Facile Cu(II) mediated conjugation of thioesters and thioacids to peptides and proteins under mild conditions

Yao Sun[a†], Zhenbin Lyu[b†], Zhiqiang Wang[b, c], Xiaodong Zeng[b], Hui Zhou[b], Fuchun Xu[d], Ziyang Chen[b], Yuling Xu[a], Ping Xu[c*], Xuechuan Hong[b,d*]

a. Key Laboratory of Pesticides and Chemical Biology, Ministry of Education, Hubei International Scientific and Technological Cooperation Base of Pesticide and Green Synthesis, Chemical Biology Center, College of Chemistry, Central China Normal University, Wuhan430079

b. State Key Laboratory of Virology, Zhongnan Hospital of Wuhan University, Wuhan University School of Pharmaceutical Sciences, Wuhan 430071, China.

c. State Key Laboratory of Proteomics, National Center for Protein Sciences Beijing, Beijing Institute of Radiation Medicine, Beijing102206, China

d. Medical College, Tibet University, Lasa, 850000, China

Corresponding Authors: Xuechuan Hong, PhD; PingXu, PhD

Email: xhy78@whu.edu.cn; xuping@mail.ncpsb.org

† These authors contributed equally to this work.

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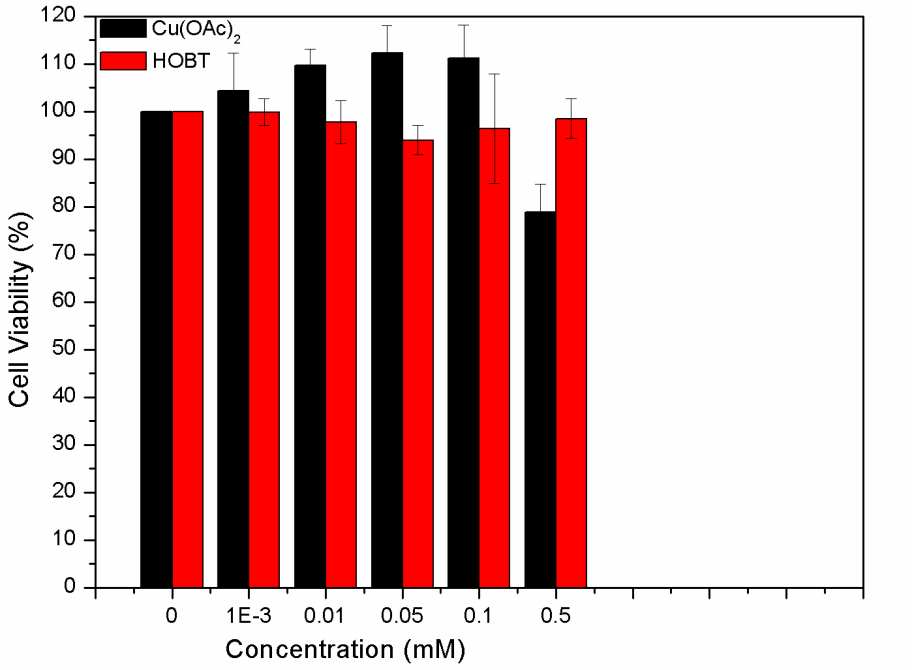


Figure S1. The MTT experiment on L929 cell lines for $Cu(OAc)_2$ and HOBt.

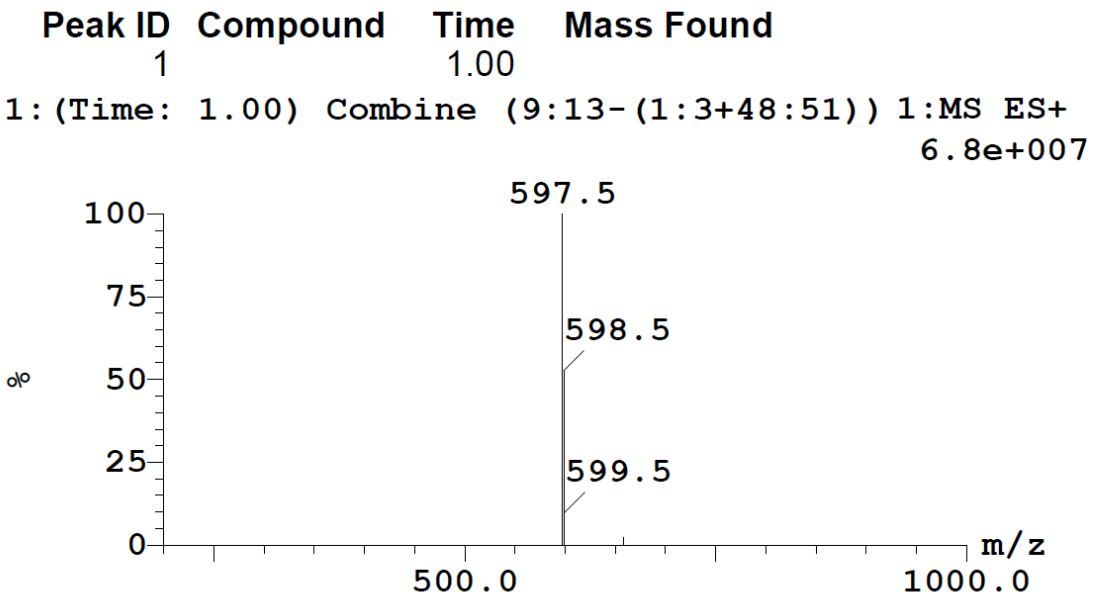


Figure S2. ESI-MS of Cy5.5COSH

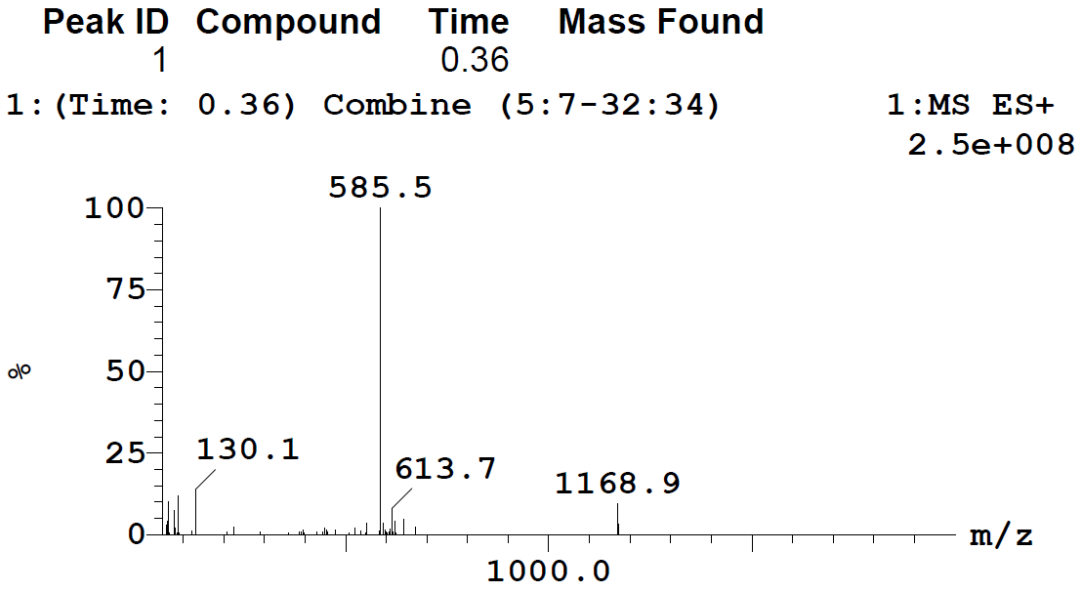


Figure S3. ESI-MS of Cy5.5-RGDfK

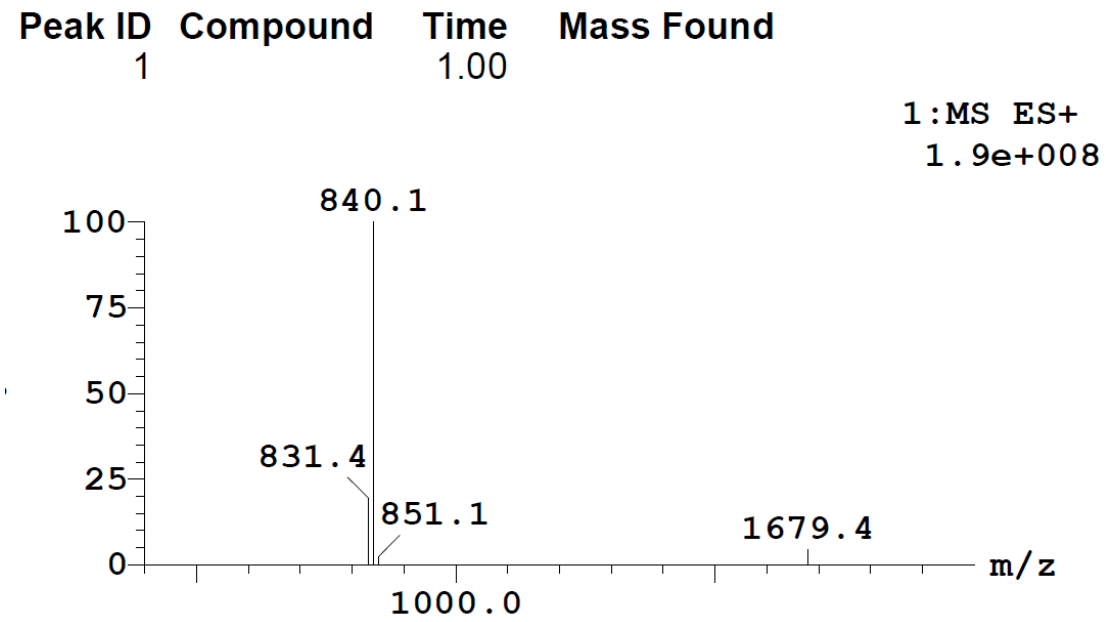


Figure S4. ESI-MS of Cy5.5-JMV594

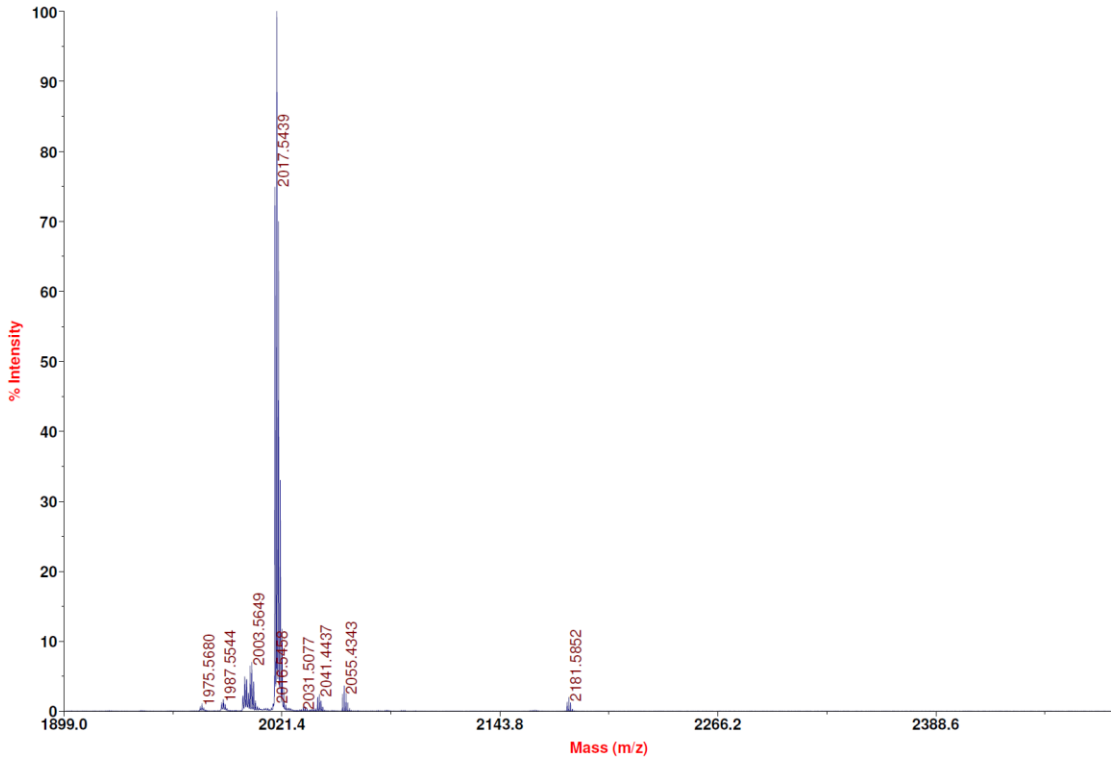


Figure S5. MALDI-TOF-MS of Cy5.5-AE105

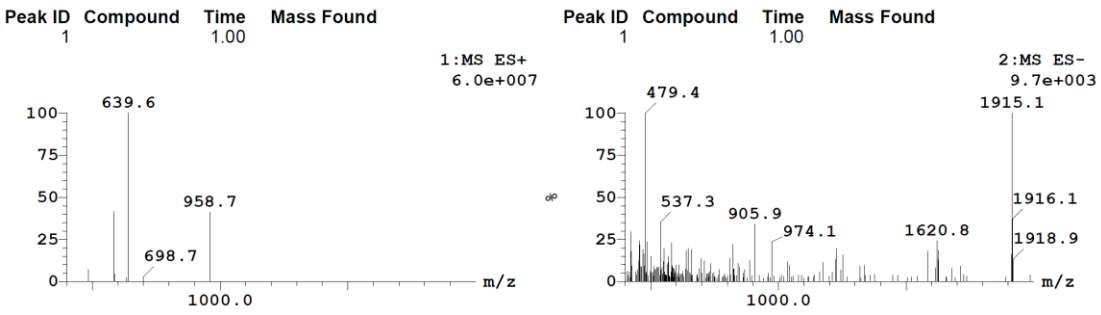


Figure S6. ESI-MS of Cy5.5-E[c(RGDyK)$_2$]

General procedure for the modification of Ub protein with thioester and the sample preparation for MS analysis.

The thioester (100 to 200 eq) and Ub (86.0 μg, 0.01 μmmol, 1 eq) were dissolved in the mixture of PBS/DMF (5:5 to 7:3, total 150 μL). Then $Cu(OAc)_2$ (2.00 μg to 4.00 μg, 1 eq to 2 eq) and HOBt (13.5 μg, 10 eq) were added and the reaction mixture was stirred at room temperature for 6 h to 16 h. The crude product was in-gel digested with trypsin overnight. The peptides mixtures were extracted with extraction buffer (5 % FA and 50 % ACN in water) and analyzed through LC-MS/MS.

The crude protein was purified by SDS-PAGE. The gel was stained with Coomassie Blue G-250 and cut into about 1 mm^3 cubes, followed by destaining and in-gel digestion with 10 ng/μL of trypsin at 37 °C overnight. The peptides were extracted with extraction buffer (5 % FA and 50 % ACN in water) and then ACN and finally dried. Peptide mixtures were loaded onto an in-house packed capillary column (75 μm I.D. and 15 cm) with 3 μm C18 reverse-phase fused-silica (Michrom Bioresources, Inc., Auburn, CA) with a flow rate of 0.3 μL/min, and eluted with a 100 minutes gradient developed as follows: 0-5 % B for 10 minutes, 5-10 % B for 10 minutes, 10-20 % B for 30 minutes, 20-45 % B for 40 minutes, and 45-80 % B for 10 minutes (Buffer A: 0.1 % FA; Buffer B: 0.1 % FA and 100 % ACN).

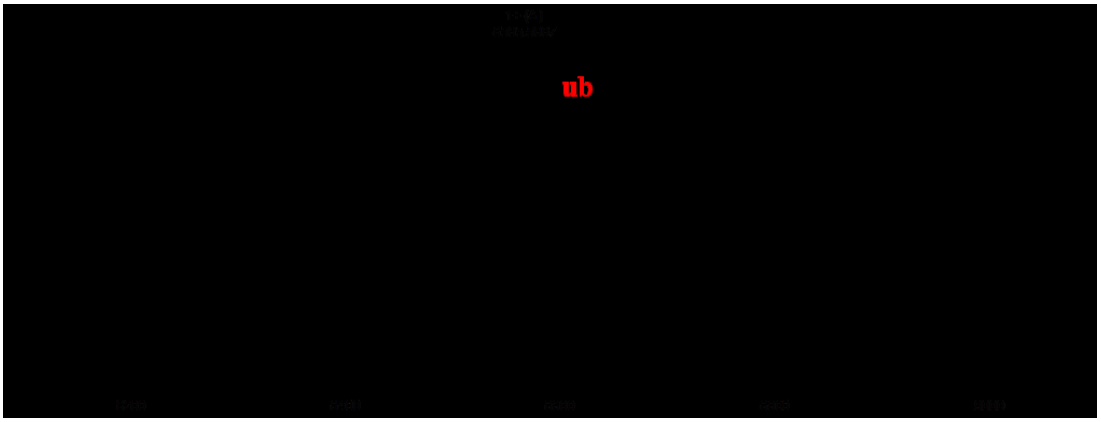


Figure S7. The result of MALDI-TOF-MS spectrum of Ub modification with 100 eq of Cbz-L-Phe thioester under 1eq $Cu(OAc)_2$ and 10eq HOBt

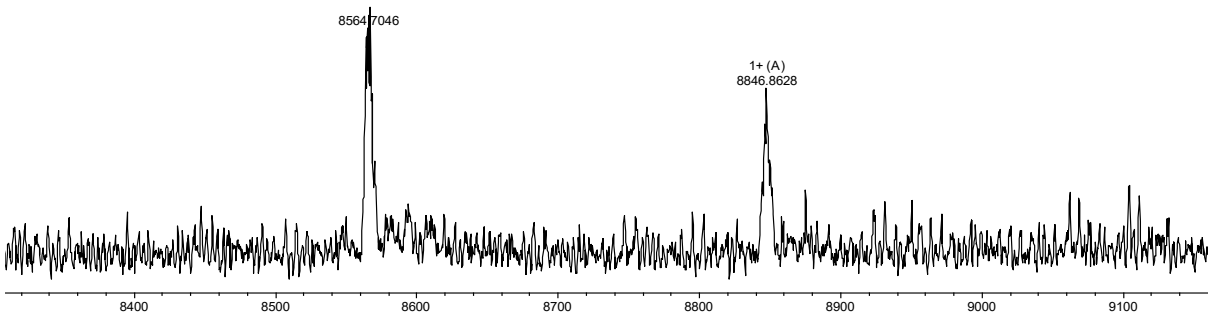


Figure S8. The result of MALDI-TOF-MS spectrum of Ub modification with Cbz-L-Phe thioester under 2eq $Cu(OAc)_2$, 200 eq thioester and 16 h conditions.

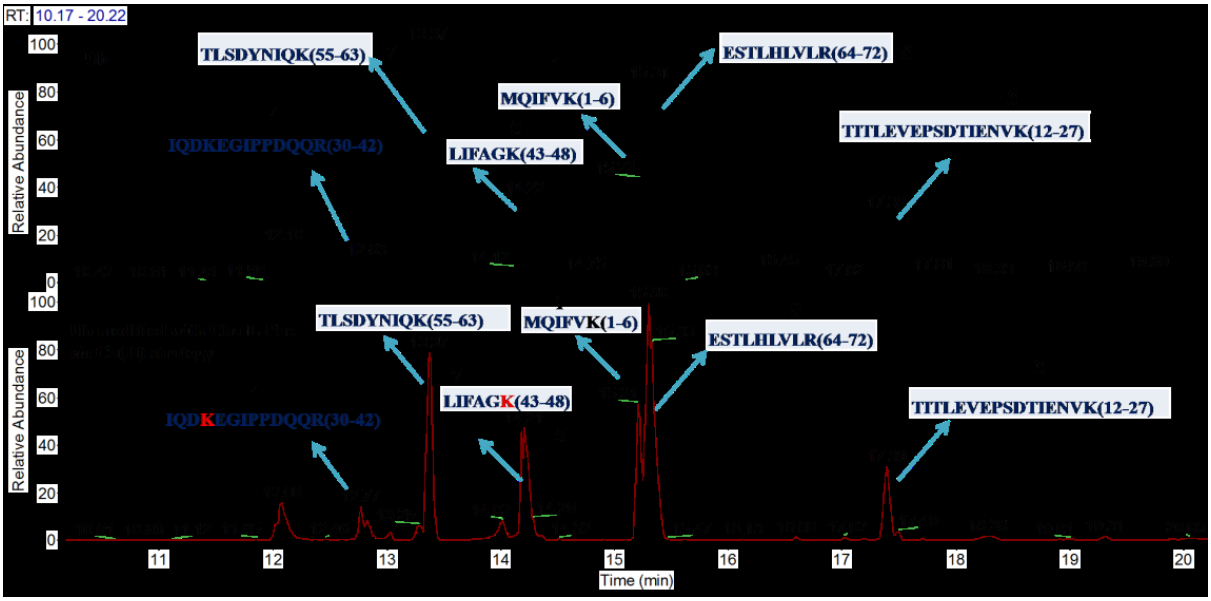


Figure S9. LC-MS spectrum of the resulting peptide fragment of trypsinized Ub conjugated with Cbz-L-Phe thioester.

Peptide sequence	Mass(z=1)	Mass(z=2)
QDK$_{33}$EGIPPDQQR	1805.761	903.380

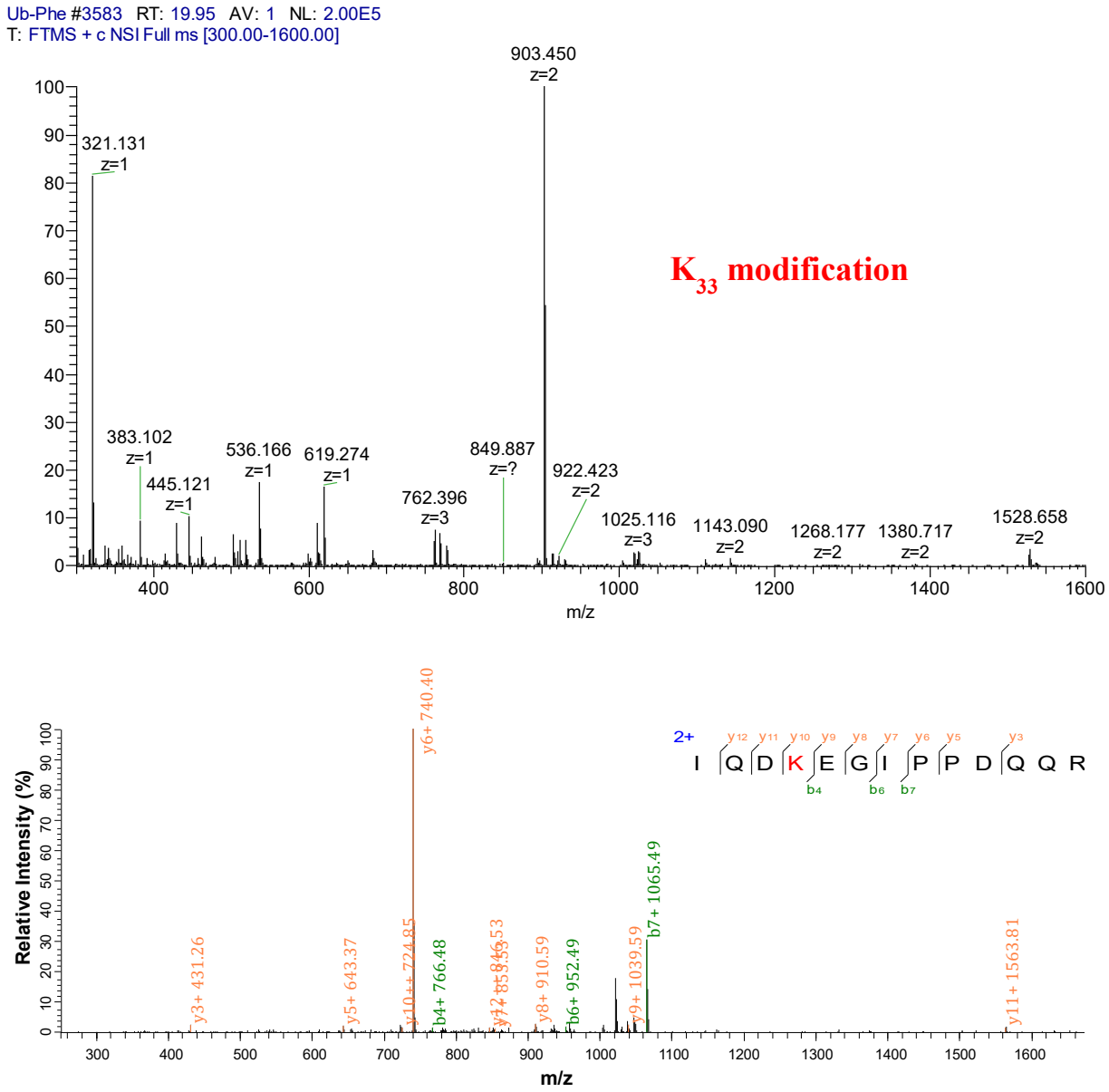


Figure S10. MS and MS_2 spectrums of the peptide fragment of Ub K_{33} modified with Cbz-L-Phe thioester.

Peptide sequence	Mass(z=1)	Mass(z=2)
LIFAGK$_{48}$QLEDGR	1628.645	814.826

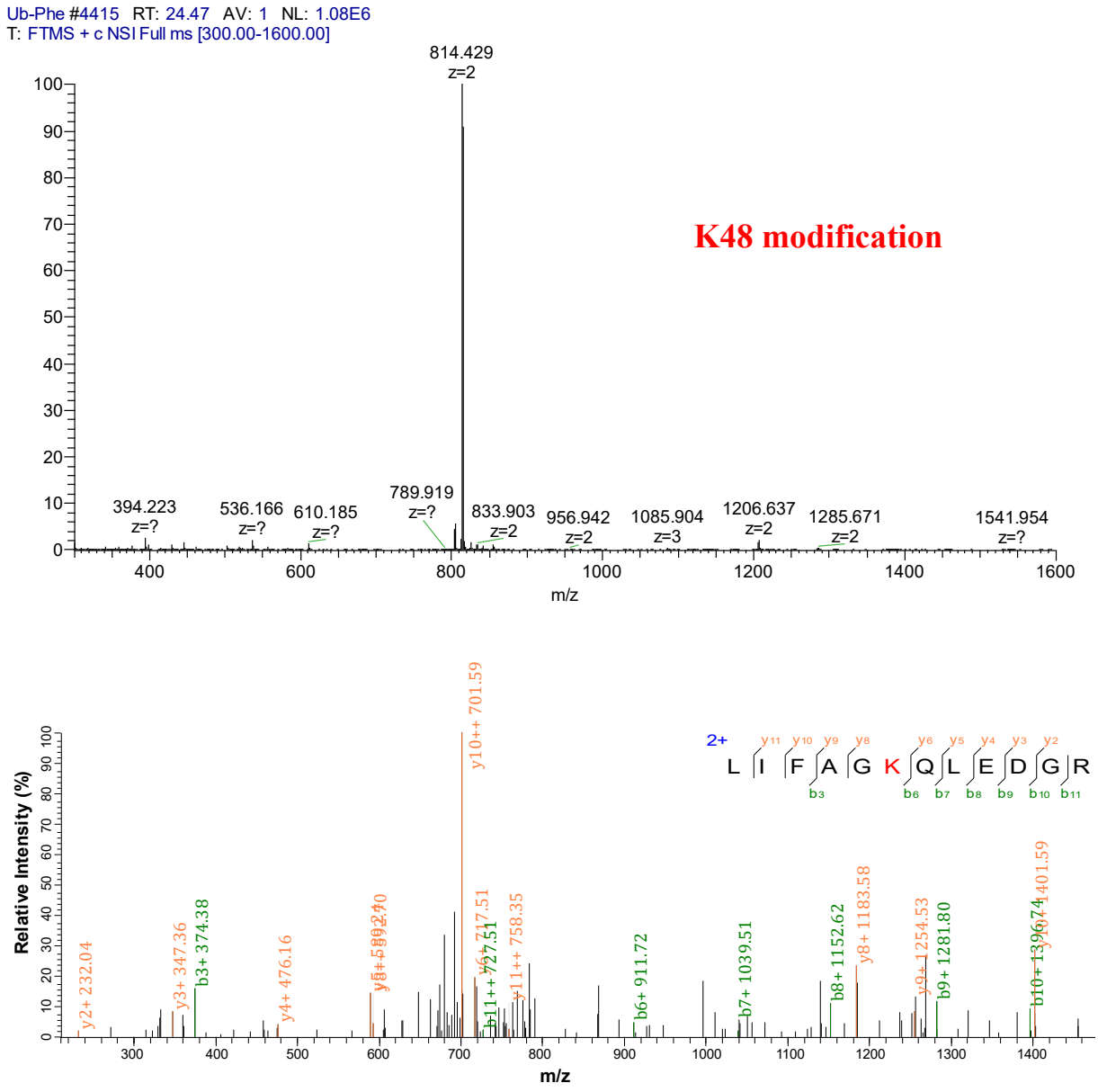


Figure S11. MS and MS_2 spectrums of the peptide fragment of Ub K_{48} modified with Cbz-L-Phe thioester.

Peptide sequence	Mass(z=1)	Mass(z=2)
MQIFVK$_6$TLTGK	1492.658	746.829

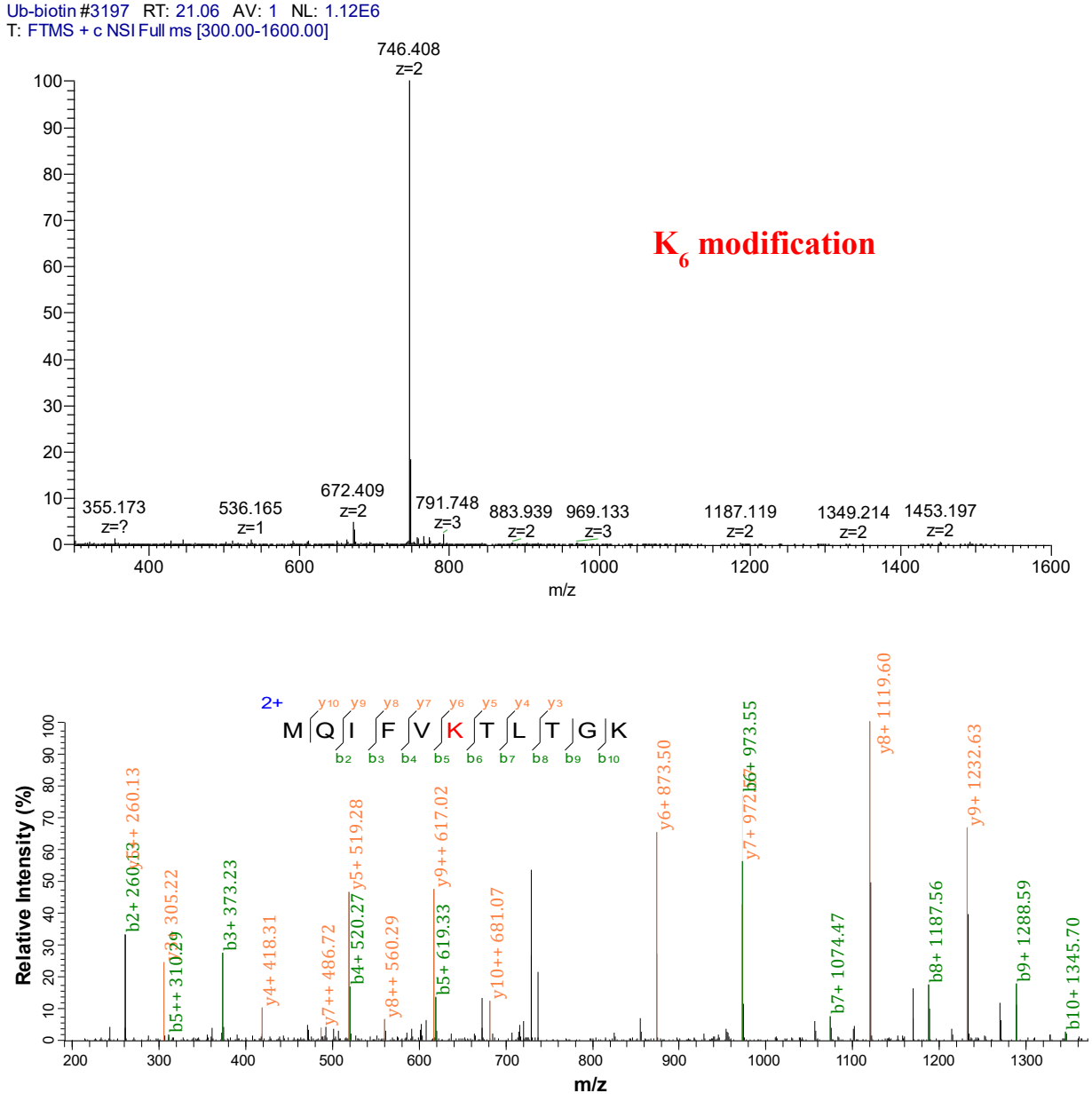


Figure S12. MS and MS_2 spectrums of the peptide fragment of Ub K_6 modified with biotin thioester.

Peptide sequence	Mass(z=1)	Mass(z=2)
IQDK$_{33}$EGIPPDQQR	1750.733	875.87

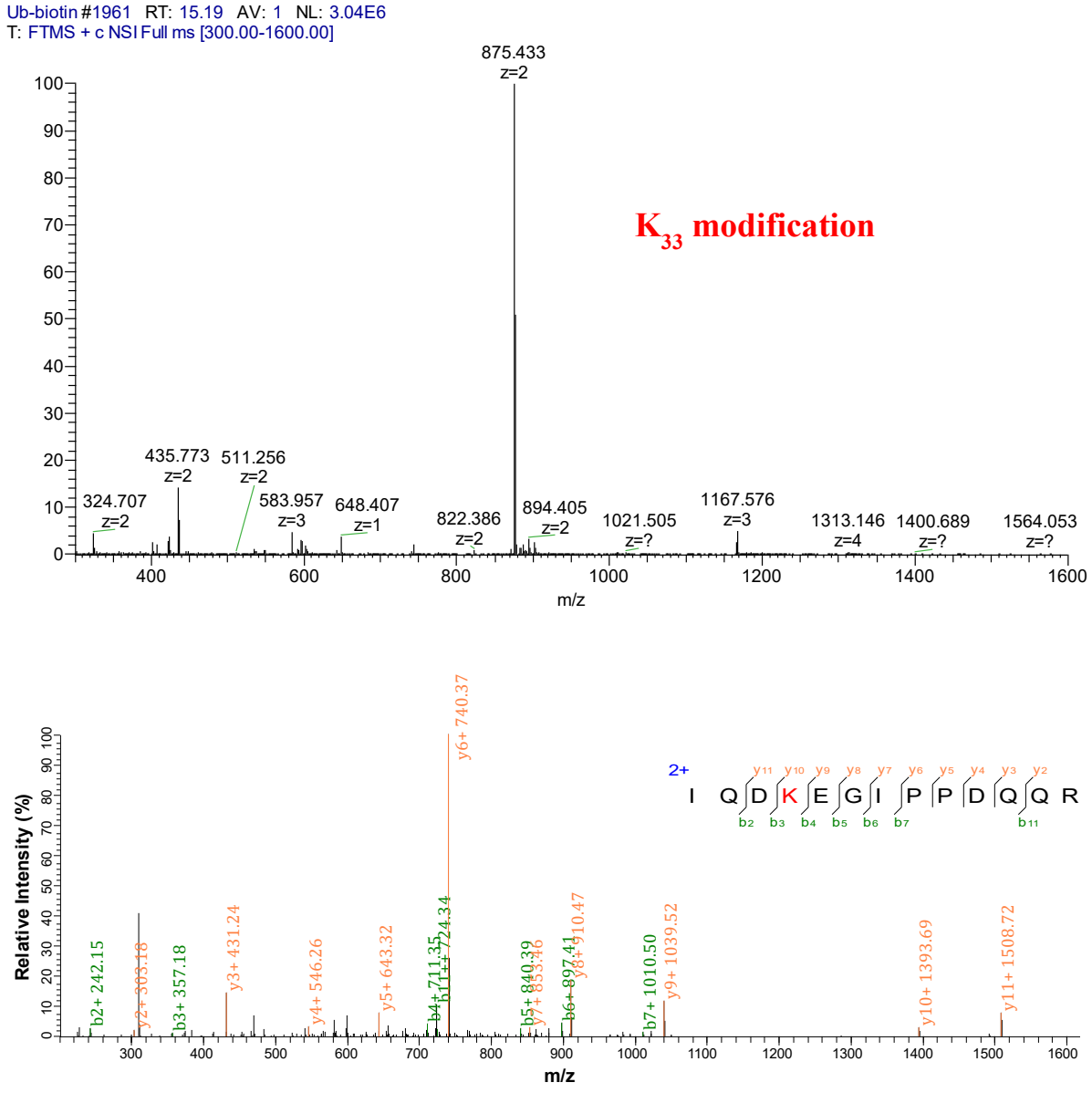


Figure S13. MS and MS_2 spectrums of the peptide fragment of Ub K_{33} modified with biotin thioester.

Peptide sequence	Mass(z=1)	Mass(z=2)
LIFAGK$_{48}$QLEDGR	1573.618	787.310

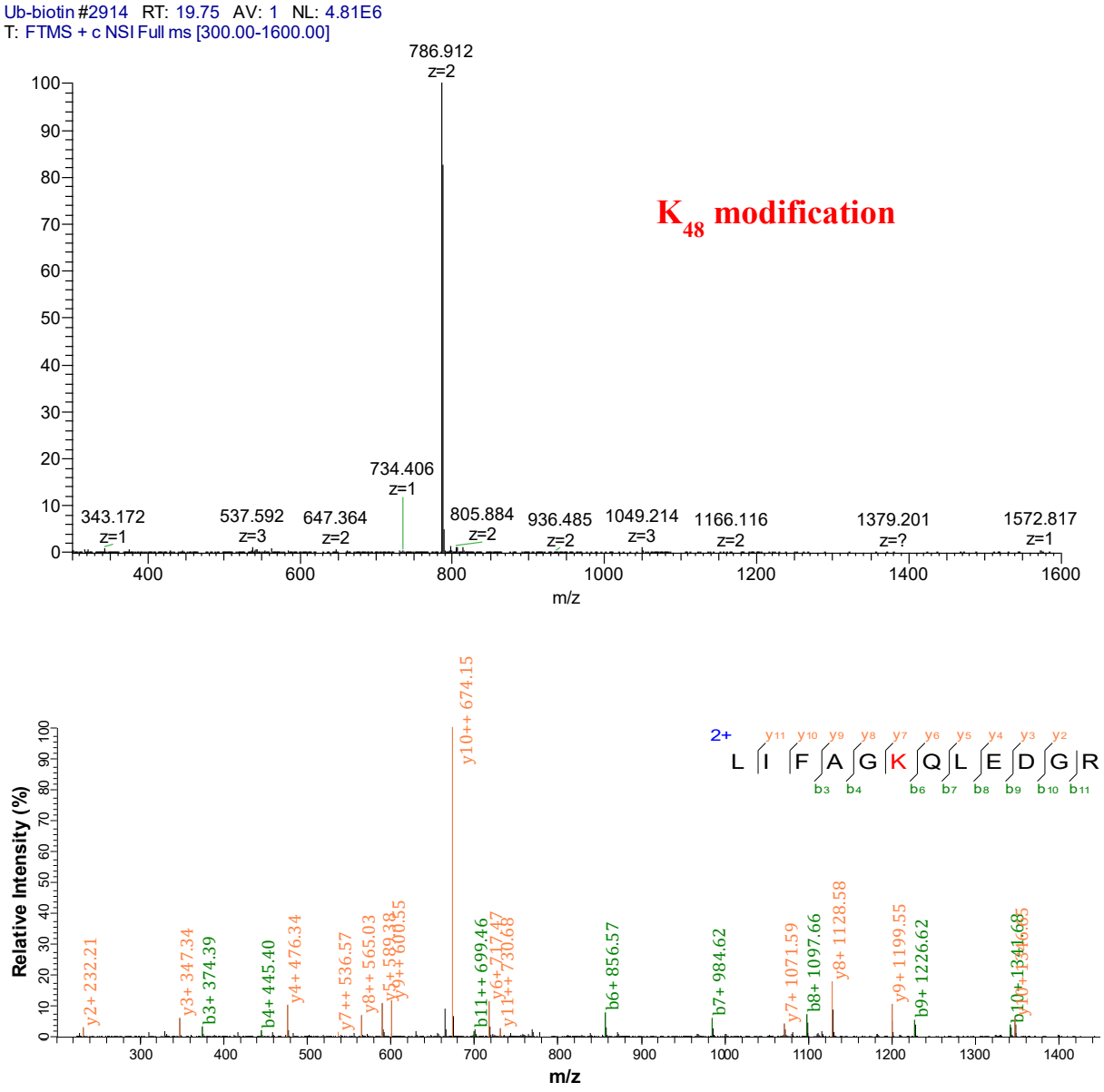


Figure S14. MS and MS_2 spectrums of the peptide fragment of Ub K_{48} modified with biotin thioester.

General procedure for the modification of Ub protein with thioacid and the sample preparation for MS analysis.

The thioacid (100 to 200 eq) and Ub (86.0 μg, 0.01 μmmol, 1 eq) were dissolved in the mixture of PBS/DMF (5:5, total 150 μL) or PBS. Then $Cu(OAc)_2$ (2.00 μg to 4.00 μg, 1 eq to 2 eq) and HOBt (2 eq to 4 eq) were added and the reaction mixture was stirred at room temperature for 6 h. The crude product was in-gel digested with trypsin overnight. The peptides mixtures were extracted with extraction buffer (5 % FA and 50 % ACN in water) and analyzed through LC-MS/MS.

The crude protein was purified by SDS-PAGE. The gel was stained with Coomassie Blue G-250 and cut into about 1 mm^3 cubes, followed by destaining and in-gel digestion with 10 ng/μL of trypsin at 37 °C overnight. The peptides were extracted with extraction buffer (5 % FA and 50 % ACN in water) and then ACN and finally dried. Peptide mixtures were loaded onto an in-house packed capillary column (75 μm I.D. and 15 cm) with 3 μm C18 reverse-phase fused-silica (Michrom Bioresources, Inc., Auburn, CA) with a flow rate of 0.3 μL/min, and eluted with a 100 minutes gradient developed as follows: 0-5 % B for 10 minutes, 5-10 % B for 10 minutes, 10-20 % B for 30 minutes, 20-45 % B for 40 minutes, and 45-80 % B for 10 minutes (Buffer A: 0.1 % FA; Buffer B: 0.1 % FA and 100 % ACN).

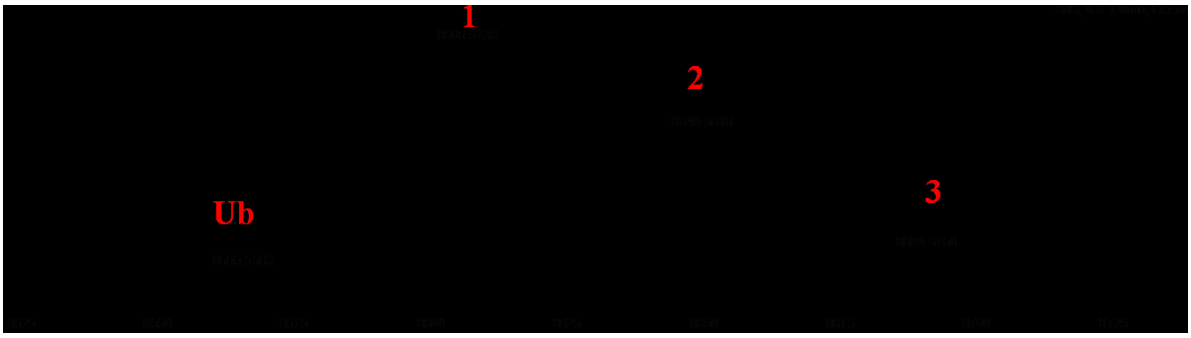


Figure S15. MALDI-TOF-MS spectrum result of Ub modification with thioacid under 1eq $Cu(OAc)_2$, 2 eq HOBt, 100 eq thioester and 6 h conditions.

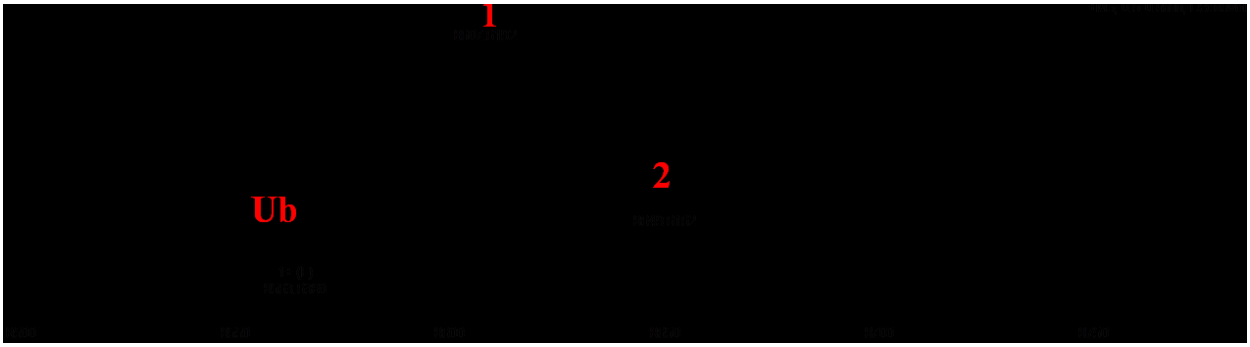


Figure S16. MALDI-TOF-MS spectrum result of Ub modification with thioacid in PBS solution.

Peptide sequence	Mass(z=1)	Mass(z=2)
*MQIFVK$_6$	849.453	425.230

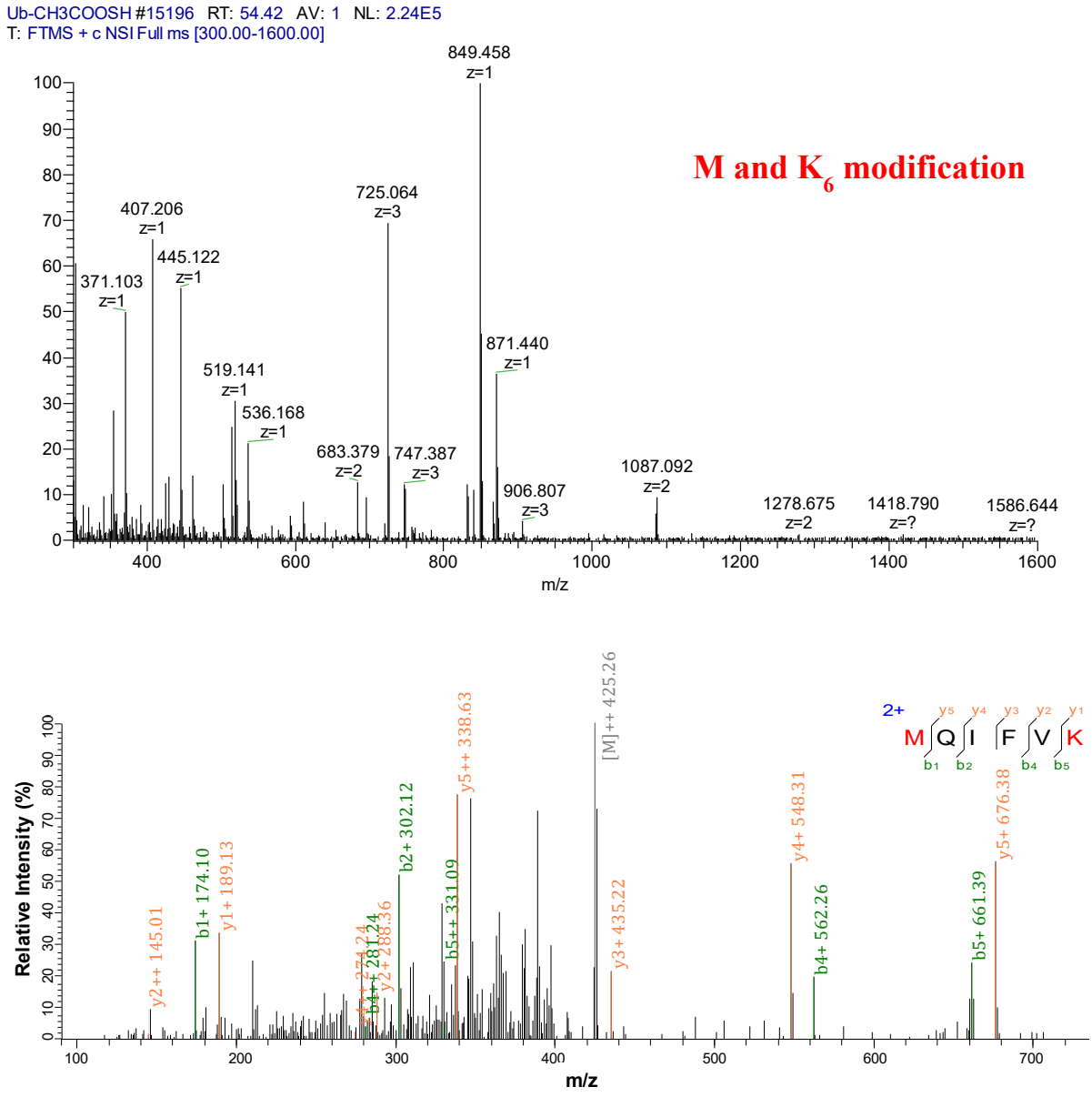


Figure S17. MS and MS_2 spectrums of the peptide fragemnts of Ub N-terminal and K_6 modified with thioacid.

Peptide sequence	Mass(z=1)	Mass(z=2)
TLTGK$_{11}$TITLEVEPSDTIENVK	2330.233	1165.620

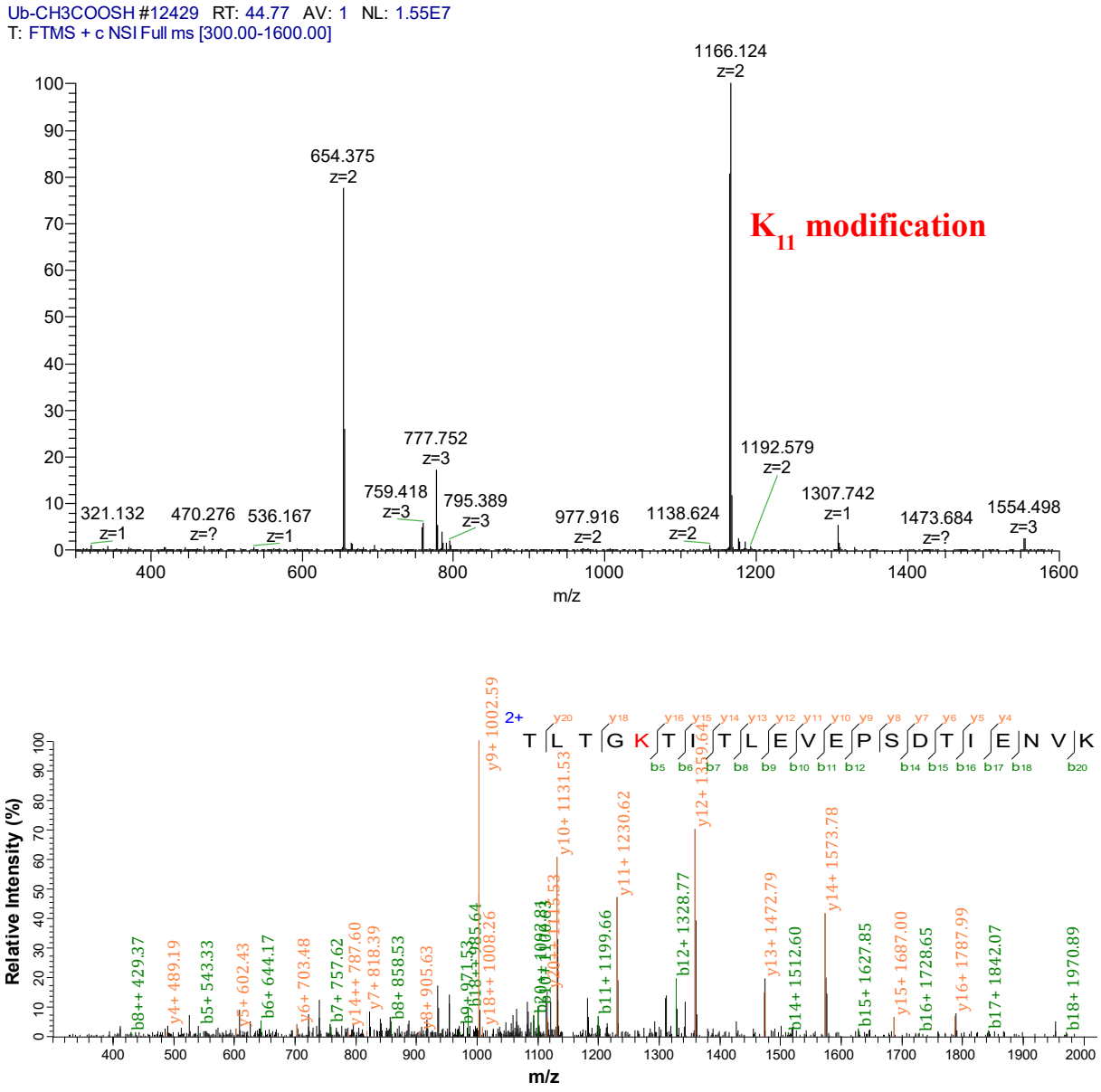


Figure S18. MS and MS_2 spectrums of the peptide fragments of Ub K_{11} modified with thioacid.

Peptide sequence	Mass(z=1)	Mass(z=2)
TITLEVEPSDTIENVKAK$_{29}$	2029.069	1015.038

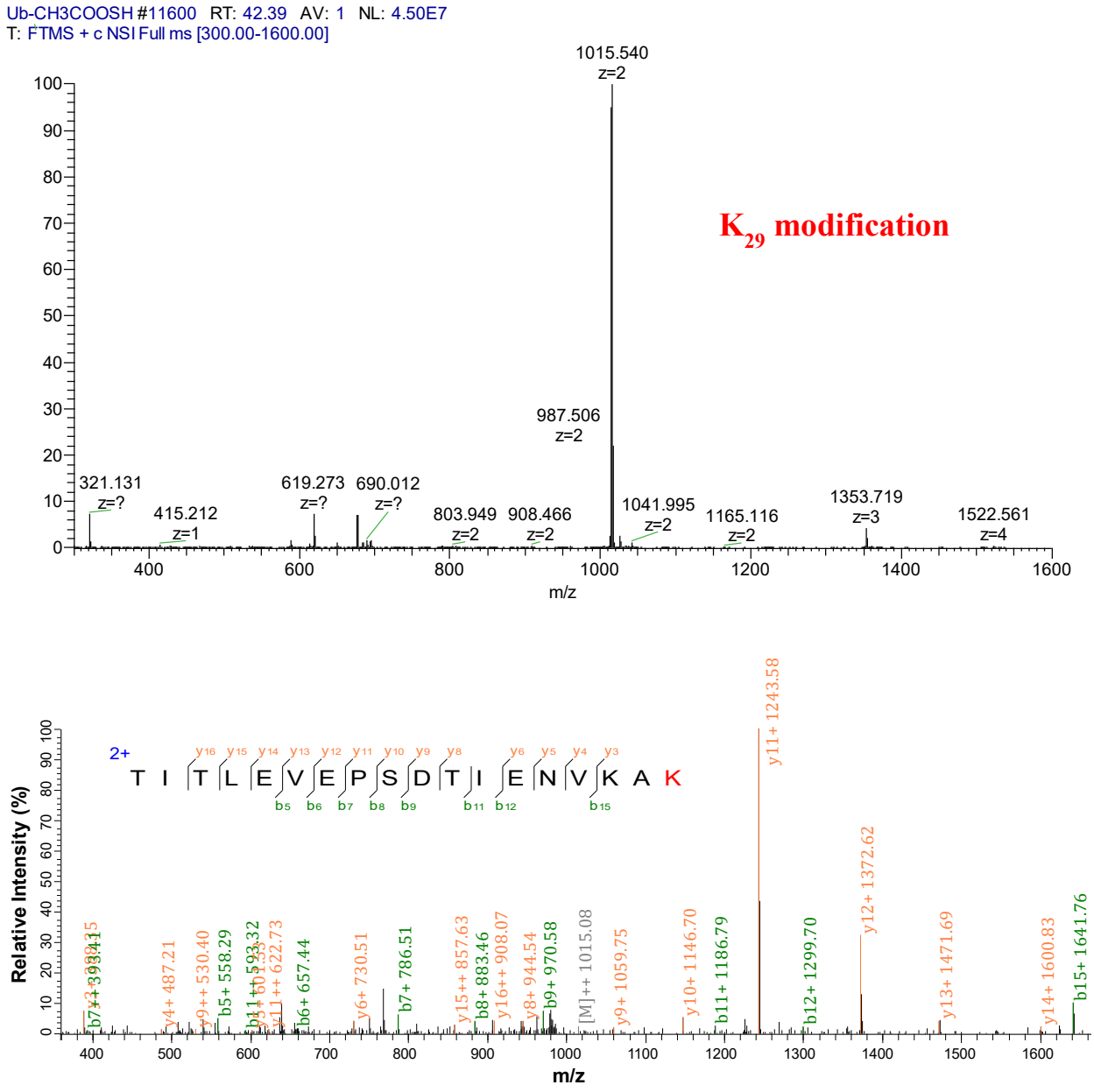


Figure S19. MS and MS_2 spectrums of the peptide fragments of Ub K_{29} modified with thioacid.

Peptide sequence	Mass(z=1)	Mass(z=2)
IQDK$_{33}$EGIPPDQQR	1565.791	783.399

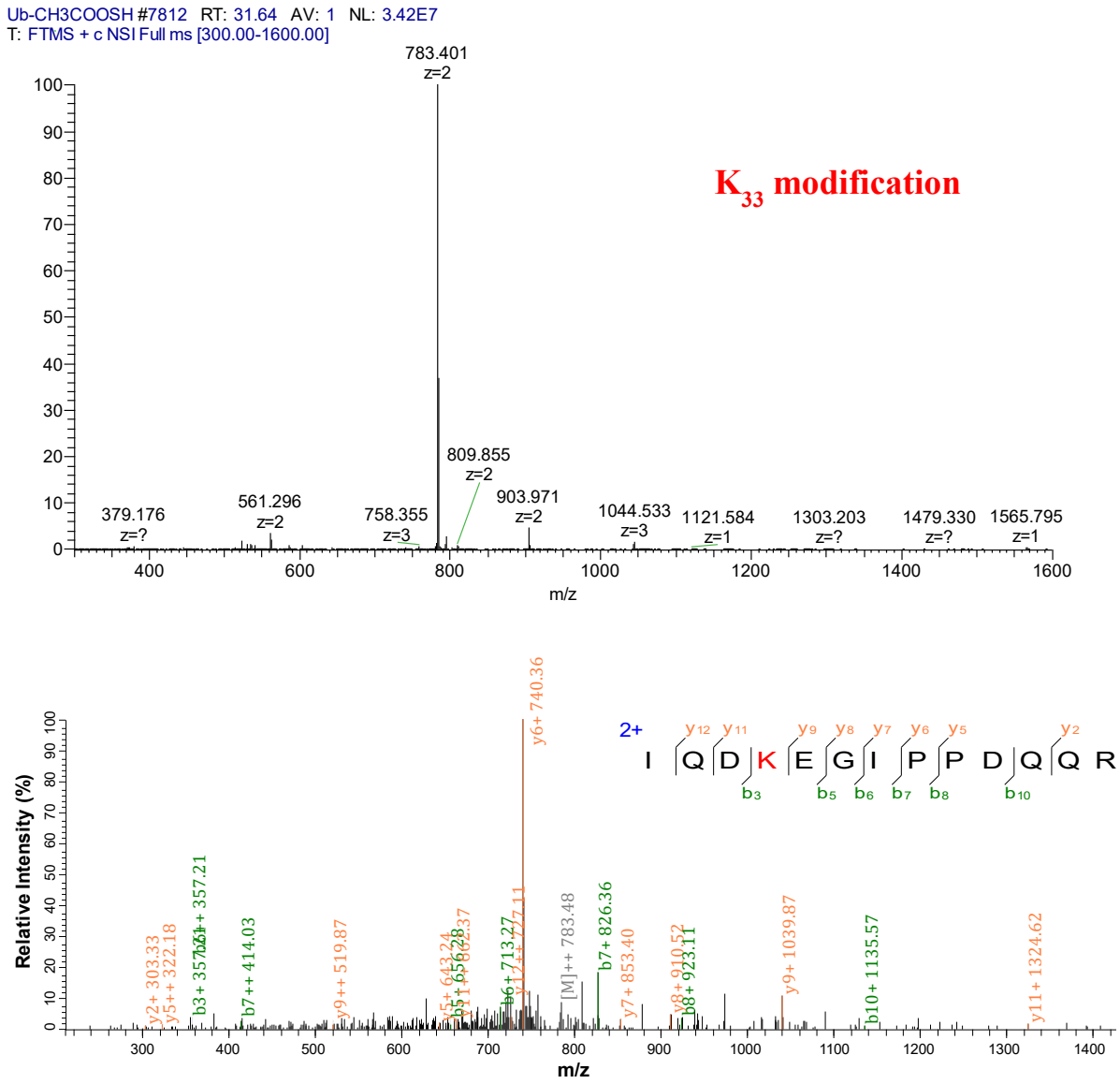


Figure S20. MS and MS_2 spectrums of the peptide fragments of Ub K_{33} modified with thioacid.

Peptide sequence	Mass(z=1)	Mass(z=2)
LIFAGK$_{48}$QLEDGR	1388.753	694.880

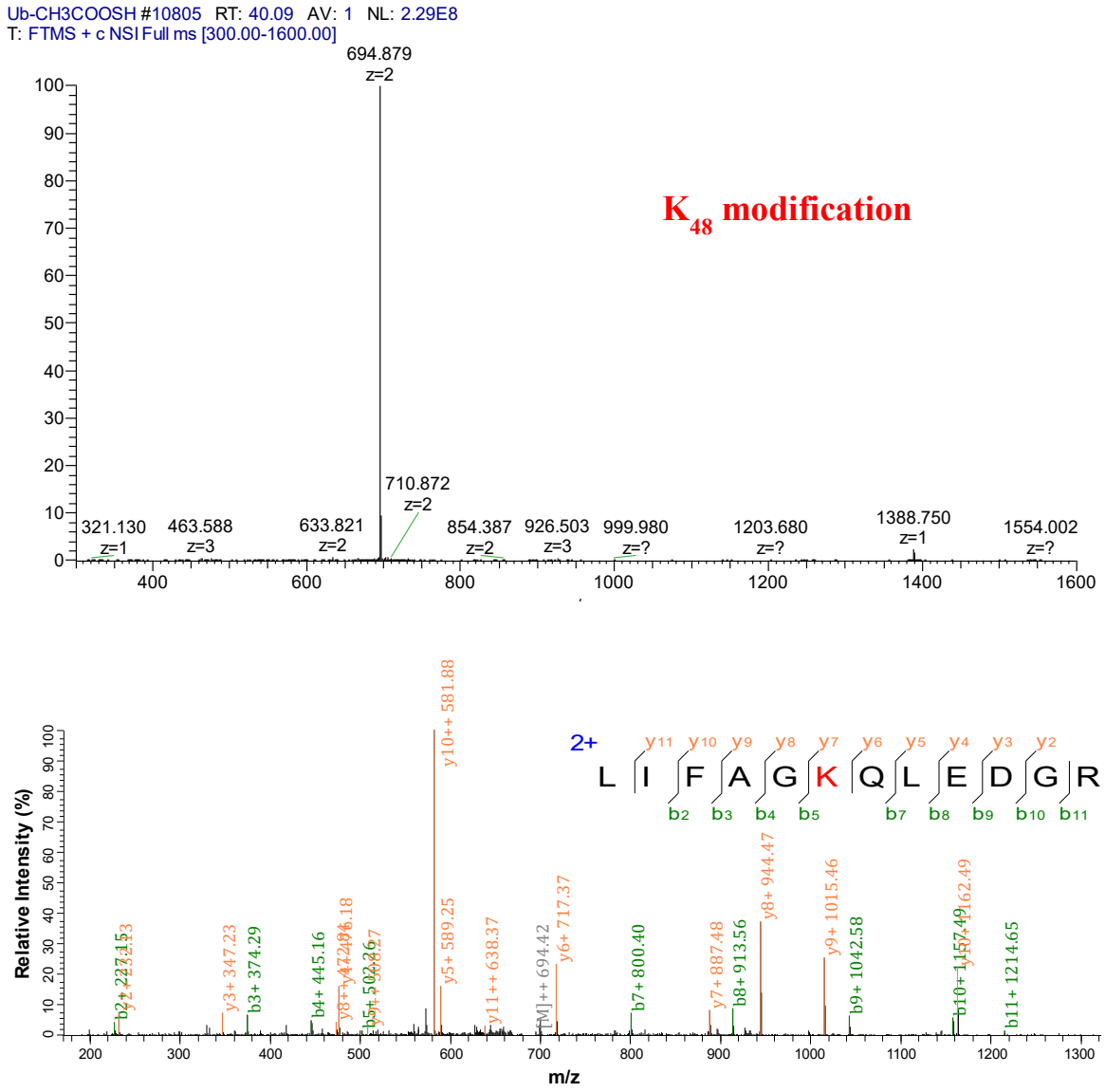


Figure S21. MS and MS_2 spectrums of the peptide fragments of Ub K48 modified with thioacid.

Peptide sequence	Mass(z=1)	Mass(z=2)
TLSDYNIQK	1123.562	562.285

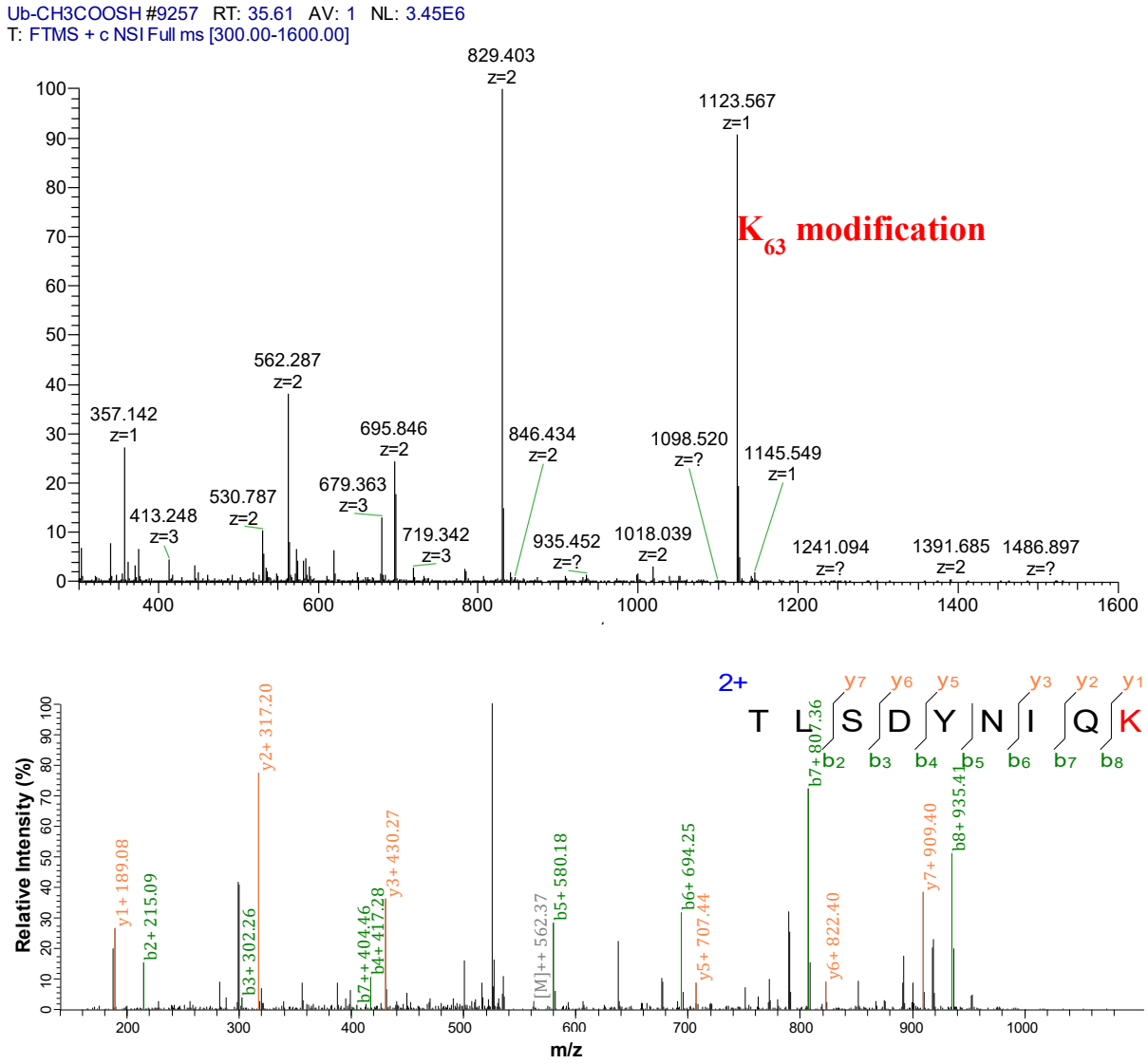
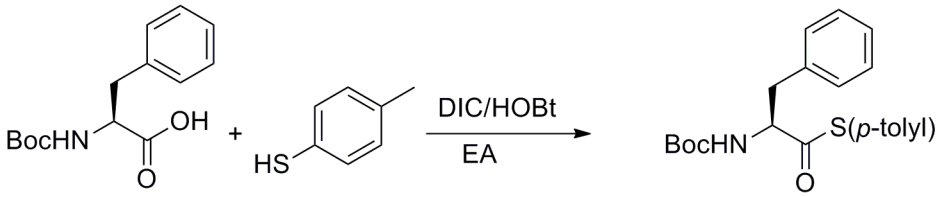


Figure S22. MS and MS_2 spectrums of the peptide fragments of Ub K63 modified with thioacid.

General methods

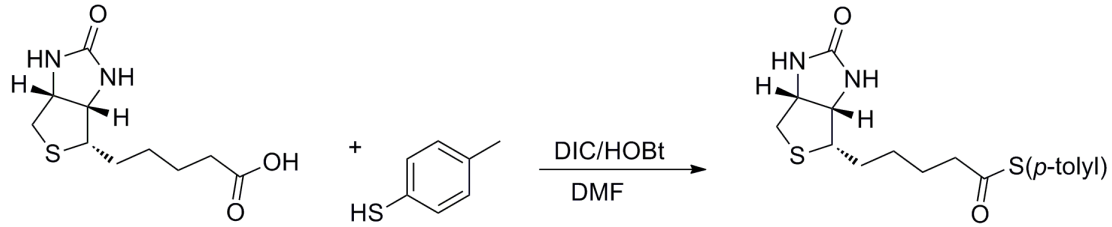
All chemicals were purchased from commercial sources (such as Aldrich, conju-probe, Lumiprobe and peptide international). The ^{1}H and ^{13}C NMR spectra were acquired on a Bruker 400 MHz magnetic resonance spectrometer. Data for ^{1}H NMR spectra are reported as follows: chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform-d (δ 7.26, s); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened); coupling constants are reported as a *J* value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. MALDI-MS spectrometric analyses were performed on an Applied Biosystems 4700 MALDI TOF mass spectrometer. HPLC was performed on a Dionex HPLC System (Dionex Corporation) and a reversed-phase C18 column was used for analysis (Phenomenax, 5 μm, 4.6 mm × 250 mm) and semi-preparation (Agilent, 5 μm, 10 mm × 250 mm).

Chemical synthesis and characterization



1 **1a**

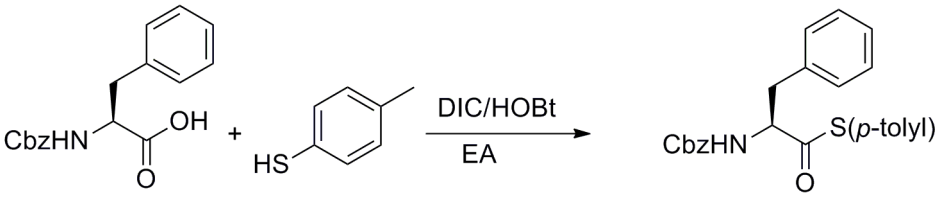
Synthesis of 1a: The mixture of Boc-Phe-*L*-amino acid (265.0 mg, 998.9 μmol), 4-Methylbenzenethiol (136.4 mg, 1.098 mmol), diisopropylcarbodiimide (189.3 mg, 1500 μmol) and 1-hydroxybenzotriazole (202.6 mg, 1500 μmol) were dissolved in ethyl acetate (2000 μL). The reaction mixture was stirred for overnight and purified by flash chromatography affording the desired product as a white solid (315.4 mg, 85 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.37-7.21 (m, 9H), 5.00 (d, *J*=12.0, 1H), 4.79-4.77 (m, 1H), 3.20-3.14 (m, 2H), 2.40 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 199.7，154.9, 139.8, 135.6, 134.5, 130.1, 129.4, 128.6, 127.1, 123.7, 119.0, 80.4, 60.8, 38.4, 28.3, 21.3; HRMS (ESI) Calcd for: $C_{21}H_{26}NO_3S$: 372.1628. Found: 372.1622 ($[M+H]^+$).



1b

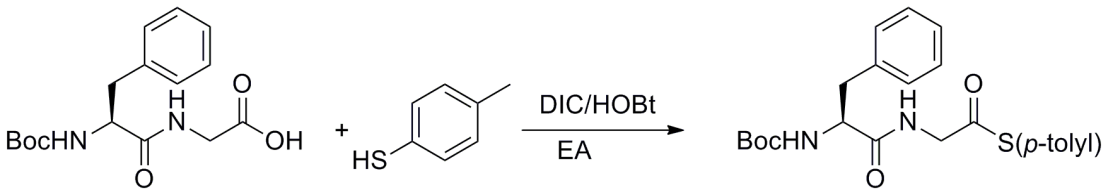
Synthesis of 1b: The mixture of biotin (244.0 mg, 998.9 μmol), 4-Methylbenzenethiol (136.4 mg, 1.098 mmol) diisopropylcarbodiimide (189.3 mg, 1500 μmol) and 1-hydroxybenzotriazole (202.6 mg, 1500 μmol) were dissolved in DMF (2000 μL). The reaction mixture was stirred for overnight and purified by flash chromatography affording the desired product as a white solid (273.1 mg, 78 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.27 (br, 1H), 7.62 (d, *J*=8.0, 1H), 7.40 (d, *J*=8.0, 1H), 7.27-7.14 (m, 6H), 7.06 (br, 1H), 5.16 (d, *J*=8.0, 1H), 4.86-4.81 (m, 1H), 3.45-3.29 (m, 2H), 2.39 (s, 3H), 1.48 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 200.6, 155.2, 139.7, 136.1, 134.6,

130.0, 127.6, 123.8, 123.2, 119.8, 119.0, 111.2, 109.7, 80.4, 60.5, 28.3, 28.1, 21.3; HRMS (ESI) Calcd for: $C_{17}H_{23}N_2O_2S_2$: 351.1195. Found: 351.1193 ($[M+H]^+$).



1 **1c**

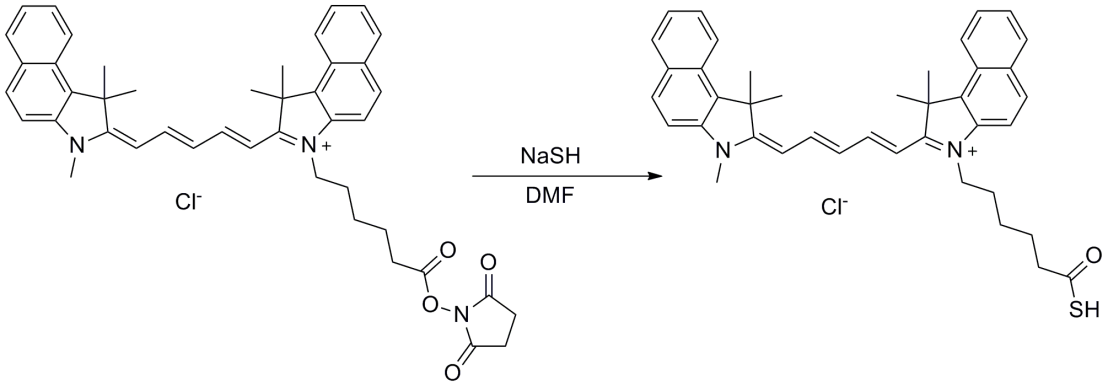
Synthesis of 1c: The mixture of Cbz-Phe-*L*-amino acid (299.0 mg, 999.0 μmol), 4-Methylbenzenethiol (136.4 mg, 1.098 mmol) diisopropylcarbodiimide (189.3 mg, 1500 μmol) and 1-hydroxybenzotriazole (202.6 mg, 1500 μmol) were dissolved in ethyl acetate (2000 μL). The reaction mixture was stirred for overnight and purified by flash chromatography affording the desired product as a white solid (344.3 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.29-7.08 (m, 14H), 5.17 (d, *J*=8.0, 1H), 5.04 (s, 2H), 4.78-4.73 (m, 1H), 3.12-3.02 (m, 2H), 2.30 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 199.1, 155.6, 140.0, 136.0, 135.3, 134.5, 130.1, 129.4, 128.7, 128.5, 128.3, 128.1, 127.3, 123.4, 67.3, 61.3, 38.4, 21.4,; HRMS (ESI) Calcd for: $C_{24}H_{24}NO_3S$: 406.1471. Found: 406.1471 ($[M+H]^+$).



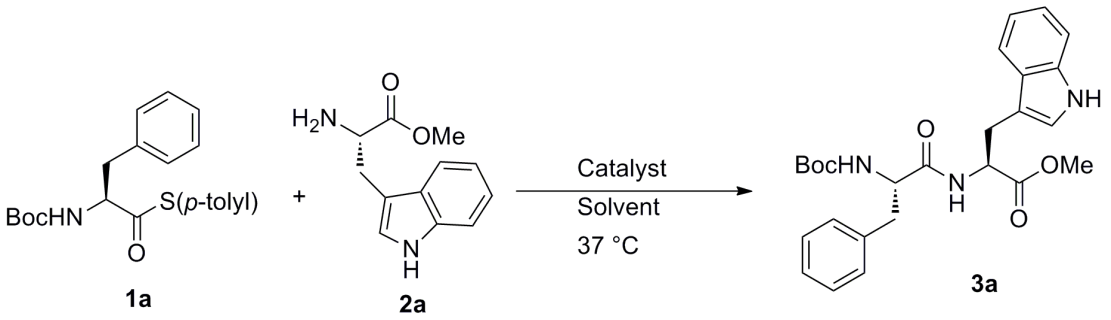
1d

Synthesis of 1d: The mixture of Boc-L-Phe-Gly-OH (322.0 mg, 999.1 μmol), 4-Methylbenzenethiol (136.4 mg, 1.098 mmol) diisopropylcarbodiimide (189.3 mg, 1500 μmol) and 1-hydroxybenzotriazole (202.6 mg, 1500 μmol) were dissolved in ethyl acetate (2000 μL). The reaction mixture was stirred for overnight and purified by flash chromatography affording the desired product as a white solid (325.4 mg, 76 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.21(s, 1H), 7.61 (d, *J*=8, 1H), 7.41 (d, *J*=8, 1H), 7.26-7.08 (m, 7H), 5.14 (d, *J*=12, 1H), 4.85-4.80 (m, 1H), 3.45-3.28 (m, 2H), 2.39 (s, 3H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 199.8，139.8, 135.6,

134.5, 130.1, 129.4, 128.7, 127.1, 123.7; HRMS (ESI) Calcd for: $C_{23}H_{28}N_2O_4S$: 429.1843. Found: 429.1843 ($[M+H]^+$).

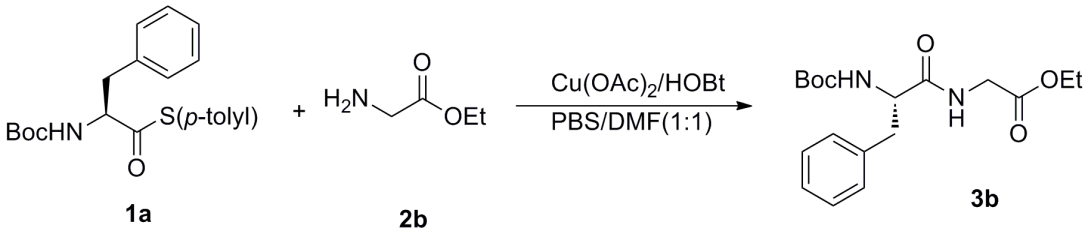


Synthesis of Cy5.5COSH: The Cy5.5-NHS (2.100 mg, 2.932 μmol) was dissolved in distilled DMF (300.0 μL). NaSH (300.0 μg, 5.355 μmol) was added to this stirring solution under N_2 atmosphere. The reaction mixture was allowed to stir for 4h. Then the reaction mixture was purified by HPLC to get the desired Cy5.5 thioacid as a blue solid . ESI-MS Calcd for:$C_{40}H_{43}N_2OS$: 599.3,Found: 599.5 ($[M+H]^+$).

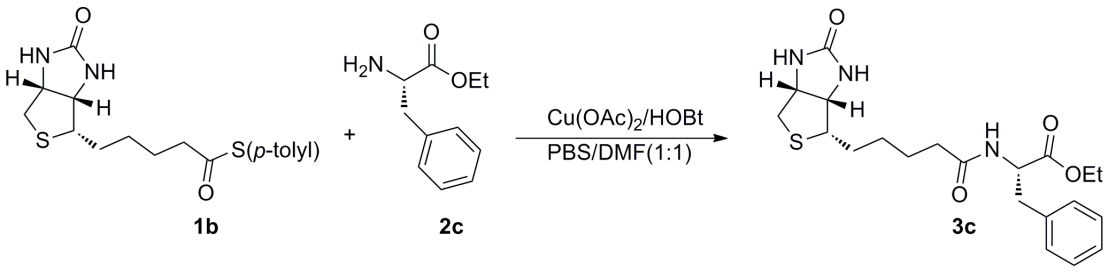


Synthesis of 3a: Boc-Phe-*p*-toluene thiol ester (371.0 mg, 998.7 μmol) was added to DMF (2000 μL) or DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added catalyst (1 eq) or catalyst and HOBt (1eq or 2eq) and Try-OMe (327.0 mg, 1.498 mmol) in it. The mixture was stirred at 37 °C, then cooled to room temperature. The solvent was evaporated under reduced pressure, and the residue dissolved in ethyl acetate (15.00 mL) then washed successively with 1M aqueous HCl, 2M aqueous NaOH and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated to dryness. The crude product was purified by flash chromatography (first use 25% ethyl acetate in petroleum ether, then use 10% MeOH in DCM wash the chromatography to get the product. The title compound was prepared as a white solid.

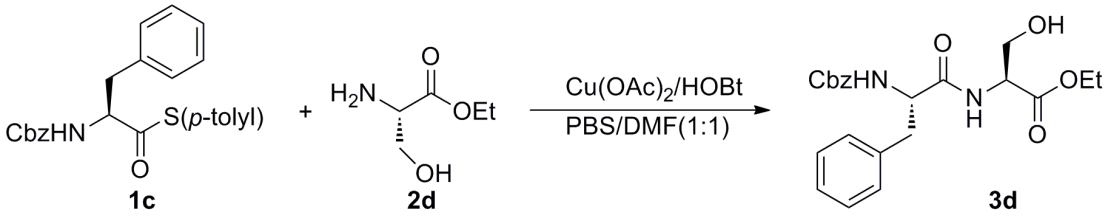
^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.20 (br, 1H),7.36-7.09 (m, 10H), 6.41 (br, 1H), 4.84-4.76 (m, 2H), 4.36 (br, 1H), 3.64 (s, 3H), 3.28-3.27 (m, 2H), 3.04-3.03 (m, 2H), 1.38 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ = 171.7, 170.8, 136.5, 136.0, 129.4, 128.6, 126.9, 122.9, 122.2, 119.6, 118.4, 111.3, 109.7, 52.9, 52.3, 38.3, 28.2, 27.6; HRMS (ESI) Calcd for: $C_{26}H_{32}N_3O_5$: 466.2336. Found: 466.2347 ($[M+H]^+$).



Synthesis of 3b: Boc-Phe-*p*-toluene thiol ester (371.0 mg, 998.7 μmol) was added to DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added $Cu(OAc)_2$/HOBt (1 eq) and Gly (116.0 mg, 1.125 mmol) in it. The mixture was stirred at 37 ºC for 1h, then cooled to room temperature. The solvent was evaporated under reduced pressure, and the residue dissolved in ethyl acetate then washed successively with 1M aqueous HCl, 2M aqueous NaOH and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated to dryness. The crude product was purified by flash chromatography (first use 25% ethyl acetate in petroleum ether, then use 10% MeOH in DCM wash the chromatography to get the product). The title compound was prepared as a white solid (308.9 mg, 88 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.22-6.79 (m, 5H), 6.79 (br, 1H), 5.25-5.22 (m, 1H), 4.40 (br, 1H), 4.13 (dd, J_1=8.0, J_2=16.0, 2H), 4.38 (br, 1H), 3.92-3.86 (m, 2H), 3.09-2.91 (m, 2H), 1.30 (s, 9H), 1.20 (t, J=4.0, 3H), ^{13}C NMR (101 MHz, $CDCl_3$) δ = 171.5, 170.4, 169.4, 136.6, 129.2, 128.5, 126.9, 61.5, 54.2, 41.3, 38.2, 23.0, 14.1; HRMS (ESI) Calcd for: $C_{16}H_{23}N_2O_5$: 351.1914. Found: 351.1914 ($[M+H]^+$).

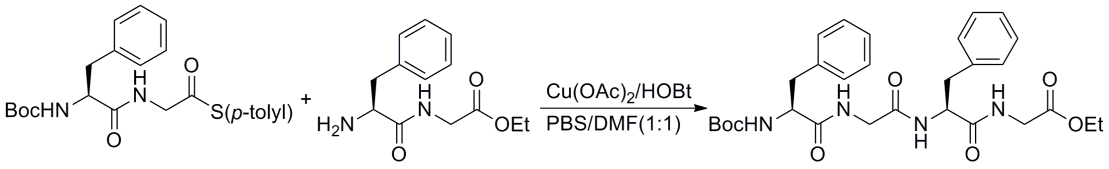


Synthesis of 3c: Biotin-*p*-toluene thiol ester (350.0 mg, 998.6 μmol) was added to DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added $Cu(OAc)_2$/HOBt (1 eq) and Phe-OEt (289.5 mg, 1.498 mmol) in it. The mixture was stirred at 37 ºC for 1h, then cooled to room temperature. The solvent was evaporated under reduced pressure, and the residue dissolved in ethyl acetate then washed successively with 1M aqueous HCl, 2M aqueous NaOH and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated to dryness. The crude product was purified by flash chromatography (first use 25% ethyl acetate in petroleum ether, then use 10% MeOH in DCM wash the chromatography to get the product). The title compound was prepared as a white solid (327.0 mg, 80 %). ^{1}H NMR (400 MHz, MeOD) δ = 7.32-7.23 (m, 5H), 4.69-4.64 (m, 1H), 4.52-4.49 (m, 1H), 4.30-4.27 (m, 1H), 4.19-4.12 (m, 2H),3.16-3.15 (m, 2H), 2.97-2.92 (m, 2H), 2.74 (d, *J*=12.0, 1H), 2.21-2.18 (m, 2H), 1.60-1.57 (m, 4H), 1.24 (t, *J*=8.0, 4H),0.94-0.90 (m, 2H), ^{13}C NMR (101 MHz, MeOD) δ = 174.5, 171.9, 136.8, 128.8, 128.1, 126.4, 61.8, 61.0, 60.2, 55.5, 53.8, 39.6, 37.0, 34.8, 28.0, 27.9, 25.3, 13.0; HRMS (ESI) Calcd for: $C_{21}H_{30}N_3O_4S$: 420.1952. Found: 420.1945 ([M+H]$^+$).



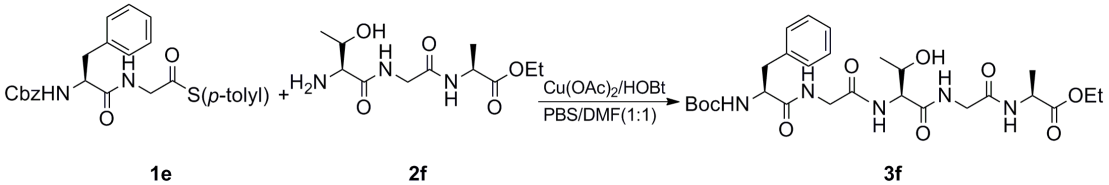
Synthesis of 3d: Cbz-Phe-*p*-toluene thiol ester (405.0 mg, 998.8 μmol) was added to DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added $Cu(OAc)_2$/HOBt (1 eq) and Ser-OEt (199.5 mg, 1.499 mmol) in it. The mixture was stirred at 37 ºC for 1h, then cooled to room temperature. The solvent was evaporated under reduced pressure, and the residue dissolved in ethyl acetate then washed successively with 1M aqueous HCl, 2M aqueous NaOH and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated to dryness. The crude product was purified by flash chromatography (first use 25% ethyl acetate in petroleum ether, then use 10% MeOH in DCM wash the chromatography to get the product). The title compound was prepared as a white solid (352.0 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.27-7.06

(m, 11H), 5.58 (d, *J*=8.0, 1H), 5.00-4.89 (m, 2H), 4.52-4.50 (m, 2H), 4.13-4.10 (m, 2H), 3.82 (s, 2H), 3.08-2.67 (m, 2H), 1.20 (t, *J*=8.0, 3H), ^{13}C NMR (101 MHz, $CDCl_3$) δ = 171.5, 170.1, 136.2, 136.0, 129.3, 128.6, 128.5, 128.2, 128.0, 127.0, 67.1, 62.8, 61.9, 56.2, 54.9, 38.4, 14.1; HRMS (ESI) Calcd for: $C_{22}H_{27}N_2O_3$: 415.1864. Found: 415.1856 ([M+H]$^+$).



1e **2d** **3e**

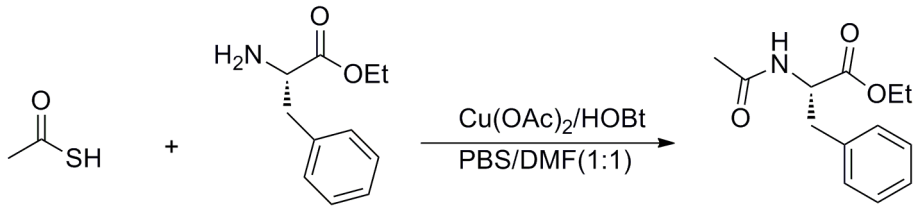
Synthesis of 3e: Boc-L-Phe-Gly-*p*-toluene thiol ester (428.0 mg, 998.9 μmol) was added to DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added $Cu(OAc)_2$/HOBt (1 eq) and L-Phe-Gly-OEt (375.0 mg, 1.498 mmol) in it. The mixture was stirred at 37 ºC for 2 h, then cooled to room temperature. The reaction mixture was purified by HPLC to get desire product as a white solid (415.0 mg, 75 %). ^{1}H NMR (400 MHz, MeOD) δ = 7.31-7.21 (m, 10H), 4.65 (s, 3H), 4.29-4.19 (m, 2H), 3.95-3.94 (m, 3H), 3.67-3.62 (m, 2H), 3.27-3.23 (m, 1H), 3.16-3.11 (m, 1H), 2.98-2.95 (m, 1H), 2.87-2.85 (m, 1H), 1.37-1.18 (m, 12H), ^{13}C NMR (101 MHz, $CDCl_3$) δ = 171.5, 170.1, 136.2, 136.0, 129.3, 128.6, 128.5, 128.2, 128.0, 127.0, 67.1, 62.8, 61.9, 56.2, 54.9, 38.4, 14.1; HRMS (ESI) Calcd for: $C_{29}H_{39}N_4O_7$: 555.2813. Found: 555.2816 ([M+H]$^+$).



1e **2f** **3f**

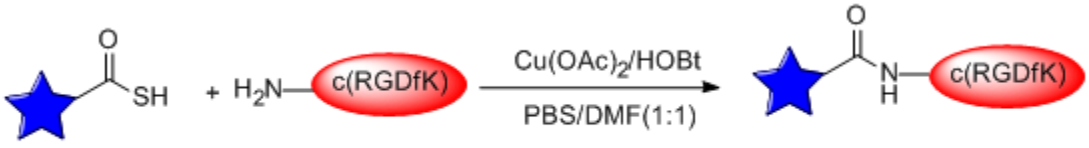
Synthesis of 3f: Cbz-L-Phe-Gly-*p*-toluene thiol ester (462.0 mg, 998.7 μmol) was added to DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added $Cu(OAc)_2$/HOBt (1 eq) and L-Thr-Gly-L-Ala-OEt (412.0 mg, 1.497 mmol) in it. The mixture was stirred at 37 ºC for 4 h, then cooled to room temperature. The reaction mixture was purified by HPLC to get desire product as a white solid (348.0 mg, 60.0 %). ^{1}H NMR (400 MHz, MeOD) δ = 8.34-8.31 (m, 1H), 8.16-8.08 (m, 2H), 7.84 (d, *J*=8.0, 1H), 7.56 (d, *J*=8.0, 1H), 7.35-7.20 (m, 10H), 4.95-4.93 (m, 2H), 4.28-4.21

(m, 3H), 4.10-4.00 (m, 3H), 3.87(t, *J*=4.0, 2H), 3.76(t, *J*=4.0, 2H), 3.07-3.02 (m, 2H), 2.79-2.73 (m, 1H), 1.74 (br, 1H), 1.28(d, *J*=8.0, 3H), 1.19(d, *J*=8.0, 3H), 1.09(d, *J*=8.0, 3H), ^{13}C NMR (101 MHz, $CDCl_3$) δ = 172.8, 170.7, 169.1, 137.4, 129.6, 128.7, 128.5, 127.9, 126.7, 67.0, 60.9, 48.1, 20.0, 17.4, 14.5; HRMS (ESI) Calcd for: $C_{27}H_{42}N_5O_9$: 580.2977. Found: 580.2971 ([M+H]$^+$).



1f **2c** **3g**

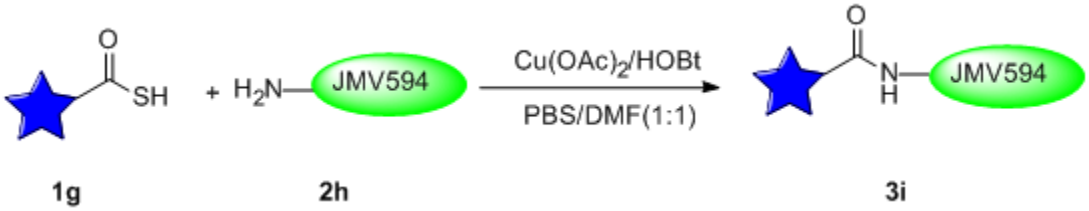
Synthesis of 3g: Thiol acid (76.00 mg, 998.4 μmol) was added to DMF/PBS (2000 μL, 1:1) in round-bottom flask, then added $Cu(OAc)_2$/HOBt (1 eq) and Phe-OEt (289.5 mg, 1.498 mmol) in it. The mixture was stirred at 37 ºC for 0.1 h, then cooled to room temperature. The solvent was evaporated under reduced pressure, and the residue dissolved in ethyl acetate then washed successively with 1M aqueous HCl, 2M aqueous NaOH and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated to dryness. The crude product was purified by flash chromatography (first use 25% ethyl acetate in petroleum ether, then use 10% MeOH in DCM wash the chromatography to get the product). The title compound was prepared as a white solid (211.0 mg, 90 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.19 (m, 3H), 7.16 – 7.05 (m, 2H), 6.11 (d, *J* = 7.4 Hz, 1H), 4.87 (dt, *J* = 7.8, 5.9 Hz, 1H), 4.18 (q, *J* = 7.1 Hz, 2H), 3.46 – 2.93 (m, 2H), 1.99 (s, 3H), 1.25 (t, *J* = 7.1 Hz, 3H),^{13}C NMR (101 MHz, $CDCl_3$) δ 171.74, 169.67, 135.95, 129.32, 128.52, 127.08, 61.52, 53.17, 37.92, 23.14, 14.12; ESI-MS Calcd for: $C_{13}H_{18}NO_3$: 236.13. Found: 235.88 ([M+H]$^+$).



1g **2g** **3h**

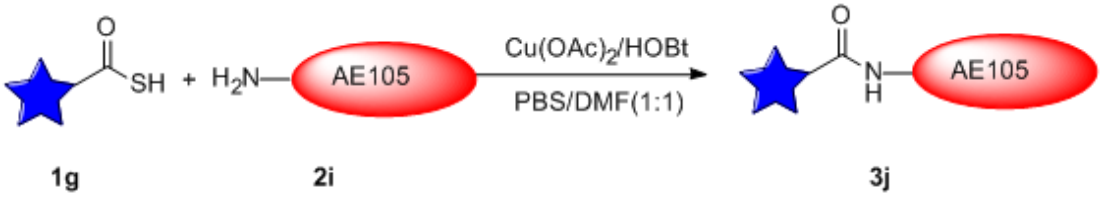
Synthesis of 3h: Cy5.5 thiol acid (635.0 μg, 999.5 μmol) was added to DMF/PBS (100.0 μL, 1:1) in EP tube, then added $Cu(OAc)_2$/HOBt (1 eq) and c(RGDfk) (900.0

μg, 1.542 μmol) in it. The mixture was stirred at 37 ºC for 0.5 h, then cooled to room temperature. The reaction mixture was purified by HPLC to get the product as a blue solid (759.4 μg, 60 %). ESI-MS Calcd for: $C_{67}H_{82}N_{11}O_8^+$: 1168.6. Found: 1168.9 ($[M+H]^+$).



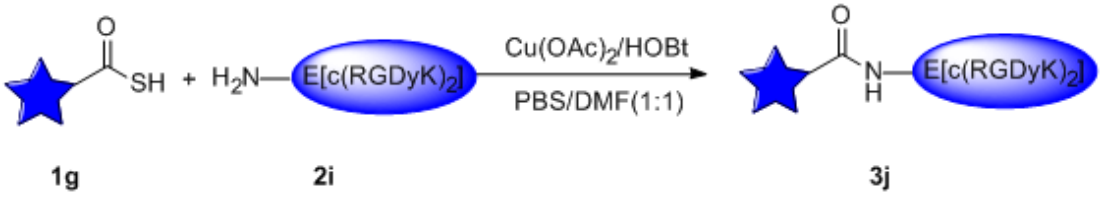
1g 2h 3i

Synthesis of 3i: Cy5.5 thiol acid (635.0 μg, 0.9995 μmol) was added to DMF/PBS (100.0 μL, 1:1) in EP tube, then added $Cu(OAc)_2$/HOBt (1 eq) and JMV594 (1600 μg, 1.438 μmol) in it. The mixture was stirred at 37 ºC for 0.5 h, then cooled to room temperature. The reaction mixture was purified by HPLC to get the product as a blue solid (1.057 mg, 63 %). ESI-MS Calcd for: $C_{95}H_{121}N_{16}O_{10}S^+$: 1677.9. Found: 1679.4 ($[M+H]^+$).



1g 2i 3j

Synthesis of 3j: Cy5.5 thiol acid (635.0 μg, 0.9995 μmol) was added to DMF/PBS (100.0 μL, 1:1) in EP tube, then added $Cu(OAc)_2$/HOBt (1 eq) and AE105 (2100 μg, 1.453 μmol) in it. The mixture was stirred at 37 ºC for 0.5 h, then cooled to room temperature. The reaction mixture was purified by HPLC to get the product as a blue solid (1170 μg, 58 %). ESI-MS Calcd for: $C_{110}H_{141}N_{18}O_{19}$: 2018.0, Found: 2017.5 ($[M+H]^+$).



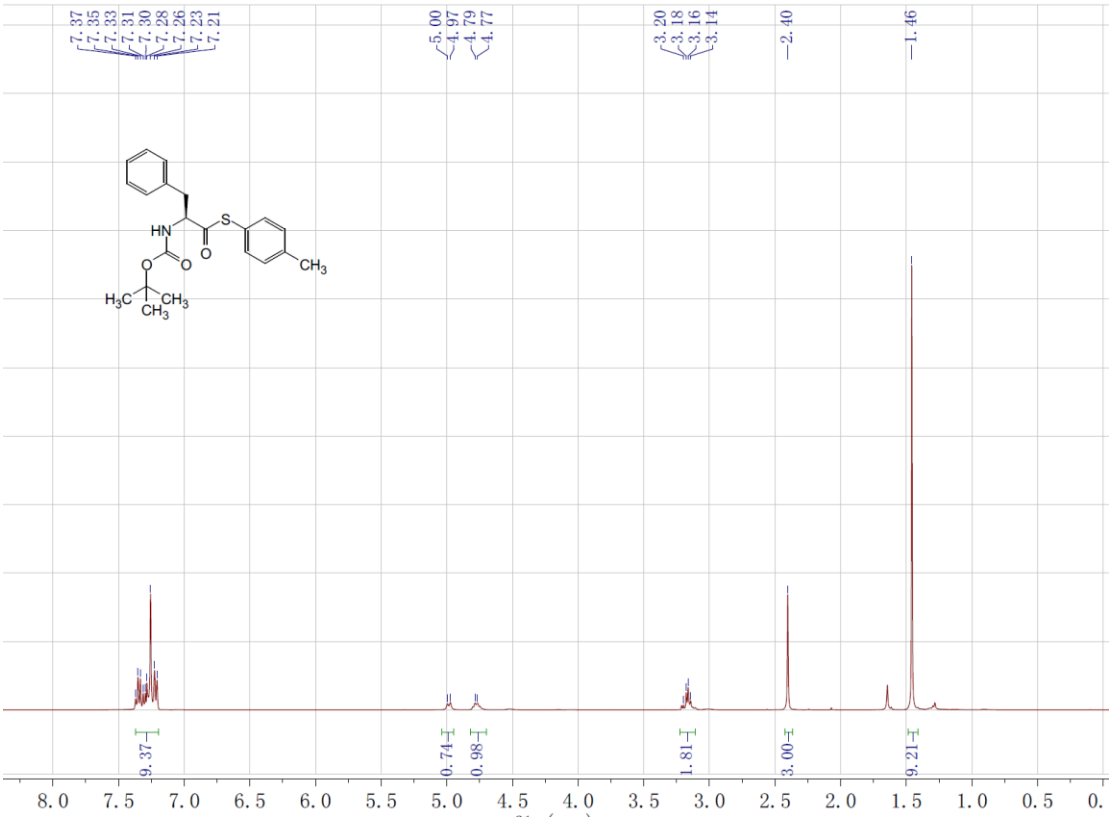
1g 2i 3j

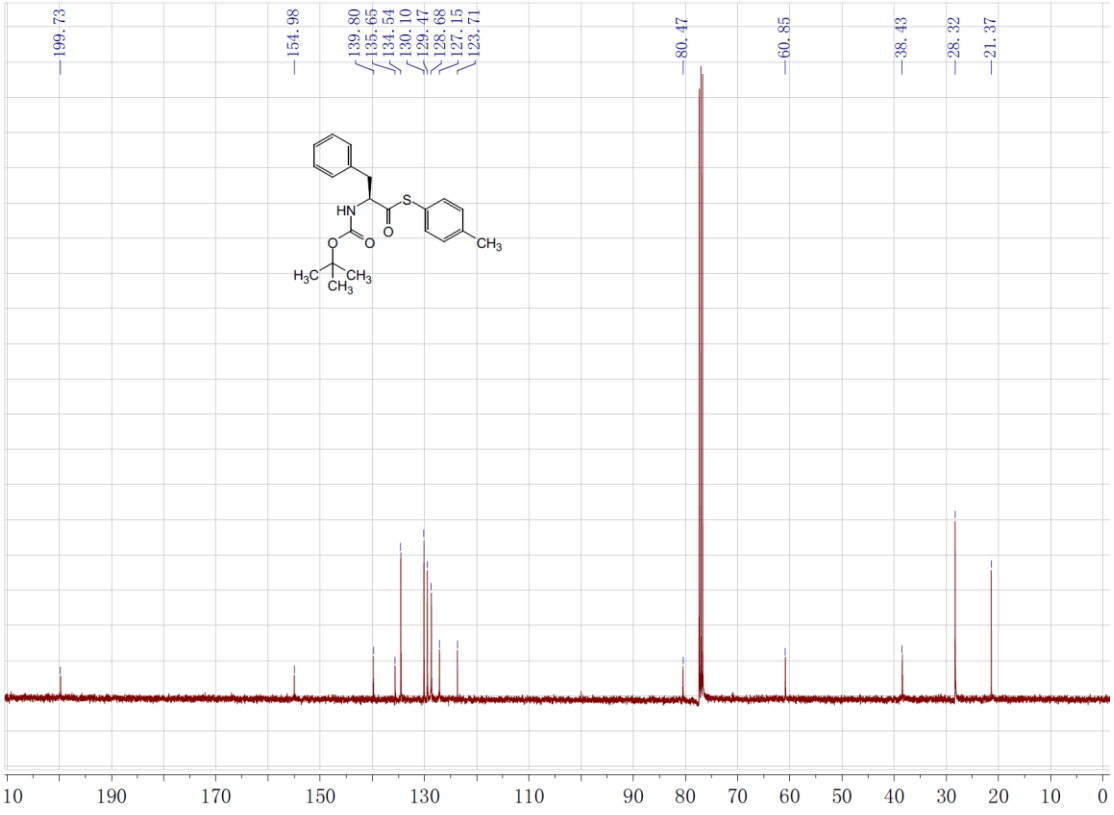
Synthesis of 3k: Cy5.5 thiol acid (635.0 μg, 0.9995 μmol) was added to DMF/PBS (100.0 μL, 1:1) in EP tube, then added $Cu(OAc)_2$/HOBt (1 eq) and E[c(RGDyK)$_2$] (2.020 mg, 1.496 μmol) in it. The mixture was stirred at 37 ºC for 0.5 h, then cooled

to room temperature. The reaction mixture was purified by HPLC to get the product as a blue solid (1.111 mg, 58 %). ESI-MS Calcd for: $C_{99}H_{129}N_{21}O_{19}^{+}$: 1915.9. Found: 1915.1 ($[M+H]^{-}$).

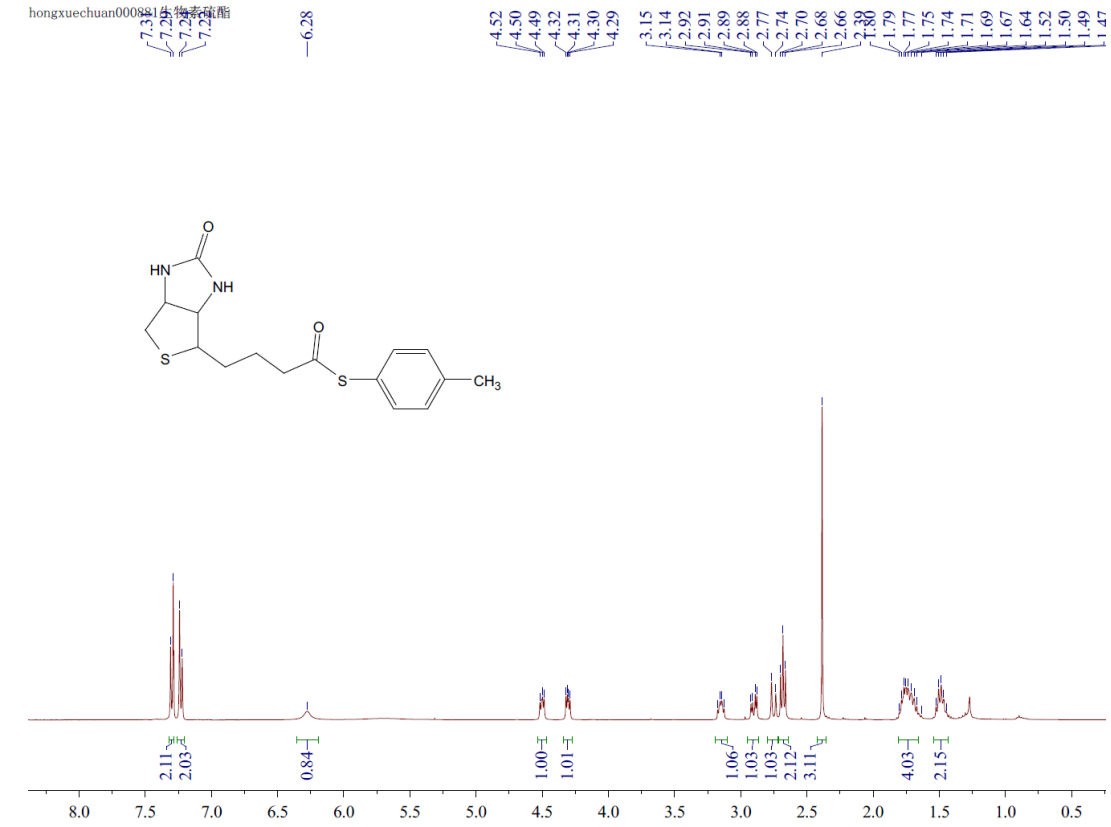
NMR Spectra

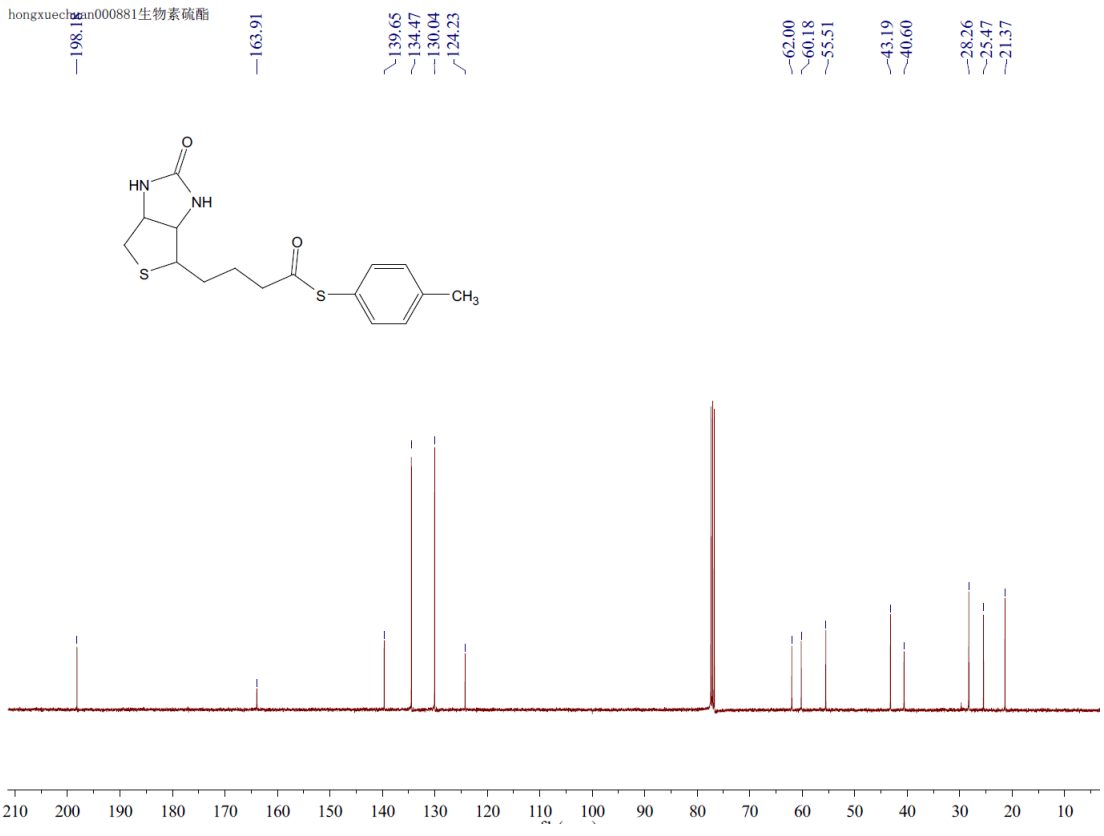
^{1}H NMR and ^{13}C NMR for 1a



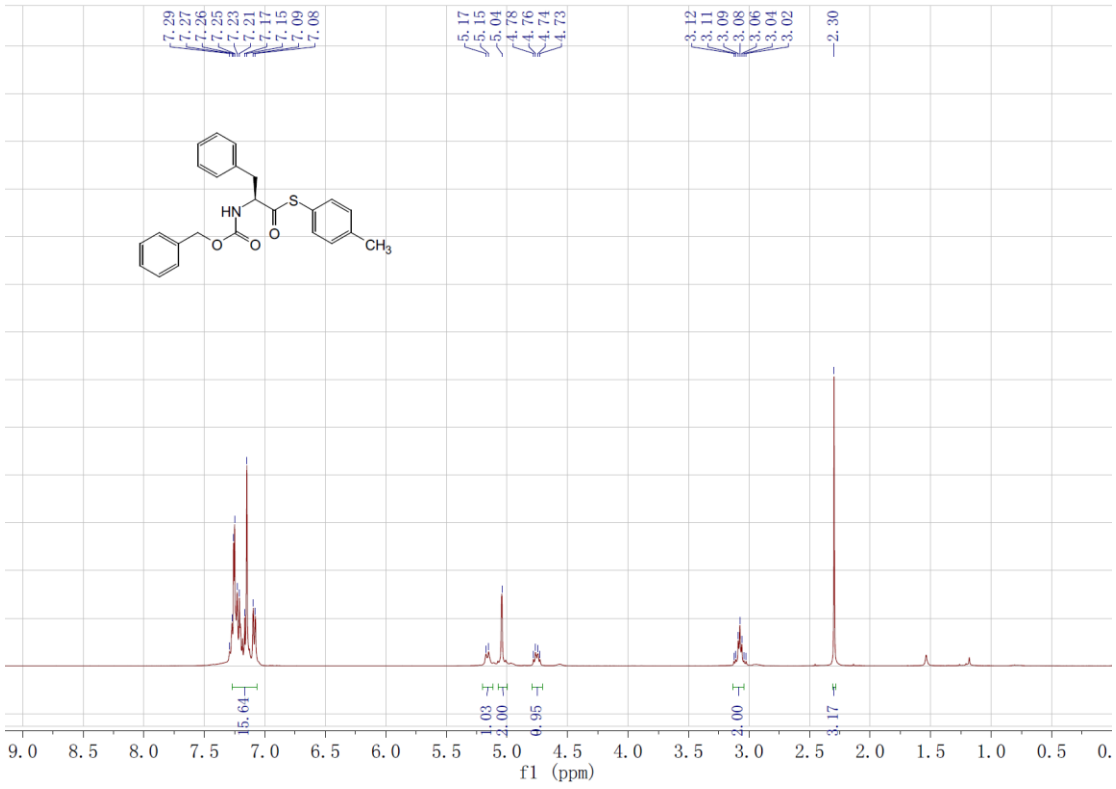


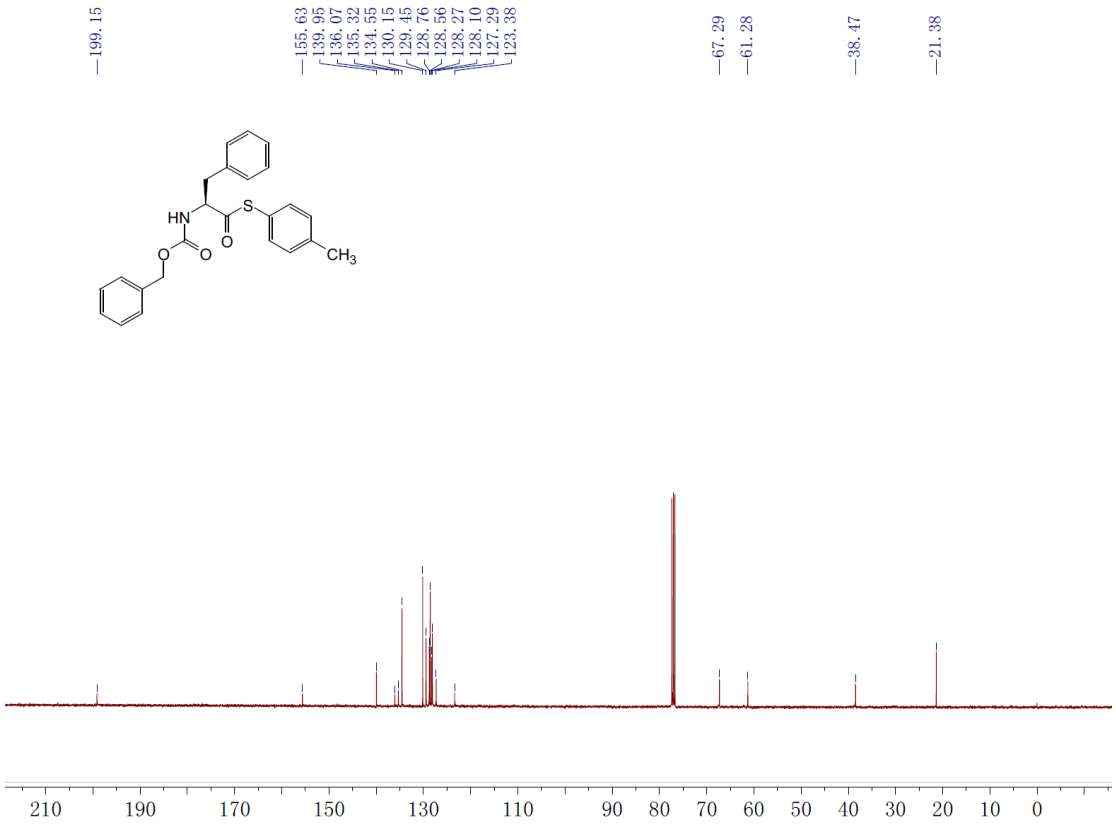
^{1}H NMR and ^{13}C NMR for 1b



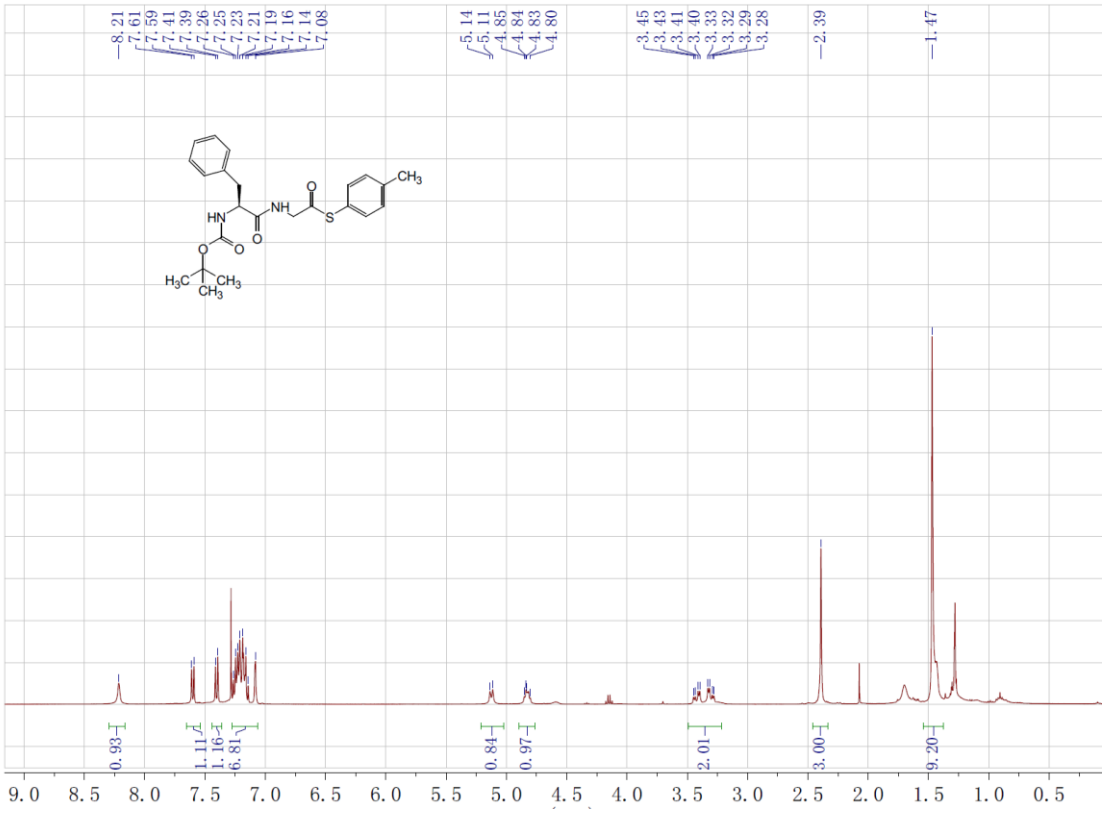


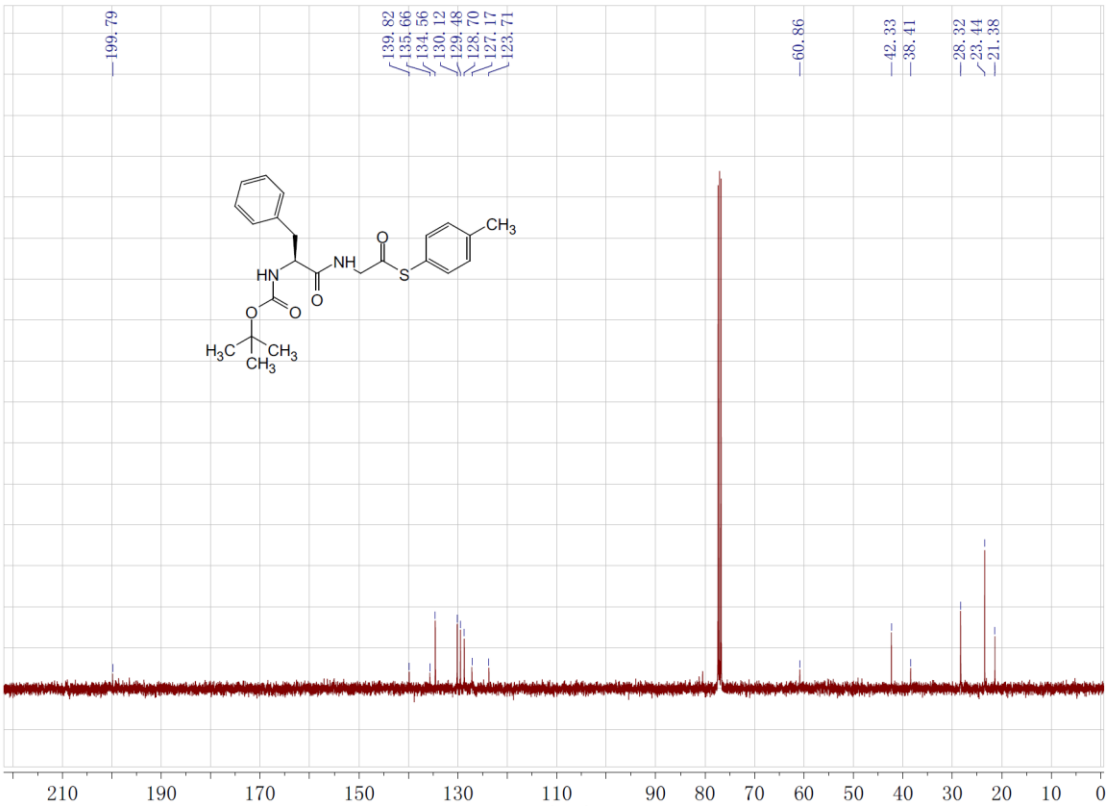
^{1}H NMR and ^{13}C NMR for 1c



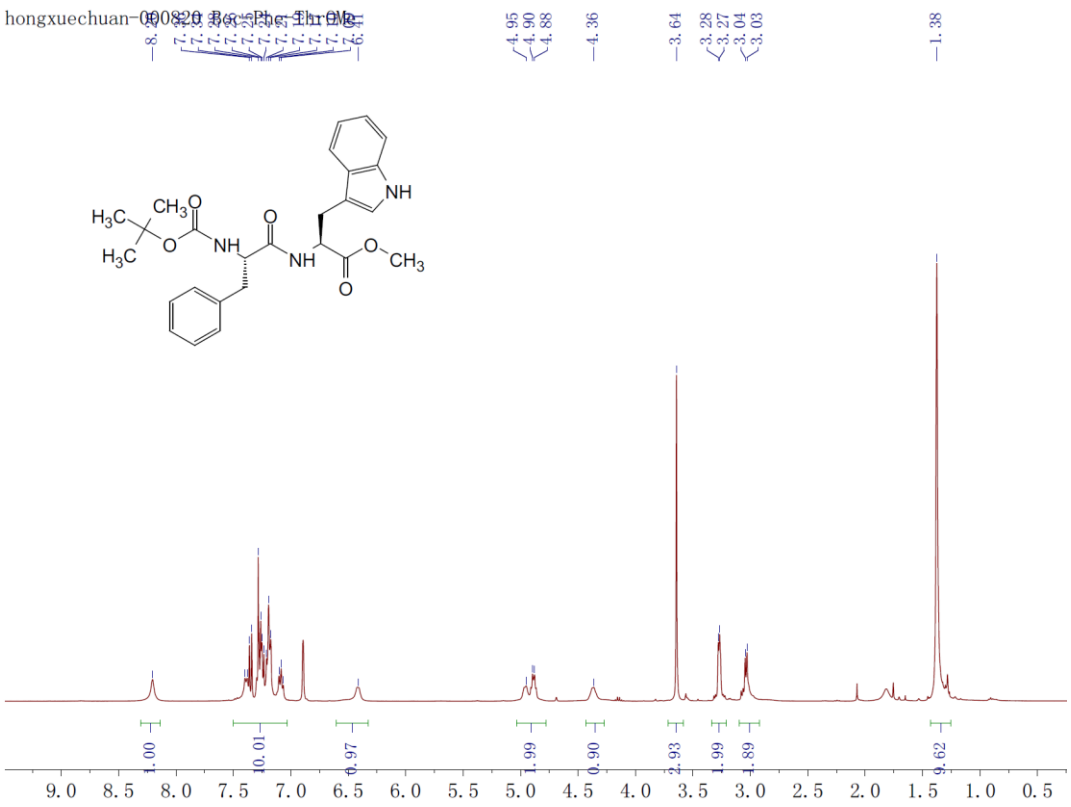


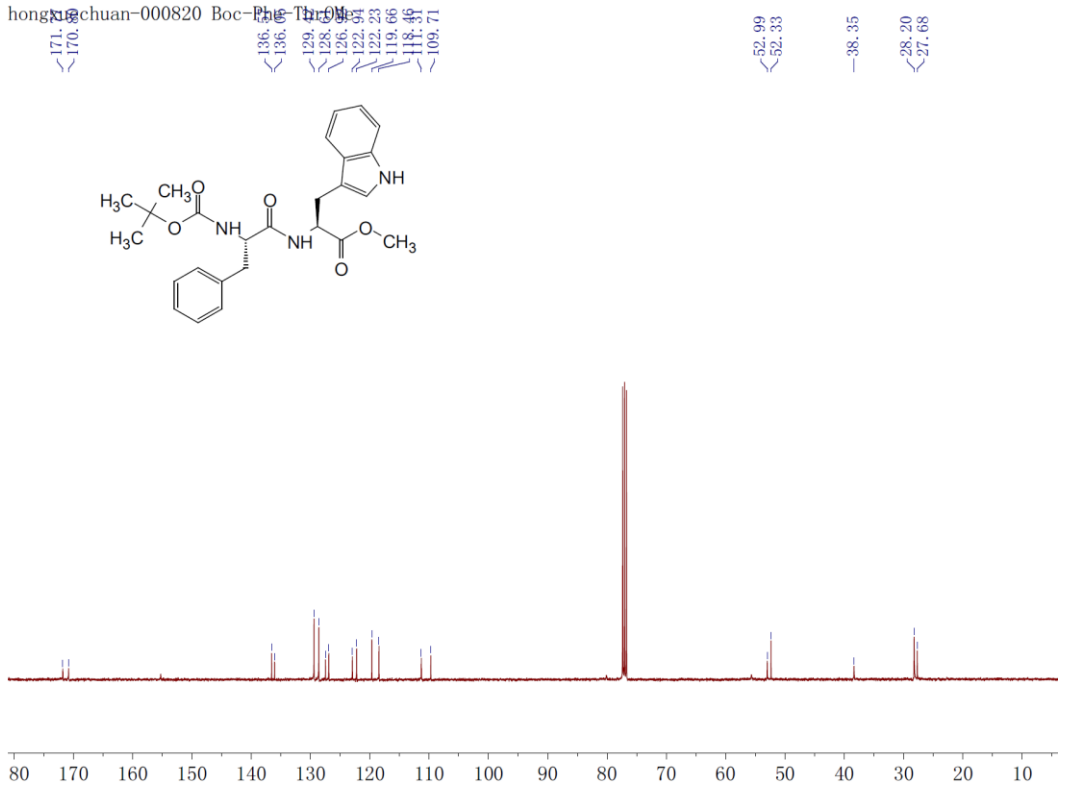
^{1}H NMR and ^{13}C NMR for 1d



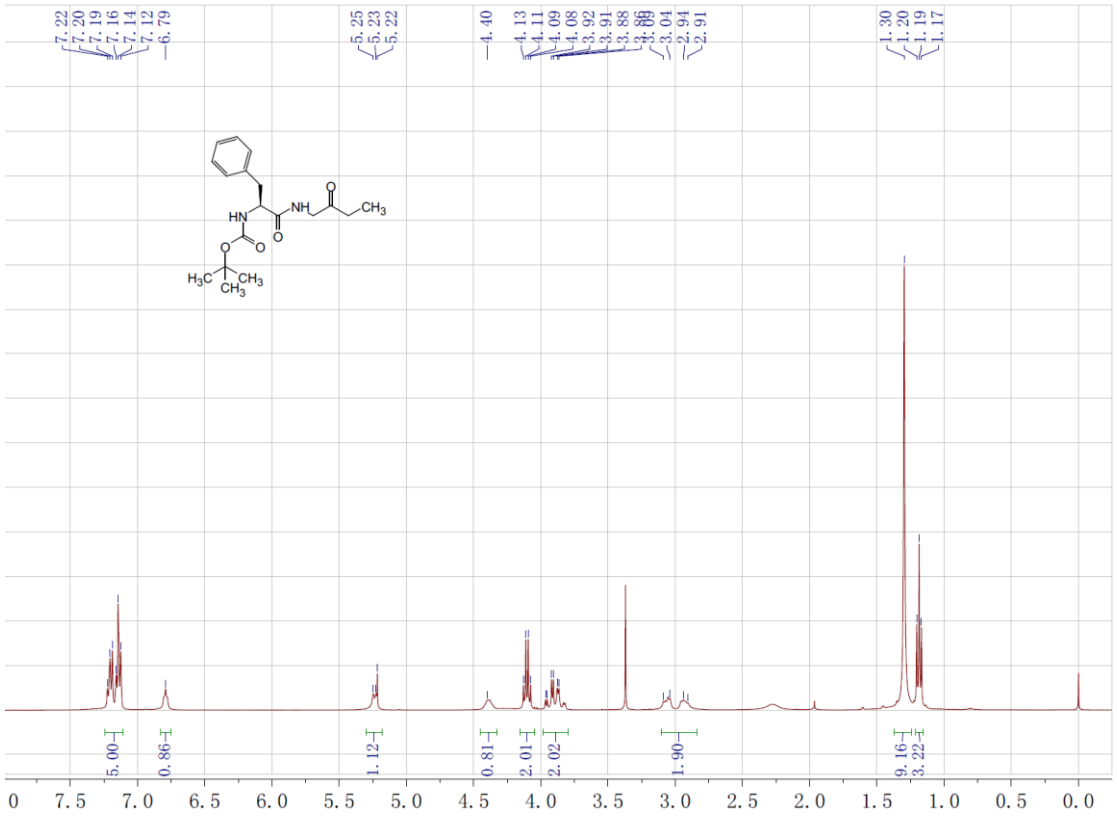


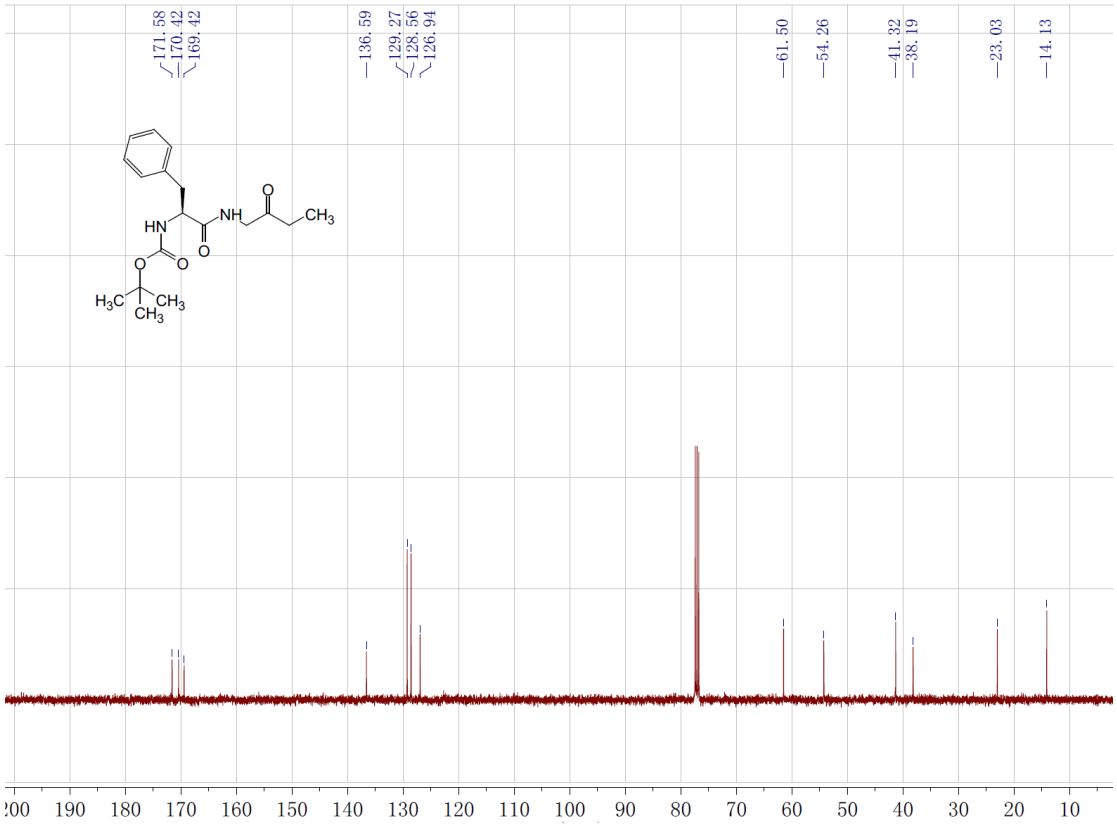
^{1}H NMR and ^{13}C NMR for 3a



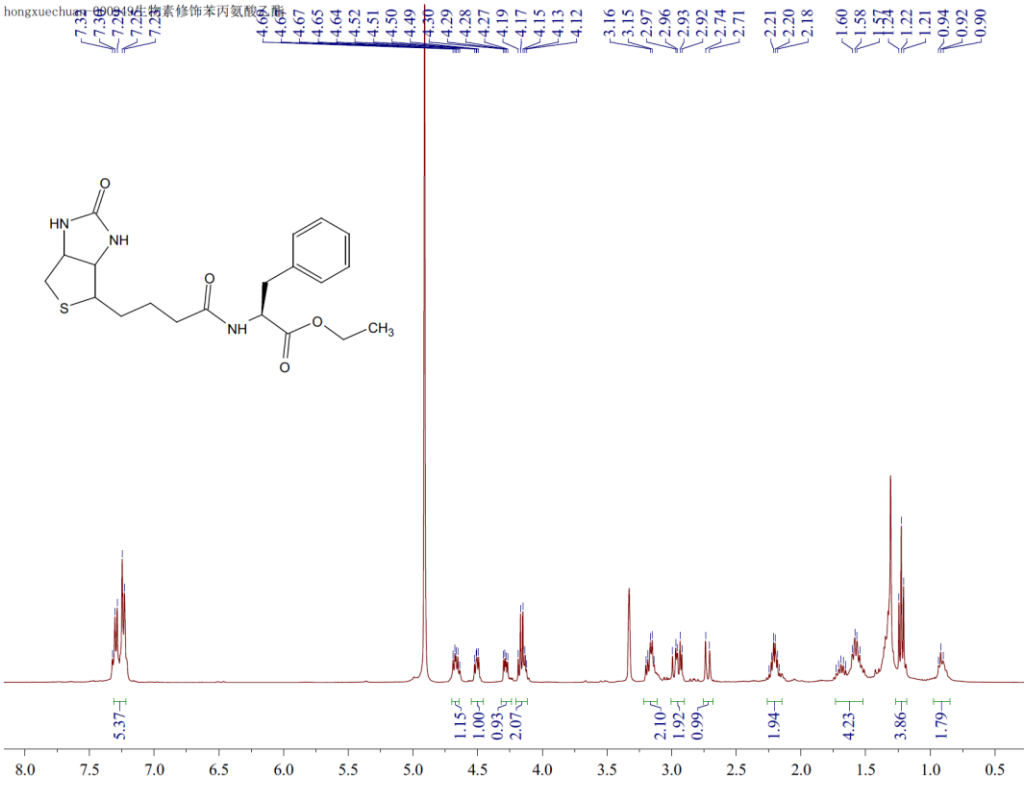


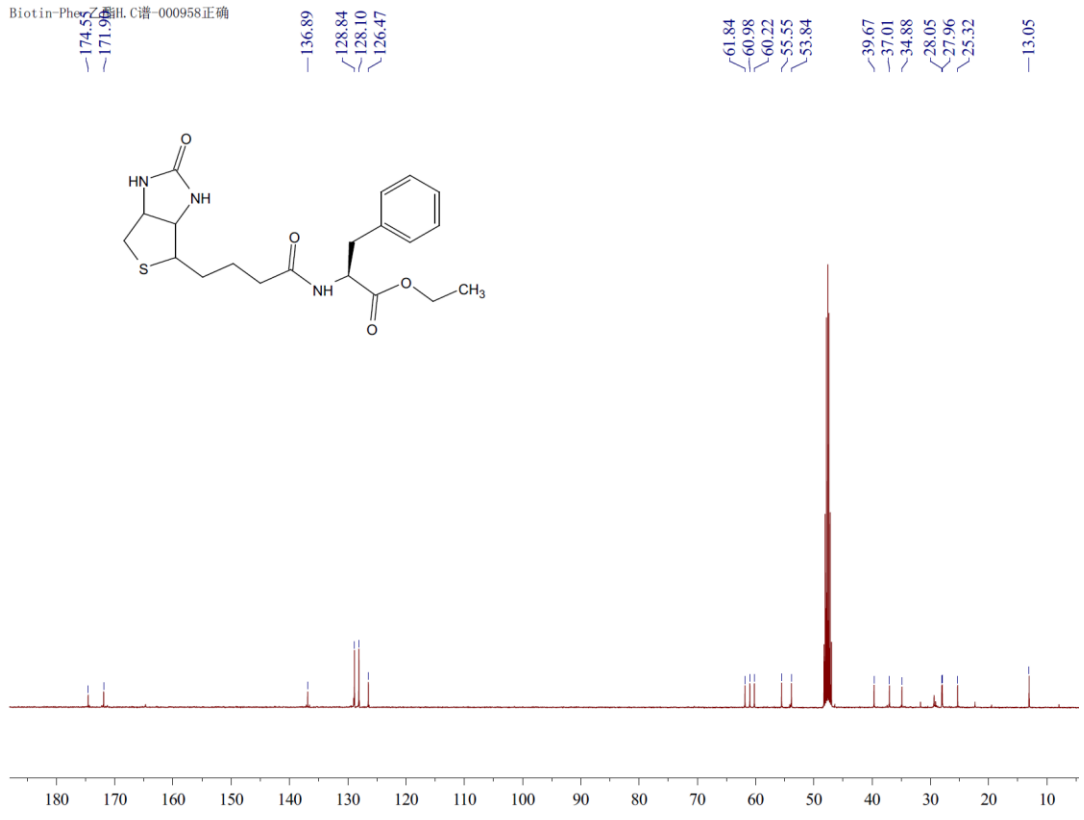
^{1}H NMR and ^{13}C NMR for 3b



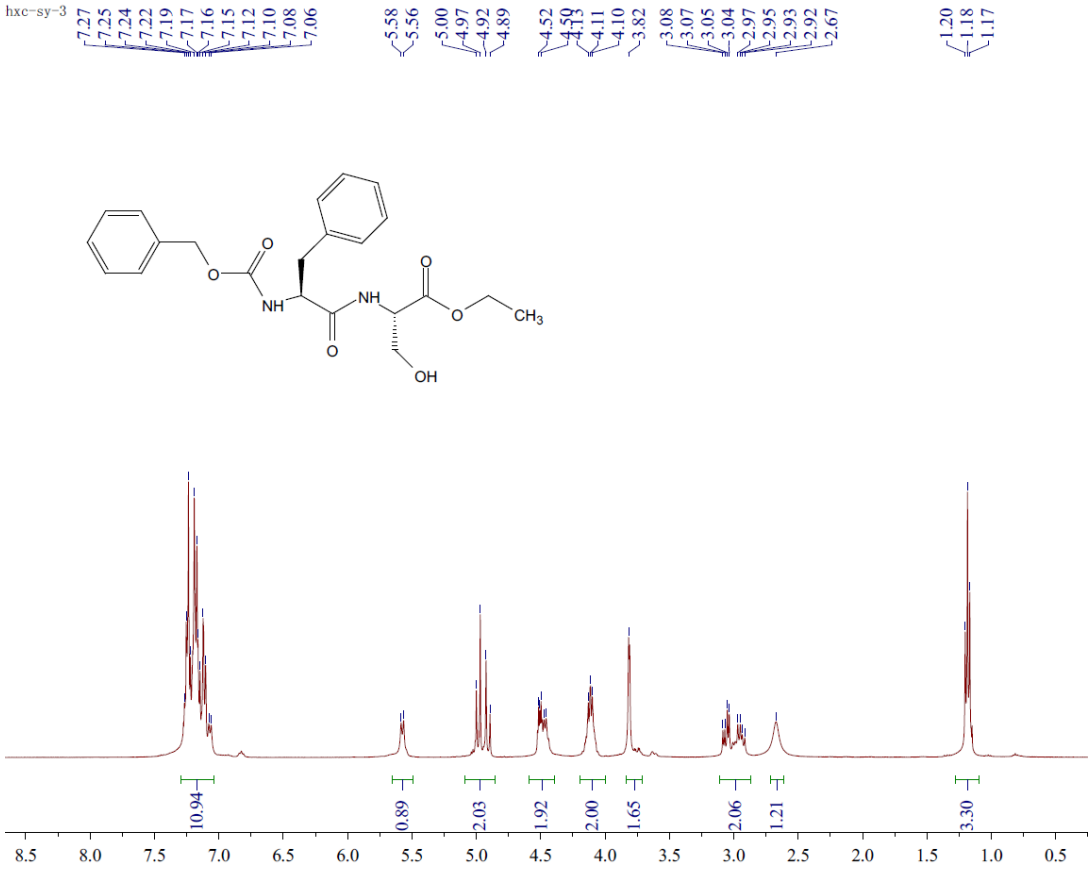


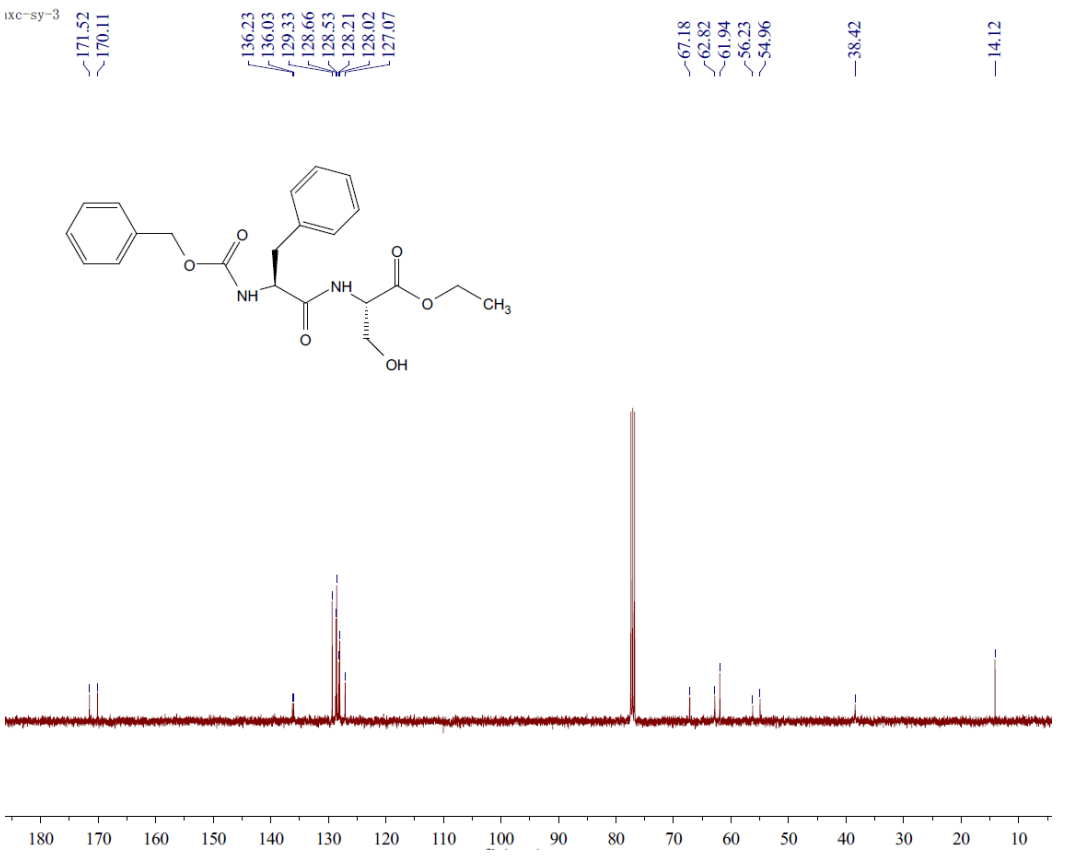
^{1}H NMR and ^{13}C NMR for 3c



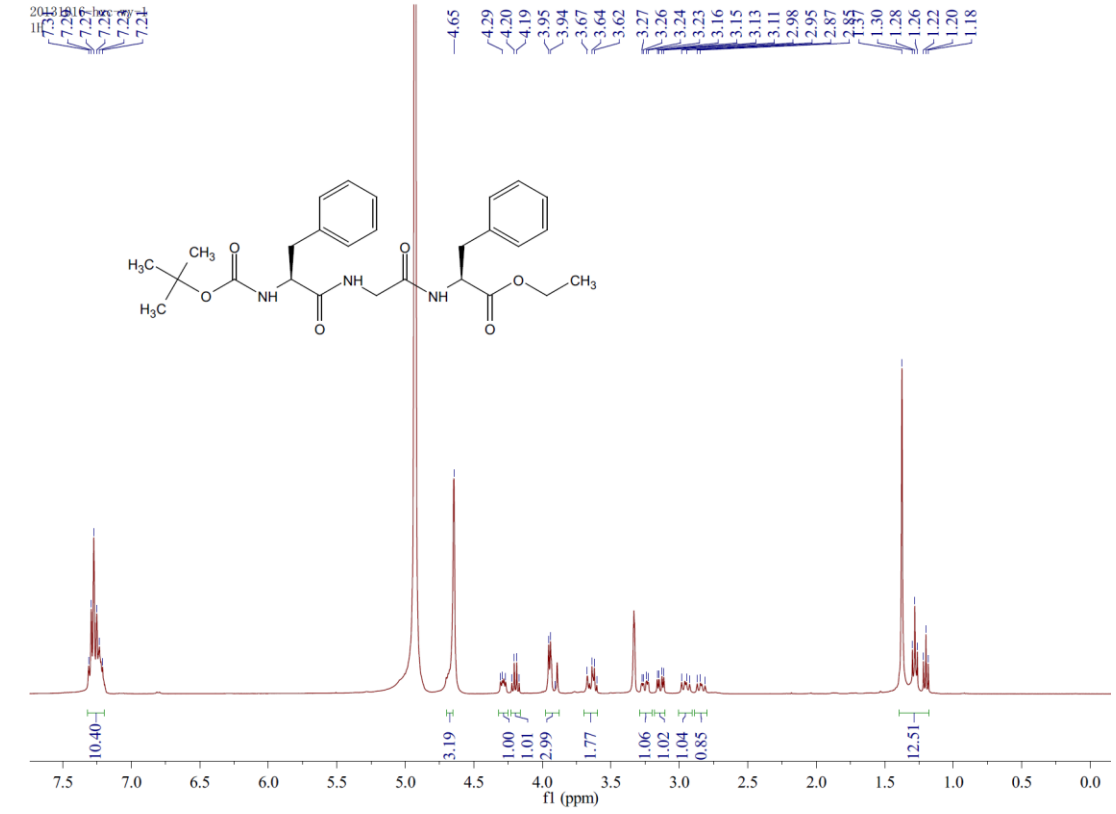


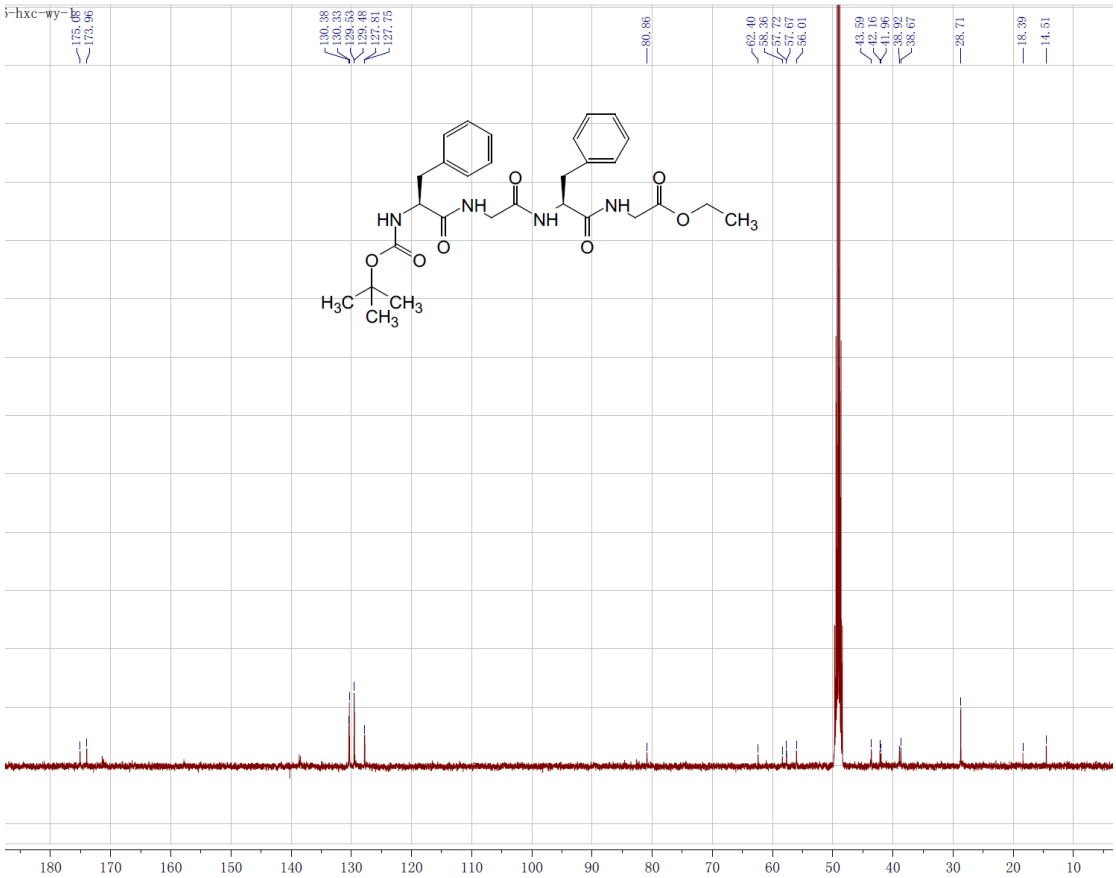
^{1}H NMR and ^{13}C NMR for 3d



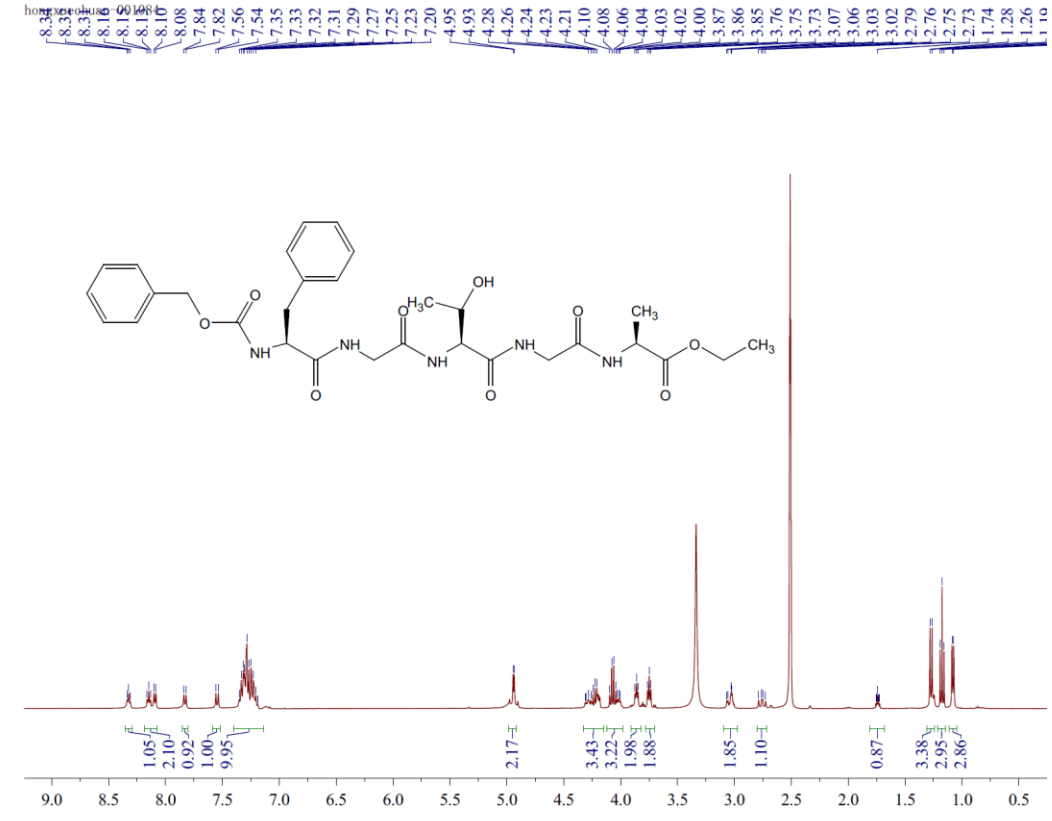


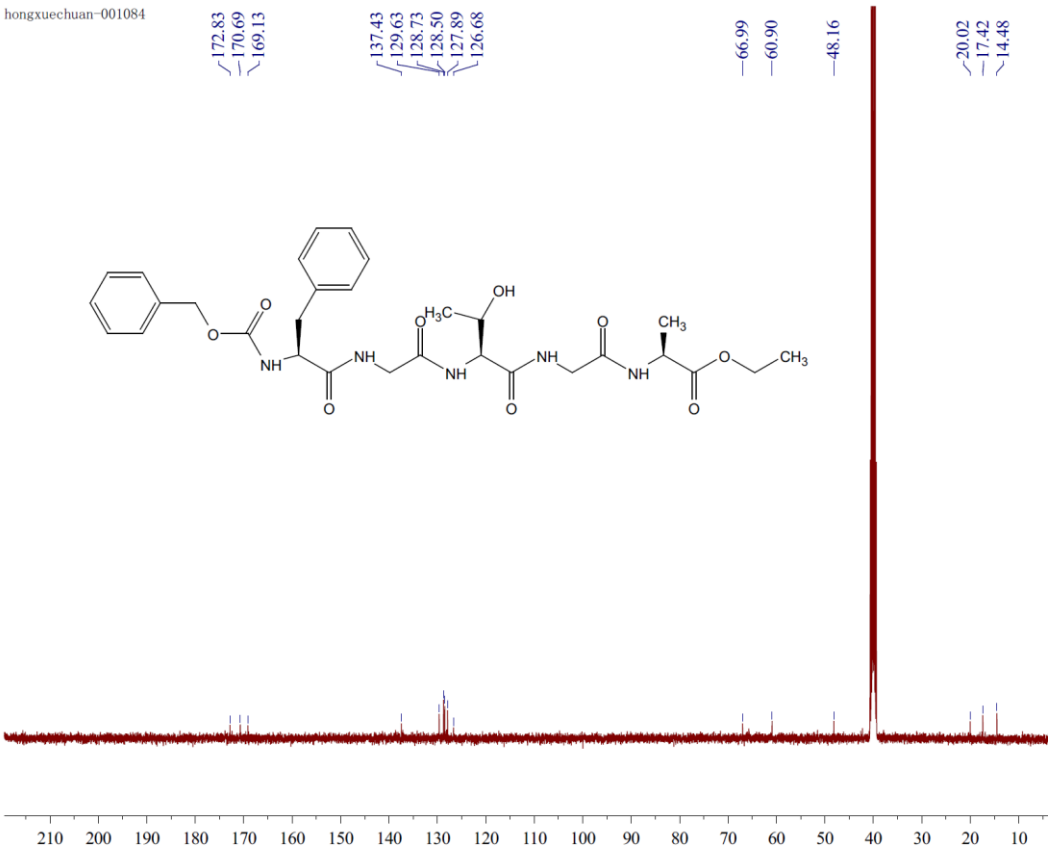
^{1}H NMR and ^{13}C NMR for 3e





^{1}H NMR and ^{13}C NMR for 3f





^{1}H NMR and ^{13}C NMR for 3g