Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supporting Information

In situ visualization and detection of protein sulfenylation responses in living cells through a dimedone-based fluorescent probe

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1. Determination of the spectroscopic characteristics of CPD and CPDDM

All pH measurements were made with a Sartorius basic pH-Meter PB-20. Fluorescence spectra were determined using a Varian Cary Eclipse fluorescence spectrometer. Absorption spectra were determined by a Varian Cary 100 UV-vis spectrophotometer. The fluorescent quantum yield was calculated according to the formula $\Phi_1=\Phi_B*I_1*A_B*\lambda_{exB}*\eta_1/I_B*A1*\lambda_{ex1}*\eta_B$. Where Φ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; λex is the excitation wavelength; η is the refractive index of the solution; the subscripts 1 and B refer to the unknown and the standard, respectively. N-butyl-4-butylamino-1,8-naphthalimide was used as a standard (Φ_F = 0.81 in absolute ethanol).[1] The exictation wavelength was 415 nm for **CPD** and 460 nm for N-butyl-4-butylamino-1,8-naphthalimide, respectively.

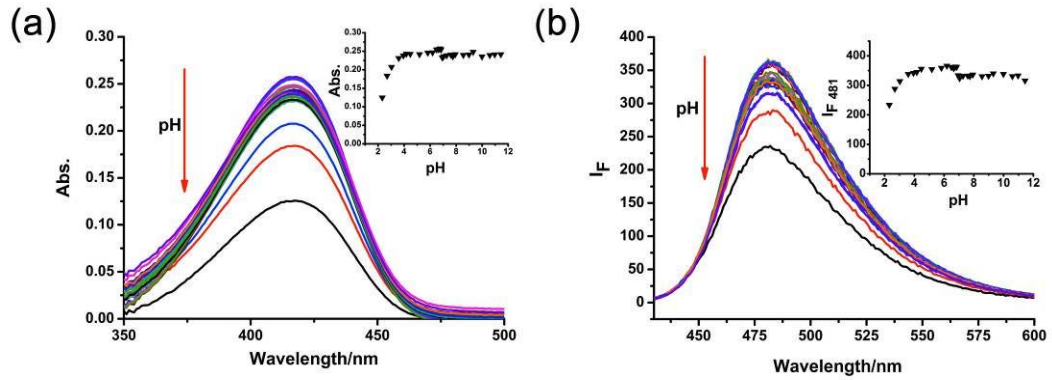


Figure S1: The pH job plot of CPD. Influences of pH on the absorbance (a) and fluorescence (b) of **CPD** (5 μM) in PBS buffer (with 1% DMSO). The inset: Absorption or fluorescence intensity as the function of pH.

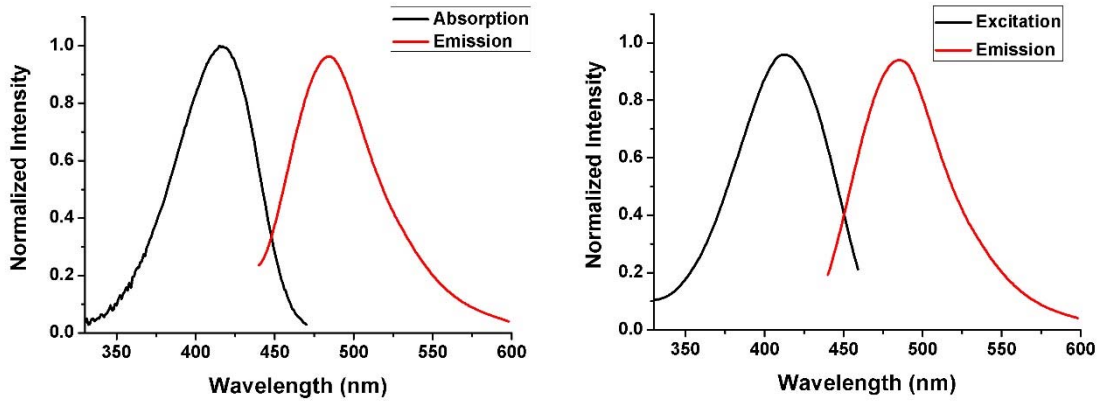


Figure S2: Normalized excitation and absorption spectra of **CPD** (5 μM) in PBS buffer (with 1% DMSO).

2. Determination whether Michael addition between CPD and sulfenic acid moiety in Trx-M affected fluorescence intensity of CPD.

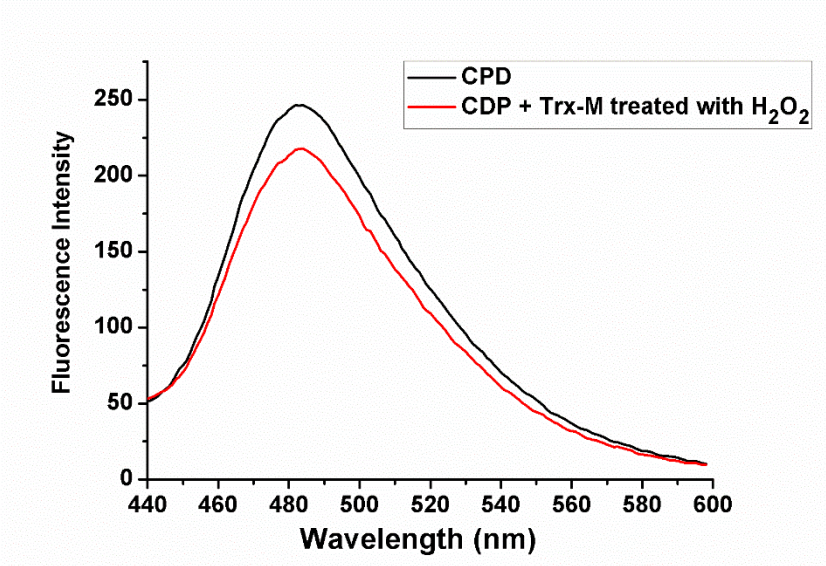


Figure S3: Fluorescence change of **CPD** before and after labeling of sufenylated protein. Trx-M was taken as the sufenylated protein model. Hydrogen peroxide (H_2O_2) was used as oxidant.

3. Determination whether fluorophore and piperazine alkyl ether linker unit of CPD induced a significant bias in probe localization and protein labeling.

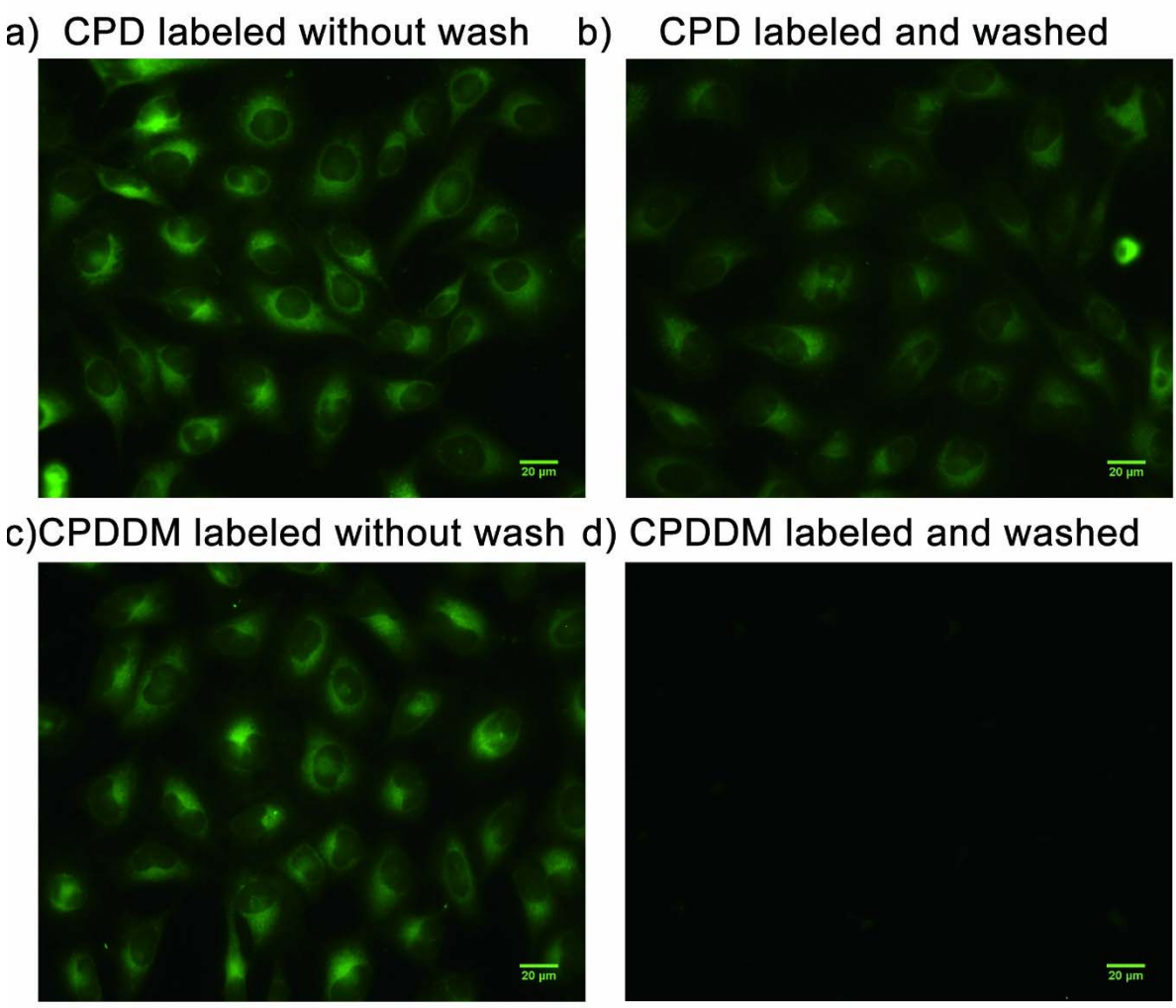


Figure S4 a-d: Fluorescent images of cells labeled with **CPD** (5 μM, a, b) **CPDDM** (5 μM, c, d). For a and c, the labeling solution was replaced by PBS before imaging without further washing. For b and d, the cells were washed six times using PBS after labeling as the standard labeling process.

4. Cellular responses of CPD labeled sulfenylated protein in HL60 cells to redox regulation.

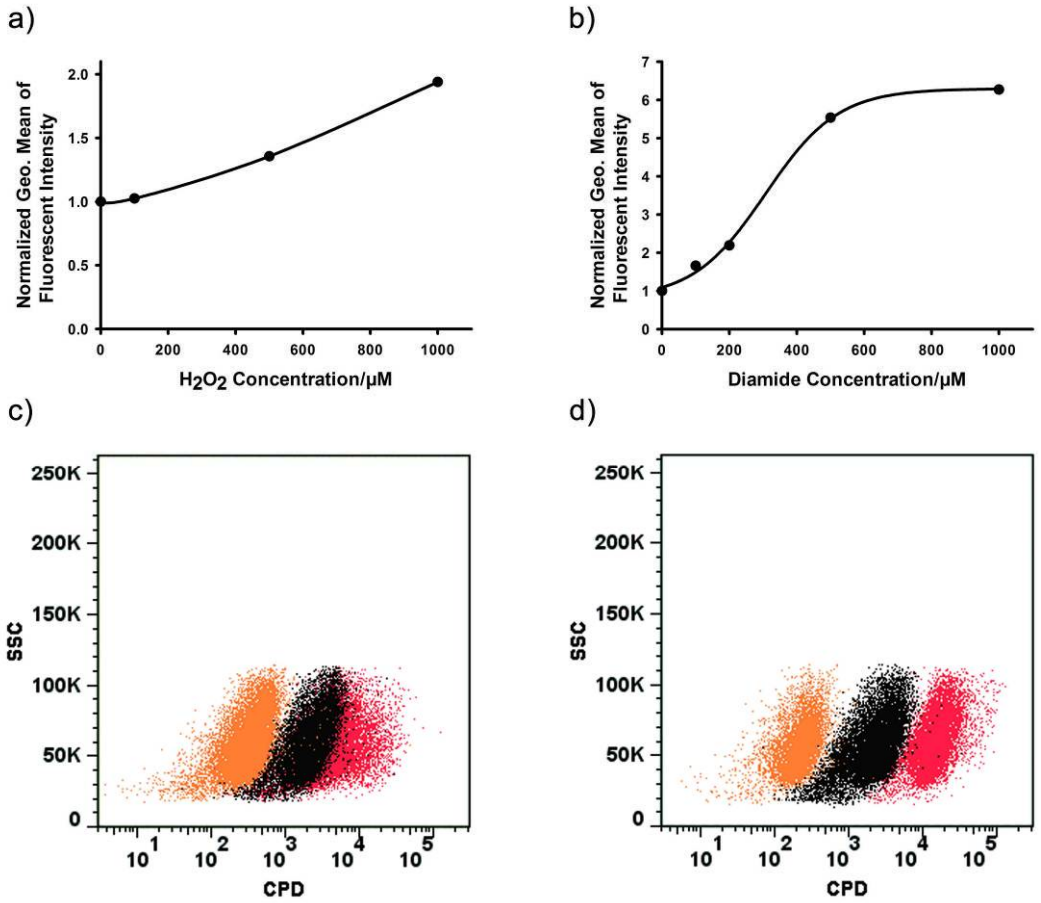


Figure S5 Cellular responses of CPD labeled sulfenylated protein in HL60 cells to redox regulation. a. Effect of different concentration of H_2O_2 (Co-incubation). b. Effect of different concentration of diamide (Co-incubation). c. Dotplot of HL 60 cells with (Red) or without (Black) 1 mM H_2O_2 co-incubation. The Dotplot of un-labeled cells were show in orange. d. Dotplot of HL 60 cells with (Red) or without (Black) 1 mM Diamide co-incubation. The Dotplot of un-labeled cells were show in orange.

5. Determination of LogP value of CPD and CPDDM

The partition coefficient (P) was determined by the shake-flask method (following OCED guidelines) according to references.[2] In details, stock solutions of CPD (or CPDDM) were prepared in phosphate buffer (pH 7.2, 1% DMSO as the co-solvent). Then the partitions with n-octanol were performed using different volumes of the two phases in the ratios of 1/1, 1/2 and 2/1 (phosphate buffer/ n-octanol) in test vials. And the partition coefficient was determined using two different stock concentrations of CPD or CPDDM (5 μM and 100μM respectively). Get the logP value of CPD and CPDDM, as shown in Table 1.

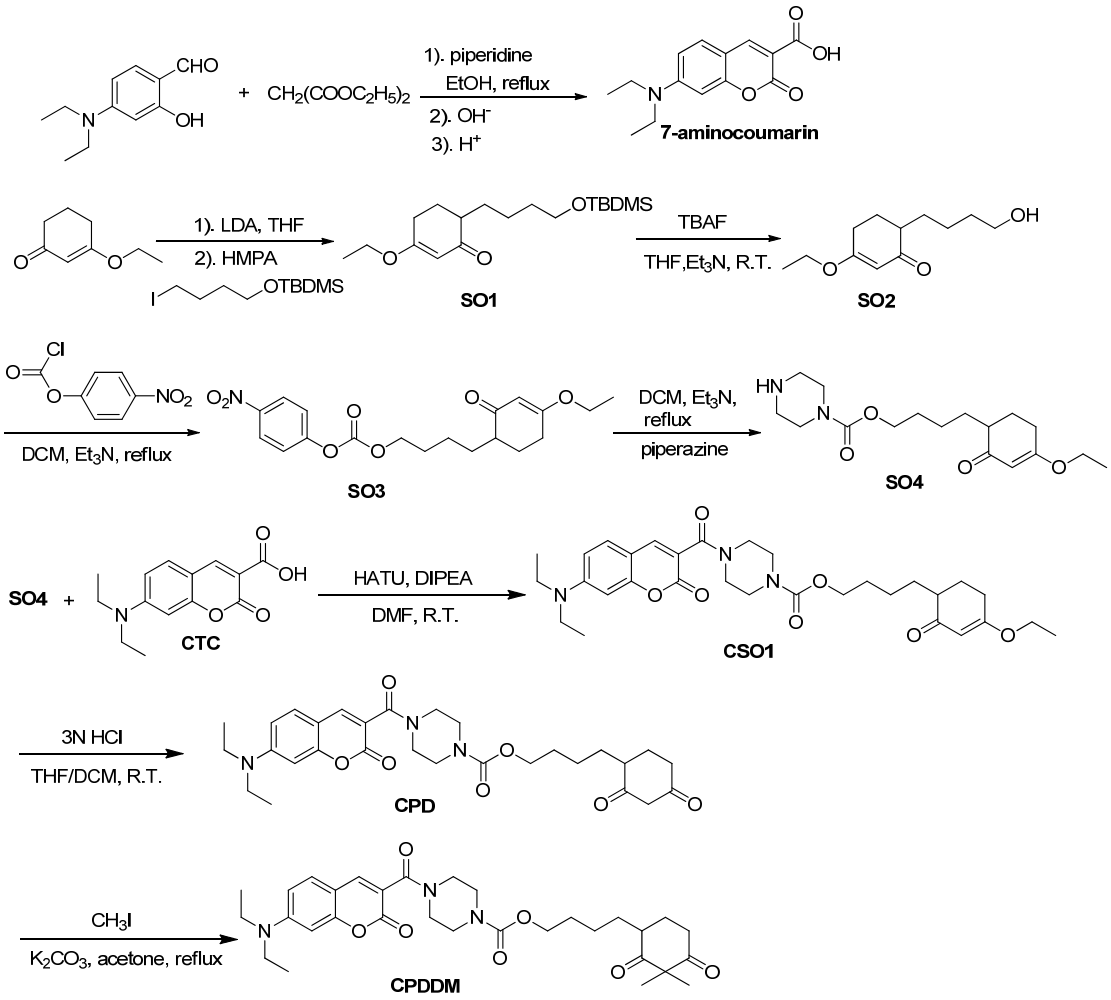
6. Experiment Methods

6.1 Synthetic scheme and detailed protocol for preparation of CPD and CPDDM

Material and methods

All chemical reagents and solvents were purchased from Sigma-Aldrich and used without further purification except for tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) which were dried according to the handbook.[3] Thin-layer chromatography (TLC) was performed on silica gel plates. Column chromatography was performed using silica gel (Hailang, Qingdao) 200-300 mesh. ^{1}H and ^{13}C NMR spectra were recorded employing a Bruker AV-400 spectrometer with chemical shifts expressed in parts per million (in deuteriochloroform, Me_4Si as internal standard). Electrospray ionization (ESI) mass spectrometry was performed in a HP 1100 LC-MS spectrometer. Melting points were determined by an X-6 micro-melting point apparatus and were uncorrected. IR spectra were recorded on a Nicolet Nexus 770 spectrometer.

General procedures for the synthesis of compounds and their characterization



Scheme S1. Synthesis of **CPD** and **CPDDM**

6-(4-((tert-butyldimethylsilyl)oxy)butyl)-3-ethoxycyclohex-2-enone (SO1)

SO1 was synthesized according to the published procedures.[4]

To a lithium diisopropylamide (LDA) solution [prepared from diisopropylamine and *n*BuLi in dry tetrahydrofuran (THF) at 0 °C] at -78 °C was added 3-ethoxy-2-cyclo-hexen-1-one (5.28 mL, 36 mmol) in dry THF (12 mL), dropwise, over 40 min. After stirring for an additional 30 min at -78 °C, hexamethyl phosphoramide (HMPA, 6.32 mL, 36 mmol) was added followed by the dropwise addition of tert-butyl (4-iodobutoxy)dimethylsilane (11.32 g, 36 mmol) in dry THF (16 mL). The resultant mixture was allowed to warm to room temperature, stirred for 6 h, and then quenched by the addition of water (20 mL). The reaction mixture was then partitioned between dichloromethane (DCM, 200 mL) and sat. NH_4Cl (80 mL). The aqueous phase was extracted with DCM (3 × 100 mL), and the organic phases were combined and washed with brine (100 mL), dried over anhydrous $MgSO_4$, and reduced to dryness. The resultant syrup was purified by chromatography on silica gel (petroleum ether: EtOAc, 4:1, v/v), afforded the target compound **SO1** as pale yellow oil (8.92 g, 76% yield). ^1H

NMR ($CDCl_3$, 400 MHz) δ 5.26 (s, 1H), 3.84 (q, 2H, J = 6.8 Hz), 3.57 (t, 2H, J = 6.4 Hz), 2.37 (t, 2H, J = 5.6 Hz), 2.16-2.10 (m, 1H), 2.07-2.00 (m, 1H), 1.84-1.80 (m, 1H), 1.70-1.65 (m, 1H), 1.52-1.29 (m, 8H), 0.84 (s, 9H), 0.00 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.5, 176.6, 102.1, 64.0, 62.9, 45.0, 32.8, 29.1, 27.8, 26.0, 25.9, 23.2, 18.2, 14.1, -5.3; GC-MS 326.

3-ethoxy-6-(4-hydroxybutyl)cyclohex-2-enone (SO2)

SO2 was synthesized according to the same published procedures.[4]

To a solution of **SO1** (0.75 g, 2.30 mmol) in dry THF (20 mL) was added TBAF (5.97 mL of a 1.0 M solution in THF, 5.97 mmol) and Et_3N (0.82 mL, 5.97 mmol). After stirring at room temperature for 2 h, the reaction was quenched by the addition of water (7 mL) and sat. NH_4Cl (7 mL). The mixture was extracted with DCM (3 × 30 mL), and the combined organic phases were dried over anhydrous $MgSO_4$ and reduced to dryness. The resultant syrup was purified by chromatography on silica gel with gradient elution (petroleum ether: EtOAc, 3:1 to 1:1, v/v) to yield **SO2** as pale yellow oil (0.478 g, 98% yield). ^1H NMR ($CDCl_3$, 400 MHz) δ 5.33 (s, 1H), 3.91 (q, 2H, J = 7.2 Hz), 3.69 (t, 2H, J = 6.4 Hz), 2.45, 2.43 (2d, 2H, J = 6.8 Hz), 2.25-2.19 (m, 1H), 2.13-2.06 (m, 1H), 1.92-1.71 (m, 2H), 1.65-1.42 (m, 5H), 1.38 (t, 3H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.8, 176.9, 101.8, 64.0, 61.9, 52.2, 44.9, 32.5, 29.0, 27.8, 25.9, 23.1, 13.9; MS (ESI+) m/z 213 [$(M+H)^+$, 100%].

4-(4-ethoxy-2-oxocyclohex-3-en-1-yl)butyl (4-nitrophenyl) carbonate (SO3)[2]

To a solution of **SO2** (272 mg, 1.26 mmol) in anhydrous DCM (10 mL, which was dried with CaH_2) was added Et_3N (0.20 mL, 1.39 mmol) and p-nitrophenyl chloroformate (280 mg, 1.39 mmol). The mixture was stirred at reflux for 4 h, then at room temperature for 72 h, before being washed with water (10 mL) and brine (10 mL). The organic phase was dried, reduced to dryness, and purified by column chromatography with gradient elution (petroleum ether : EtOAc, 6:4 to 1:1) to yield the pure product as a white solid (347 mg, 73%). Melting point (mp) 110-113 °C. ^1H NMR ($CDCl_3$, 400 MHz) δ 8.29 (dt, 2H, J = 7.2, 2.0 Hz), 8.18 (dt, 2H, J = 7.2, 2.0 Hz), 5.36 (s, 1H), 4.32 (t, 2H, J = 6.4 Hz), 3.92 (q, 2H, J = 7.2 Hz), 2.48, 2.46 (2d, 2H, J = 7.2 Hz), 2.30-2.23 (m, 1H), 2.15-2.07 (m, 1H), 1.98-1.74 (m, 4H), 1.59-1.44 (m, 3H), 1.38 (t, 3H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100MHz) δ 177.8, 161.7, 155.5, 152.5, 145.3, 125.3, 121.8, 102.0, 69.3, 64.5, 45.5, 29.1, 28.6, 28.1, 26.2, 23.2, 14.1; IR (KBr, cm^{-1}): 3415, 2958, 2852, 2364, 2317, 1766, 1654, 1610, 1519, 1336, 1220, 1183, 1117, 1020, 868; MS (ESI+) m/z 378 [$(M+H)^+$, 100%].

4-(4-ethoxy-2-oxocyclohex-3-en-1-yl)butyl piperazine-1-carboxylate (SO4)

To a solution of **SO3** (177mg, 0.49 mmol) in anhydrous DCM (5 mL) was added Et_3N (0.14 mL, 1 mmol) and anhydrous piperazine (253 mg, 2.94 mmol). The mixture was stirred at room temperature overnight. The reaction solution was reduced to dryness and purified by column chromatography on silica gel with gradient elution (DCM: MeOH, 50:1 to 30:1) to yield **SO4** as white solid (61 mg, 38%). Melting point (mp) 120-125 °C. ^1H NMR ($CDCl_3$, 400 MHz) δ 5.30 (s, 1H), 4.08 (t, 2H, J = 6.4 Hz), 3.88

(q, 2H, *J* = 6.8 Hz), 3.43 (t, 4H, *J* = 4.4 Hz), 2.81 (br, 4H), 2.41 (t, 2H, *J* = 6.4 Hz), 2.18-2.04 (m, 2H), 1.90-1.60 (m, 4H), 1.46-1.33 (m, 6H); ^{13}C NMR (CDCl3, 100 MHz) δ 201.3, 176.6, 155.6, 102.1, 65.3, 64.1, 45.8, 45.0, 29.2, 29.1, 27.9, 26.2, 23.6, 14.1; IR (KBr, cm^{-1}): 3468, 2964, 2849, 1694, 1600, 1550, 1419, 1382, 1234, 1194, 1138, 1022; MS (ESI+) m/z 325 [(M+H)$^+$, 100%].

7-diethylamino-2-oxo-2H-chromen-3-carboxylic acid (7-aminocoumarin)

The 7-diethylamino-2-oxo-2H-chromen-3-carboxylic acid was synthesized according to the published procedures.[5]

To a solution of 4-diethylaminosalicylaldehyde (3.86 g, 0.02 mol) in absolute ethanol (60 mL) were added diethylmalonate (6.4 g, 0.04 mol) and piperidine (2 mL). The mixture was stirred for reflux for 6 h, then 10% NaOH (60 mL) solution was added and the mixture was continued to reflux for 15 min. The reaction was cooled to room temperature and acidification to pH 2 using concentrated hydrochloric acid under ice bath gave a crystalline deposit which was filtered, washed with water, dried, then recrystallized with ethanol to give 7-aminocoumarine as yellow crystalline powder (5.22 g, 89%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.36 (s, 1H), 8.65 (s, 1H), 7.46 (d, 1H, *J* = 9.2 Hz), 6.73 (d, 1H, *J* = 8.4 Hz), 6.54 (s, 1H), 3.51 (q, 4H, *J* = 6.8 Hz), 1.28 (t, 6H, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.5, 164.4, 158.0, 153.7, 150.2, 131.9, 110.9, 108.5, 105.5, 96.8, 45.3, 12.3.

4-(4-ethoxy-2-oxocyclohex-3-en-1-yl)butyl 4-(7-(diethylamino)-2-oxo-2H-chromene 3-carbo-nyl)piperazine-1-carboxylate (CSO1)

To a solution of 7-aminocoumarine (84 mg, 0.32 mmol) in anhydrous DMF (5 mL) were added **SO4** (104 mg, 0.32 mmol), HATU (121 mg, 0.32 mmol) and N,N-Diisopropylethylamine (DIPEA, 121 mg, 0.32 mmol). The mixture was stirred at room temperature under argon atmosphere overnight. The reaction solution was reduced to dryness and purified by column chromatography on silica gel (with DCM : MeOH, 200:1) to yield **SO4** as pale yellow solid (122 mg, 67%). Melting point (mp) 136-140 ^{o}C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.82 (s, 1H), 7.27 (d, 1H, *J* = 8.8 Hz), 6.56 (dd, 1H, *J* = 8.8, 2.4 Hz), 6.41 (d, 1H, *J* = 2.0 Hz), 5.24 (s, 1H), 4.09-4.03 (m, 2H), 3.86-3.81 (m, 2H), 3.67 (br, 2H), 3.51 (br, 4H), 3.39 (q, 6H, *J* = 7.2 Hz), 2.38 (t, 2H, *J* = 6.0 Hz), 2.15-2.01 (m, 2H), 1.87-1.60 (m, 4H), 1.38-1.29 (m, 6H), 1.71 (t, 6H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.4, 176.8, 171.0, 165.2, 159.1, 157.2, 155.3, 151.7, 145.3, 129.9, 115.5, 109.4, 107.6, 102.0, 96.8, 65.5, 64.1, 60.3, 44.9, 44.9, 29.1, 29.0, 27.9, 26.2, 23.5, 14.1, 12.3; IR (KBr, cm^{-1}): 3462, 2924, 2864, 1707, 1610, 1522, 1457, 1413, 1353, 1234, 1194, 1131, 1075, 1000; MS (ESI+) m/z 568 [(M+H)$^+$, 100%].

6.2 Detailed protocol for in vitro and in living cells assay

Materials and Reagents.

Dithiothreitol (DTT) (Cat. D0632), DMSO (Cat. D2650), Diamide (Cat. D3648) were purchased from Sigma. Mito-tracker Deep Red (Cat. M22426), and H_2DCFDA (Cat. D339) were from Invitrogen. Dulbecco's Phosphate Buffered Saline (DPBS) powder (Cat. SH30013.04), DMEM (High glucose) medium (Cat. SH30243.01B) RPMI 1640

medium (Cat. SH30809.01B) were from Hyclone. Foetal Bovine Serum (FBS) (Cat. SFBS) was from Bovogen. pDsRed2-ER Vector (Cat. 632409) was from Clontech. Other materials were of local biological and analytical grade.

Expression and Purification of Thioredoxin 2 Mutant (Trx). Human thioredoxin 2 (Signal peptide removed) gene with a N-terminal hexahistidine tag was constructed on pET-28a vector (Novogen). To obtain a single thioredoxin mutant, the Cys_{69} was mutated to Glycine on pET-28a vector. Thioredoxin 2 C69G mutation (Trx-M) protein was expressed in Escherichia coli strain BL21 (DE 3). After 4 hours of induction with 0.1 mM IPTG, the E. coli cells were harvested by centrifugation. Lysis was obtained by ultrasound, and then centrifuged at 10,000 ×g for 30 min at 4 ºC. Trx-M was purified from the supernatant using affinity chromatography on Histrap HP column (GE Healthcare) according to the manufacturer's instructions. And the buffer for storage was inter-changed to DPBS using Amicon Ultrafiltration device from Millipore. The purity of the protein was verified by 15% SDS-PAGE to reach 95%. And the purified protein was stored at -80 ºC until further use.

Preparation of reduced forms of Trx-M.
For the single thiol model of Trx-M, 10 mM DTT was used to reduce the cysteine in the protein. After reduction, excess reductant was removed by gel filtration on Sephadex G-25 against PBSE buffer (PBS buffer prepared by DPBS powder, 5 mM EDTA, pH 7.4).

The status of the thiols in model proteins was verified using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), after incubating proteins with an excess of DTNB in PBSE buffer for 30 min in the dark at room temperature. An absorption coefficient at 412 nm of 14.12 $mM^{-1}•cm^{-1}$ was used to quantify the 5-thio-3-nitrobenzoate anion, with the absorbance of the DTNB solution and the intrinsic low absorbance of proteins at this wavelength accounted for. The molar ratio of thiol quantified by DTNB and protein quantified by absorbance at 280 nm was about 0.9-1.1.

SDS-PAGE and fluorescence imaging of gels.
The selectivity of CPD was verified by 15% SDS-PAGE electrophoresis. Samples were labeled in the PBS buffer at 37 ºC for 30 min, with a final concentration of protein at 50 μM, CPD at 100 μM and different concentration of chemicals. Samples were firstly oxidized by aimed amout of oxidant at 37ºC for 30 min, then 100x concentrated chemical and CPD solution was added into the labeling system. After labeling, the samples were desalted using Bio-rad Micro Bio-Spin Columns with Bio-Gel P-6 in SSC buffer (Cat. No. 732-6201). Then, the desalted samples were mixed with SDS-PAGE loading buffer without β-mercaptoethanol. Protein concentrations were quantified using Bradford method. Different volume of samples were loaded to make sure the same quantity proteins were loaded. And then, the electrophoresis was started immediately. The gel was imaged by Carestream In Vivo Imaging FX System (Excitation: 420 nm, Emission: 480 nm). The same gel was also stained by Coomassie brilliant blue (CBB) after the fluorescent image was obtained.

Cell Culture.

Chang liver and HL60 cells were obtained from American Type Culture collection. Changliver / HL60 cells were grown in DMEM (High glucose) medium / RPMI 1640 medium supplemented with 10% FBS. Changliver cells were typically passaged with sub-cultivation ratio of 1:4 every two days. And HL 60 cells were typically passaged with sub-cultivation ratio of 1:10 every two days. Cells were incubated in a 5% CO_2 humidified incubator at 37 ^oC.

Detection of Changliver cellular protein sulfenylation response to different chemicals.

Cells were seeded to GE 96 well glass matriplate (Cat. 28-9323-99) the day before detection with about 80% intensity. During detection, the cells were washed twice using DPBS (Containing Ca, Mg) buffer 100 µl per well each time. Then, the cells were co-incubated with 5 µM CPD and different concentration of chemicals, (100µl per well in all) in DPBS (containing 1% DMSO) for 30 min in a 5% CO_2 humidified incubator at 37 ^oC. After that, the cells washed for six times using PBS (Containing Ca, Mg) buffer 100 µl per well each time. Then 100 µl PBS for each well was added into each well for detection. And then, the fluorescent intensity was quantified by Biotek Synergy 2 microplate reader with the 420 nm excitation filter (bandpass 10 nm) and 485 nm emission filter (bandpass 20 nm).

***In situ* Imaging.**

Chang liver cells were labeled with 5 µM CPD in DPBS (containing 1% DMSO) at 37^oC. For H_2O_2, DTT, or dimedone co-incubated sample, 1 mM H_2O_2, 2 mM DTT or 1 mM dimedone was introduced into the labeling system. For CPDDM sample, same concentration of CPDDM was used instead of CPD. After 30 min, the cells were washed for six times to remove unbounded CPD before *in situ* imaging with a Nikon A1R confocal laser scanning microscope using Plan Apochromat violet corrected (VC) 60X WI (N.A. 1.20; W.D. 0.27) objective, with excitation by 405 nm laser, and 425-475 nm emission light was collected.

Colocalization.

For CPD co-localization with Mito-tracker sample: Cells were pre-washed twice, and labeled by 50 nM Mito-tracker Deep Red for 15 min at first, and then 5 µM CPD as described above.

For CPD co-localization with ER-tracker sample: The pDsRed2-ER Vector was transfected into Chang liver cells using Calcium Phosphate Cell Transfection Kit (Beyotime, Cat. C0508) the day before detection. And then, the cells were labeled by 5 µM CPD as described above.

To exclude the interference to the fluorescent signal: The excitation of CPD is 405 nm, and 425-475 nm emission light was collected. The excitation of DsRed-2 is 561 nm, and 570-620 nm emission light was collected. The excitation of Mito-Tracker Deep red is 638 nm, and > 650 nm emission light was collected.

Detection of HL60 cellular protein sulfenylation response to different chemicals.
$2.5x10^7$ HL60 cells were centrifuged at 800 rpm for 5 min, and washed twice using PBS buffer. Then the cells were resuspended by 1 ml DPBS buffer containing 5 μM CPD and different concentration of chemicals. After the 30 min incubation in a rotary shaker in the 5% CO_2 humidified incubator, the cells were washed by 1 ml DPBS per time for six times. Then the cells were resuspended in 500μl DPBS buffer for Aria I Flow Cytometry detection. The fluorescent signal excited by 407 nm laser and 430-470 nm emission light was collected. The flow cytometry data was processed by BD FACSDiva and FlowJo software.

Cell Viability Test
Chang liver cells were seeded to Coring 96 well plate the day before detection with about 80% intensity. During detection, cells were treated with different concentration of dimedone or CPD as described above, and then the cell viability was tested using Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit.

H_2DCFDA Test
Cells were seeded to GE 96 well glass matriplate (Cat. 28-9323-99) the day before detection with about 80% intensity. During detection, cells were labeled with different concentration of dimedone for 30 min as described before. Then 100 μL of 5 μM H_2DCFDA/PBS solution was added to each well. After 30 min incubation and twice washes, the fluorescent intensity was measured by Biotek Synergy 2 Microplate Reader (Ex 485 nm, Em 528 nm (bandpass 20 nm)).

References

1 Z. Xu, X. Qian, J. Cui, *Org. Lett.* **2005**, 7, 3029-3032.
2 L. B. Poole, C. Klomsiri, S. A. Knaggs, C. M. Furdui, K. J. Nelson, M. J. Thomas, J. S. Fetrow, L. W. Daniel, S. B. King, *Bioconjug. Chem.* **2007**, 18, 2004-2017; A. Berthod, S. Carda-Broch, *J. Chromatogr. A* **2004**, 1037, 3-14.
3 W. L. F. Armarego, D. D. Perrin, *Purification of Laboratory Chemicals (4th Edition)*, Elsevier, **1997**.
4 L. B. Poole, B. B. Zeng, S. A. Knaggs, M. Yakubu, S. B. King, *Bioconjug. Chem.* **2005**, 16, 1624-1628.
5 G. He, D. Guo, C. He, X. Zhang, Zhao, X., C. Duan, *Angew Chem Int Ed* **2009**, 48, 6132-6135.

7. Amino Acid sequence of recombination Trx-M (human Thioredoxin-2 C69G mutation).

M G S S H H H H H H S S G L V P R G S H M A S M T G G Q Q M G R G S E F R M T T F N I Q D G P D F Q D R V V N S E T P V V V D F H A Q W G G P C K I L G P R L E K M V A K Q H G K V V M A K V D I D D H T D L A I E Y E V S A V P T V L A M K N G D V V D K F V G I K D E D Q L E A F L K K L I G

8. NMR spectra of CPD and CPDDM.

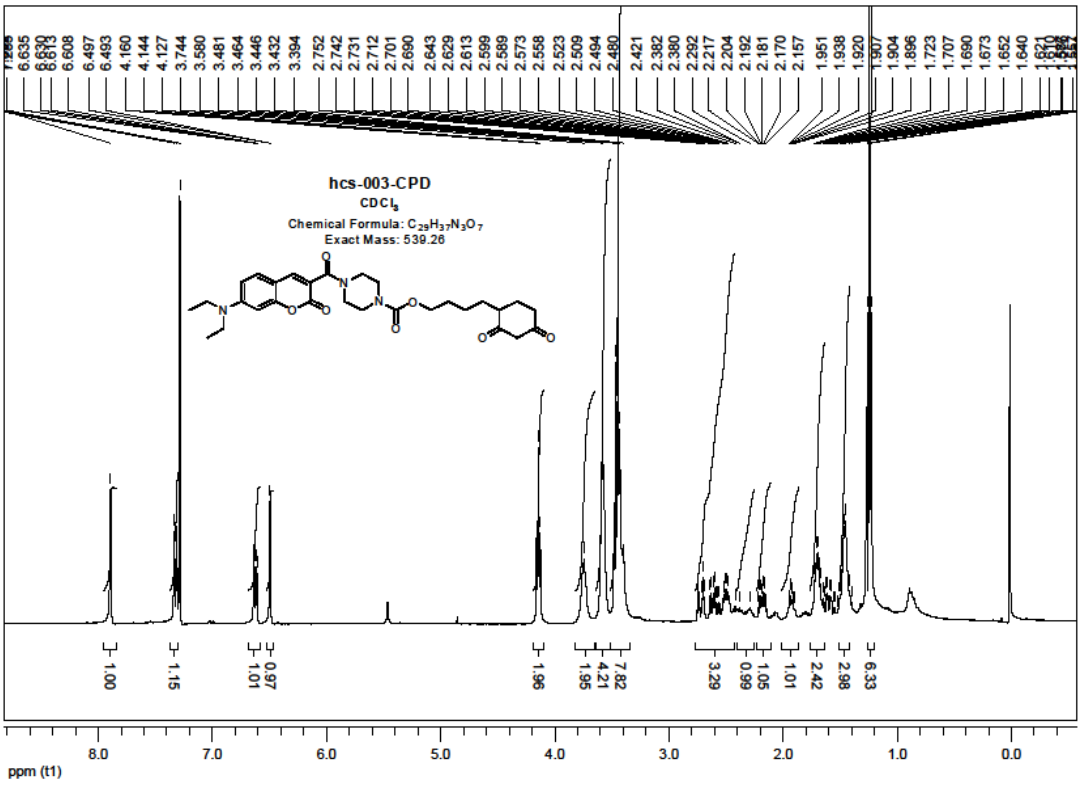


Figure S6. ^{1}H NMR spectra of CPD

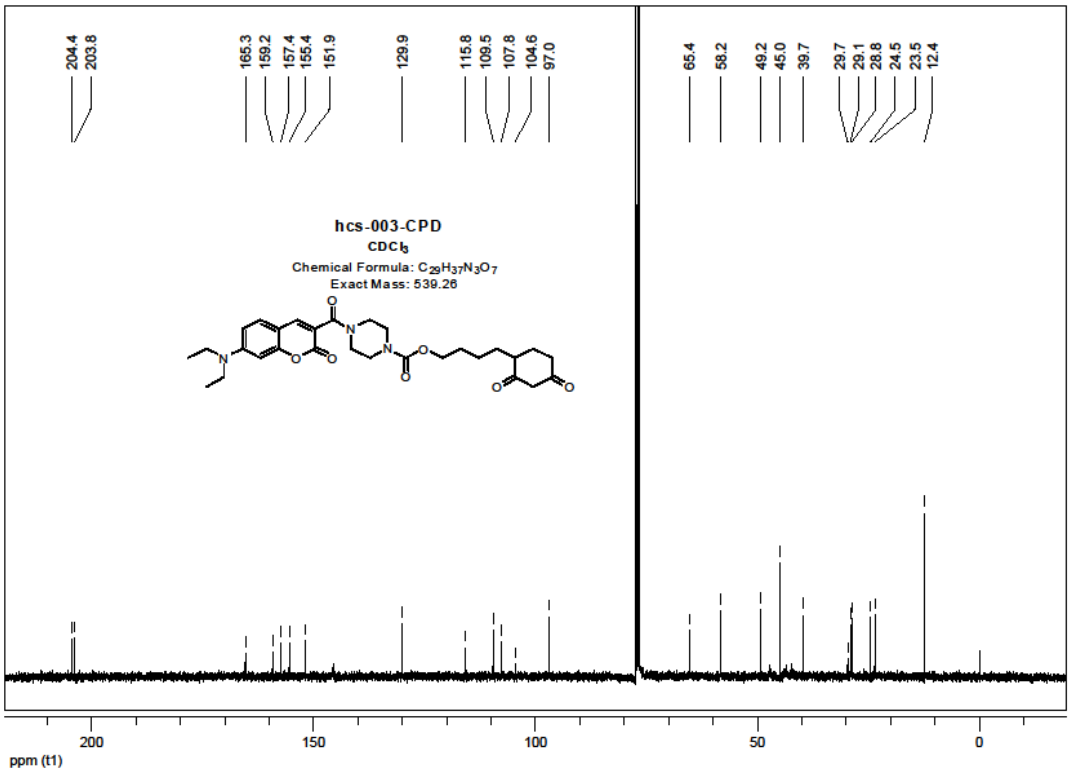


Figure S7. ^{13}C NMR spectra of CPD

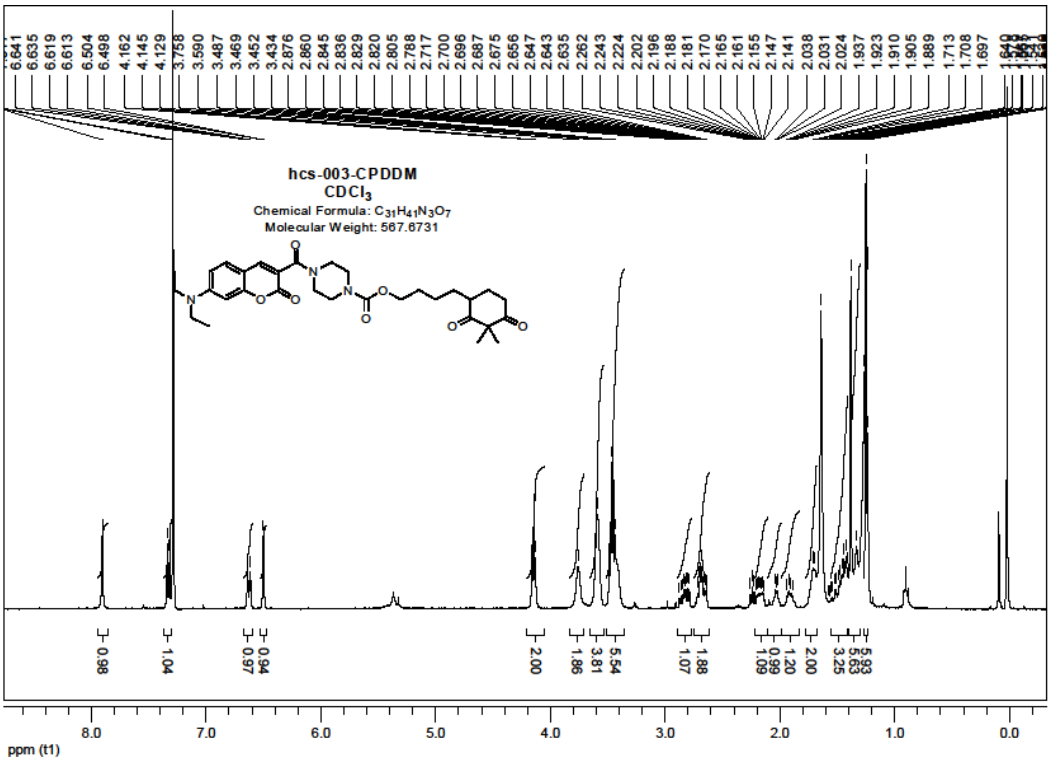


Figure S8. ^{1}H NMR spectra of CPDDM

9. ESI-Mass spectra of CPD and CPDDM.

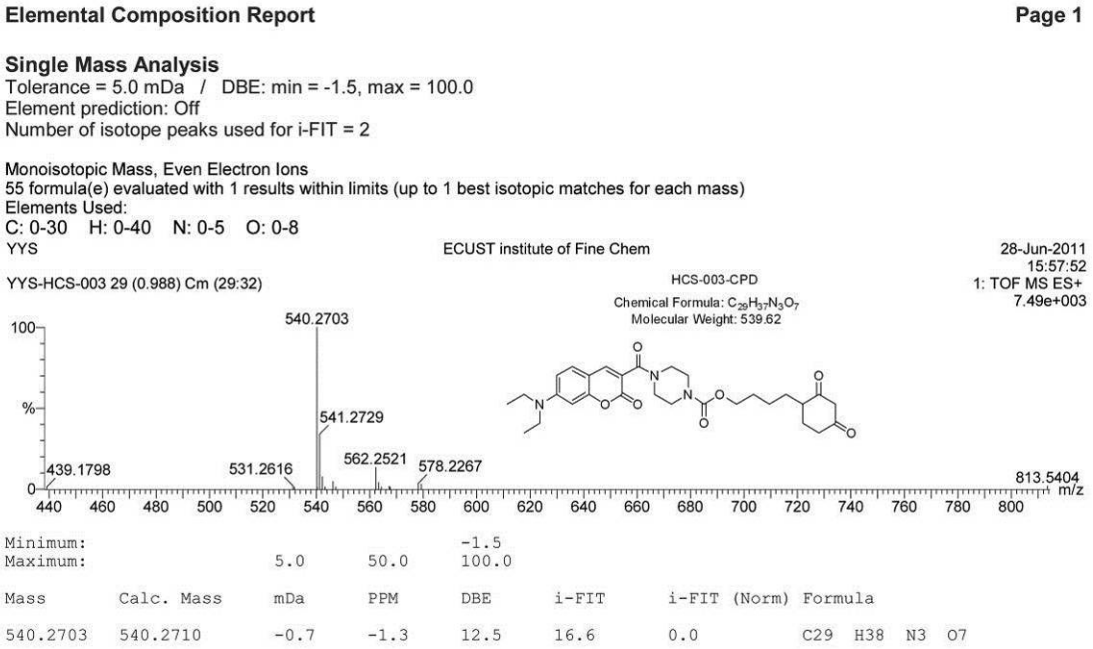


Figure S9. ESI-Mass spectrum of CPD

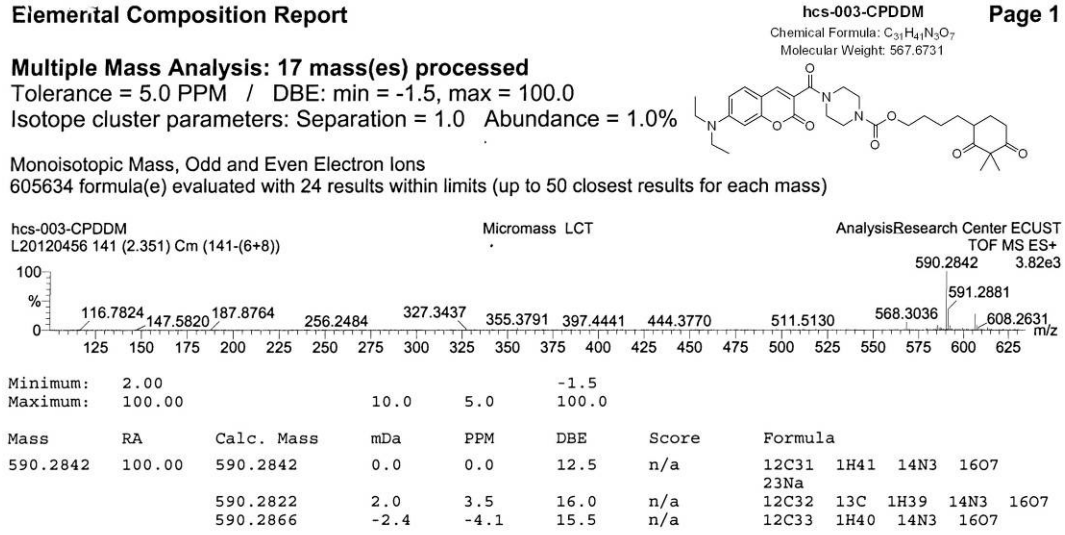


Figure S10. ESI-Mass spectrum of CPDDM

10. HPLC analysis of CPD and CPDDM.

The purity of the final compounds (**CPD** & **CPDDM**) were evaluated through HPLC analysis. The purity was above 95% (monitored both at 254 nm and 415 nm, respectively). In particular, when the detection signal was at 415 nm, the purity of **CPD** & **CPDDM** was above 98%, which suggested that no coumarin based byproduct existed and there was no interference on the labeled protein fluorescence during the labeling application. Chromatographic conditions, A: 50 mM ammonium acetate buffer, pH 6.5; B: Acetonitrile. Gradient condition: B 10% to 100%. Monitored at 415 nm.

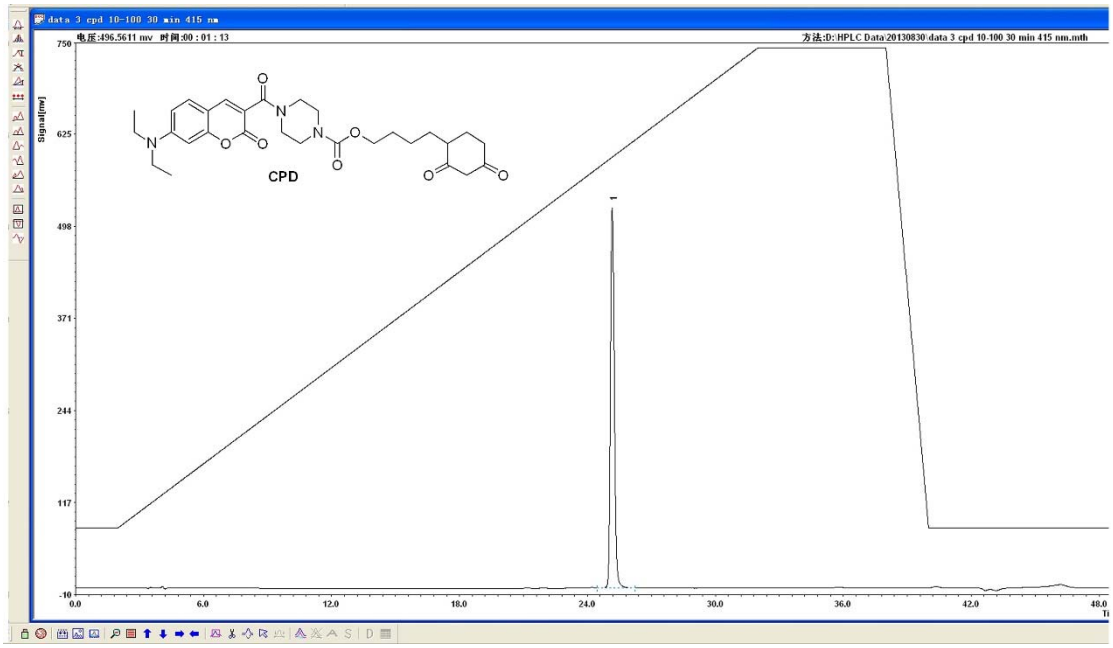


Figure S11. Selected HPLC analysis of CPD.

11. Selected Infrared (IR) spectra.

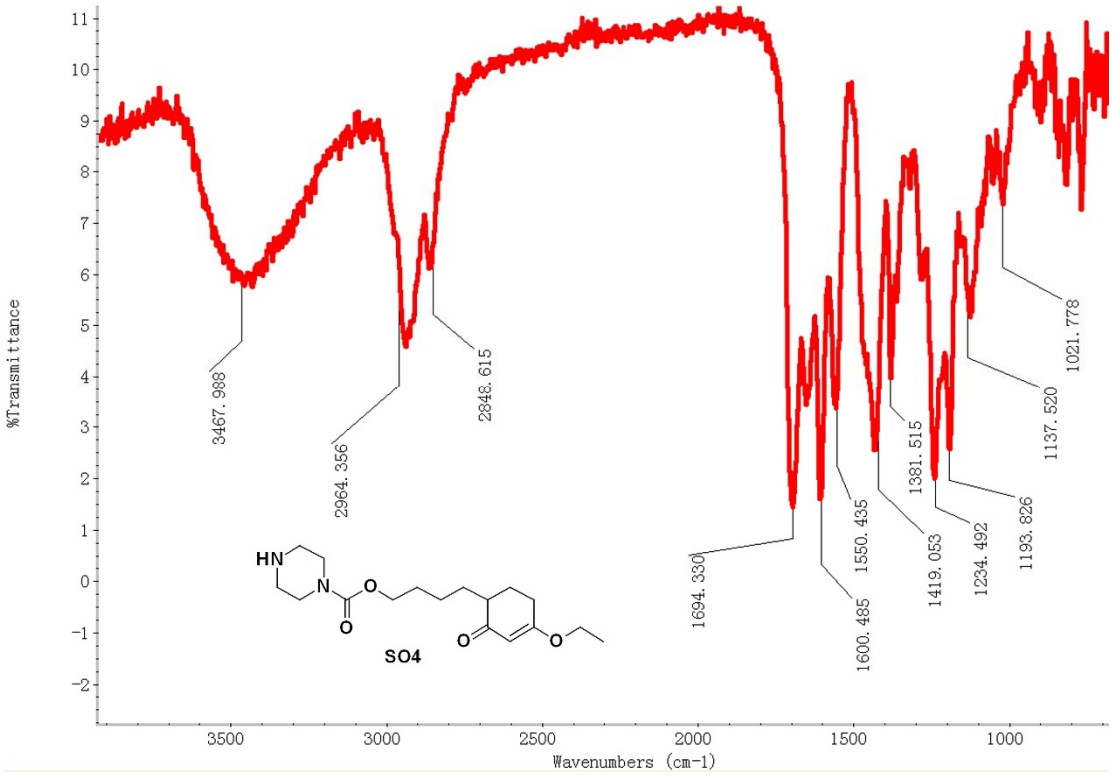


Figure S12. Selected IR spectrum of SO4.

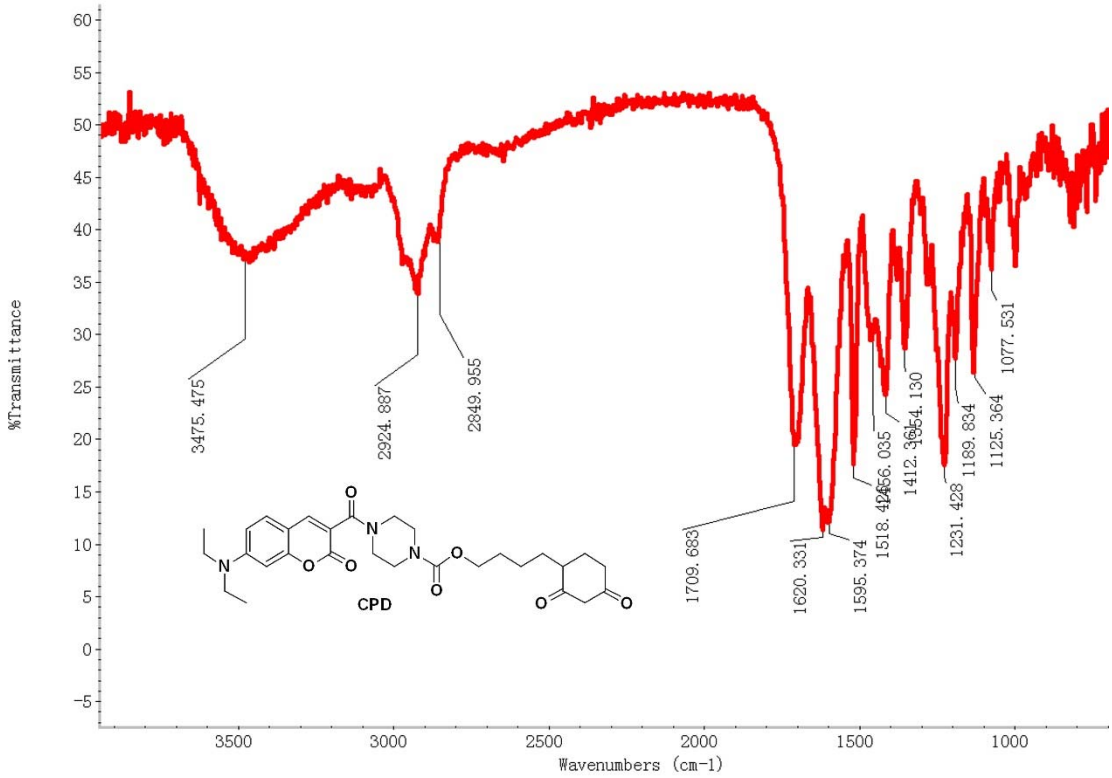


Figure S13. Selected IR spectrum of CPD.