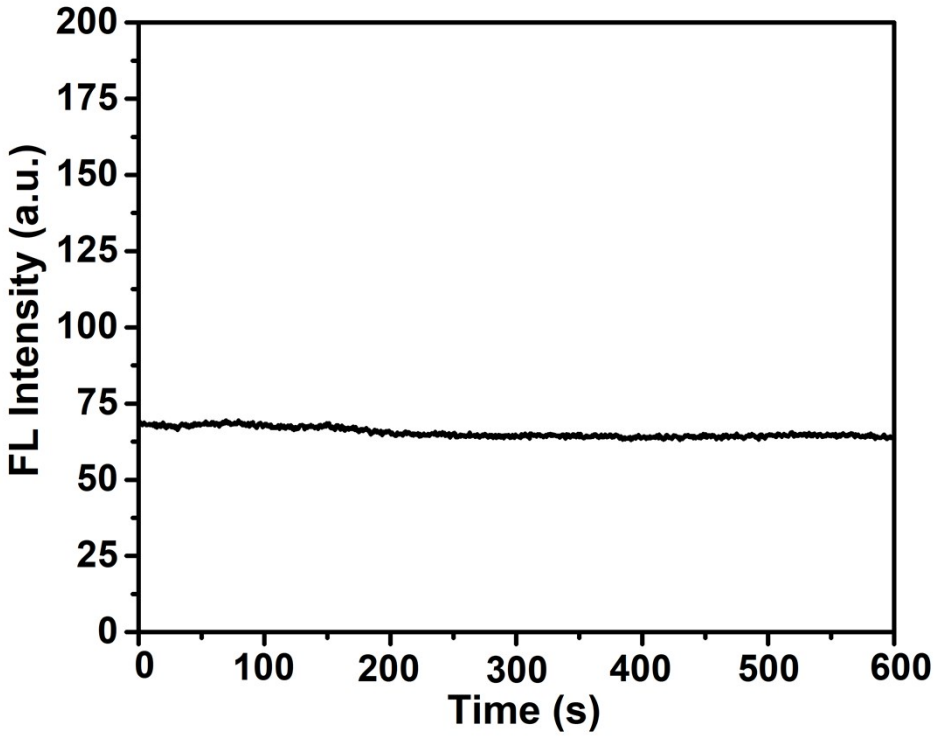


Figure. S1 The selectivity experiment of **DP-1** with analytes (0. Probe, 1. K^+, 2. Na^+, 3. Ca^{2+}, 4. Mg^{2+}, 5. S^{2-}, 6. SO_3^{2-}, 7. H_2O_2, 8. $ONOO^-$, 9. Glu, 10. Asp, 11. Gly, 12. Pro, 13. Ile, 14. Tyr, 15. Ser, 16. Lys, 17. Ala, 18. Arg, 19. Met, 20. Phe, 21. Cys, 22. Hcy, 23. GSH, 24. PGP-1), λ_{ex} = 500 nm.



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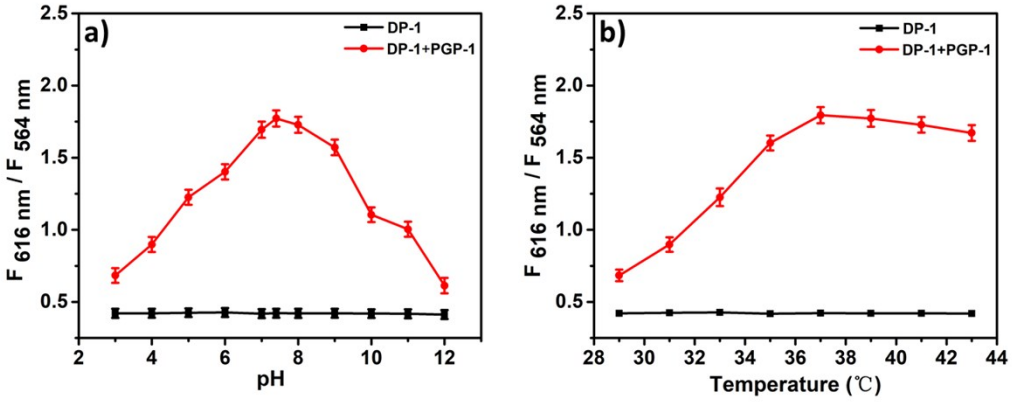
133 **Figure. S2** The light stability test of **DP-1** in 600 s, **DP-1** = 10 μM. λ_{ex} = 500 nm.



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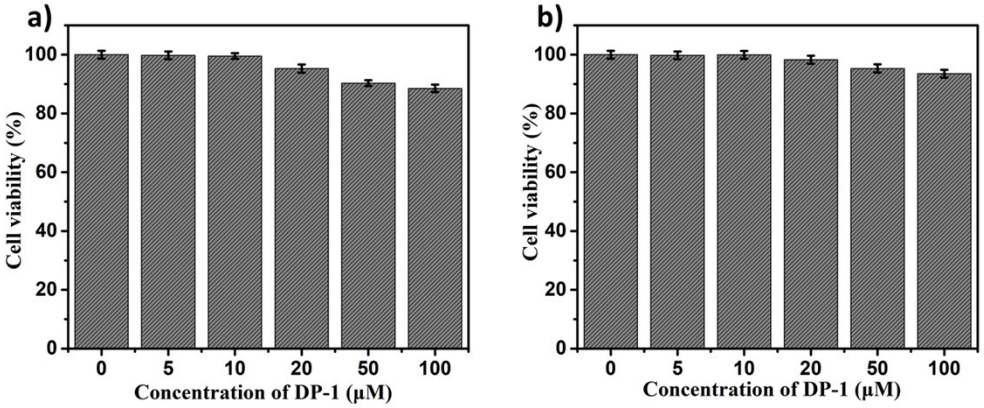
135 **Figure. S3** Effects of (a) pH and (b) reaction temperature on the fluorescence of **DP-1**

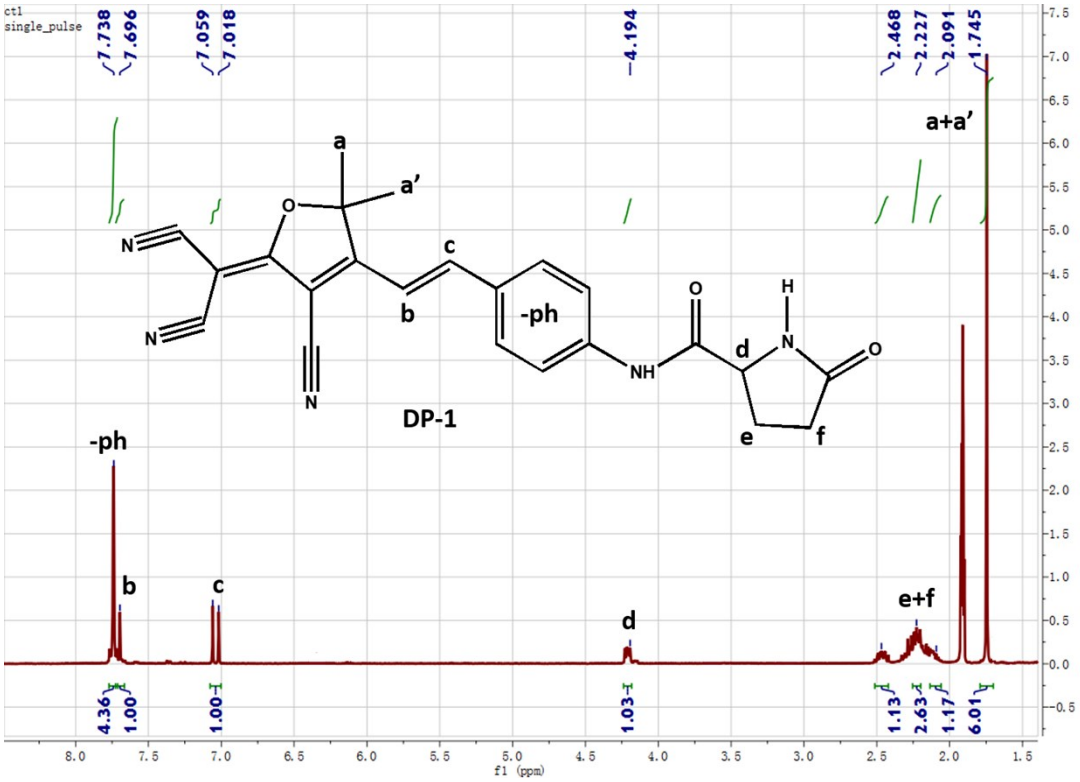
136 without and with PGP-1 (100 ng / mL), λ_{ex} = 500 nm.



137

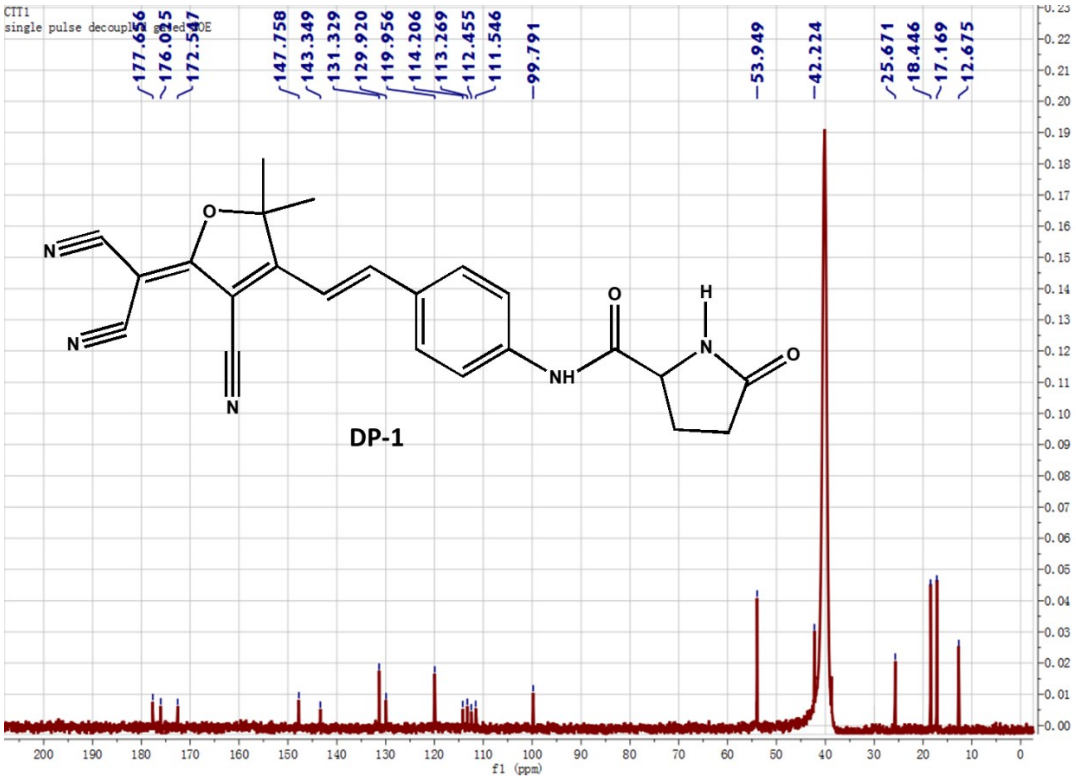
138 **Figure. S4** Effects of **DP-1** at varied concentrations on the viability of a) HepG2 and

139 b) RAW264.7 cells. The cell viability data were checked five times.



140

141 **Figure. S5** ^{1}H NMR (400 MHz) spectrum of **DP-1**.



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143 **Figure. S6** ^{13}C NMR (400/4 MHz) spectrum of **DP-1.**

Generic Display Report

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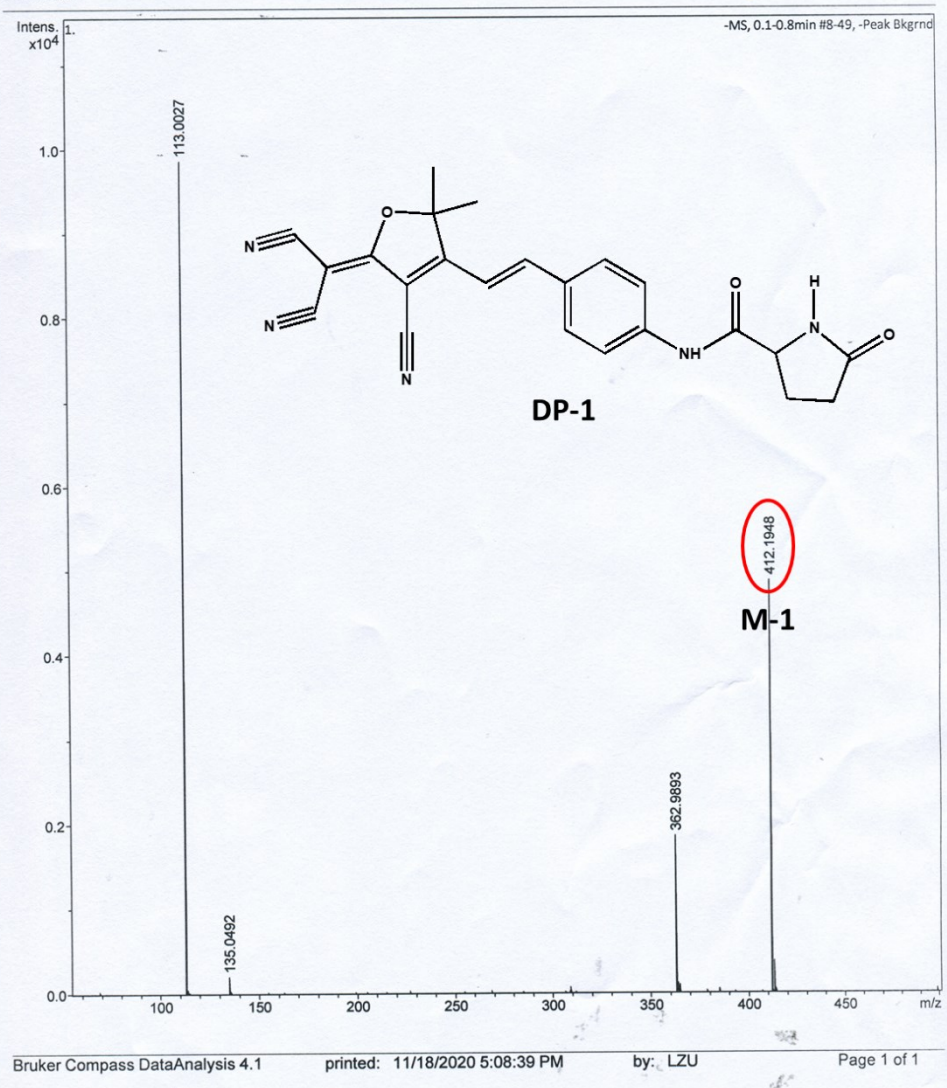

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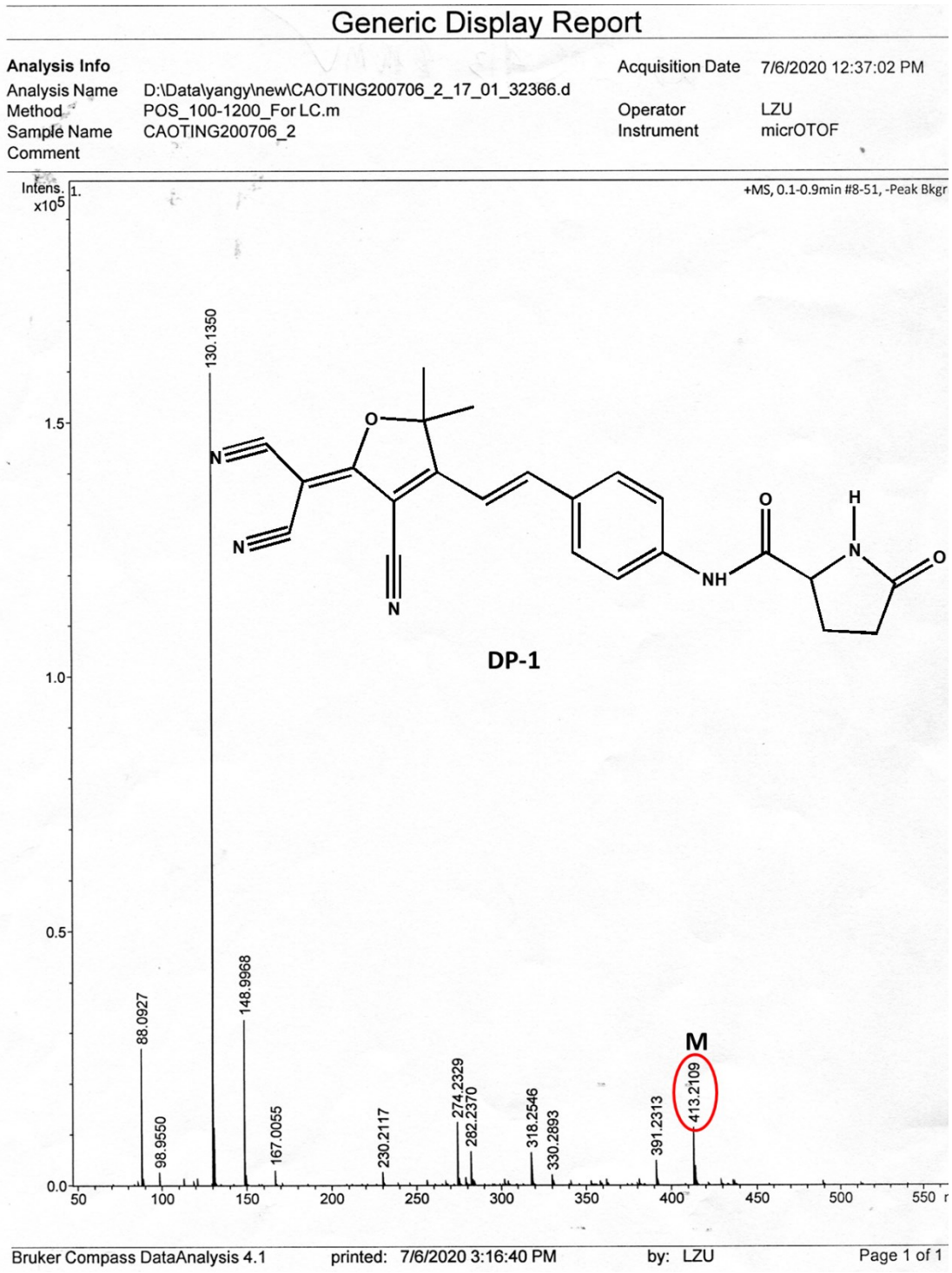

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146 **Figure. S7** MS Spectrum of **DP-1** ($C_{23}H_{18}N_5O_3$, calculated M, 413.14; found, 412.19

147 M-1 and 413,21 M).

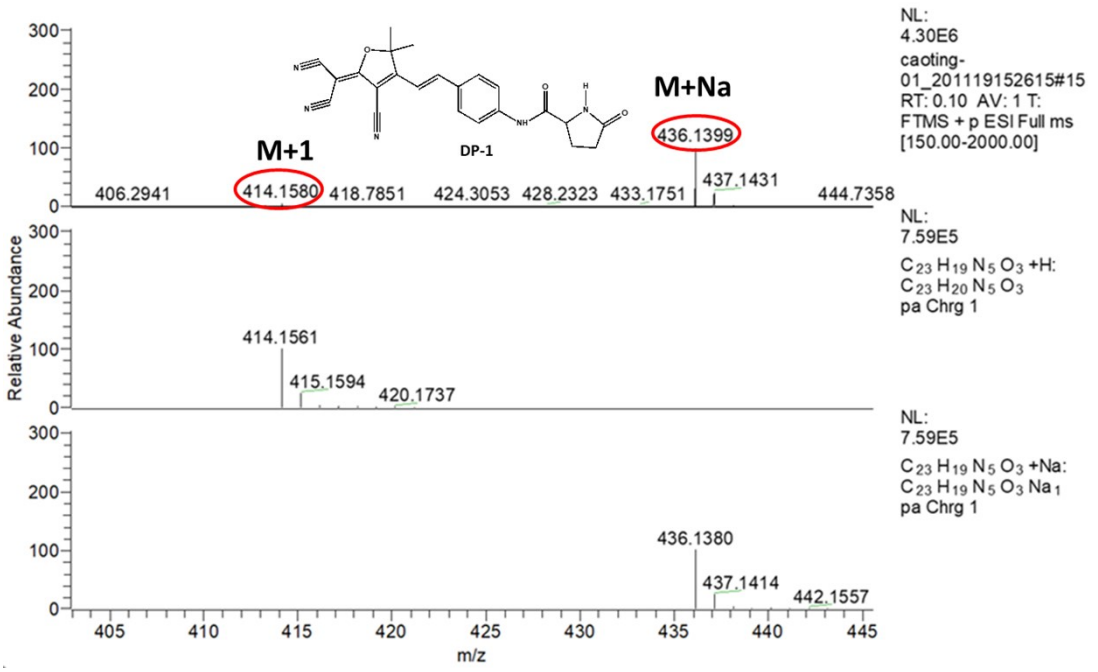

Figure. S8 HRMS Spectrum of **DP-1** ($C_{23}H_{18}N_5O_3$, calculated M, 413.1488; found 414.1580 M+1 and 436.1399 M+Na).

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