Supporting Information

A unique reaction of diphenylcyclopropenone and 1,2-aminothiol with releasing the thiol for multiple bioconjugation

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1. Reagents and instruments

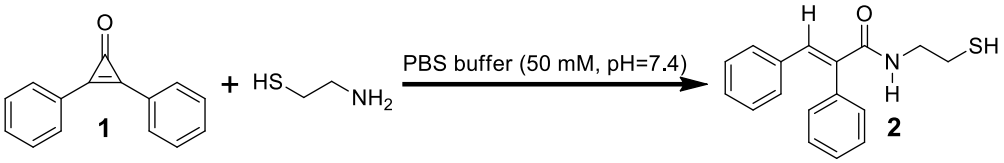
All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiment without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by UV light. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.50 ppm, CD_3CN = 1.96 ppm). ^{1}H NMR coupling constants (*J*) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), dd (doublet doublet), t (triplet), m (multiplet). High-resolution mass spectrum (HRMS) was obtained on an Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS. The UV-visible spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer (METASH, China). AGELA TECHNOLOGIES HPLC LC-10F with C18 column (4.6 mm x 250 mm) was employed for HPLC analysis, and different detection wavelengths (254 nm, 300 nm) were used for different compounds.

2. Insights into the reaction of diphenylcyclopropenone and 2-aminoethanethiol

All measurements were performed in degassed phosphate buffers (PBS, 50 mM, pH = 7.4, containing 50% CH_3CN). Diphenylcyclopropenone (**DPCP**, **1**) and **DPCP** derivatives were dissolved into CH_3CN to prepare the stock solutions with concentrations of 1-10 mM. All spectra measurements were performed in a 3 mL cuvette with 2 mL solution, and the reaction mixture was shaken uniformly before the measurement.

Water-stability of 1 in PBS: 0.5 mM **1** in PBS (50 mM, pH = 7.4, cotaining 50% CH_3CN) was checked by time-dependent HPLC tests. Conditions: detection wavelength: 300 nm; flow: 1 mL/min; Buffer A: 0.1% (v/v) trifluoroacetic acid in

water; buffer B: methanol; elution conditions: 0-3 min, buffer B: 5-70%; 3-28 min, buffer B: 70-80%; 28-30 min, buffer B: 80-5%.



Isolation and characterization of the product: compound **1** (101 mg, 0.5 mM) was dissolved in 100 mL degassed, sealed PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN), and 2-aminoethanethiol (Cy, 80 mg, 0.6 mM) was added to the solution subsequently. After stirring for 3 h, the solution was extracted with ethyl acetate (50 mL × 3). Then the collected organic layer was washed with water, brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography to give a white solid **2** (42 mg, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.48-7.41 (m, 3H), 7.28-7.26 (m, 2H), 7.19-7.10 (m, 3H). 7.00-6.98 (m, 2H), 5.89 (bs, 1H), 3.49-3.45 (m, 2H), 2.68-2.63 (m, 2H), 1.23 (t, J = 8.5 Hz, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 167.1, 137.4, 136.1, 134.9, 134.1, 130.4, 129.9, 129.7, 128.7, 128.2, 42.9, 24.5. HRMS (ESI): m/z calculated for $C_{17}H_{18}NOS^+$: 284.1104; found: 284.1110.

HPLC analysis for the reaction process: the reaction of **1** (0.5 mM) and Cy (2.0 mM) was incubated in PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN), and the time-dependent HPLC were checked. After reaction for 30 min, tris(2-carboxyethyl)phosphine (TCEP, 0.5 mM) was added to the solution for another 30 min of incubation; and the peak for possible dimer of **2** via disulfide bond (within the red dot frame, Fig. S4) was disappeared. As a control, compound **2** (0.2 mM) was also monitored by HPLC. The HPLC conditions were used as description above.

HRMS analysis of the reaction in D_2O-based PBS buffer: 1 (5 mM) and Cy (4 mM) were dissolved in PBS buffer (prepared by deuteroxide (D_2O), 100 mM, pH = 7.4, containing 50% CD_3CN) for 4 h of incubation before HRMS tests.

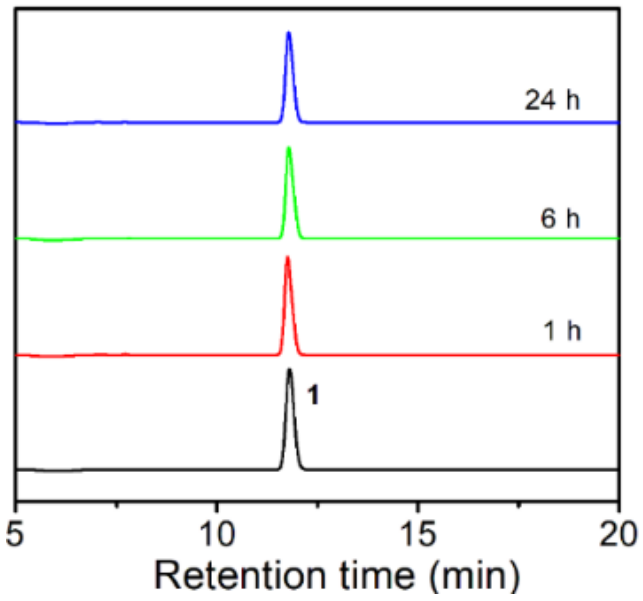


Figure S1. Time-dependent HPLC traces of **1** in PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN) at room temperature. Detection wavelength: 300 nm.

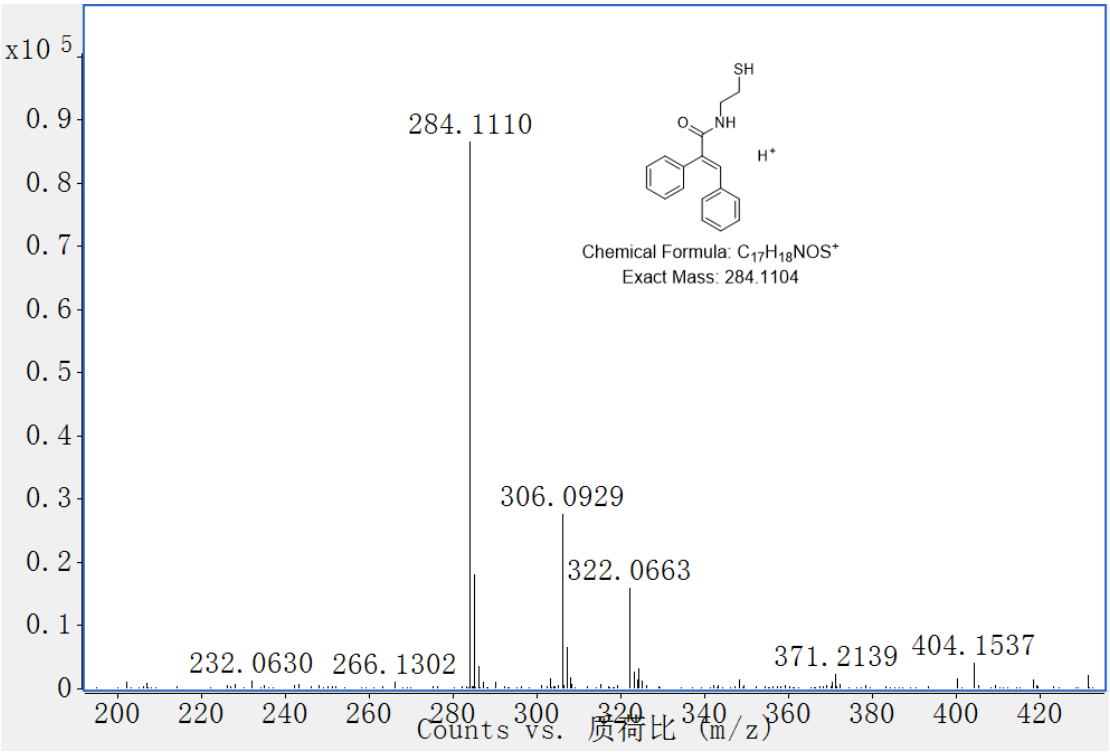


Figure S2. HRMS analysis of the reaction between **1** (2 mM) with cysteamine (5 mM) in PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN) for 4 h of incubation.

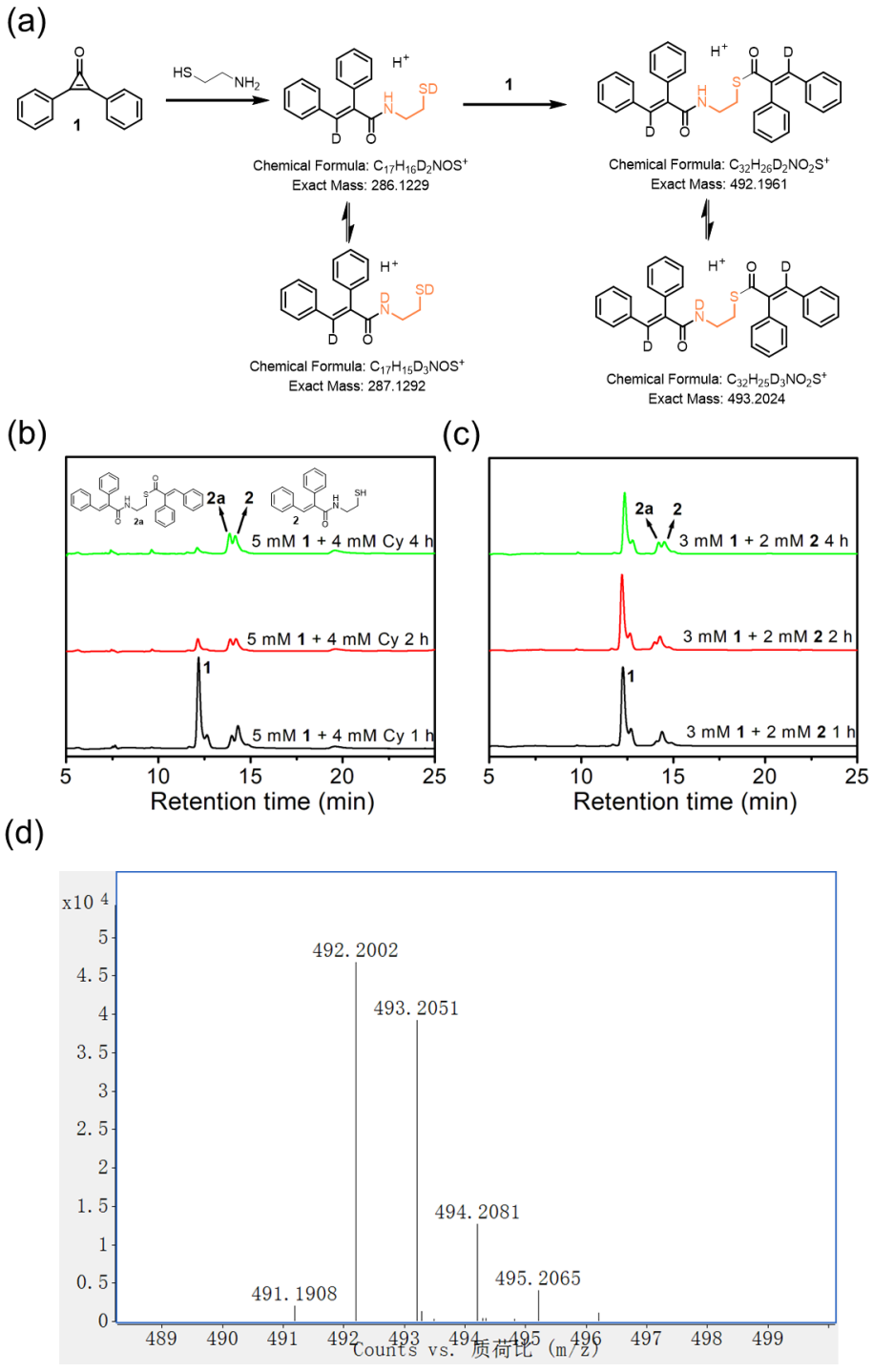


Figure S3. (a) Possible reaction products of excess **1** and Cy in a D_2O-based buffer and their exact mass values. (b) HPLC traces for the reaction of **1** (5 mM) with Cy (4 mM) in PBS at room temperature. Detection wavelength: 300 nm. (c) HPLC traces for the reaction of **1** (3 mM) with product **2** (2 mM) in PBS at room temperature. Detection wavelength: 300 nm. (d) HRMS analysis of the reaction aliquot of **1** (5 mM) with Cy (4 mM) in a D_2O-based PBS buffer (100 mM, pH 7.4, containing 50% CD_3CN) for 4 h of incubation.

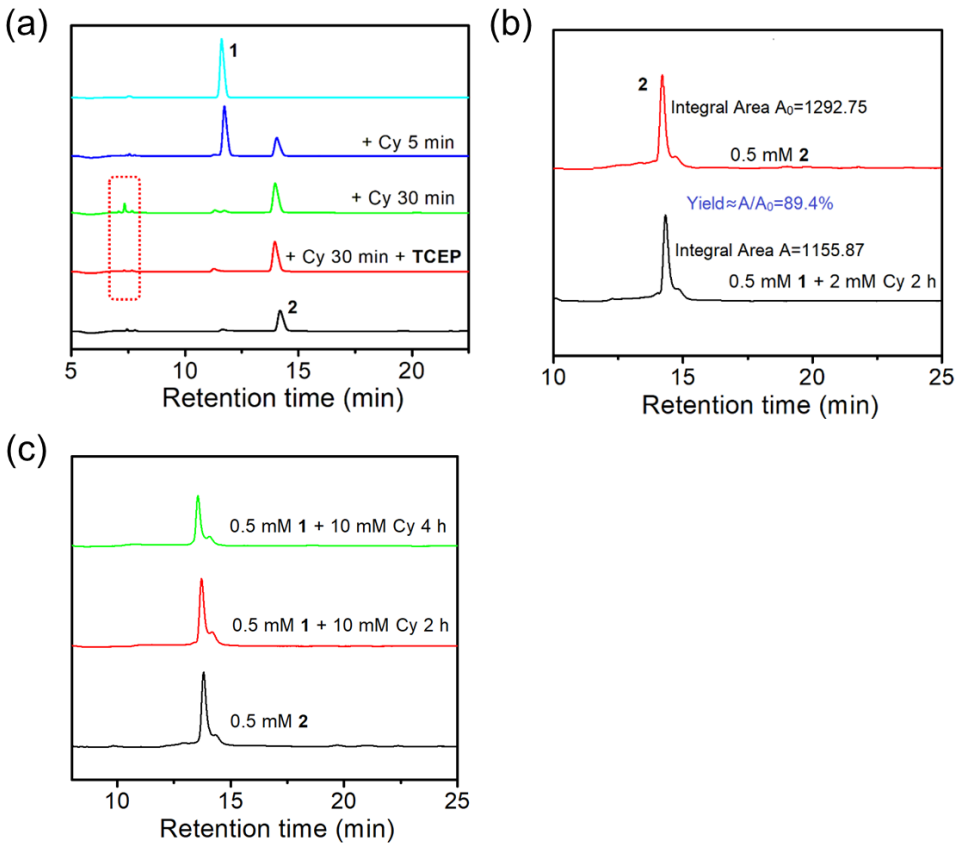


Figure S4. (a) HPLC traces of the reaction of **1** (0.5 mM) with Cy (2.0 mM) in PBS buffer at room temperature. The incubation time was indicated inset for the traces. Control traces of pure compounds **1** and **2** are also shown. (b) HPLC trace for **1** (0.5 mM) with Cy (2 mM) in PBS for 2 h of incubation. (c) HPLC traces for the reaction of **1** (0.5 mM) with Cy (10 mM) in PBS buffer at room temperature. Detection wavelength: 300 nm.

3. Kinetic and selective studies of 1 with nucleophiles

UV-Vis spectra for kinetic and selective analysis: 1 (10 μM) was incubated with different nucleophiles including Cy, 2-mercaptoethanol (βME), ethylamine, ethanol, amino acids at 25 ºC, and the absorbance profiles were recorded at different time points. The pseudo-first-order rates, k_{obs}, were obtained by fitting the time-dependent absorbance data with a single exponential function, and the linear fitting of k_{obs} versus nucleophile concentrations yielded the kinetic rate k_2.[1] The selectivity was measured by absorbance responses at 296 nm of **1** (10 μM) with various amino acids (1 mM) in the absence or presence of Cys for 1 h incubation. Since some amine acids have a certain absorption at 220 nm - 320 nm, the relative absorption data of $(A_0\text{-}A)/A_0$ were used for the selective tests.

HRMS analysis: 1 (5 mM) and βME (4 mM) were dissolved in PBS buffer (prepared by H_2O or deuteroxide (D_2O), pH = 7.4, 100 mM, containing 50% CD_3CN) for 4 h of incubation before HRMS tests. For dual-labelling, the reaction of **1** (5.0 mM) with Cy (4.0 mM) was incubated in PBS for 1 h and then with 6,7-methylenedioxy-4-methyl-3-maleimidocoumarin (**Cour-Mlm**, 6.0 mM) for another 2 h in one pot before HRMS tests.

HPLC analysis: the reaction mixture of **1** (0.5 mM) with ethylamine (20 mM) was incubated in PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN), and the time-dependent HPLC were checked. The conditions were used as description above. For control studies, the reaction mixture of **1** with **Cour-Mlm** or tryptophan (Trp) was incubated for different times and then tested by time-dependent HPLC analysis as description. For dual-labelling, the reaction mixture of **1** (1.8 mM) and Cy (1.5 mM) was incubated in PBS for 1.5 h and then trapped in one pot by **Cour-Mlm** (3.0 mM) for 2 h. The reaction solutions were directly monitored by HPLC tests as description above.

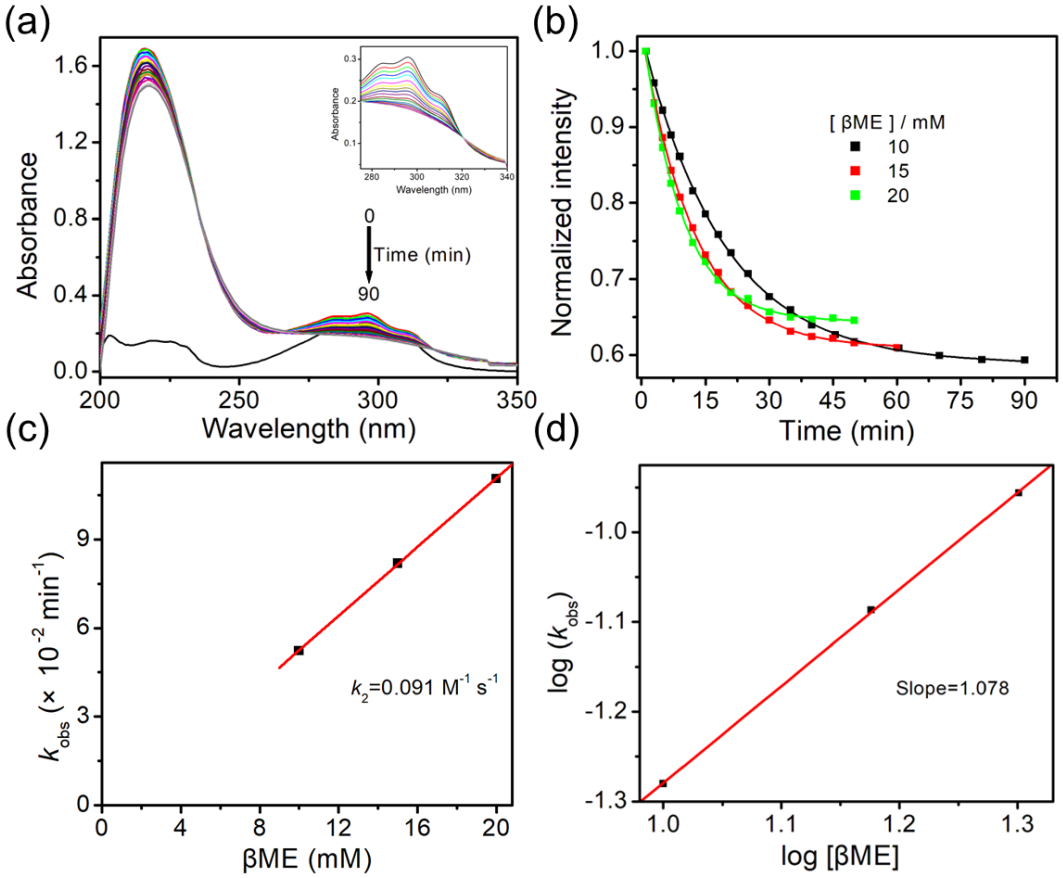


Figure S5. (a) Time-dependent absorbance spectra of **1** (10 μM) in the presence of βME (10 mM) in PBS. (b) Time-dependent normalized absorbance signals at 296 nm of **1** (10 μM) towards different concentrations of βME in PBS. The solid lines represent the best fitting with a single exponential function to give k_{obs}. (c) The linear-relationship plots of k_{obs} versus the concentration of βME give reaction rate of k_2 = 0.09 $M^{-1}s^{-1}$. (d) The linear-relationship plots of log(k_{obs}) versus log([βME]). The slope of 1.078 supports a first-order dependence in βME for the reaction.

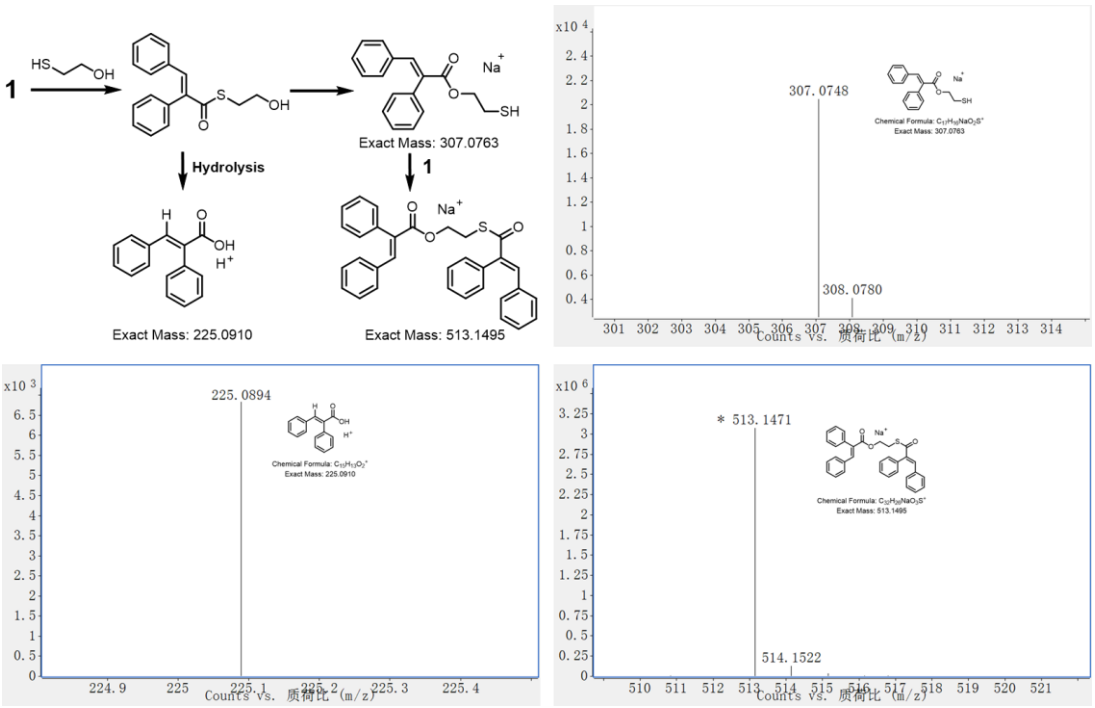


Figure S6. HRMS analysis of the reaction aliquot of **1** (5 mM) with βME (4 mM) in PBS for 4 h of incubation and a possible reaction pathway for the mass results.

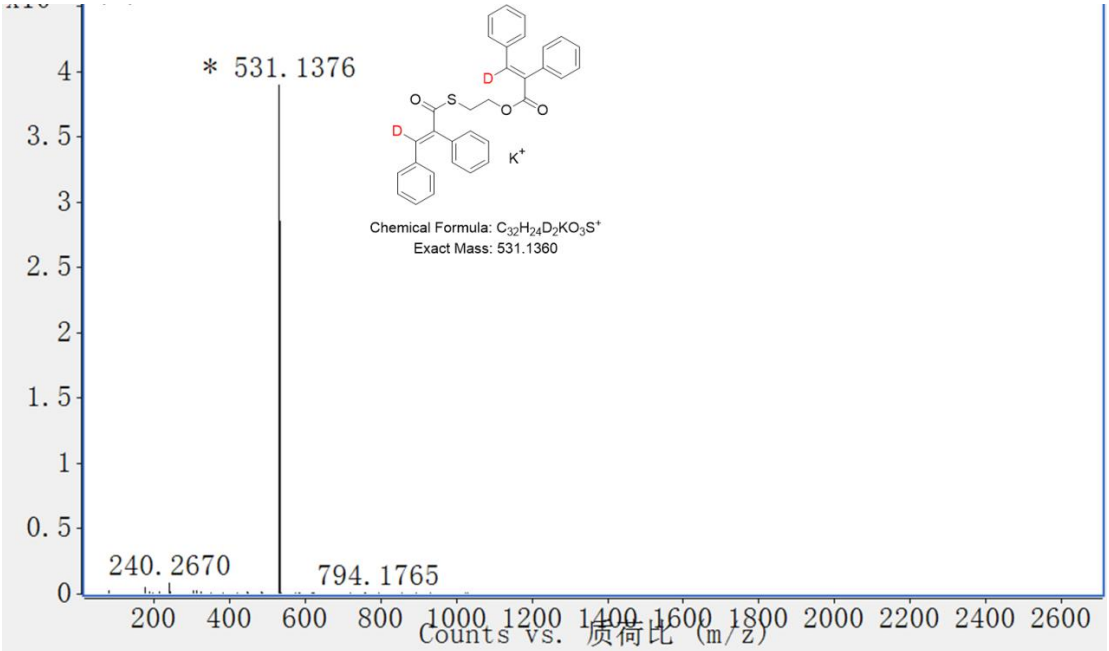


Figure S7. HRMS analysis of the reaction aliquot of **1** (5 mM) with βME (4 mM) in a D_2O-based PBS for overnight incubation.

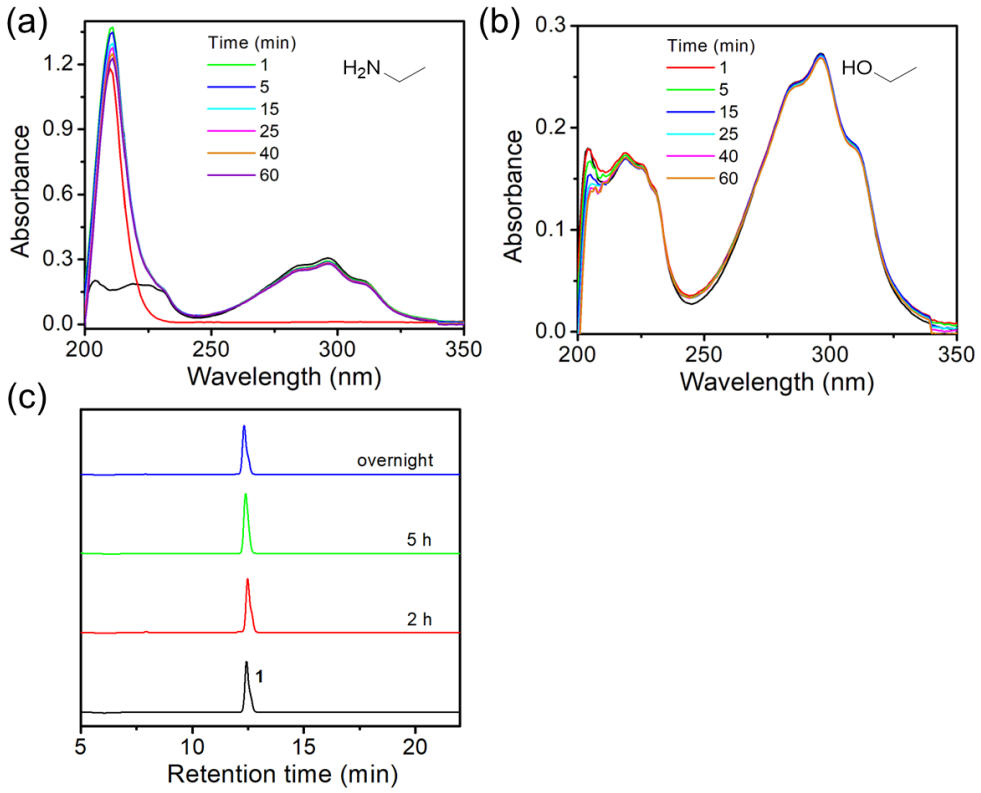


Figure S8. (a) Time-dependent absorbance spectra of **1** (10 μM) in the presence of ethylamine (50 mM) in PBS. The red line represents 50 mM ethylamine only. (b) Time-dependent absorbance spectra of **1** (10 μM) in the presence of ethanol (50 mM) in PBS buffer at room temperature. (c) Time-dependent HPLC traces of **1** (0.5 mM) with ethylamine (20 mM) in PBS buffer. Detection wavelength: 300 nm.

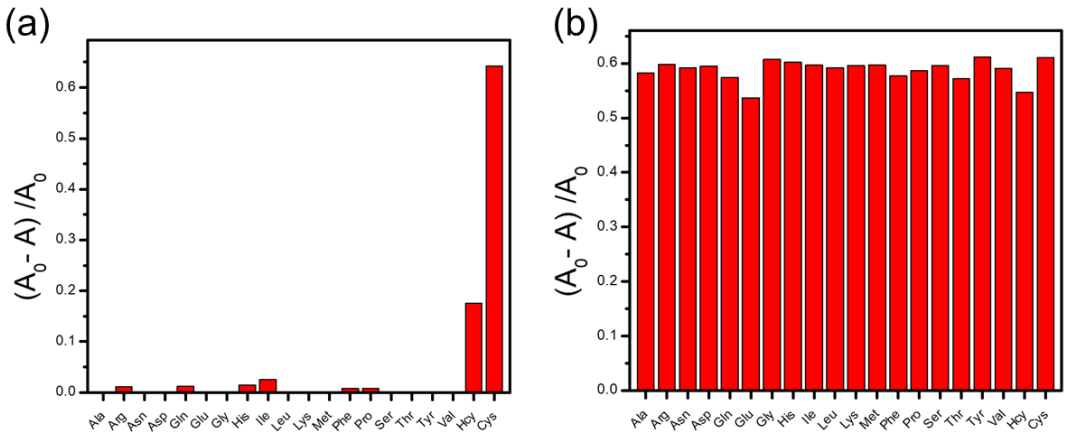


Figure S9. (a) Relative absorbance signals at 296 nm of **1** (10 μM) toward 1 mM different amino acids in PBS for 1 h of incubation. (b) Relative absorbance signals at 296 nm of **1** (10 μM) toward 1 mM different amino acids in the presence of 1 mM Cys for 1 h incubation.

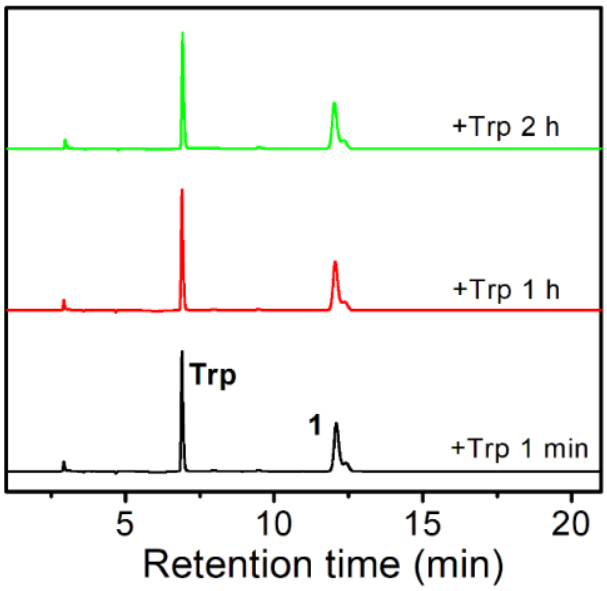


Figure S10. Time-dependent HPLC traces of **1** (0.4 mM) + Trp (1 mM) in PBS. Detection wavelength: 280 nm.

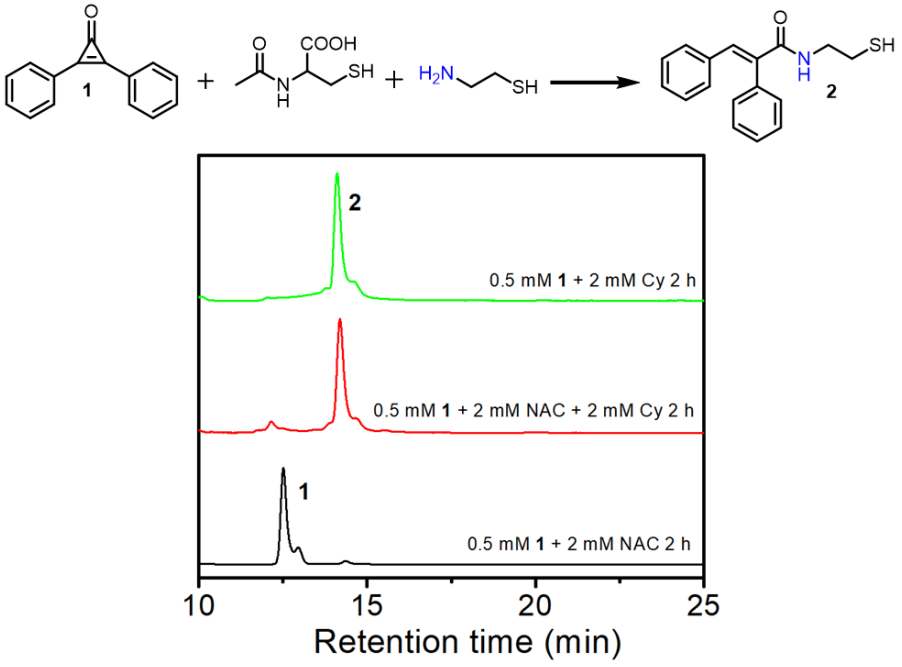


Figure S11. HPLC traces for **1** (0.5 mM) with N-Acetyl-L-cysteine (NAC, 2 mM), or Cy (2 mM), or NAC (2 mM) + Cy (2 mM) for 2 h. Detection wavelength: 300 nm.

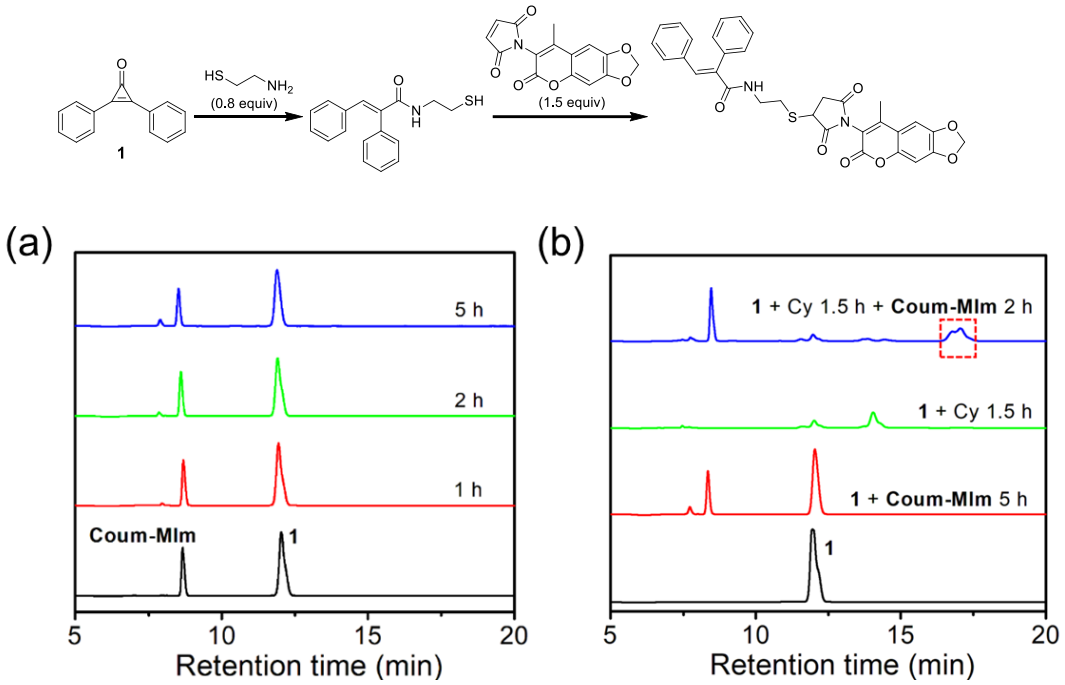


Figure S12. (a) HPLC traces of **1** (0.6 mM) with **Cour-Mlm** (1.0 mM) in PBS at room temperature. The incubation times were indicated inset for the traces. (b) HPLC traces of **1** (1.8 mM) reacting with Cy (1.5 mM) in PBS for 1 h, and then **Coum-Mlm** (3.0 mM) was added to the reaction system for another 2 h. The possible dual-labelling peak is highlighted by red dot frame. Detection wavelength: 300 nm.

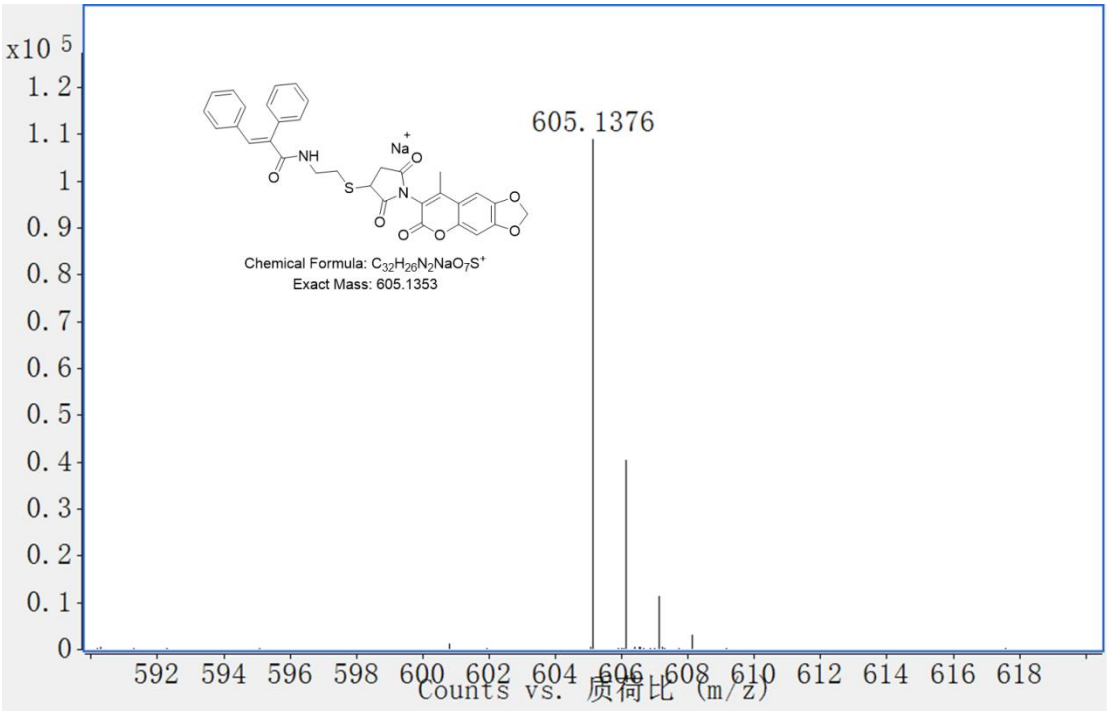
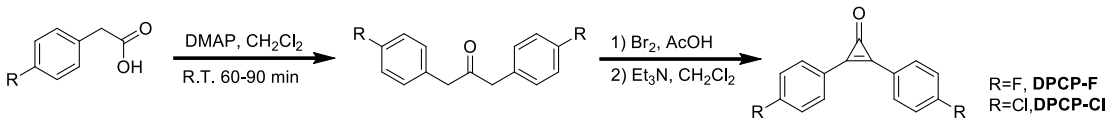
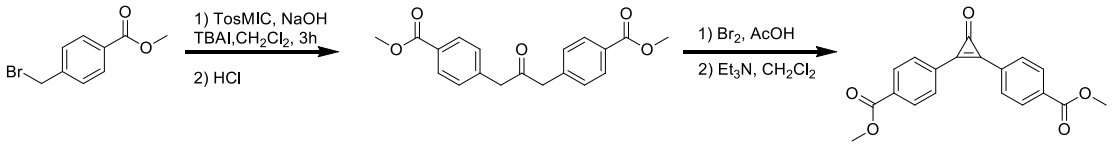


Figure S13. HRMS analysis of the reaction solution of **1** with Cy followed by **Coum-Mlm**.

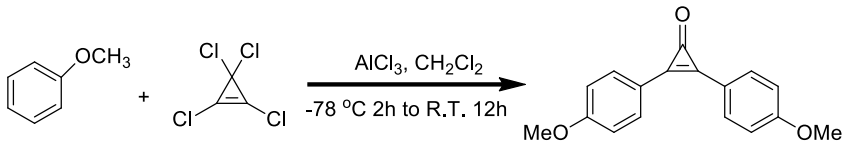
4. Synthesis of DPCP derivatives and their reactions with Cys



Synthesis of substituted cyclopropenones (**DPCP-F**, **DPCP-Cl**) was performed based on previous methods.[2,3] The substituted phenylacetic acid (-F, or -Cl) was added to a stirring solution of DCC (1.5 eq) and DMAP (0.3 eq) in THF at room temperature. After 60 minutes, the reaction was filtered through celite, and the diaryl ketone was purified by flash chromatography on silica gel. The resulted ketone was dissolved in DCM, and then a solution of bromine (2.05 eq) in acetic acid was added dropwise. After stirring for 6 hours, the reaction was poured into saturated sodium bicarbonate solutions, and the solvent was removed under reduced pressure. The resulted residue was added to a stirring solution of triethylamine (2.5 eq) in DCM at room temperature for 1 hour. The reaction mixture was washed by brine, dried with sodium sulfate, and concentrated under reduced pressure. The product was purified by silica gel column chromatography. For **DPCP-F**, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00-7.97 (m, 4H), 7.30-7.27 (m, 4H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 165.1 (d, $^{1}J_{C\text{-}F}$ = 256.6 Hz), 154.9, 146.2, 133.9 (d, $^{3}J_{C\text{-}F}$ = 9.4 Hz), 120.5, 116.9 (d, $^{2}J_{C\text{-}F}$ = 22.3 Hz). ^{19}F NMR (377 MHz, $CDCl_3$) δ -103.42. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{15}H_9F_2O^+$: 243.0616; found: 243.0625. For **DPCP-Cl**, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, J = 8.4 Hz, 4H), 7.57 (d, J = 8.4 Hz, 4H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 154.9, 147.3, 139.3, 132.6, 129.9, 122.2. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{15}H_9Cl_2O^+$: 275.0025; found: 275.0036.



DPCP-COOMe was synthesized based on our previous work.[3] ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, J = 8.2 Hz, 4H), 8.05 (d, J = 8.3 Hz, 4H). 3.99 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 165.8, 155.3, 149.8, 133.8, 131.3, 130.6, 127.1, 52.7.



DPCP-OMe was synthesized based on a published procedure.[2] Tetrachlorocyclopropene (1 eq.) was added to a stirring slurry of $AlCl_3$ (1.05 eq.) in CH_2Cl_2 at -78 °C for 10 min stirring, and anisole (2 eq.) in CH_2Cl_2 solution was then added, and the reaction was allowed to warm to room temperature overnight. The resulted reaction mixture was poured into water, extracted into CH_2Cl_2, washed with brine, dried with anhydrous sodium sulfate, and concentrated under reduced pressure. The cyclopropenone was purified by column chromatography and recrystallized from hexanes to yield a white crystalline. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, J = 8.8 Hz, 4H), 7.02 (d, J = 8.8 Hz, 4H), 3.87 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 162.7, 155.2, 143.9, 133.4, 116.9, 114.6, 55.5. HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{17}H_{15}O_3^+$: 267.1016; found: 267.1016.

Kinetic analysis: 1 (10 μM) or diphenylcyclopropenone (**DPCP**) derivatives was incubated with Cys at 25 ºC, and the time-dependent absorbance profiles were recorded. Time-dependent normalized UV-Vis signals towards different concentrations of Cys were used for kinetic fitting.

HPLC Analysis: Water stability of **DPCP-Cl**, **DPCP-F**, **DPCP-COOMe** and **DPCP-OMe** in PBS (50 mM, pH 7.4, cotaining 50% CH_3CN) was tested by time-dependent HPLC analysis. The reactivity of **DPCP-OMe** (0.5 mM) with Cys (20 mM) in PBS was checked by time-dependent HPLC. Conditions: detection wavelength: 300 nm; flow: 1 mL/min; Buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: methanol; elution conditions: 0-3 min, buffer B: 5-70%; 3-28 min, buffer B: 70-80%; 28-30 min, buffer B: 80-5%.

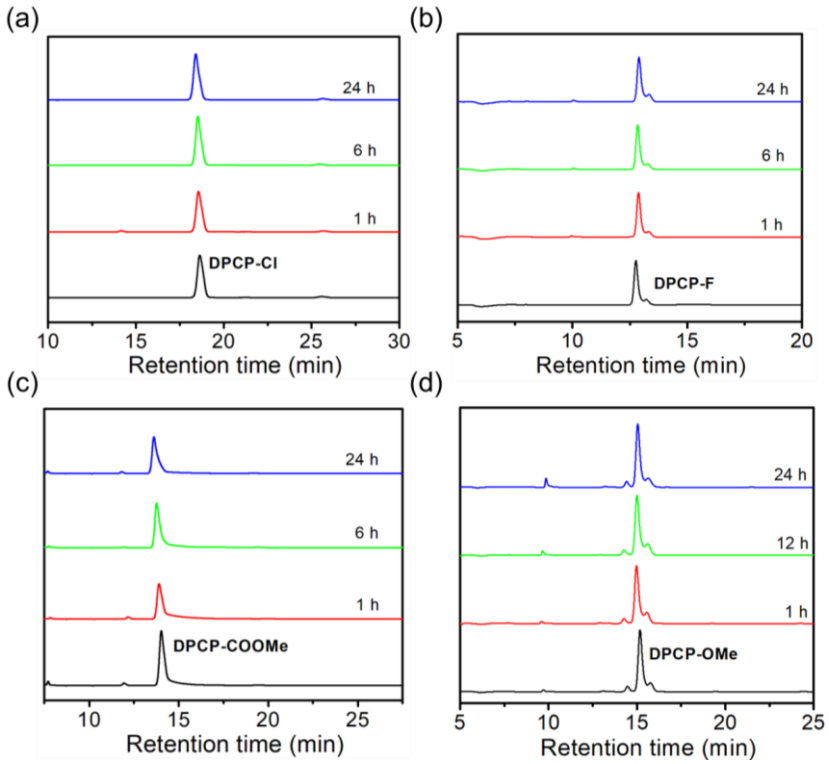


Figure S14. Time-dependent HPLC traces of **DPCP-Cl**, **DPCP-F**, **DPCP-COOMe**, and **DPCP-OMe** in PBS buffer (50 mM, pH 7.4, containing 50% CH_3CN) at room temperature. Detection wavelength: 300 nm.

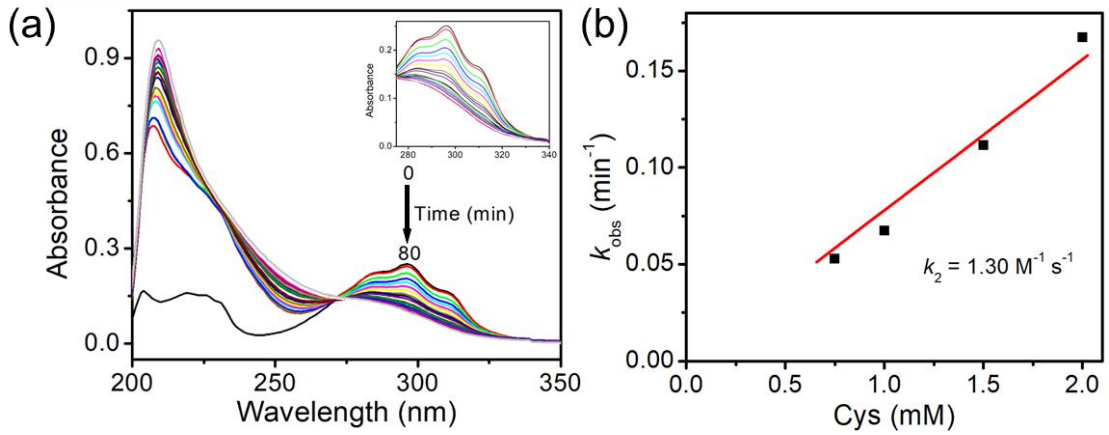


Figure S15. (a) Time-dependent absorbance spectra of **1** (10 μM) in the presence of Cys (1 mM) in PBS at 25 ºC. (b) The linear relationship plots of k_{obs} versus the concentration of Cys gives reaction rate of k_2 = 1.30 $M^{-1}s^{-1}$.

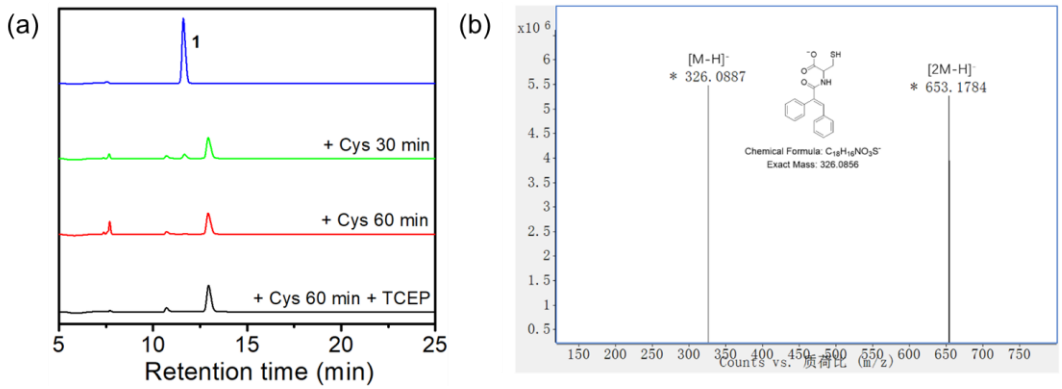


Figure S16. (a) Time-dependent HPLC traces for **1** (0.5 mM) with Cys (2 mM) in PBS at room temperature. Detection wavelength: 300 nm. (b) HRMS analysis of the reaction between **1** (2 mM) with Cys (3 mM) in PBS for 4 h of incubation.

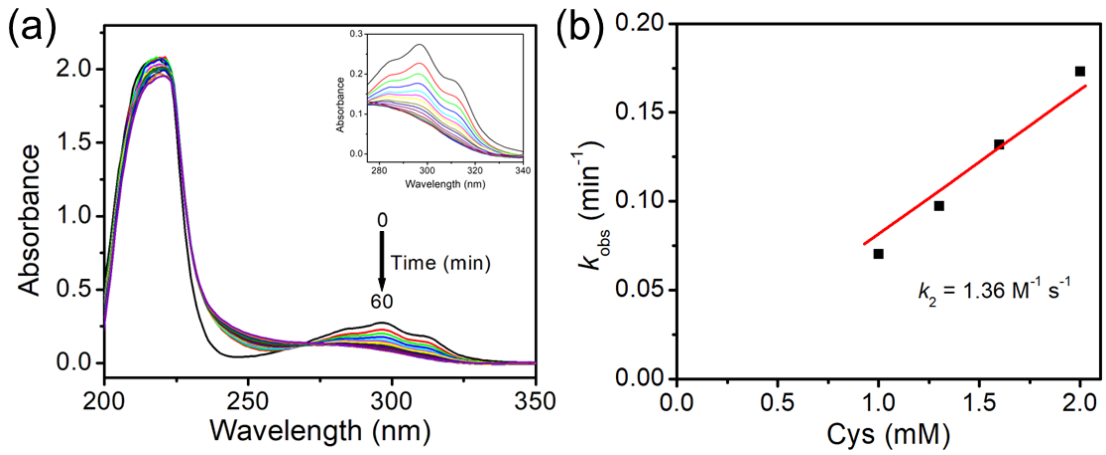


Figure S17. (a) Time-dependent absorbance spectra of **DPCP-F** (10 μM) in the presence of Cys (1 mM) in PBS at 25 °C. (b) The linear relationship plots of k_{obs} versus the concentration of Cys gives reaction rate of k_2 = 1.36 $M^{-1}s^{-1}$.

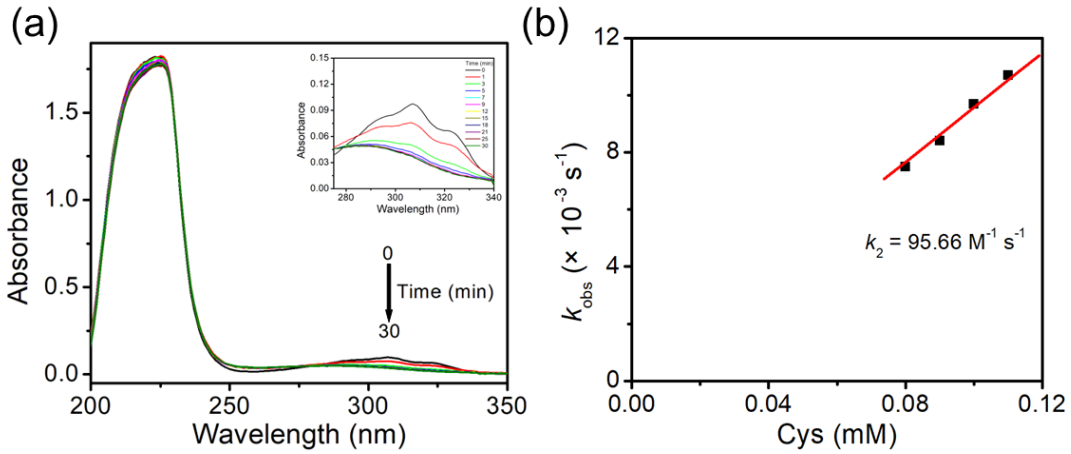


Figure S18. (a) Time-dependent absorbance spectra of **DPCP-Cl** (5 μM) in the presence of Cys (100 μM) in PBS at 25 °C. (b) The linear relationship plots of k_{obs} versus the concentration of Cys gives reaction rate of k_2 = 95.66 $M^{-1}s^{-1}$.

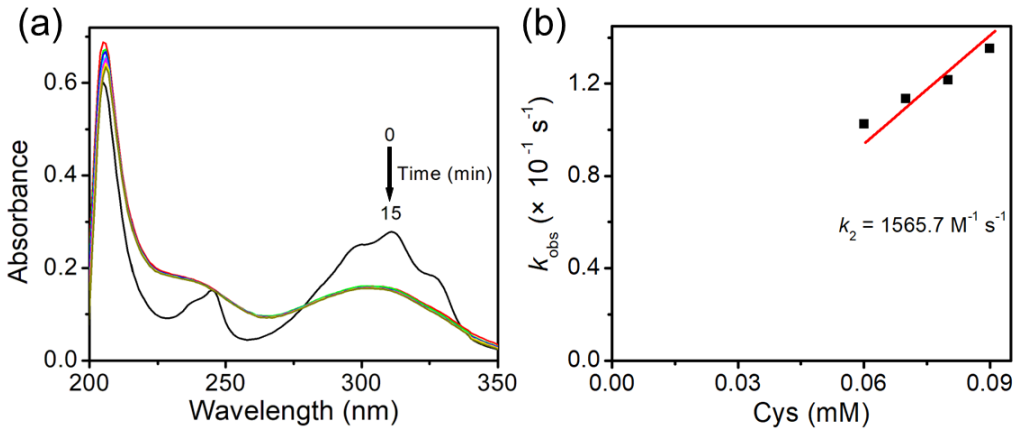


Figure S19. (a) Time-dependent absorbance spectra of **DPCP-COOMe** (10 μM) in the presence of Cys (100 μM) in PBS at 25 °C. (b) The linear relationship plots of k_{obs} versus the concentration of cysteine gives reaction rate of k_2 = 1565.7 $M^{-1}s^{-1}$.

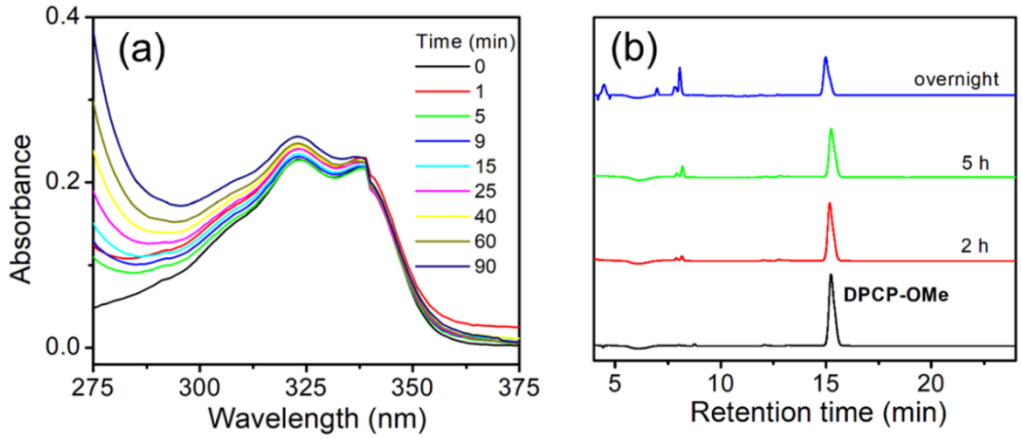


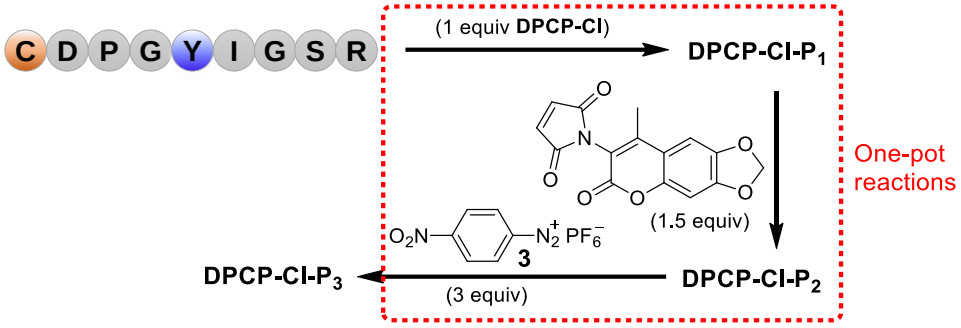
Figure S20. (a) Time-dependent absorbance spectra of **DPCP-OMe** (5 μM) in the presence of Cys (40 mM) in PBS at 25 °C. (b) HPLC traces of the reaction of **DPCP-OMe** (0.5 mM) with Cys (20 mM) in PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN) at room temperature.

5. Multiple labelling of *N*-Cys molecules via DPCPs

Selectivity analysis: The selectivity was measured by absorbance responses of **DPCP-Cl** (10 μM) with various amino acids (100 μM) in the absence or presence of Cys (100 μM) for 1 h incubation. All analytes were prepared as stock solutions in degassed water. Relative absorbance signals at 296 nm were used for data analysis.

HRMS analysis for dual-labelling: the reaction of **DPCP-Cl** (5.0 mM) with Cy (4.0 mM) was incubated in PBS (50 mM, pH = 7.4, containing 50% CH_3CN) for 1 h and then with **Cour-Mlm** (6.0 mM) for another 2 h in one pot before HRMS tests. The reaction of **DPCP-Cl** (0.5 mM) and Cys (1.0 mM) was performed in PBS for 1 h,

and then the released thiol was trapped in one pot by **Cour-Mlm** (1.0 mM) for 2 h before HRMS tests.



HRMS analysis for one-pot triple-labelling: as shown in the above schematic drawing, the reaction of **DPCP-Cl** (0.6 mM) and the CDPGYIGSR peptide (0.6 mM) were incubated in PBS buffer (50 mM, pH = 7.4, containing 50% CH_3CN) for 1 h, and then the released thiol was trapped in one pot sequentially by **Cour-Mlm** (0.9 mM) for 2 h, and then the 4-nitrobenzenediazonium salt (**3**, 1.8 mM) was added for another 4 h of incubation. Each step of reaction solutions was directly checked by HRMS tests.

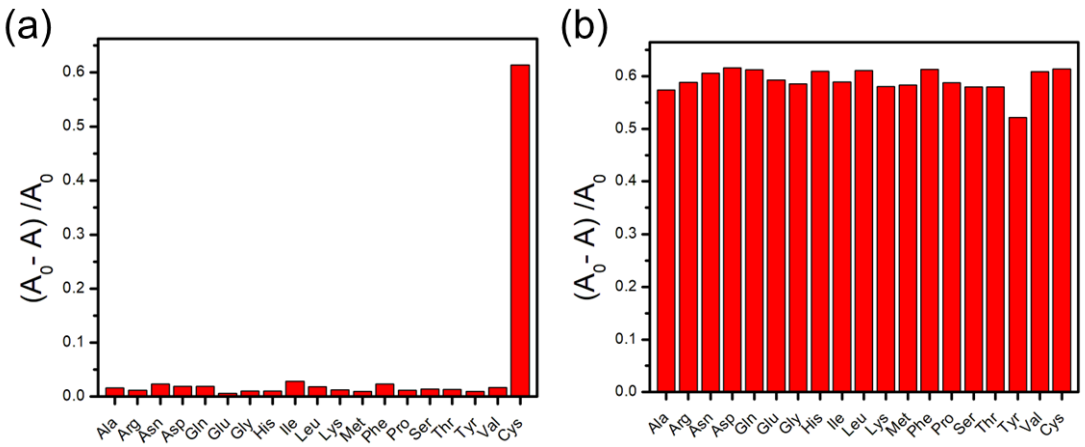


Figure S21. (a) Relative absorbance signals at 296 nm of **DPCP-Cl** (10 μM) toward 100 μM different species of amino acids in PBS for 1 h of incubation. (b) Relative absorbance signals at 296 nm of **DPCP-Cl** (10 μM) toward various species of amino acids in the presence of 100 μM Cys for 1 h of incubation.

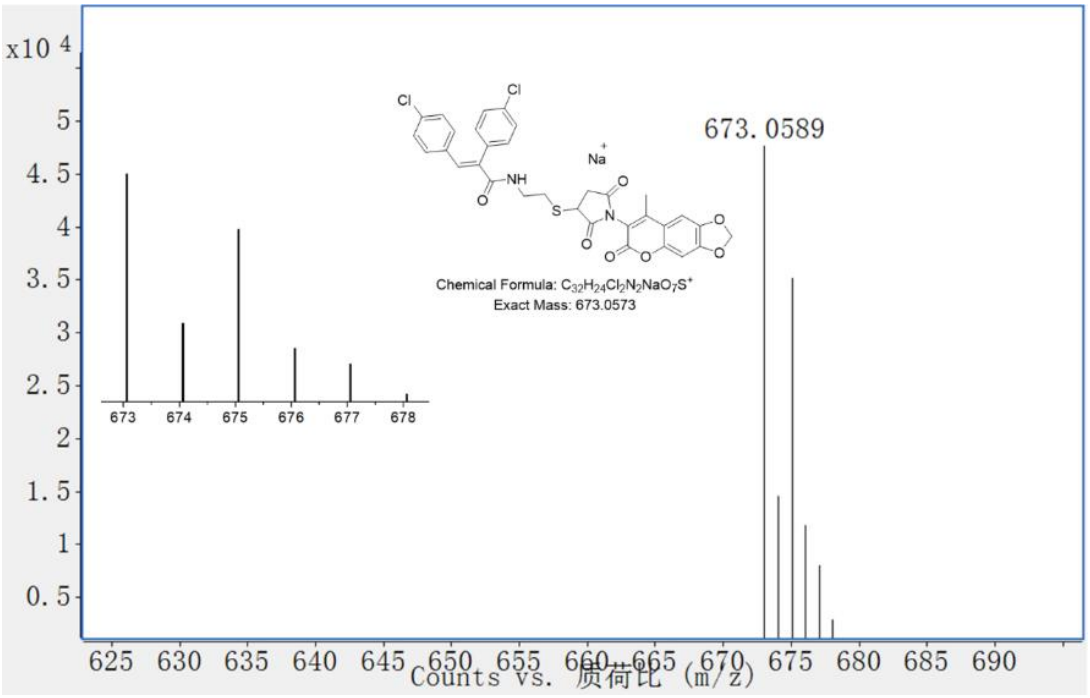


Figure S22. HRMS analysis of the reaction solution of **DPCP-Cl** (5 mM) with Cy (4 mM) for 1 h and then **Coum-Mlm** (6 mM) in one pot for 2 h. The isotope distributions for the labelling product are shown inset, which are well matched with the observed patterns.

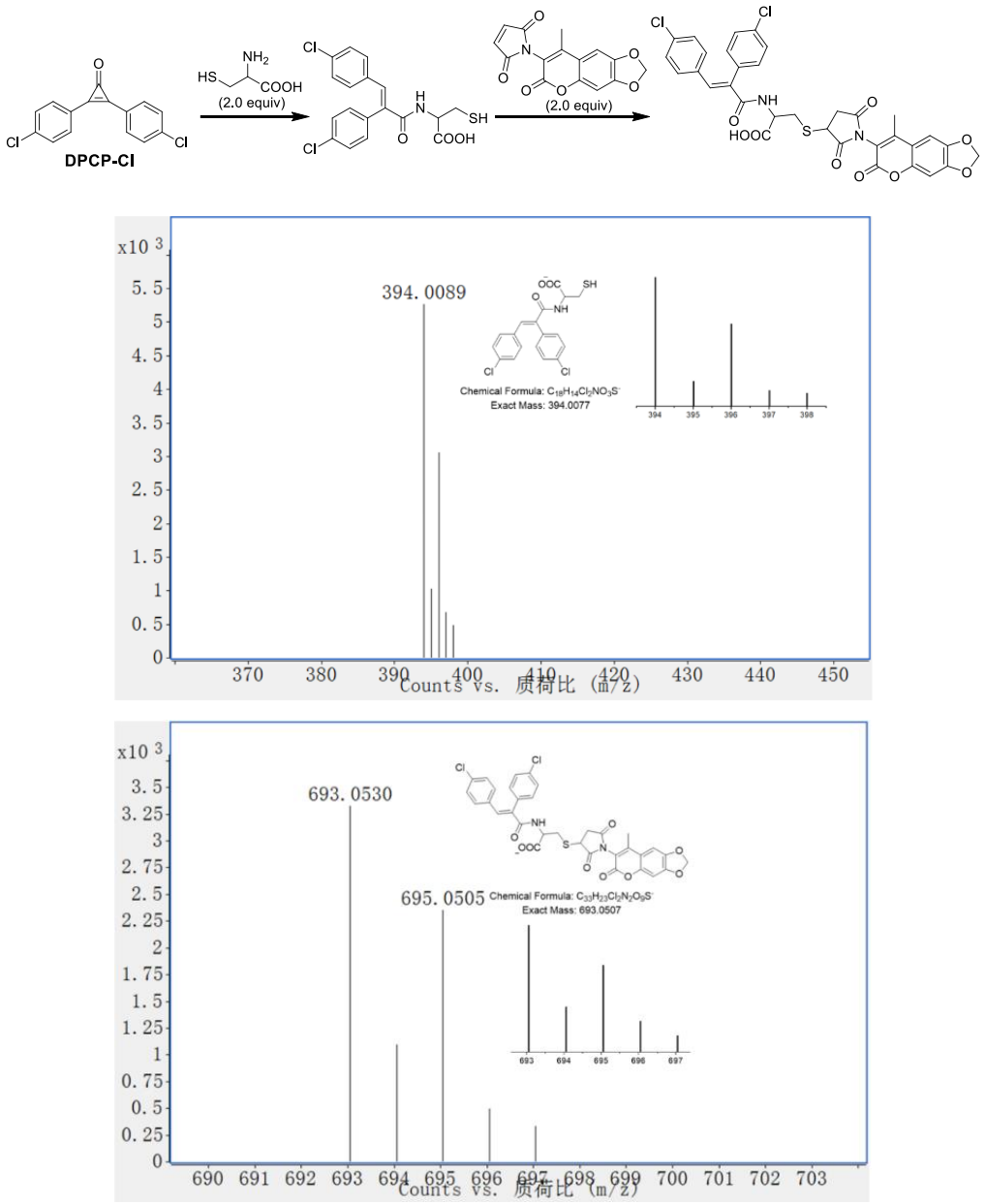


Figure S23. HRMS spectra (negative ion motif) of the reaction solution of **DPCP-Cl** (0.5 mM) with 1 mM Cys and further reaction with 1 mM **Coum-Mlm**. The isotope distributions for each labelling product are shown inset, which are well matched with the observed patterns.

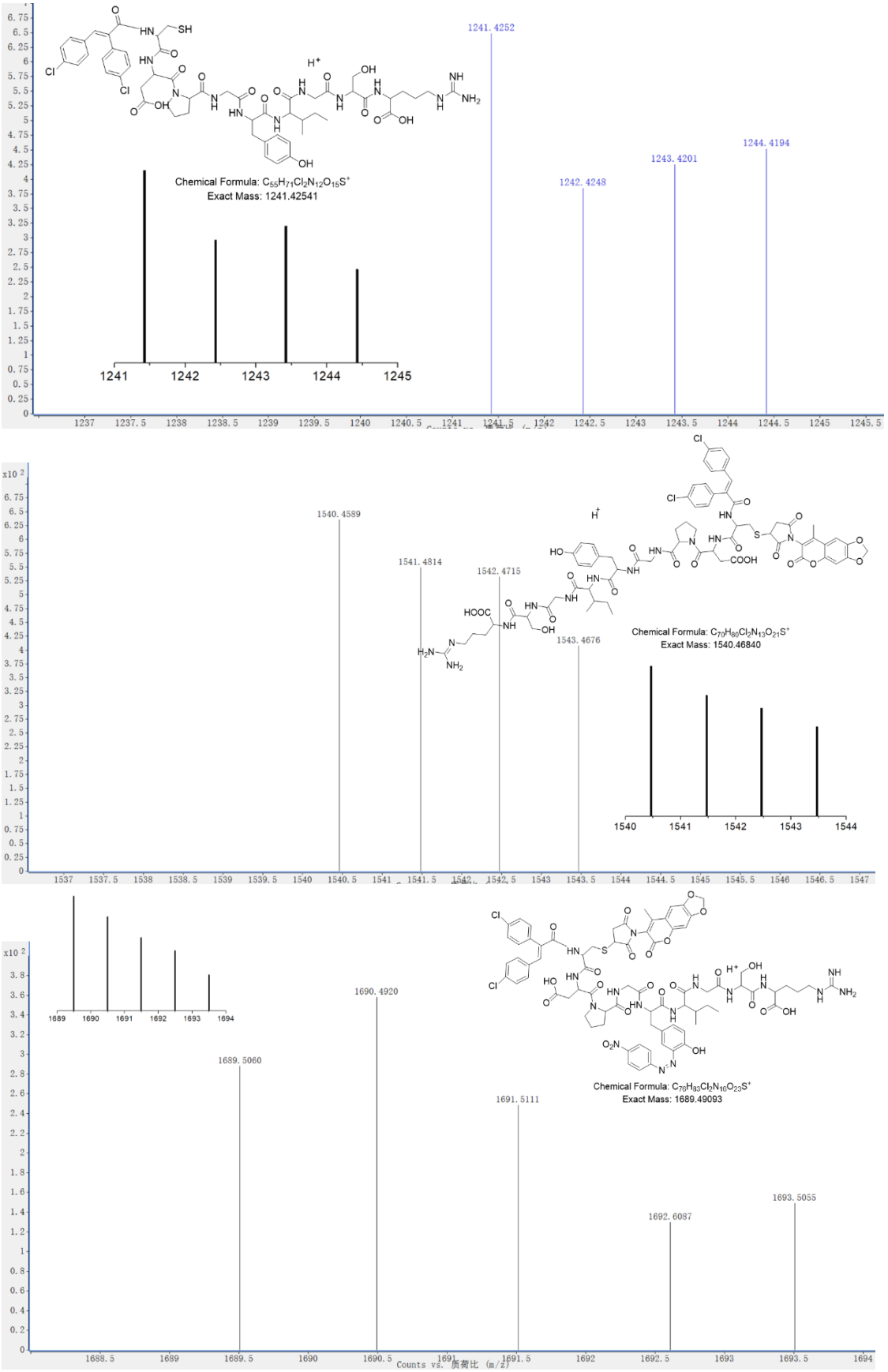


Figure S24. HRMS analysis of the reaction solution of the peptide with **DPCP-Cl**, **Coum-Mlm** and **3**. The isotope distributions for the labelling products are shown inset.

6. Supplementary NMR and HRMS spectra

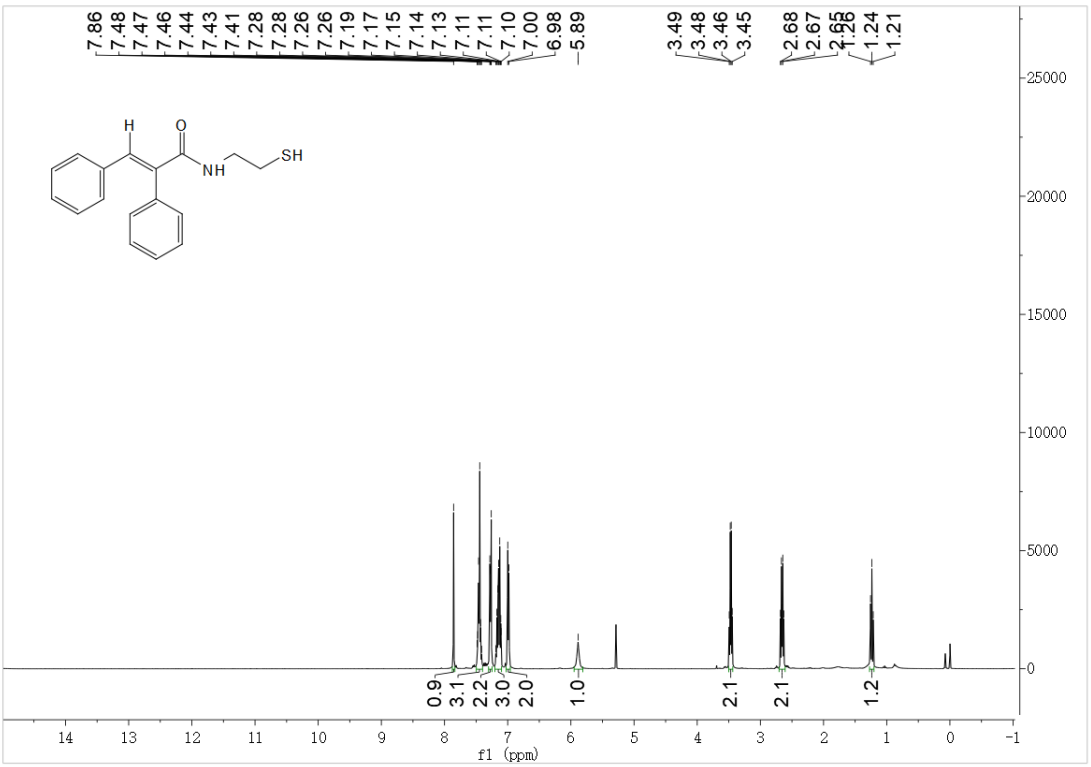


Figure S25. ^{1}H NMR spectrum of **2**.

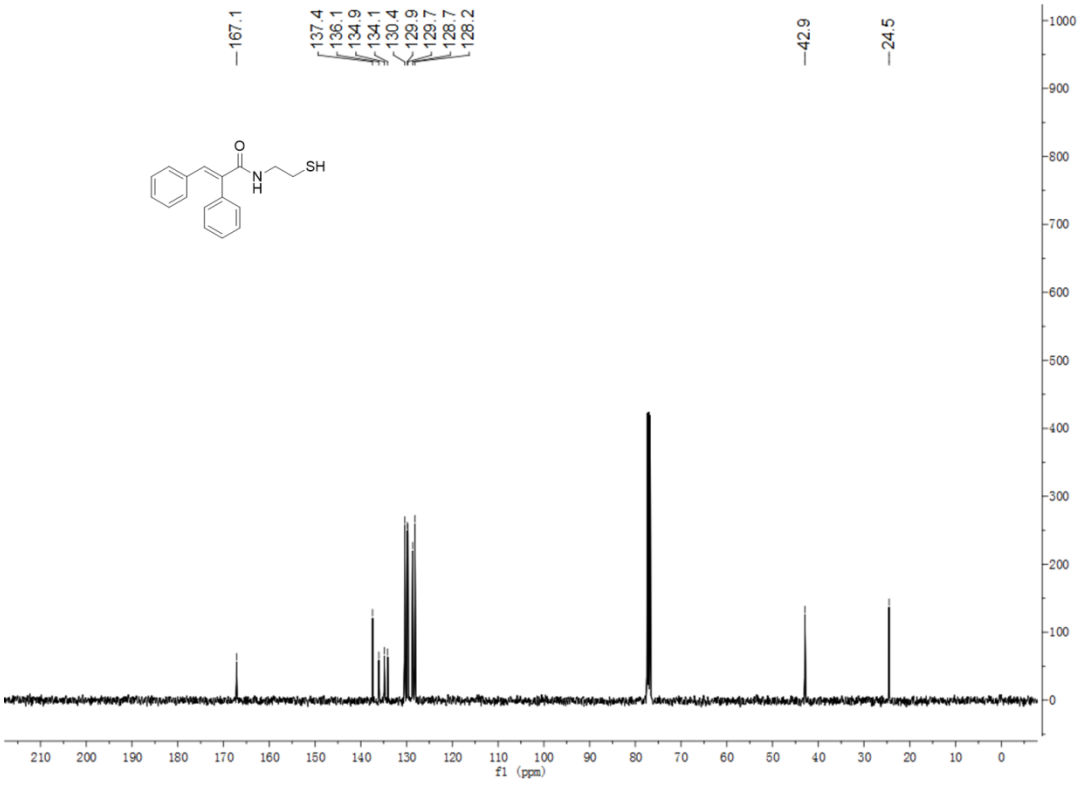


Figure S26. ^{13}C NMR spectrum of **2**.

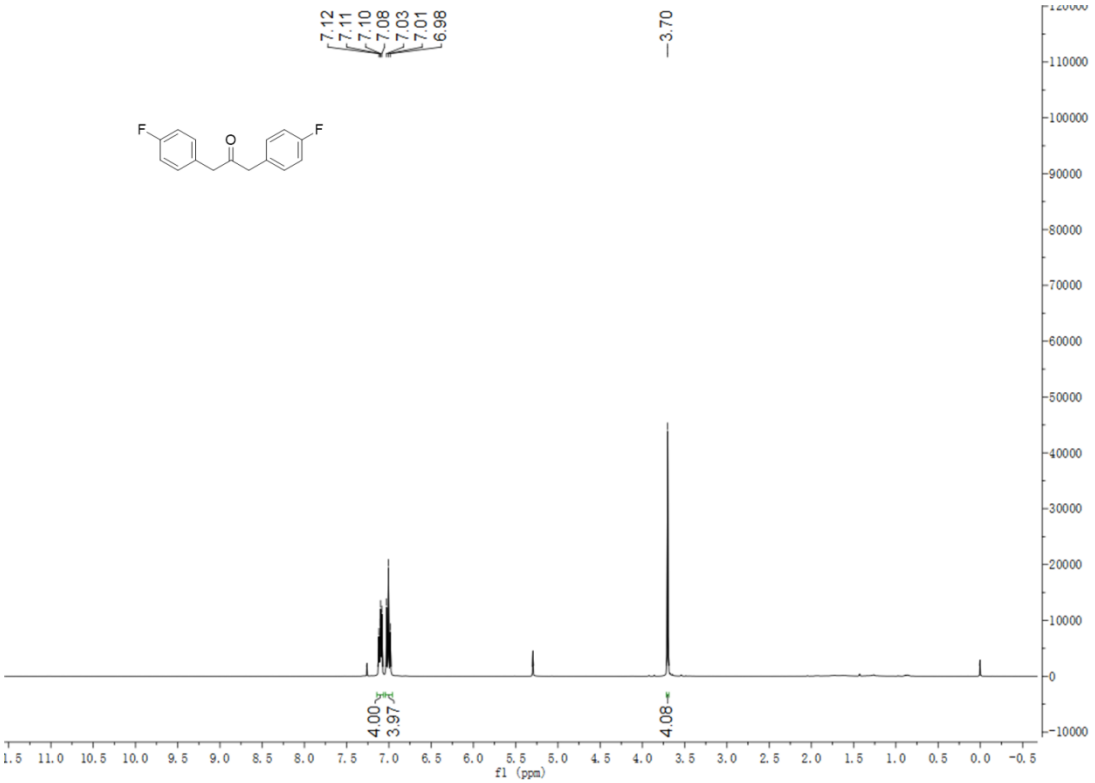


Figure S27. ^{1}H NMR spectrum of 1,3-bis(4-fluorophenyl)propan-2-one.

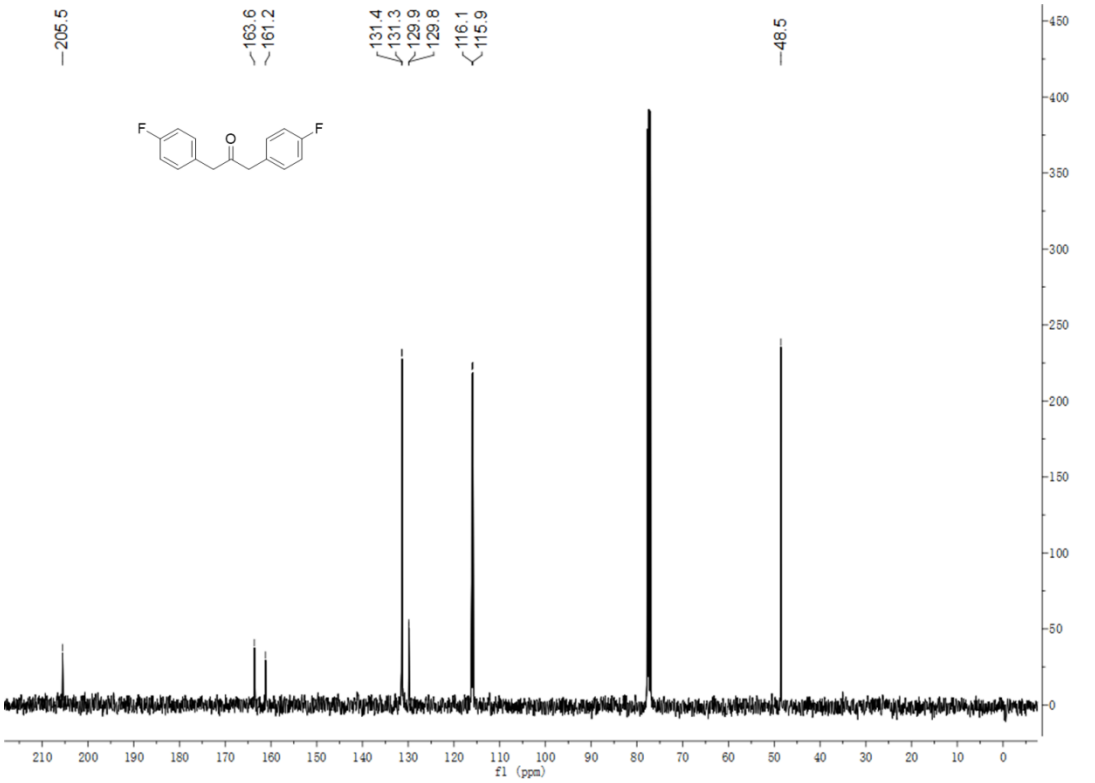


Figure S28. ^{13}C NMR spectrum of 1,3-bis(4-fluorophenyl)propan-2-one.

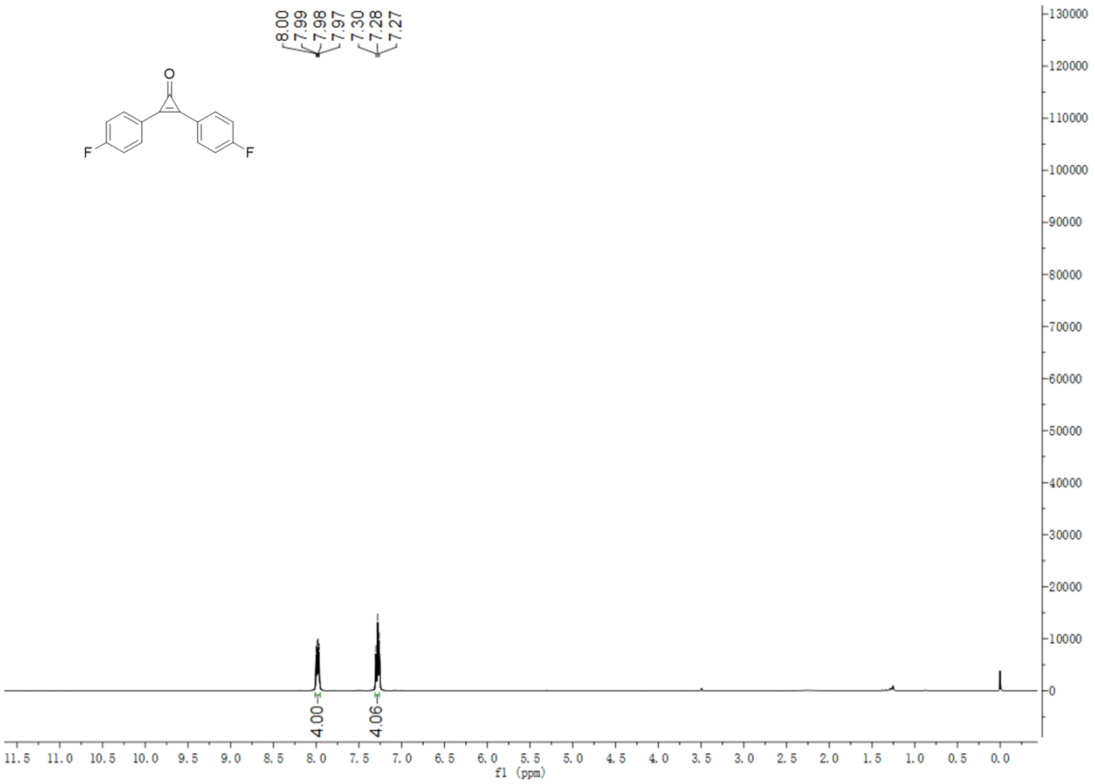


Figure S29. ^{1}H NMR spectrum of **DPCP-F**.

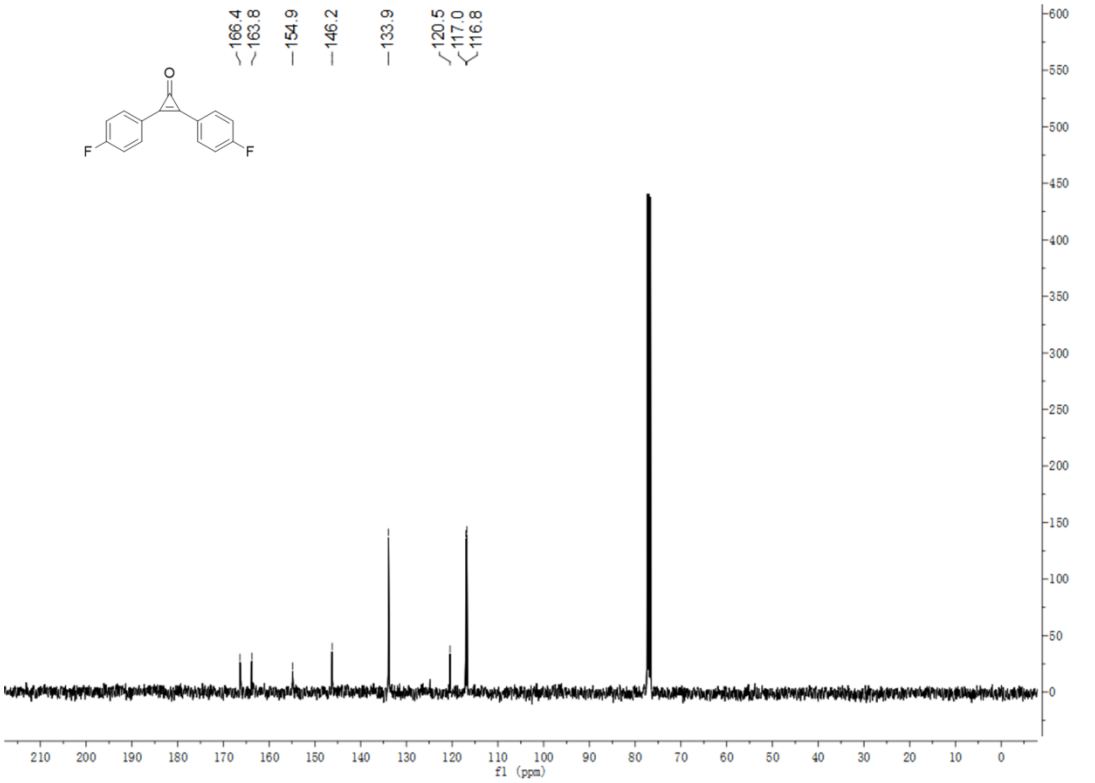


Figure S30. ^{13}C NMR spectrum of **DPCP-F**.

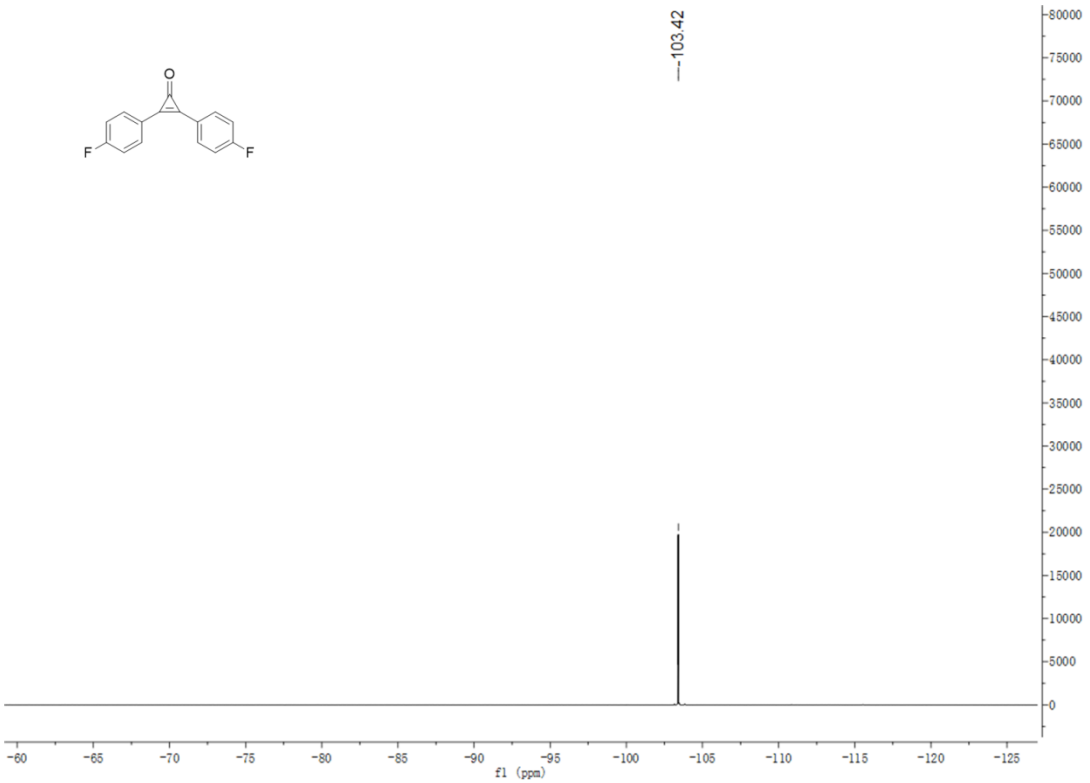


Figure S31. ^{19}F NMR spectrum of **DPCP-F**.

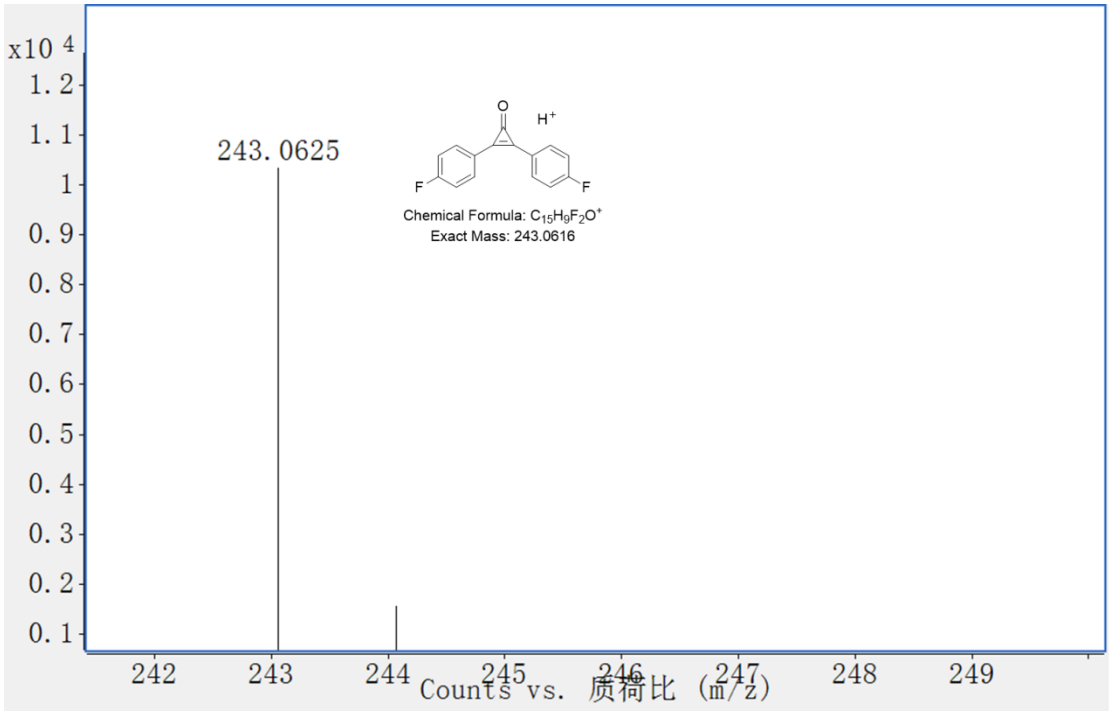


Figure S32. HRMS spectrum of **DPCP-F**.

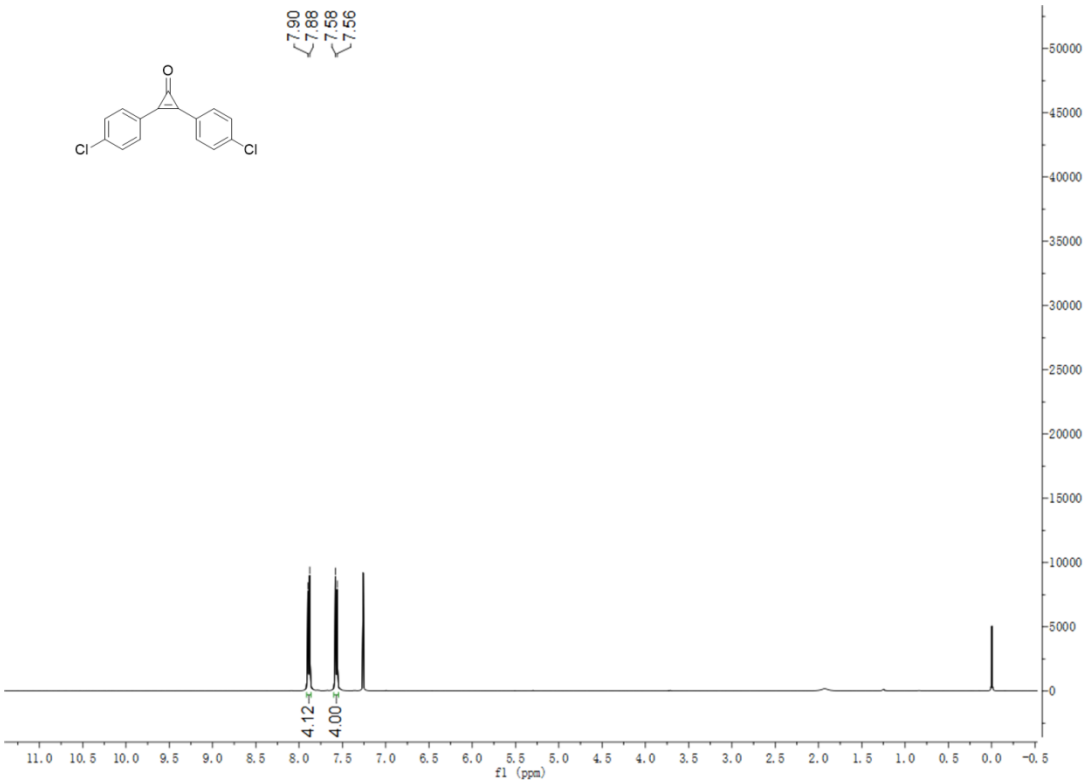


Figure S33. ^{1}H NMR spectrum of **DPCP-Cl**.

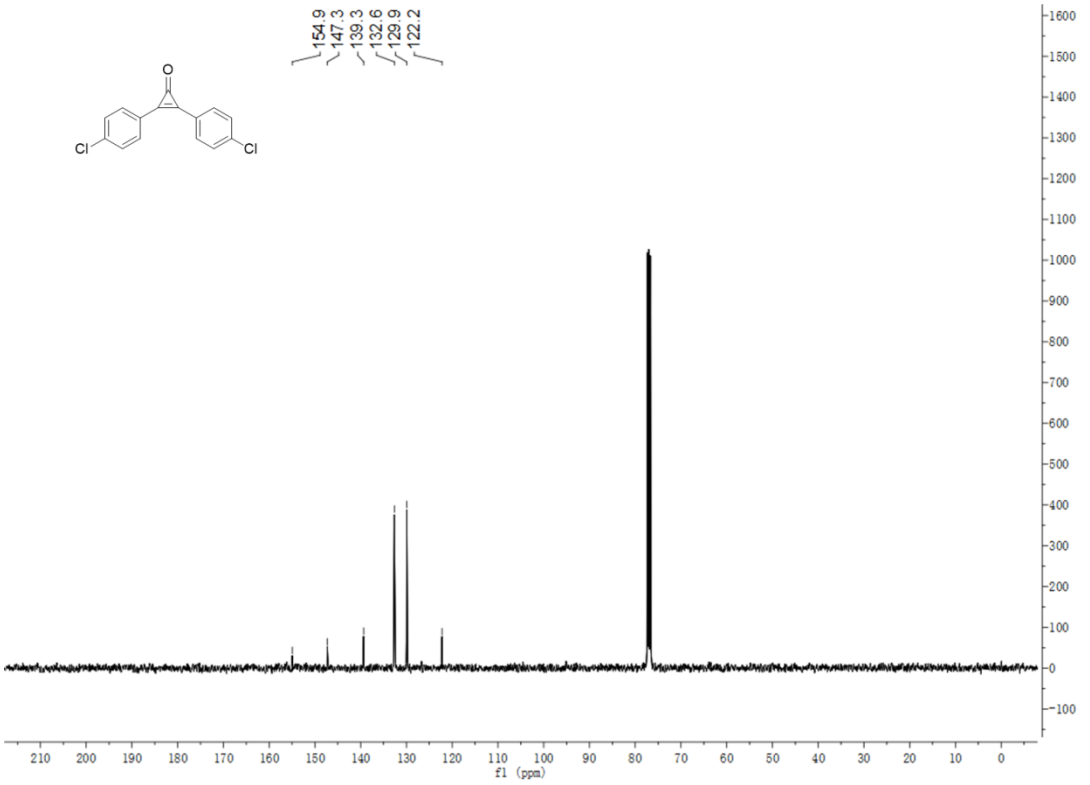


Figure S34. ^{13}C NMR spectrum of **DPCP-Cl**.

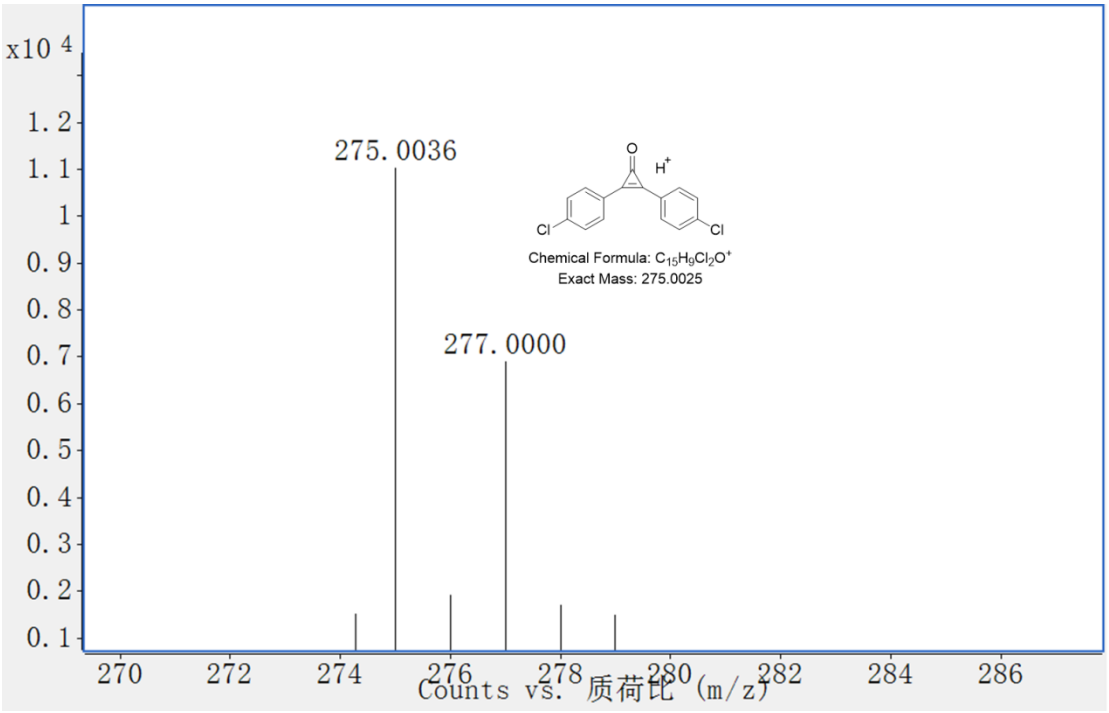


Figure S35. HRMS spectrum of **DPCP-Cl**.

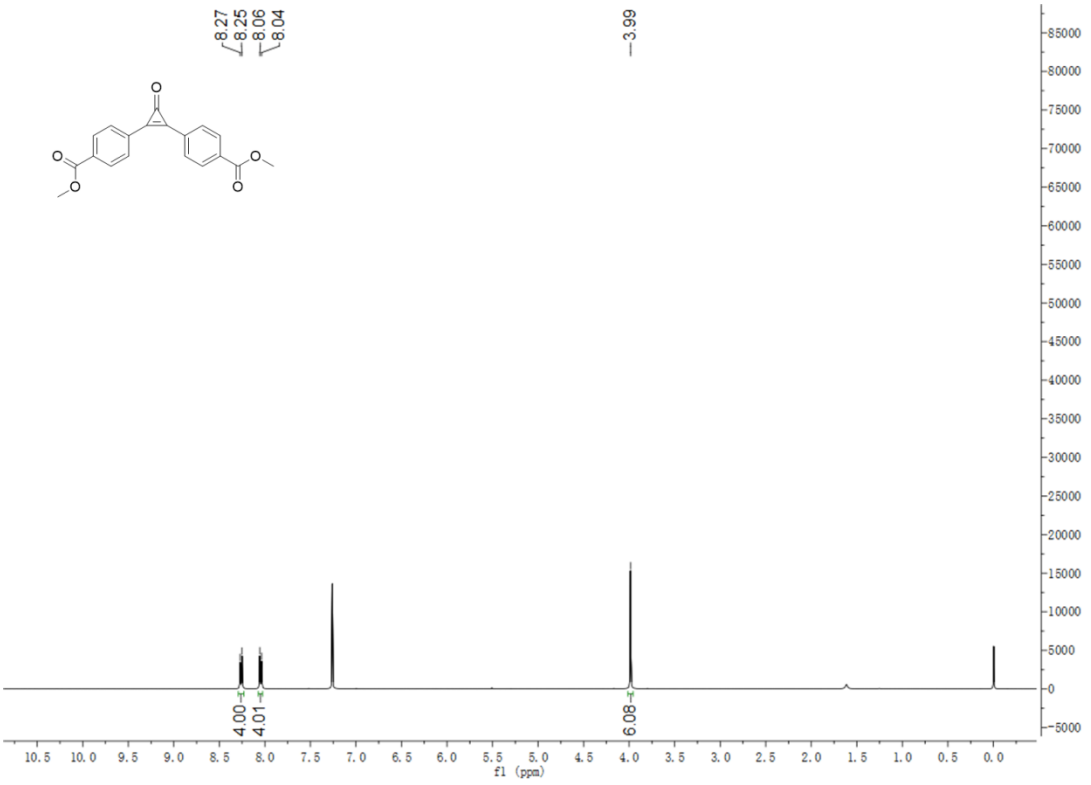


Figure S36. ^{1}H NMR spectrum of **DPCP-COOMe**.

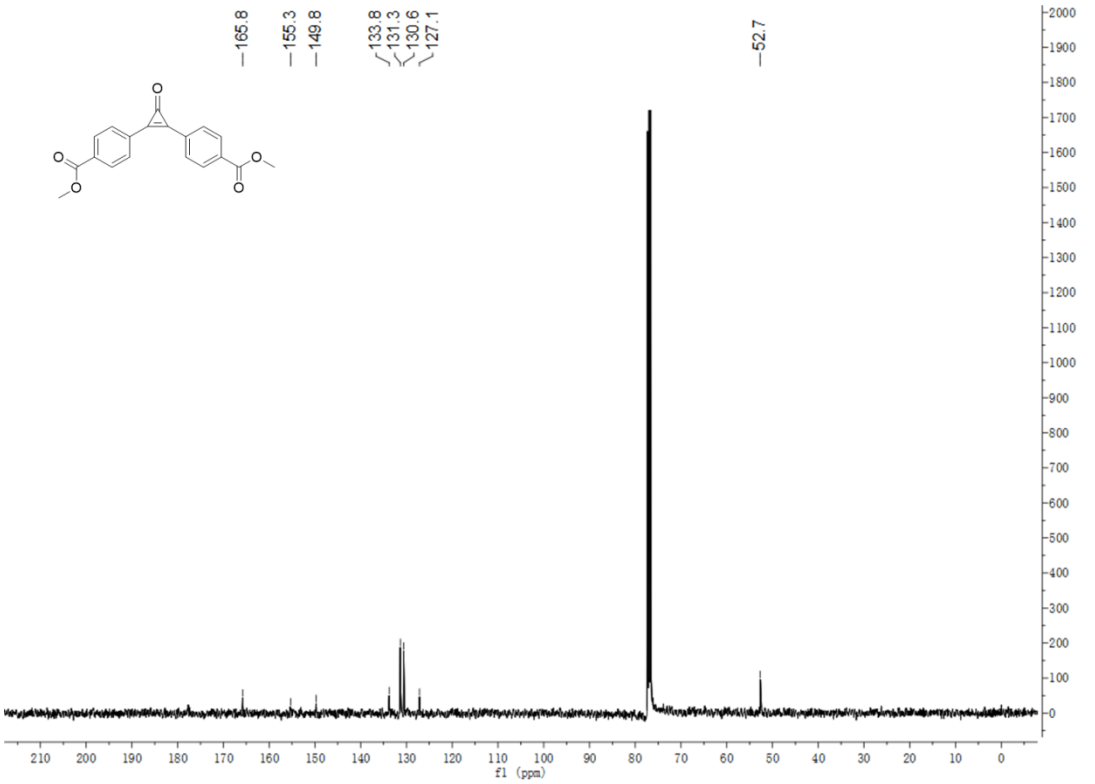


Figure S37. ^{13}C NMR spectrum of **DPCP-COOMe**.

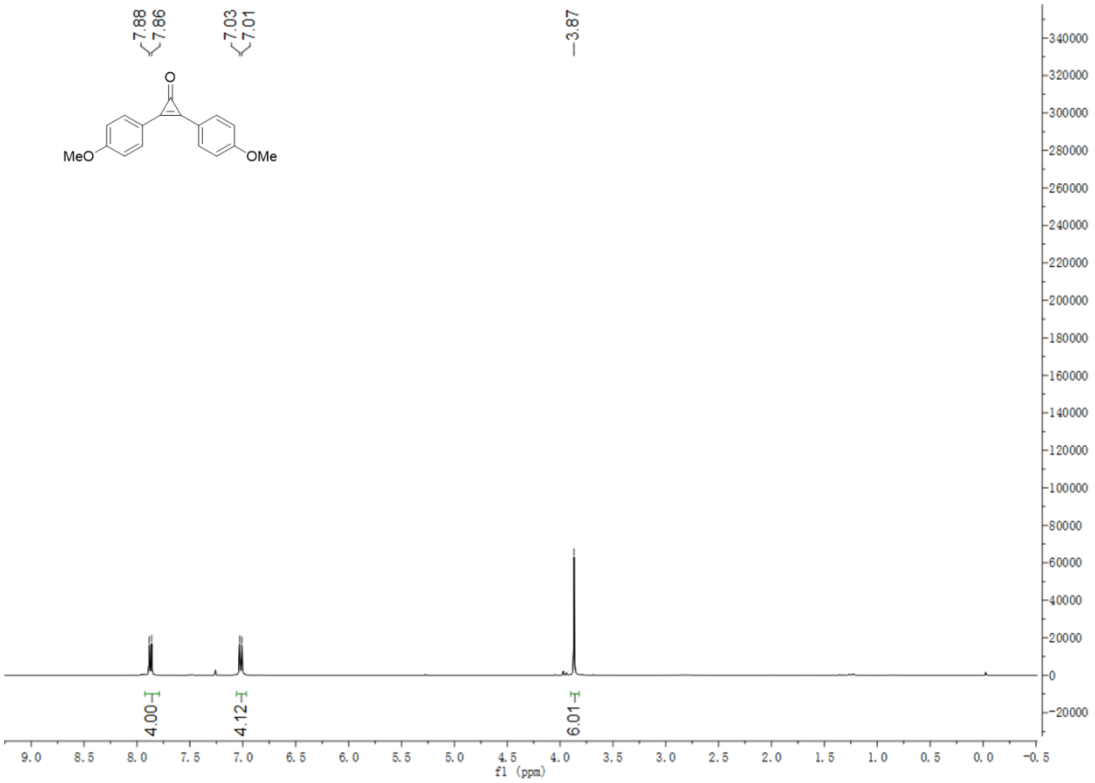


Figure S38. ^{1}H NMR spectrum of **DPCP-OMe**.

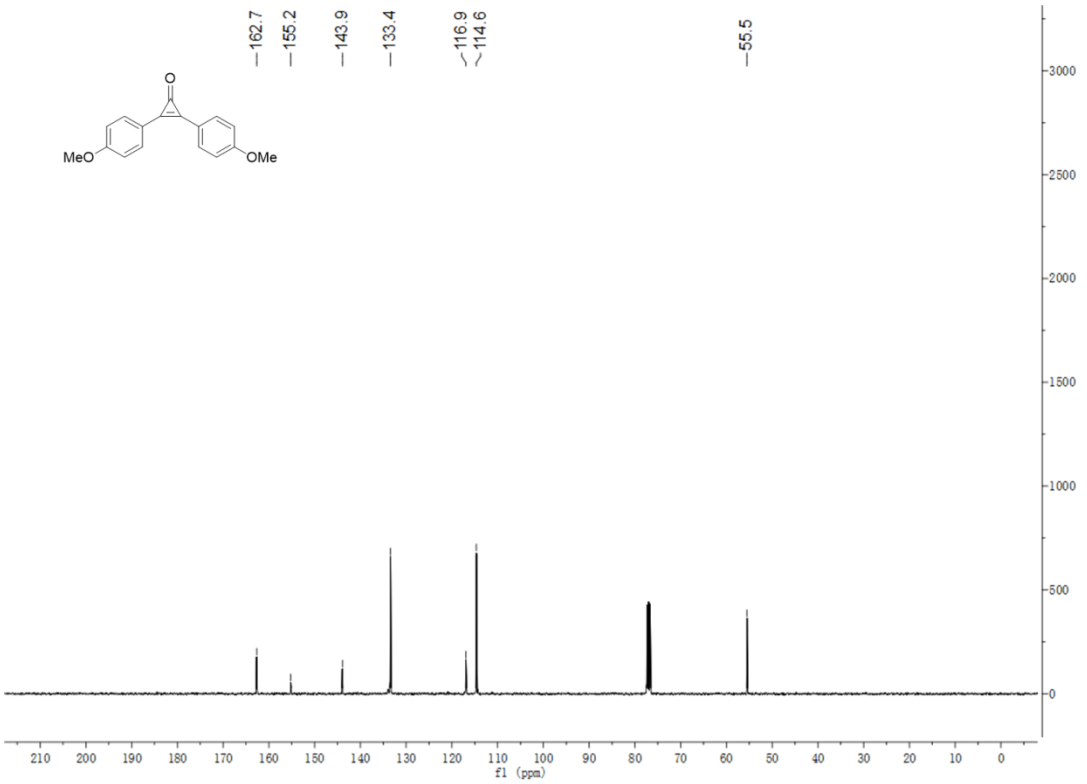


Figure S39. ^{13}C NMR spectrum of **DPCP-OMe**.

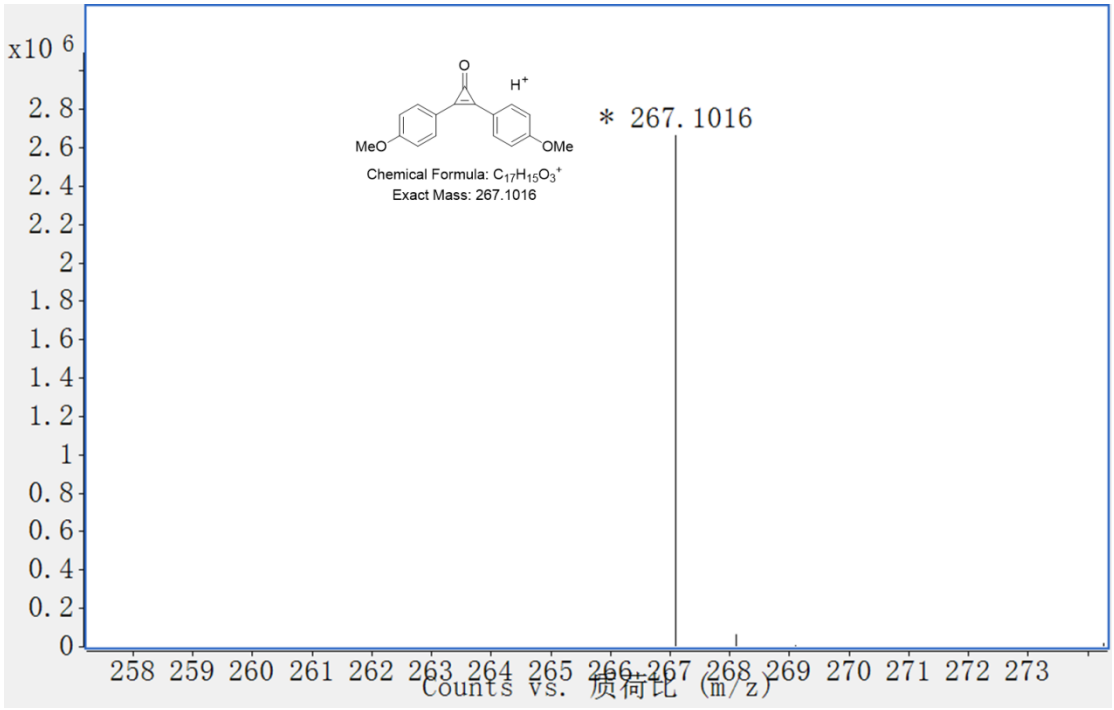


Figure S40. HRMS spectrum of **DPCP-OMe**.

7. Reference

1. I. Ismail, Z. Chen, L. Sun, X. Ji, H. Ye, X. Kang, H. Huang, H. Song, S. G. Bolton, Z. Xi, M. D. Pluth and L. Yi, *Chem. Sci.*, 2020, **11**, 7823-7828.

2. (a) C. M. Vanos and T. H. Lambert, *Angew. Chem. Int. Ed*., 2011, **50**, 12222-12226. (b) X. Li, C. Han, H. Yao and A. Lin, *Org. Lett.*, 2017, **19**, 778-781.

3. X. Kang, H. Huang, C. Jiang, L. Chen, Y. Sang, X. Cai, Y. Dong, L. Sun, X. Wen, Z. Xi and L. Yi, *J. Am. Chem. Soc.*, 2022, **144**, 3957-3967.