Supporting Information

Insights into self-degradation of cysteine esters and amides under physiological conditions yield new cleavable chemistry

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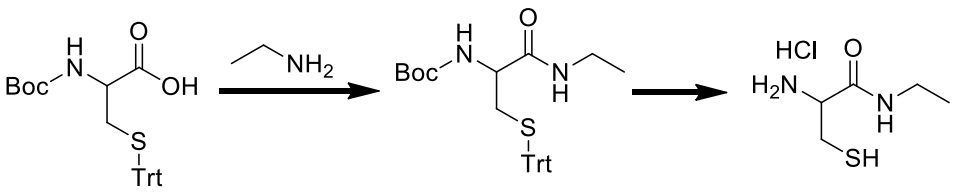
1. General chemicals and instruments

All chemicals and solvents used for synthesis or tests were purchased from commercial suppliers and applied directly in the experiments without further purification. The progress of the reaction was monitored by TLC on precoated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by basic $KMnO_4$, UV light or iodine. Merck silica gel 60 (70-200 mesh) was used for general column chromatography purification. All ^{1}H, $^{13}C\{^{1}H\}$, $^{19}F\{^{1}H\}$, DEPT-135, NOESY, and HMBC NMR spectra were recorded on a Bruker 400 (400 MHz for ^{1}H NMR and 101 MHz for ^{13}C NMR) spectrometer at room temperature. 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; CD_3OD = 4.87 ppm; DMSO-d6 = 2.50 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), q (quartet), bs (broad singlet), m (multiplet). High-resolution mass spectra (HRMS) were recorded on an Agilent 6540 UHD Accurate-Mass Q-TOFLC/MS with positive and/or negative ion modes. The UV-visible spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer (METASH, China). Fluorescence studies were performed using F-280 spectrophotometer (Tianjin Gangdong Sci & Tech., Development. Co., Ltd). AGELA TECHNOLOGIES HPLC LC-10F with C18 column (4.6 mm x 250 mm) was employed for HPLC analysis, and different detection wavelengths (215 nm, 254 nm, 353 nm) were used for different compounds. Circular dichroism (CD) spectra were obtained on circular dichroism spectrometer J-815.

2. Discovery of spontaneous H_2S release from cysteine derivatives



Synthesis of CysNHEt. To a solution of boc-L-Cys(trt)-OH (928 mg, 2 mmol) in 5 mL DMF, 2-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU, 1.14 g, 3 mmol) and 3 mL $EtNH_2$ (2 M in THF) were added for 1 h of reaction at r.t. The reaction solution was mixed with 50 mL water, and the product was exacted by EtOAc. The 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 with EtOAc/ petroleum ether (1/6) to give a white solid boc-Cys(trt)-NHEt (573 mg, 58%). Boc-Cys(trt)-NHEt and triethylsilane (TES, 390 μL) were dissolved in 3 mL TFA/CH_2Cl_2 (1:1) for 30 min of reaction. After removing the solvent under reduced pressure, the residue was purified by silica gel column chromatography with MeOH/CH_2Cl_2 (1/10) containing 0.5% HCl (from 4 N in dioxane) to give a white solid L-CysNHEt•HCl. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.80 (t, J = 4.9 Hz, 1H), 8.50 (bs, 2H), 3.94 (bs, 1H), 3.17-3.09 (m, 2H), 3.03-2.93 (m, 2H), 2.86 (bs, 1H), 1.05 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 166.3, 53.9, 33.8, 25.1, 14.5. HRMS (ESI) for $[M+H]^+$: m/z 149.0762 (calcd for $C_5H_{13}N_2OS^+$, 149.0743).

Methylene Blue Assay (MBA) calibration curve. Normally, H_2S concentration was quantitatively determined via the standard MBA.[1] The stock solutions of 1% $Zn(OAc)_2$ in water, 30 mM $FeCl_3$ in 1.2 M HCl, 20 mM *N*,*N*-dimethyl-*p*-phenylene diamine in 7.2 M HCl were prepared and stored at 4 ºC. The methylene blue cocktail solution was freshly prepared by mixing $Zn(OAc)_2$, $FeCl_3$, *N*,*N*-dimethyl-*p*-phenylene diamine solutions in 1:2:2 (v:v:v). A solution (1.8 mL) containing 0.9 mL of the methylene blue cocktail and 0.9 mL degassed PBS in cuvette was used as MBA background solution. A 250 mM stock solution of Na_2S in degassed PBS was prepared on ice and diluted to 1-10 mM. Immediately after the dilution, Na_2S solution was added to the 1.8 mL MBA solution for final concentrations of 10, 20, 30, 40, and 50 μM, respectively. Solutions were mixed thoroughly and incubated at 25 ºC for 1 h. Absorbance values at 670 nm were measured for the MBA calibration curve.

H_2S release trapped by MBA. All Cys derivatives were dissolved in degassed PBS buffer directly and freshly before the tests. Different Cys derivatives at 2 mM were

dissolved in PBS (10-200 mM, pH 6.0-9.0) and incubated at 25 ºC. At a certain time-point, 0.9 mL of the reaction solution was mixed with 0.9 mL of the methylene blue cocktail for another 1 h of incubation. The resulted solution was checked by absorbance at 670 nm to determine the H_2S concentration. In addition, high concentrations (10, 15, and 20 mM) of L-Cys or L-CysNHEt were incubated in PBS (50 mM, pH 7.4) at 25 ºC for 4 h before the MBA tests. For time-dependent tests, 15 mM L-CysNHEt was incubated for 2, 4, 6, or 8 h in PBS (50 mM, pH 7.4). For a commercialized dipeptide L-Cys-Gly, high concentration solutions (10, 15, 20 mM) in PBS (50 mM, pH 7.4) were incubated at 25 ºC for 2 h, or 15 mM solutions were incubated for different times (0.5, 1, 2, 3 h), and then each 0.3 mL of the reaction solution was mixed with 0.3 mL the methylene blue cocktail for 1 h of incubation. Each experiment was performed in triplicate unless stated otherwise.

H_2S release detected by a H_2S-specific fluorescent probe. We also detected the H_2S release from these Cys derivatives by fluorescent spectra based on a fluorescence probe **Cy7-NBD**.[2] Different analytes (5 mM) were dissolved in degassed PBS buffers (50 mM, pH = 7.4) for 0, 4, or 8 h of incubation, and then **Cy7-NBD** (10 μM) was added for another 5 min of incubation. The fluorescence spectra of all solutions were recorded with excitation at 730 nm.

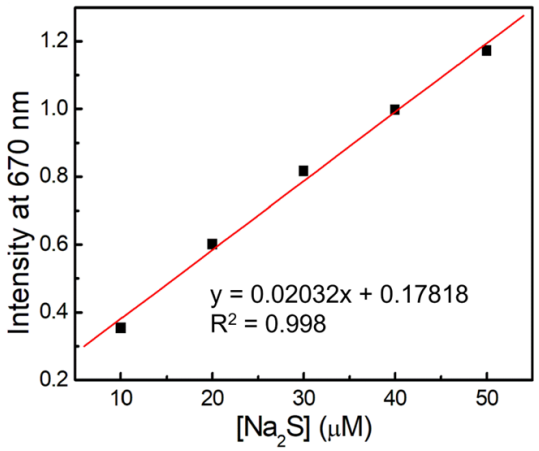


Fig. S1. MBA calibration curve of the intensities at 670 nm versus the Na_2S concentrations.

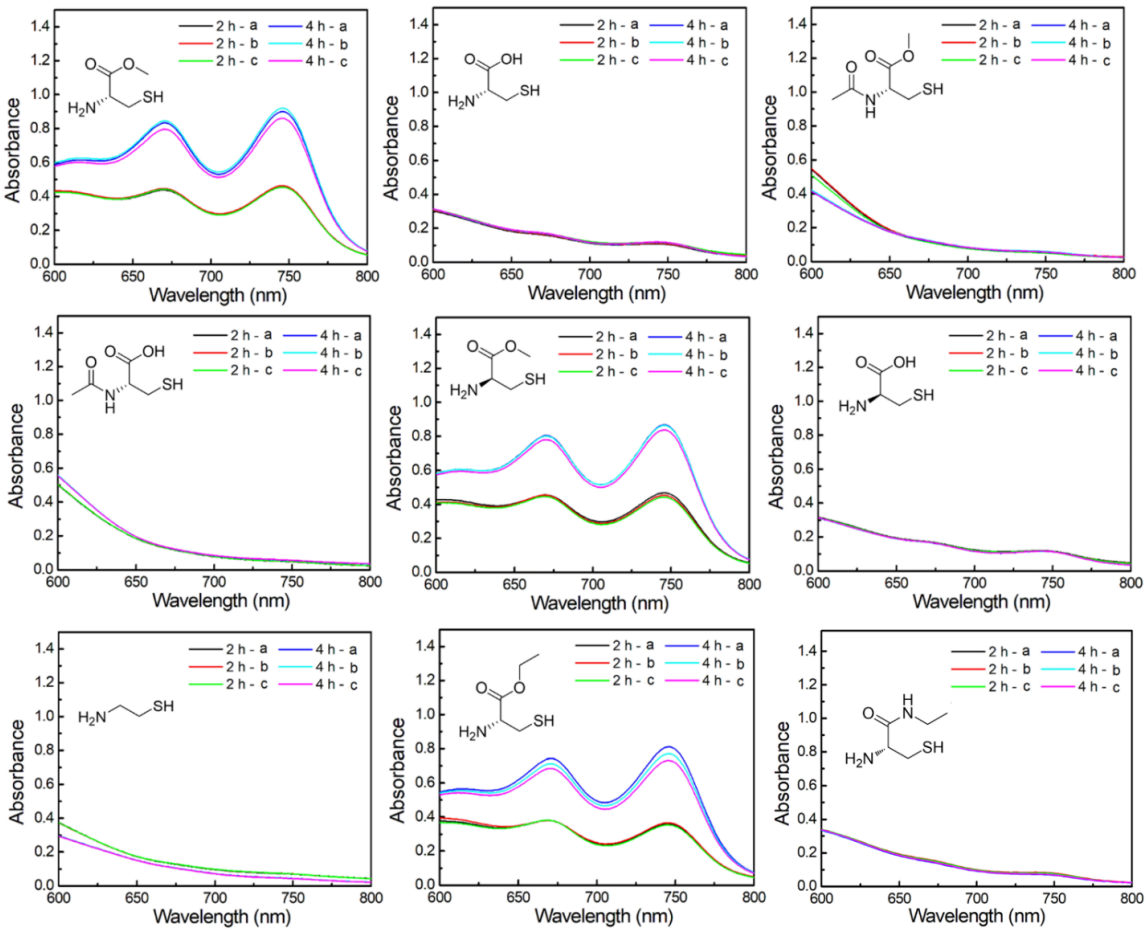


Fig. S2. Absorbance spectra of MBA tests for 2 mM analytes (each structure inset) in PBS buffer (200 mM, pH = 7.4) after 2 or 4 h of incubation. The tests were performed in triplicate. L-CysOMe, D-CysOMe, and L-CysOEt resulted in a large absorbance at 670 nm, and other species did not result in obvious absorbance at 670 nm. The intensities at 670 nm were used to calculate the H_2S concentrations based on the calibration curve in Fig. S1.

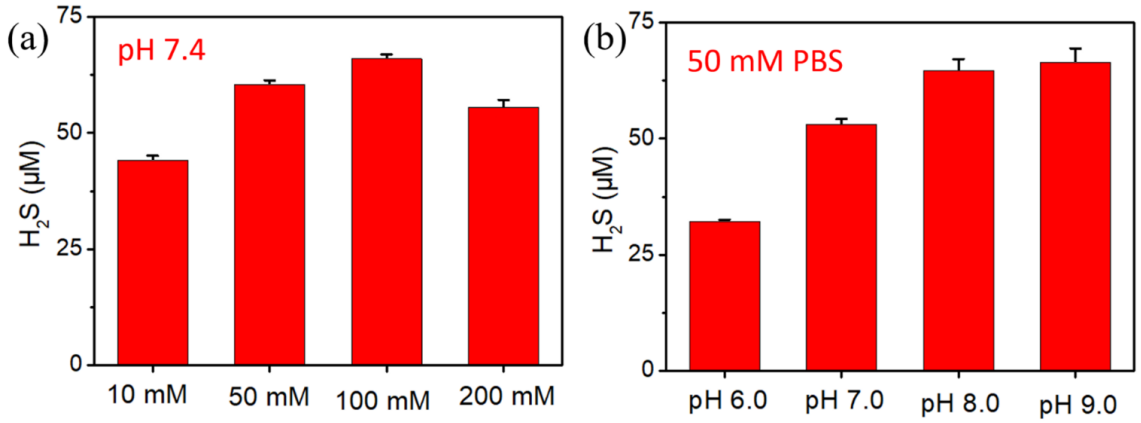


Fig. S3. (a, b) Quantitative determination of the H_2S generation from 2 mM CysOEt in a) different concentrations of PBS (pH = 7.4) or b) different pH values of PBS (50 mM) at 25 ºC for 4 h of incubation via MBA. The results are expressed as mean ± S.D. (n = 3).

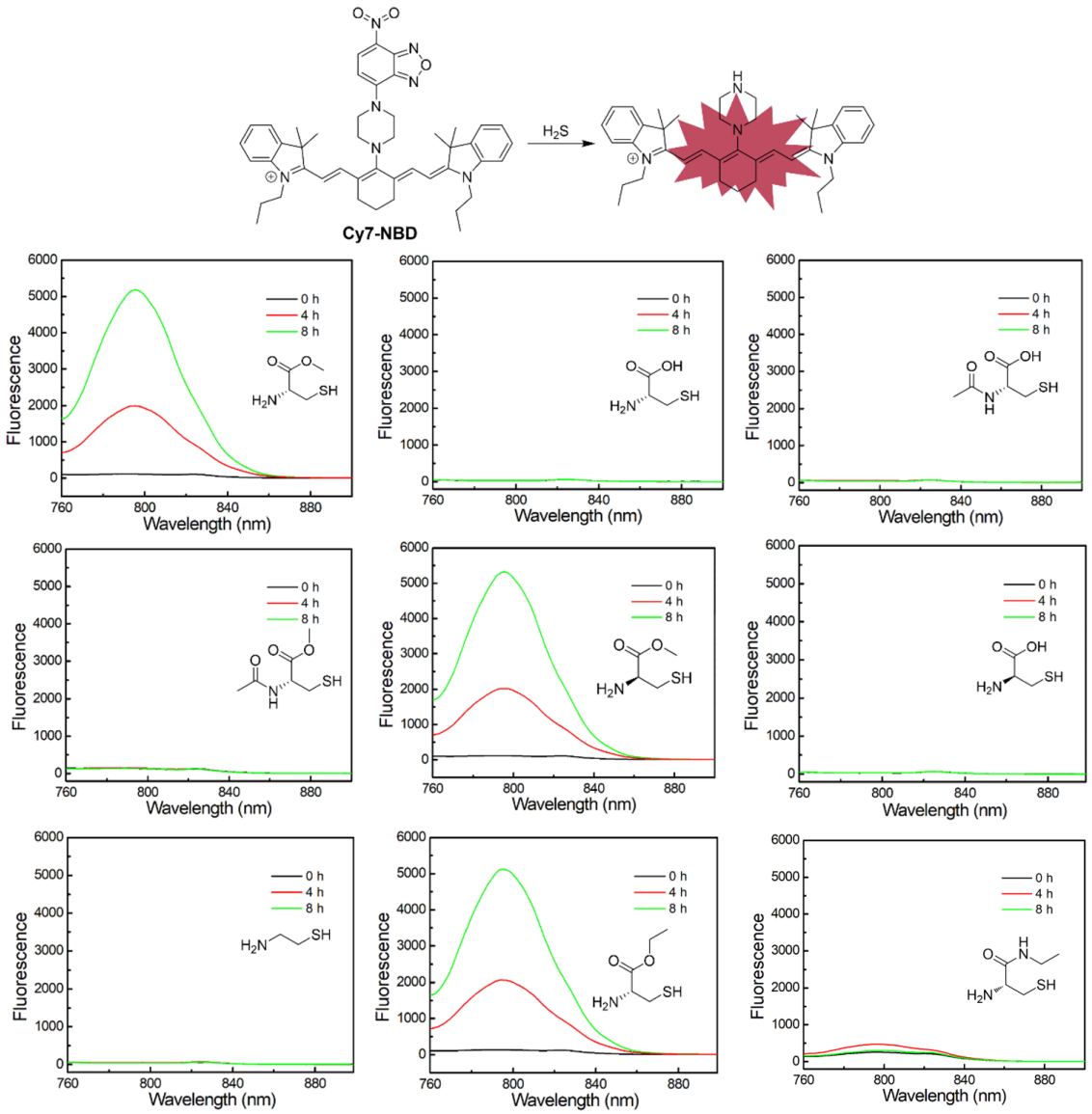


Fig. S4. Emission spectra of a H_2S-specific probe **Cy7-NBD**[2] (10 μM) in the presence of 5 mM analytes (each structure inset) at 0, 4 or 8 h of incubation in PBS buffer (50 mM, pH = 7.4). L-CysOMe, D-CysOMe, and L-CysOEt resulted in a large fluorescence enhancement; L-CysNHEt resulted in a slight fluorescence enhancement; other species did not result in noticeable fluorescence enhancement. The results imply that the H_2S release is major from L-CysOMe, D-CysOMe, and L-CysOEt, as well as minor from L-CysNHEt.

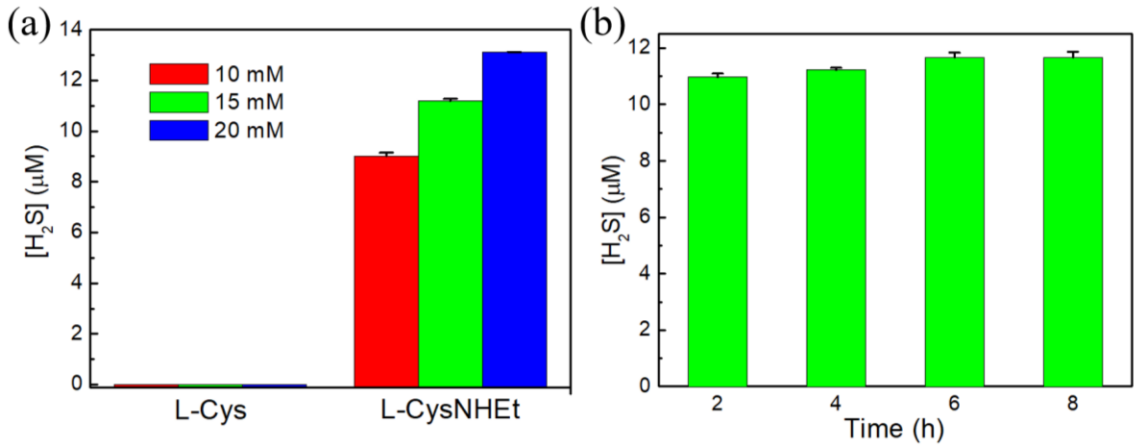


Fig. S5. (a) Quantitative determination of the H_2S generation from high concentrations (10, 15, 20 mM) of Cys or CysNHEt in PBS (50 mM, pH = 7.4) for 4 h of incubation at 25 ºC via MBA. (b) Quantitative determination of time-dependent H_2S generation from 15 mM L-CysNHEt in PBS (50 mM, pH = 7.4) at 25 ºC via MBA. The results are expressed as mean ± S.D. (n = 3).

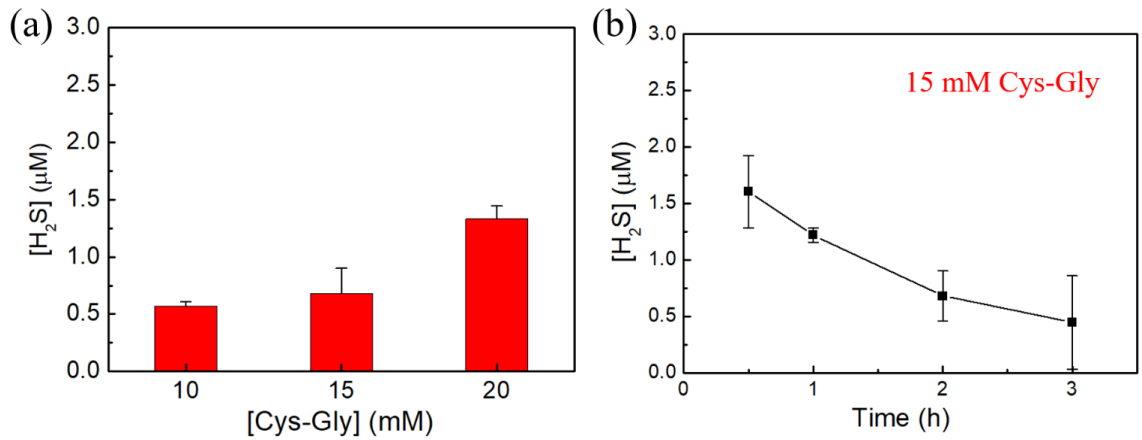


Fig. S6. (a) Quantitative determination of the H_2S generation from high concentrations (10, 15, 20 mM) of dipeptide Cys-Gly in sealed PBS (50 mM, pH = 7.4) for 2 h of incubation at 25 ºC via MBA. (b) Quantitative determination of time-dependent H_2S generation from 15 mM Cys-Gly in PBS (50 mM, pH = 7.4) at 25 ºC via MBA. The results are expressed as mean ± S.D. (n = 3).

3. Insights into the self-degradation of esterified cysteines

3.1 HRMS analysis of the self-degraded reactions

20 mM L-CysOMe or L-CysOEt was dissolved in PBS (200 mM, pH 7.4) for 2 days of incubation at room temperature, and each resulted aliquot was analyzed by HRMS directly.

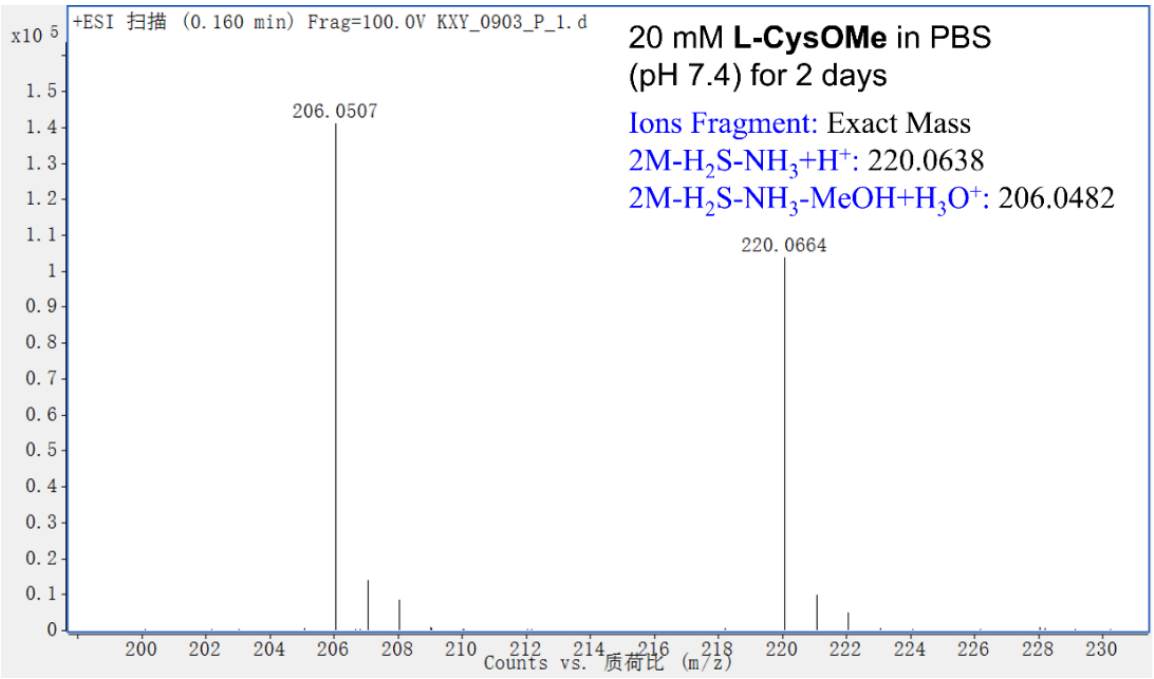


Fig. S7. HRMS analysis of CysOMe (20 mM) in PBS (pH = 7.4) after 2 days of incubation at room temperature.

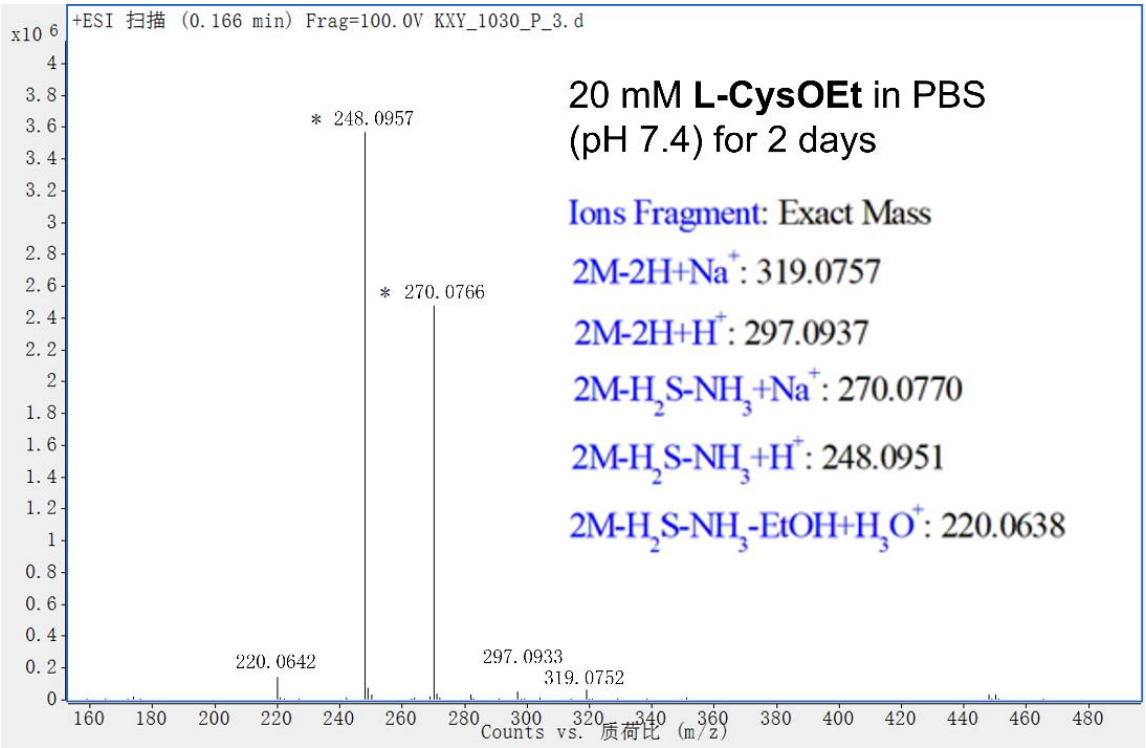


Fig. S8. HRMS analysis of CysOEt (20 mM) in PBS (pH = 7.4) after 2 days of incubation at room temperature.

3.2 NH_3 release trapped by the Indophenol Blue Assay (IBA)

IBA calibration curve.[3] NH_4Cl sample solution was used as the tested NH_3 source. 0.5 mL Phenol (150 mM) solution containing 5 mg sodium nitroprusside was added to 1 mL NH_4Cl solutions (20-200 μM) in a polystyrene centrifuge tube. Then 0.5 mL of hypochlorite (HOCl) solution (300 mM HOCl : 100 mM NaOH = 1: 2 (v:v)) was added to the mixture. The final solution was stored at 25 ºC in the dark for 1 h of incubation. Absorbance values at 640 nm were measured for the calibration curve.

Determination of the release of NH_3 from esterified Cys by IBA. Different concentrations (5 mM or 20 mM) of Cys derivatives were dissolved in PBS buffer (50 mM, pH 7.4) for 2 days of incubation. For determination of high concentration NH_3, the dilution (for 5 mM CysOR: 0.1 mL solution was added to 0.9 mL PBS for 10 times dilution; for 20 mM CysOR: 25 μL solution was added to 975 μL PBS for 40 times dilution) should be required by using the IBA calibration curve. The final NH_3 concentration was estimated by 10×[diluted NH_3 concentration] or 40×[diluted NH_3 concentration] for 5 mM or 20 mM analytes, respectively.

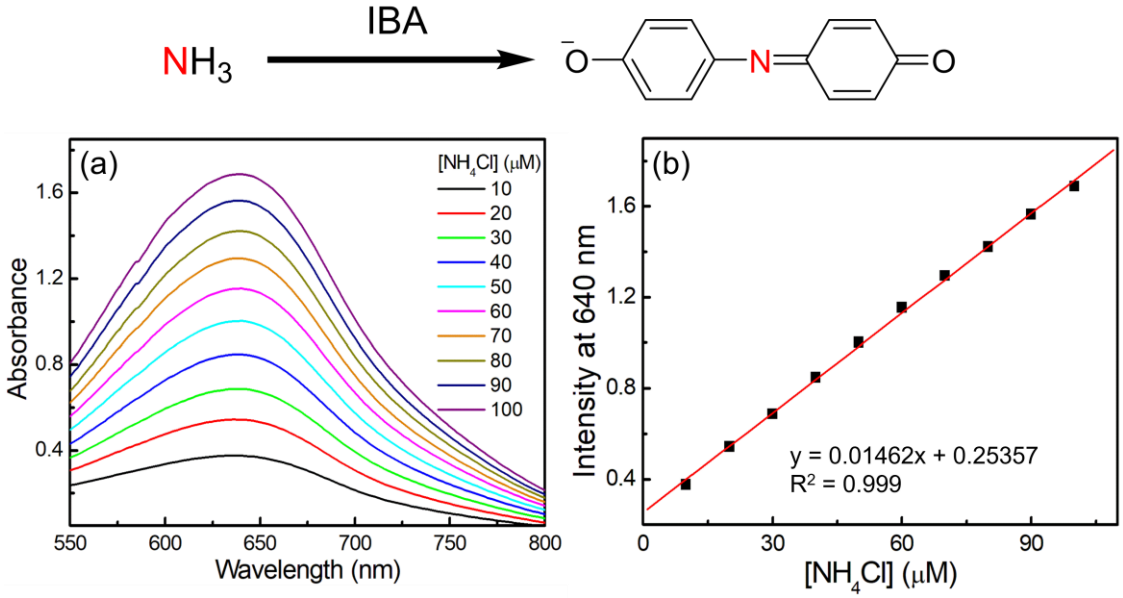


Fig. S9. IBA calibration curve generated by using known concentrations of NH_4Cl. (a) The absorbance spectra of different concentrations of NH_4Cl (10-100 μM) in PBS buffer (pH = 7.4) processed by IBA. (b) IBA calibration curve of the intensities at 640 nm versus the NH_4Cl concentrations.

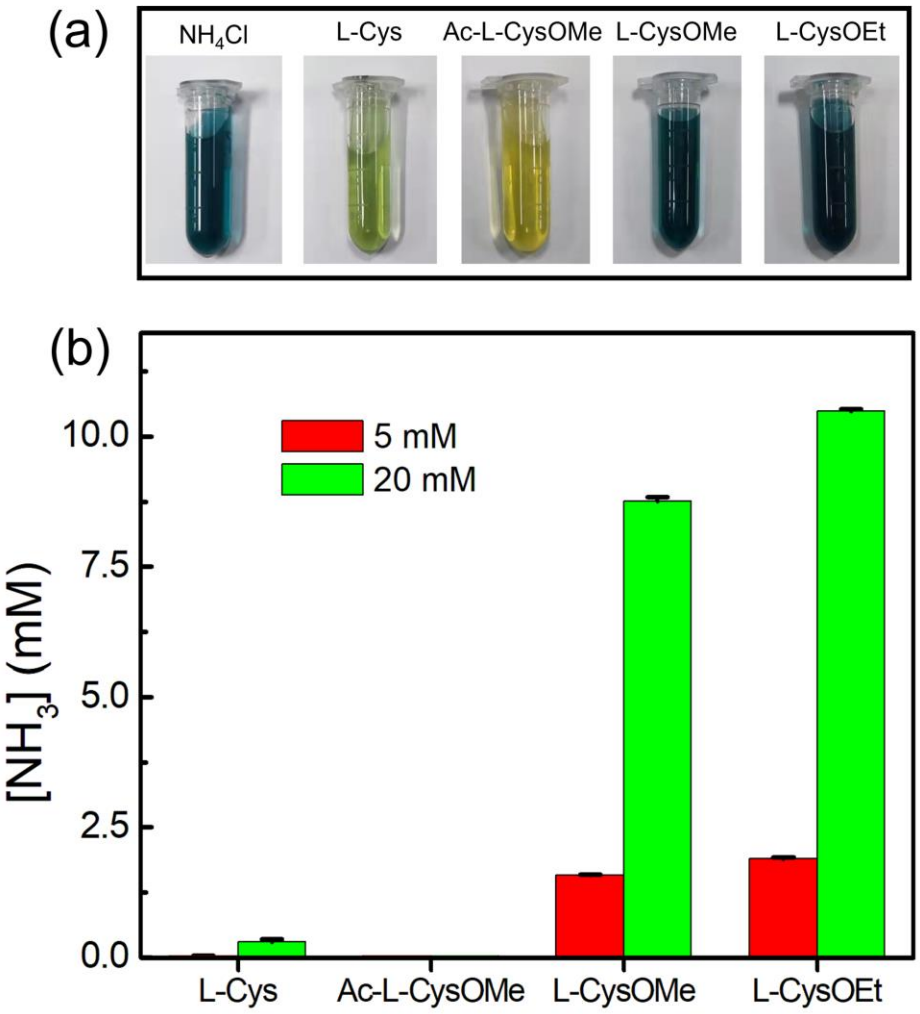
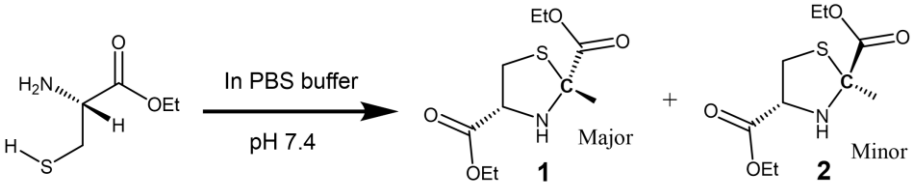


Fig. S10. (a) Photos of indophenol blue generated by 20 mM analytes (indicated inset) in PBS buffer (pH = 7.4) after 2 days of incubation. All reaction solutions were diluted 40 times before tests; blue color for the NH_3 release was observed for CysOR, but not for Ac-L-CysOMe, implying the release of NH_3 from CysOR. (b) Quantitative determination of the NH_3 release from the analytes (at 5 and 20 mM) in PBS buffer (pH = 7.4) after 2 days of incubation; the reaction solution was diluted (10 times for 5 mM; or 40 times for 20 mM) before tests. The results are expressed as mean ± SD (n = 3). It is noted that Cys may degrade slowly in alkaline solutions under the IBA conditions to generate a small amount of NH_3.

3.3 Characterization of organic products from the self-degradation of L-CysOEt



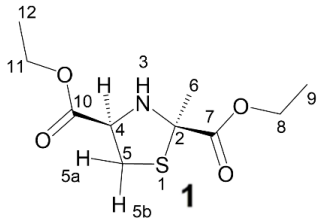
L-Cysteine ethyl ester hydrochloride (1.116 g, 6.0 mmol) was dissolved in 300 mL degassed, sealed PBS buffer (200 mM, pH 7.4). After stirring for 2 days, the solution was extracted with ethyl acetate. Then the collected organic layer was washed with water, brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (100/1) to give colorless oil of diethyl (2*R*,4*R*)-2-methylthiazolidine-2,4-dicarboxylate **1** and diethyl (2*S*,4*R*)-2-methylthiazolidine-2,4-dicarboxylate **2**, with overall isolated yield of 24.9%. HRMS (ESI): m/z 248.0957 $[M+H]^+$ (calcd for $C_{10}H_{18}NO_4S^+$, 248.0951).

For **1** (124 mg, 16.7%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.30-4.15 (m, 4H), 3.95 (dd, J = 10.4, 5.5 Hz, 1H), 3.57 (bs, 1H), 3.32 (dd, J = 10.1, 5.5 Hz, 1H), 2.86-2.76 (m, 1H), 1.64 (s, 3H), 1.33-1.19 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.6, 170.1, 74.6, 65.7, 62.3, 61.7, 40.2, 28.3, 14.2, 14.0.

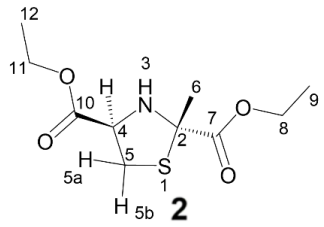
For **2** (61 mg, 8.2%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.42-4.32 (m, 1H), 4.30-4.12 (m, 4H), 3.42-3.32 (m, 1H), 3.10-3.00 (m, 1H), 2.92 (bs, 1H), 1.86 (s, 3H), 1.37-1.20 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.4, 171.4, 76.2, 65.9, 61.8, 61.7, 39.3, 25.4, 14.2, 14.0.

HPLC analysis: L-CysOEt (20 mM) was dissolved in PBS buffer (200 mM, pH 7.4) for 2 days of incubation at room temperature, and the resulted solution was directly analyzed by HPLC. Conditions: detection wavelength: 254 nm; flow 1.0 mL/min; buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: methanol; elution condition: 0-2 min, buffer B: 5-55%; 2-18 min, buffer B: 55-85%; 18-20 min, buffer B: 85-5%. The solutions of **1** (10 mM) and **2** (10 mM) were also monitored by HPLC under the same conditions. The yield of **1** + **2** from the self-degradation of L-CysOEt was estimated by the ratio of peak integral areas from HPLC traces (Fig. S12).

Circular Dichroism (CD) spectroscopy: Compounds **1** and **2** were respectively dissolved in H_2O to prepare 0.5 mg/mL of solution. Then CD & UV-vis spectra were detected by circular dichroism spectrometer J-815 simultaneously.



Position	δ_H (mult., J_{H-H} in Hz)	δc
2		74.6
3	3.57 (bs, 1H)	
4	3.95 (dd, J = 10.4, 5.5 Hz, 1H)	65.7
5	5a (2.86-2.76 (m, 1H) 5b (3.32 (dd, J = 10.1, 5.5 Hz, 1H))	40.2
6	1.64 (s, 3H)	28.3
7		172.6
8	4.30 – 4.15 (m, 4H)	62.3
9	1.33-1.19 (m, 6H)	14.2
10		170.1
11	4.30 – 4.15 (m, 4H)	61.7
12	1.33-1.19 (m, 6H)	14.0



Position	δ_H (mult., J_{H-H} in Hz)	δc
2		76.2
3	2.92 (bs, 1H)	
4	4.42 – 4.32 (m, 1H)	65.9
5	5a (3.10-3.00 (m, 1H)) 5b (3.42-3.32 (m, 1H))	39.3
6	1.86 (s, 3H)	25.4
7		172.4
8	4.30 – 4.12 (m, 4H)	61.8
9	1.37-1.20 (m, 6H)	14.2
10		171.4
11	4.30 – 4.12 (m, 4H)	61.7
12	1.37-1.20 (m, 6H)	14.0

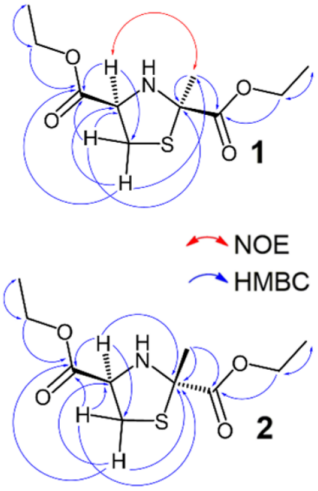


Fig. S11. Key NOESY and HMBC correlations, and full assessments of NMR spectroscopic data for **1** and **2**.

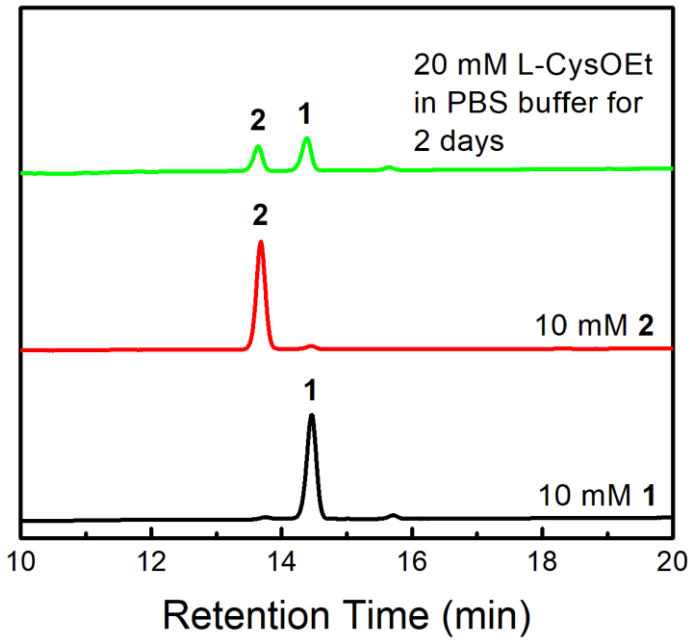


Fig. S12. Comparative HPLC traces of the L-CysOEt (20 mM) solution in PBS buffer (pH 7.4) after 2 days of incubation (green line), and the solutions of isolated **1** (10 mM) (black line) and **2** (10 mM) (red line). The overall yield (~53%) of organic products (**1** + **2**) is estimated as (the ratio of integral areas of **1** to that of 10 mM **1**) + (the ratio of integral areas of **2** to that of 10 mM **2**) from HPLC analysis. Detection wavelength: 254 nm.

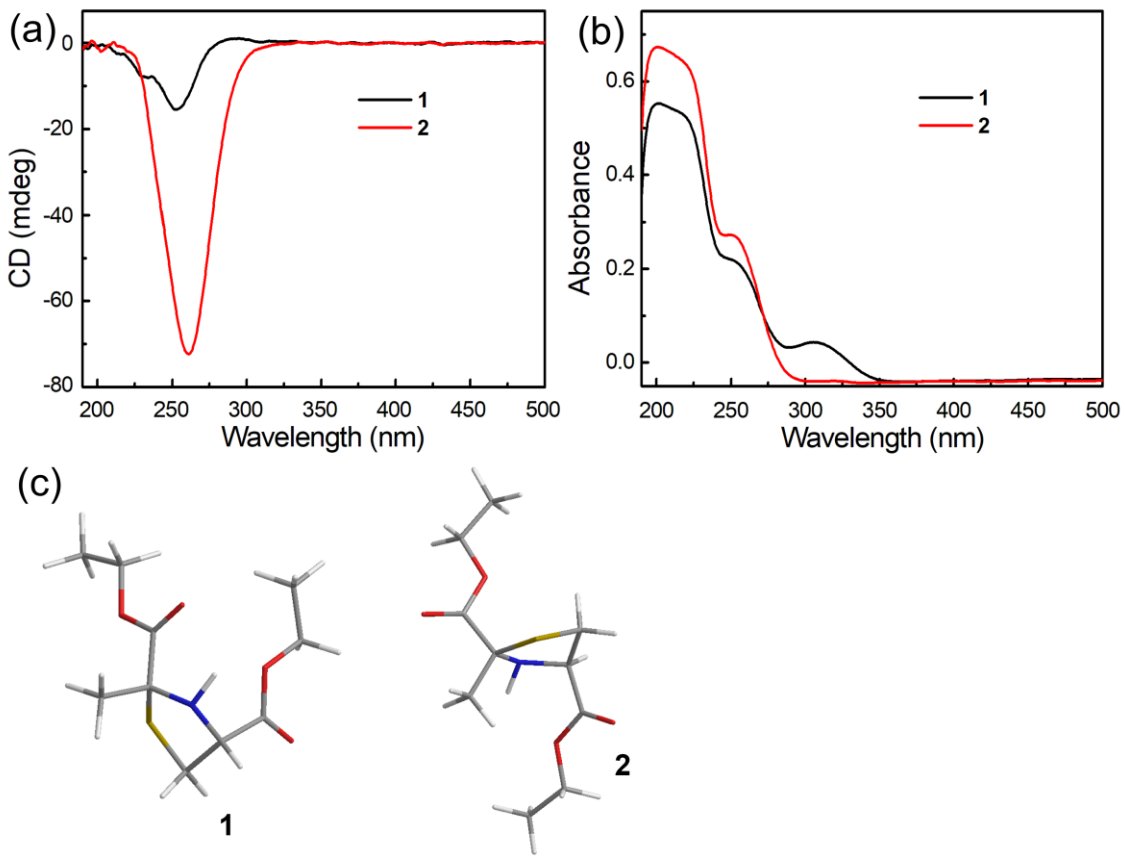
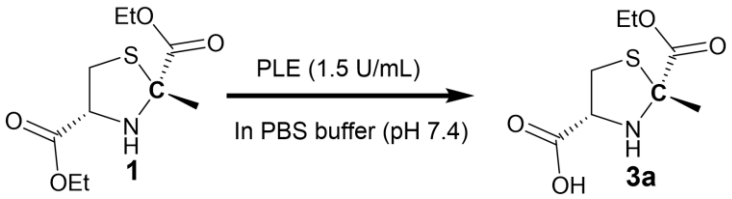


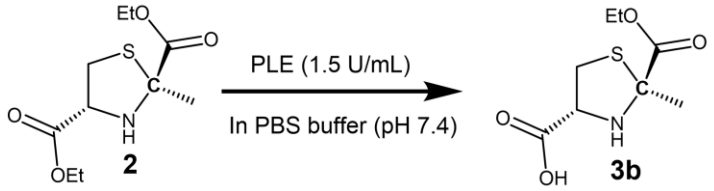
Fig. S13. (a) CD and (b) absorbance spectra of **1** and **2** (0.5 mg/mL in H_2O), respectively. (c) ChemDraw 3D models (MM2 energy-minimized)[4] of **1** and **2** suggest a partly left-hand helical distortion in **2**; white, grey, blue, red, and yellow sticks represent H, C, N, O, and S atoms, respectively.

3.4 Characterization of enzyme-deesterified products from 1 and 2



To a solution of **1** (200 mg, 0.81 mmol) in 50 mL PBS (50 mM, pH 7.4), porcine liver esterase (PLE, final 1.5 U/mL) was added. After 3 h of incubation at room temperature, the pH of the solution was adjusted to 2-3 by HCl (1 N aqueous solution), and the resulted solution was extracted by EtOAc. The collected organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (100/5) to give a white solid (2*R*,4*R*)-2-methylthiazolidine-2,4-dicarboxylic acid 2-ethyl ester **3a** (72 mg, isolated yield 41%).

The PLE-catalyzed hydrolysis yields monocarboxylic acid product, probably due to steric congestion of another ester function.[5] ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.55 (bs, 2H), 4.33 (dd, J = 8.9, 6.8 Hz, 1H), 4.26 (q, J = 7.1 Hz, 2H), 3.49 (dd, J = 11.4, 6.7 Hz, 1H), 3.18 (dd, J = 11.3, 9.0 Hz, 1H), 1.81 (s, 3H), 1.31 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.5, 173.3, 75.0, 66.3, 62.9, 38.3, 25.9, 14.1. HRMS (ESI): m/z 218.0505 $[M-H]^-$ (calcd for $C_8H_{12}NO_4S^-$, 218.0493).



To a solution of **2** (120 mg, 0.49 mmol) in 30 mL PBS (50 mM, pH 7.4), PLE (final 1.5 U/mL) was added at room temperature. After 3 h reaction, the pH of the solution was adjusted to 2-3 by HCl (1 N). Then the resulted solution was extracted by EtOAc. The collected organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (100/5) to give a white solid (2*S*,4*R*)-2-methylthiazolidine-2,4-dicarboxylic acid 2-ethyl ester **3b** (58 mg, isolated yield 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50-7.37 (m, 2H), 4.49-4.41 (m, 1H), 4.18 (q, J = 7.1 Hz, 2H), 3.38 (dd, J = 10.5, 7.5 Hz, 1H), 3.25 (dd, J = 10.4, 6.7 Hz, 1H), 1.87 (s, 3H), 1.27 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.1, 172.5, 75.4, 65.6, 62.3, 37.9, 25.4, 14.1. HRMS (ESI): m/z 218.0505 $[M-H]^-$ (calcd for $C_8H_{12}NO_4S^-$, 218.0493).

The above reaction mixtures of **1** or **2** with PLE were also monitored by time-dependent HPLC analysis (Fig. S14). Conditions: detection wavelength: 215 nm; flow 1.0 mL/min; buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: methanol; elution condition: 0-2 min, buffer B: 5-55%; 2-18 min, buffer B: 55-85%; 18-20 min, buffer B: 85-5%.

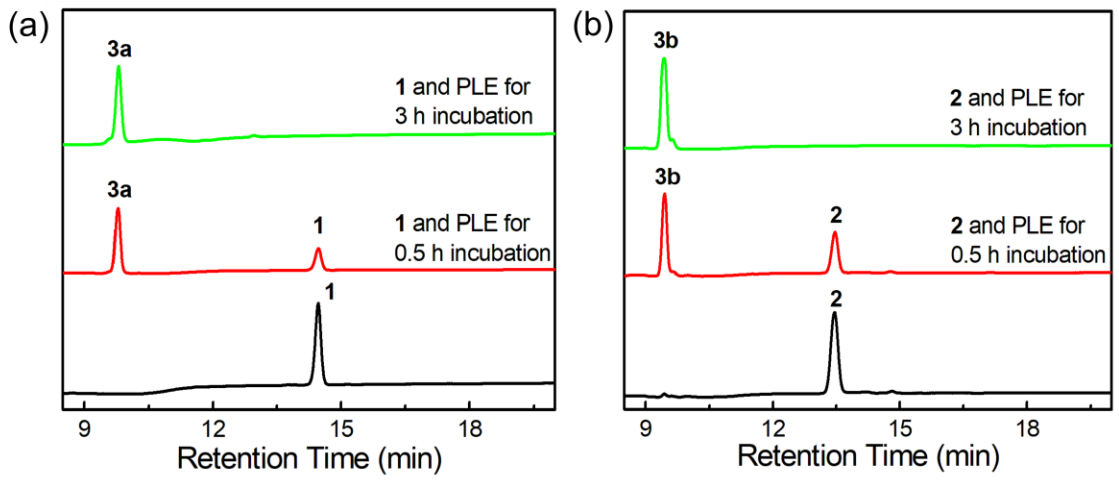
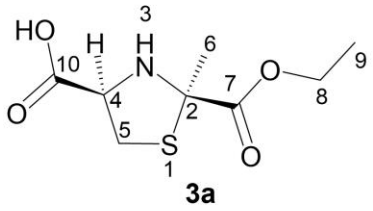
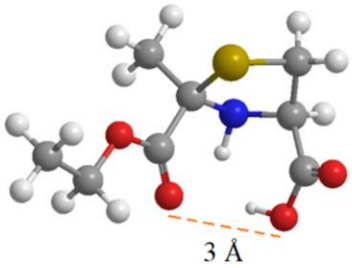
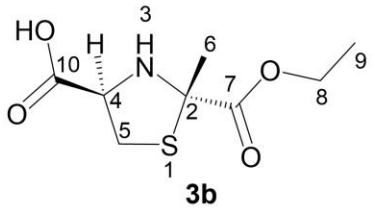


Fig. S14. Time-dependent HPLC traces of the reactions of 4 mg/mL **1** (a) or **2** (b) in the presence of PLE (1.5 U/mL) in PBS buffer (50 mM, pH 7.4). The control traces of pure **1** and **2** are also shown. Detection wavelength: 215 nm.



Position	δc (**3a**)	δc (**1**)
2	75.0	74.6
4	66.3	65.7
5	38.3	40.2
6	25.9	28.3
* 7	173.3	172.6
8	62.9	62.3
9	14.1	14.2
* 10	173.5	170.1





Position	δc (**3b**)	δc (**2**)
2	75.4	76.2
4	65.6	65.9
5	37.9	39.3
6	25.4	25.4
* 7	172.5	172.4
8	62.3	61.8
9	14.1	14.2
* 10	174.1	171.4

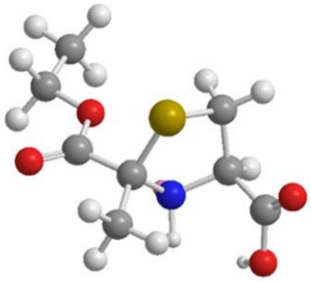


Fig. S15. Comparative analysis of the ^{13}C NMR data of **3a** and **3b** and their ChemDraw 3D models (MM2 energy-minimized).[4] For compound **3b**, we observed an obvious change of the ^{13}C chemical shift after hydrolysis of the ester C10, but not for the nonhydrolysis ester C7. While for compound **3a**, we observed changes of the signals for both C7 and C10, which may be due to possible intramolecular hydrogen bonding interaction, as suggested by the ChemDraw 3D model. White, grey, blue, red, and yellow spheres represent H, C, N, O, and S atoms, respectively.

3.5 Insights into the self-degradated processes of L-CysOEt

UV-vis analysis of the self-degradation of L-CysOEt. 20 mM L-CysOEt was dissolved in PBS buffer (200 mM, pH 7.4) in a sealed cuvette, and then time-dependent absorbance spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer.

HRMS analysis of the self-degradation of L-CysOEt in a D_2O-based buffer. L-CysOEt (20 mM) was dissolved in PBS buffer (prepared by deuteroxide (D_2O), pH 7.4, 200 mM) for 2 days of incubation before HRMS test. In a control experiment, compound **1** (10 mM) was incubated in the D_2O-based PBS buffer for 2 days before HRMS test.

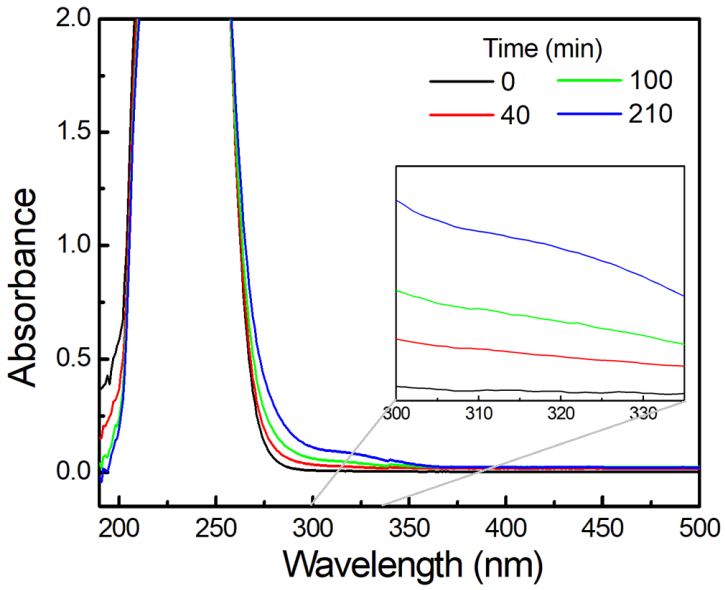


Fig. S16. Time-dependent UV-vis spectra of CysOEt (20 mM) in PBS (pH = 7.4) at 25 ºC.

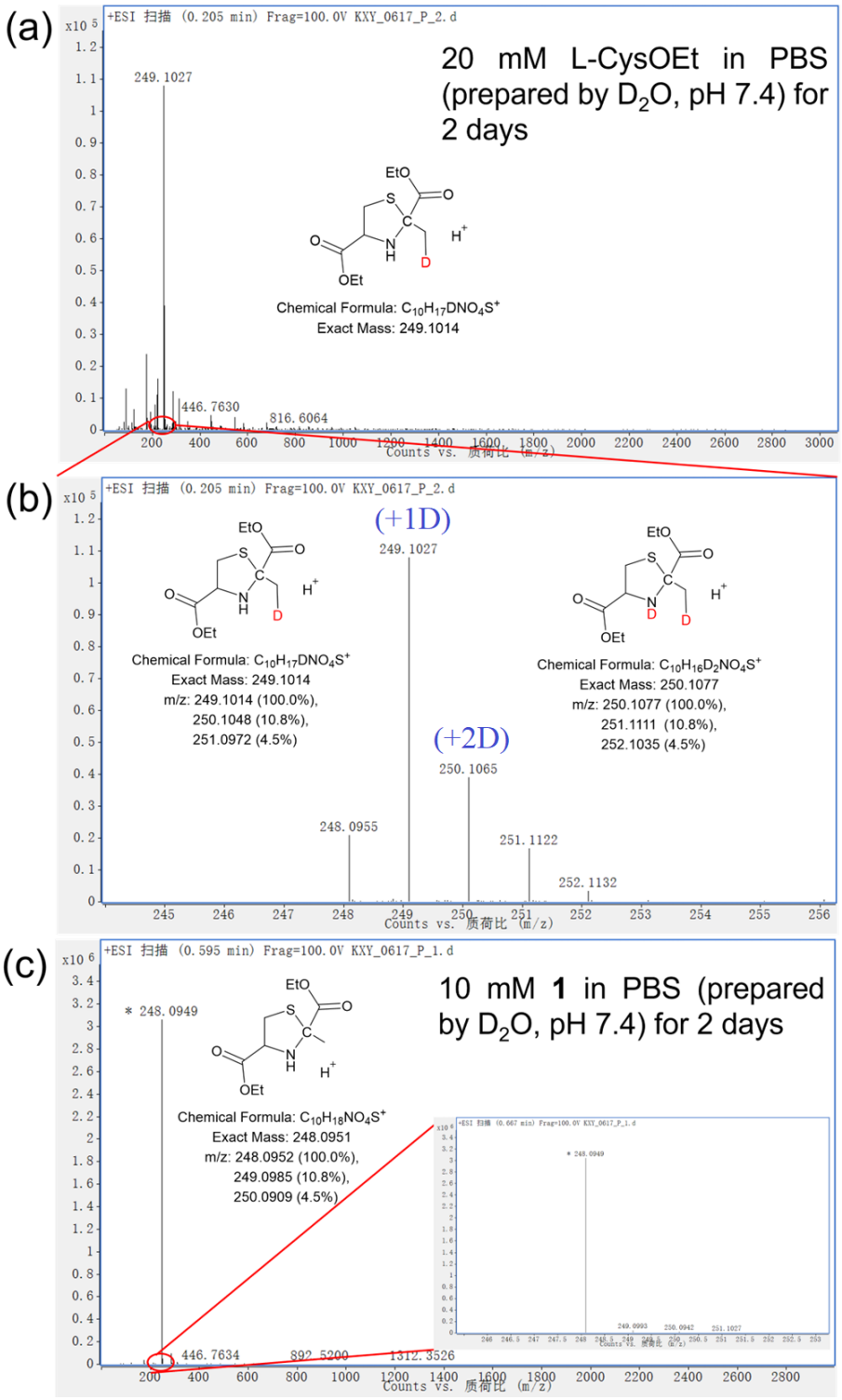


Fig. S17. (a, b) HRMS analysis of the reaction aliquot of L-CysOEt (20 mM) in a D_2O-based PBS buffer (pH = 7.4) after 2 days of incubation. (c) HRMS analysis of **1** (10 mM) in a D_2O-based PBS buffer (pH = 7.4) after 2 days of incubation. The m/z peaks as well as the unusual isotope distribution in (b) suggest the formation of deuterium-incorporated products. The major mass peak in (c) suggests the deuterium-exchange should be slow, which further supports the products in (b) should be covalently deuterium-incorporated at the 2-methyl position, as shown inset.

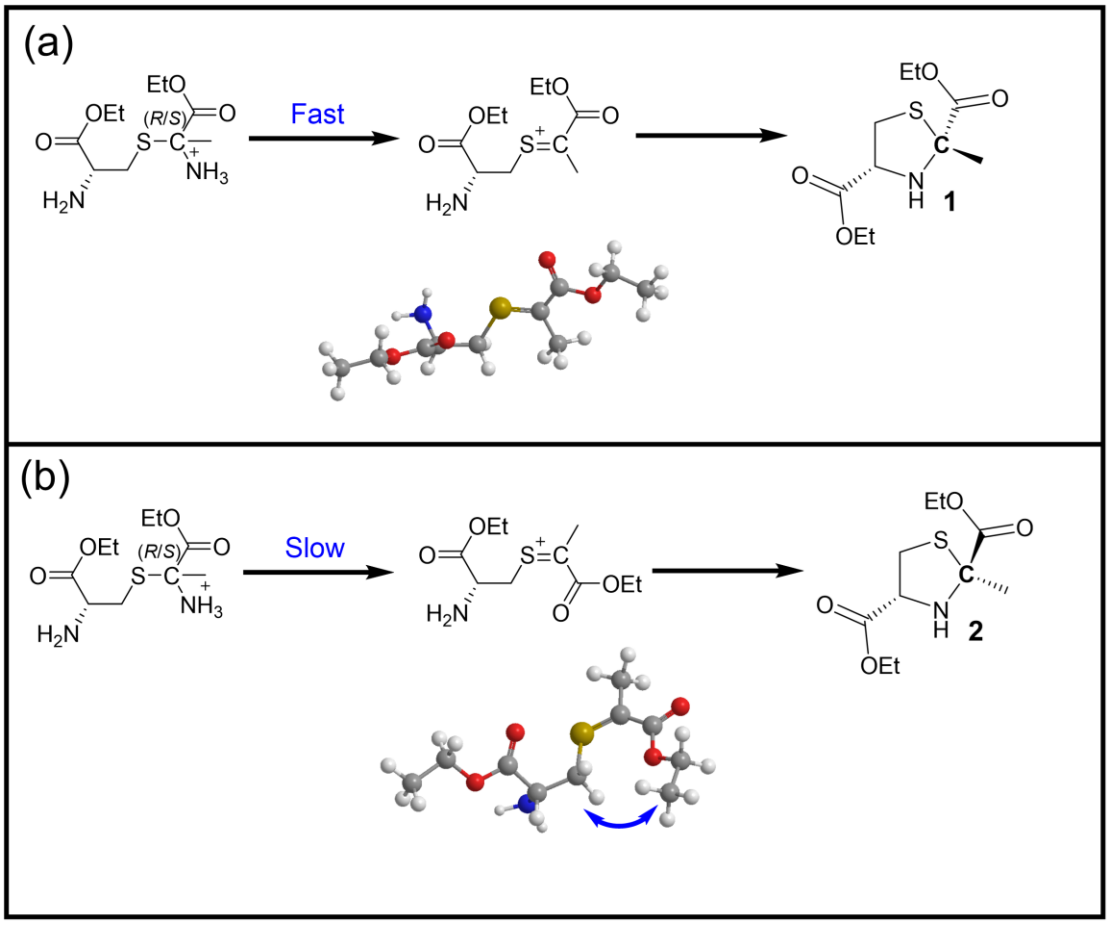
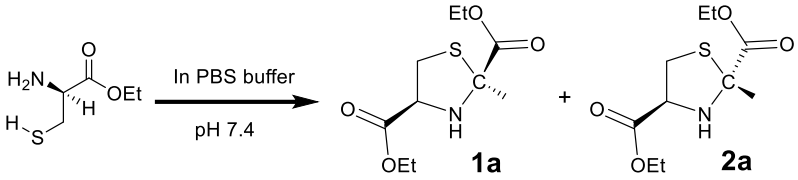


Fig. S18. (a, b) Analysis of the products **1** and **2** via two potential pathways based on the ChemDraw 3D models (MM2 energy-minimized), respectively.[4] The S=C double bond with *E*-configuration should be more favorable than the *Z*-configuraiton due to the possibly steric crowding among side chains (highlighted by a double-headed arrow) in b). As expected, the yield of **1** is higher than **2** (**1**:**2** = 2:1). White, grey, blue, red, and yellow spheres represent H, C, N, O, and S atoms, respectively.

4. Characterization of the organic products from self-degradation of D-CysOEt

Synthesis of D-CysOEt.[6] Thionyl chloride (1.5 mL, 20 mmol) was slowly added to a solution of D-cysteine (600 mg, 5 mmol) in ethanol (10 mL) with stirring and cooling in an ice bath. After stirring at reflux for 6 h, the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH (100/10) containing 0.5% HCl (4 N in dioxane) to give a white solid D-cysteine ethyl ester hydrochloride (D-CysOEt•HCl, 668 mg, 73%). ^{1}H NMR (400 MHz,

MeOD) δ 4.56-4.36 (m, 1H), 4.28 (q, J = 6.6 Hz, 2H), 3.56-3.44 (m, 1H), 3.42-3.21 (m, 1H), 1.30 (t, J = 6.6 Hz, 3H).



D-CysOEt•HCl (1.1 g, 5.9 mmol) was dissolved in 300 mL PBS buffer (200 mM, pH 7.4). After stirring for 3 days at room temperature, the solution was extracted with EtOAc. The collected organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (100/1) to give colorless oil diethyl (2*S*,4*S*)-2-methylthiazolidine-2,4-dicarboxylate (**1a**) and diethyl (2*R*,4*S*)-2-methylthiazolidine-2,4-dicarboxylate (**2a**), with overall isolated yield 12.6%. HRMS (ESI): m/z 248.0950 $[M+H]^+$ (calcd for $C_{10}H_{18}NO_4S^+$, 248.0951).

For **1a** (62 mg, 8.4%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.35-4.17 (m, 4H), 4.00 (dd, J = 10.5, 5.6 Hz, 1H), 3.36 (dd, J = 10.3, 5.6 Hz, 1H), 2.90-2.81 (m, 1H), 1.69 (s, 3H), 1.35-1.26 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7, 170.2, 74.7, 65.8, 62.5, 61.9, 40.3, 28.4, 14.3, 14.1.

For **2a** (31 mg, 4.2%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.42-4.34 (m, 1H), 4.32-4.13 (m, 4H), 3.42-3.33 (m, 1H), 3.05 (dd, J = 10.3, 8.0 Hz, 1H), 1.87 (s, 3H), 1.35-1.23 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.4, 171.5, 76.3, 66.0, 61.9, 61.8, 39.3, 25.5, 14.3, 14.1.

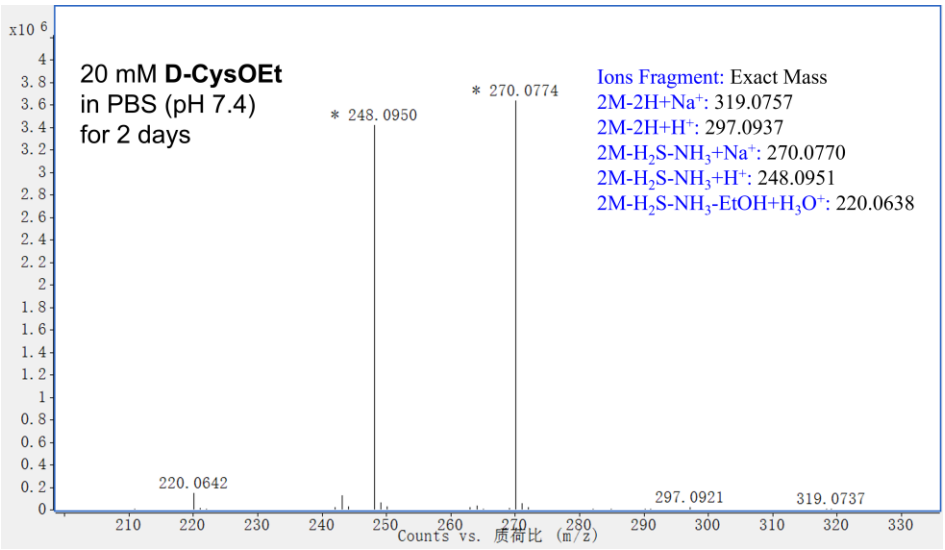


Fig. S19. HRMS analysis of D-CysOEt (20 mM) in PBS buffer (pH 7.4) for 2 days of incubation at room temperature. The mass peaks are consistent with that of L-CysOEt.

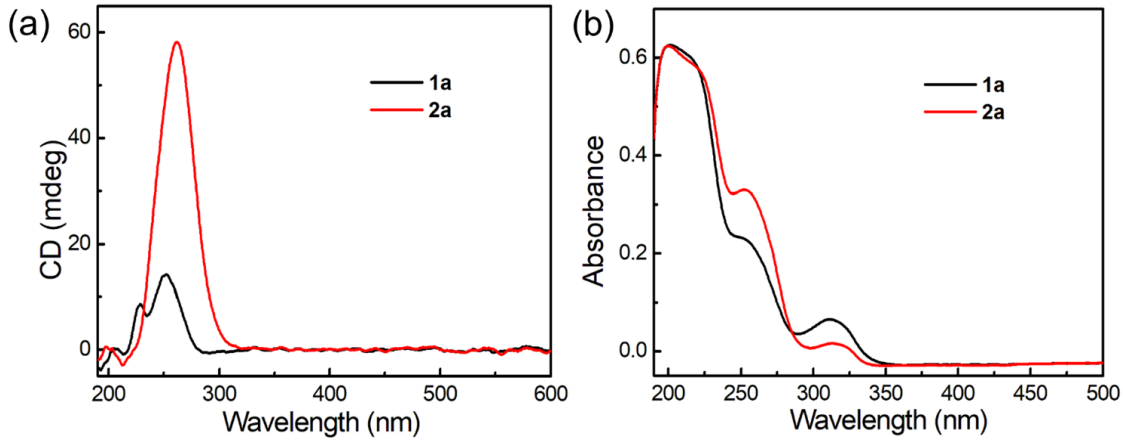


Fig. S20. (a) CD and (b) absorbance spectra of **1a** and **2a** (0.5 mg/mL, dissolved in H_2O).

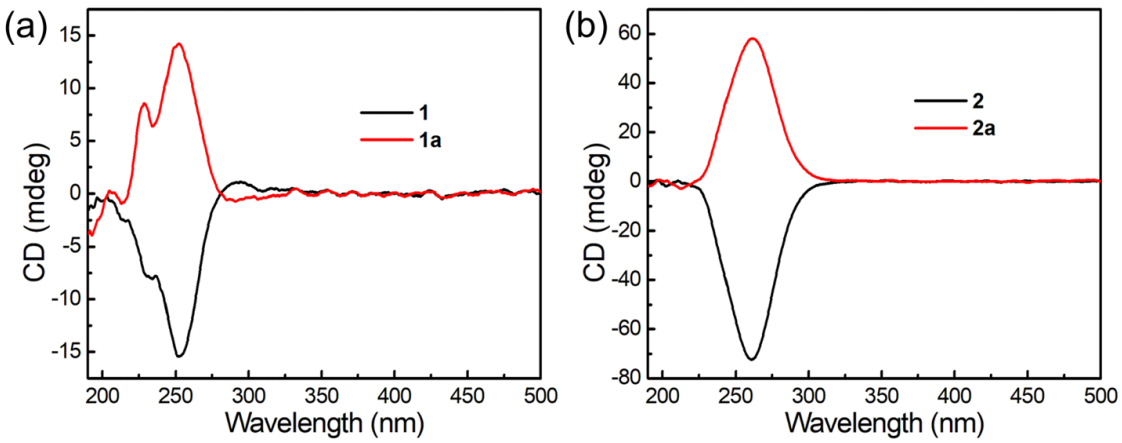


Fig. S21. Comparison of CD spectra of the two pairs of enantiomers.

5. Development of esterase-resistant H_2S donors

5.1 Comparison on the stability of Cys esters in the presence of esterase

0.3 mM *S*-Trityl-L-cysteine ethyl ester or *S*-trityl-L-cysteine tert-butyl ester in PBS (50 mM, pH 7.4, containing 10% CH_3CN) in the presence of PLE (final 1 U/mL) was checked by time-dependent HPLC analysis. Conditions: detection wavelength: 215 nm; flow 1.0 mL/min; buffer A: 0.1% (v/v) trifluoroacetic acid in water; buffer B: methanol; elution condition: 0-2 min, buffer B: 5-55%; 2-28 min, buffer B: 55-85%; 28-30 min, buffer B: 85-5%.

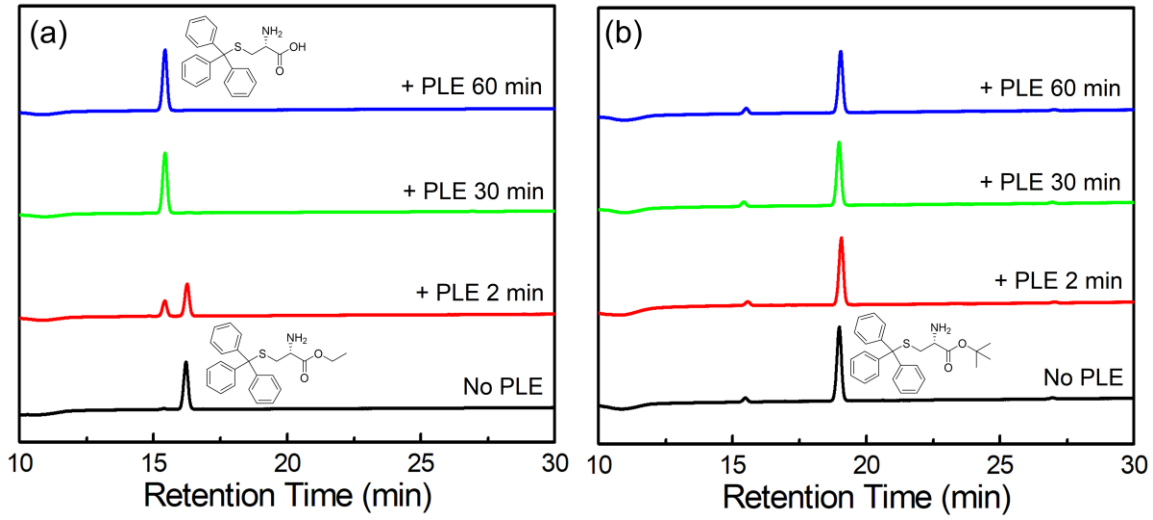
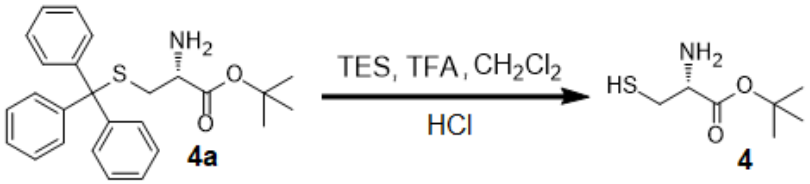


Fig. S22. Time-dependent HPLC traces of *S*-trityl-L-cysteine ethyl ester (a) or *S*-trityl-L-cysteine tert-butyl ester (b) in PBS (50 mM, pH 7.4, containing 10% CH_3CN, 1 U/mL PLE). *S*-Trityl-L-cysteine ethyl ester was fast hydrolyzed to *S*-trityl-L-cysteine, but *S*-trityl-L-cysteine tert-butyl ester was esterase-resistant.[7]

5.2 Synthesis of tert-butyl esters 4-6



To a solution of *S*-trityl-L-cystine tert-butyl ester hydrochloride **4a** (600 mg, 1.3 mmol) in 4 mL CH_2Cl_2, triethylsilane (TES, 250 μL) was added, and then a solution (1 mL, trifluoroacetic acid/CH_2Cl_2 = 1/1) was added dropwise under the protection of N_2. After stirring for 10 min, the reaction was monitored by TLC plate. When **4a** was completely

disappeared, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH (100/3) containing 0.5% HCl (from 4 N in dioxane) to give a white solid **4** (235 mg, 84%). ^{1}H NMR (400 MHz, MeOD) δ 4.24-4.19 (m, 1H), 3.09-3.05 (m, 2H), 1.54 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 166.3, 84.4, 54.6, 26.7, 23.9. HRMS (ESI): m/z 178.0904 $[M+H]^+$ (calcd for $C_7H_{16}NO_2S^+$, 178.0896).

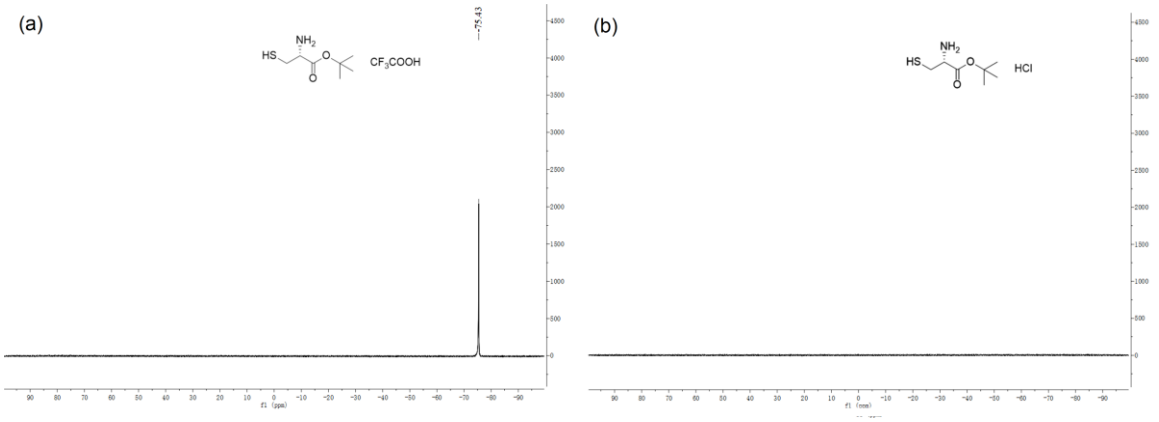
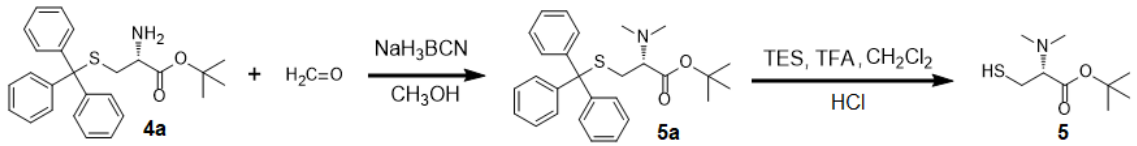
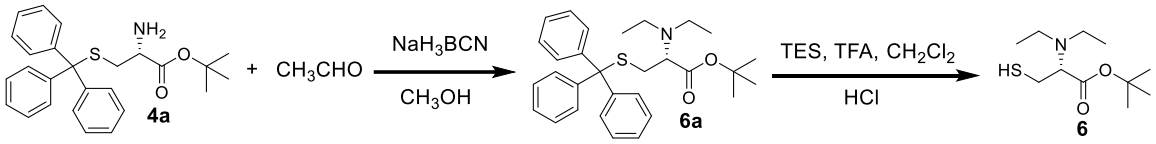


Fig. S23. Comparison on ^{19}F NMR spectra for the same concentration (70 mM) of L-CysOtBu in complex with trifluoroacetate (a) or hydrochloride (b). Our method (0.5% HCl in elution for column chromatography) was demonstrated to completely remove the trifluoroacetate.



To a cooled suspension (0 ºC) of **4a** (1.0 g, 2.2 mmol) and $NaBH_3CN$ (0.41 g, 6.6 mmol) in MeOH (6 ml) was added 37% formaldehyde solution (520 μL, 6.6 mmol). The resulting solution was allowed to stir at room temperature for 4 h, after which the solution was concentrated in vacuo, and the residue was dissolved in EtOAc. The 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 with CH_2Cl_2/EtOAc (100/4) to give a colorless oil **5a** (697 mg, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53-7.44 (m, 6H), 7.34-7.26 (m, 6H), 7.25-7.18 (m, 3H), 2.79-2.72 (m, 1H), 2.57 (dd, J = 12.6, 7.5 Hz, 1H), 2.41 (dd, J = 12.6, 7.8 Hz, 1H), 2.24 (s, 6H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.9, 144.9, 129.7, 127.9, 126.6, 81.3, 67.2, 66.7, 41.5, 31.6, 28.3. HRMS (ESI): m/z 448.2289 $[M+H]^+$ (calcd for $C_{28}H_{34}NO_2S^+$, 448.2305).

To a solution of **5a** (340 mg, 0.76 mmol) in 4 mL CH_2Cl_2, TES (250 μL) was added, and then a solution (1.0 mL, trifluoroacetic acid/CH_2Cl_2 =1/1) was added dropwise under the protection of N_2. After stirring for 10 min, the reaction was monitored by TLC plate. When **5a** was completely disappeared, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH (100/3) containing 0.5% HCl (from 4 N in dioxane) to give a white solid **5** (146 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.00-3.82 (m, 1H), 3.51-3.32 (m, 1H), 3.20-3.00 (m, 1H), 2.90 (s, 6H), 1.97 (t, J = 8.6, 1H), 1.55 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.7, 85.9, 68.9, 43.9, 38.3, 28.1, 22.3. HRMS (ESI): m/z 206.1220 $[M+H]^+$ (calcd for $C_9H_{20}NO_2S^+$, 206.1209).



To a cooled suspension (0 ºC) of **4a** (228 mg, 0.5 mmol) and $NaBH_3CN$ (93 mg, 1.5 mmol) in MeOH (4 ml) was added acetaldehyde (84 μL, 1.5 mmol). The resulting solution was allowed to stir at room temperature for 5 h, after which the solution was concentrated in vacuo, and the residue was dissolved in EtOAc. The 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 with CH_2Cl_2 to give a colorless oil **6a** (180 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50-7.42 (m, 6H), 7.34-7.27 (m, 6H), 7.24-7.17 (m, 3H), 3.12-2.99 (m, 1H), 2.65-2.48 (m, 3H), 2.47-2.31 (m, 3H), 1.42 (s, 9H), 0.97 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2, 145.1, 129.8, 127.9, 126.6, 81.0, 66.8, 63.0, 44.7, 32.2, 28.3, 14.2. HRMS (ESI): m/z 476.2578 $[M+H]^+$ (calcd for $C_{30}H_{38}NO_2S^+$, 476.2618).

To a solution of **6a** (340 mg, 0.71 mmol) in 4 mL CH_2Cl_2, TES (250 μL) was added, and then a mixed solution (1 mL, trifluoroacetic acid/CH_2Cl_2 =1/1) was added dropwise under the protection of N_2. After stirring for 10 min, the reaction was monitored by TLC plate. When **6a** was completely disappeared, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography with CH_2Cl_2/MeOH (100/2) containing 0.5% HCl (from 4 N in dioxane) to give a white solid

6 (107 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10-3.91 (m, 1H), 3.59-3.26 (m, 3H), 3.15-2.85 (m, 3H), 1.95-1.81 (m, 1H), 1.60-1.46 (m, 15H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.9, 85.5, 65.8, 48.9, 46.4, 28.2, 22.6, 11.3, 10.3. HRMS (ESI): m/z 234.1532 $[M+H]^+$ (calcd for $C_{11}H_{24}NO_2S^+$, 234.1522).

5.3 H_2S release from the tert-butyl esters

H_2S release from 4 and 5 at pH 7.4. 2 mM H_2S donor **4** or **5** was dissolved in PBS buffer (pH 7.4) for 2 h or 4 h of incubation at 25 °C. Then 0.9 mL H_2S donor solution was mixed with 0.9 mL of the methylene blue cocktail. After another 1 h of incubation, the resulted solution was checked by absorbance at 670 nm to determine the H_2S concentration.

H_2S release in the presence of PLE. 2 mM H_2S donor L-Cys-OEt, **4** or **5** was dissolved in PBS buffer (pH 7.4, containing 1.0 U/mL PLE) for 4 h of incubation. Then 0.9 mL H_2S donor solution was mixed with 0.9 mL of the methylene blue cocktail for 1 h of incubation to determine the H_2S concentration.

H_2S release in the presence of GSH. 2 mM H_2S donor **4** or **5** was dissolved in PBS buffer (pH 7.4, containing 5 mM GSH) for 4 h of incubation. Then 0.9 mL H_2S donor solution was mixed with 0.9 mL of the methylene blue cocktail for 1 h of incubation to determine the H_2S concentration.

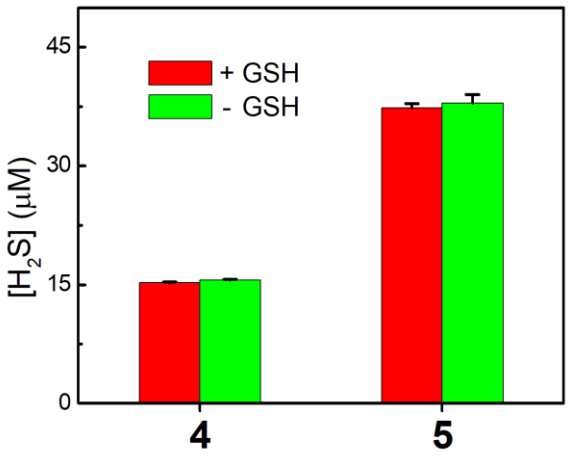


Fig. S24. Comparison on the H_2S release from 2 mM **4** or **5** in the presence of 5 mM GSH in PBS buffer (pH 7.4) at 4 h of incubation at 25 ºC. The results are expressed as mean ± SD (n = 3). The results suggest that H_2S-releasing efficiencies are not influenced by GSH.

5.4 Further insights into the self-degradated pathways of Cys esters

UV-vis analysis of the self-degradation of 4 or 5. 1 mM H_2S donor (**4** or **5**) was dissolved in PBS buffer (pH 7.4) in sealed cuvette, and then time-dependent absorbance spectra were recorded.

HRMS analysis of the self-degradation of 4-6. 4 (20 mM) was dissolved in PBS buffer (pH 7.4) overnight or for 2 days of incubation before HRMS test. 10 mM **5** or **6** was dissolved in PBS buffer (pH 7.4, containing 50% CH_3CN for dissolving the donors) for 4 h or 2 days of incubation before HRMS test.

Quantitative determination of the release of both H_2S and NH_3 from the same solution of 4. The self-degradation of **4** (2 mM) in 40 mL PBS buffer (pH 7.4) was tested in a sealed 50 ml flask precharged with argon gas. At a certain time-point, 0.9 mL H_2S donor solution was drawn off and mixed with 0.9 mL of the methylene blue cocktail to determine the H_2S concentration. At the same time, 1 mL H_2S donor solution was drawn off and mixed with phenol solution of 0.5 mL (150 mM) containing 5 mg of sodium nitroprusside, and then 0.5 mL of hypochlorite solution to determine the NH_3 concentration.

HRMS analysis of the self-degradation of 6 in D_2O-based PBS buffer. 6 (10 mM) was dissolved in PBS buffer (prepared by D_2O, pH 7.4, 200 mM, containing 50% CH_3CN) for 2 days of incubation before HRMS test.

Long-acting H_2S release in aqueous buffer. Probe **BODIPY-NBD**[8] (50 μM) was incubated with or without 100 μM **5** in PBS (pH 7.4, containing 20% EtOH for dissolving the probe, 5 mM GSH) for 48 h, and the time-dependent fluorescence spectra were recorded. The excitation wavelength was 469 nm.

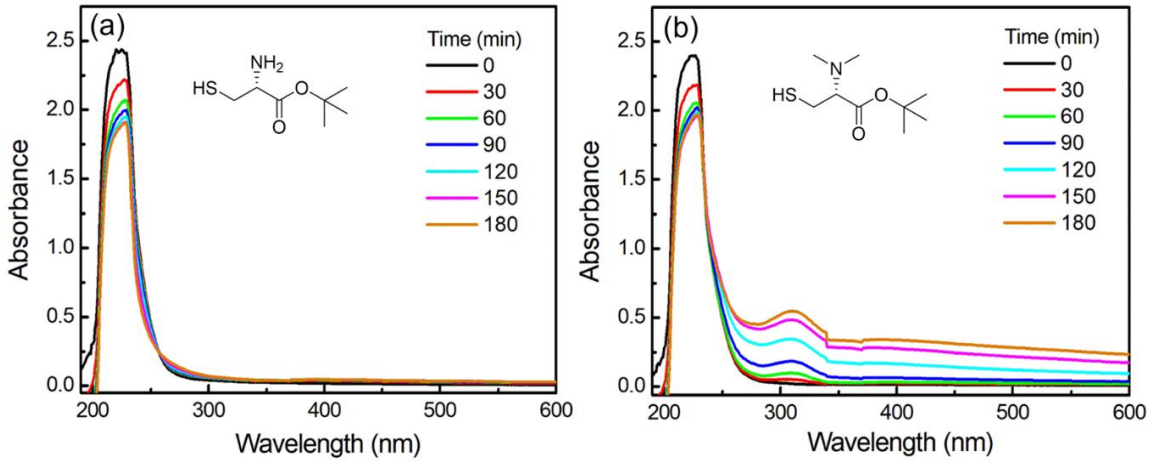


Fig. S25. Time-dependent absorbance spectra of 1 mM **4** (a) or **5** (b) in PBS (pH 7.4) at 25 ºC. The increase at 315 nm for **5** is more obvious than **4**, probably because 1) the H_2S release from **5** is faster than from **4**; 2) the metastable immediate **b** analog in **4** is more reactive than that in **5**.

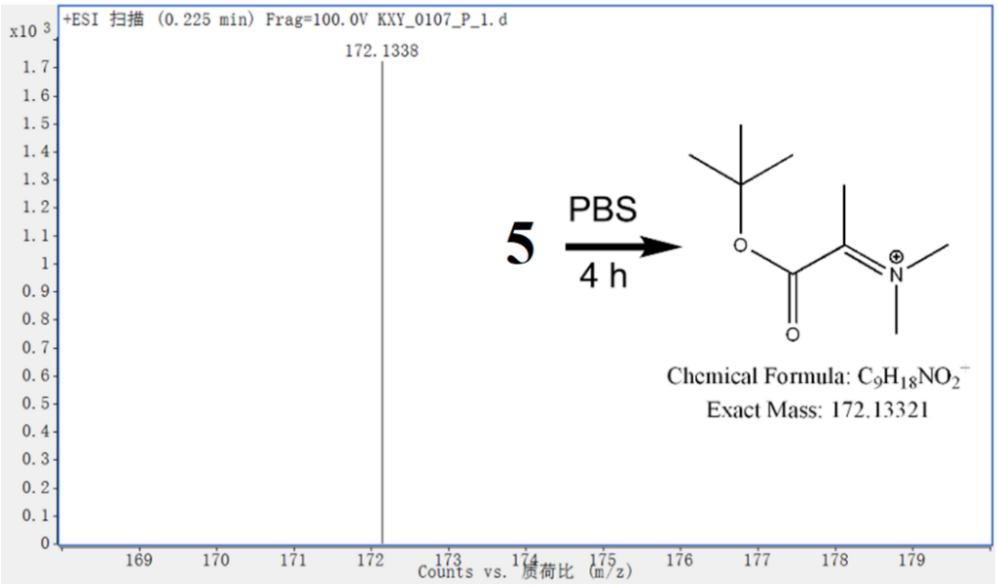
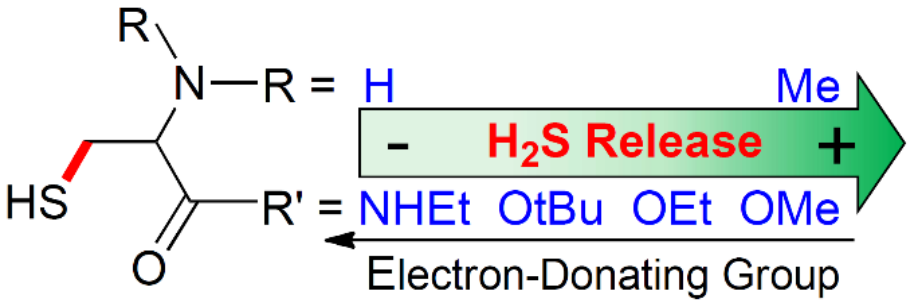


Fig. S26. HRMS analysis of **5** (10 mM) in PBS buffer (pH = 7.4, containing 50% CH_3CN) after 4 h of incubation.



Scheme S1. Illustration of tunable motifs for the H_2S release at physiological pH. The cleavage bond is highlighted by red bold line.

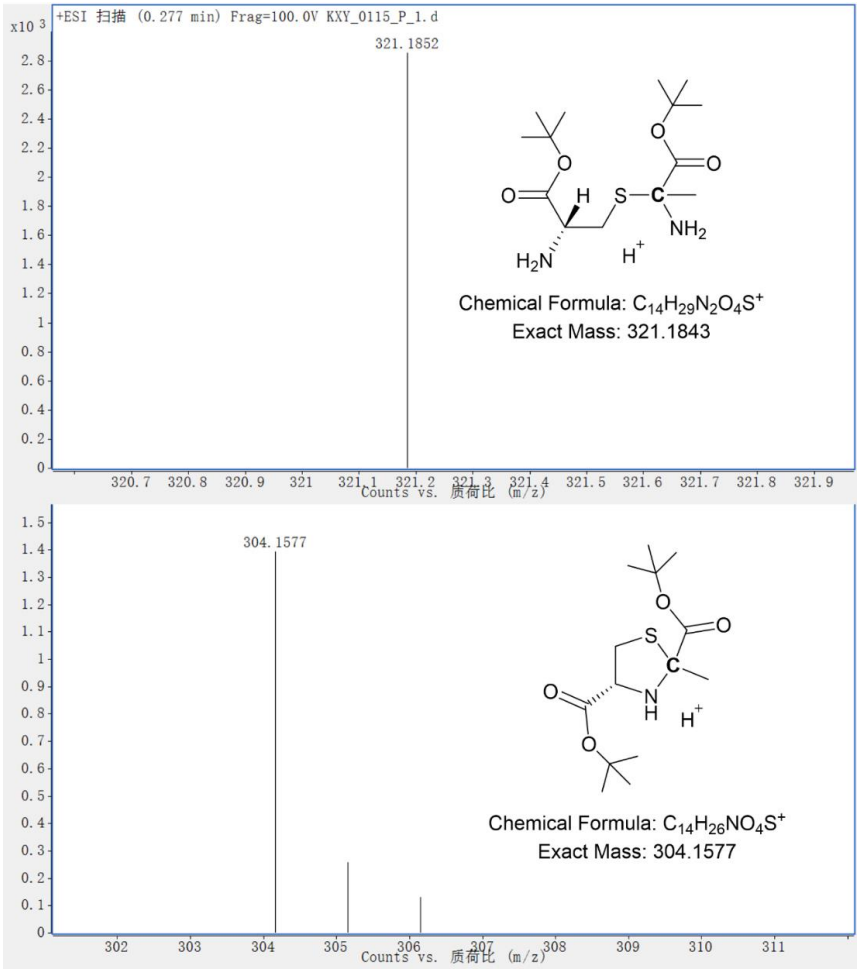


Fig. S27. HRMS analysis of **4** (20 mM) in PBS buffer (pH = 7.4) after overnight incubation. The possibly observed intermediate **d** analog may be due to slowly intramolecular five-membered ring formation from sterically hindered tBu esters.

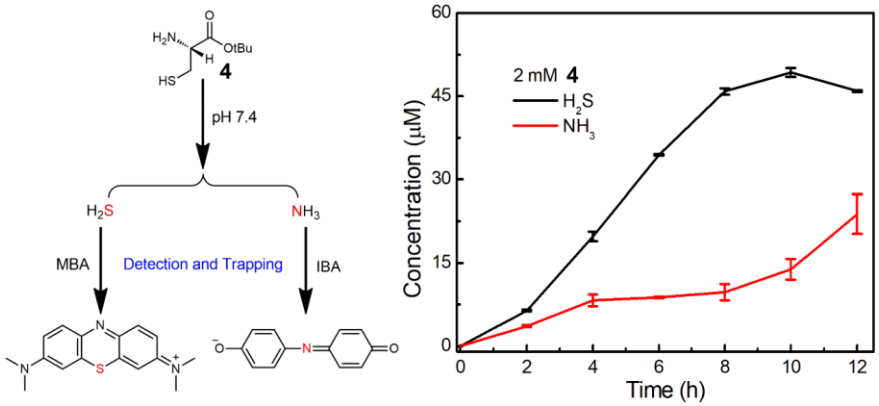


Fig. S28. Quantitative comparison on the time-dependent release of both H_2S and NH_3 from the same solution of **4** (2 mM) in a degassed, sealed PBS (pH = 7.4). The results are expressed as mean ± SD (n = 2). The long-term H_2S concentrations should be reduced because H_2S oxidizes and volatilizes after its generation.[1b] The existence of an induction period for the NH_3 release suggests that an intermediate required for its generation may initially accumulate during the H_2S release process. The NH_3 release is smaller than the H_2S release at the early reaction time is consistent with the HRMS results in Fig. S27, where the possible intermediate **d** analog with a loss of H_2S but without a loss of NH_3 was observed.

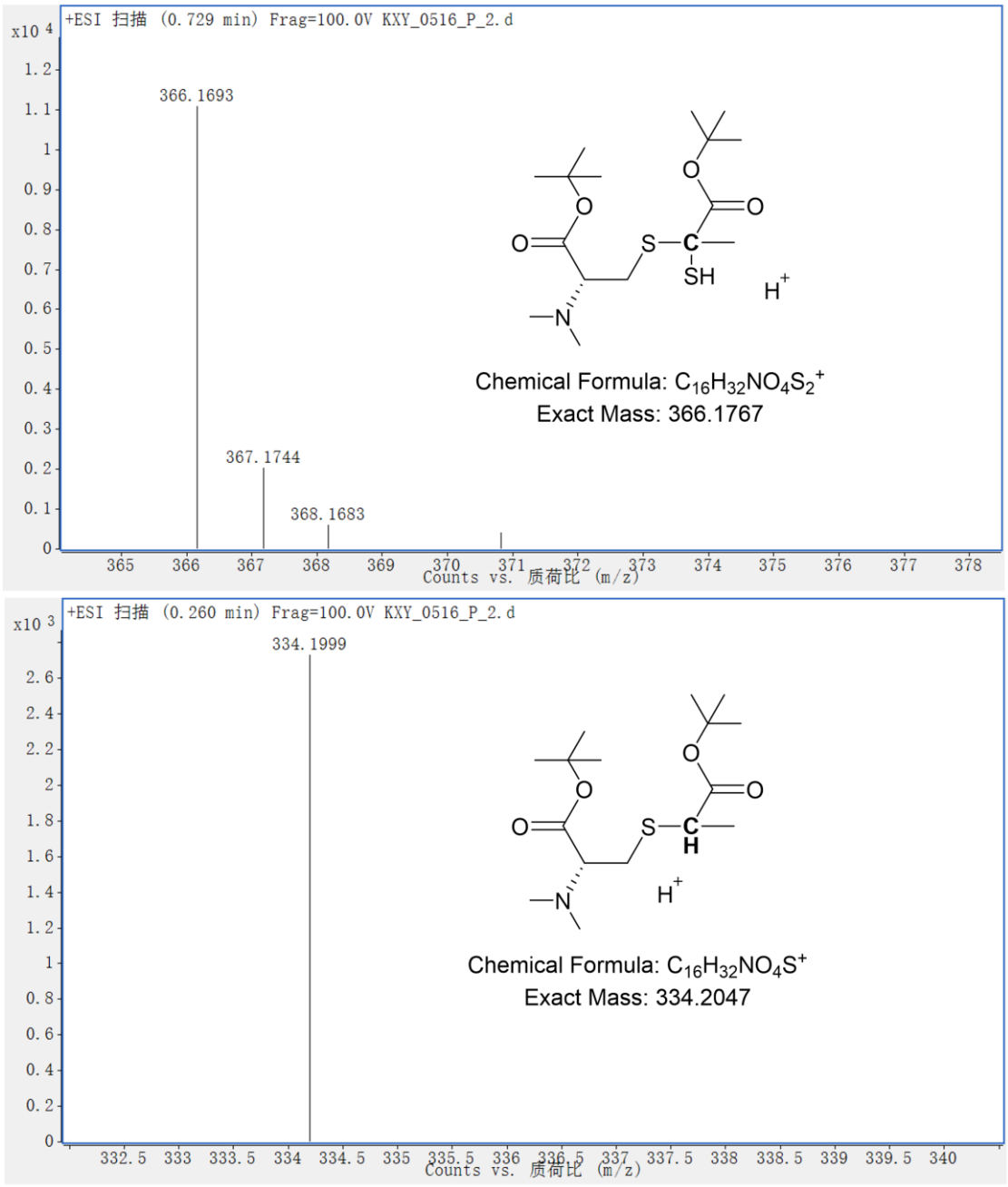


Fig. S29. HRMS analysis of **5** (10 mM) in PBS buffer (pH 7.4, containing 50% CH_3CN) for 2 days of incubation. The proposed structures with exact mass are also shown inset.

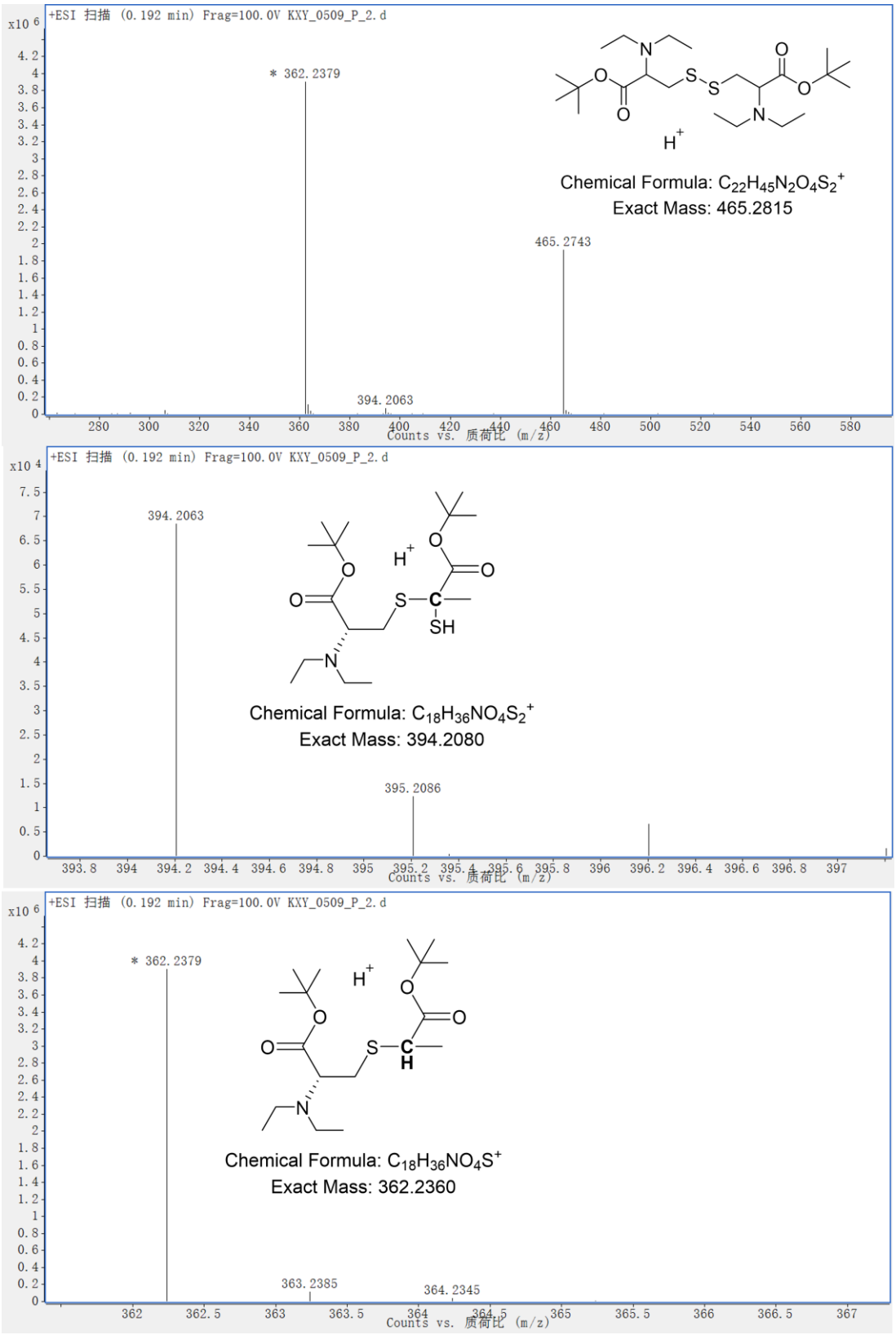


Fig. S30. HRMS analysis of **6** (10 mM) in PBS buffer (pH 7.4, containing 50% CH_3CN) for 2 days of incubation. The proposed structures with exact mass are also shown inset.

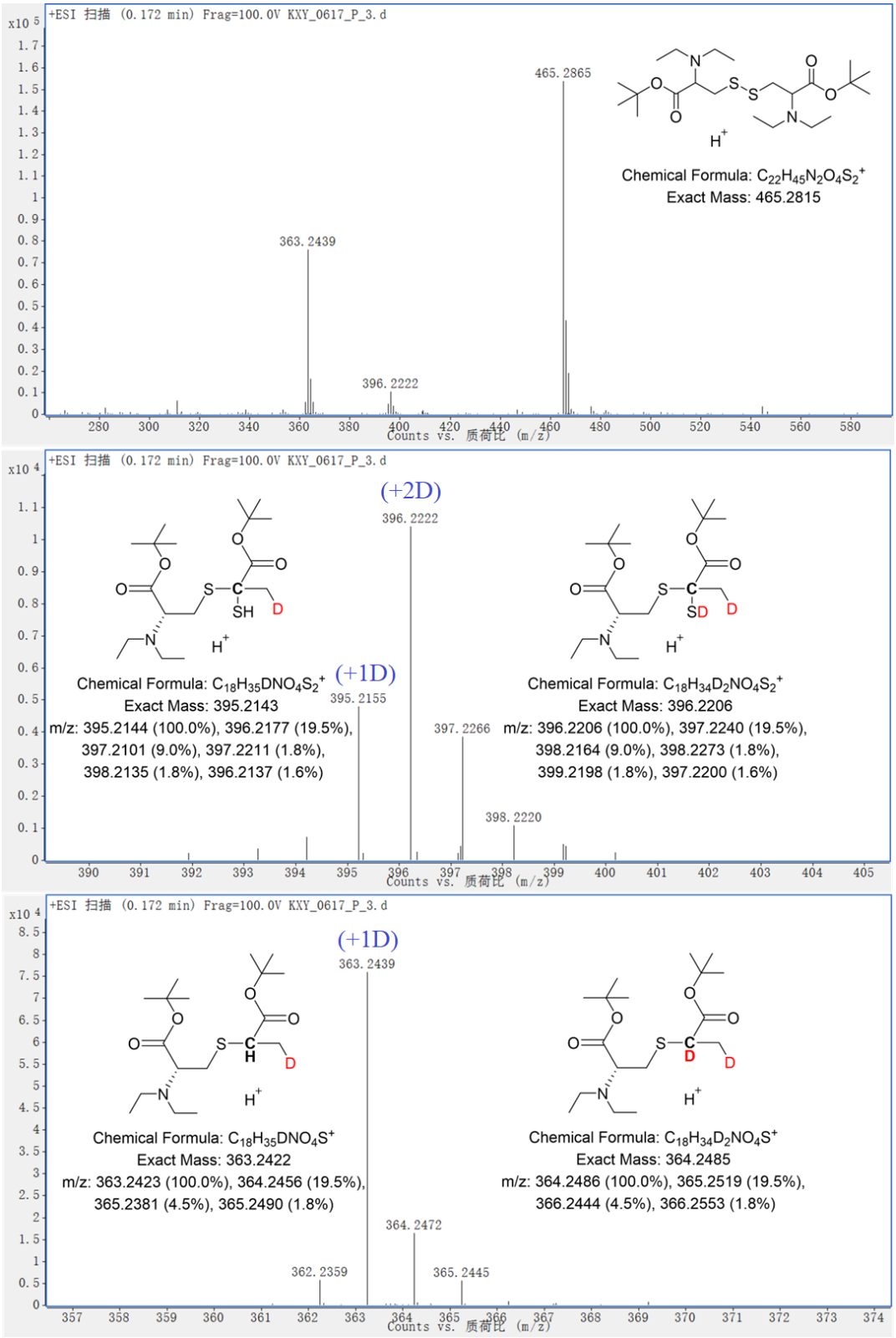


Fig. S31. HRMS analysis of the reaction aliquot of **6** (10 mM) in a D_2O-based PBS buffer (pH 7.4, containing 50% CH_3CN) for 2 days of incubation. The unusual isotope distribution suggested the proposed D-incorporated structures as shown inset.

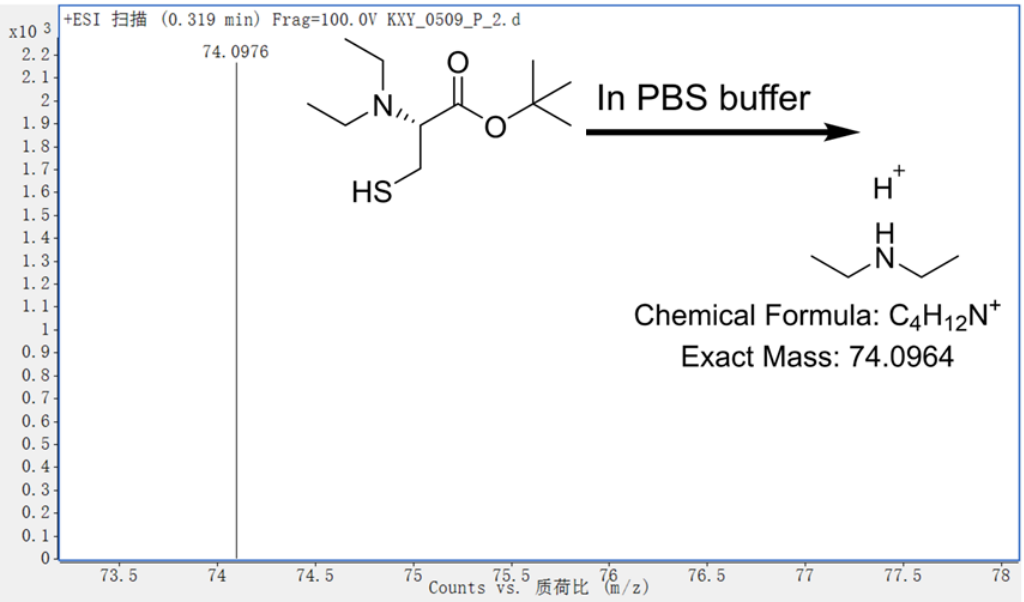


Fig. S32. HRMS analysis of **6** in PBS buffer (pH 7.4) for 2 days of incubation.

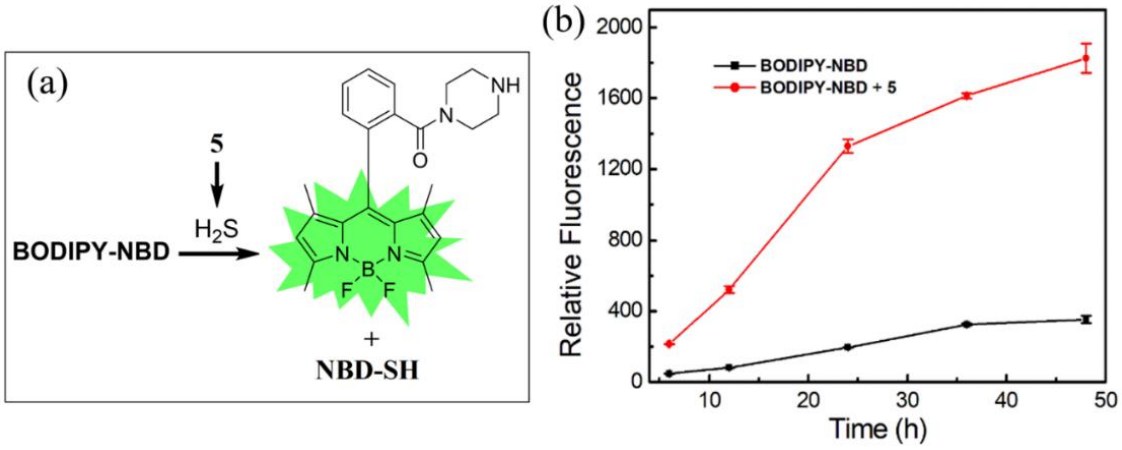
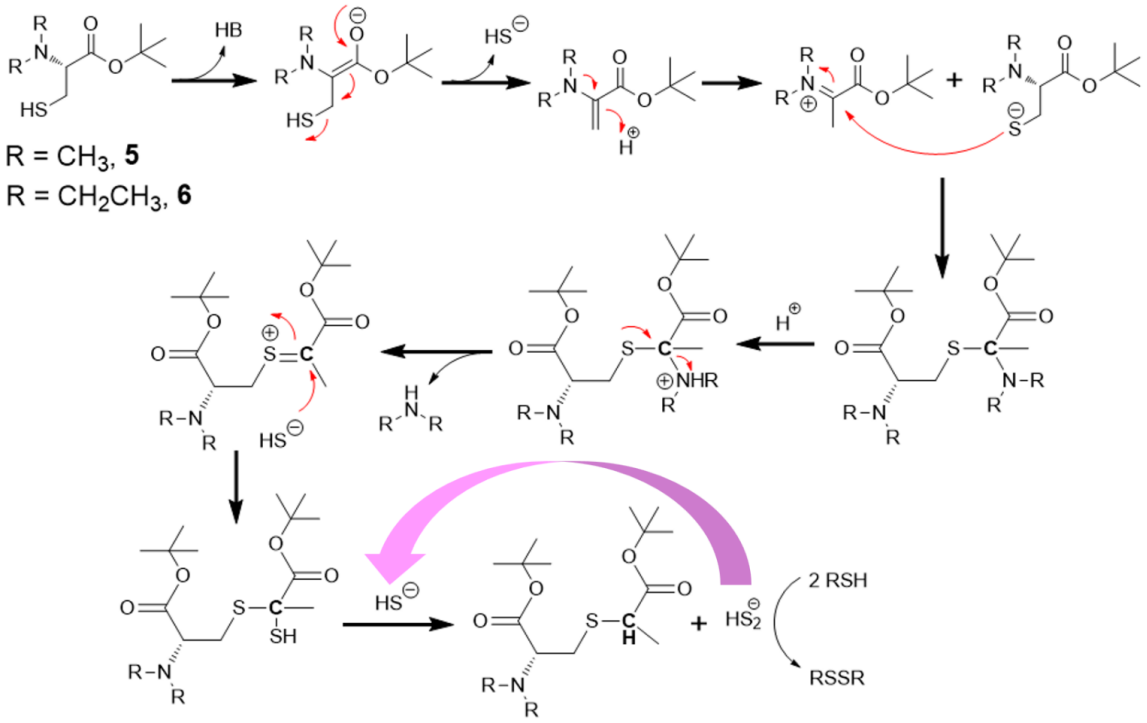


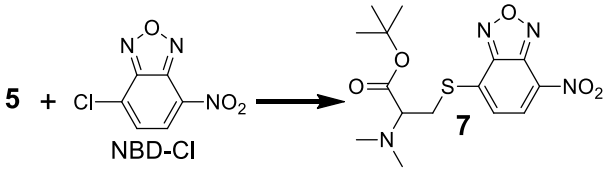
Fig. S33. (a) Schematic drawing for trapping and fluorescent detection of the released H_2S from **5** in buffers. (b) Time-dependent emission intensities at 520 nm of **BODIPY-NBD**[8] (50 μM) in PBS (pH 7.4, containing 5 mM GSH) in the absence (black line) or presence (red line) of **5** (100 μM) for 48 h of incubation at 25 ºC. The data are expressed as mean ± SD (n = 3).



Scheme S2. Schematic drawing of a proposed reaction pathway for the self-degradation of **5** and **6** in PBS buffer (pH = 7.4). After the elimination of HS^- at physiological pH, the enamine-to-imine tautomerization followed by a bimolecular addition may occur. Then dual-alkylated amines are released, and the intermediate with a S=C double bond can further react with HS^- to form a dimer with a mass of (2M-R_2NH). Based on the reported H_2S-mediated reductions from Pluth and co-workers,[9] we assigned two possible dimers structures for the observed m/z peaks of (2M-R_2NH-S) as well as (2M-2H).

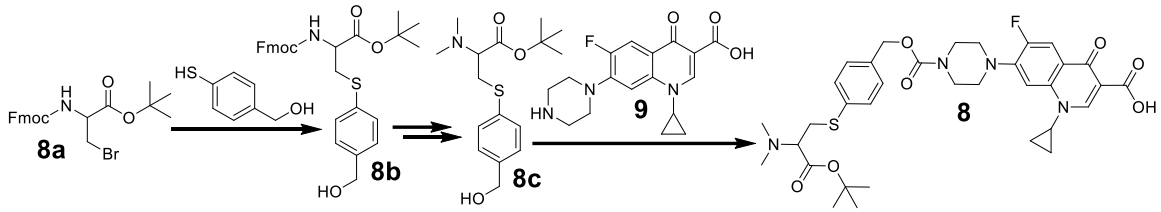
6. Sustained-release thiol and drug from esterified Cys derivatives

6.1 Synthesis of NBD thioether 7 and prodrug 8



To a solution of **5** (50 mg, 0.2 mmol) in 5 mL CH_2Cl_2, NBD-Cl (100 mg, 0.5 mmol) and *N*,*N*-diisopropylethylamine (82 µL, 0.5 mmol) were added for stirring 2 h. Then the solvent was removed under reduced pressure, and the crude residue was purified by silica gel column chromatography with EtOAc/petroleum ether (1/4) to give a pale brown solid **7** (32 mg, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 7.9 Hz, 1H), 7.28 (d, J = 7.8

Hz, 1H), 3.62-3.51 (m, 3H), 2.47 (s, 6H), 1.53 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.8, 149.3, 142.5, 132.7, 130.7, 121.0, 82.8, 66.0, 41.5, 32.3, 28.4. HRMS (ESI) for $[M+H]^+$: m/z 369.1268 (calcd for $C_{15}H_{21}N_4O_5S^+$, 369.1227).



Compound **8a** was synthesized based on a previous work.[10] Commerically available (*S*)-tert-Butyl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-hydroxypropanoate (Fmoc-Ser-OtBu, 1.00 g, 2.61 mmol) and tetrabromomethane (CBr_4, 1.06 g, 3.20 mmol) were dissolved under argon in dry CH_2Cl_2 (15 mL) and cooled to 0 °C. To the stirred solution was added PPh_3 (1.09 g, 4.17 mmol) in several portions within 15 min. The resulted solution was allowed to warm to room temperature and stirred for an additional 1 h. The solvent was removed under reduced pressure, and the residual product was purified by flash column chromatography with petroleum ether/ EtOAc (100/15) to give a colorless oil **8a** (1.10 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 7.5 Hz, 2H), 7.64 (d, J = 7.4 Hz, 2H), 7.44-7.40 (m, 2H), 7.36-7.32 (m, 2H), 5.82 (d, J = 6.5 Hz, 1H), 4.77-4.69 (m, 1H), 4.46-4.37 (m, 2H), 4.28-4.24 (m, 1H), 3.86-3.78 (m, 2H), 1.54 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.8, 155.6, 143.8, 141.3, 127.8, 127.1, 125.2, 120.1, 83.6, 67.4, 54.6, 47.1, 34.4, 28.0. HRMS (ESI) for $[M+H]^+$: m/z 468.0807 (calcd for $C_{22}H_{24}BrNNaO_4^+$, 468.0781).

To a solution of **8a** (200 mg, 0.45 mmol) and Cs_2CO_3 (134 mg, 0.41 mmol) in DMF (5 mL) at 0 °C was added 4-mercaptobenzyl alcohol (57 mg, 0.41 mmol), and the reaction was stirred for 30 min. The resulted solution was worked-up by addition of H_2O (20 mL) and extracted with EtOAc (3×15 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography with petroleum ether/ EtOAc (7/3) to give a colorless oil **8b** (106 mg, 51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.58-7.53 (m, 2H), 7.42-7.38 (m, 4H), 7.33-7.28 (m, 2H), 7.25-7.19 (m, 2H), 5.66 (d, J = 7.0 Hz, 1H), 4.58-4.55 (m, 1H), 4.52 (s, 2H), 4.29 (d, J = 7.2 Hz, 2H), 4.14 (t, J = 7.1

Hz, 1H), 3.53-3.48 (m, 1H), 3.34-3.29 (m, 1H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.4, 155.6, 143.8, 141.3, 139.8, 134.5, 130.4, 127.8, 127.6, 127.1, 125.3, 120.1, 83.1, 67.2, 64.6, 54.6, 53.5, 47.1, 36.9, 28.0. HRMS (ESI) for $[M+H]^+$: m/z 528.1835 (calcd for $C_{29}H_{31}BNNaO_5S^+$, 528.1815).

Compound **8b** (180 mg, 0.34 mmol) was dissolved in a mixture of CH_2Cl_2 (4 mL) and piperidine (1 mL) for 1 h stirring, and the solution was removed under reduced pressure. The residue was fast purified by silica gel column chromatography with MeOH/CH_2Cl_2 (1/20) to give a colorless oil, which was dissolved in MeOH (4 mL) containing $NaBH_3CN$ (93 mg, 1.0 mmol) and 37% formaldehyde solution (81 mg, 1.0 mmol). After stirring overnight, the resulted solution was concentrated, and the crude residue was purified by silica gel column chromatography with 3% MeOH in CH_2Cl_2 to give a colorless oil **8c** (60 mg, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35-7.32 (m, 2H), 7.27-7.25 (m, 2H), 4.61 (s, 2H), 4.25 (bs, 1H), 3.20-3.11 (m, 2H), 2.33 (s, 6H), 1.47 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.7, 139.4, 135.0, 130.1, 127.7, 82.0, 67.5, 64.7, 48.6, 41.7, 34.0, 28.4. HRMS (ESI) for $[M+H]^+$: m/z 312.1659 (calcd for $C_{16}H_{26}NO_3S^+$, 312.1628).

To a solution of **8c** (60 mg, 0.19 mmol) in dry CH_2Cl_2 (5 mL) was added *N*,*N*'-disuccinimidyl carbonate (75 mg, 0.29 mmol) and Et_3N (80 μL, 0.58 mmol). The resulted mixture was stirred at room temperature overnight and then concentrated. The residue was fast purified by silica gel column chromatography with 2% MeOH in CH_2Cl_2 to give the NHS activated ester, which was dissolved in dry CH_2Cl_2 (20 mL) containing ciprofloxacin (**9**, 39 mg, 0.12 mmol) and Et_3N (34 μL, 0.24 mmol). After stirring for another 12 h, the reaction solution was concentrated under reduced pressure, and the crude residue was purified by silica gel column chromatography with 2% MeOH in CH_2Cl_2 containing 0.1% Et_3N to give a white solid **8** (31 mg, 24%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.98 (bs, 1H), 8.70 (s, 1H), 7.96 (d, J = 12.8 Hz, 1H), 7.35-7.33 (m, 3H), 7.29-7.27 (m, 2H), 5.11 (s, 2H), 3.75-3.70 (m, 4H), 3.57-3.51 (m, 1H), 3.32-3.25 (m, 5H), 3.18-3.16 (m, 2H), 2.37 (s, 6H), 1.47 (s, 9H), 1.24-1.21 (m, 2H), 1.19-1.16 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.1, 166.9, 155.1, 152.5, 147.6, 145.8, 139.1, 134.5, 129.5, 128.9, 120.2, 112.7, 112.4, 108.2, 105.3, 82.0, 67.3, 67.1, 49.7, 45.9, 41.6, 35.4, 33.8,

29.8, 28.4, 8.7, 8.3. HRMS (ESI) for $[M+H]^+$: m/z 669.2794 (calcd for $C_{34}H_{42}FN_4O_7S^+$, 669.2752).

6.2 Sustained-release of NBD-SH from 7

UV-vis spectra for the sustained-release NBD-SH. 100 μM Compound **7** was dissolved in PBS (50 mM, pH 7.4, containing 10% CH_3CN) in a sealed cuvette at 25 ºC, and then time-dependent absorbance spectra were recorded on a UV-6000 UV-VIS-NIR-spectrophotometer. The self-degradated reaction yielded a unique color of NBD-SH,[11] and the rate constant *k* was determined by fitting the time-dependent intensities at 534 nm to a single exponential function.

HRMS analysis. 10 mM Compound **7** was dissolved in PBS (pH 7.4, containing 50% CH_3CN) for 4 h of incubation, and the resulted aliquot was analyzed by HRMS directly in both positive and negative ion modes.

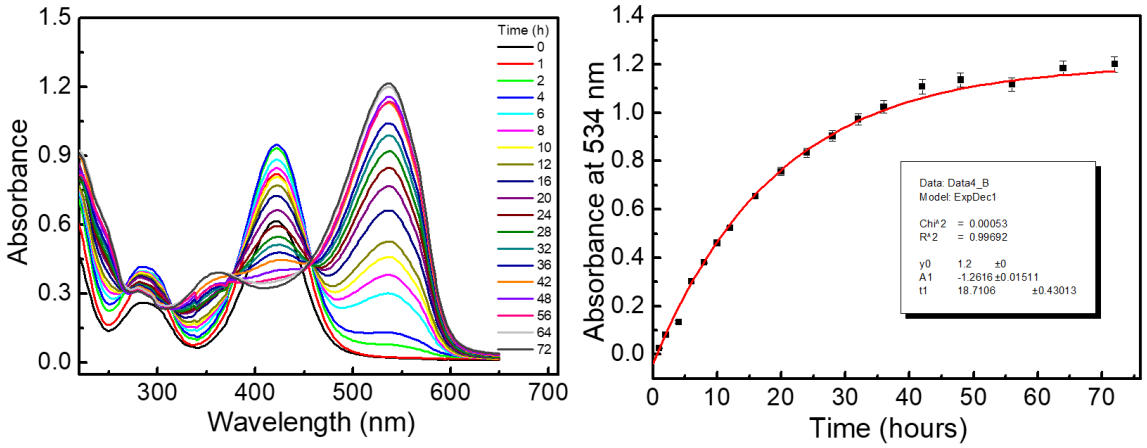


Fig. S34. (a) Representative time-dependent absorbance spectra of **7** in PBS buffer (pH 7.4) at 25 ºC. (b) Time-dependent absorbance intensities at 534 nm of **7** (100 μM) in PBS buffer (pH 7.4) at 25 ºC. The results are expressed as mean ± SD (n = 3). The pseudo-first-order rate, $k = 1.48 \times 10^{-5}$ s^{-1}, was determined by fitting the data with a single-exponential function (solid red line), and the half-life ($t_{1/2}$) is calculated as 13.0 h.

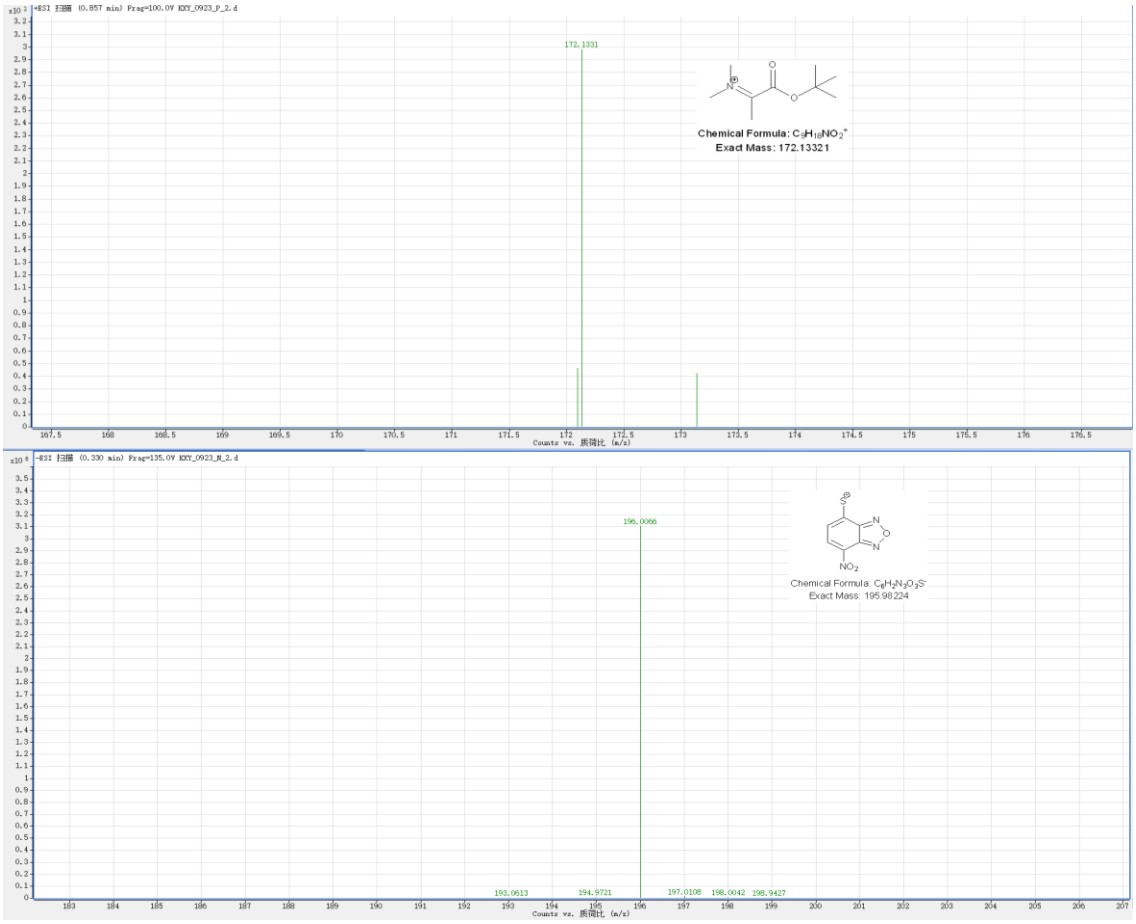


Fig. S35. HRMS analysis of the self-degradated reaction aliquot of **7** in PBS buffer (pH 7.4, containing 50% CH_3CN) after 4 h of incubation. The positive (top) and negative (bottom) ion modes suggest the cleavage reaction to generate the both products as shown inset.

6.3 Sustained-release of drug from 8

HPLC analysis for the sustained-release drug. Compound **8** was dissolved in DMSO to obtain a 10 mM stock solution. Prodrug **8** (100 μM) was incubated in PBS buffer (50 mM, pH 7.4, containing 20% CH_3CN) at 37 ºC, and then time-dependent HPLC tests were recorded. Conditions: ANGELA TECHNOLOGIES HPLC LC-10F; C18 column with 4.6 mm x 250 mm; detection wavelength: 353 nm; flow 1.0 mL/min; buffer A: 0.1% TFA in water; buffer B: MeOH; elution condition: 0-3 min, B: 5-50%; 3-13 min, B: 50-80%; 13-25 min, B: 80-95%; 25-30 min, B: 95-5%. The rate constant *k* was determined by fitting the time-dependent peak areas of **8** or **9** from the HPLC traces to a single exponential function.

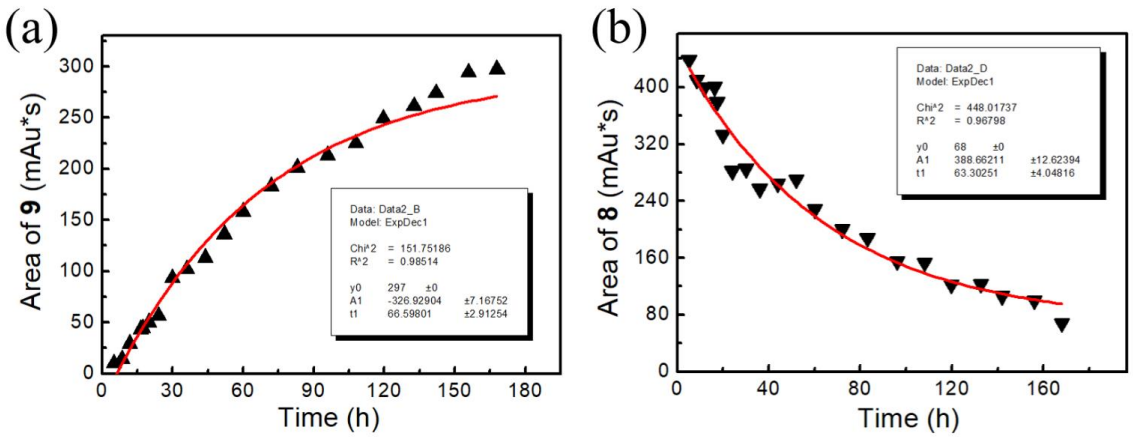
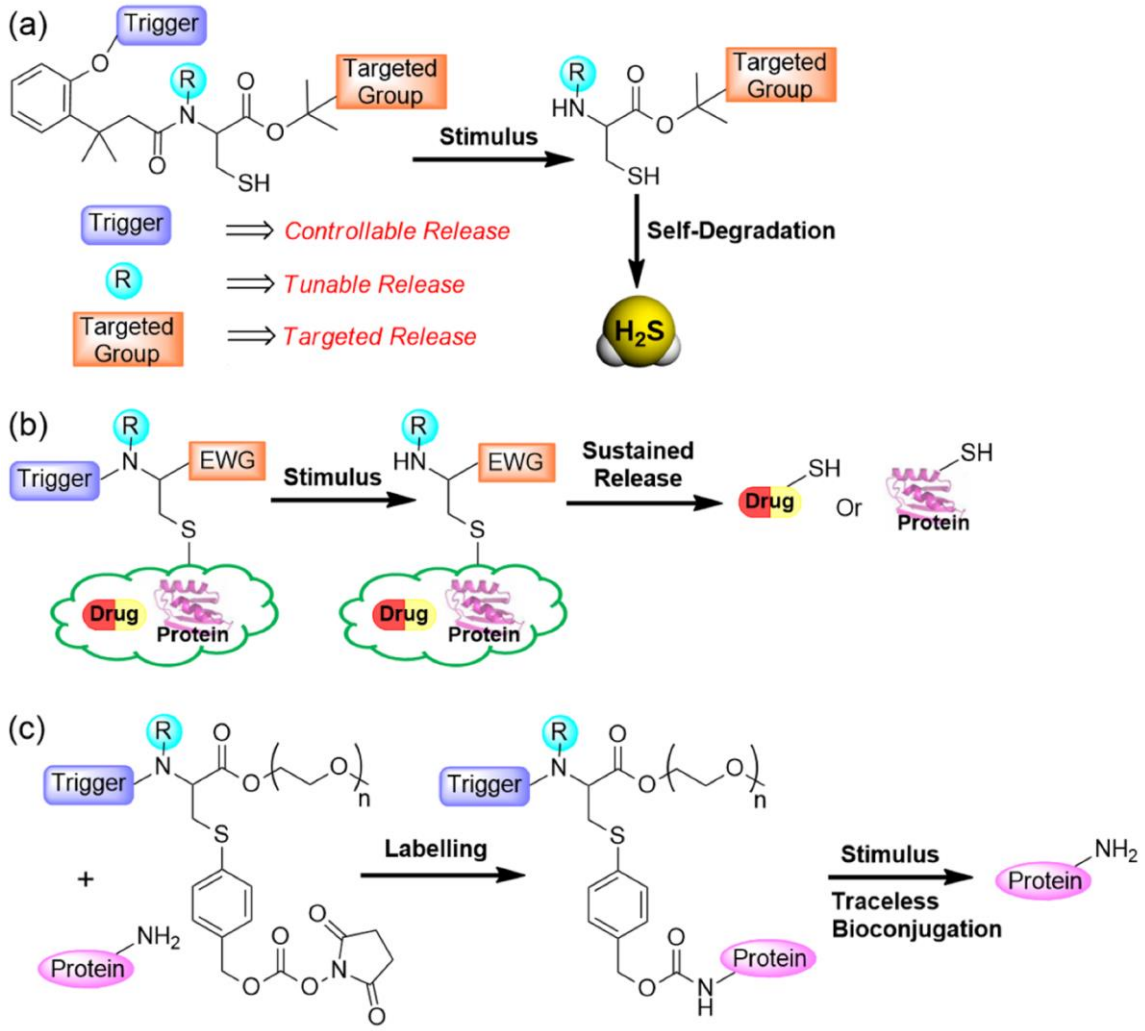
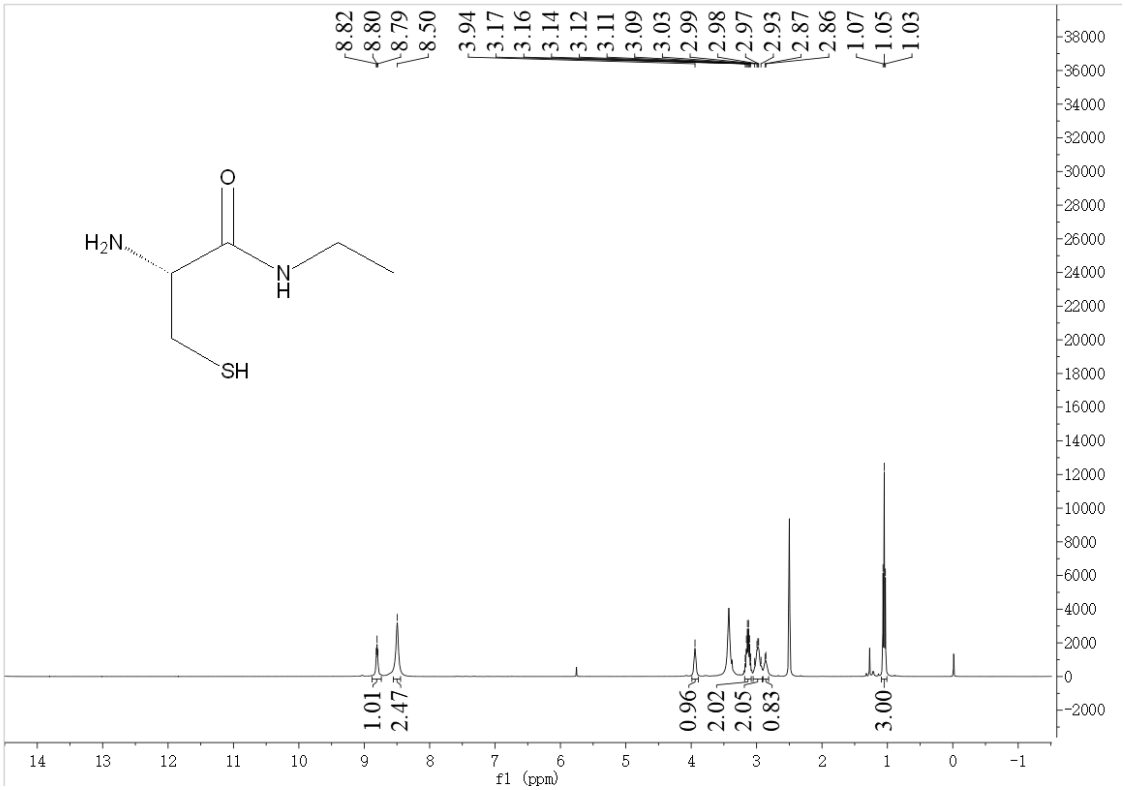


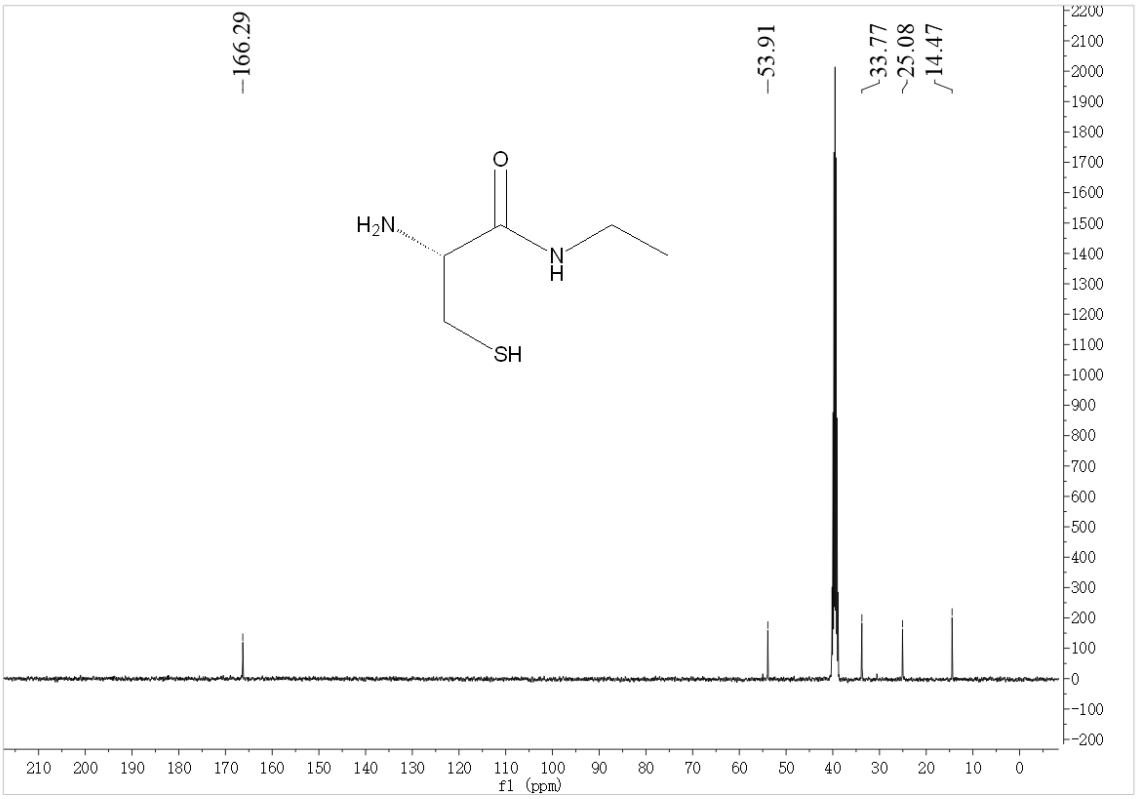
Fig. S36. (a, b) Time-dependent peak areas of **9** or **8** from the HPLC analysis of the self-degradated **8** in PBS (pH 7.4), respectively. The solid lines represent the best fitting with a single-exponential function. The half-life ($t_{1/2}$) for the drug release is calculated as 46.1 h from (a).

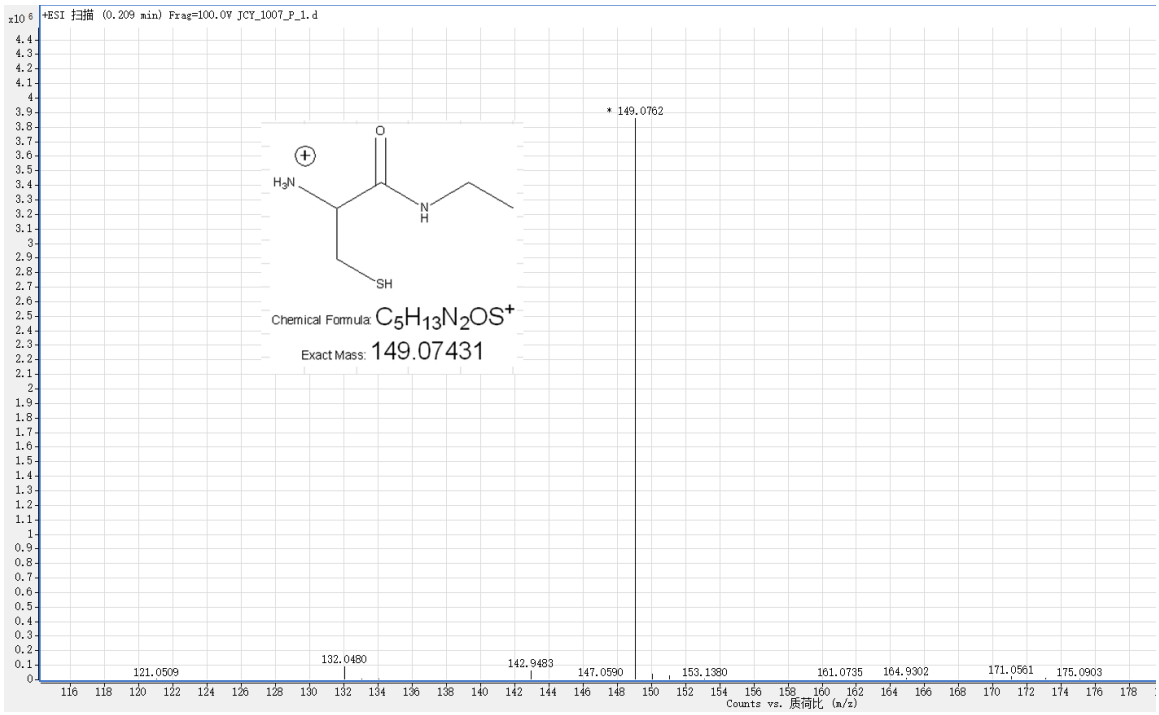


Scheme S2. Future prospects for further applications of our cleavage reactions. a) Inspired by a series of elegant works on triggering strategies including triggers of enzymes, reactive oxygen species (ROS), light, or bioorthogonal reactions, et al,[12] new controllable, targeted H_2S donor motifs may be developed; b) Based on the masked, self-degradated structural motif, new prodrugs may be developed for thiol-containing small molecules and proteins.[13] In addition, we envision that the ester group might be replaced by other more electron-withdrawing groups (EWGs) to develop faster cleavage chemistries under physiological conditions. c) Peptide/protein-polymer bioconjugates may be constructed by coupling the masked self-degradated structural motif with biomolecules through a self-immolative linker, or using 2-azidoacrylates to label the cysteine residue.[14] After activation, spontaneous cleavage will lead to traceless protein release from the conjugates in a controllable fashion.[15]

7. Supporting NMR and HRMS spectra






+ESI 扫描 (0.209 min) Frag=100.0V JCY_1007_P_1.d
* 149.0762
Chemical Formula: C5H13N2OS+
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132.0480
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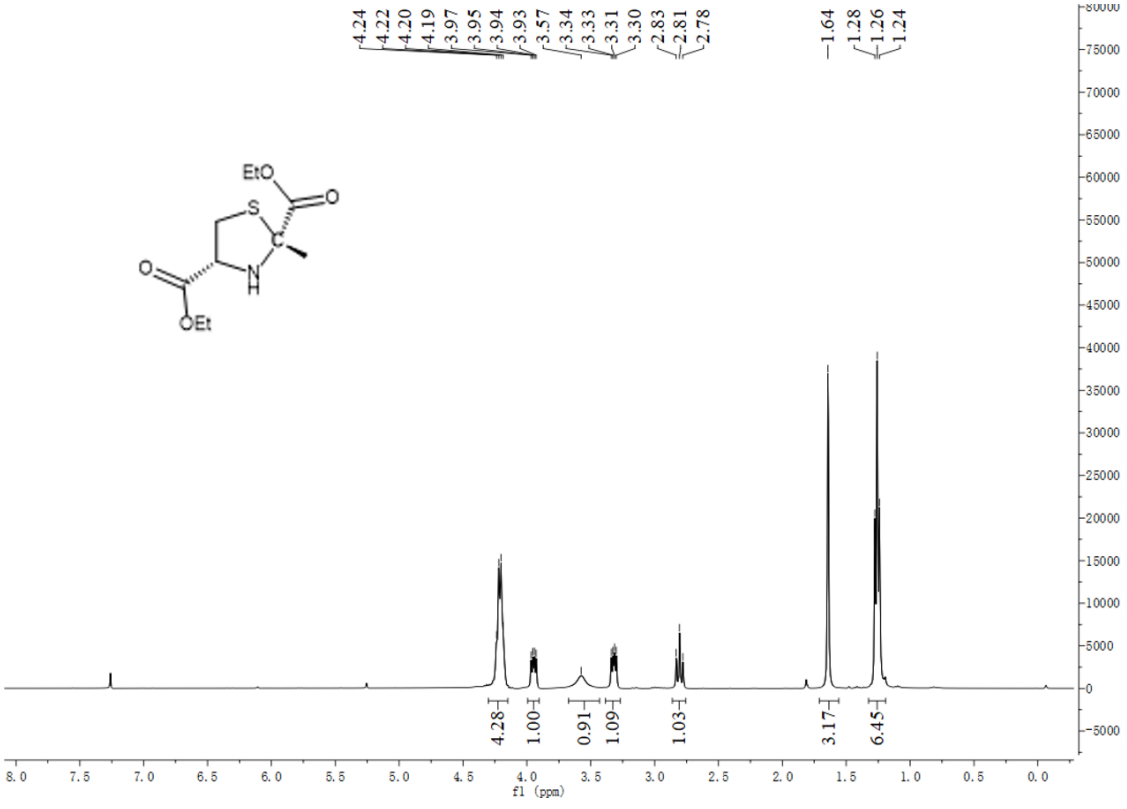
153.1380
161.0735
164.9302
171.0561
175.0903
Counts vs. 质荷比 (m/z)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3.94
3.93
3.57
3.34
3.33
3.31
3.30
2.83
2.81
2.78
1.64
1.28
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OEt
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1.00
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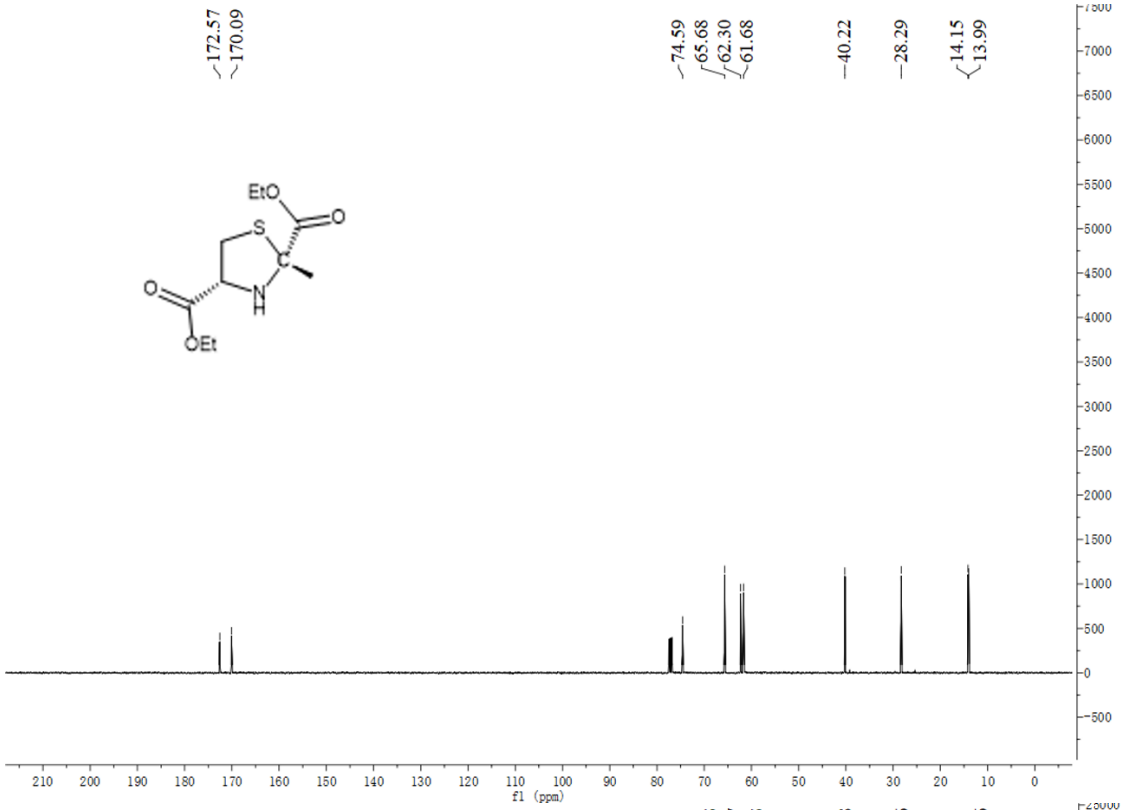
1.09
1.03
3.17
6.45
f1 (ppm)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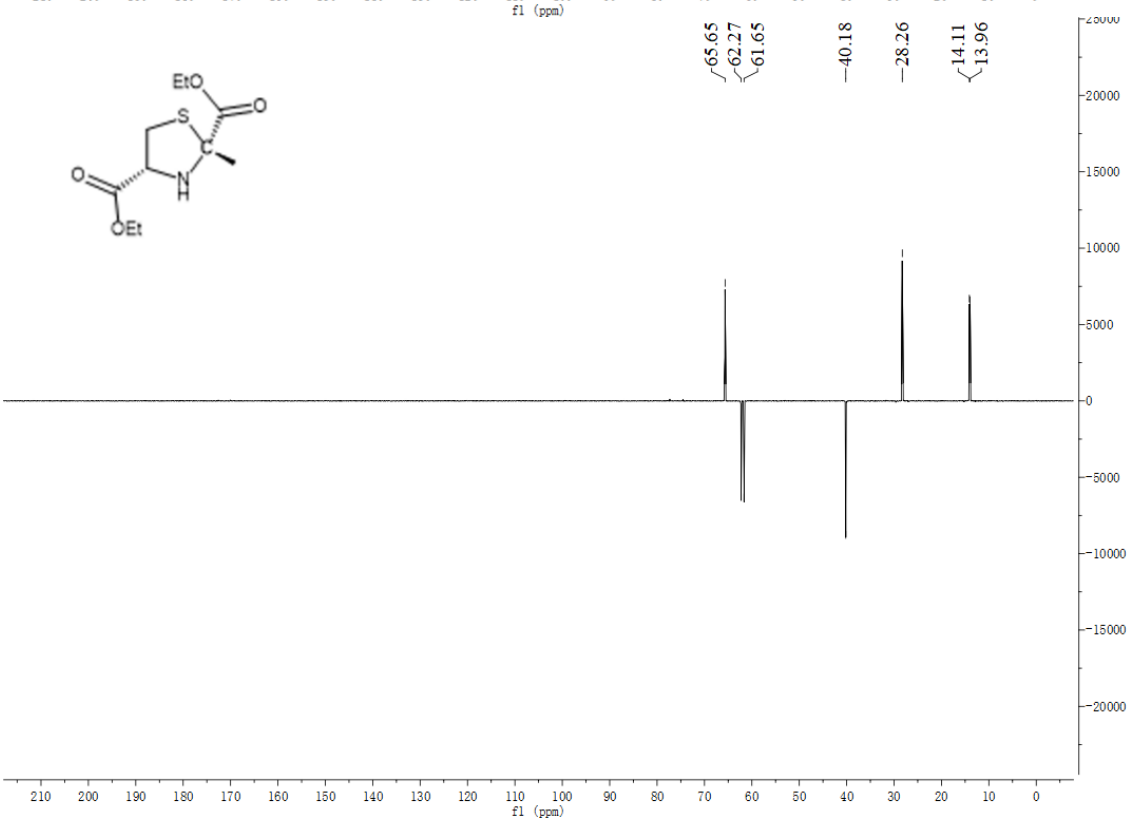
H
O
OEt
f1 (ppm)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EtO
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C
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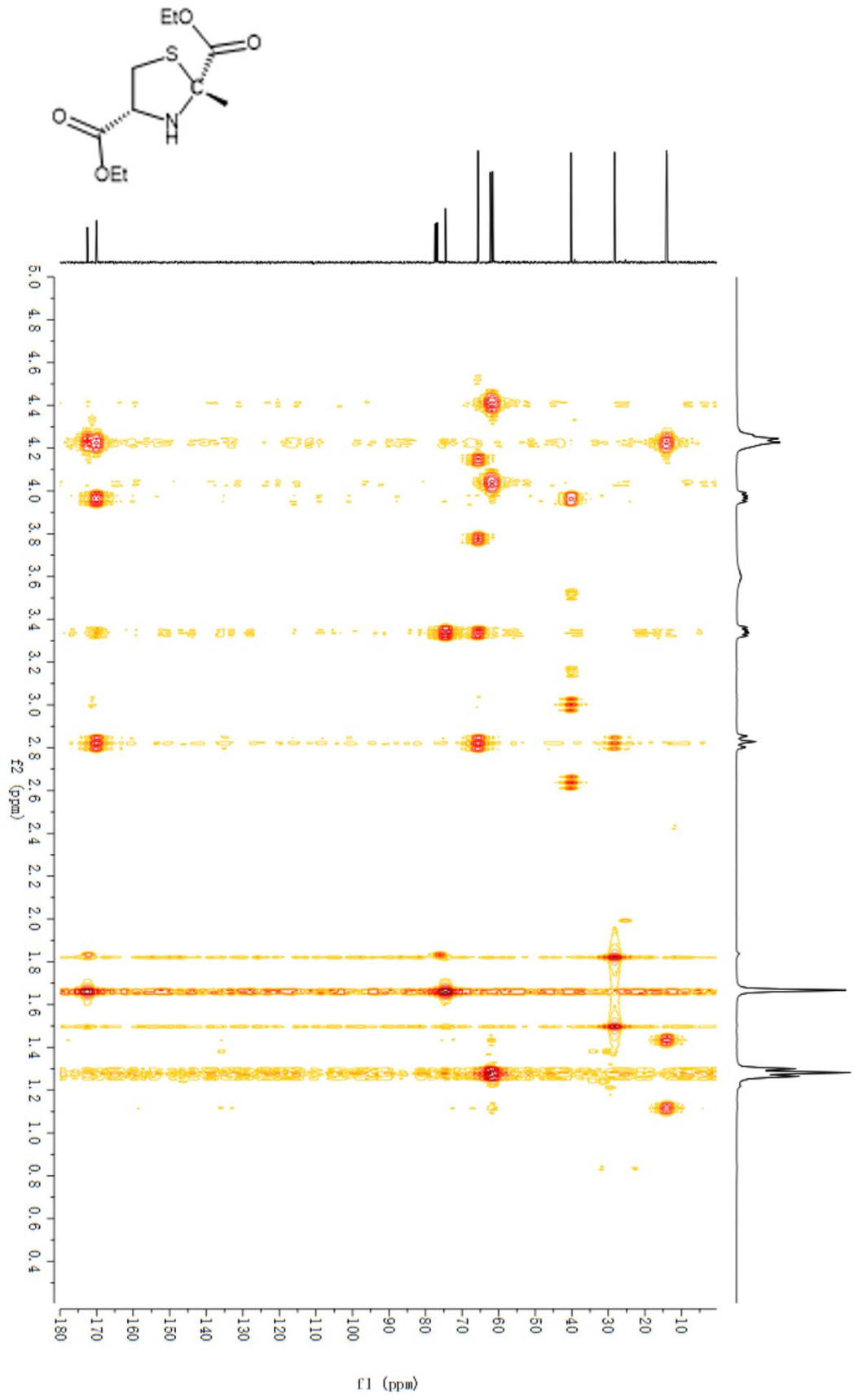
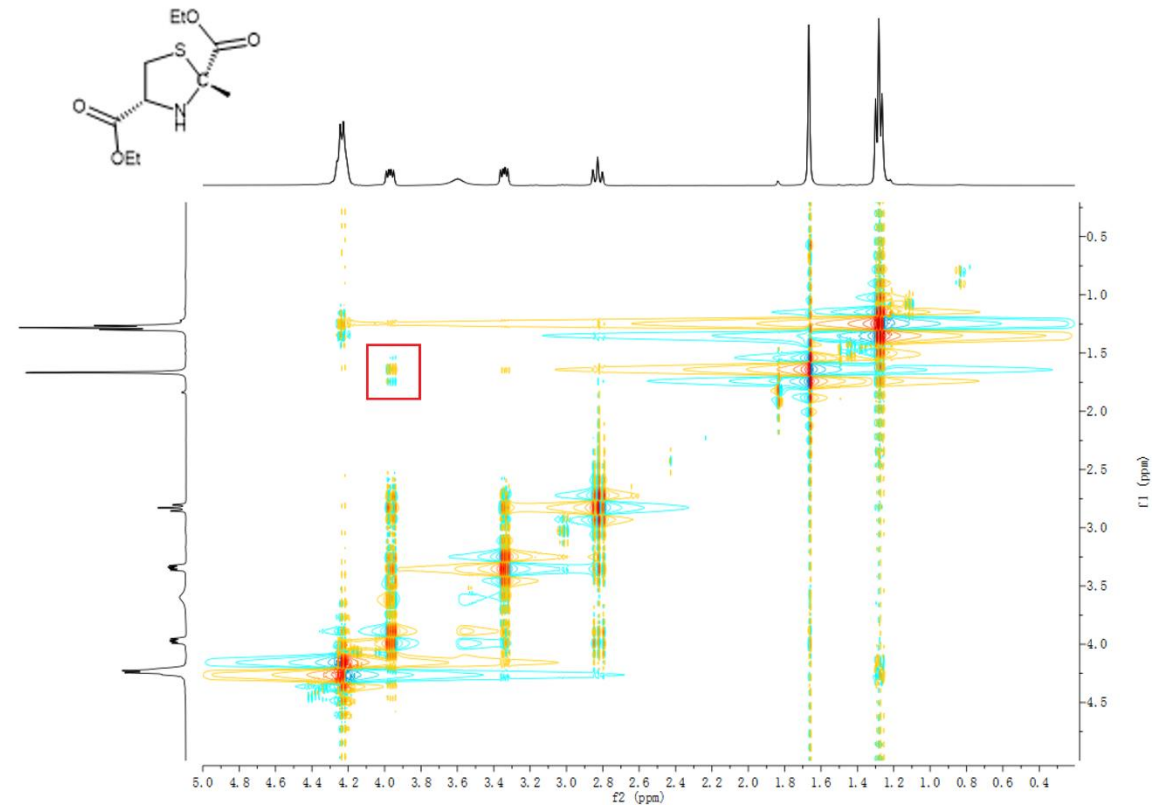
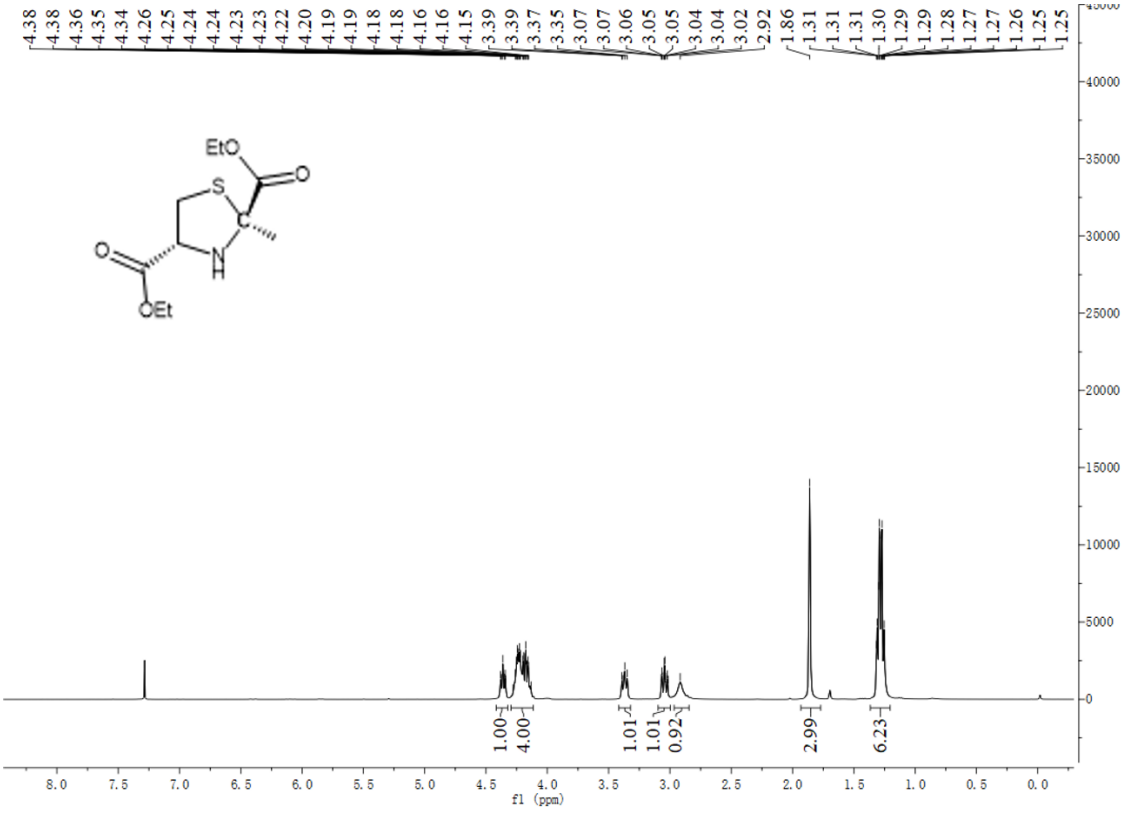
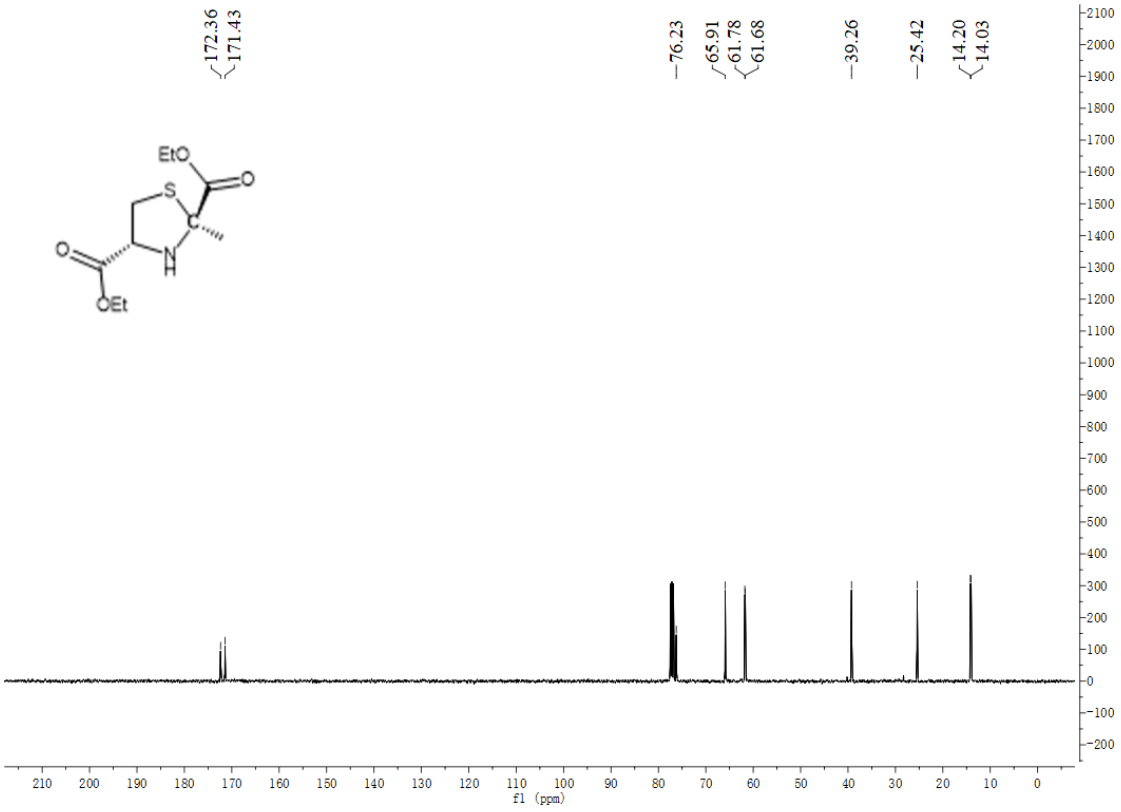
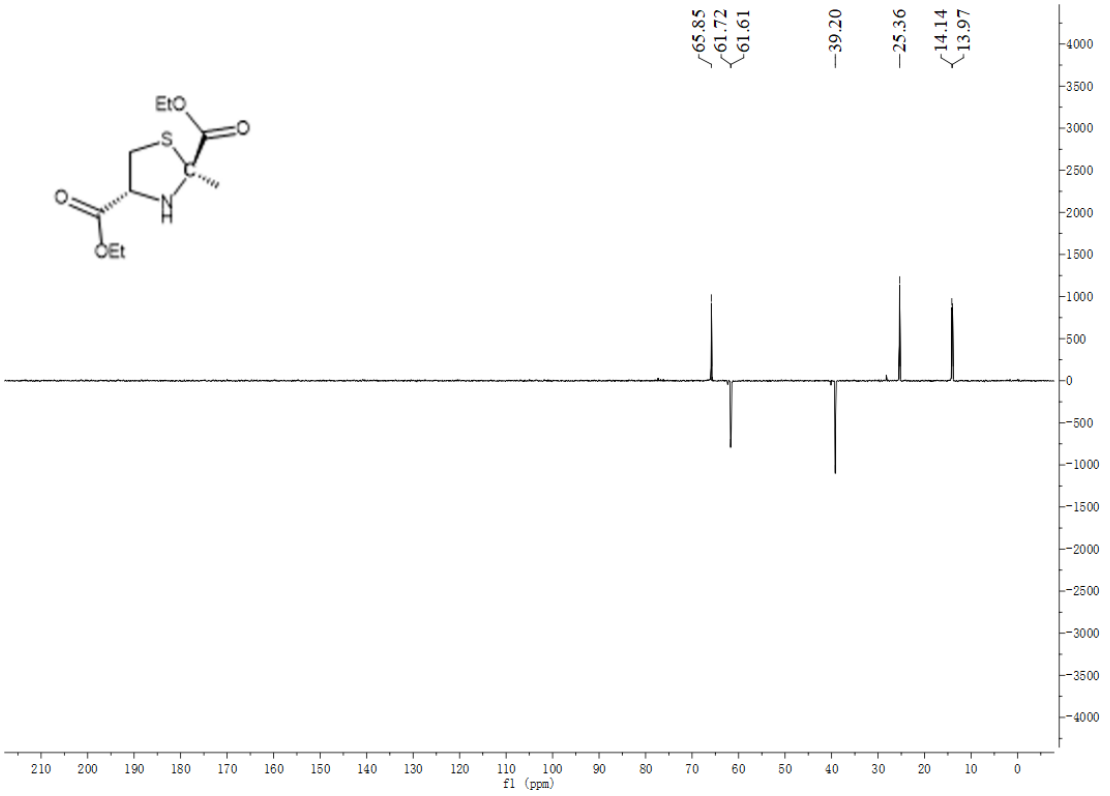
H
O
OEt
f1 (ppm)

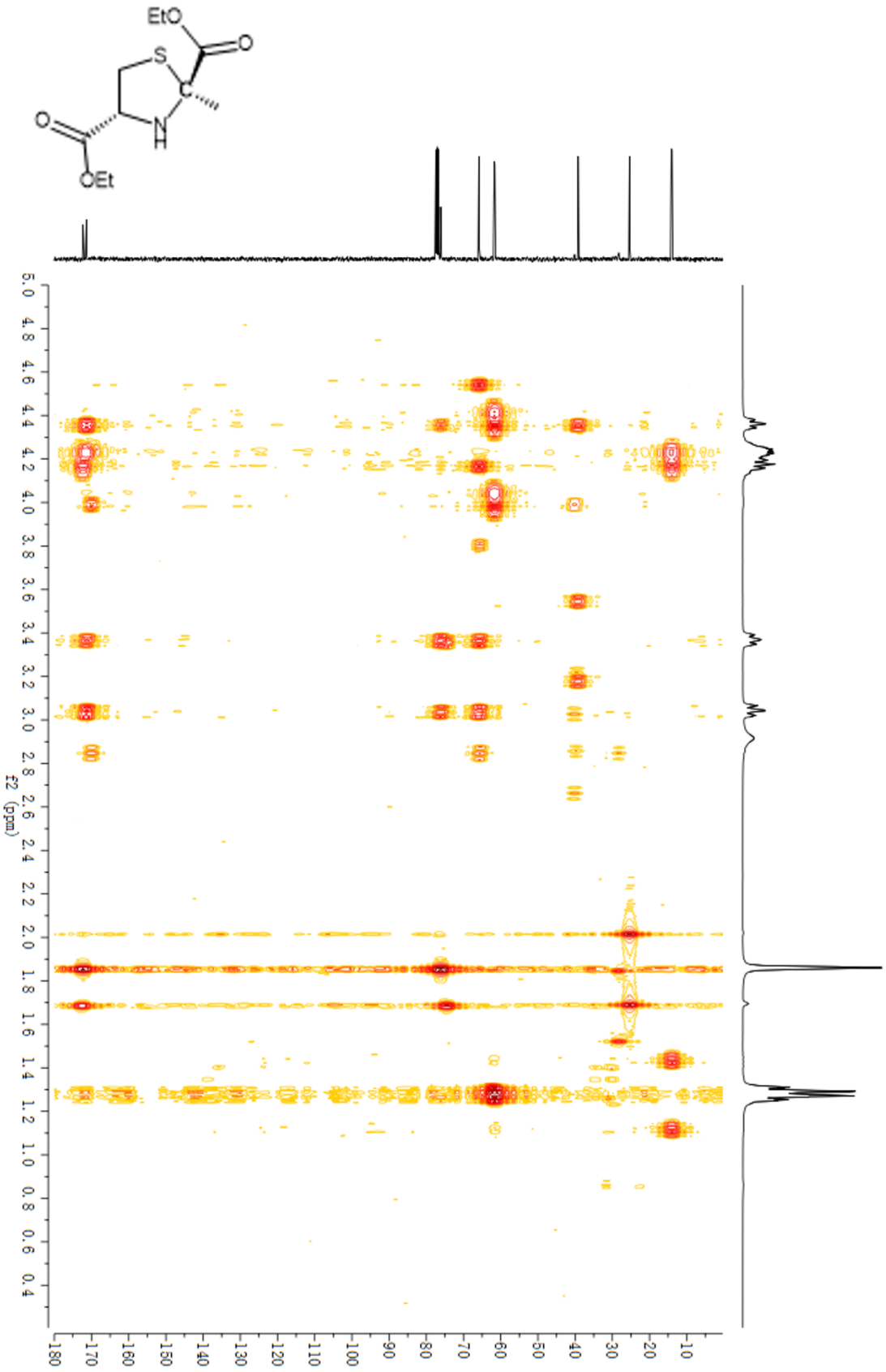

EtO
O
S
C
O
N
H
OEt
f2 (ppm)
f1 (ppm)

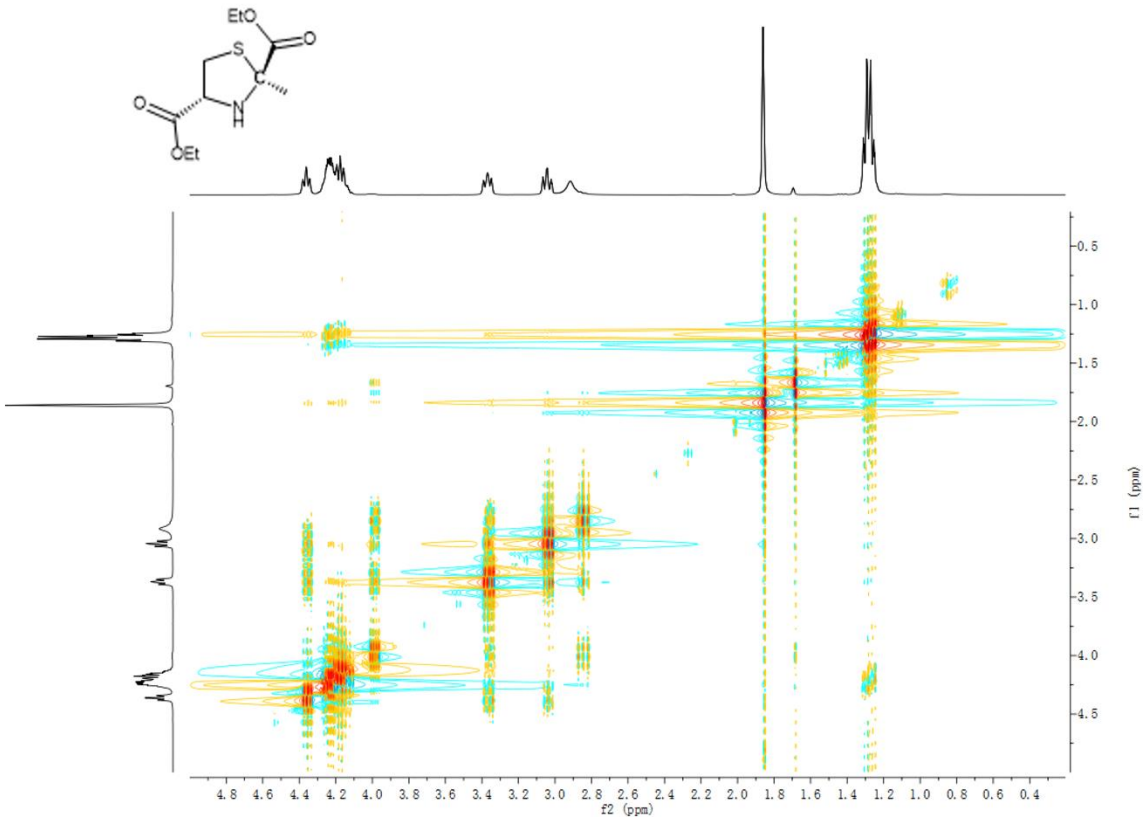

EtO
O
S
C
N
H
O
OEt
f2 (ppm)
f1 (ppm)

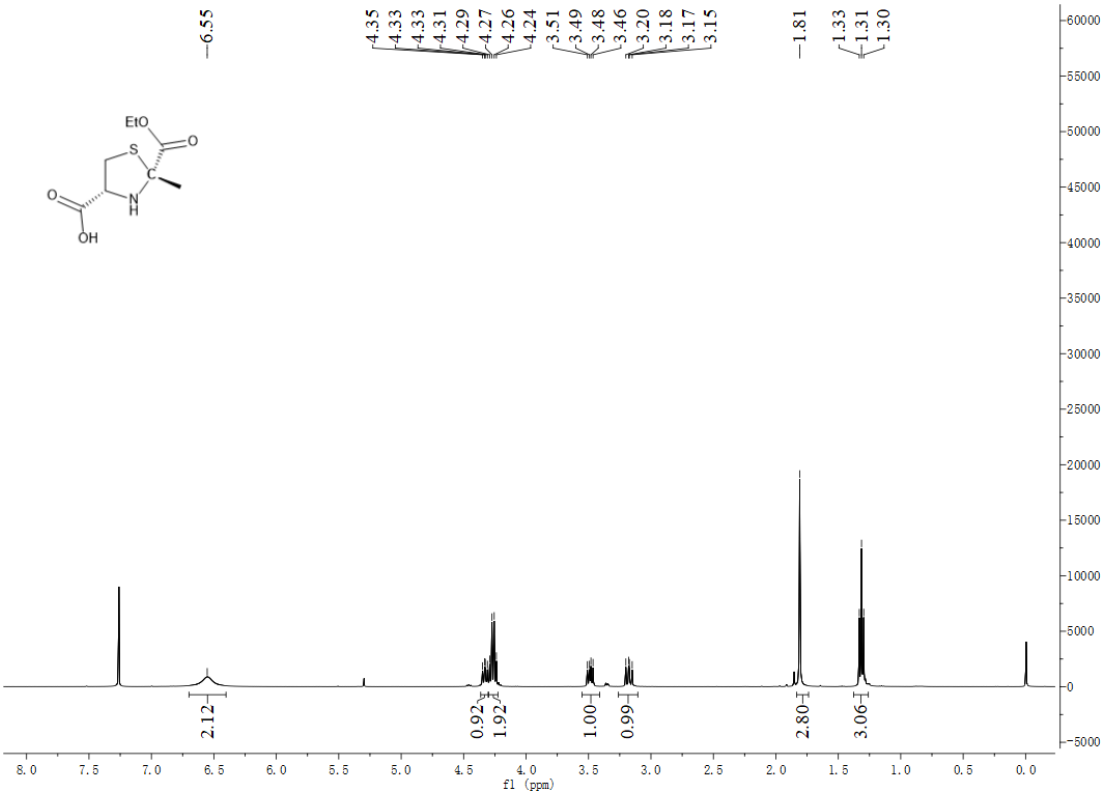

4.38 4.38 4.36 4.35 4.34 4.26 4.25 4.24 4.24 4.23 4.23 4.22 4.20 4.19 4.19 4.18 4.18 4.16 4.16 4.15 3.39 3.39 3.37 3.35 3.07 3.07 3.06 3.05 3.05 3.04 3.04 3.02 2.92 1.86 1.31 1.31 1.31 1.30 1.29 1.29 1.28 1.27 1.27 1.26 1.25 1.25
EtO
O
S
C
N
H
O
OEt
1.00
4.00
1.01
1.01
0.92
2.99
6.23
f1 (ppm)

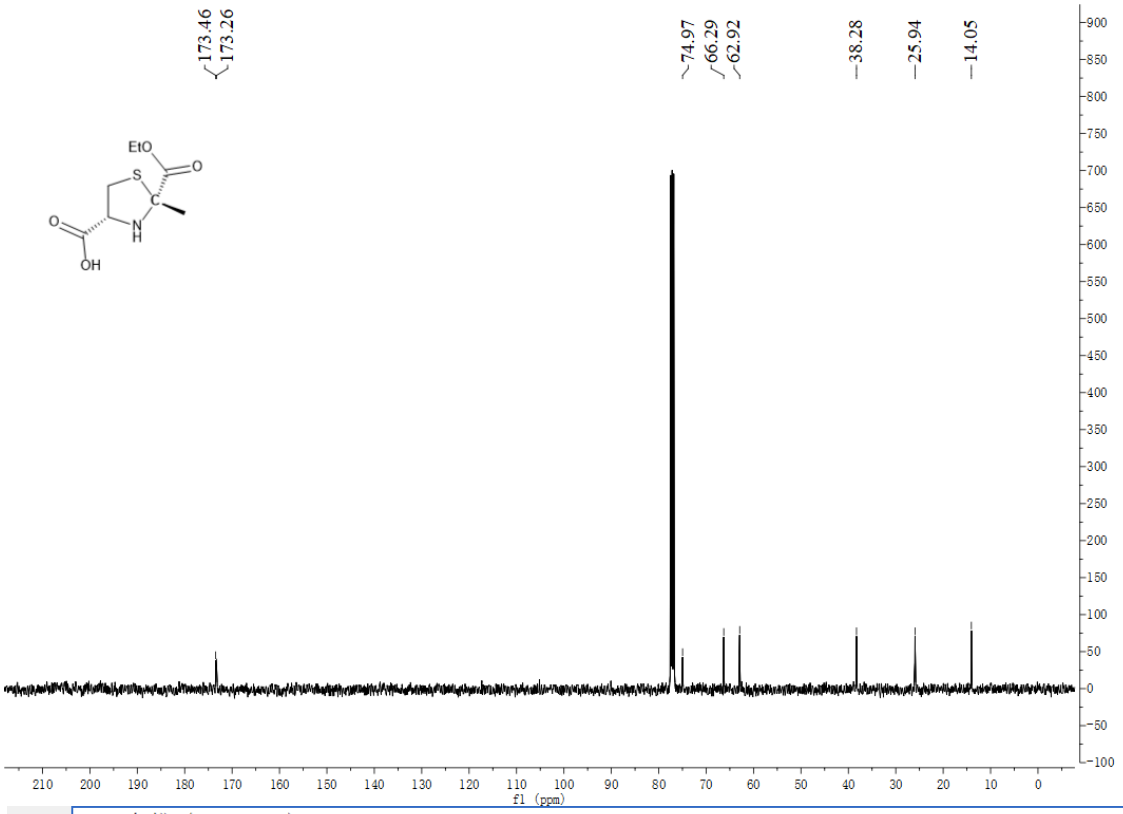

172.36
171.43
76.23
65.91
61.78
61.68
39.26
25.42
14.20
14.03
EtO
O
S
C
N
H
O
OEt
f1 (ppm)

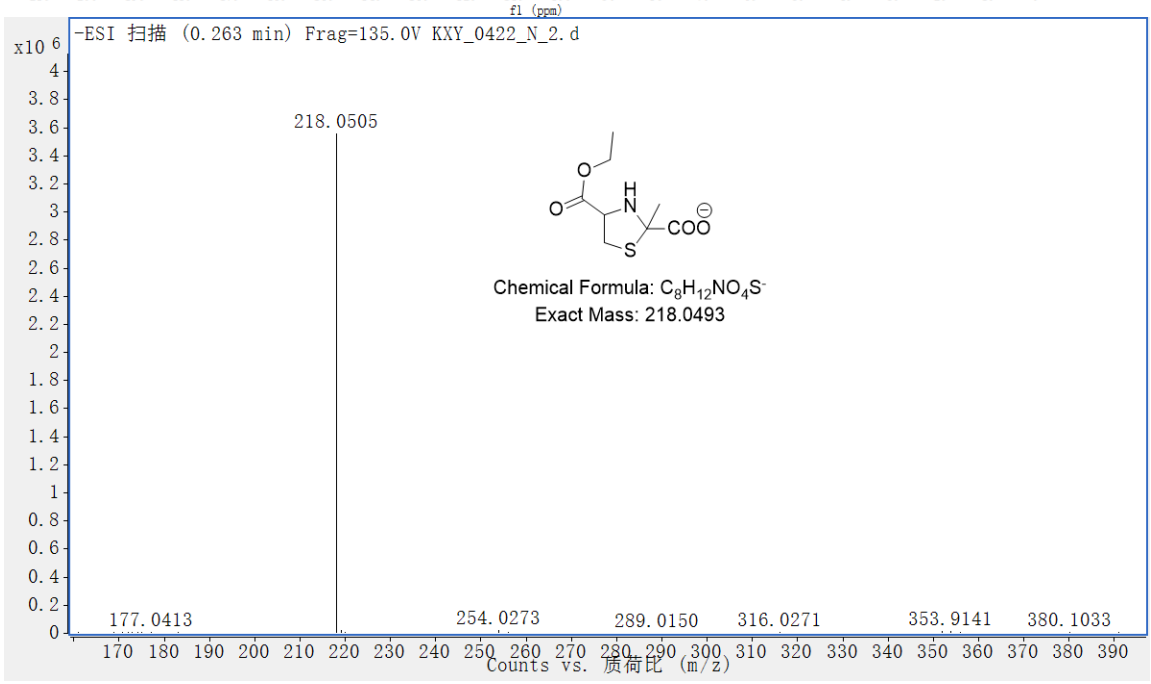

65.85
61.72
61.61
39.20
25.36
14.14
13.97
EtO
O
S
C
N
H
O
OEt
f1 (ppm)

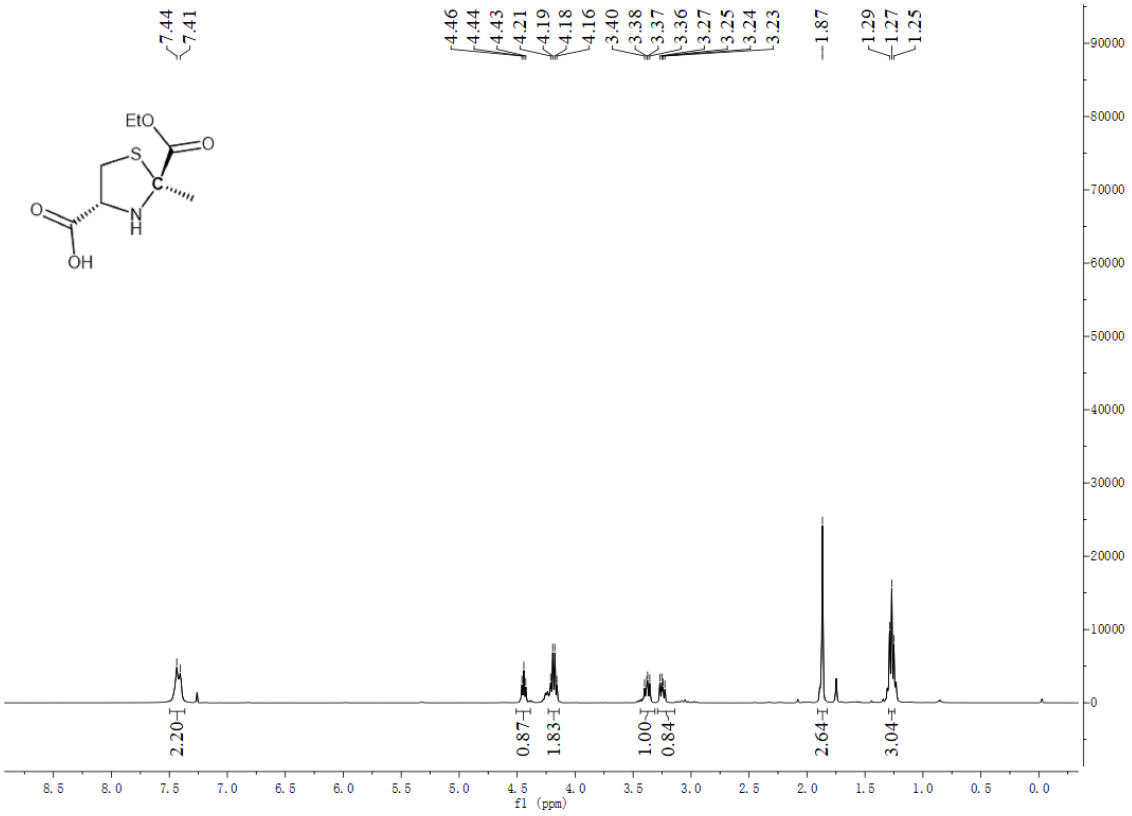

EtO
O
S
C
O
N
H
OEt
f2 (ppm)

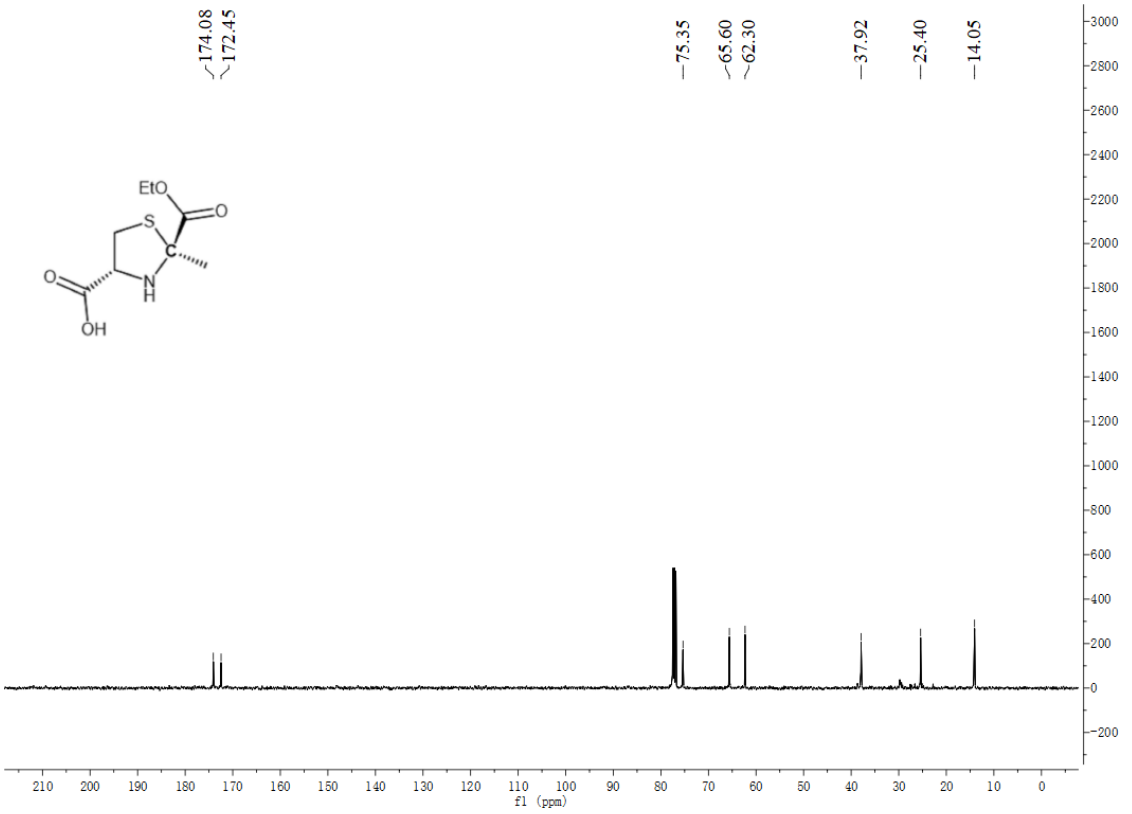

EtO
O
S
C
N
H
O
OEt
f1 (ppm)
f2 (ppm)

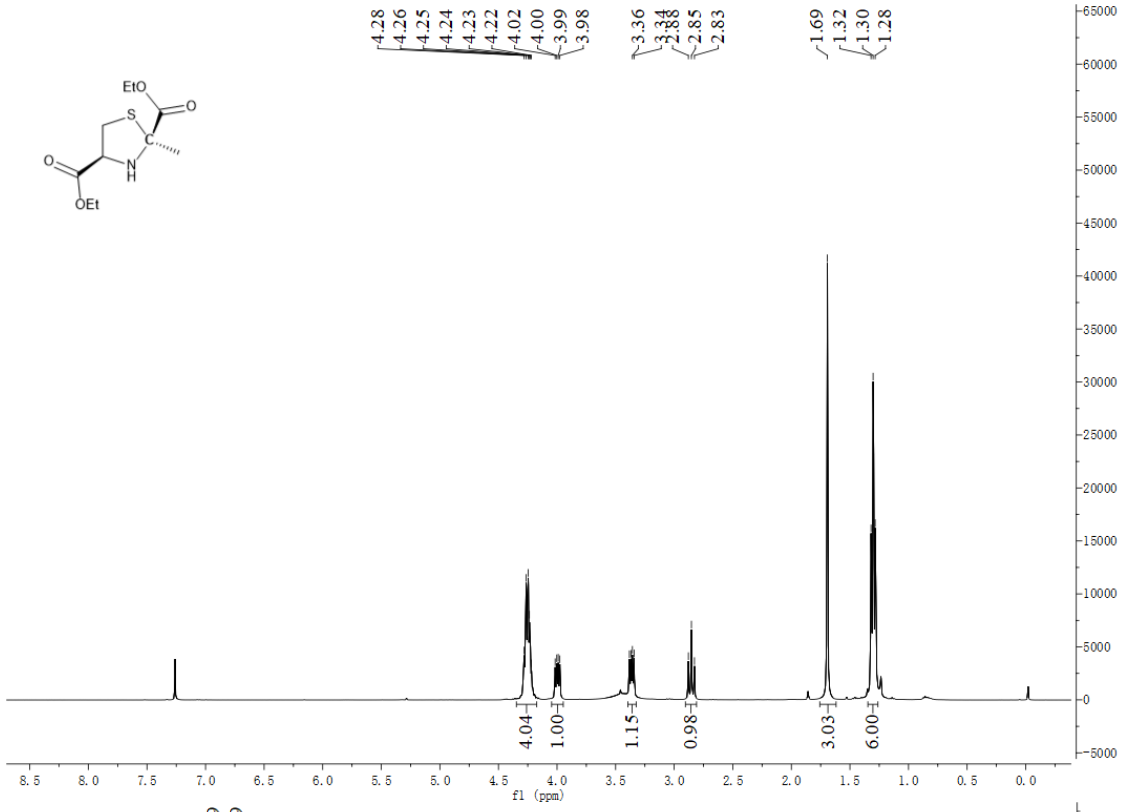

6.55
4.35
4.33
4.33
4.31
4.29
4.27
4.26
4.24
3.51
3.49
3.48
3.46
3.20
3.18
3.17
3.15
1.81
1.33
1.31
1.30
EtO
O
S
C
N
H
O
OH
2.12
0.92
1.92
1.00
0.99
2.80
3.06
f1 (ppm)

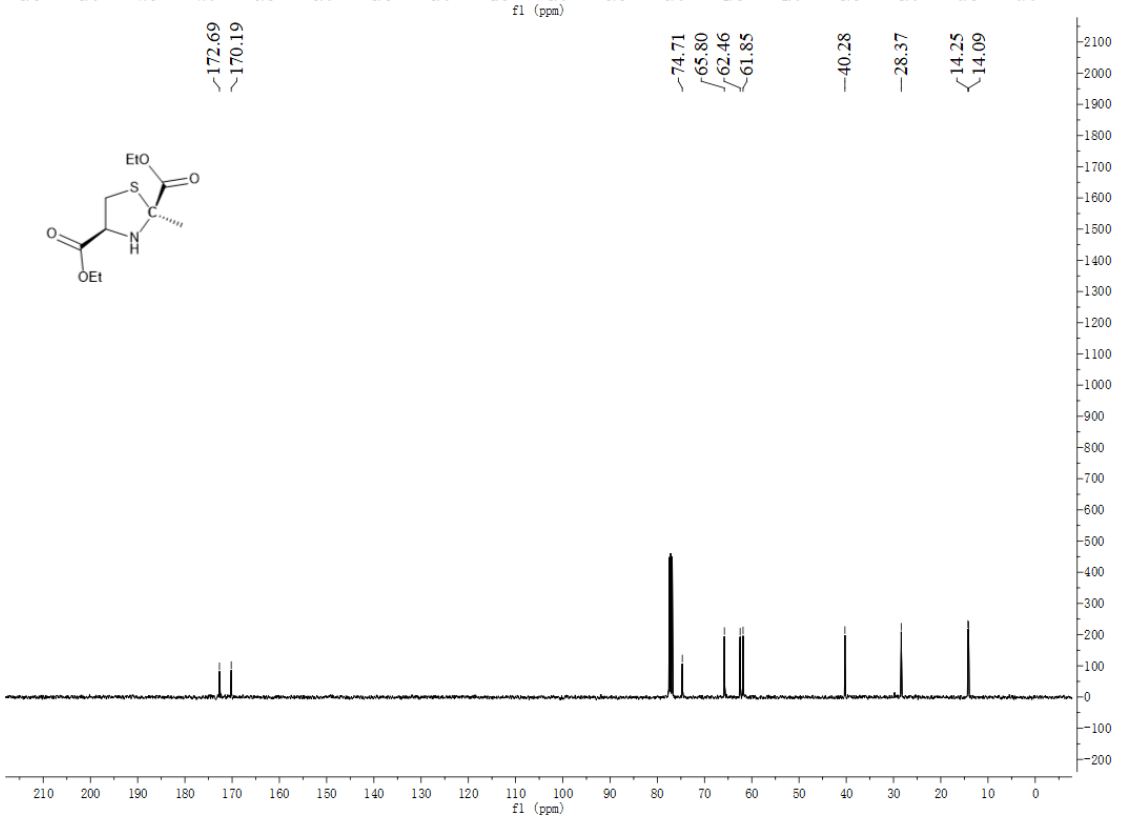

173.46
173.26
74.97
66.29
62.92
38.28
25.94
14.05
EtO
O
S
C
N
H
O
OH
f1 (ppm)

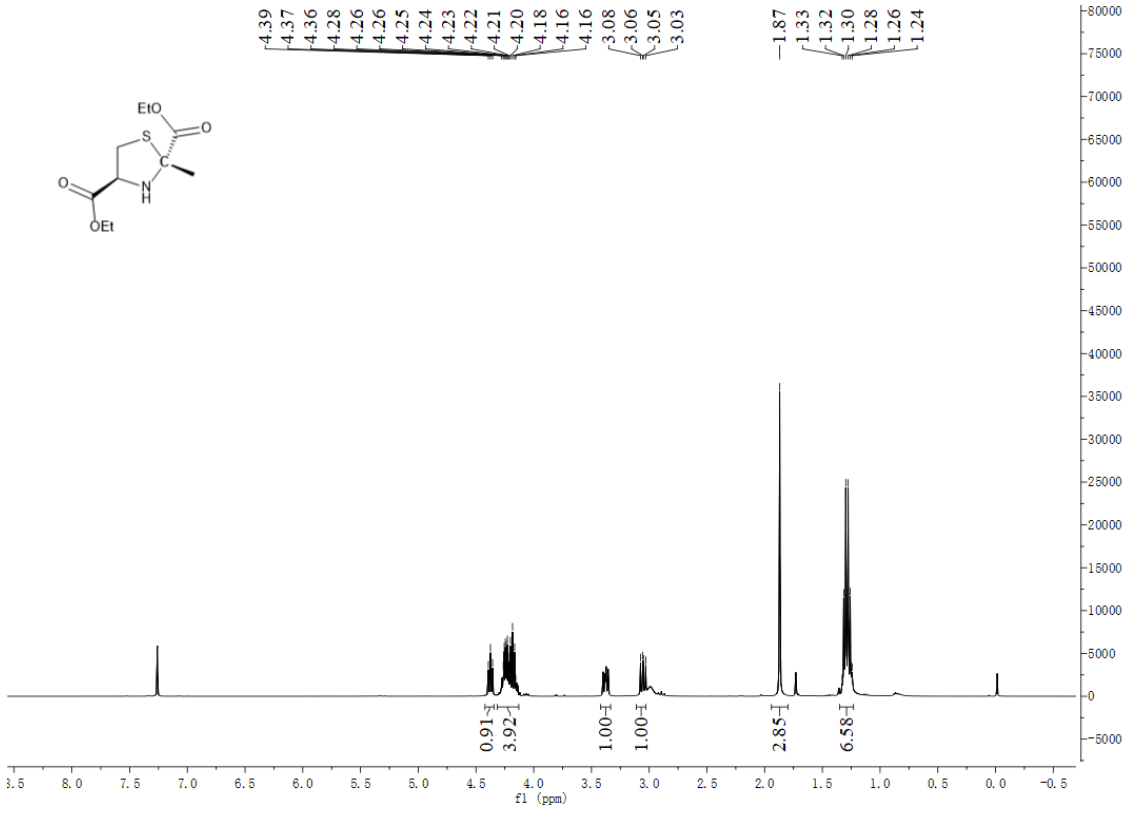

-ESI 扫描 (0.263 min) Frag=135.0V KXY_0422_N_2.d
x10 6
218.0505
177.0413
254.0273
289.0150
316.0271
353.9141
380.1033
Counts vs. 质荷比 (m/z)
Chemical Formula: C8H12NO4S-
Exact Mass: 218.0493

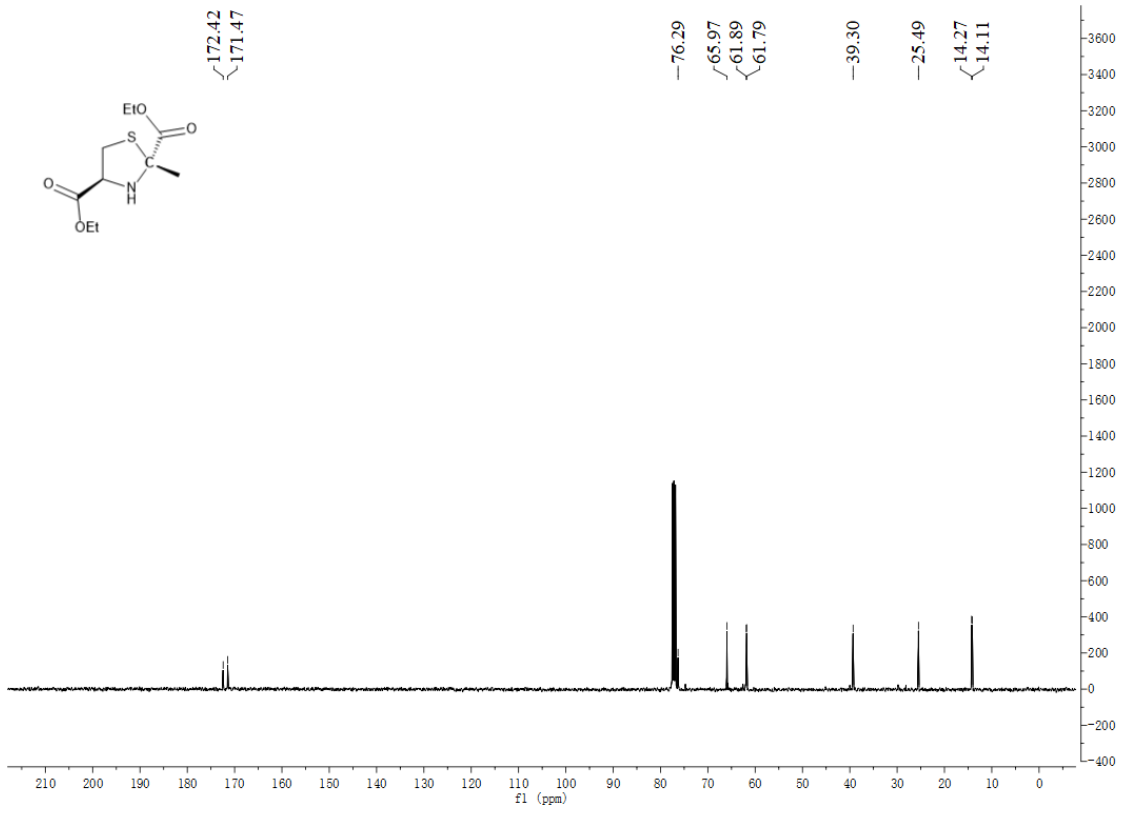

7.44
7.41
4.46
4.44
4.43
4.21
4.19
4.18
4.16
3.40
3.38
3.37
3.36
3.27
3.25
3.24
3.23
1.87
1.29
1.27
1.25
EtO
O
S
C
N
H
O
OH
2.20
0.87
1.83
1.00
0.84
2.64
3.04
f1 (ppm)

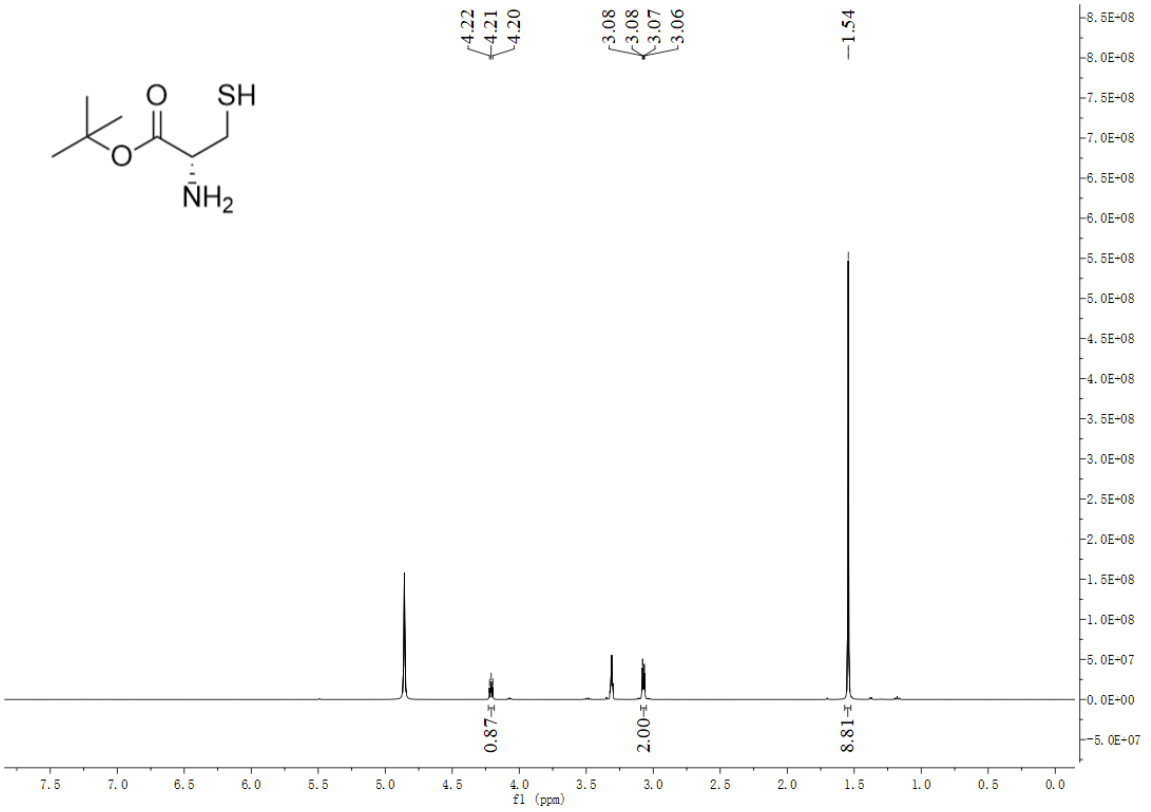

174.08
172.45
75.35
65.60
62.30
37.92
25.40
14.05
EtO
O
S
C
N
H
O
OH
f1 (ppm)

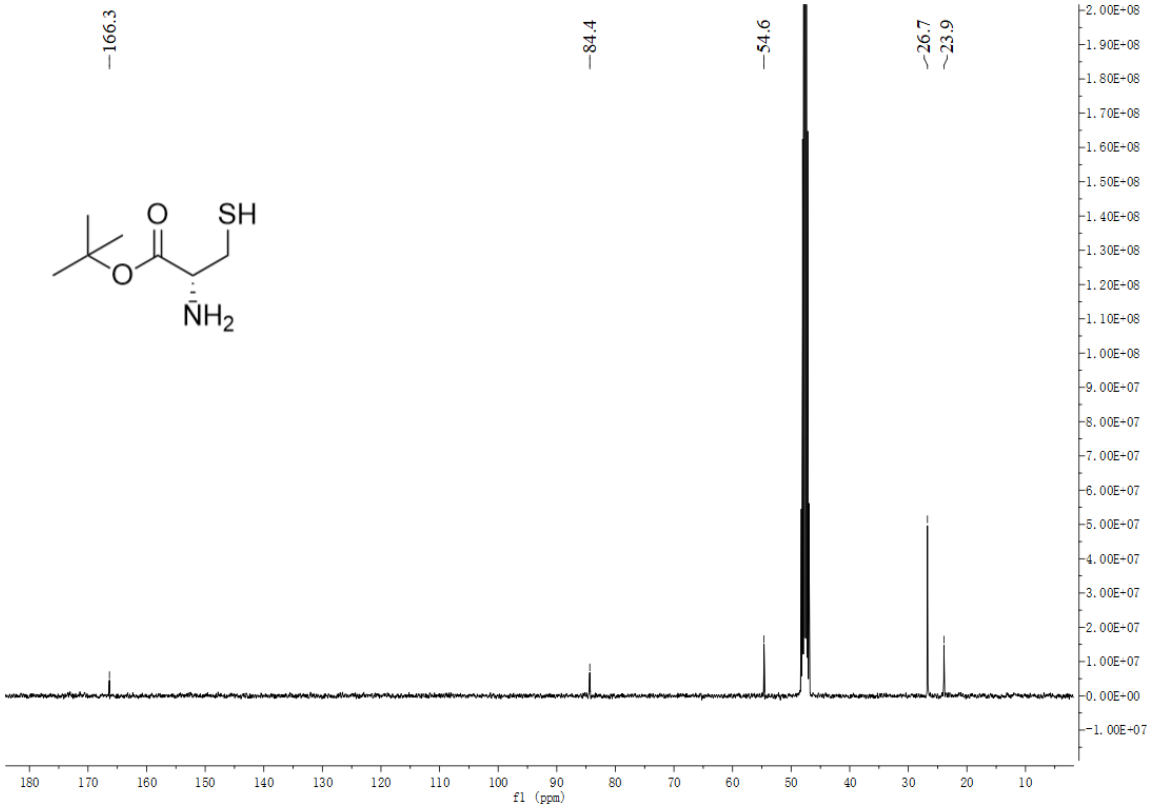

4.28
4.26
4.25
4.24
4.23
4.22
4.02
4.00
3.99
3.98
3.36
3.34
2.88
2.85
2.83
1.69
1.32
1.30
1.28
EtO
O
S
C
N
H
O
OEt
4.04
1.00
1.15
0.98
3.03
6.00
f1 (ppm)

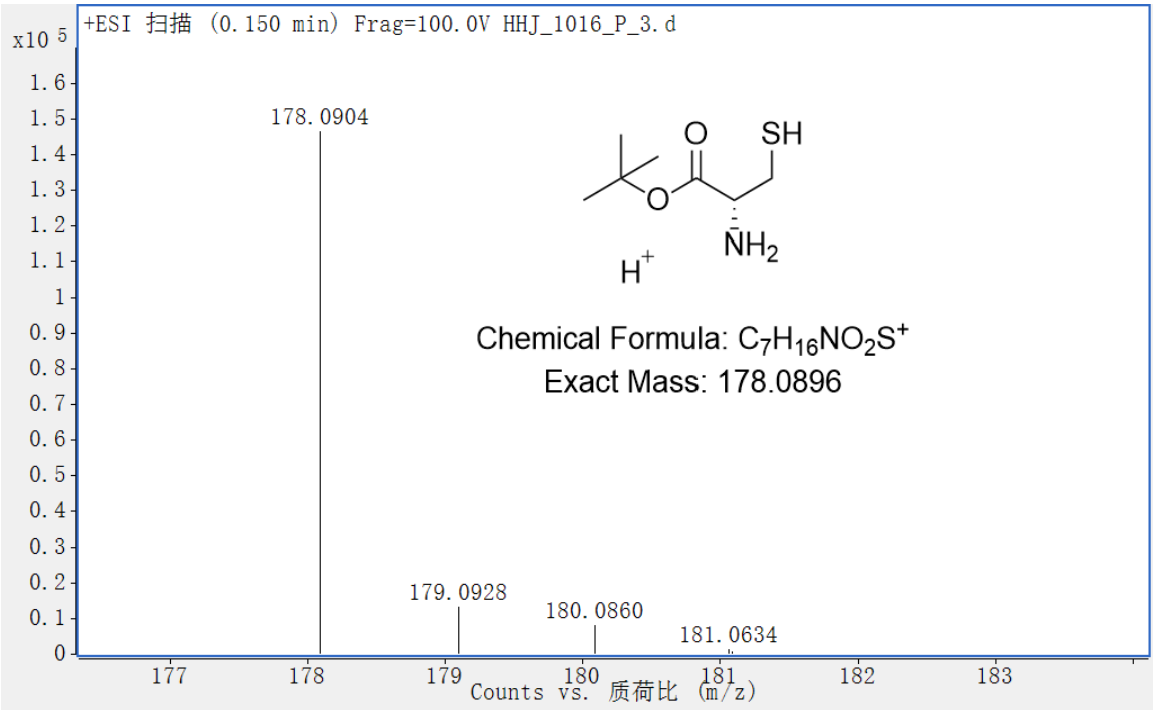

172.69
170.19
74.71
65.80
62.46
61.85
40.28
28.37
14.25
14.09
EtO
O
S
C
N
H
O
OEt
f1 (ppm)

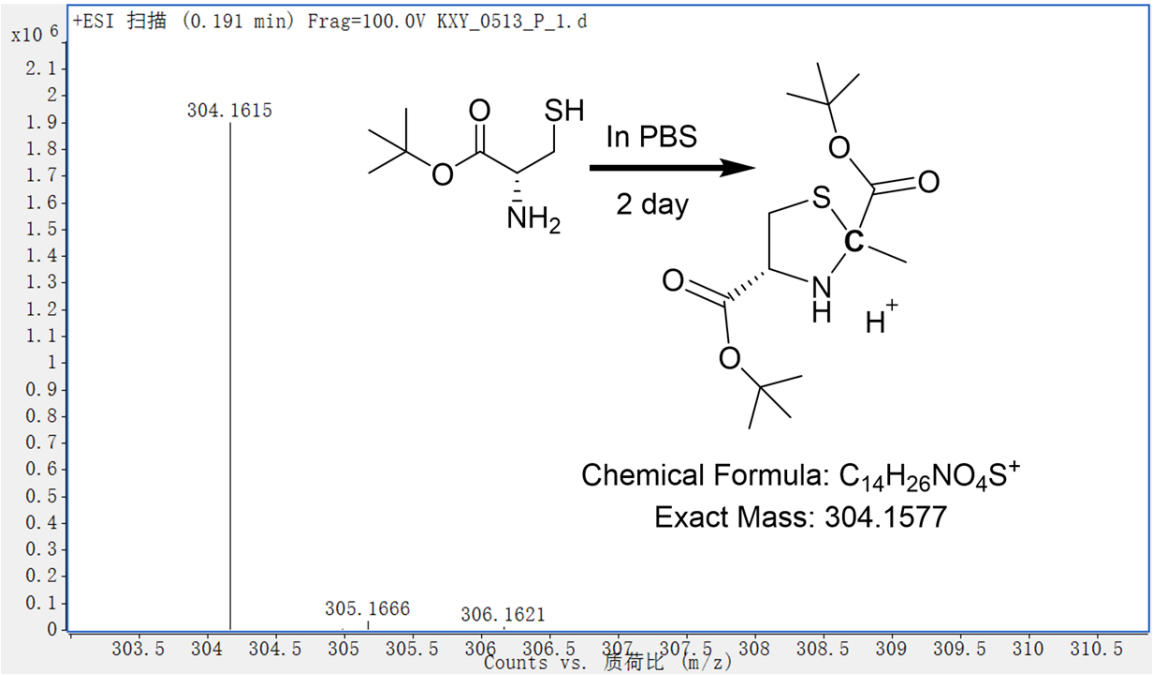

4.39
4.37
4.36
4.28
4.26
4.26
4.25
4.24
4.23
4.22
4.21
4.20
4.18
4.16
4.16
3.08
3.06
3.05
3.03
1.87
1.33
1.32
1.30
1.28
1.26
1.24
EtO
O
S
C
N
H
O
OEt
0.91
3.92
1.00
1.00
2.85
6.58
f1 (ppm)

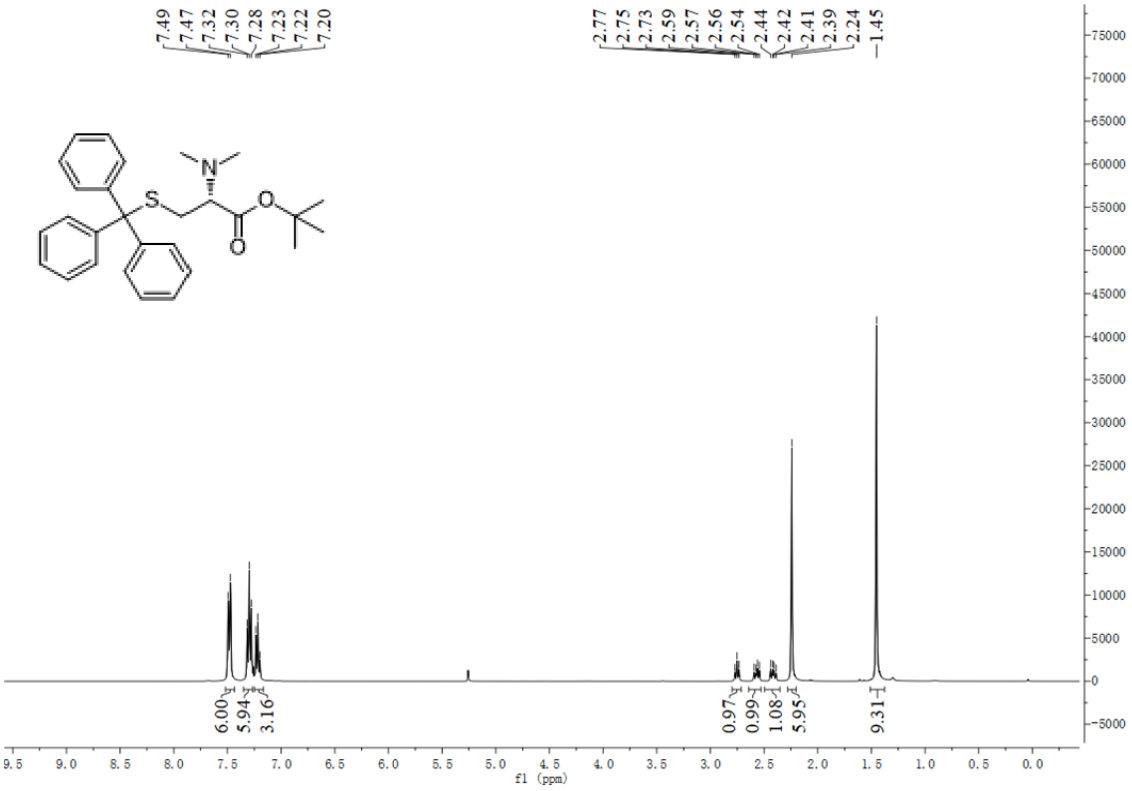

172.42
171.47
76.29
65.97
61.89
61.79
39.30
25.49
14.27
14.11
EtO
O
S
C
N
H
O
OEt
f1 (ppm)

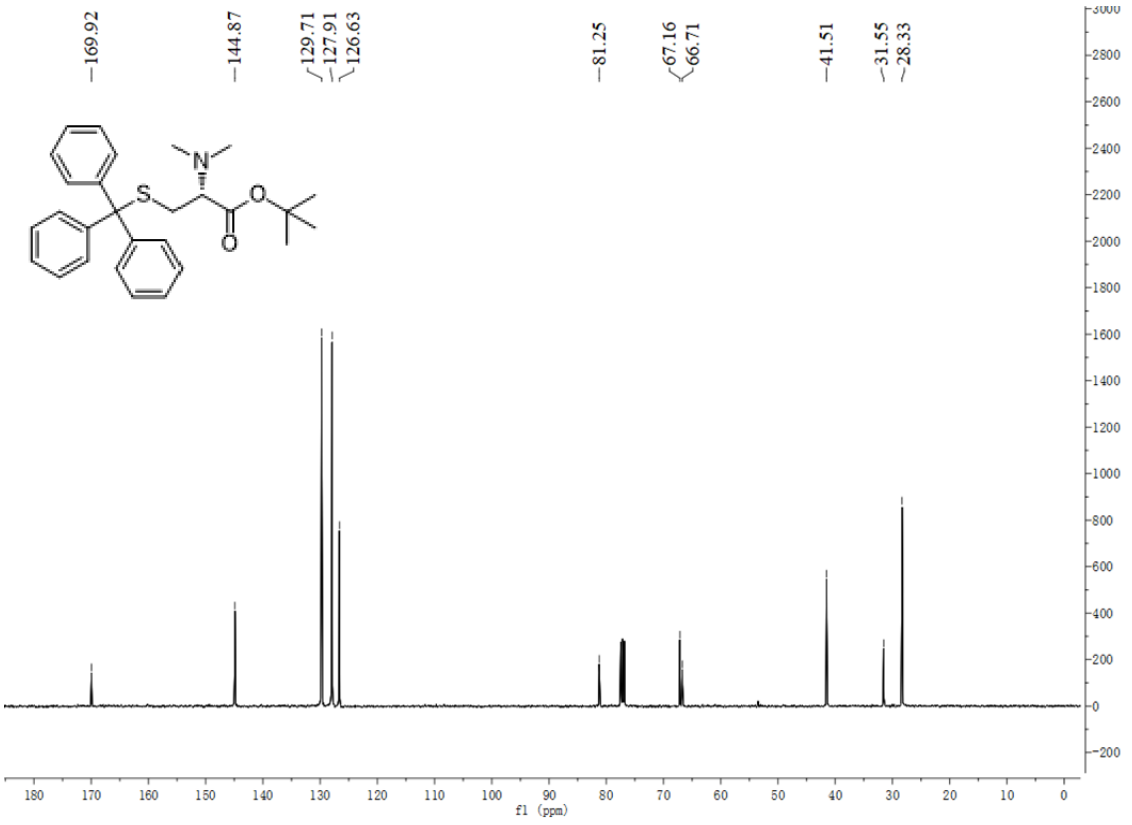

4.22
4.21
4.20
3.08
3.08
3.07
3.06
1.54
O
SH
O
NH2
0.87
2.00
8.81
f1 (ppm)

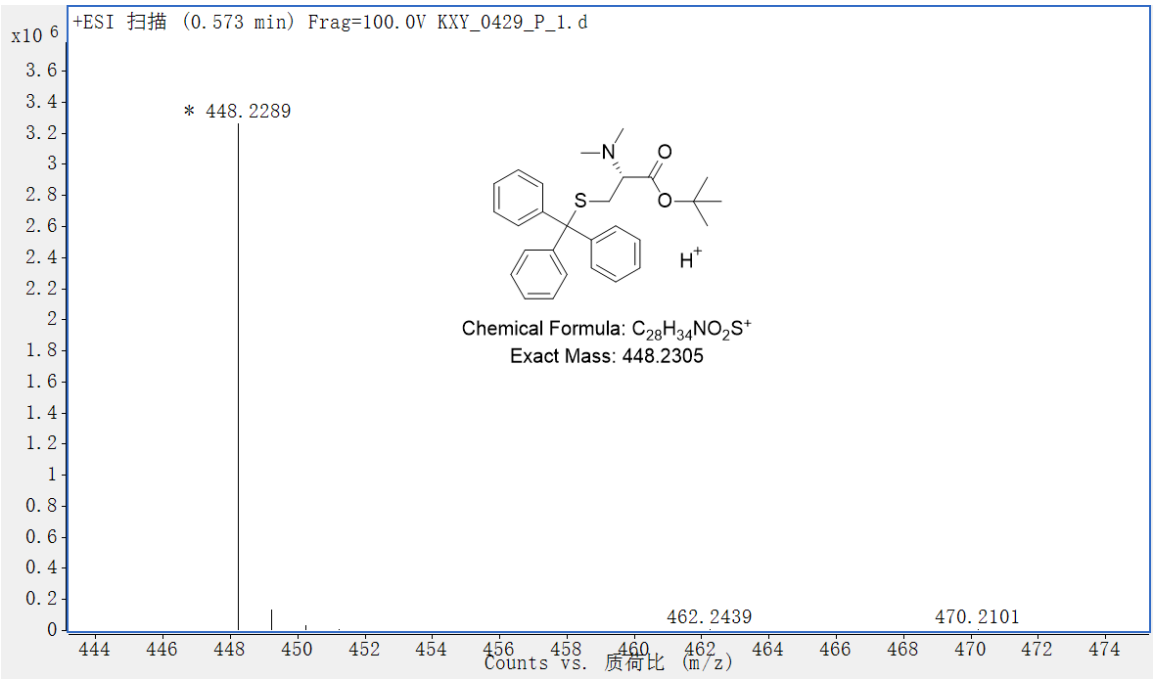

166.3
84.4
54.6
26.7
23.9
O
SH
O
NH2
f1 (ppm)

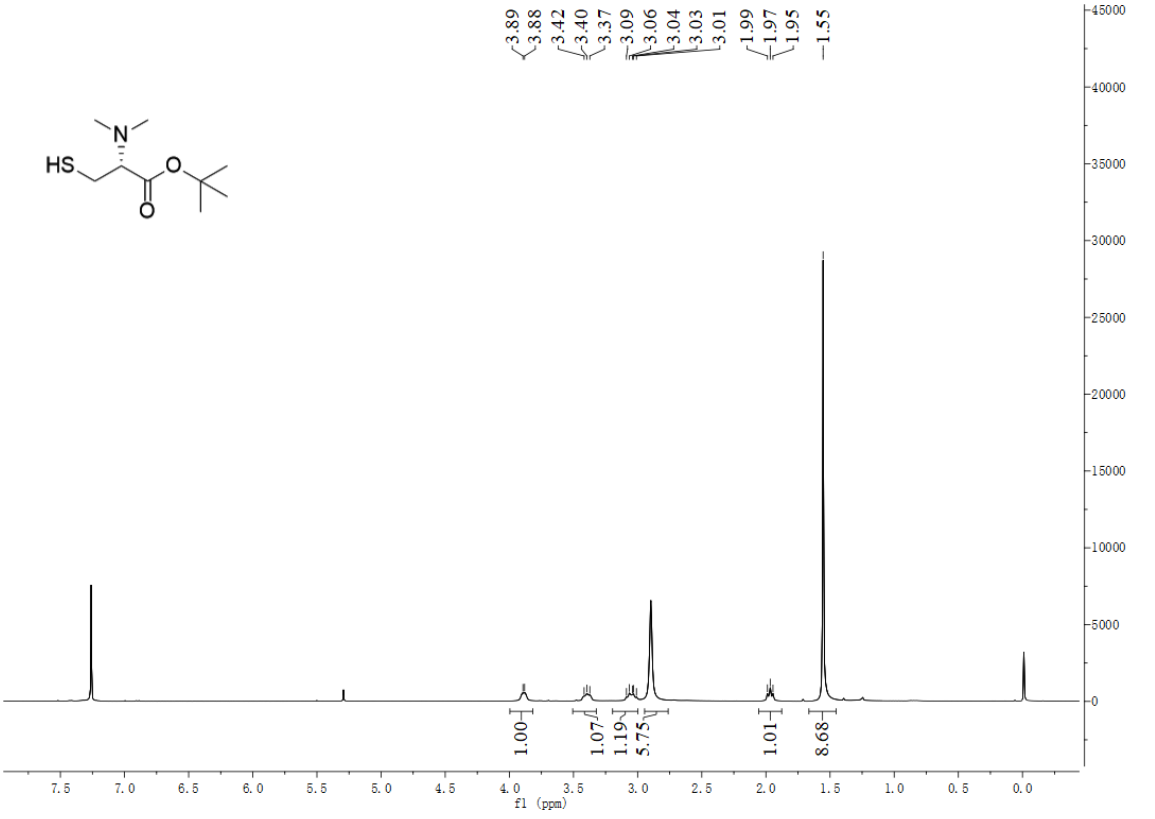

+ESI 扫描 (0.150 min) Frag=100.0V HHJ_1016_P_3.d
x10 5
178.0904
179.0928
180.0860
181.0634
Chemical Formula: C7H16NO2S+
Exact Mass: 178.0896
Counts vs. 质荷比 (m/z)

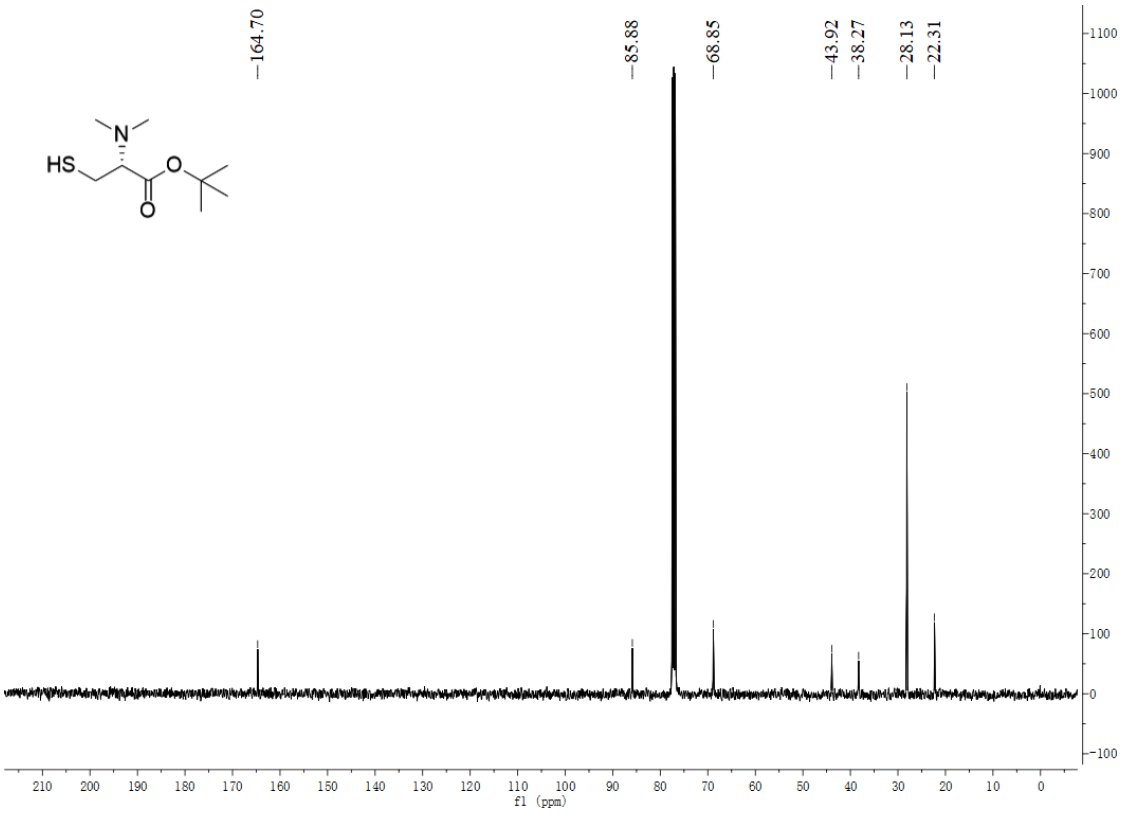

+ESI 扫描 (0.191 min) Frag=100.0V KXY_0513_P_1.d
x10 6
304.1615
305.1666
306.1621
In PBS
2 day
Chemical Formula: C14H26NO4S+
Exact Mass: 304.1577
Counts vs. 质荷比 (m/z)

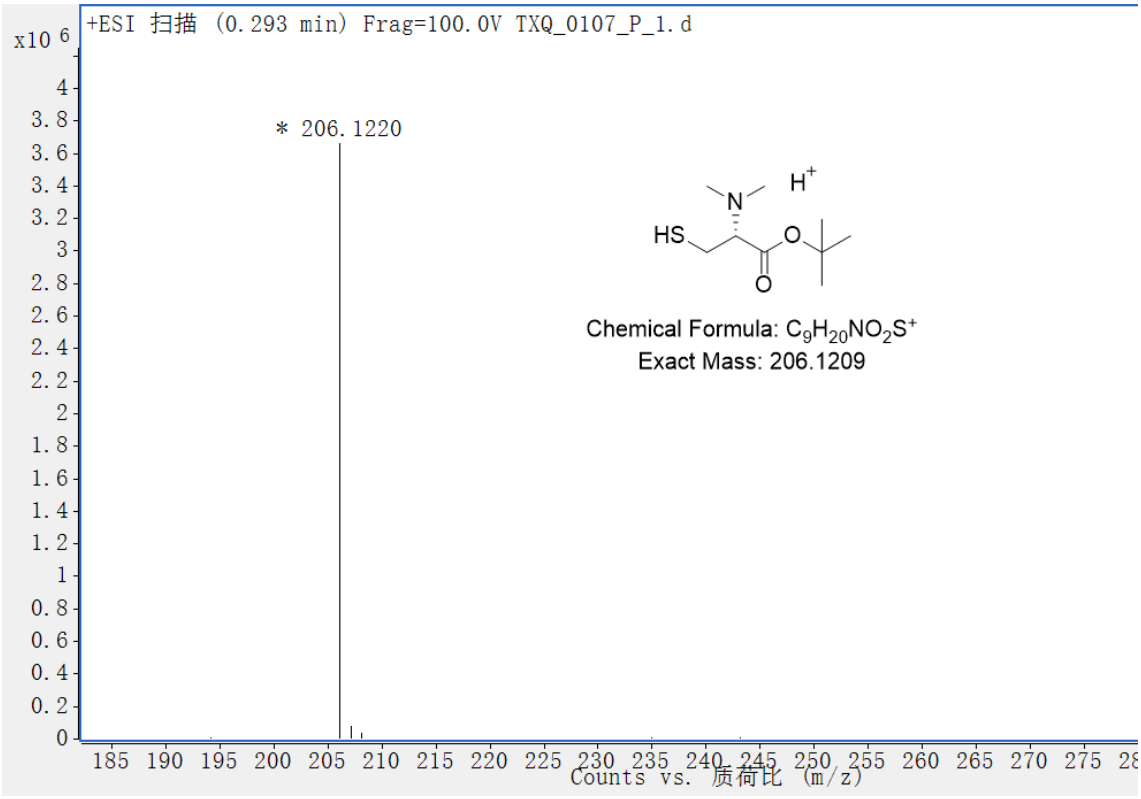

7.49
7.47
7.32
7.30
7.28
7.23
7.22
7.20
2.77
2.75
2.73
2.59
2.57
2.56
2.54
2.44
2.42
2.41
2.39
2.24
1.45
6.00
5.94
3.16
0.97
0.99
1.08
5.95
9.31
f1 (ppm)

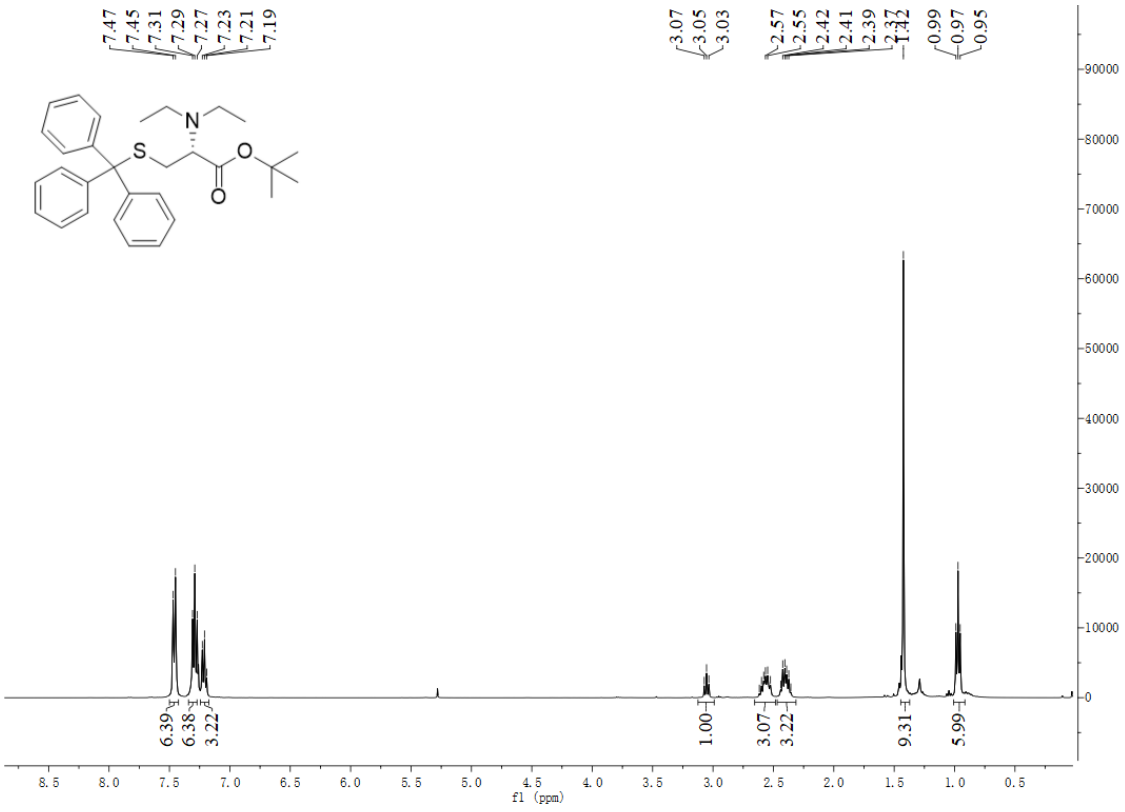

169.92
144.87
129.71
127.91
126.63
81.25
67.16
66.71
41.51
31.55
28.33
f1 (ppm)

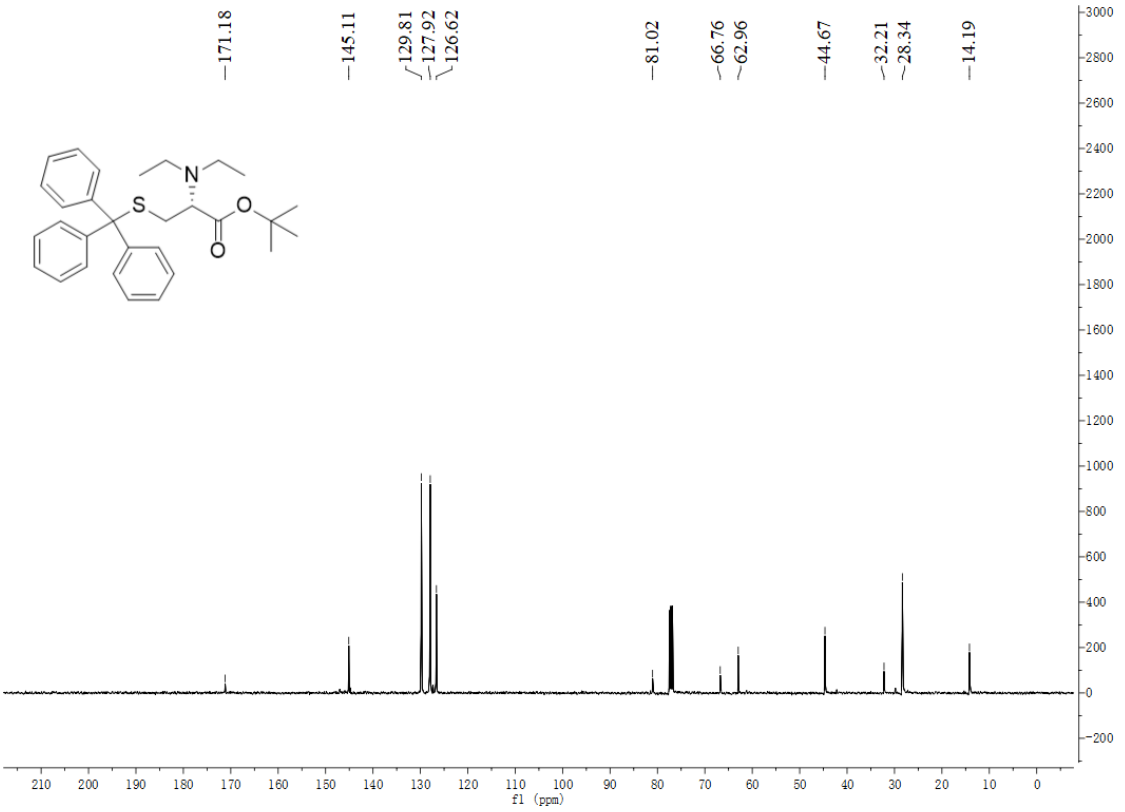

+ESI 扫描 (0.573 min) Frag=100.0V KXY_0429_P_1.d
x10 6
* 448.2289
462.2439
470.2101
Chemical Formula: C28H34NO2S+
Exact Mass: 448.2305
Counts vs. 质荷比 (m/z)

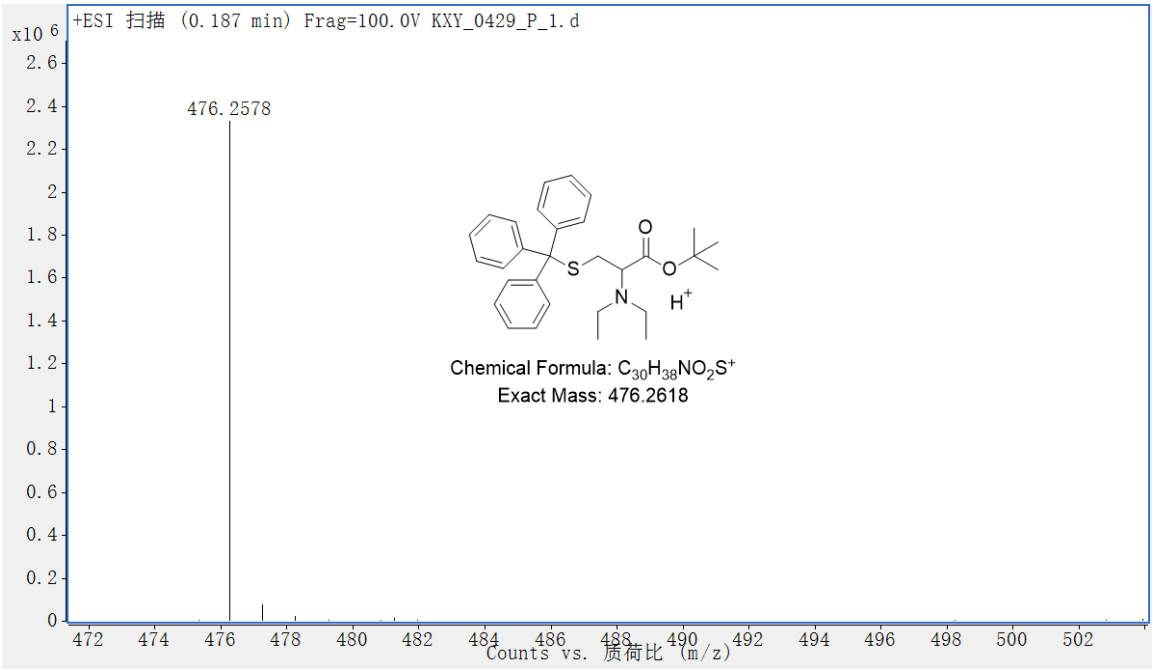

3.89
3.88
3.42
3.40
3.37
3.09
3.06
3.04
3.03
3.01
1.99
1.97
1.95
1.55
1.00
1.07
1.19
5.75
1.01
8.68
f1 (ppm)

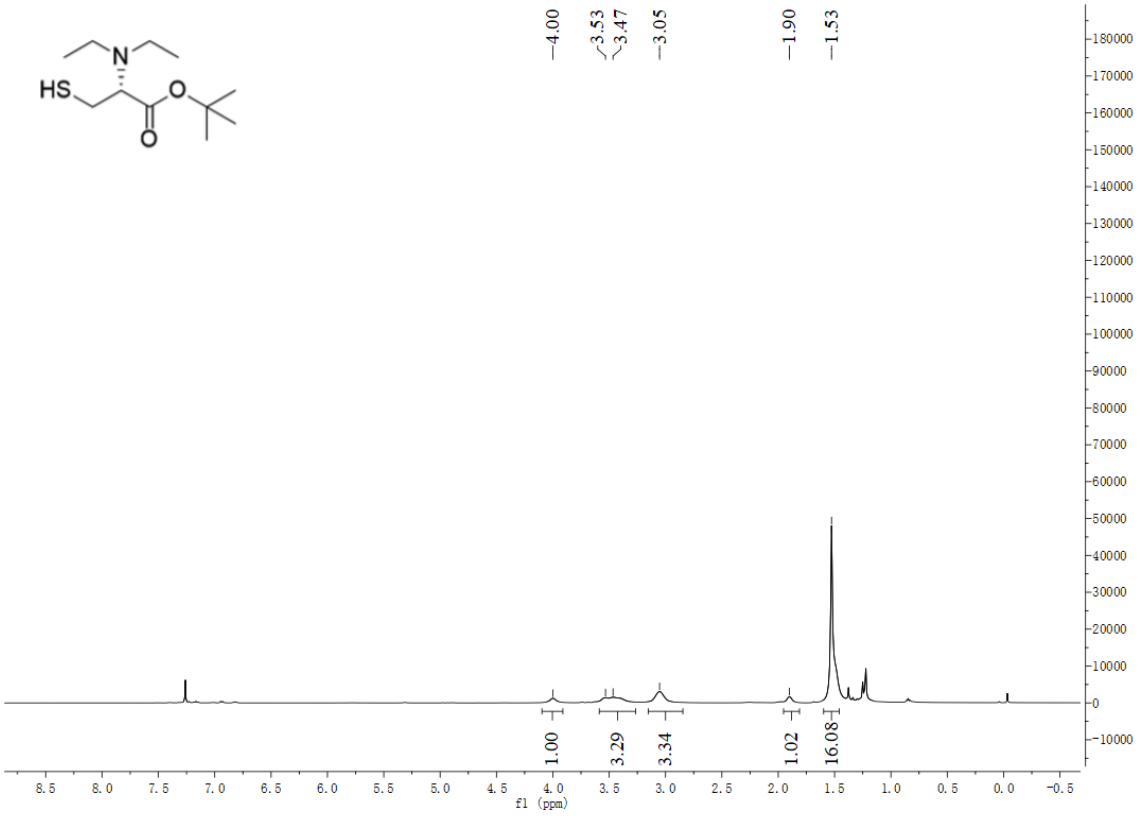

164.70
85.88
68.85
43.92
38.27
28.13
22.31
HS
N
O
O
f1 (ppm)

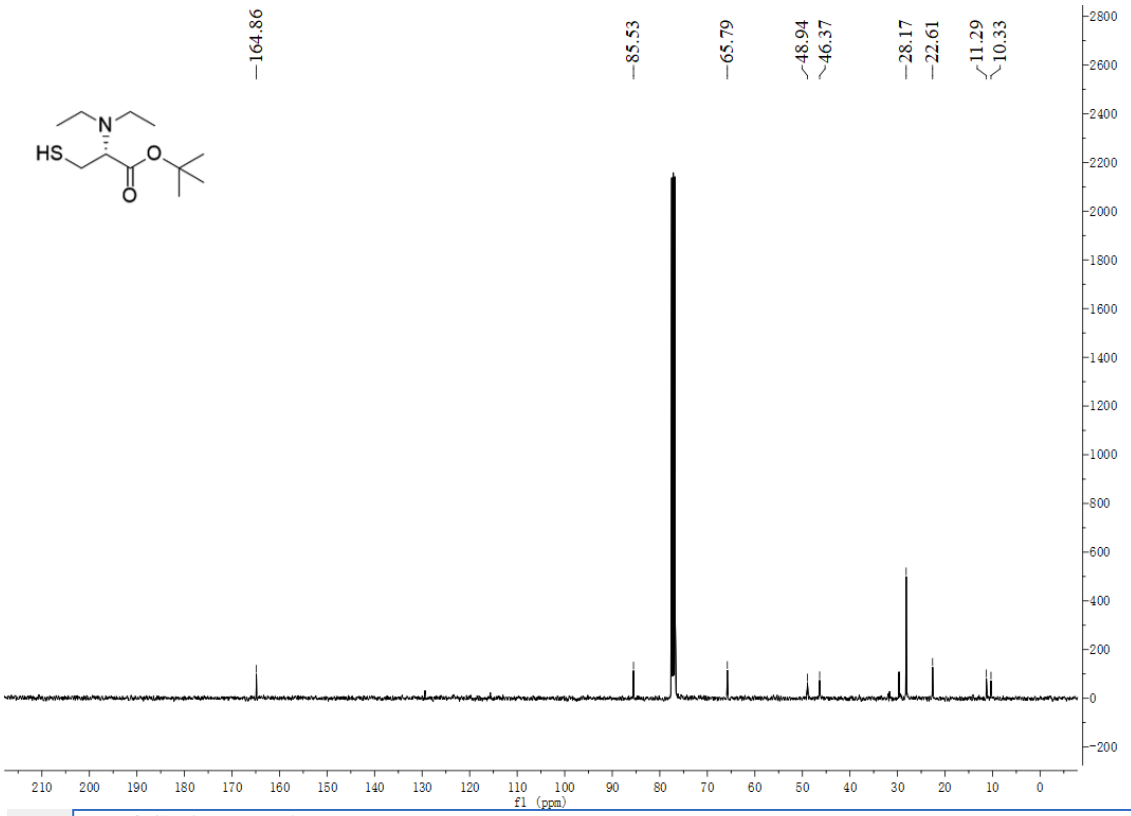

+ESI 扫描 (0.293 min) Frag=100.0V TXQ_0107_P_1.d
x10 6
* 206.1220
HS
N
H+
O
O
Chemical Formula: C9H20NO2S+
Exact Mass: 206.1209
Counts vs. 质荷比 (m/z)

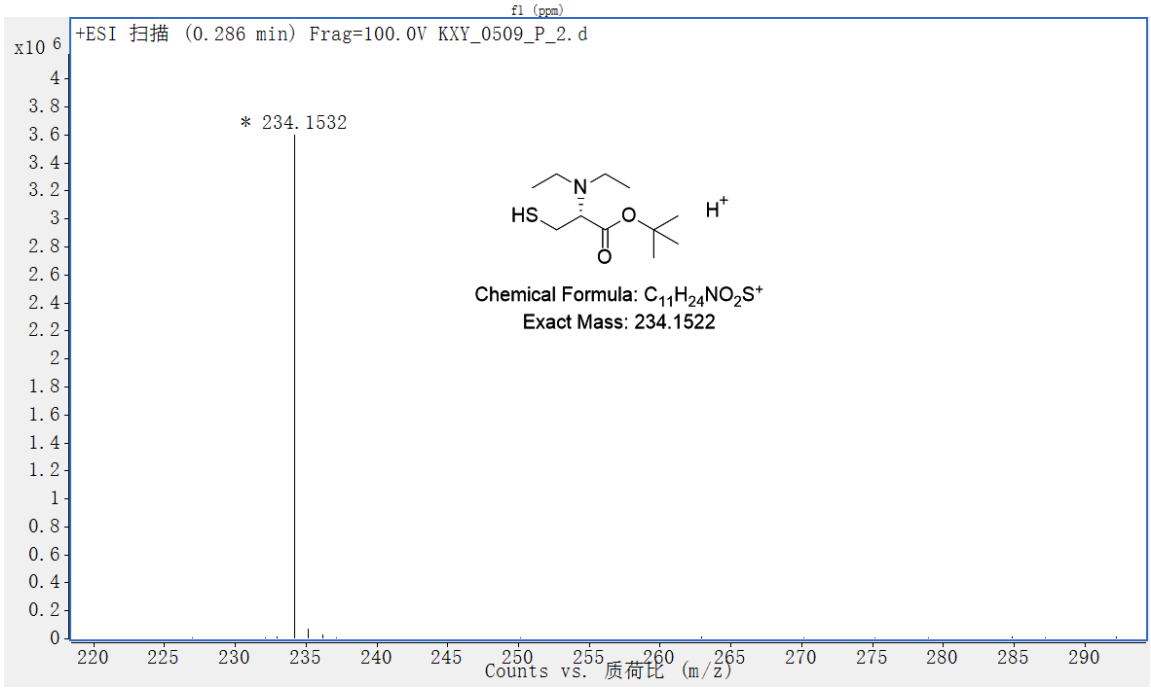

7.47
7.45
7.31
7.29
7.27
7.23
7.21
7.19
3.07
3.05
3.03
2.57
2.55
2.42
2.41
2.39
2.37
1.42
0.99
0.97
0.95
6.39
6.38
3.22
1.00
3.07
3.22
9.31
5.99
f1 (ppm)

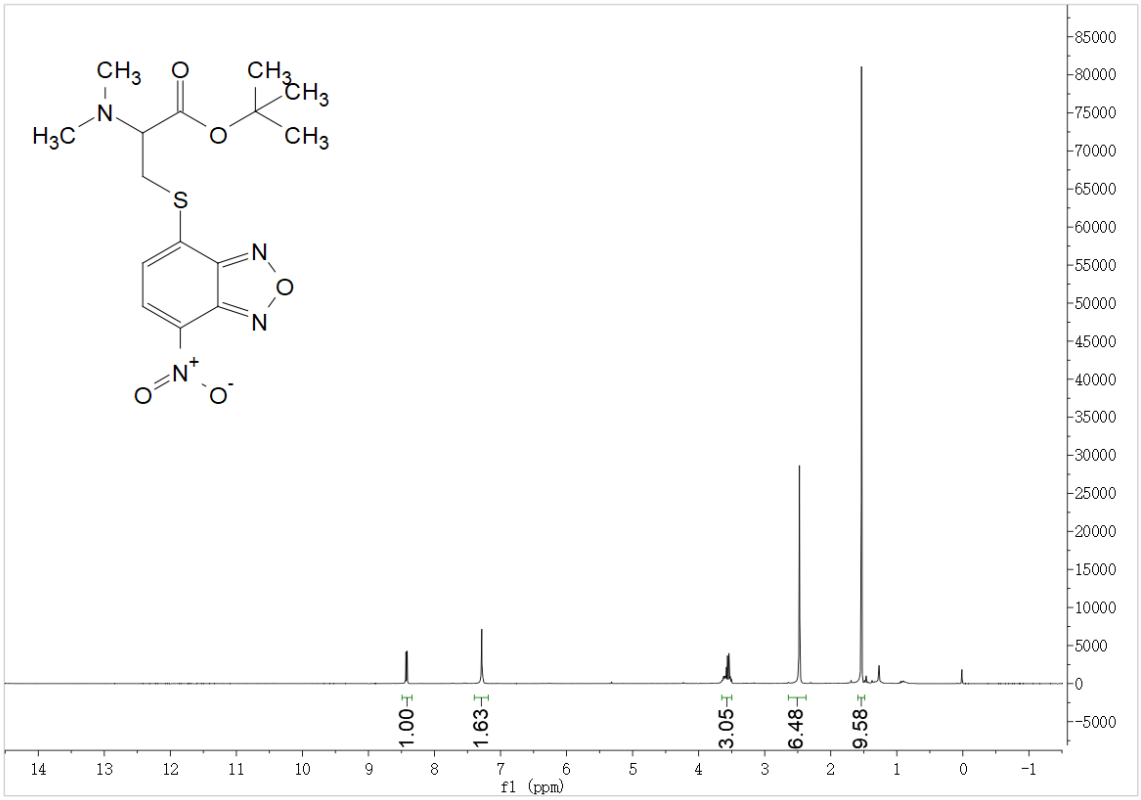

171.18
145.11
129.81
127.92
126.62
81.02
66.76
62.96
44.67
32.21
28.34
14.19
f1 (ppm)

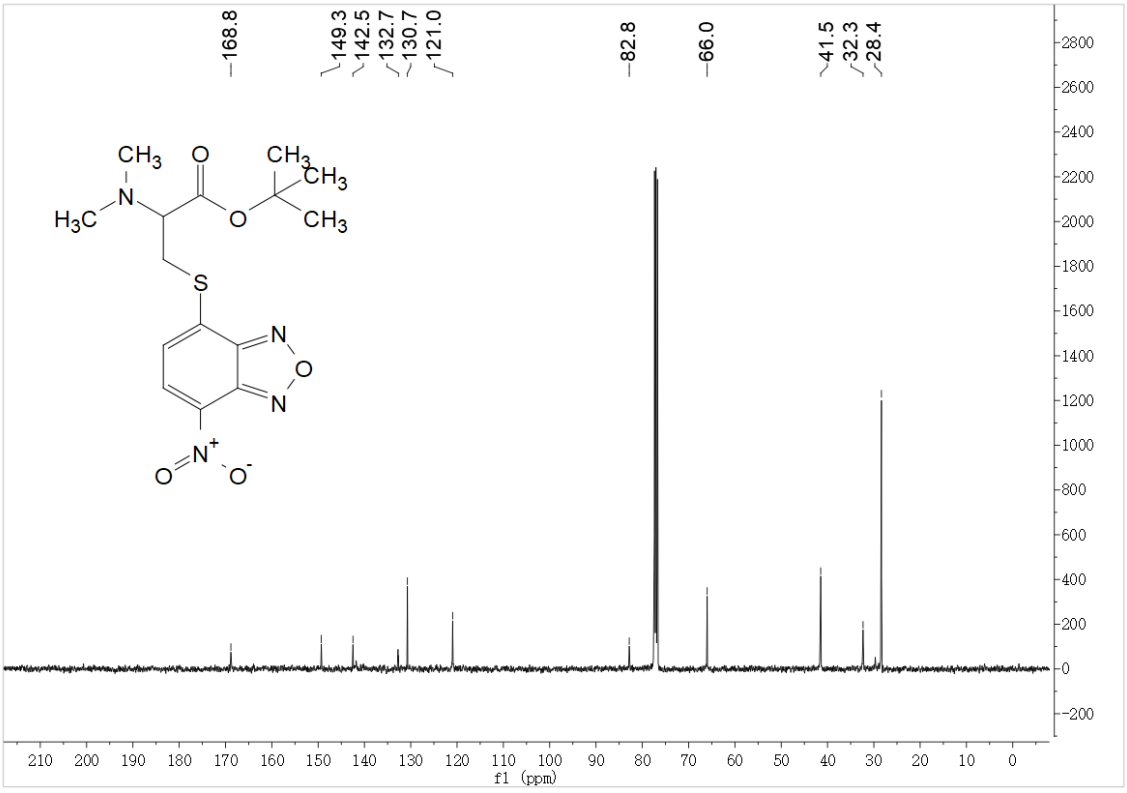

+ESI 扫描 (0.187 min) Frag=100.0V KXY_0429_P_1.d
x10 6
476.2578
Chemical Formula: C30H38NO2S+
Exact Mass: 476.2618
Counts vs. 质荷比 (m/z)

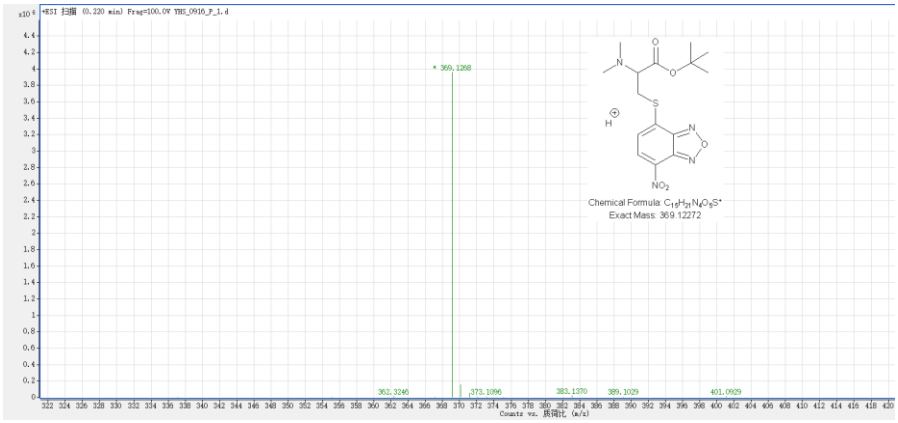

4.00
3.53
3.47
3.05
1.90
1.53
1.00
3.29
3.34
1.02
16.08
f1 (ppm)

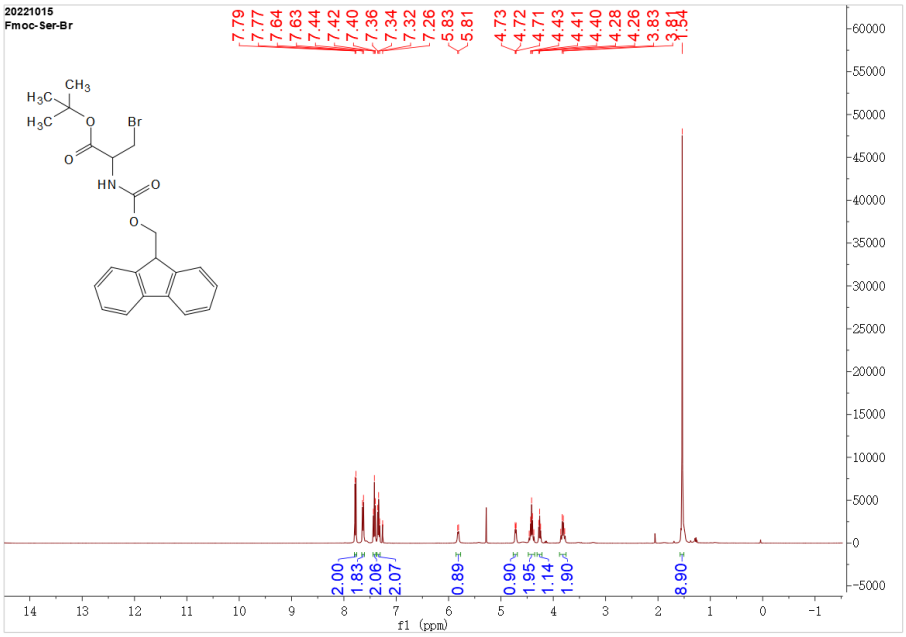

164.86
85.53
65.79
48.94
46.37
28.17
22.61
11.29
10.33
HS
N
O
O
f1 (ppm)

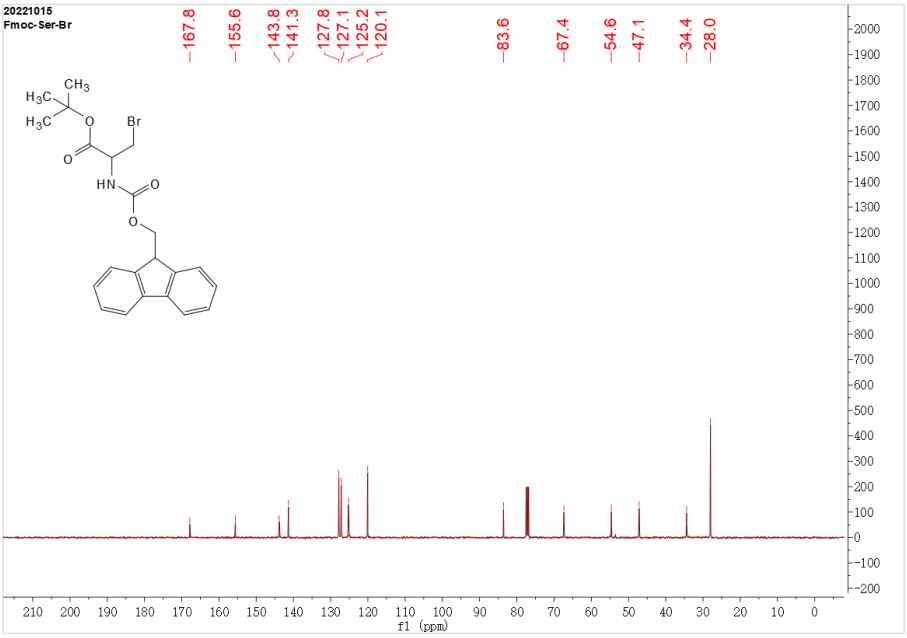

+ESI 扫描 (0.286 min) Frag=100.0V KXY_0509_P_2.d
x10 6
* 234.1532
HS
N
O
O
H+
Chemical Formula: C11H24NO2S+
Exact Mass: 234.1522
Counts vs. 质荷比 (m/z)

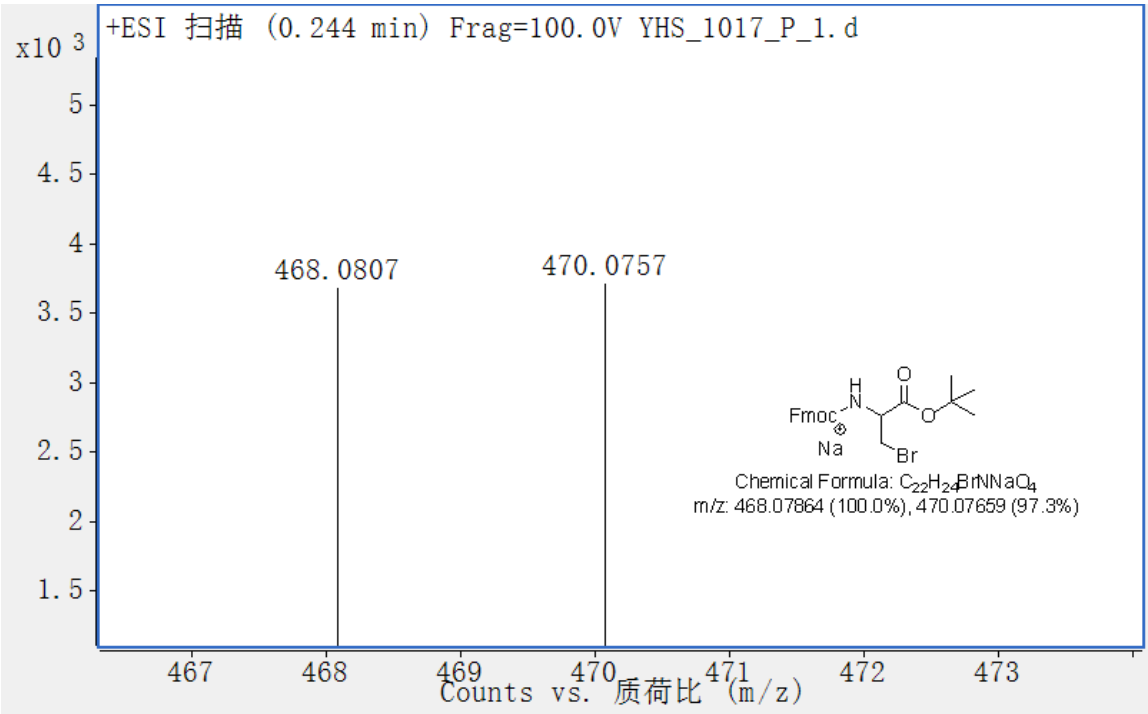

1.00
1.63
3.05
6.48
9.58
f1 (ppm)

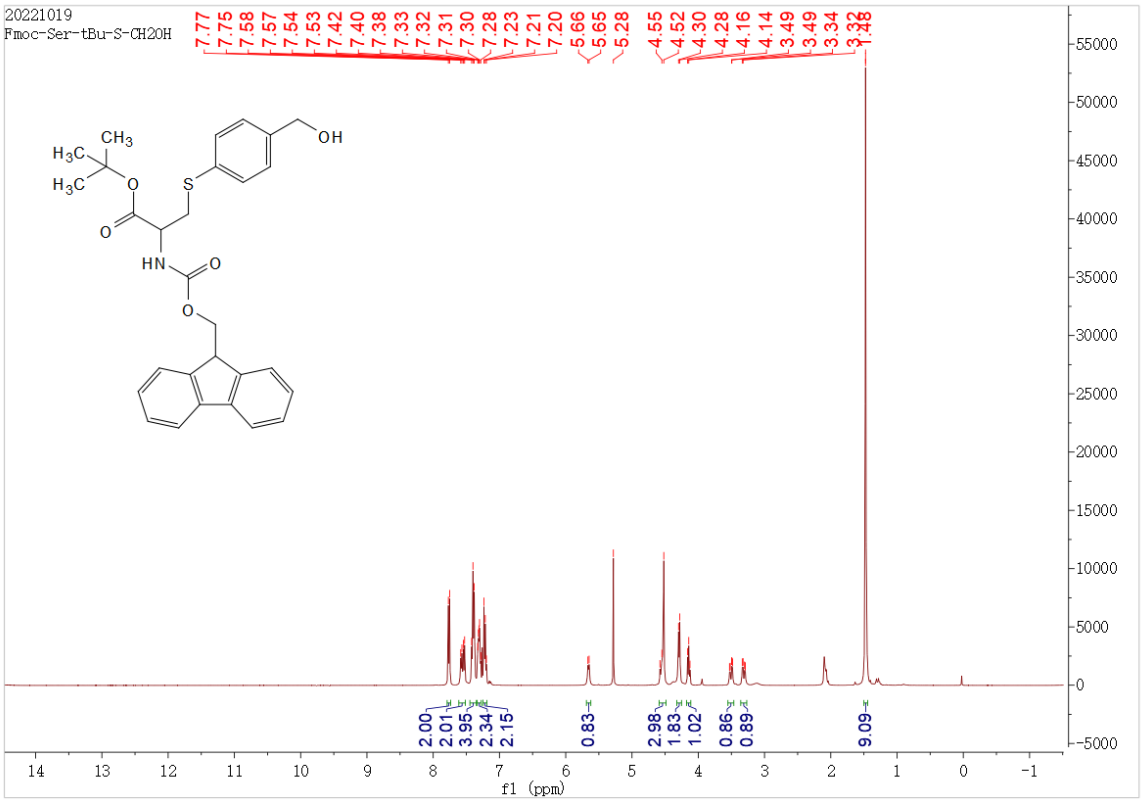

168.8
149.3
142.5
132.7
130.7
121.0
82.8
66.0
41.5
32.3
28.4
f1 (ppm)

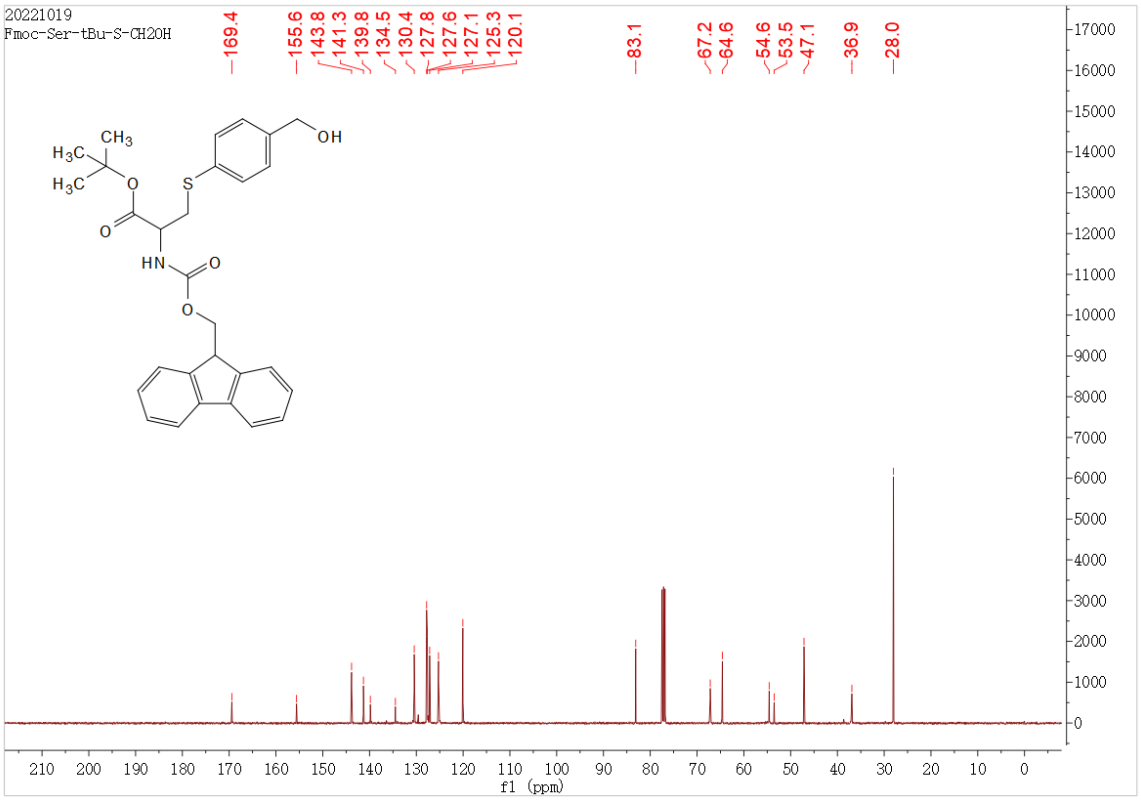

Chemical Formula: $C_{16}H_{21}N_4O_5S^+$
Exact Mass: 369.12272

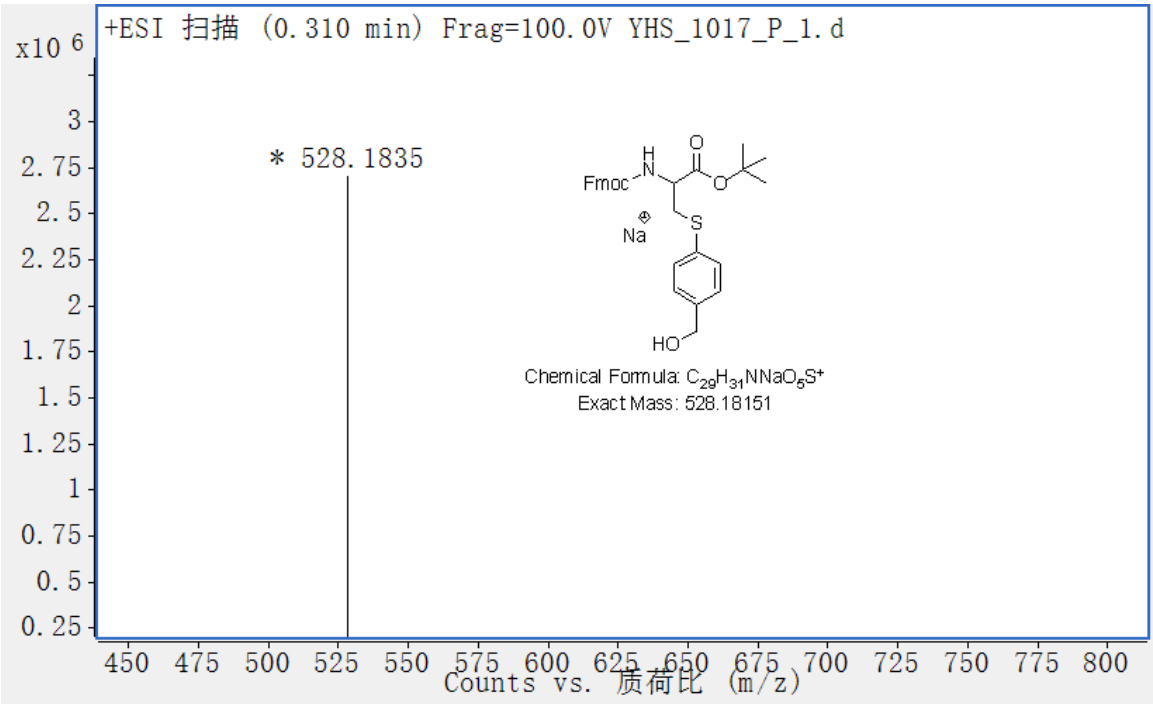

20221015
Fmoc-Ser-Br
f1 (ppm)

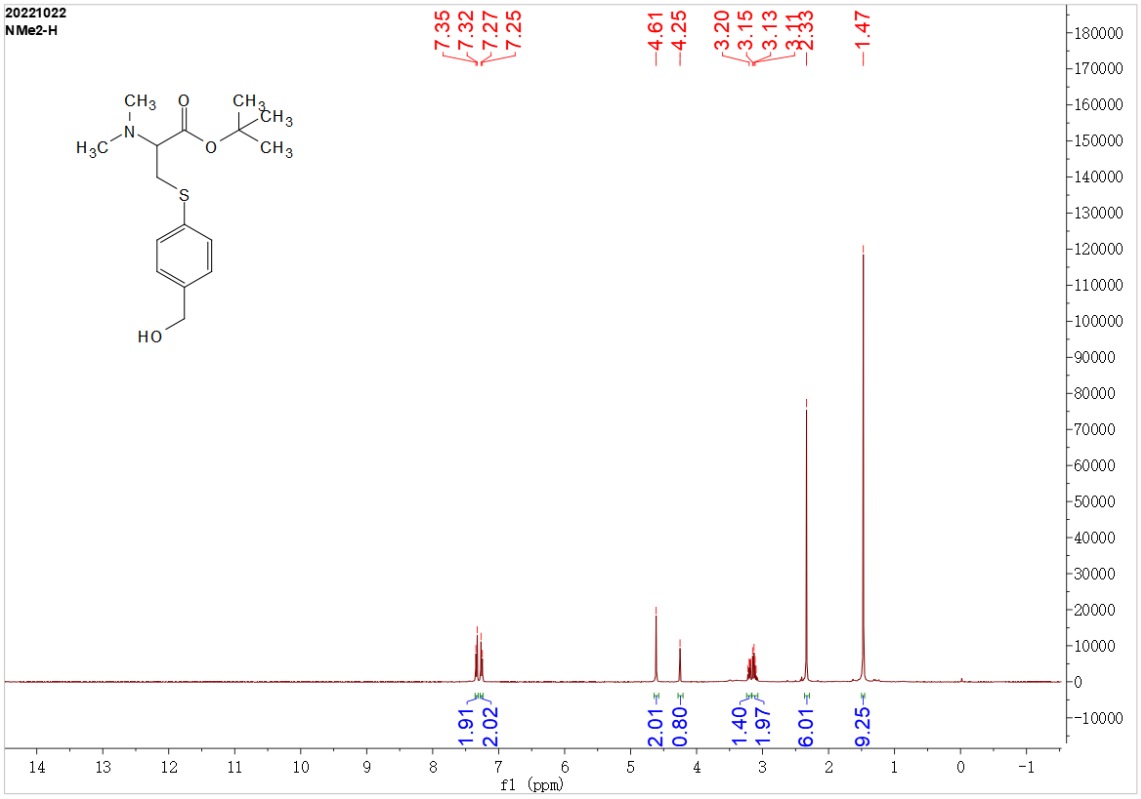

20221015
Fmoc-Ser-Br
f1 (ppm)

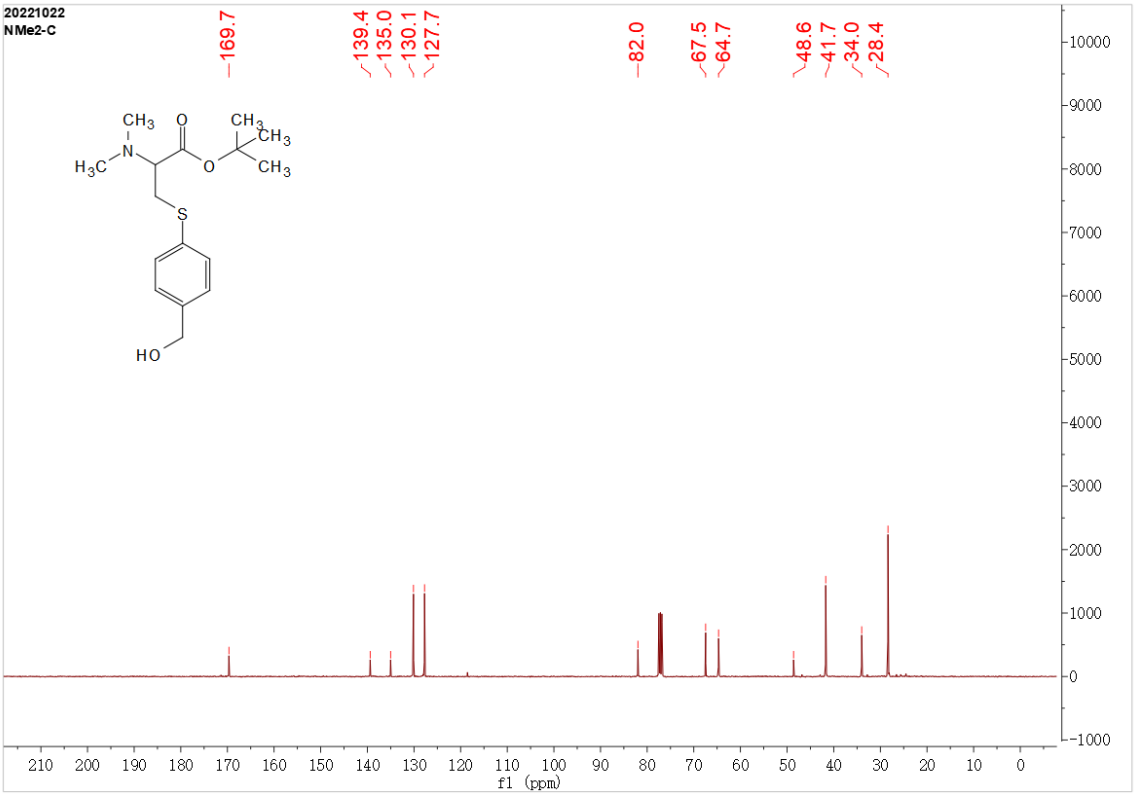

+ESI 扫描 (0.244 min) Frag=100.0V YHS_1017_P_1.d
x10 3
5
4.5
4
3.5
3
2.5
2
1.5
468.0807
470.0757
Fmoc
H
N
O
O
Na
Br
Chemical Formula: C22H24BrNNaO4
m/z: 468.07864 (100.0%), 470.07659 (97.3%)
467
468
469
470
471
472
473
Counts vs. 质荷比 (m/z)

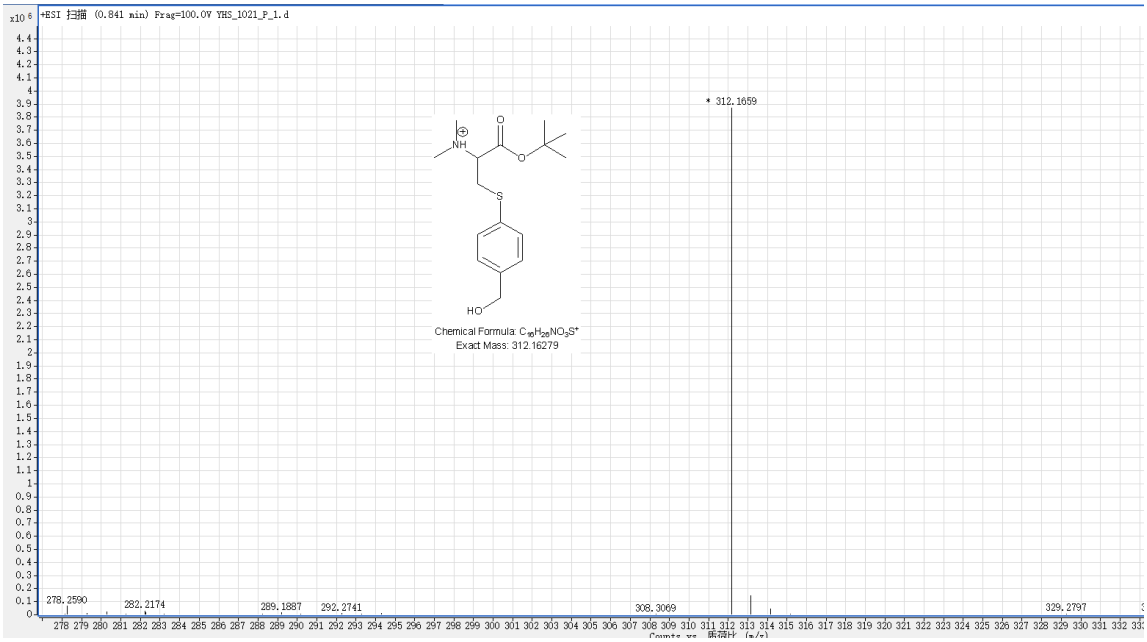

20221019
Fmoc-Ser-tBu-S-CH2OH
7.77
7.75
7.58
7.57
7.54
7.53
7.42
7.40
7.38
7.33
7.32
7.31
7.30
7.28
7.23
7.21
7.20
5.66
5.65
5.28
4.55
4.52
4.30
4.28
4.16
4.14
3.49
3.49
3.34
3.32
1.48
CH3
H3C
H3C
O
S
OH
O
HN
O
O
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000
2.00
2.01
3.95
2.34
2.15
0.83
2.98
1.83
1.02
0.86
0.89
9.09
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
f1 (ppm)

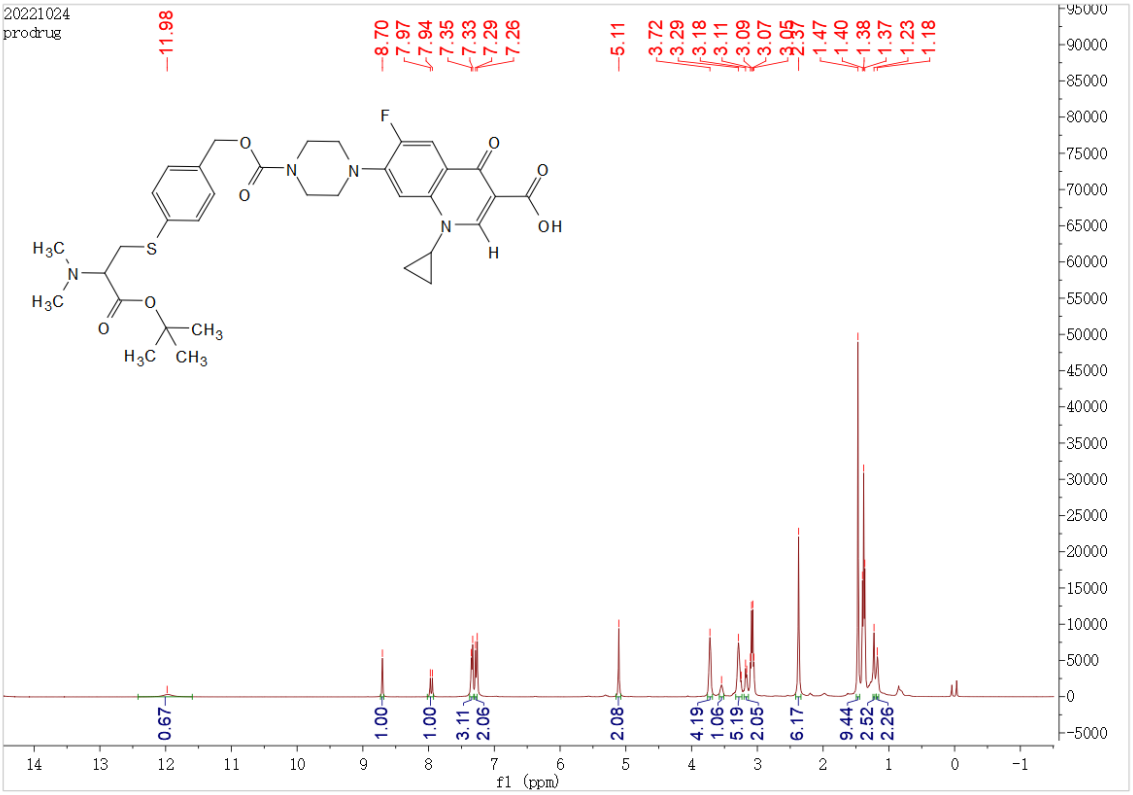

20221019
Fmoc-Ser-tBu-S-CH2OH
169.4
155.6
143.8
141.3
139.8
134.5
130.4
127.8
127.6
127.1
125.3
120.1
83.1
67.2
64.6
54.6
53.5
47.1
36.9
28.0
f1 (ppm)

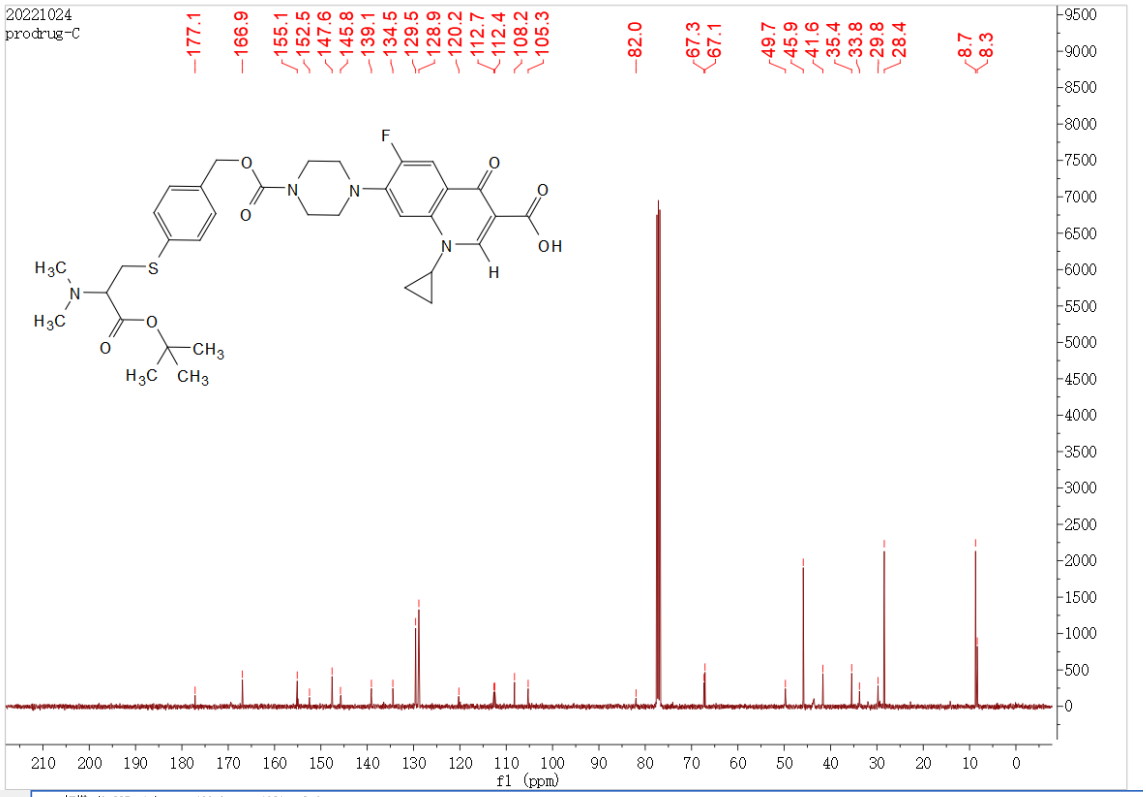

+ESI 扫描 (0.310 min) Frag=100.0V YHS_1017_P_1.d
x10 6
* 528.1835
Chemical Formula: C29H31NNaO5S+
Exact Mass: 528.18151
Counts vs. 质荷比 (m/z)

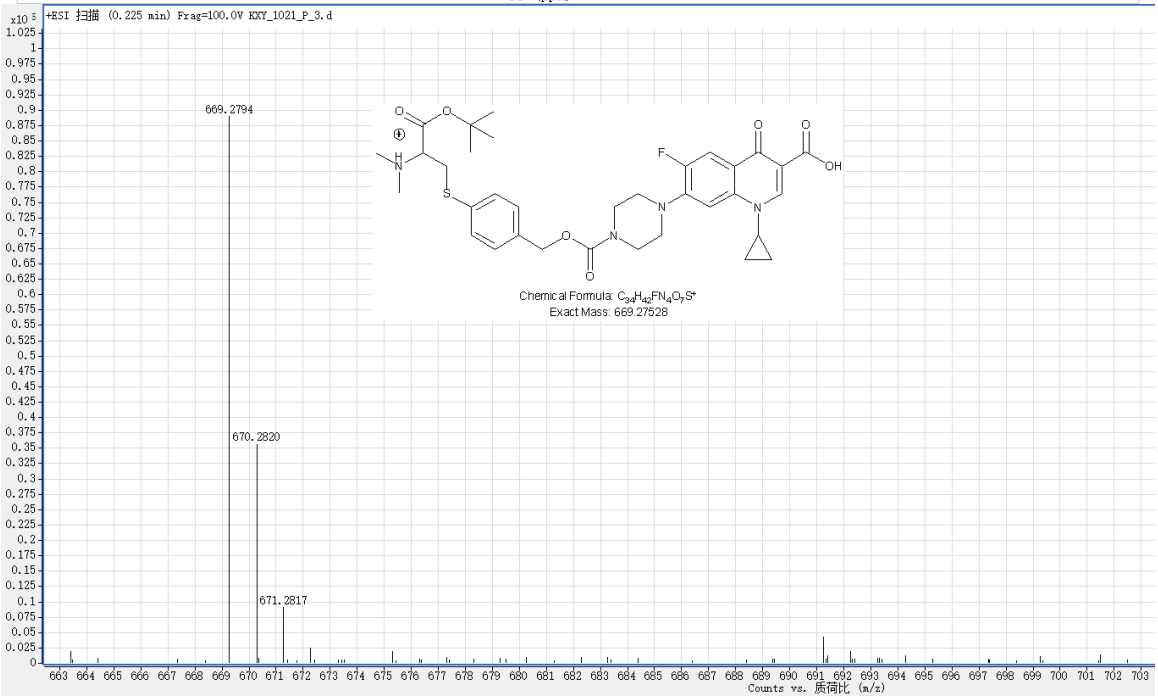

20221022
NMe2-H
7.35
7.32
7.27
7.25
4.61
4.25
3.20
3.15
3.13
3.11
2.33
1.47
1.91
2.02
2.01
0.80
1.40
1.97
6.01
9.25
f1 (ppm)


20221022
NMe2-C
169.7
139.4
135.0
130.1
127.7
82.0
67.5
64.7
48.6
41.7
34.0
28.4
f1 (ppm)


+ESI 扫描 (0.841 min) Frag=100.0V YHS_1021_P_1.d
* 312.1659
Chemical Formula: $C_{16}H_{26}NO_3S^+$
Exact Mass: 312.16279
278.2590
282.2174
289.1887
292.2741
308.3069
329.2797


20221024
prodrug
11.98
8.70
7.97
7.94
7.35
7.33
7.29
7.26
5.11
3.72
3.29
3.18
3.11
3.09
3.07
2.97
1.47
1.40
1.38
1.37
1.23
1.18
0.67
1.00
1.00
3.11
2.06
2.08
4.19
1.06
5.19
2.05
6.17
9.44
2.52
2.26
f1 (ppm)


20221024
prodrug-C
177.1
166.9
155.1
152.5
147.6
145.8
139.1
134.5
129.5
128.9
120.2
112.7
112.4
108.2
105.3
82.0
67.3
67.1
49.7
45.9
41.6
35.4
33.8
29.8
28.4
8.7
8.3
f1 (ppm)


+ESI 扫描 (0.225 min) Frag=100.0V KXY_1021_P_3.d
669.2794
670.2820
671.2817
Chemical Formula: C36H42FN4O7S+
Exact Mass: 669.27528
Counts vs. 质荷比 (m/z)

8. Supporting reference

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