Supporting Information

Fmoc Synthesis of Peptide Thioesters without Post-Chain Assembly Manipulation

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1. General Information

1.1 Materials.

All reagents and solvents were bought from Sinopharm Chemical Reagent Co. Ltd or Alfa Aesar and were purified when necessary. THF and Et_2O were distilled from sodium-benzophenone ketyl immediately prior to use. DMF was distilled under reduced pressure from sodium sulfate and stored over 4 Å molecular sieves. CH_2Cl_2, pyridine and Et_3N were distilled from calcium hydride immediately prior to use. All other commercially obtained reagents and solvents were used as received without further purification unless otherwise indicated. All organic extracts were dried over sodium sulfate and concentrated under rotary evaporator. TLC was carried out on plates pre-coated with silica gel 60 F254. Visualization was accomplished using UV light, iodine vapors, ninhydrin solution, permanganate solution and/or phosphomolybdic acid (PMA) solution. Flash column chromatographic purification of products was accomplished using forced-flow chromatography on Silica Gel (300-400 mesh).

1.2 NMR Spectroscopy.

^{1}H-NMR, ^{13}C-NMR spectra were recorded on a JOEL JNM-ECA300 nuclear magnetic resonance spectrometer instrument at room temperature in $CDCl_3$ unless otherwise indicated. Data for ^{1}H-NMR are reported as follows: chemical shift δ is reported in parts per million (ppm) relative to TMS (^{1}H 0.00 ppm) or chloroform (^{1}H 7.26 ppm). Proton NMR splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin), sextet (sex), septet (sep), multiplet (m), apparent (ap), and broad (br) with the coupling constant *J* reported in hertz (Hz). Data for ^{13}C-NMR are reported in terms of chemical shift (δ ppm) relative to chloroform (^{13}C 77.16 ppm).

1.3 Peptide Synthesis.

Side-chain Fmoc protected amino acids used were : Arg(Pbf), Asn(Trt), Asp(O^{t}Bu), Cys(Trt), His(Trt), Glu(O^{t}Bu), Gln(Trt), Lys(Boc), Ser(^{t}Bu), Thr(^{t}Bu), Trp(Boc), and Tyr(^{t}Bu). Fmoc amino acids, DIEA, HOBt, HBTU, Wang resin and Rink amide resin were from GL Biochem (Shanghai) Ltd, all other reagents from Bo Mai JieTechnology Co, Ltd. Peptides were synthesized by manual Fmoc-SPPS using HBTU activation procedure. After chain assembly was completed, the peptide was

deprotected and cleaved from the resin by treatment with TFA cocktails at room temperature. The peptides were precipitated with Et_2O, and purified by semi-preparative RP-HPLC. Peptide identity was confirmed by MALDI-TOF/MS.

1.4 Reversed-Phase HPLC, UV Spectrometer and Mass Spectrometry.

Analytical and semipreparative HPLC was performed using a Prominence LC-20AT with SPD-20A UV/Vis detector. A Vydac C18 column (5 μm, 4.6 mm×150 mm, 4.6 mm×250 mm) with a 1 mL/min flow rate was used for analytical scale HPLC, and a Vydac C18 column (10 μm, 10 or 25 mm×250 mm) with a 4mL/min or 10mL/min flow rate was used for semi-preparative HPLC. In all cases, linear gradients solvent A (0.1% TFA in water) and solvent B (0.1% TFA in CH_3CN) were utilized. Data were recorded and analyzed using the software system LC Solution.

UV was performed on a U-3900 UV-Vis Spectrophotometer.

ESI-MS was performed on a Bruker Daltonics Inc. APEX II Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer in the Institute of Chemistry, Chinese Academy of Sciences or a Bruker Daltonics Inc. APEX IV FT-ICR Mass Spectrometer in Peking University (Analytical Instrumentation Center). MALDI-TOF/MS was performed on Bruker Daltonics Inc. autoflex I MALDI-TOF mass spectrometer or an Applied Biosystems 4800PLUS MALDI-TOF/TOF mass spectrometer in Center of Biomedical Analysis, Tsinghua University. The matrix used for MALDI-TOF was α-cyano-4-hydroxycinnamic acid.

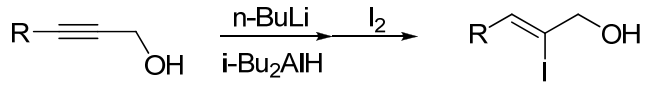
1.5 Ligation Reaction.

Aliquots of this solution were treated with equal volumes of 10% TCEP (pH 7.0) for 3-5 minutes to completely hydrolyse any thiol adduct before HPLC analysis. The ligation reaction was monitored by HPLC using a gradient starting from 20% of B and with an increase of 1% B per minute when a baseline resolution of the different products was required.

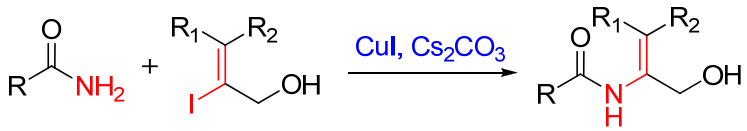
2. Experimental Section

2.1 Synthesis of the model enamide peptides and the N-methyl enamide building block

a. General Synthesis Procedure

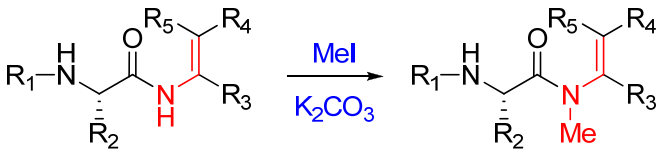


General method for the synthesis of vinyl iodide [1,2] (1): The propargylic alcohol (1 equiv) in anhydrous THF (1 mmol/ml) at -23℃ under argon was treated with n-BuLi (1.1 equiv, a 1.6 *M* cyclohexane solution) and the white slurry was stirred vigorously for 10min. A solution of i-Bu_2AlH in CH_2Cl_2 (3 equiv) was added, and the colorless solution was heated at 35℃ for 60 h. Excess hydride was decomposed with anhydrous ethyl acetate (1.5 ml, 15 mmol) at 0℃. Iodine (5 equiv) in THF was added at -78℃. The mixture had been allowed to stir over 20 min. The mixture was poured into a mixture of sat. aq $Na_2S_2O_3$, sat. aq K_2CO_3, and sat. aq Rochelle salt. The product was extracted with ether, dried over Na_2SO_4 and purification by flash chromatography provided vinyl iodide.



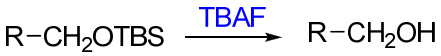
General method for the synthesis of enamide [3-5] (2): A pressure tube was charged with amide (2 equiv), copper(I) iodide (2 equiv), and Cs_2CO_3 (2 equiv). The tube was evacuated under high vacuum, backfilled with argon, and closed with a rubber septa. Vinyl iodide (1 equiv) and *N*,*N'*-dimethylethylenediamine (2 equiv) in dry and degassed dioxane or THF were next added, and the light blue suspension was sonicated for 2 min. The mixture was stirred at 50-60℃ until TLC indicated complete conversion of the vinyl iodide. The reaction mixture was cooled to rt, filtered over a plug of silica gel, washed with AcOEt or CH_2Cl_2, and concentrated. The crude residue was purified by flash chromatography over silica gel to give the desired enamide.

General Procedure of Mitsunobu reaction[6] (3): Diisopropyl azodicarboxylate (DIAD) in CH_2Cl_2 (1.5 equiv) was added dropwise to a stirred solution of alcohol (1 equiv) in dry CH_2Cl_2 containing PPh_3 (1.5 equiv) and triphenylmethyl thiol (1.5 equiv) at room temperature under argon. The mixture was heated at reflux for 5-10 h under argon. After cooling, the reaction mixture was concentrated in vacuo to afford a residue, which was purified by column chromatography to give the desired product.



General procedure for the methylation of the enamide[7,8] (4): The The methylation of the enamide(1 equiv) was achieved stereoselectively on the nitrogen atom of enamide moiety by an excess of methyl iodide(10 equiv) and potassium carbonate(10 equiv) in DMF. The reaction mixture was stirred for 48 h at room temperature. The reaction was diluted with CH_2Cl_2, washed with sat. $NaHCO_3$ and sat. brine, and dried over $MgSO_4$. The crude product was purified with column chromatography to give the N-Me product.

General procedure for the deprotection of the TBS[9](5):



To a solution of TBS-protected alcohol (1 equiv) in THF was added TBAF (1.2equiv) at 0 ºC under argon. After being stirred at the same temperature until TLC indicated total consumption of raw material, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on silica gel to give the alcohol.

Reference

(1) Corey, E. J.; Kirst, H. A.; Katzenel.Ja *Journal of the American Chemical Society* **1970**, *92*, 6314.

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(4) Toumi, M.; Couty, F.; Evano, G. *Journal of Organic Chemistry* **2008**, *73*, 1270-1281.

(5) Wang, J.; Schaeffler, L.; He, G.; Ma, D. W. *Tetrahedron Letters* **2007**, *48*, 6717-6721.

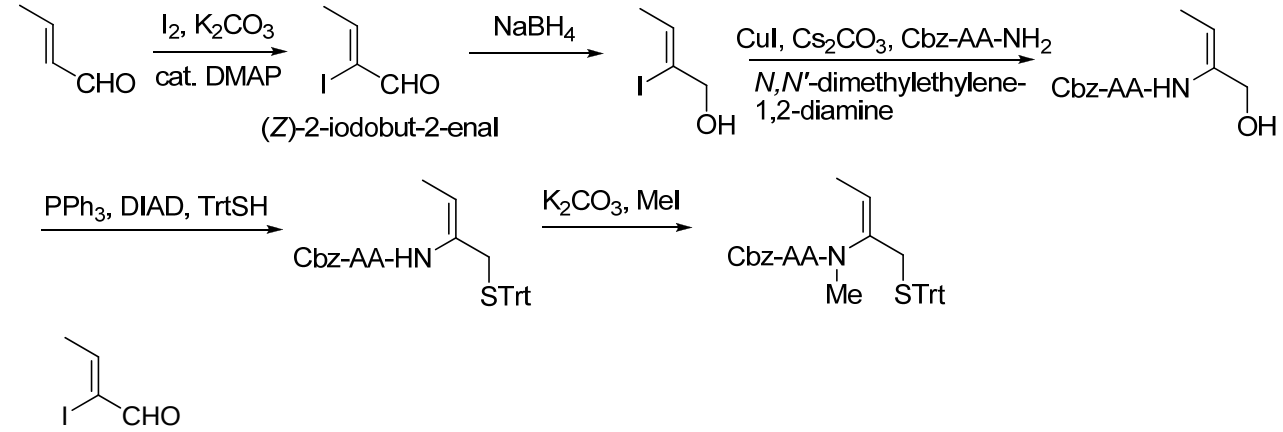
(6) Takizawa, T.; Watanabe, K.; Narita, K.; Oguchi, T.; Abe, H.; Katoh, T. *Chemical Communications* **2008**, 1677-1679.

(7) Loiseau, N.; Cavelier, F.; Noel, J. P.; Gomis, J. M. *Journal of Peptide Science* **2002**, *8*, 335-346.

(8) Jimenez, J. C.; Chavarria, B.; Lopez-Macia, A.; Royo, M.; Giralt, E.; Albericio, F. *Organic Letters* **2003**, *5*, 2115-2118.

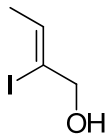
(9) Doi, T.; Numajiri, Y.; Munakata, A.; Takahashi, T. *Organic Letters* **2006**, *8*, 531-534.

b. General Synthesis Procedure Of the Model (N-alkyl) Enamide Peptides



(*Z*)-2-iodobut-2-enal

According to the literature procedure[1], to a solution of crotonaldehyde (7.0 g, 100 mmol) in a mixture of 250 mL THF and 250 mL water was added K_2CO_3 (16,6 g, 120 mmol), I_2 (38.1 g, 150 mol) and DMAP (2.4 g, 20 mmol) successively. The mixture was stirred for 3 h and then was diluted with EtOAc and washed with sat. $NaHSO_3$ and 0.1 M HCl successively. The organic layers were dried with Na_2SO_4, and the crude product obtained after evaporation was used in the next step without further purification.



(*Z*)-2-iodobut-2-en-1-ol

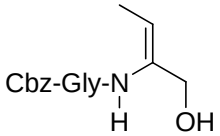
According to the literature procedure[1],a solution of crude 2-iodo-2-butenal in MeOH (300 mL) was cooled in ice and $NaBH_4$ (5.7 g, 150 mmol) was added in portions. After stirring at 0°C for 2 h, the solvent was evaporated and the residu was dissolved in EtOAc and washed with 1 M NaOH. The

product was isolated by flash column chromatography (EtOAc/PE, 3:1) gave pure butenol (11.9 g, 60 mmol, 60% over 2 steps).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.80 (d, 3H, *J*=6.5Hz), 4.25 (s, 2H), 5.99 (q, 1H, *J*=6.5Hz).

Reference

(1) Wanner, M. J.; Boots, R. N. A.; Eradus, B.; Gelder, R. d.; van Maarseveen, J. H.; Hiemstra, H. *Organic Letters* **2009**, *11*, 2579-2581.

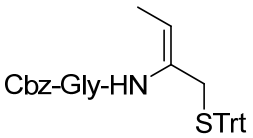


(Z)-benzyl 2-(1-hydroxybut-2-en-2-ylamino)-2-oxoethylcarbamate

The compound was prepared using the preceding procedure (2): The model compound (yield: 67%) was purified by flash chromatography (PE/EtOAc 1:2 to 1:3) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.57(d, 3H, *J*=6.8Hz), 3.95(d, 2H, *J*=5.9Hz), 4.15(s, 2H), 5.11(q, 1H, *J*=6.9Hz), 5.16(s, 2H), 5.37(br,1H), 7.2-7.36(m, 5H), 7.73(br,1H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 11.7, 44.9, 64.0, 67.3, 116.0, 128.1, 128.3, 128.6, 134.9, 136.0, 156.9, 168.6



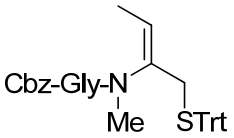
(Z)-benzyl 2-oxo-2-(1-(tritylthio)but-2-en-2-ylamino)ethylcarbamate

The compound was prepared using the preceding procedure (3): The model compound (yield: 65%) was purified by flash chromatography (PE/EtOAc/CH_2Cl_2 6:1:2 to 4:1:2) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.39(d, 3H, *J*=6.9Hz), 3.14(s, 2H), 3.86(d, 2H, *J*=5.5Hz), 5.05(q, 1H, *J*=6.9Hz), 5.13(s, 2H), 5.35(br,1H), 6.77(br,1H), 7.24-7.37(m, 20H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 12.8, 36.9, 44.6, 66.9, ,67.1, 120.0, 126.6, 127.8, 128.0, 128.4, 129.5, 129.7, 136.1, 144.5, 156.7, 167.3

Mass spectrum: calcd for $[C_{33}H_{32}N_2O_3S+Na]^+$: 559.2, found 559.1



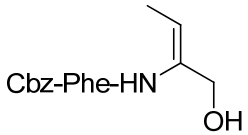
(Z)-benzyl 2-(methyl(1-(tritylthio)but-2-en-2-yl)amino)-2-oxoethylcarbamate

The compound was prepared using the preceding procedure (4): The model compound (yield: 57%) was purified by flash chromatography (PE/EtOAc 5:1 to 4:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.52(d, 3H, J=6.9Hz), 2.88(s, 3H), 2.89-3.09(m, 2H), 3.69(m, 2H), 5.09(s, 2H), 5.53(m, 2H), 7.26-7.36(m, 20H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 13.1, 34.8, 36.6, 42.6, 66.8, 67.8, 127.0, 127.7, 128.2, 128.6, 129.7, 135.9, 136.7, 144.4, 156.2, 168.2

Mass spectrum: calcd for $[C_{34}H_{34}N_2O_3S+H]^+$: 551.2, found 551.4, $[C_{34}H_{34}N_2O_3S+Na]^+$: 573.2, found 573.3

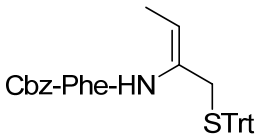


(S,Z)-benzyl 1-(1-hydroxybut-2-en-2-ylamino)-1-oxo-3-phenylpropan-2-ylcarbamate

The compound was prepared using the preceding procedure (2): The model compound (yield:59%) was purified by flash chromatography (PE/EtOAc 4:1 to 2:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.32(d, 3H, J=6.9Hz), 3.08(dd, 1H, J_1=7.6Hz, J_2=13.1Hz), 3.17(dd, 1H, J_1=6.5Hz, J_2=13.4Hz), 4.10(s, 2H), 4.49(q, 1H, J=7.1Hz), 5.05(q, 1H, J=7.1Hz), 5.10(s, 2H), 5.30(br, 1H), 7.22-7.33(m, 10H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 11.4, 38.4, 56.8, 64.0, 67.3, 115.1, 127.3, 128.1, 128.4, 128.6, 128.9, 129.3, 135.3, 136.0, 136.2, 156.3, 170.0



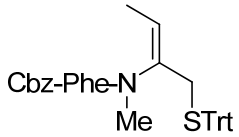
(S,Z)-benzyl 1-oxo-3-phenyl-1-(1-(tritylthio)but-2-en-2-ylamino)propan-2-ylcarbamate

The compound was prepared using the preceding procedure (3): The model compound (yield: 85%) was purified by flash chromatography (PE/EtOAc10:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.23(d, 3H, J=6.5Hz), 3.07(d, 2H, J=6.9Hz), 3.08(s, 2H), 4.48(q, 1H, J=7.2Hz), 4.90(q, 1H, J=6.9Hz), 5.08(s, 2H), 5.28 (br, 1H), 6.66 (br, 1H), 7.22-7.35(m, 25H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 12.8, 36.9, 38.4, 56.4, 67.0, 67.2, 120.3, 126.7, 127.1, 127.9, 128.1, 128.2, 128.6, 128.8, 129.4, 129.7, 136.1, 136.4, 144.7, 156.0, 169.0.

Mass spectrum: calcd for $[C_{40}H_{38}N_2O_3S+Na]^+$: 649.3, found649.2, $[C_{40}H_{38}N_2O_3S+K]^+$: 665.3, found665.2



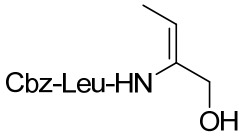
(S,Z)-benzyl 1-(methyl(1-(tritylthio)but-2-en-2-yl)amino)-1-oxo-3-phenylpropan-2-ylcarbamate

The compound was prepared using the preceding procedure (4): The model compound (yield: 69%) was purified by flash chromatography (PE/EtOAc 8:1 to 6:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.12-1.29(m, 3H), 2.29(0.55H), 2.60-3.23(4.45H), 4.12(0.18H), 4.51-5.52(4.82H), 7.02-7.35(25H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 12.6, 12.9, 34.2, 35.8, 36.9, 38.8, 39.9, 40.6, 52.4, 53.2, 66.6, 67.6, 126.9, 127.0, 127.3, 128.0, 128.5, 129.6, 136.0, 136.7, 137.3, 144.5, 155.2, 171.2, 171.8.

Mass spectrum: calcd for $[C_{41}H_{40}N_2O_3S+Na]^+$: 663.3, found663.3, $[C_{41}H_{40}N_2O_3S+K]^+$: 679.3, found679.2

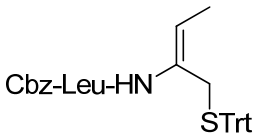


(S,Z)-benzyl 1-(1-hydroxybut-2-en-2-ylamino)-4-methyl-1-oxopentan-2-ylcarbamate

The compound was prepared using the preceding procedure (2): The model compound (yield: 60%) was purified by flash chromatography (PE/EtOAc 1:3) .

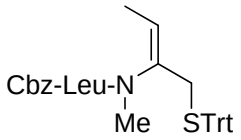
^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.95(d, 6H, J=6.2Hz), 1.57-1.71 (m, 6H), 4.13 (s, 2H), 4.32 (t, 1H, *J*=7.42Hz), 5.12 (s, 2H), 5.43(br, 1H), 7.30 (m, 5H), 8.00 (br, 1H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 11.6, 22.0, 23.0, 24.9, 40.6, 54.0, 64.2, 67.5, 114.6, 128.2, 128.4, 128.7, 135.7, 136.0, 156.7, 171.2



(S,E)-benzyl 4-methyl-1-oxo-1-(1-(tritylthio)but-2-en-2-ylamino)pentan-2-ylcarbamate

The compound was prepared using the preceding procedure (3): The model compound (yield: 65%) was purified by flash chromatography (PE/EtOAc/CH_2Cl_2 6:1:2 to 4:1:2) .



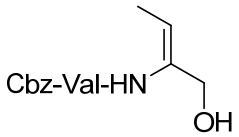
(S,Z)-benzyl 4-methyl-1-(methyl(1-(tritylthio)but-2-en-2-yl)amino)-1-oxopentan-2-ylcarbamate

The compound was prepared using the preceding procedure (4): The model compound (yield: 76%) was purified by flash chromatography (PE/EtOAc 4:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.73-1.59 (m, 12H), 2.72-3.22 (m, 5H), 4.33 (dt, 0.45H, J_1=9.61Hz, J_2=2.73Hz), 4.33 (dt, 0.55H, J_1=10.3Hz, J_2=2.73Hz),4.87-5.11(m, 2H), 5.23 (t, 1H, *J*=8.07Hz), 5.47 (q, 0.55H, *J*=6.98Hz), 5.23 (q, 0.45H, *J*=6.87Hz), 7.15-7.40 (m, 20H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 12.6, 13.6, 21.4, 23.6, 24.4, 34.9, 36.7, 37.3, 38.6, 43.3, 49.3, 50.4, 66.6, 67.3, 67.5, 126.8, 127.0, 128.1, 128.2, 128.5, 128.6, 129.6, 129.7, 135.9, 136.7, 137.5, 144.5, 155.7, 172.7, 173.1.

Mass spectrum: calcd for $[C_{38}H_{42}N_2O_3S+K]^+$: 645.3, found 645.5.

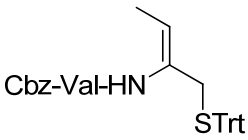


(S,Z)-benzyl 1-(1-hydroxybut-2-en-2-ylamino)-3-methyl-1-oxobutan-2-ylcarbamate

The compound was prepared using the preceding procedure (2): The model compound (yield: 54%) was purified by flash chromatography (PE/EtOAc/CH2Cl_2 1:1:1 to 1:1:2) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.97(d, 3H, *J*=6.8Hz), 1.01(d, 3H, *J*=6.8Hz), 1.57(d, 3H, *J*=6.9Hz), 2.22(m, 1H), 4.02(dd, 1H, J_1=8.2Hz, J_2=6.5Hz), 4.13(s, 2H), 5.13-5.18(m, 3H), 5.28(br,1H), 7.26-7.35(m, 5H), 7.47(m, 1H).

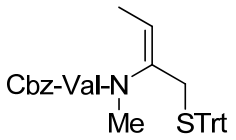
^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 11.8, 18.0, 19.5, 30.6, 61.1, 64.3, 67.5, 115.0, 128.2, 128.5, 128.7, 135.6, 136.0, 156.7, 170.5



(S,Z)-benzyl 3-methyl-1-oxo-1-(1-(tritylthio)but-2-en-2-ylamino)butan-2-ylcarbamate

The compound was prepared using the preceding procedure (3): The model compound (yield:78%) was purified by flash chromatography with CH_2Cl_2.

^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.93(d, 3H, *J*=6.9Hz), 1.02(d, 3H, *J*=6.9Hz), 1.40(d, 3H, *J*=6.9Hz), 2.18(m, 1H), 3.05(d, 1H, *J*=13.0Hz), 3.15(d, 1H, *J*=13.0Hz), 4.01(q, 1H, *J*=7.2Hz), 5.00(q, 1H, *J*=7.6Hz), 5.10(s, 2H), 5.31(br, 1H), 7.26-7.37(m, 20).



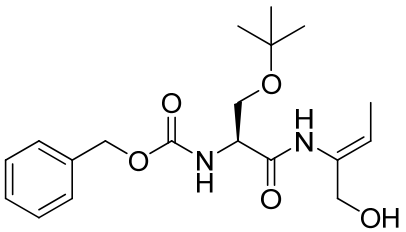
(S,Z)-benzyl 3-methyl-1-(methyl(1-(tritylthio)but-2-en-2-yl)amino)-1-oxobutan-2-ylcarbamate

The compound was prepared using the preceding procedure (4): The model compound (yield: 82%) was purified by flash chromatography (PE/EtOAc/CH_2Cl_2 8:1:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ0.66-1.05 (m, 6H), 1.37-1.52 (m, 3H), 1.75 (m, 1H, *J*=6.51Hz), 2.67-3.17 (m, 5H), 4.15 (dd, 0.43H, J_1=9.95Hz, J_2=6.54Hz), 4.33 (dt, 0.57H, J_1=9.96Hz, J_2=6.54Hz),4.74-5.06(m, 2H), 5.23 (t, 1H, *J*=8.94Hz), 5.47 (q, 0.57H, *J*=6.87Hz), 5.23 (q, 0.43H, *J*=6.87Hz), 7.15-7.40 (m, 20H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 12.6, 13.8, 17.2, 19.9, 32.1, 34.8, 37.1, 37.3, 39.3, 55.9, 56.8, 66.7, 67.4, 126.9, 128.1, 128.6, 129.6, 136.0, 136.7, 137.5, 144.5, 155.9, 171.8, 172.2

Mass spectrum: calcd for $[C_{37}H_{40}N_2O_3S+Na]^+$: 615.3, found 615.4, $[C_{37}H_{40}N_2O_3S+K]^+$: 631.3, found 631.4

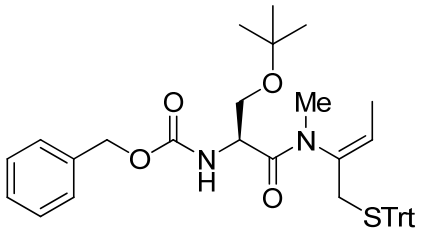


(S,Z)-benzyl 3-tert-butoxy-1-(1-hydroxybut-2-en-2-ylamino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (2): The model compound (yield: 55%) was purified by flash chromatography (PE/EtOAc 3:2) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.17(s, 9H), 1.60(d, 3H, *J*=7.2Hz), 3.45(t, 1H, *J*=8.4Hz), 4.16(s, 2H), 4.27(d, 2H, *J*=6.5Hz), 5.11(m, 3H), 5.75(br,1H), 7.26-7.37(m, 5H) , 8.20(br,1H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 11.7, 27.3, 55.1, 61.7, 63.9, 67.2, 74.6, 114.0, 128.1, 128.2, 128.5, 135.6, 136.0, 156.0, 169.1



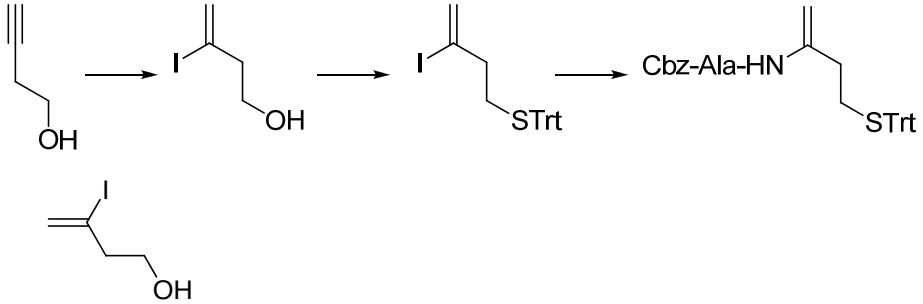
(*S*,*Z*)-benzyl 3-*tert*-butoxy-1-(methyl(1-(tritylthio)but-2-en-2-yl)amino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (4): The model compound (yield: 57%) was purified by flash chromatography (PE/EtOAc 8:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ0.99-1.15 (m, 9H), 1.38 (d, 1.7H, *J*=6.18Hz), 1.56 (d, 1.3H, *J*=6.18Hz), 2.89-3.43 (m, 7H), 4.36 (dd, 0.45H, *J*=6.87Hz), 4.33 (dt, 0.55H, *J*=6.87Hz), 4.81-5.52(m, 4H), 7.15-7.38 (m, 20H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 12.4, 13.6, 27.3, 34.2, 36.4, 38.6, 50.9, 52.0, 62.7, 63.2, 66.7, 67.6, 73.3, 126.8, 128.0, 128.6, 129.6, 136.5, 136.7, 137.1, 144.5, 155.3, 170.2, 170.9

Mass spectrum: calcd for $[C_{39}H_{44}N_2O_4S+H]^+$: 637.4, found 637.6, $[C_{39}H_{44}N_2O_4S+Na]^+$: 659.4, found659.6



3-iodobut-3-en-1-ol

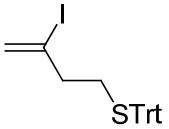
According to the Takayuki Shioiri group[1], NaI (6.0 g, 40 mmol) was dissolved in CH_3CN (30 mL) at rt and then to the mixture was added TMSCl (5.08 mL, 40 mmol) followed by H_2O (360 mL, 20 mmol). After 10 min, a solution of 3-butyn-1-ol (1.4 g, 20 mmol) in CH_3CN (5.0 mL) was added and the resulting mixture was allowed to react for 1 h at room temperature. The reaction was quenched with H_2O (60 mL) and the mixture was extracted with ether. Drying over Na_2SO4, filtration, and evaporating ether gave crude the iodo alcohol (3.2 g, 81%) as a reddish brown oil.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.63(t, 2H, J=5.8Hz), 3.74(t, 2H, J=5.8Hz), 5.84(s, 1H), 6.18(s,1H);

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 48.0, 60.9, 107.5, 128.4

Reference

(1) Sugiyama H., Yokokawa, F. and Shioiri T. *Organic Letters* **2000**, *2*, 2149-2152.

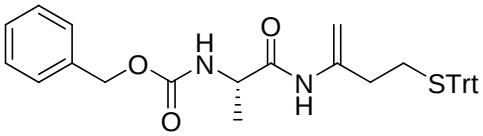


(3-iodobut-3-en-1-yl)(trityl)sulfane

The compound was prepared using the preceding procedure (3): The model compound (yield: 75%) was purified by flash chromatography (PE/EtOAc 200:1 to 150:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.30(t, 2H,J=6.2), 2.34(t, 2H, J=6.2), 5.65(s, 1H), 5.91(s, 1H), 7.26-7.45(m, 15H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 31.4, 44.4, 66.8, 109.4, 126.7, 127.9, 129.6, 133.7, 144.7



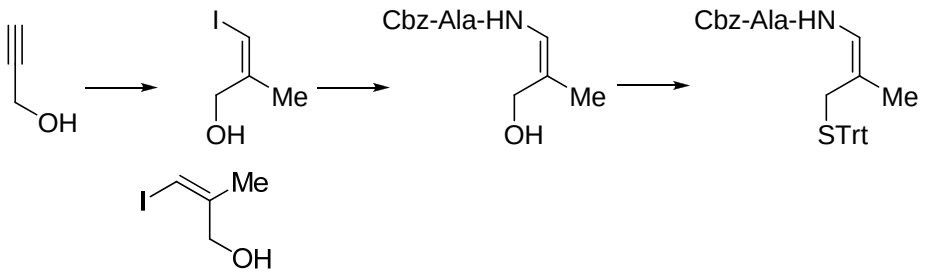
(*S*)-benzyl 1-oxo-1-(4-(tritylthio)but-1-en-2-ylamino)propan-2-ylcarbamate

The compound was prepared using the similar preceding procedure (2): The model compound (yield: 16%) was purified by flash chromatography (PE/EtOAc 2:1 to 1:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.36(d, 3H, J=7.2Hz), 1.98(m, 2H), 2.25(d, 2H, J=3.0Hz), 4.23(m, 1H), 4.31(m, 1H), 4.66(q, 1H), 5.10(s, 2H), 7.26-7.42(m, 20H), 7.75(br, 1H), 7.80(br, 1H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 17.8, 25.2, 31.7, 50.5, 66.8, 67.4, 110.3, 126.8, 128.0, 128.2, 128.4, 128.7, 129.7, 136.0, 144.9, 146.9, 156.5, 169.6

Mass spectrum: calcd for $[C_{34}H_{34}N_2O_3S+Na]^+$: 573.2, found573.2, $[C_{34}H_{34}N_2O_3S+K]^+$: 589.2, found 589.2

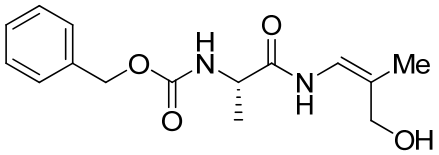


(*Z*)-3-iodo-2-methylprop-2-en-1-ol

According to the literature procedure[1], to a suspension of propargyl alcohol (1.0 g, 17.8 mmol) and CuI (3.4 g, 17.8 mmol) in Et_2O (50 ml) was added MeMgBr (3.0 M in Et_2O, 12.4 ml, 37.4 mmol) at

–5 °C. The mixture was gradually allowed to warm to rt and stirred for 2 h. After addition of ICl (2.89 g, 17.8 mmol) at –5 °C, the mixture was gradually allowed to warm to rt and stirring was continued for additional 18 h. The reaction was quenched with saturated NH_4Cl at 0 °C. The reaction mixture was filtered through Celite pad and the filtrate was extracted with Et_2O. The extract was washed with brine, dried over Na_2SO_4 and chromatographed (PE/AcOEt 5:1 to 4:1) to give (*Z*)-3-iodo-2-methylprop-2-en-ol (1.60 g, 45%) as a pale yellow oil.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.95(s, 3H), 4.21(s, 2H), 5.96(s, 1H)

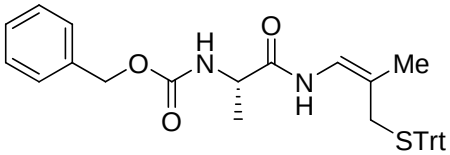


(*S*,*Z*)-benzyl 1-(3-hydroxy-2-methylprop-1-enylamino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (2): The model compound (yield: 67%) was purified by flash chromatography (PE/EtOAc 4:1 to 3:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.39(d, 3H, J=6.9Hz), 1.60(s, 3H), 4.12(q, 1H, J=7.2Hz), 4.21(s, 2H), 5.10(s, 2H), 6.61(d, 1H, J=10.3Hz), 7.26-7.35(m, 5H)

Mass spectrum: calcd for $[C_{15}H_{20}N_2O_4+Na]^+$:315.2, found 315.2



(*S*,*Z*)-benzyl 1-(2-methyl-3-(tritylthio)prop-1-enylamino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (3): The model compound (yield: 75%) was purified by flash chromatography (PE/EtOAc 4:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.38(d, 3H, J=7.2Hz), 1.54(s, 3H), 2.81(s, 2H), 4.13(m, 1H), 5.12(s, 2H), 6.51(d, 1H, J=10.6Hz), 7.27-7.41(m, 20H)

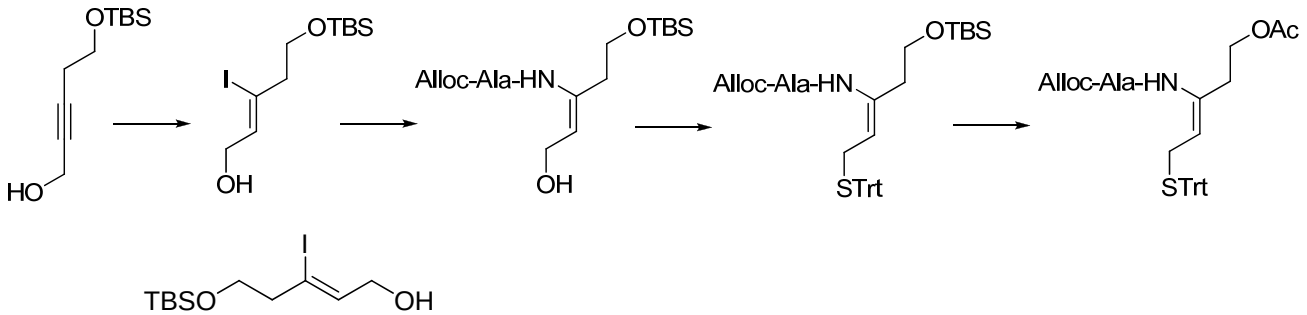
^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 14.5, 22.0, 39.3, 55.8, 67.1, 67.5, 114.6, 120.0, 126.8, 128.0, 128.3, 128.5, 128.8, 129.8, 136.0, 144.9, 156.5, 169.0

Mass spectrum: calcd for $[C_{34}H_{34}N_2O_3S+Na]^+$:573.2, found573.2, $[C_{34}H_{34}N_2O_3S+K]^+$: 589.2, found589.2

Reference

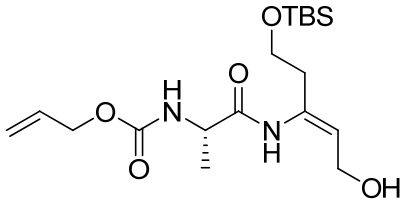
(1) Onyango, E. O.; Tsurumoto, J.; Imai, N.; Takahashi, K.; Ishihara, J.; Hatakeyama, S. *Angewandte*

Chemie-International Edition **2007**, *46*, 6703-6705.



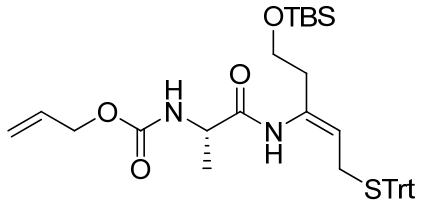
(*Z*)-5-(*tert*-butyldimethylsilyloxy)-3-iodopent-2-en-1-ol

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.06(s, 6H), 0.88(s, 9H), 2.71(t, 2H, J=6.3Hz), 3,74(t, 2H, J=6.2Hz), 4.18(t, 2H, J=5.5Hz), 5.91(t, 1H, J=5.7Hz).



(*S*,*Z*)-allyl 1-(5-(*tert*-butyldimethylsilyloxy)-1-hydroxypent-2-en-3-ylamino)-1-oxopropan-2-ylcarbamate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.06(s, 6H), 0.90(s, 9H), 1.42(d, 3H, J=6.9Hz), 2.38(t, 2H, J=5.7Hz), 3.77(t, 2H, J=5.8Hz), 3.94(d, 2H, J=7.5Hz), 4.11(q, 1H, J=7.2Hz), 4.57(d, 2H, J=5.1Hz), 5.24(m, 2H), 5.41(t, 1H, J=7.2Hz), 5.90(m, 1H)

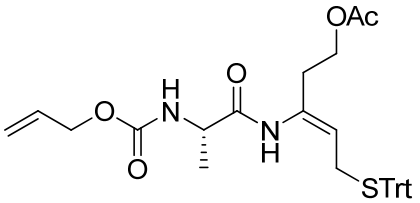


(*S*,*Z*)-allyl 1-(5-(*tert*-butyldimethylsilyloxy)-1-(tritylthio)pent-2-en-3-ylamino)-1-oxopropan-2-ylcarbamate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.06(s, 6H), 0.89(s, 9H), 1.35(d, 3H, J=6.8Hz), 2.20(t, 2H, J=5.9Hz), 2.74(d, 2H, J=8.6Hz), 3.68(d, 2H, J=5.8Hz), 4.12(q, 1H, J=7.04Hz), 4.55(d, 2H, J=5.5Hz), 5.22(m, 2H), 5.89(m, 1H), 6.29(t, 1H, J=8.6Hz), 7.25-7.42(m, 15H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 18.4, 21.8, 26.0, 29.7, 32.2, 60.5, 63.2, 65.9, 67.0, 110.0, 117.9, 126.7, 128.0, 129.7, 132.7, 136.7, 144.9, 162.0, 170.3

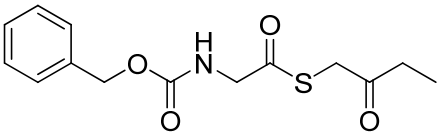
Mass spectrum: calcd for $[C_{37}H_{48}N_2O_4SSi+Na]^+$: 667.3, found 667.5



(*S*,*Z*)-3-(2-(allyloxycarbonylamino)propanamido)-5-(tritylthio)pent-3-enyl acetate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.38(d, 3H, J=7.2Hz), 2.00(s, 3H), 2.42(t, 2H, 6.3Hz), 2.75(d, 2H, J=8.6Hz), 4.06(t, 2H, J=6.4Hz), 4.08(m, 2H), 4.57(d, 2H, J=5.5Hz), 5.25(m, 2H), 5.92(m, 1H), 7.26-7.42(m, 15H)

Mass spectrum: calcd for $[C_{33}H_{36}N_2O_5S+Na]^+$: 595.2, found 595.3

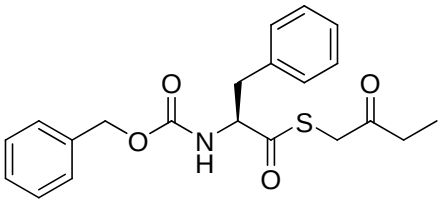


S-2-oxobutyl 2-(benzyloxycarbonylamino)ethanethioate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.09(t, 3H, J=7.4Hz), 2.58(q, 2H, J=6.5Hz), 3.78(s, 2H), 4.16(d, 2H, J=6.2Hz), 5.16(s, 2H), 7.26-7.37(m, 5H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 7.6, 34.9, 38.0, 50.3, 67.1, 127.8, 128.0, 128.4, 136.0, 156.4, 197.4, 204.4

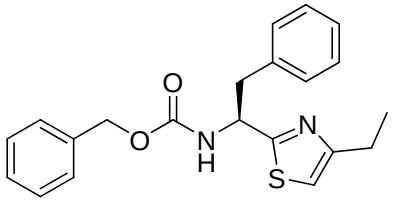
Mass spectrum: calcd for $[C_{14}H_{17}NO_4S+Na]^+$: 317.1, found 317.2.



(*S*)-*S*-2-oxobutyl 2-(benzyloxycarbonylamino)-3-phenylpropanethioate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.09(t, 3H, J=7.2Hz), 2.54(q, 2H, J=7.4 Hz), 3.13(m, 2H), 3.73(m, 2H), 4.75(q, 1H), 5.09(s, 2H), 7.26-7.33(m, 10H)

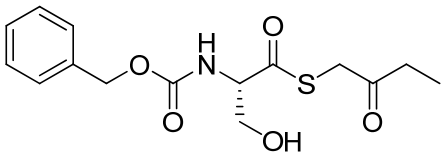
Mass spectrum: calcd for $[C_{21}H_{23}NO_4S+Na]^+$: 408.1, found 408.1, $[C_{21}H_{23}NO_4S+K]^+$: 424.1, found 424.1



(*S*)-benzyl 1-(4-ethylthiazol-2-yl)-2-phenylethylcarbamate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.28(t, 3H, J=7.6Hz), 2.78(q, 2H, J=7.6Hz), 3.28(d, 2H, J=6.2Hz), 5.09(s, 2H), 5.32(m, 1H), 6.75(s, 1H), 7.21-7.33(m, 10H)

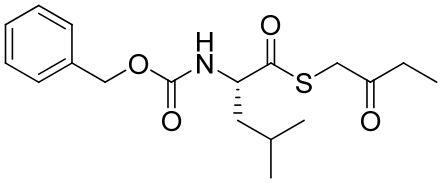
Mass spectrum: calcd for $[C_{21}H_{22}N_2O_2S+Na]^+$: 389.1, found 389.1, $[C_{21}H_{22}N_2O_2S+K]^+$: 405.1, found 405.1



(*S*)-*S*-2-oxobutyl 2-(benzyloxycarbonylamino)-3-hydroxypropanethioate

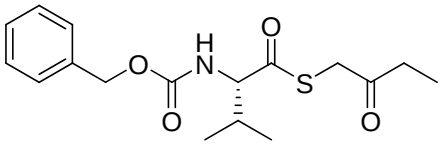
^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.25(t, 3H, J=7.2Hz), 2.58(q, 2H, J=7.6Hz), 4.11(m, 4H), 4.30(t, 1H, J=6.7Hz), 5.17(s, 2H), 7.25-7.37(m, 5H)

Mass spectrum: calcd for $[C_{15}H_{19}NO_5S+Na]^+$: 348.1, found 348.4



(*S*)-*S*-2-oxobutyl 2-(benzyloxycarbonylamino)-4-methylpentanethioate

Mass spectrum: calcd for $[C_{18}H_{25}NO_4S+Na]^+$: 374.3, found 374.2.

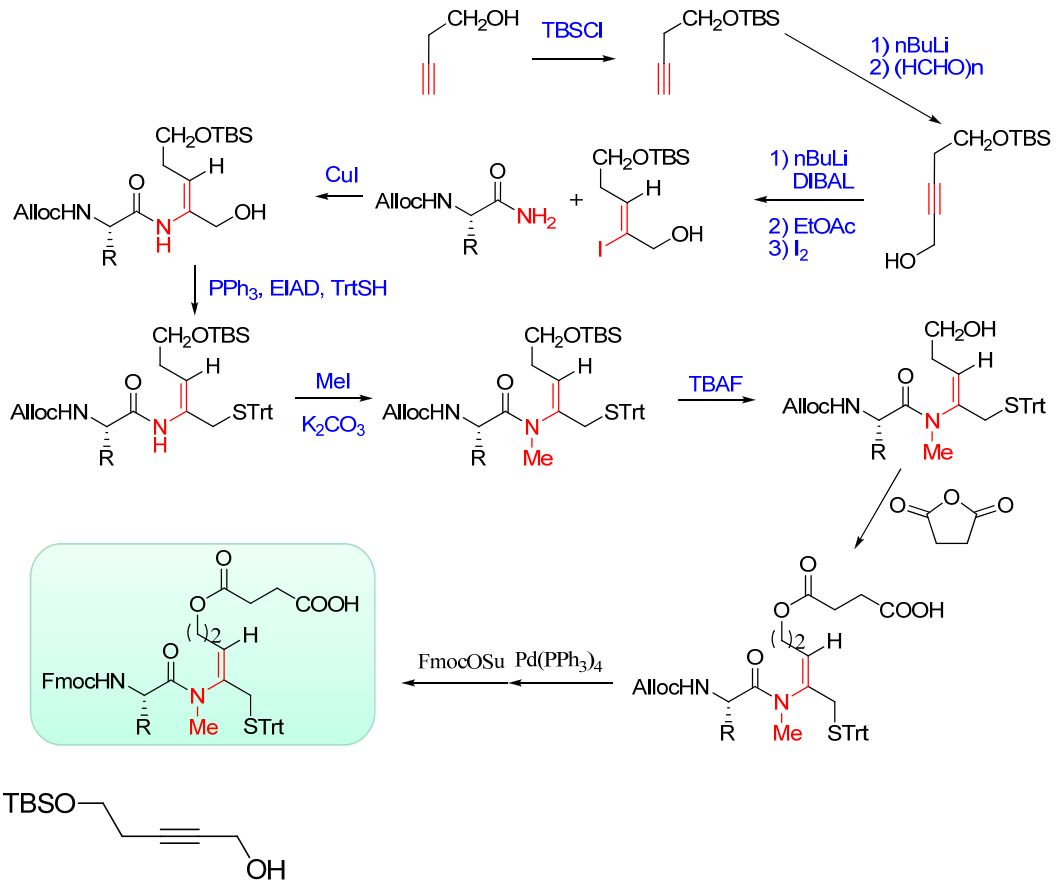


(*S*)-*S*-2-oxobutyl 2-(benzyloxycarbonylamino)-3-methylbutanethioate

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.9(d, 3H, J=3.5Hz), 1.01 (d, 3H, J=3.5Hz), 1.09(t, 3H, J=3.6Hz), 2.37(m, 1H), 2.57(q, 2H, J=3.6Hz), 3.74(m, 2H), 4.39(d, 1H, J=4.5Hz), 5.15(s, 2H), 7.26-7.39(m, 5H)

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 7.7, 16.8, 19.2, 31.0, 34.9, 38.4, 65.8, 67.2, 127.8, 128.0, 128.8, 136.1, 156.2, 199.9, 204.2

Mass spectrum: calcd for $[C_{17}H_{23}NO_4S +Na]^+$: 374.3, found 374.2.



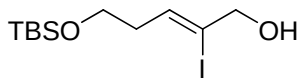
5-((*tert*-butyldimethylsilyl)oxy)pent-2-yn-1-ol

According to the literature[1,2], TBDMSCl (7.24 g, 48 mmol) was added to a solution of but-3-yn-1-ol (2.8 g, 40 mmol), DMAP (49mg, 0.4 mmol) and imidazole (6.0 g, 88 mmol) in CH_2Cl_2 (50mL). The mixture was stirred 12 h at room temperature, and then H_2O was added. The mixture was extracted with CH_2Cl_2, washed with 1M HCl and brine, dried over Na_2SO_4 and concentrated *in vacuo* to afford the product (>99%) as an oil which was used without further purification.

To a solution of the above alkyne in THF (100 mL) at –78 ºC was added 1.6 M *n*-BuLi in cyclohexane (36 mL, 57.6 mmol). The mixture was stirred for 0.5 h at -78 ºC, and was added $(CH_2O)_n$ (4.32 g,144 mmol). The reaction mixture was stirred for 5 h at rt, and the reaction was quenched with sat. aq. NH_4Cl (100 mL) and the aqueous layer was extracted with Et_2O, washed with brine, and dried over $MgSO_4$. The crude product was purified with column chromatography (10% EtOAc-PE) to give the alcohol as a colorless oil (5.66 g,26.4 mmol, 55% two steps yield).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.01(s, 6H), 0.84(s, 9H), 2.37(tt, 2H, J_1=7.1Hz, J_2=7.1Hz), 2.98(br, 1H), 3.66(t, 2H, J=7.2Hz), 4.15(s, 2H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): -5.3, 18.3, 23.1, 25.9, 50.9, 61.9, 79.8, 82.8.

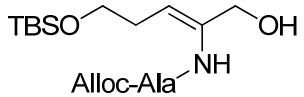


(*Z*)-5-(*tert*-butyldimethylsilyloxy)-2-iodopent-2-en-1-ol

The synthesis of vinyl iodide was achieved by the similar procedure of (1): propargylic alcohol (5.57g, 26mmol). After purification by flash column chromatography (PE/EtOAc 10:1) the product (3.65g, 41%) was obtained as an colorless oil.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.05(s, 6H), 0.89(s, 9H), 1.92(t, 1H, *J*=6.7Hz), 2.40(q, 2H, *J*=6.5Hz), 3.68(t, 2H, *J*=6.5Hz), 4.25(d, 2H, *J*=6.5Hz), 6.00(t, 1H, *J*=6.5Hz).

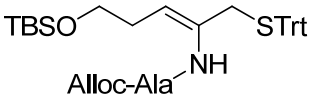
^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): -5.1, 18.4, 26.0, 39.3, 61.5, 71.6, 109.8, 132.



(*Z*)-allyl 1-(5-(*tert*-butyldimethylsilyloxy)-1-hydroxypent-2-en-2-ylamino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (2): The compound (yield: 64%) was purified by flash chromatography (PE/EtOAc 3:2) .

Mass spectrum: calcd for $[C_{18}H_{34}N_2O_5Si+Na]^+$: 409.2, found 409.3; $[C_{18}H_{34}N_2O_5Si+K]^+$: 425.2, found 425.2



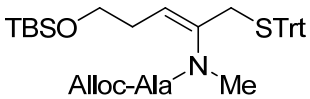
(*Z*)-allyl 1-(5-(*tert*-butyldimethylsilyloxy)-1-(tritylthio)pent-2-en-2-ylamino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (3): The model compound (yield: 90%) was purified by flash chromatography (PE/EtOAc 10:1) .

^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.06(s, 6H), 0.89(s, 9H), 1.35(d, 3H, *J*=6.8Hz), 2.20(t, 2H, *J*=5.9Hz), 2.74(d, 2H, *J*=8.6Hz), 3.68(d, 2H, *J*=5.8Hz), 4.12(q, 1H, *J*=7.04Hz), 4.55(d, 2H, *J*=5.5Hz), 5.22(m, 2H), 5.89(m, 1H), 6.29(t, 1H, *J*=8.6Hz), 7.25-7.42(m, 15H).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 18.4, 21.8, 26.0, 29.7, 32.2, 60.5, 63.2, 65.9, 67.0, 110.0, 117.9, 126.7, 128.0, 129.7, 132.7, 136.7, 144.9, 162.0, 170.3.

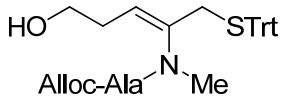
Mass spectrum: calcd for $[C_{37}H_{48}N_2O_4SSi+Na]^+$: 667.3, found 667.4; $[C_{37}H_{48}N_2O_4SSi+K]^+$: 683.3, found 683.2



(*Z*)-allyl 1-((5-(*tert*-butyldimethylsilyloxy)-1-(tritylthio)pent-2-en-2-yl)(methyl)amino)-1-oxopropan-2-ylcarbamate

The compound was prepared using the preceding procedure (4): The model compound (yield: 86%) used for the next step without purification.

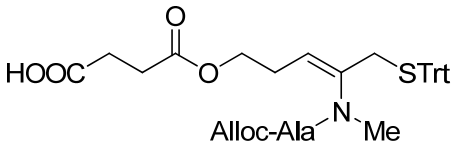
Mass spectrum: calcd for $[C_{38}H_{50}N_2O_4SSi+Na]^+$: 681.3, found 681.5; $[C_{38}H_{50}N_2O_4SSi+K]^+$: 697.3, found 697.5



(*Z*)-allyl 1-((5-hydroxy-1-(tritylthio)pent-2-en-2-yl)(methyl)amino)-1-oxopropan-2-ylcarbamate

TBAF (1.0 M solution in THF, 2.5 ml, 2.5 mmol) was added to a solution of N-Me enamide (1.28g, 2 mmol) in THF (15 ml) at 0℃. After being stirred at room temperature for 1h, the reaction mixture was diluted with EtOAc and quenched with sat. NH_4Cl (aq.) and water. The aqueous layer was separated and extracted with EtOAc. The combined organic layers were dried over $MgSO_4$ and concentrated. The crude product was was purified by flash chromatography (PE/EtOAc 1:1) . (1.02 g, 1.88 mmol, 94%).

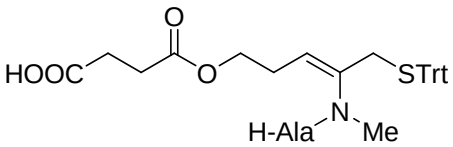
Mass spectrum: calcd for $[C_{32}H_{36}N_2O_4S+H]^+$: 545.3, found 545.3; $[C_{32}H_{36}N_2O_4S+Na]^+$: 567.3, found 567.4.



(*Z*)-7,9-dimethyl-5,8,15-trioxo-10-(tritylthiomethyl)-4,14-dioxa-6,9-diazaoctadeca-1,10-dien-18-oic acid

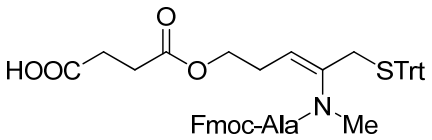
According to the literature[3], to a solution of alcohol (0.82 g, 1.5 mmol), succinic anhydride (0.45 g, 4.5 mmol, 3 eq.) and DMAP (8 mg) in CH_2Cl_2 (30 mL) was added triethylamine (0.63 g, 4.5 mmol, 3 eq.) at r.t. After 4h the solvent was removed under vacuum, the residue was taken up in 10% HCl solution (10 mL) and was extracted with CH_2Cl_2 (2 × 25 mL). The organic layer was washed with water (10 mL), then with brine (10 mL), dried over Na_2SO_4, and evaporated to dryness which was purified by column chromatography over silica gel(EtOAc-PE 1:1 to 2:1) to furnish acid (0.82 g, 85% yield).

Mass spectrum: calcd for $[C_{36}H_{40}N_2O_7S-H]^-$: 643.3, found 643.5.



(*Z*)-4-(4-(2-amino-*N*-methylpropanamido)-5-(tritylthio)pent-3-enyloxy)-4-oxobutanoic acid

According to the literature[4], Alloc-protected compound (773 mg, 1.2 mmol) was dissolved in THF (20 mL). Pd $(PPh_3)_4$ (139 mg, 0.12 mmol) and N-methylaniline (1.3 mL, 12 mmol) were added, and then the reaction mixture was stirred at room temperature. Once the reaction was done, the solution was concentrated under vacuum. The crude product was purified by flash column chromatography (EtOAc/PE 1:1 then MeOH/CH_2Cl_2 1:10) to give pure compound (619 mg,1.1 mmol, 92%).



(*Z*)-4-(4-(2-(4-((9*H*-fluoren-9-yl)methoxy)-3,4-dioxobutan-2-yl)-1-methylhydrazinyl)-5-(tritylthio)pent-3-enyloxy)-4-oxobutanoic acid

According to the literature[5], Fmoc-OSu (404 mg, 1.2 mmol) was added to the amine (561 mg, 1 mmol) dissolved in anhydrous, amine-free dimethylformamide (DMF) (3 mL). Upon addition of NEt_3 (166 μL, 1.2 mmol). The solution was stirred at room temperature for 12 h.Then, CH_2Cl_2 and 1M HCl are added, the organic layer is separated, washed with brine and dried over Na_2SO_4. Evaporation of the solvent and purification by column chromatography, using CH_2Cl_2:MeOH as eluent, affords the pure products as solid(680 mg,0.87 mmol, 87%).

^{13}C-NMR (300MHz, $CDCl_3$, δ ppm): 19.5, 20.2, 26.9, 27.5, 30.1, 31.2, 35.4, 36.8, 37.1, 38.2, 46.8, 47.2, 47.8, 62.7, 66.9, 67.7, 120.0, 125.0, 126.9, 127.1, 128.0, 128.2, 129.6, 137.5, 138.6, 141.3, 144.3, 155.2, 155.4, 172.9, 174.1, .

Mass spectrum: calcd for $[C_{47}H_{46}N_2O_7S+H]^+$: 783.3, found783.6; $[C_{47}H_{46}N_2O_7S+Na]^+$: 805.3, found 805.7; $[C_{47}H_{46}N_2O_7S+K]^+$: 821.3, found 821.4

Reference

(1) Jensen, T.; Pedersen, H.; Bang-Andersen, B.; Madsen, R.; Jorgensen, M. Angewandte Chemie-International Edition 2008, 47, 888-890.

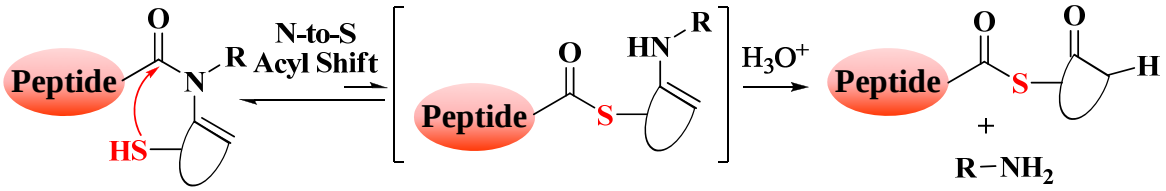
(2) Ohtsuki, K.; Matsuo, K.; Yoshikawa, T.; Moriya, C.; Tomita-Yokotani, K.; Shishido, K.; Shindo, M. *Organic Letters* **2008**, *10*, 1247-1250.

(3) Singh, C.; Chaudhary, S.; Kanchan, R.; Puri, S. K. *Organic Letters* **2007**, *9*, 4327-4329.

(4) Yang, Y. Y.; Ficht, S.; Brik, A.; Wong, C. H. *Journal of the American Chemical Society* **2007**, *129*, 7690-7701.

(5) Yang, Y. Y.; Ficht, S.; Brik, A.; Wong, C. H. *Journal of the American Chemical Society* **2007**, *129*, 7690-7701.

2.2 The structural optimization of peptide enamide motiety for the peptide thioesters



Scheme S1. The general synthetic strategy of peptide α-thioesters through N-to-S acyl shift.

Recently, peptide and protein thioester synthsis through N-to-S acyl shift, that is mechanistically more similar to the intein-medated protein splicing, is an emerging area. Aimoto et al. utilized the autoactivating cysteinyl prolyl ester (CPE) unit for peptide ligation. In this interesting strategy, the amino group released via N-to-S acyl shift is captured by diketopiperazine formation.

Herein, we report an efficient Fmoc-SPPS approach for the peptide α-thioesters through N-to-S acyl transfer mediated with hydrolysis of N-alkyl enamine derivatives (Scheme S1). Our investigation began with examining the model enamide peptide **1** which was designed by the concept described above. Compound **1** was treated with a mixture of TFA/TIPS/H_2O (95/2.5/2.5, v/v/v) at rt to remove the thiol protecting group as well as to initiate the N-to-S acyl transfer reaction. Unfortunately, the reaction gave the target product **1a** and the by-product **1b** in the yield **17%** and **83%** respectively within 20mins (Figure S1).

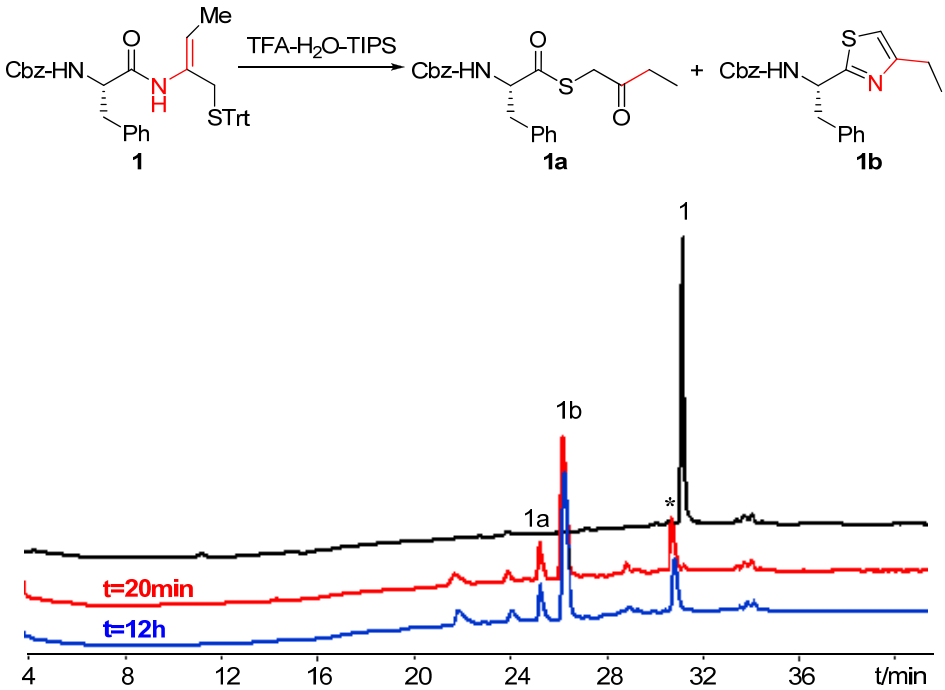
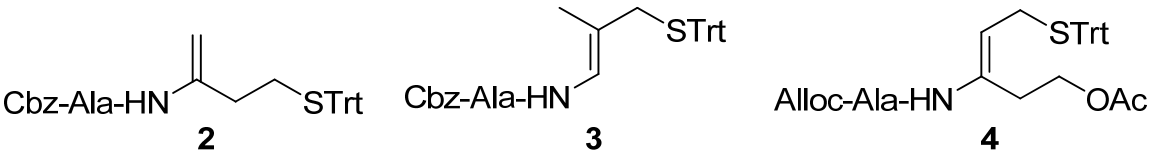


Figure S1. HPLC data for the N-to-S acyl transfer reaction of **1** at 0 min, 20 min and 12 h.

* is related with Trt-deprotection.

We reasoned that the favored thiazole derivatives formation is driven by the aromaticity and such aromatic structure should be suppressed to obtain target peptide thioesters. There are two tactics to optimize the enamide structure moiety: N-to-S acyl shift via a cyclic six-membered intermediate or N-alkyl enamide derivatives.



It was found that all enamide peptides (**2-4**) which would employ an N-to-S acyl transfer via a six-member ring intermediate could not provide any target peptide thioesters by ESI-MS test. Subsequently we focused on the N-alkyl enamide peptides. When the N-alkyl enamide peptides **5** was tested, we obtained a very high peptide thioesters in 86% yield at rt for 12h. Then compound **5** was subjected to 20% piperidine in DMF to determine the stability of the N-alkyl enamide moiety under standard Fmoc-deprotection condition. It exhibited little decomposition after 24h (Figure S2).

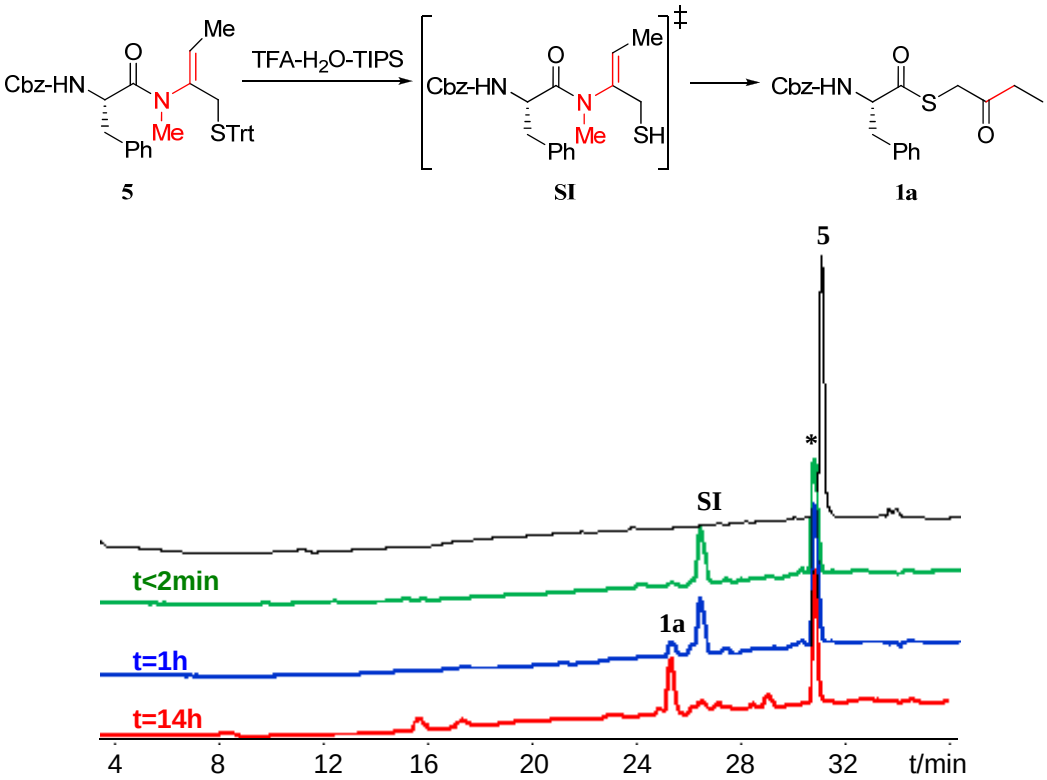


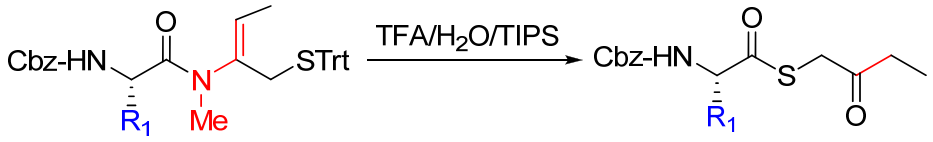
Figure S2. HPLC data for the N-to-S acyl transfer reaction of **5** at 0 min, 20 min and 12 h.

* is related with Trt-deprotection.

In order to research the scope of the method for peptide thioester, a series of N-methyl enamide peptides with Gly, Phe, Ser, Leu and Val in the C-terminal position are synthesized and examined the

efficiency for peptide thioesters (Table 1). The rearrangement reaction was performed efficiently with C-terminal unhindered amino acids (entries 1,2). Similarly, it works very well for trifunctional amino acids (entries 3). For the sterically demanding amino acids, the desired peptide thioesters was obtained in 55-70% yield (entries 4,5). Whereas the conversion by N-alkyl cysteine-assisted thioesterification was reported to take 2-3 days to generate peptide thioesters having C-terminal Leu in only 7% yield[1].

Table 1. The efficiency for peptide thioesters of N-methyl enamide peptides by N-to-S acyl shift



Entry	1	2	3	4	5
R_1	H	Ph	OH		
	Gly	**Phe**	**Ser**	**Leu**	**Val**
Yield /%	93%	86%(62%[b])	79%	70%	55%

[a] All the values were measured by using HPLC. [b] Isolated yield.

The above results show that the N-alkyl enamide moiety is compatible with standard Fmoc chemistry and could be used for the synthesis of peptide thioesters.

Moreover, our test of compound **5** with the conditions for Fmoc deprotection shows that **5** remains intact after treatment with 20% piperidine in DMF for 48 hours. It's indicated that the N-alkyl enamide moiety is stable under standard Fmoc-deprotection condition.

Reference

(1) H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.* **2007**, 48, 25-28.

2.3 (Glyco-)peptide preparation by the Fmoc-based solid phase peptide synthesis

In Boc-SPPS protocol, the extremely stro ng acidic conditions such as HF or TFMSA, are generally incompatible with posttranslational modification proteins, such as phospho- and glycopeptides. The novel Fmoc-based SPPS strategy, which allows the generation of peptide thioesters by N-to-S acyl

shift of N-alkyl enamide peptide fragments, should be a popular method for the chemical synthesis of modification proteins with high biological value.

a. General procedures for the Fmoc solid phase peptide synthesis

All peptides were prepared by SPPS on a 0.1 mmol scale and were manually synthesized. The amino acid residues were attached to the proline residues with a double coupling procedure. The Fmoc-deprotection time of second amino acid residues should be shorter (2×3min) to suppress diketopiperazine formation when Wang resin used.

(a) Standard pre-activation of resin Protocol: The resin before use was swollen in CH_2Cl_2/DMF mixture solvent for 1-2 h.

(b) Standard Fmoc-Deprotection Protocol: After treatment with 20% piperidine/DMF (2×5min) the resin was washed (5×DMF, 5×CH_2Cl_2, 5×DMF).

(c) Standard Coupling Protocol: After pre-activation of 4 equiv of Fmoc-protected amino acid in DMF for 5 min using 3.6-3.8 equiv of HBTU, 4 equiv of HOBt and 8 equiv of DIEA, the solution was added to the resin. After 60-90 min, the resin was washed with DMF (5×), CH_2Cl_2 (5×), and DMF (5×). The coupling reaction was monitored with the ninhydrin test.

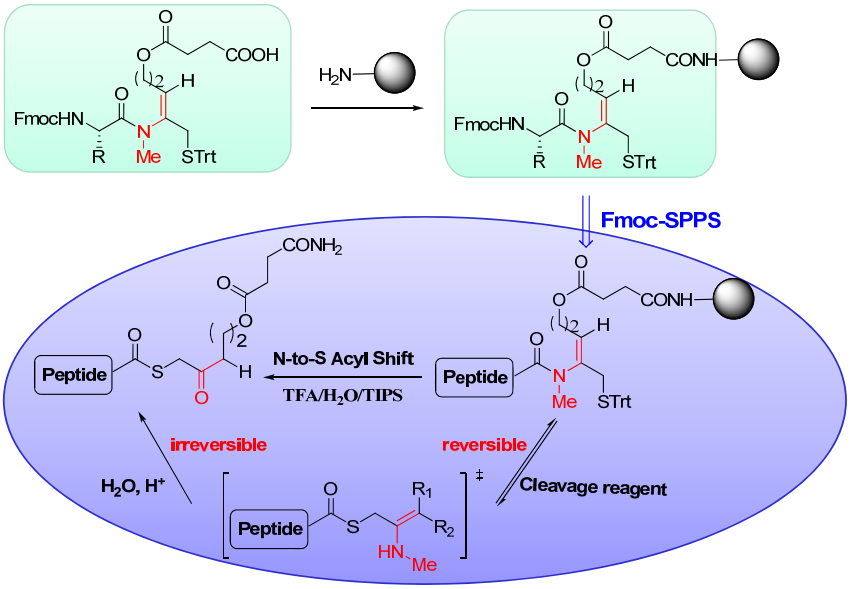
(d) Standard Capping Protocol: Ac_2O/DIEA/DMF (1:1:8) was added to the resin. After 5 min the resin was washed with DMF (5×), CH_2Cl_2 (5×), and DMF (5×).

(e) Standard Cleavage Protocol: A mixture of TFA/TIPS/H_2O (95:2.5:2.5) was added. Cleavage was performed for 1-2 h. The cleavage cocktail was collected and the resin was washed with the TFA cleavage cocktail (3×).

(f) Workup: The above TFA solution was concentrated by N_2 pumping and then the crude peptides were precipitated by the addition of a chilled diethyl ether to give white precipitates. The resulting peptide suspensions were centrifuged for 5 min at 5000 rpm and the above clear solution was decanted. The precipitation, centrifugation and decantation operations were repeated twice. The resulting white residues were dissolved in CH_3CN/H_2O, purified by semi-preparative HPLC, and analyzed by MALDI-TOF/MS (The matrix used： α-cyano-4-hydroxycinnamic acid).

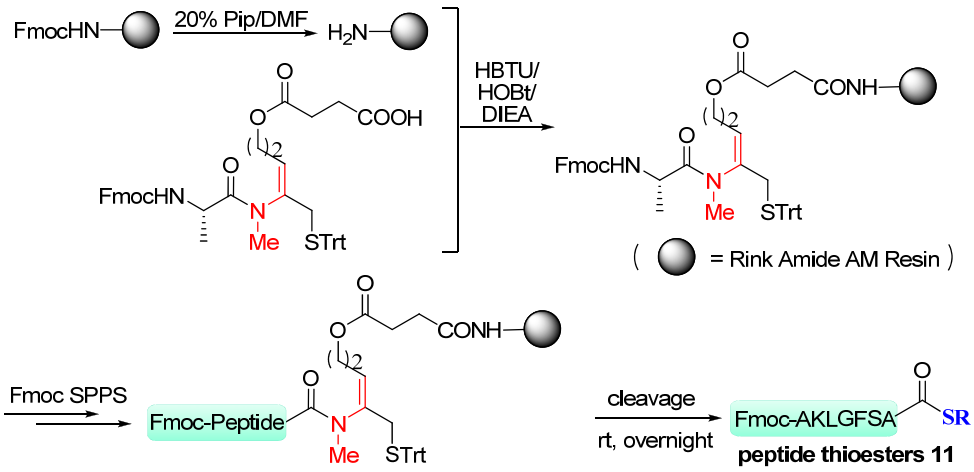
b. Synthesis of peptide thioesters segments by N-to-S acyl shift Mediated with Hydrolysis of N-alkyl Enamine Derivatives

The Rink amide-AM Resin was swollen in CH_2Cl_2/DMF. After 1 h the resin was washed (3×DMF, 3×CH_2Cl_2, 3×DMF), followed by removal of the Fmoc group by treating it with 20% piperidine/DMF (2×5 min) and another washing step. The amino acid N-methyl enamide building blocking was coupled onto Rink amide-AM Resin with HBTU, HOBt and DIEA. Following an on-resin removal of the Fmoc group, the peptide-chain assembly was then performed using standard Fmoc-SPPS protocols, and the peptide was cleaved from the resin with TFA tocktails to obtain the target peptide thioesters (Scheme S2). In all cases, the desired peptide thioesters was determined by MALDI-TOF/MS.



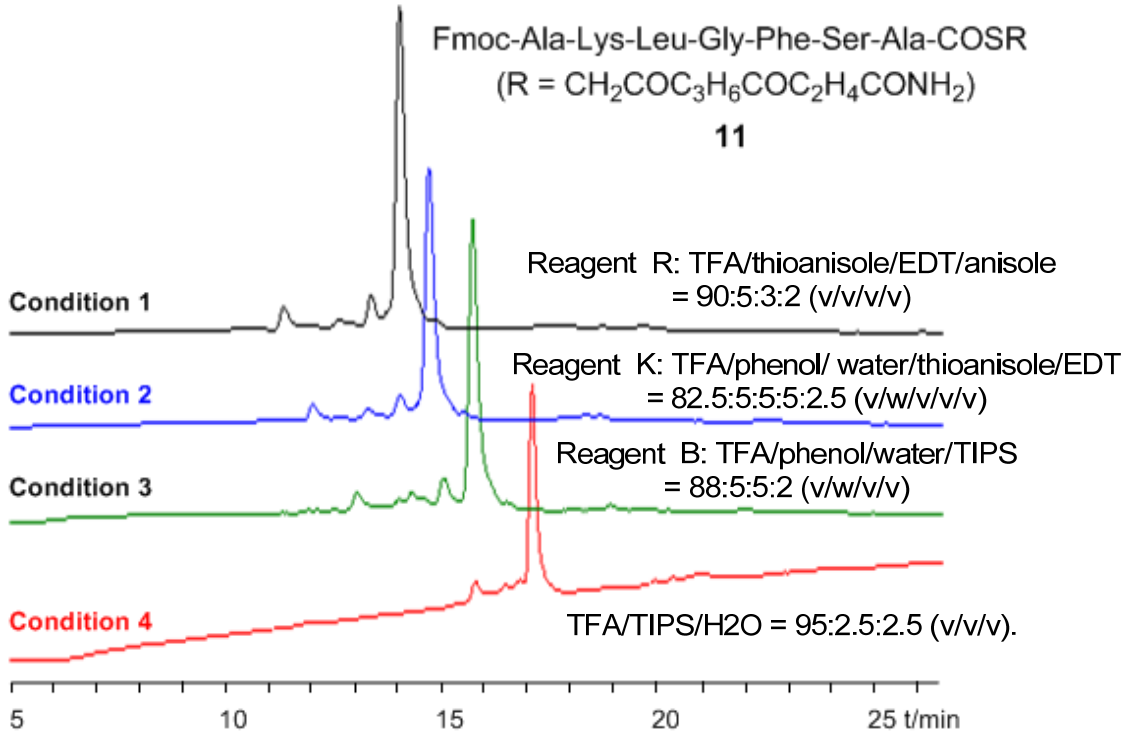
Scheme S2. The Strategy for Fmoc-Based Synthesis of Peptide Thioesters via N-to-S Acyl Shift

Synthesis of model peptide thioester 11: Fmoc-Ala-Lys-Leu-Gly-Phe-Ser-Ala-SR



Scheme S3. The General Route for Fmoc-Based Synthesis of peptide thioester 11.

The N-methyl enamide building blocking **10** (86 mg, 0.11 mmol, 1.1 equiv relative to resin substitution) was added to a solution of HBTU (41 mg, 0. 11 mmol, 1.1 equiv relative to resin loading) and HOBt (15 mg, 0. 11 mmol, 1.1 equiv relative to resin loading) in DMF, followed by DIEA (diisopropylethylamine) (52μL, 0.30 mmol, 3.0 equiv relative to resin) to pre-activate the acid. After 3-5 min, the solution was added to Rink amide-AM resin which the Fmoc-group was deprotected by the operation described above and stirred at room temperature for 3 h. Remaining free amines were acetylated by standard capping protocol twice. Subsequent steps were completed with standard HBTU/HOBt/DIEA coupling and Fmoc-deprotection protocols for Fmoc solid-phase peptide chemistry. The N-methyl enamide peptide resin was divided into four equal portions. Each was treated with four kinds of TFA cocktails at room temperature overnight to obtain the crude peptide thioesters(cleaving cocktails including Reagent R: TFA-thioanosole-EDT-anisole=90:5:3:2, v/v/v/v; Reagent K: TFA-phenol-H_2O- thioanosole-EDT=82.5:5:5:5:2.5, v/w/v/v/v; Reagent B : TFA-phenol-H_2O-TIPS=88:5:5:2, v/w/v/v or TFA-TIPS-H_2O=95:2.5:2.5, v/v/v). The major peak was determinated by MALDI-TOF/MS: m/z=1130.6 ($[M+H]^+$ calculated 1130.5).



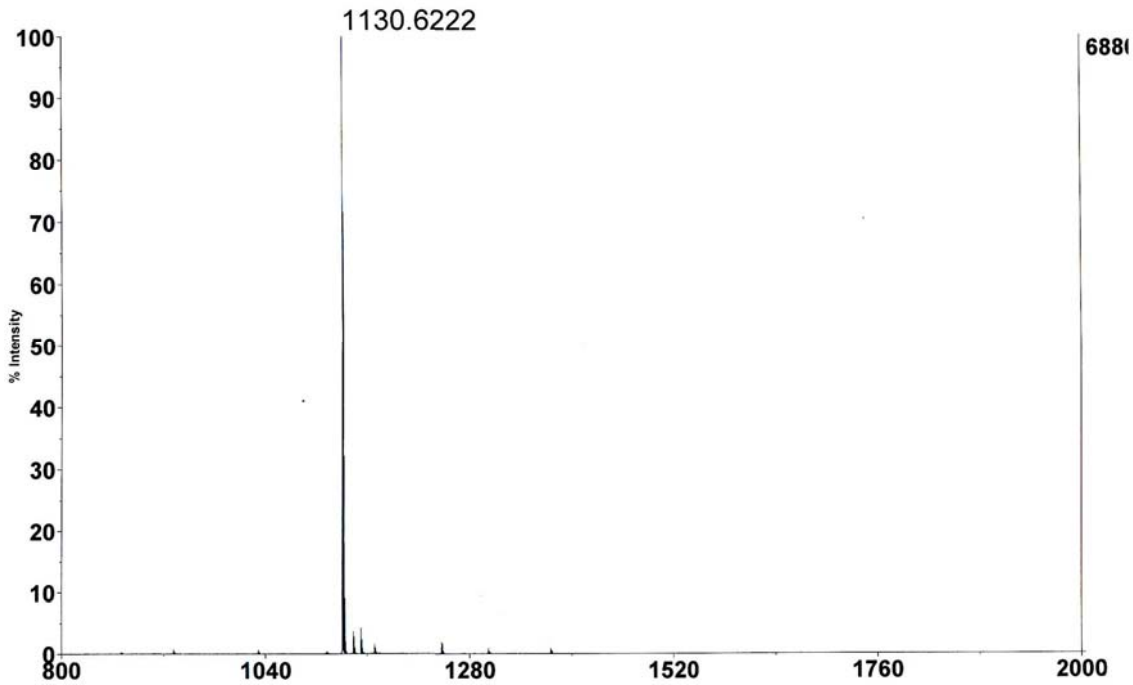


Figure S3. HPLC analysis of crude peptide thioesters **11** by different cleavage reagents and the MALDI-TOF/MS of major peak. Curve 1-4 represents recpectively the HPLC chromatogram of **11** treated with reagent R, reagent K, reagent B and TFA-TIPS-H_2O. Gradient: 20-80% buffer B in 30 min.

As shown in Figure S3, the desired crude peptide thioesters Fmoc-AKLGFASA-SR (**11**, R= -$CH_2COC_3H_6OCOC_2H_4CONH_2$) was obtained in 85-90% purity by HPLC analysis with various cleavage reagents and the isolated yields under the four cleavage conditions are 81%(0.016mmol, 18mg), 76%(0.015mmol, 17mg), 79%(0.016mmol, 18mg), and 84%(0.017mmol, 19mg), respectively. The compatibility of N-alkyl enamide moiety with different cleavage conditions is very useful because it is usually necessary to optimize the acidolytic cleavage cocktails in order to maximize the desired peptide formation, especially for the peptide with Met, Trp et al. amino acids which are easy to alkylation.

Synthesis of model peptide thioesters 12 to determinate the epimerization at the chiral center

The diastereomer Fmoc-AKLGFS-[D]A-SR peptide **12** was synthesized in the same way as peptide **11**(0.05mmol Rink amide AM resin). The convertion proceeded without significant side reactions using reagent R and D-Ala peptide obtained in 75% yield(0.031mmol, 35mg). In comparision to the two diastereomers by HPLC, epimerization at the C-terminal chiral center was less than 1% (Figure S4). These data show that the new method is applicable to the preparation of peptide thioesters

carrying C-terminal chiral amino acids in little epimerization ratio.

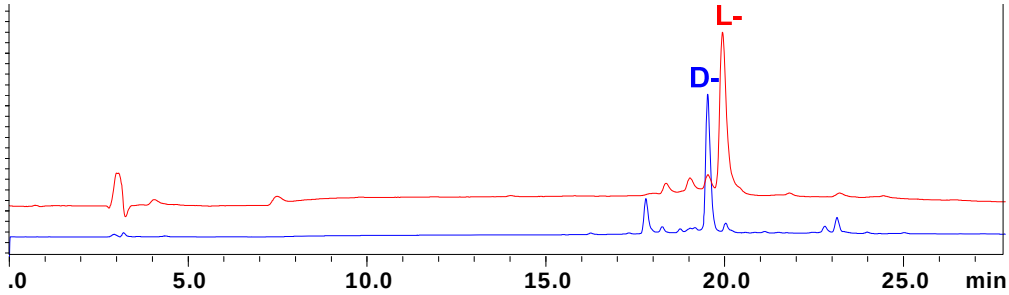
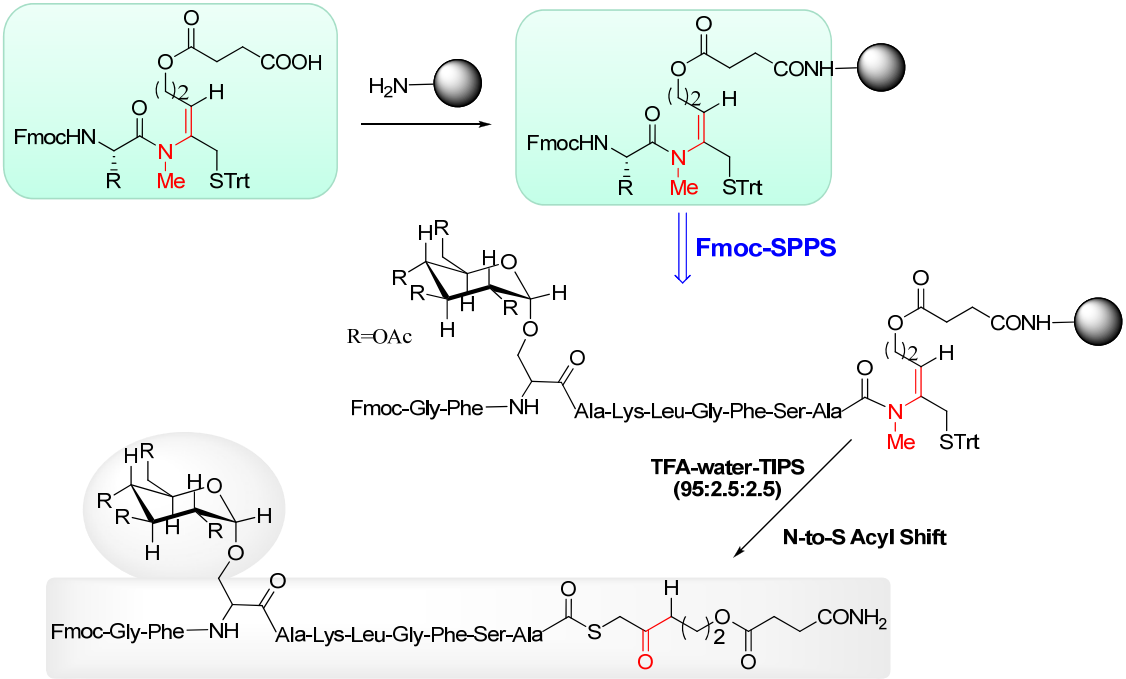


Figure S4. HPLC analysis for the epimerization of the C-terminal amino acid. Gradient: 20-80% buffer B in 30 min. Peak L-, peptide thioester **11**, t_R=19.90 min; peak D-, peptide thioester **12**, t_R=19.52 min.

Synthesis of Model (Glyco-)peptide Thioesters 13 and 14

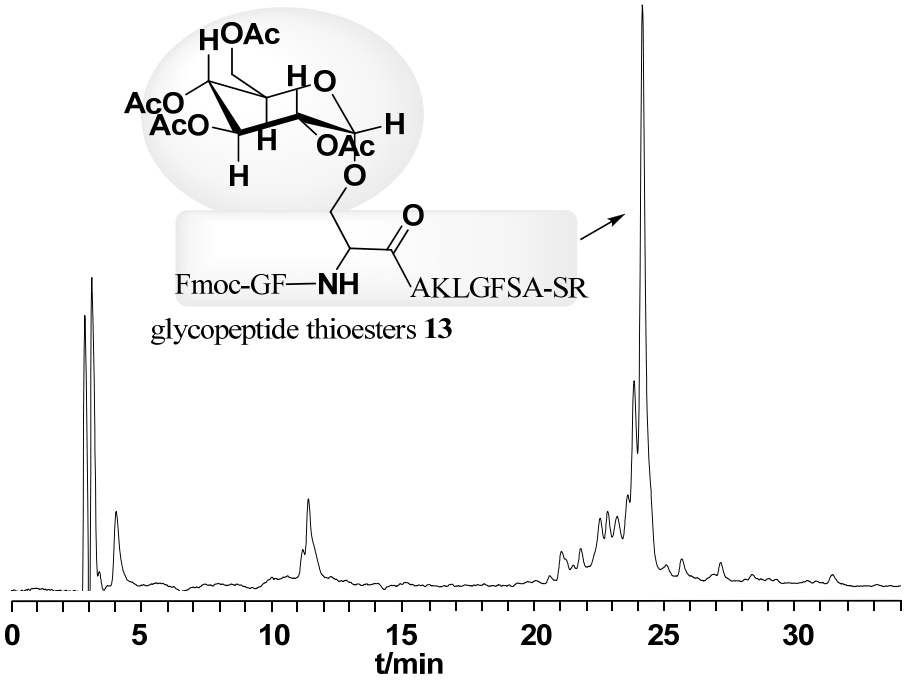


Scheme S4. The General Route for Fmoc-Based Synthesis of peptide thioesters **13**.

The N-methyl enamide building blocking **10** (86 mg, 0.11 mmol, 1.1 equiv relative to resin substitution) was added to a solution of HBTU (41 mg, 0. 11 mmol, 1.1 equiv relative to resin loading) and HOBt (15 mg, 0. 11 mmol, 1.1 equiv relative to resin loading) in DMF, followed by DIEA (diisopropylethylamine) (52μL, 0.30 mmol, 3.0 equiv relative to resin) to pre-activate the acid. After 3-5 min, the solution was added to Rink amide-AM resin which the Fmoc-group was deprotected by the operation described above and stirred at room temperature for 3 h. Remaining free amines were acetylated by standard capping protocol twice. Subsequent steps were completed with

standard HBTU/HOBt/DIEA coupling and Fmoc-deprotection protocols for Fmoc solid-phase peptide chemistry expect that the amino acid Fmoc-Ser-[β-D-Glc(OAc)$_4$]-OH (1.1 equiv relative to resin substitution) was coupled twice. The N-methyl enamide glycopeptide resin was treated with Reagent R cocktails at room temperature overnight to obtain the crude glycopeptide thioesters. The major peak was determinated by MALDI- TOF/MS: m/z=1751.8 ([M+H]$^+$ calculated 1751.7).

To verify the broad applicability of this new strategy, two glycopeptide thioesters Fmoc-GFS-[β-D-Glc(OAc)$_4$]-AKLGFSA-SR (**13**) and the glycopeptide thioesters with more amino acid residues Fmoc-QRMKYVCGFS-[β-D-Glc(OAc)$_4$]-AKLGFSA-SR (**14**) were synthesized in 56%(0.023mmol, 40mg) and 49%(0.021mmol, 56mg) isolated yield respectively (Figure S5, S6). Noteworthily, the HPLC chromatogram of crude product after the TFA cleavage step shows that a good purity of desired glycopeptide thioester.This indicates that the new method is a particularly viable method for the synthesis of glyco- peptide and protein.



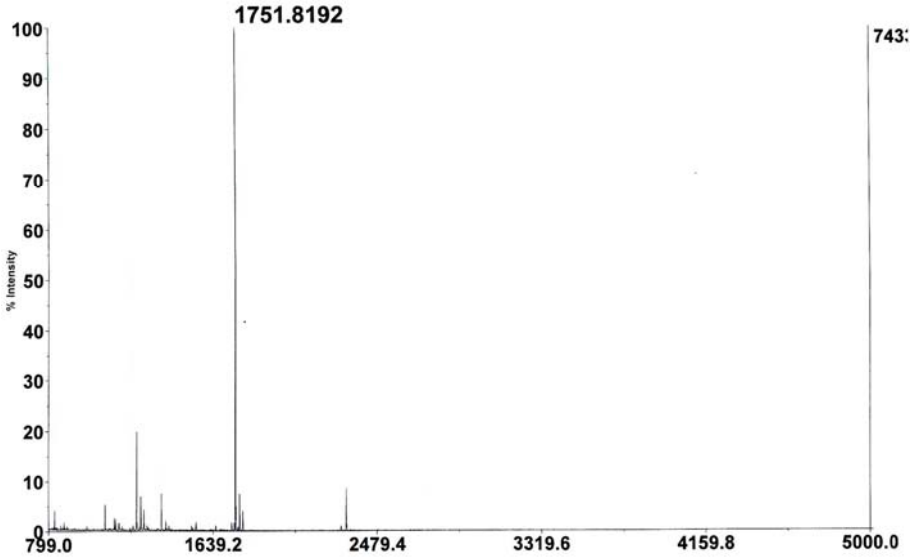
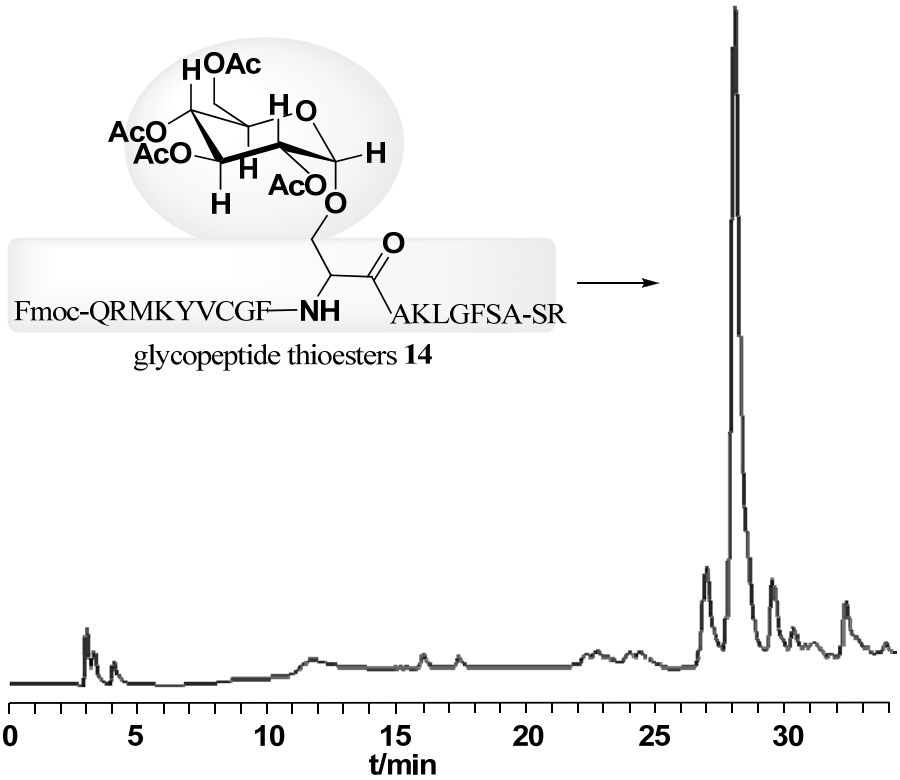


Figure S5. HPLC analysis of crude glycopeptide thioesters **13** and MALDI-TOF/MS of the major peak. Gradient: 20-80% buffer B in 30 min.



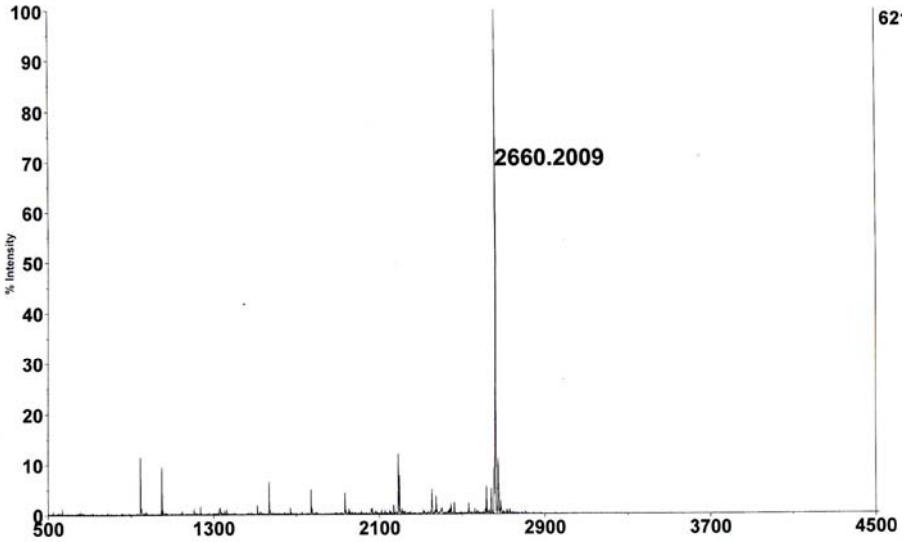
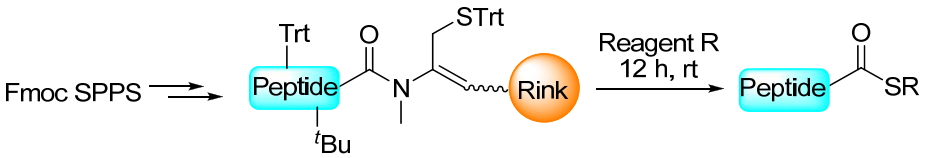


Figure S6. HPLC analysis of crude glycopeptide thioesters **14** and MALDI-TOF/MS of the major peak. Gradient: 20-80% buffer B in 30 min.

Moreover, we have made the enamide-containing amino acids for Gly, Lys, Phe, Leu, Val, and Ile. With these special amino acids in hand we can readily prepare the corresponding peptide thioesters in good yields and purities (Table 2).

Table 2. Fmoc SPPS synthesis of peptide thioesters.



Entry	target peptides	purity %[a]	yield %[b]	Pure peptide /mmol	Pure peptide /mg
1	Fmoc-AKEAEKITT**G**-SR[c]	91	70	0.041	42
2	Fmoc-AKLGFS**A**-SR	88	81	0.016	18
3	Fmoc-IKEYFYTSG**K**-SR	85	64	0.043	46
4	H-AVRTTGI**F**-SR	82	68	0.041	30
5	Fmoc-GGAGSAQAMP**L**-SR	91	78	0.041	(9+38)[d]
6	Fmoc-MFVFAVRTTGI**F**-SR	75	60	0.041	51
7	Fmoc-GDSKDVRKF**I**-SR[e]	85	70	0.046	52
8	Fmoc-AELVDALQF**V**-SR[e]	56	32	0.043	21

[a] Purity of crude peptides based on HPLC detection trace at 214 nm. [b] Isolated yield based upon the loading of the frist enamide-containing amino acid. [c] R = $CH_2COC_3H_6COC_2H_4$- $CONH_2$. [d] 9mg of Met[O] and 38mg of Met, [e] Cleavage for 24 h at 30 °C.

The HPLC analysis of crude thioesters for different amino acids, and MALDI-TOF/MS of the major peak as follow:

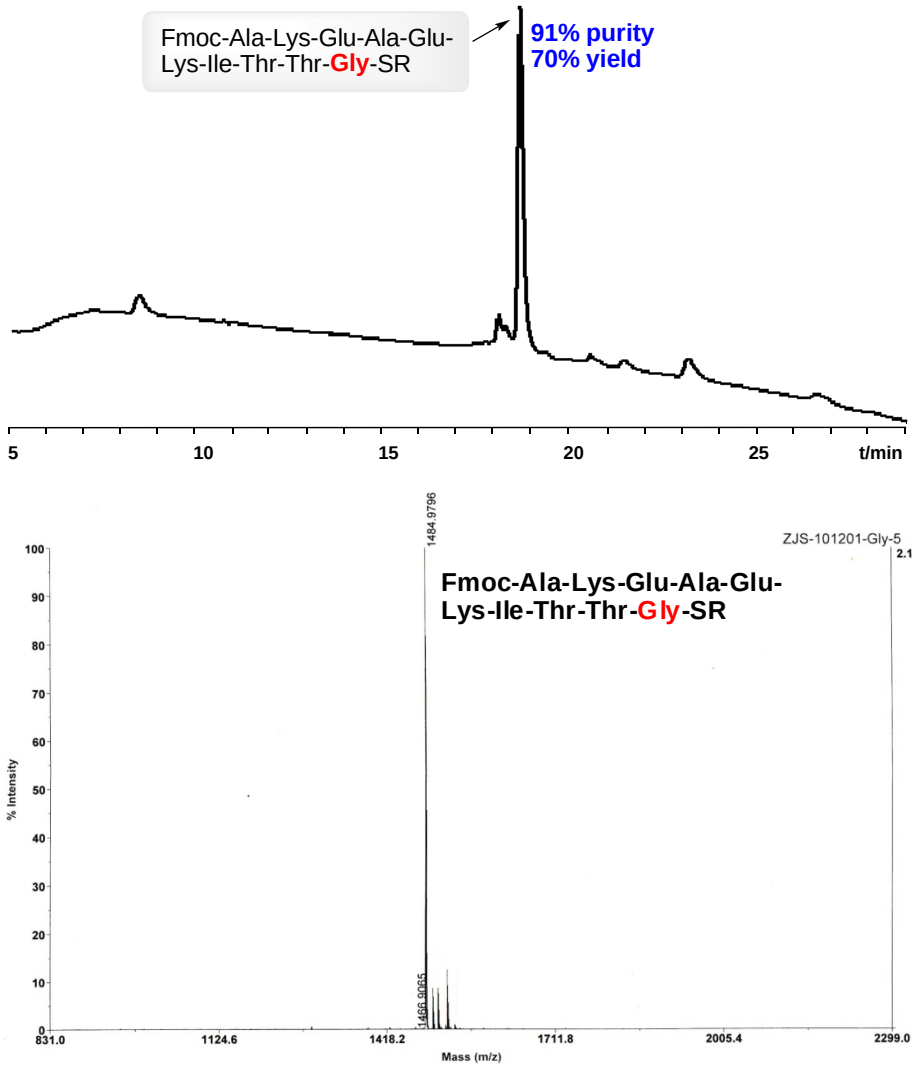
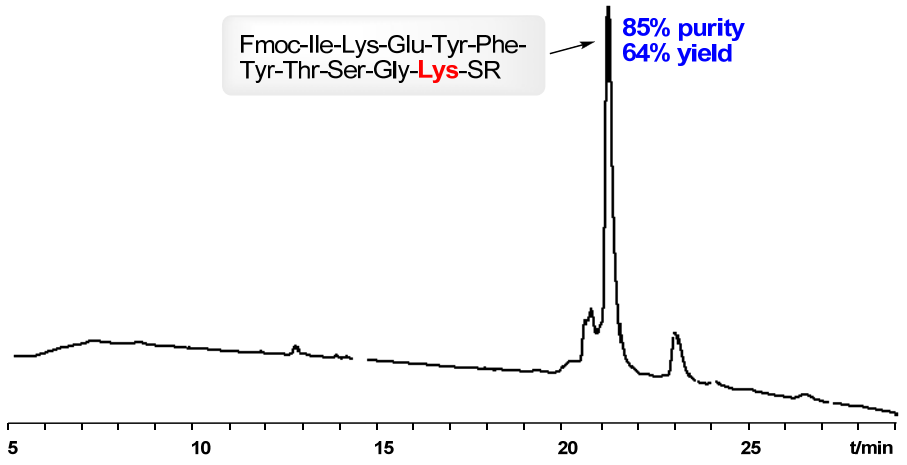


Figure S7. HPLC analysis of crude Gly-thioesters and MALDI-TOF/MS of the major peak. Gradient: 10-60% buffer B in 25 min.



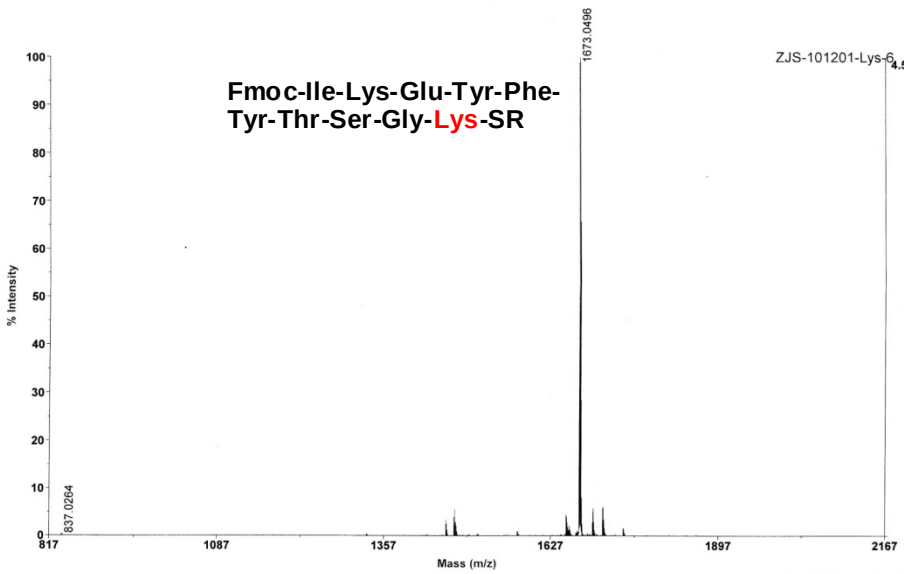
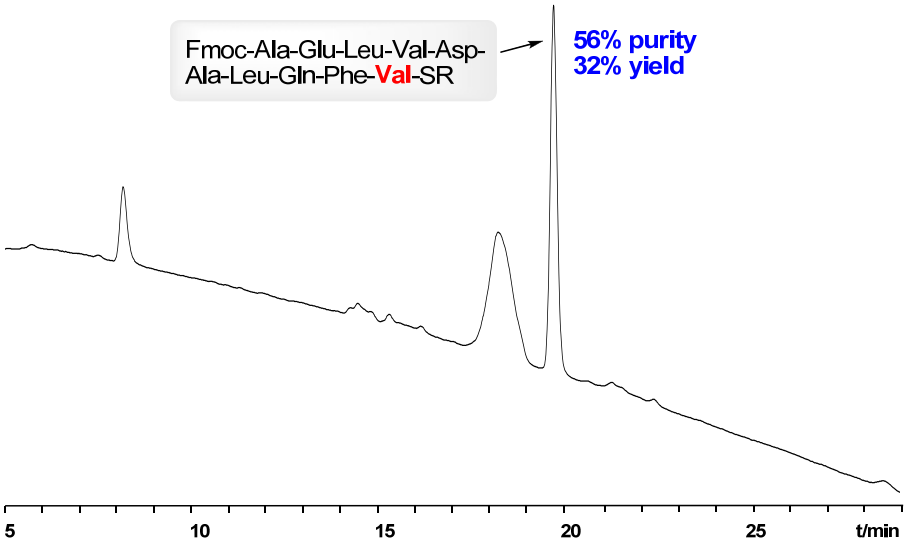


Figure S8. HPLC analysis of crude Lys-thioesters and MALDI-TOF/MS of the major peak. Gradient: 10-60% buffer B in 25 min.



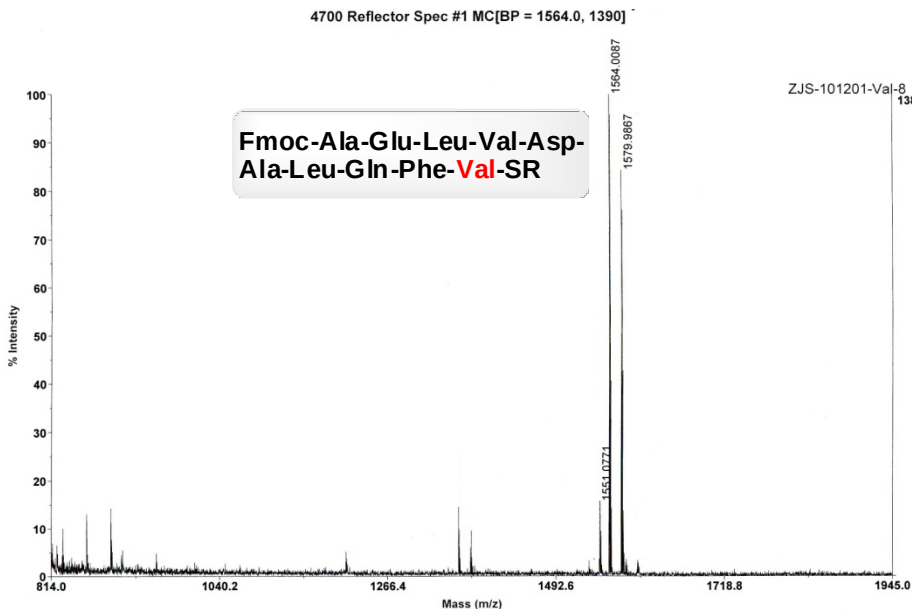


Figure S9. HPLC analysis of crude Val-thioesters and MALDI-TOF/MS of the major peak. Gradient: 40-70% buffer B in 30 min.

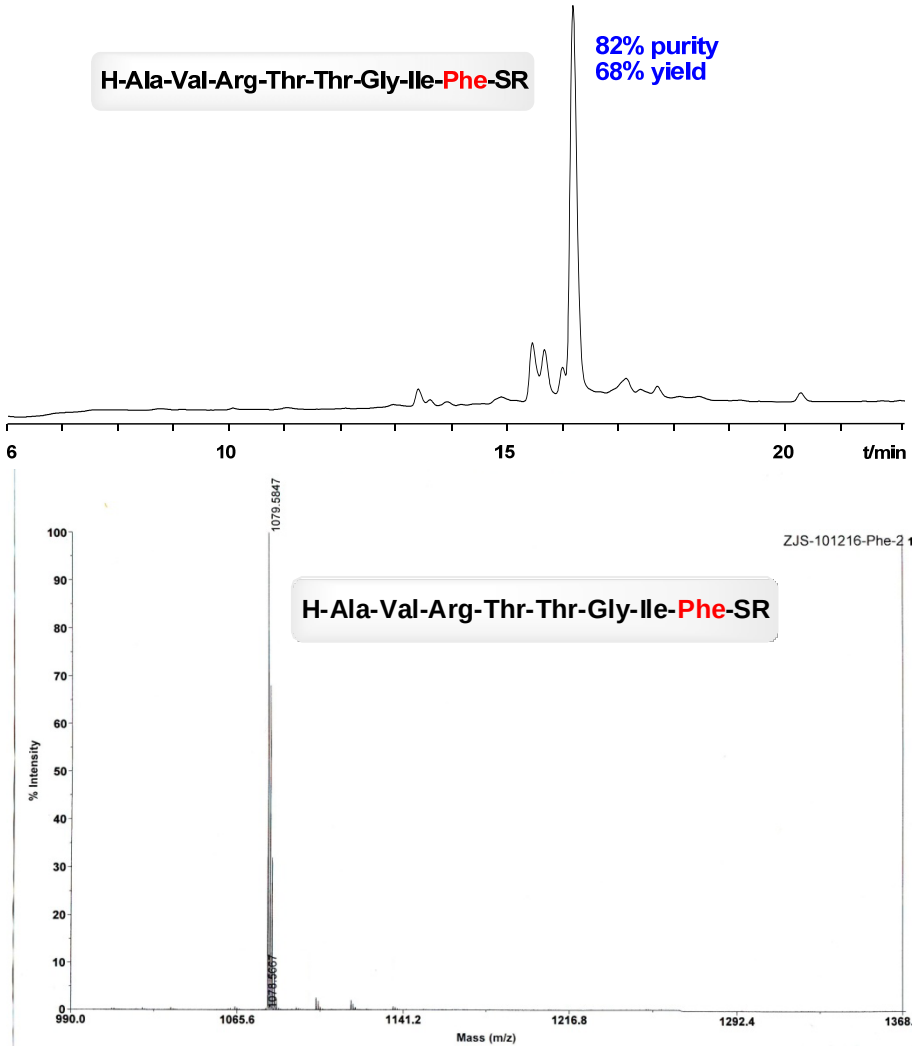
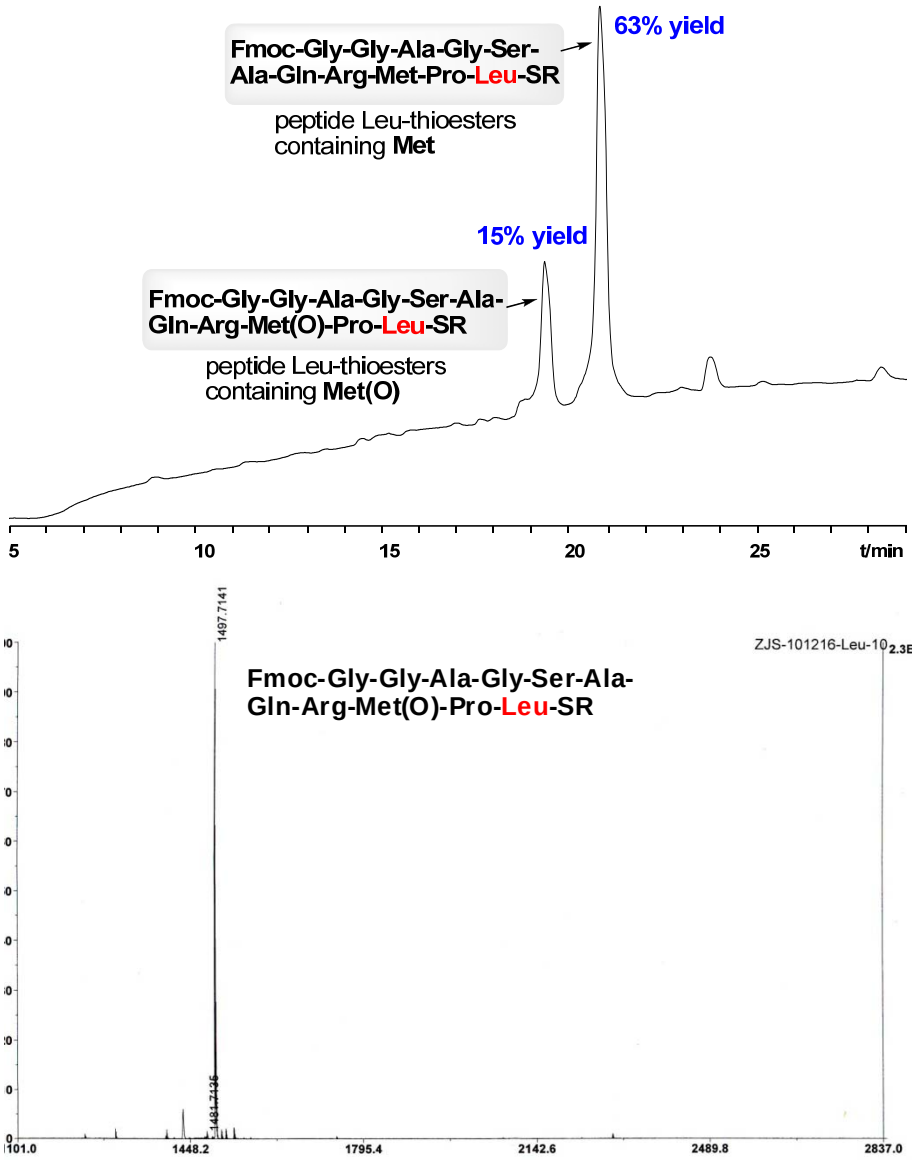


Figure S10. HPLC analysis of crude Phe-thioesters and MALDI-TOF/MS of the major peak.

Gradient: 40-70% buffer B in 30 min,C4.



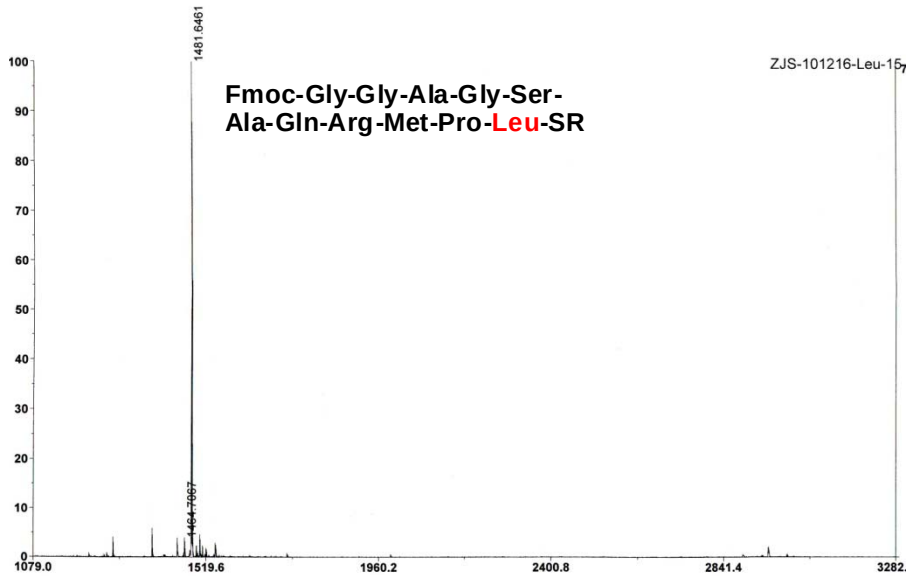


Figure S11. HPLC analysis of crude Leu-thioesters and MALDI-TOF/MS of the major peak.

Gradient: 10-60% buffer B in 25 min.

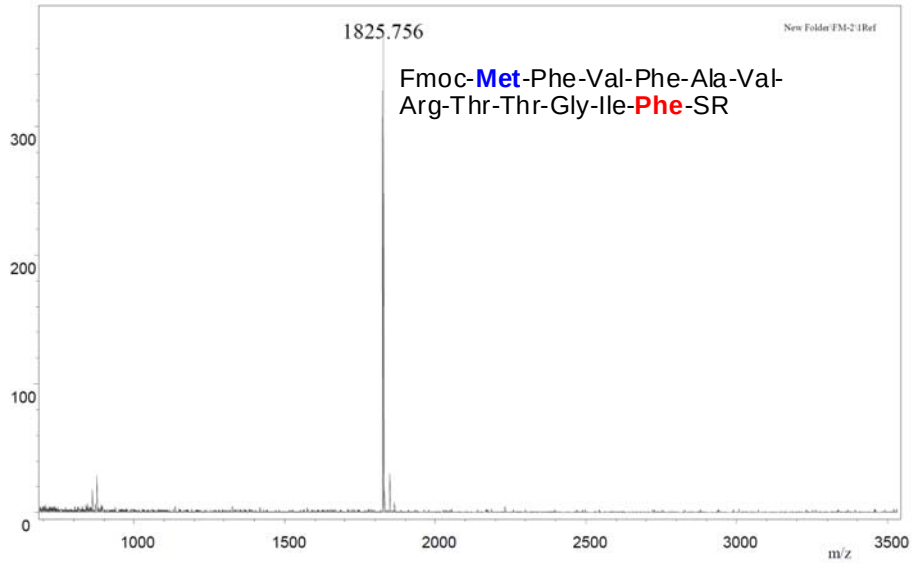


Figure S12. HPLC analysis of crude Phe-thioesters and MALDI-TOF/MS of the major peak.

Gradient: 10-60% buffer B in 25 min.

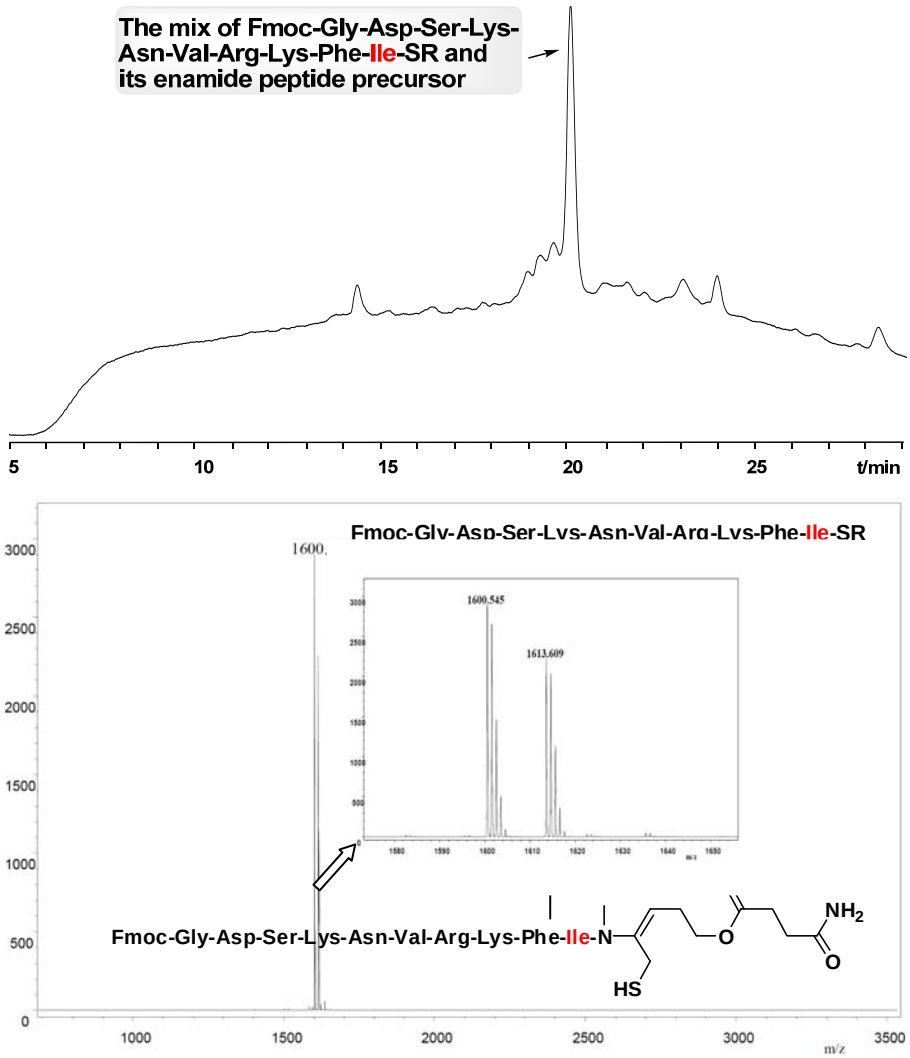


Figure S13. HPLC analysis of crude Ile-thioesters and MALDI-TOF/MS of the major peak. Gradient: 10-60% buffer B in 25 min. Cleavage for 10h at 30℃.

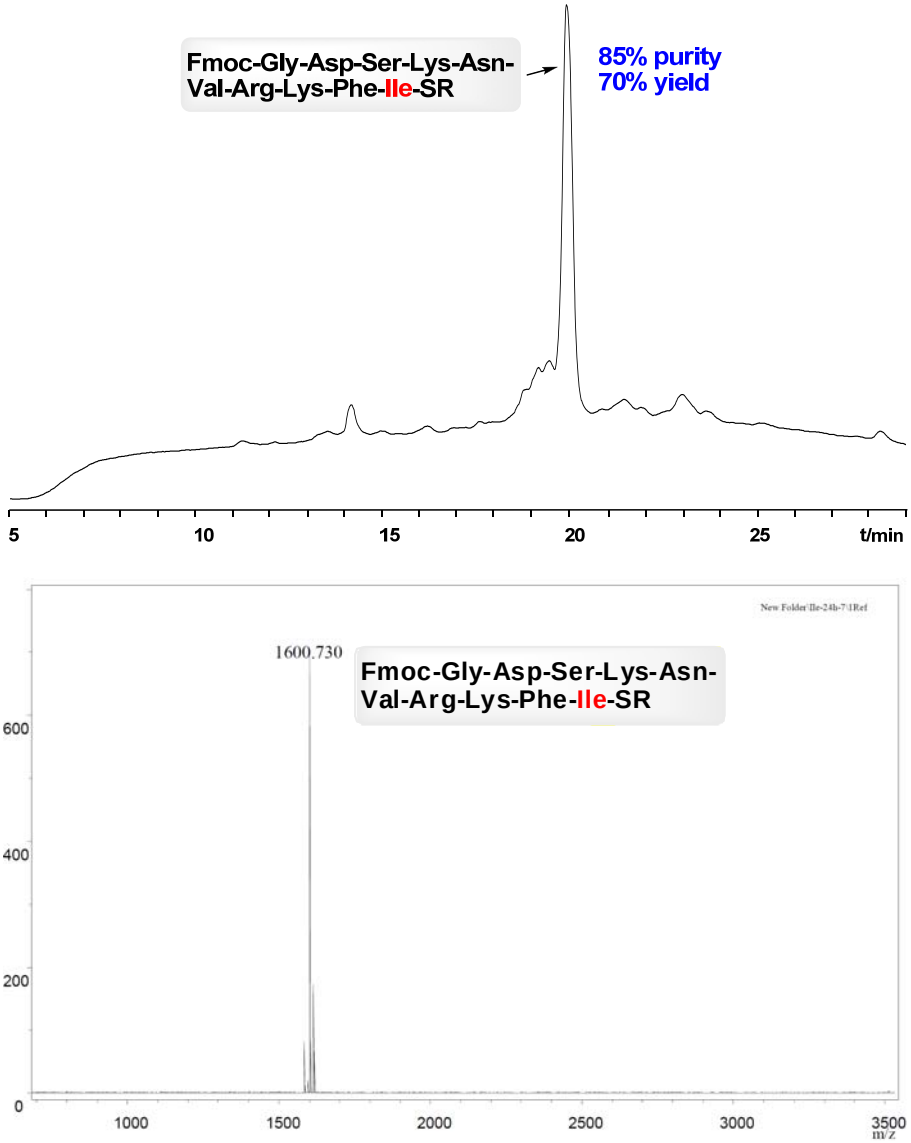
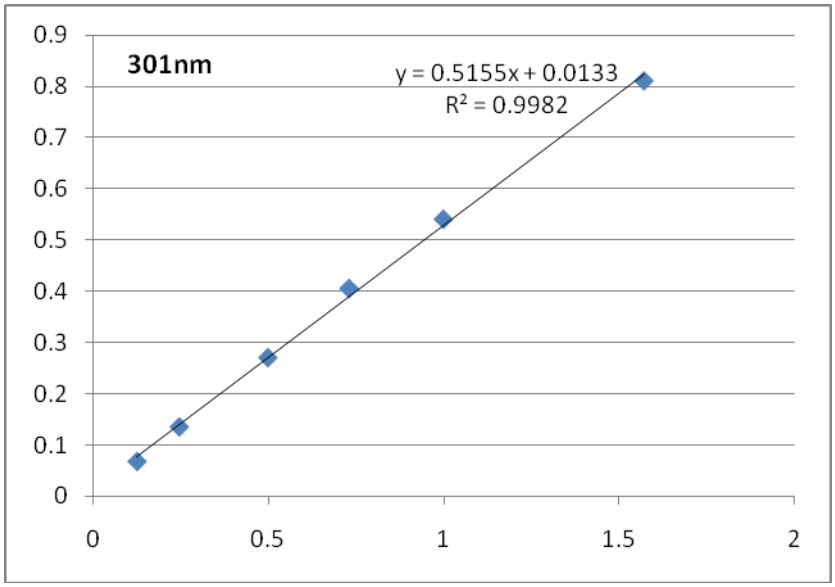


Figure S14. HPLC analysis of crude Ile-thioesters and MALDI-TOF/MS of the major peak. Gradient: 10-60% buffer B in 25 min.Cleavage for 24h at at 30℃.

The resin loading of the first enamide-containing amino acid by Fmoc determination

In addition, the estimation of level of first residue attachment was achieved by quantifying the amount of Fmoc removed at deprotection cycle via UV/Vis spectrophotometry.

Frist, we Standard Curve of Fmoc-protected resin at 301 nm using the commercial Rink amide AM resin (loading=0.27mmol/g). 0.25mg, 0.5mg, 1.0mg, 1.5 mg, 2 mg and 3 mg of Rink resin was weighted exactly into 4ml of Piperidine/DMF(1:4) respectively. The mixture was shaken thoroughly and left to settle for 25 to 30min in order to the complete Fmoc deprotection.After UV determination, we got the standard curve as follow:



Standard Curve of Fmoc-Rink resin at 301 nm.

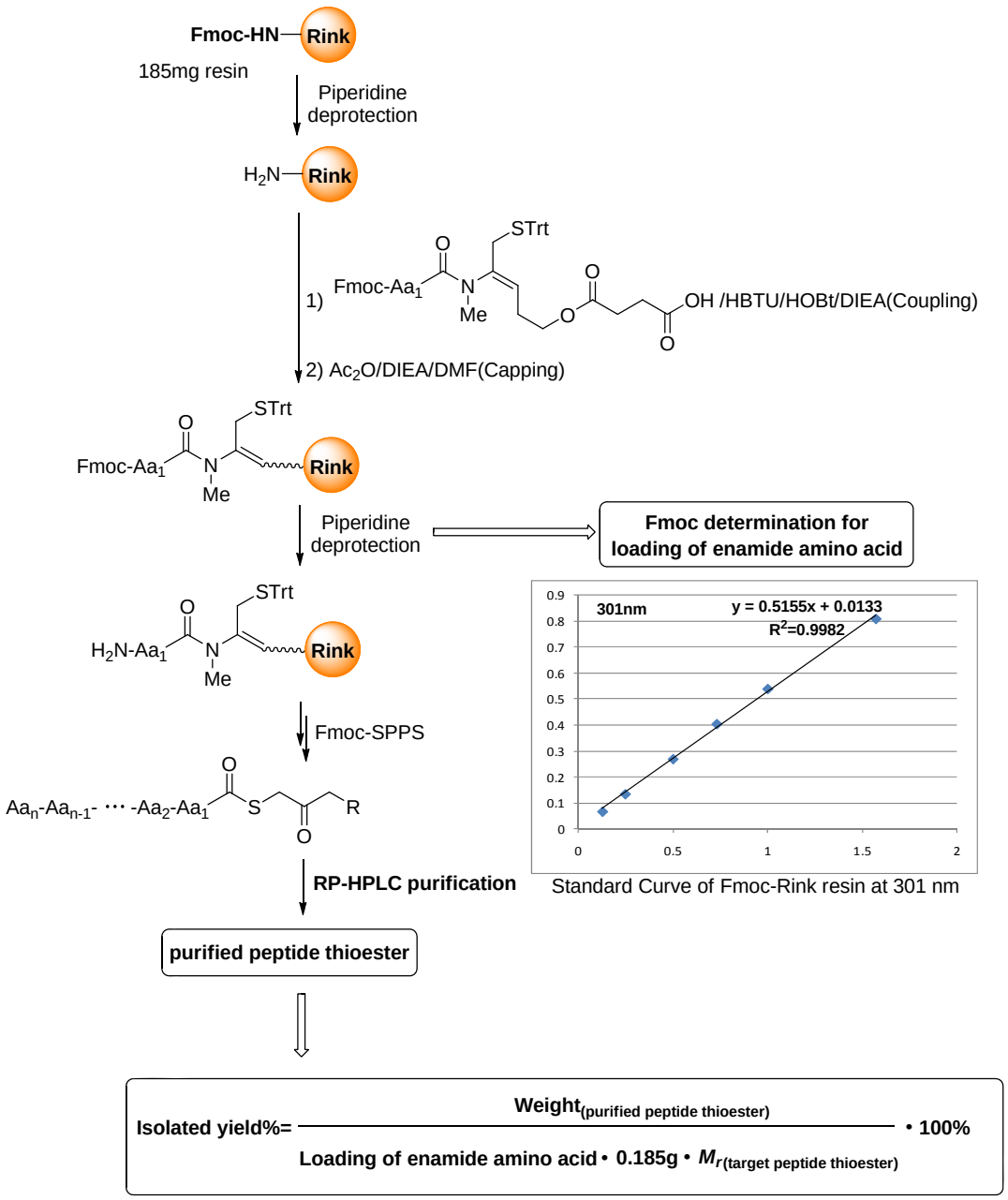
Second, we measured the coupling efficiency of all enamide amino acid in this work with Rink amide resin. In brief, after the enamide-containing amino acid was coupled onto the Rink amide AM resin, the resin was washed 4×DMF, 4×CH_2Cl_2, 4×DMF and 4×CH_2Cl_2 or ether, and dried to constant weight.

1. 0.8-1.4mg dried enamide resin was weighted exactly into a small flask or glass vial;
2. 4ml Piperidine/DMF(1:4) was added and the mixture was shaken thoroughly and left to settle for 25 to 30min in order to the complete Fmoc deprotection;
3. The resin is filtered off and the absorbance of the filtrate is collected;
4. Filled the UV cell with 2.0mL Piperidine/DMF(1:4) (reference solution), placed the cell into the spectrophotometer and zeroed at 301 nm;
5. 2.0 mL solution prepared at step 3 was added into the cell and measured the absorbance at λ=301 nm;
6. Calculate the loading using the following equation:

Loading (mmol/g) = $0.5155 \times Abs_{sample} + 0.0133$[a]

[a] λ=301 nm; 4ml deblocking solution .

The General approach for determination of loading of enamide amino acid and isolated yield of peptide thioesters is shown in Scheme S5.



Scheme S5. The General approach for determination of loading of enamide amino acid and isolated yield of peptide thioesters.

Following the method described above, the loading of enamide amino acid of Gly, Ala, D-Ala, Phe, Lys, Leu,Val and Ile were measured at λ=301 nm(Table 3).

Table 3. The resin loading of the first enamide-containing amino acid**.**

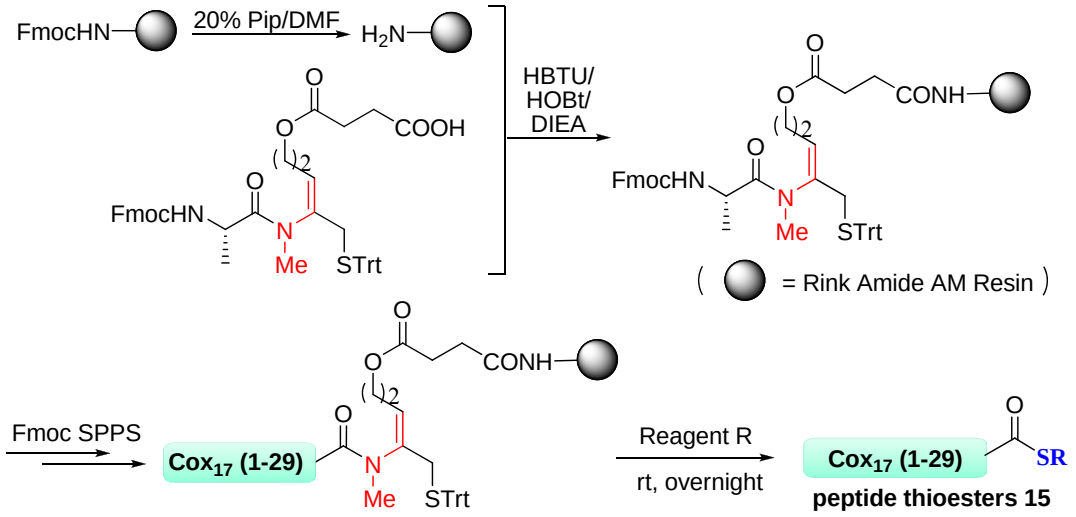
	mg·(4ml deblocking solution)$^{-1}$	Abs $_{\lambda=301nm}$	Resin loading of the frist enamide amino acid/mmol·g^{-1}
Gly	0.8	0.317	0.22
Ala	1.2	0.491	0.22
D-Ala	1.4	0.568	0.22
Phe	1.0	0.411	0.23
Lys	1.4	0.596	0.23
Leu	1.0	0.406	0.22
Val	0.8	0.332	0.23
Ile	0.8	0.358	0.25

0.22-0.25 mmol/g of loading (the resin loading=0.27mmol/g) is an indication of effective coupling of the frist enamide amino acid.

Synthesis of the Human Cox17 (1-29) peptide thioesters 15

Amino acid sequence of Human Cox17 (1-29):

Gly-Ser-Phe-Thr-Met-Pro-Gly-Leu-Val-Asp-Ser-Asn-Pro-Ala-Pro-Pro-Glu-Ser-Gln-Glu-Lys-Lys-Pro-Leu-Lys-Pro-Cys-Cys-Ala-SR (R=-$CH_2COC_3H_6OCOC_2H_4CONH_2$)



Scheme S6. The General Route for Fmoc-Based Synthesis of peptide thioesters 15.

The N-methyl enamide building blocking **10** (86 mg, 0.11 mmol, 1.1 equiv relative to resin substitution) was added to a solution of HBTU (41 mg, 0. 11 mmol, 1.1 equiv relative to resin loading) and HOBt (15 mg, 0. 11 mmol, 1.1 equiv relative to resin loading) in DMF, followed by

DIEA (diisopropylethylamine) (52μL, 0.30 mmol, 3.0 equiv relative to resin) to pre-activate the acid. After 3-5 min, the solution was added to Rink amide-AM resin which the Fmoc-group was deprotected by the operation described above and stirred at room temperature for 3 h. Remaining free amines were acetylated by standard capping protocol twice. Subsequent steps were completed with standard HBTU/HOBt/DIEA coupling and Fmoc-deprotection protocols for Fmoc solid-phase peptide chemistry. The N-methyl enamide peptide resin was treated with Reagents R at room temperature overnight to obtain the crude peptide thioesters. The product thioesters was purified with a Vydac C18 column (10 μm, 25 mm×250 mm) with a 10mL/min flow rate (Gradient: 20-35% buffer B over 30 min) in 42% isolated yield (37umol, 121mg). It was determinated by MALDI-TOF/MS: m/z=3243.7 ($[M+H]^+$ calculated 3243.5).

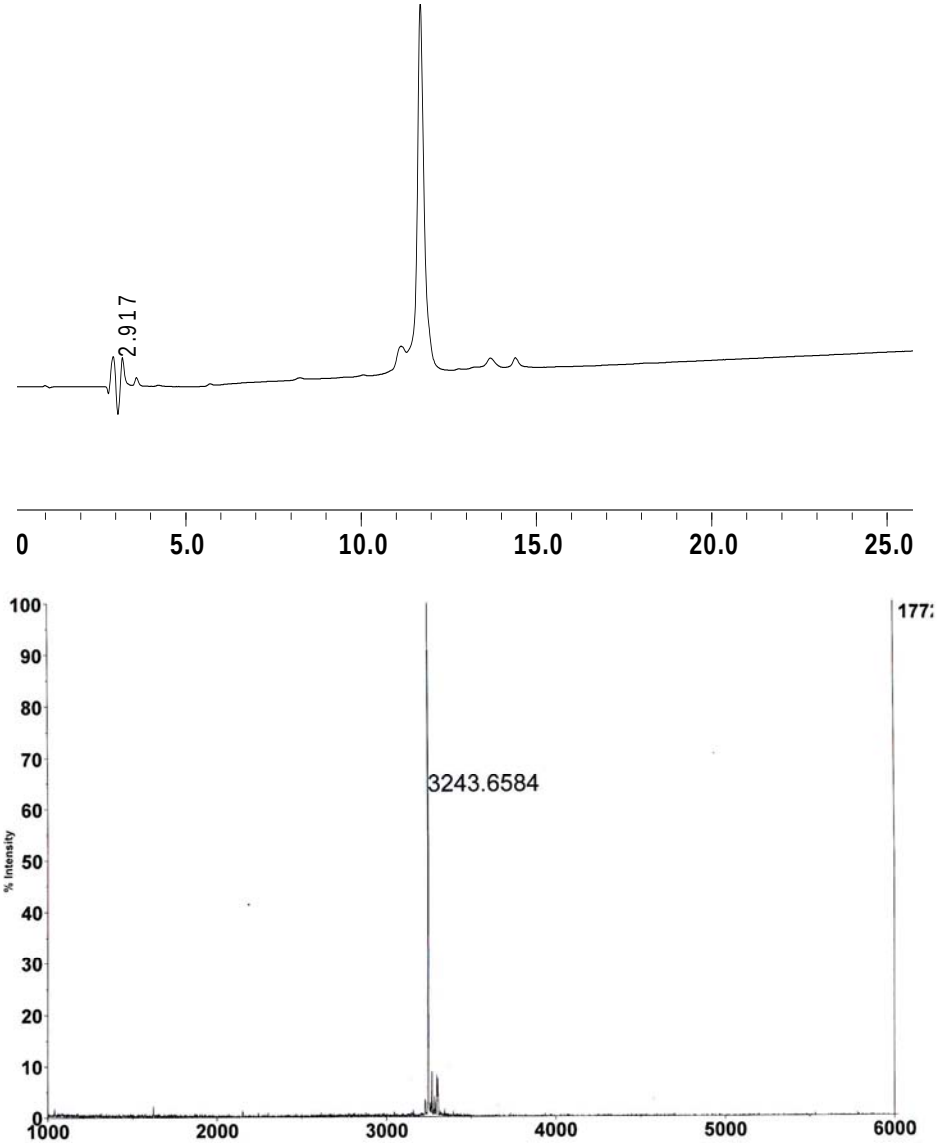


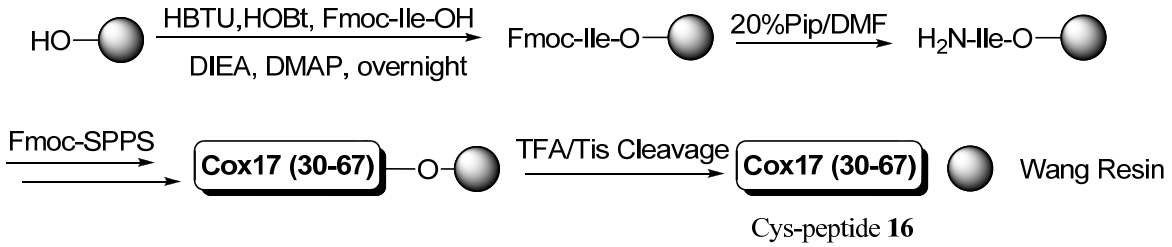
Figure S15. HPLC analysis and MALDI-TOF/MS of peptide thioesters **15**. gradient: 20–40% of

buffer B in 25 min

c. Synthesis of N-Cys Human Cox17 (30-67)

Amino acid sequence of Human Cox17 (30-67):

Cys-Pro-Glu-Thr-Lys-Lys-Ala-Arg-Asp-Ala-Cys-Ile-Ile-Glu-Lys-Gly-Glu-Glu-His-Cys-Gly-His-Leu-Ile-Glu-Ala-His-Lys-Glu-Cys-Met-Arg-Ala-Leu-Gly-Phe-Lys-Ile



Scheme S7. The General Route for Fmoc-Based Synthesis of N-Cys peptide **16**.

Fmoc-Ile-OH (0. 4mmol, 4 equiv relative to resin loading) was added to a solution of HBTU (0. 39 mmol, 3.9 equiv relative to resin loading), DMAP (0.1 equiv relative to Fmoc-Ile-OH and HOBt (0. 4mmol, 4 equiv relative to resin loading) in DMF, followed by DIEA (0.8mmol, 8 equiv relative to resin) to pre-activate the acid. After 5 min, the solution was added to pre-active Wang resin and stirred at room temperature overnight. Remaining free hydroxyl groups were acetylated by standard capping protocol. Subsequent steps were completed with HBTU/HOBt/DIEA coupling of each amino acid twice if necessary and Fmoc-deprotection protocols for Fmoc solid-phase peptide chemistry with an exception that the last amino acid Boc-Cys(Trt)-OH was coupled with standard method. The N-Cys peptide **16** was synthesized according to the general procedure in 18% yield (18umol, 43mg) after purification with a Vydac C18 column (10 μm, 25 mm×250 mm) with a 10mL/min flow rate (Gradient: 20-35% buffer B over 30 min). It's noted that the Cys-peptide should be kept under argon gas. MALDI-TOF/MS: m/z=4292.5 ($[M+H]^+$, calculated 4294.1).

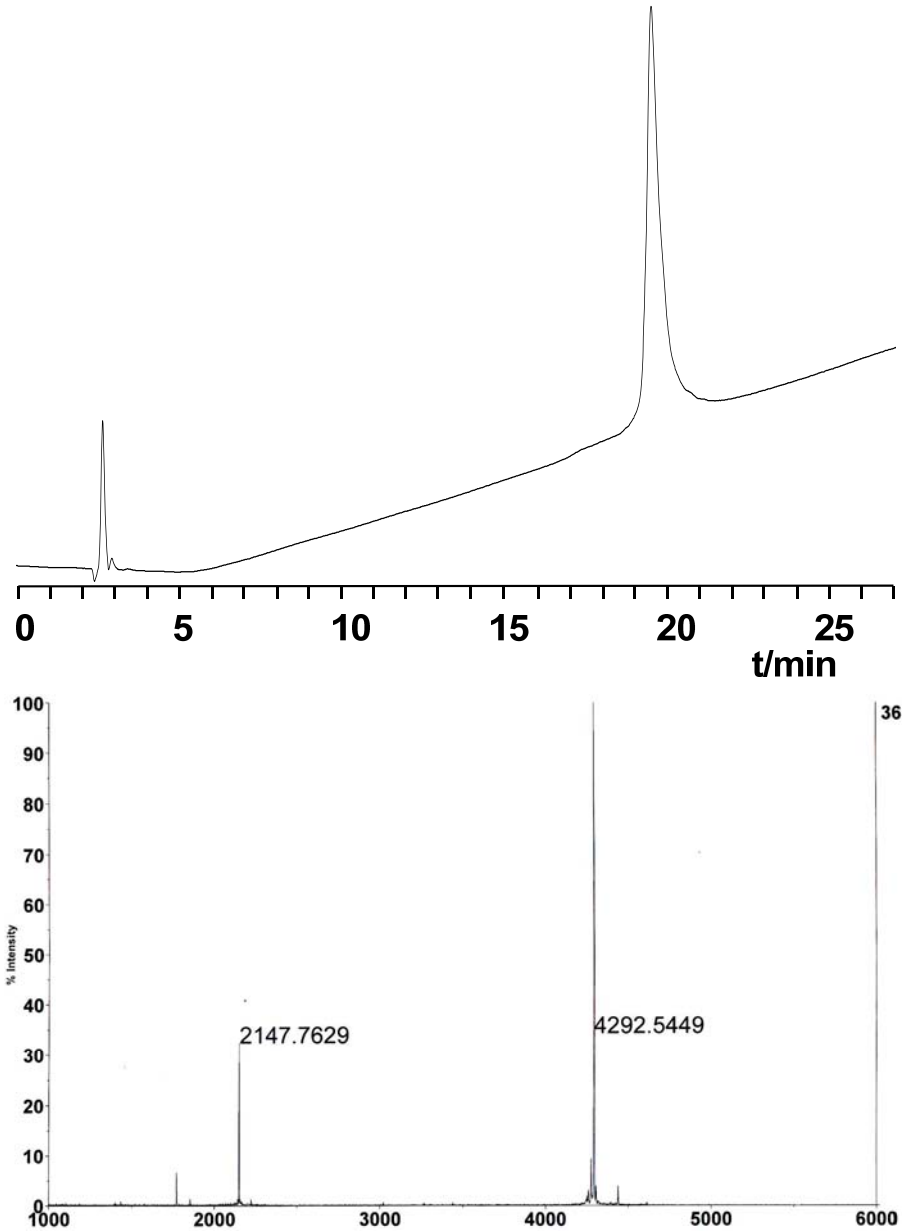
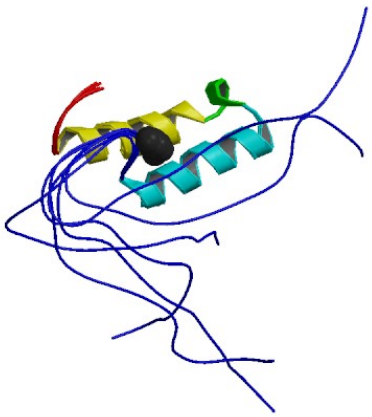


Figure S16. HPLC analysis of peptide thioesters **16** and MALDI-TOF/MS of the Cys-peptide **16**. gradient: 20–35% of buffer B in A over 30 min

2.4 The New Peptide Thioesters Strategy for Chemical Synthesis of the Human Cox 17 Protein

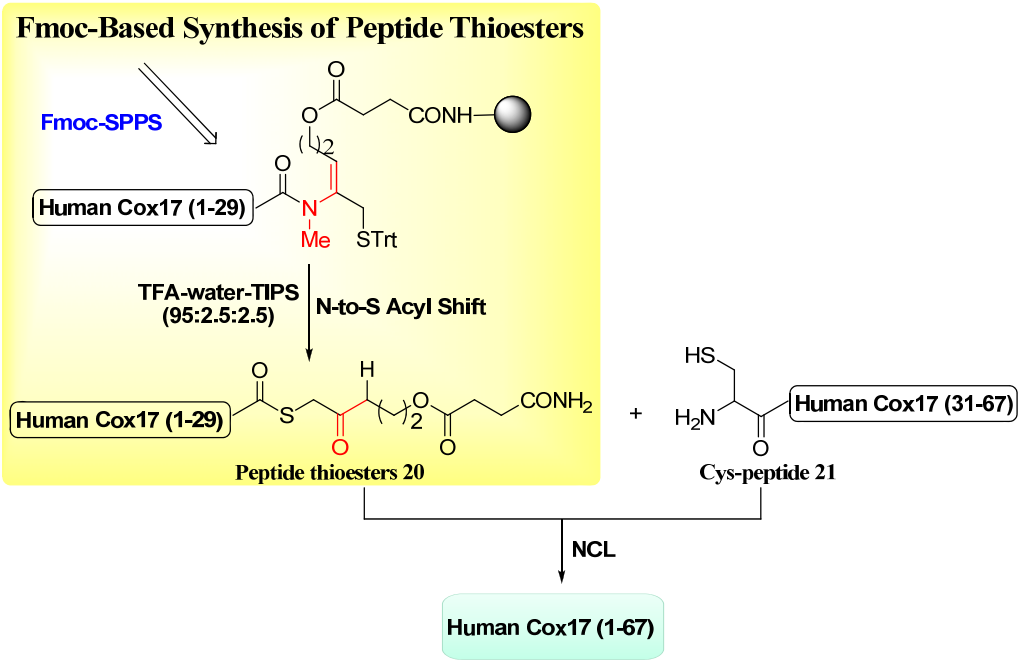


PDB accession no. 2RNB

Gly[1]-Ser-Phe-Thr-Met-Pro-Gly-Leu-Val-Asp[10]-Ser-Asn-Pro-Ala-Pro-Pro-Glu-Ser-Gln-Glu[20]-Lys-Lys-Pro-Leu-Lys-Pro-Cys-Cys-Ala-Cys[30]-Pro-Glu-Thr-Lys-Lys-Ala-Arg-Asp-Ala-Cys[40]-Ile-Ile-Glu-Lys-Gly-Glu-Glu-His-Cys-Gly[50]-His-Leu-Ile-Glu-Ala-His-Lys-Glu-Cys-Met[60]-Arg-Ala-Leu-Gly-Phe-Lys-Ile[67]

Figure S17. The Molecular structure of the Human Cox17 and target amino acid sequence.

The Human Cox17 protein, which is a 67-residue protein and contains six conserved cysteines, is a key mitochondrial copper chaperone responsible for supplying copper ions to cytochrome *c* oxidase. In the mitochondrial intermembrane space (IMS) Cox17 can exist in three oxidation states: from the fully reduced state where no disulfide bonds to a partially oxidized form with two disulfide bonds or to a fully oxidized protein with three disulfide bonds are present. The partially oxidized form with with two S-S bonds and two reduced Cys is involved in copper transfer to Sco1 and Cox11. [1-5]



Scheme S8. The New Peptide Thioesters Strategy for Chemical Synthesis of Human Cox17.

The synthetic strategy of Human Cox17 protein is shown in Scheme S8. The *C*-terminal Human Cox17 (1-29) peptide thioesters **15** was synthesized by the new Fmoc-based N-to-S acyl shift strategy described above with 42% isolated yield. The *C*-terminal peptide thioesters **15** (1.8mg, 1equiv) and the Cys-peptide **16** (2.4mg, 1.1equiv) were dissolved in 500μL freshly degassed ligation buffer (200mM sodium phosphate, 6M Gn·HCl, 25mM MPAA, 50mM TCEP·HCl, pH=7.15). The reaction was complete within six hours at room temperature under argon gas. The ligation gave almost exclusively (with >95% yield by HPLC analysis) the objective Human Cox17 protein **17** (Figure S10). The product was characterized by MALDI-TOF analysis (observed mass=7307.9Da, calculated mass = 7308.5Da).

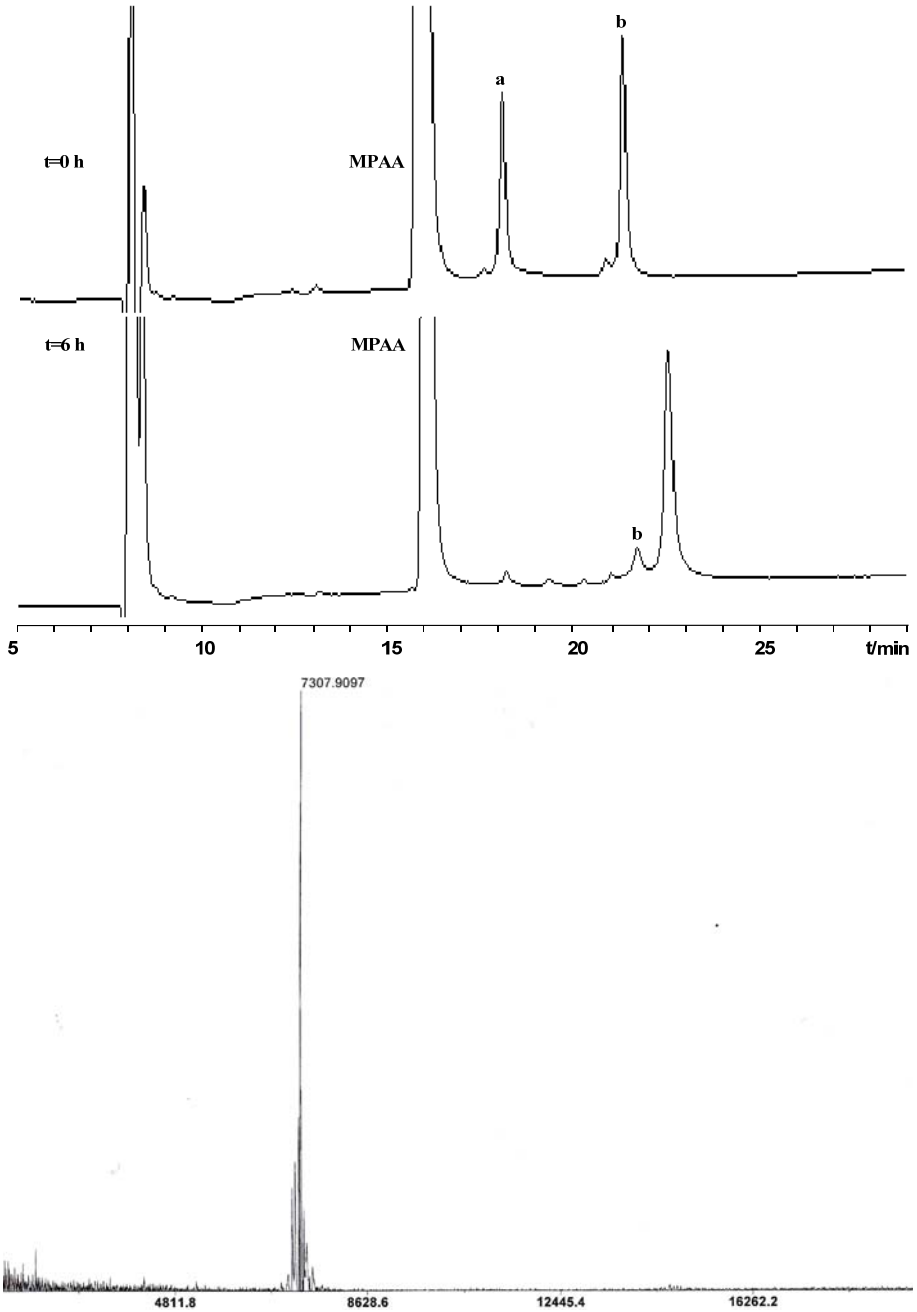


Figure S18. Analytical HPLC data for the synthesis of Human Cox17 protein at 0 and 6 h. The chromatographic separations were performed using a linear gradient (20–40%) of buffer B in buffer A over 20 min (buffer A=0.1% TFA in water; buffer B=0.1% TFA in CH_3CN). The product was confirmed by MALDI-TOF.

a = The Human Cox17 (1-29) peptide thioesters **15**; b = The Human Cox17 (30-67) Cys-peptide **16**; c = The Human Cox17 protein **17**.

Reference

(1) Y. C. Horng, P. A. Cobine, A. B. Maxfield, H. S. Carr, and D. R. Winge, *J. Biol. Chem.* **2004**, *279*, 35334.

(2) P. Palumaa, L. Kangur, A. Voronova, and R. Sillard, *Biochem. J.* **2004**, *382*, 307.

(3) A. Voronova, J. Kazantseva, M. Tuuling, N. Sokolova, R. Sillard, and P. Palumaa, *Protein Expression Purif.* **2007**, *53*, 138.

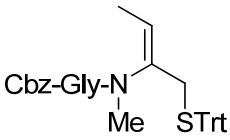
(4) L. Banci, I. Bertini, S. Ciofi-Baffoni, A. Janicka,M. Martinelli, H. Kozlowski, and P. Palumaa, *J. Bio. Chem.*, **2008**, *283*, 7912.

(5) L. Banci, I. Bertini, S. Ciofi-Baffoni, T. Hadjiloi, M. Martinelli, and P. Palumaa, *PNAS*, **2008**, *105*, 6803.

Abbreviations

Ac: acyl; ACN or CH_3CN: acetonitrile; Ac_2O: acetic anhydride; Bn: benzyl; Boc: *tert*-butoxycarbonyl; Cbz or Z: benzyloxycarbonyl; $CHCl_3$:chloroform; CH_2Cl_2: dichloromethane; DIEA: *N*,*N*-diisopropylethylamine; DMAP: 4- *N*,*N*-dimethylaminopyridine; DMF: N,N-dimethylformamide; DMSO: dimethyl sulfoxide; EDC.HCl: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Et_2O: diethyl ether; EtOAc: ethyl acetate; FCC: flash column chromate-graphy; Fmoc: 9-fluorenylmethoxycarbonyl; HBTU: *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate; HOBt: 1-hydroxybenzotriazole; Imi: imidazole; LAH: lithium aluminum hydride; Me: methyl; MeOH: methyl alcohol; Ms: methanesulfonyl; NaH: sodium hydride; NCL: native chemical ligation; NEt_3: triethylamine; NMM: *N*-methylmorpholine; NMP: N-methyl-2-pyrrolidone; NMR: nuclear magnetic resonance; Py.: pyridine; PBS: Phosphate Buffered Saline; PE: petroleum ether; RP-HPLC: reversed-phase high performance liquid chromatography; R_t: retention time; SPPS: solid phase peptide synthesis; TBAB: tetrabutylammonium bromide; TBAF: etrabutylammonium fluoride; TBS or TBDMS: *t*-butyldimethylsilyl; TCEP·HCl: tris (2-carboxyethyl) phosphine hydrochloride; TFA: trifluoroacetic acid; THF: tetrahydrofuran; TMS: tetramethylsilane; TIPS: triisopropylsilane; TLC: thin layer chromatography; Tris: Tris-(hydroxy methyl)amino methane; Ts: *4*-toluenesulfonyl

3. Copies of 1H-NMR, 13C-NMR and MS Spectra



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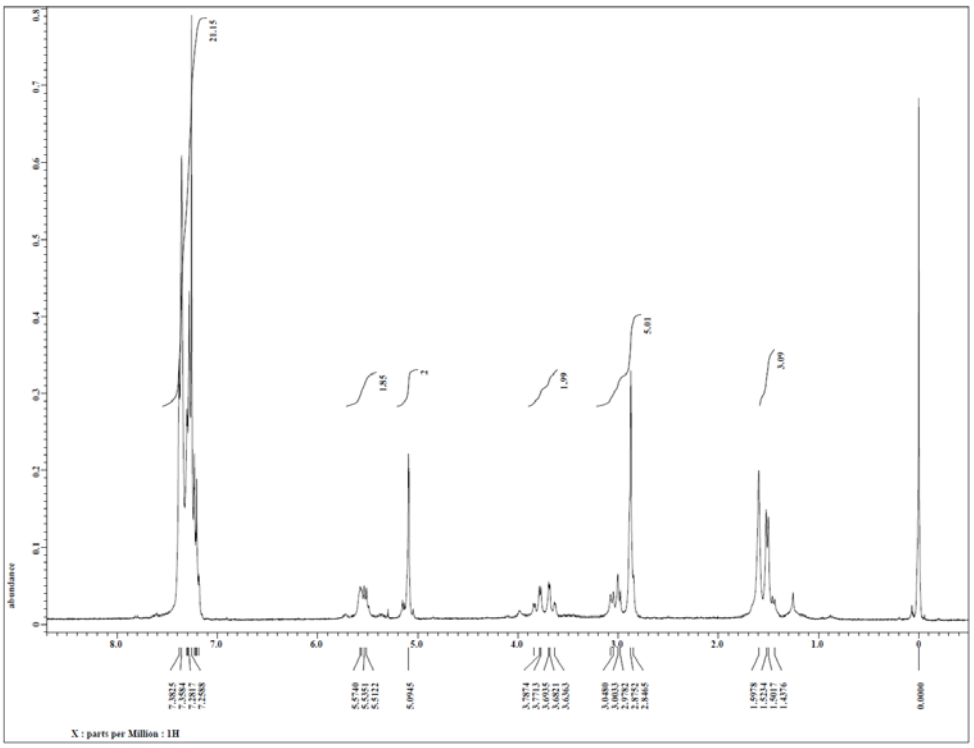

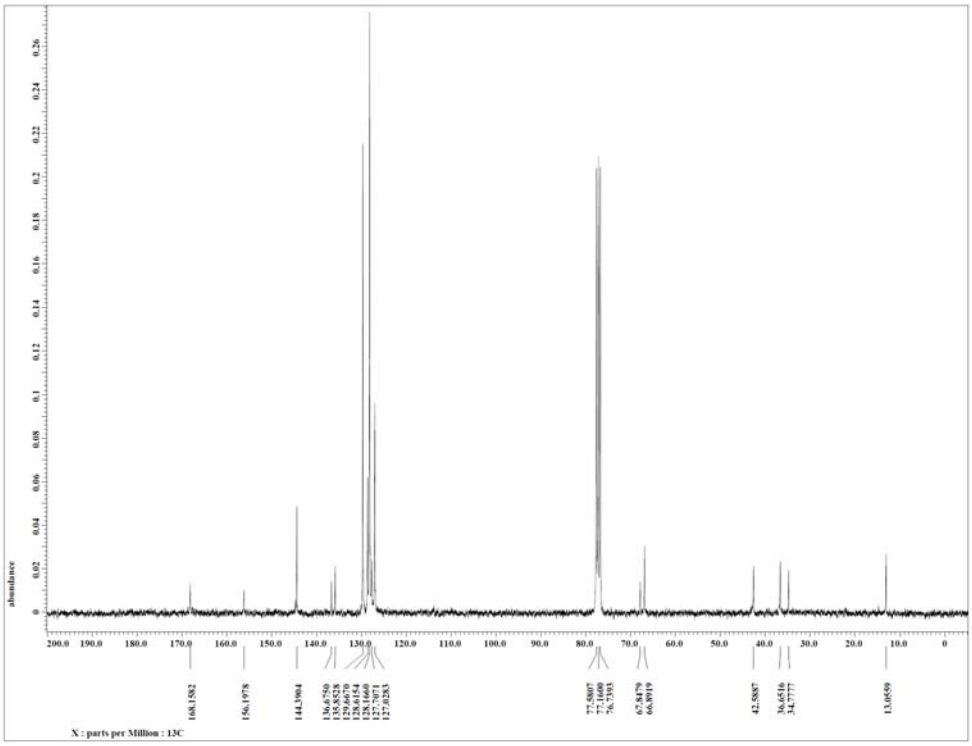

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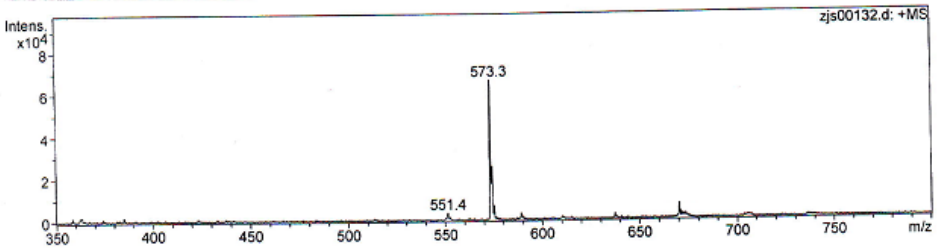

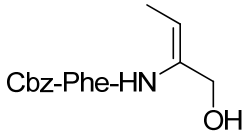

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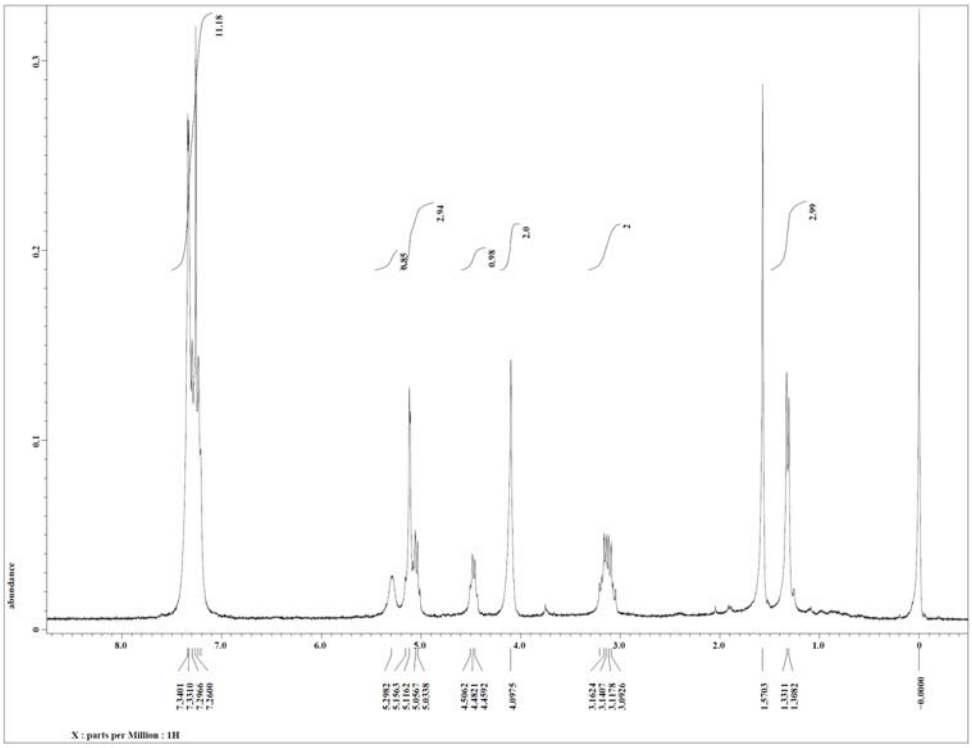

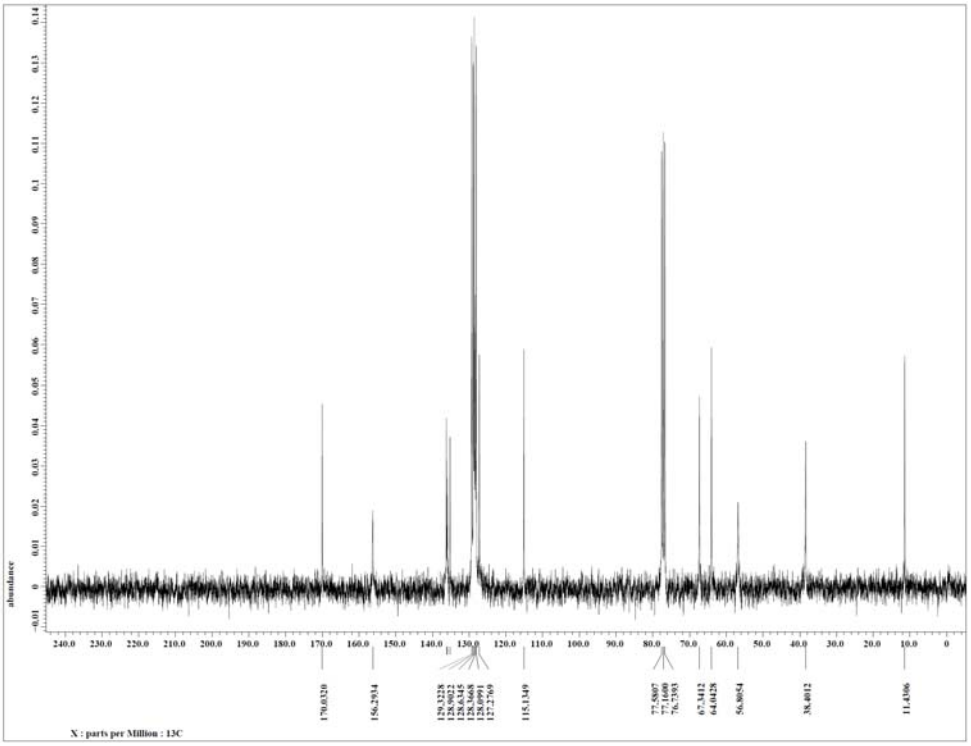
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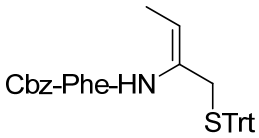
X : parts per Million : 13C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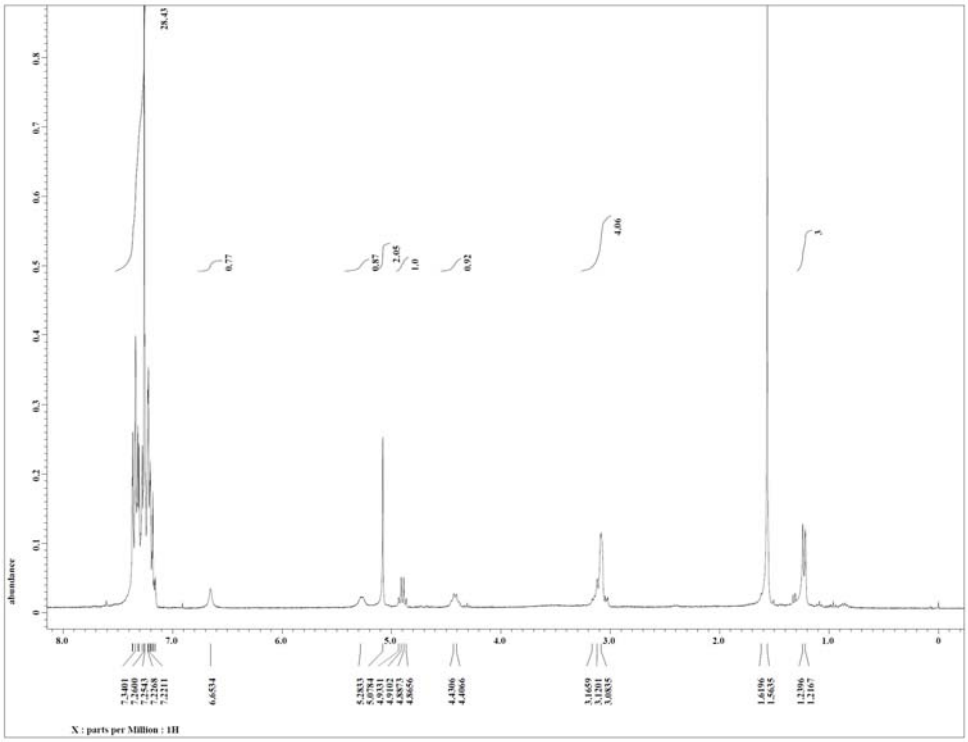
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4.9331
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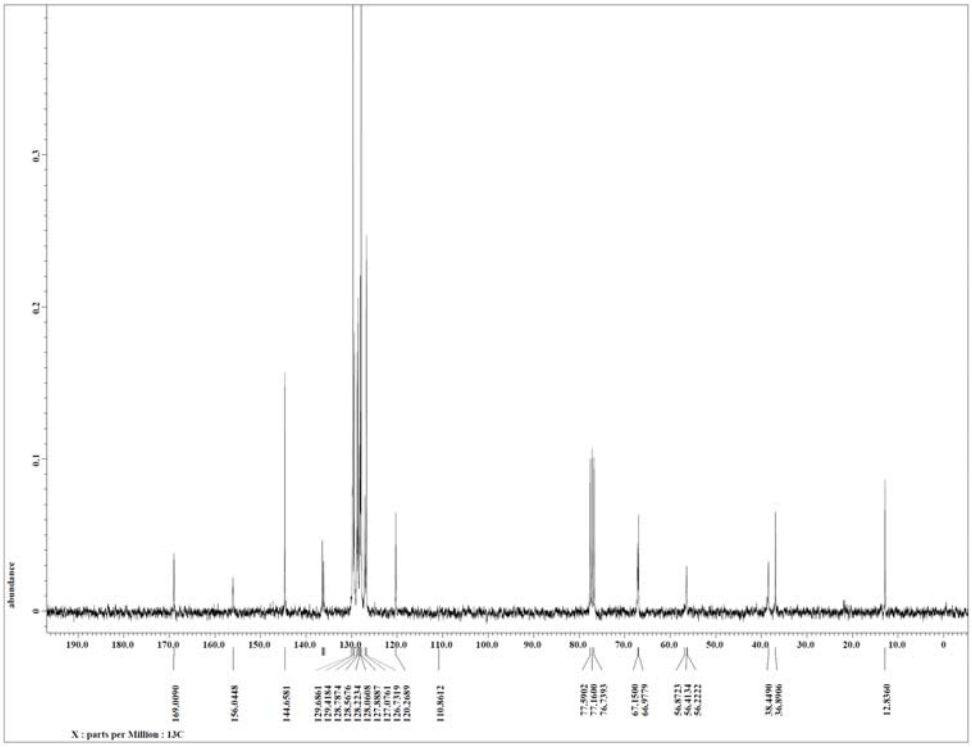
X : parts per Million : 1H



Peking University Mass Spectrometry Sample Analysis Report

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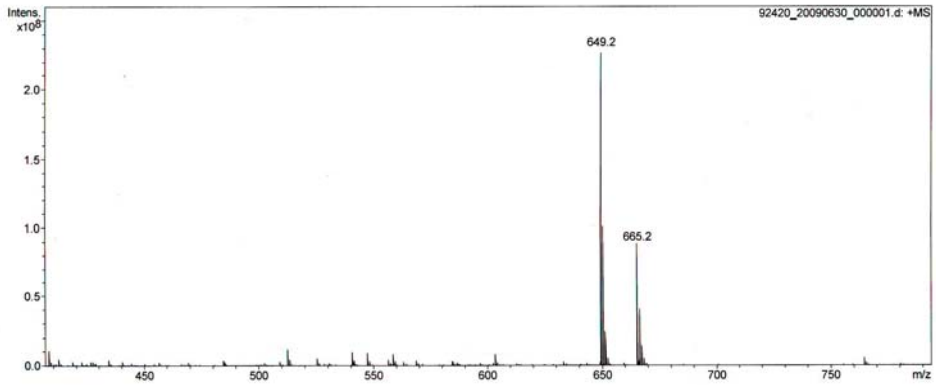

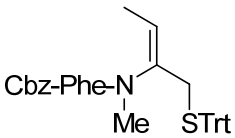

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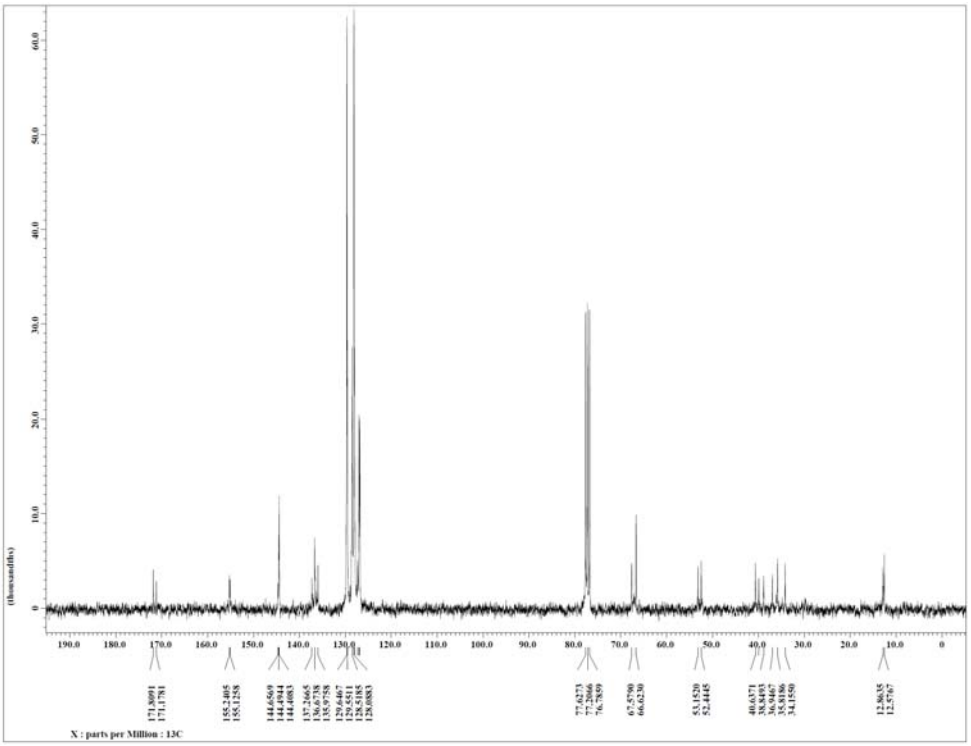

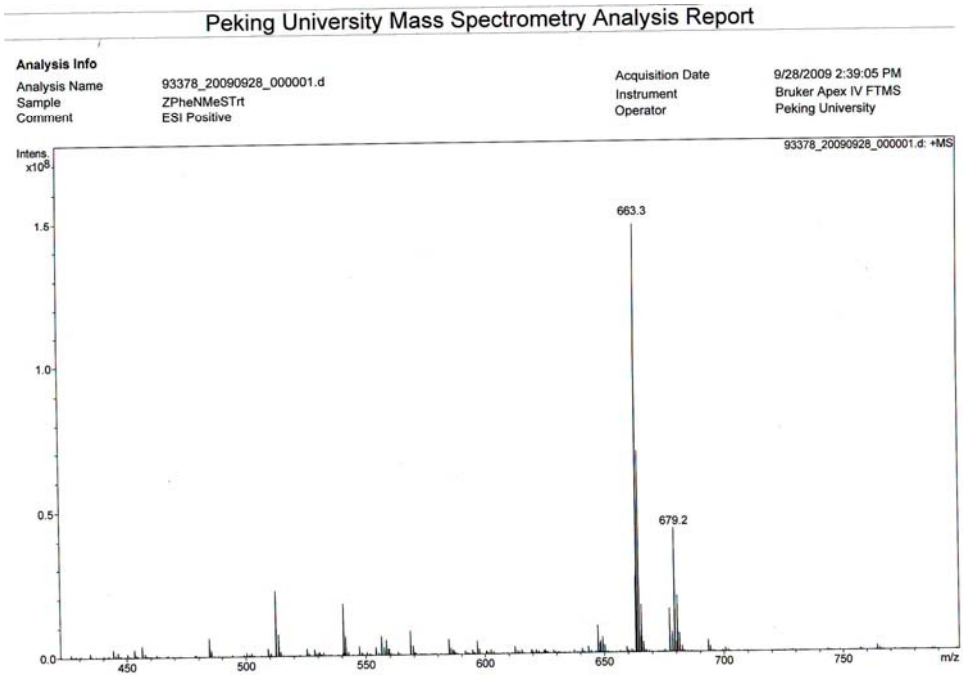

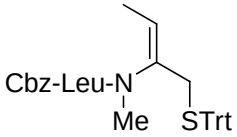

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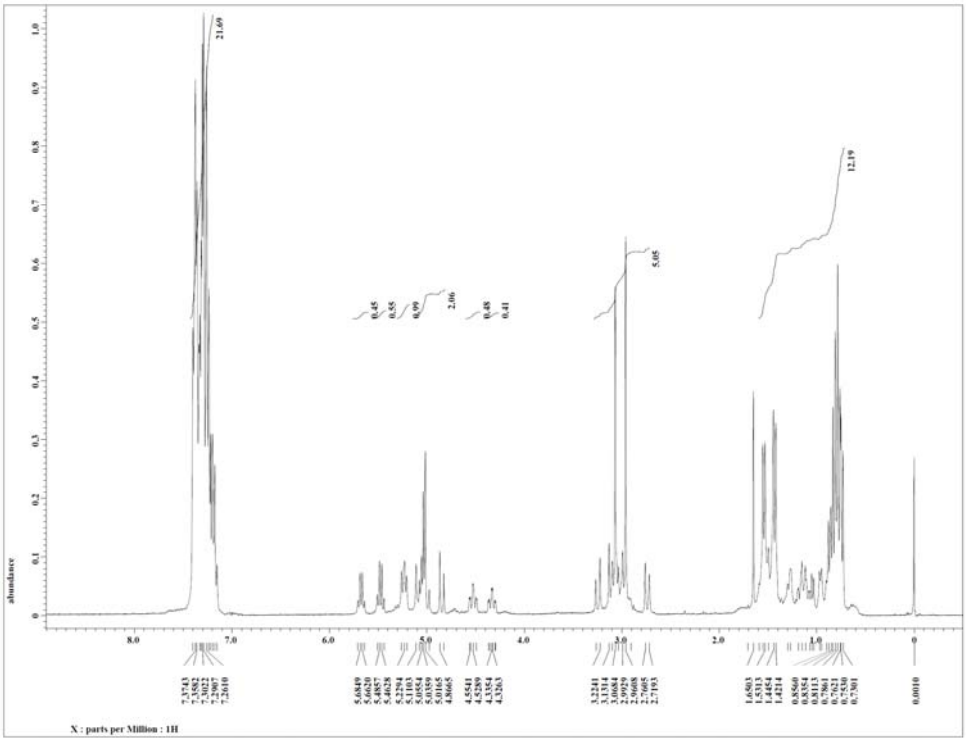
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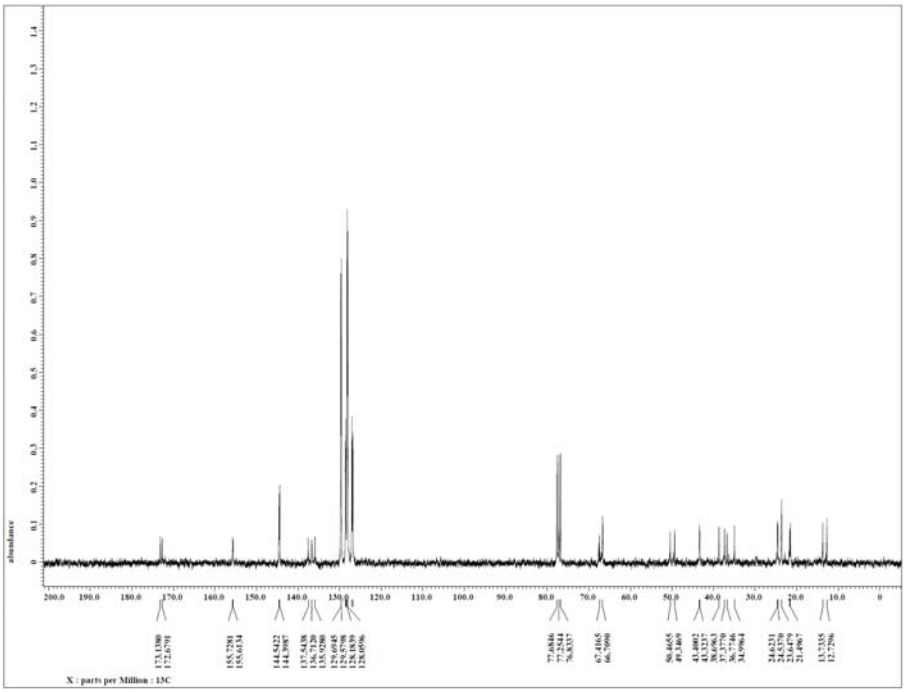
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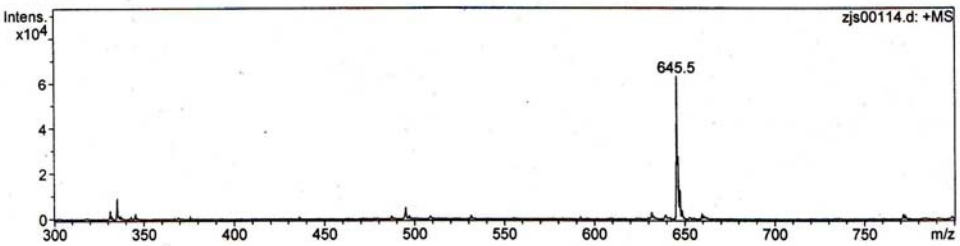

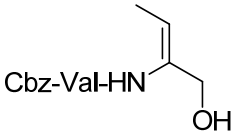

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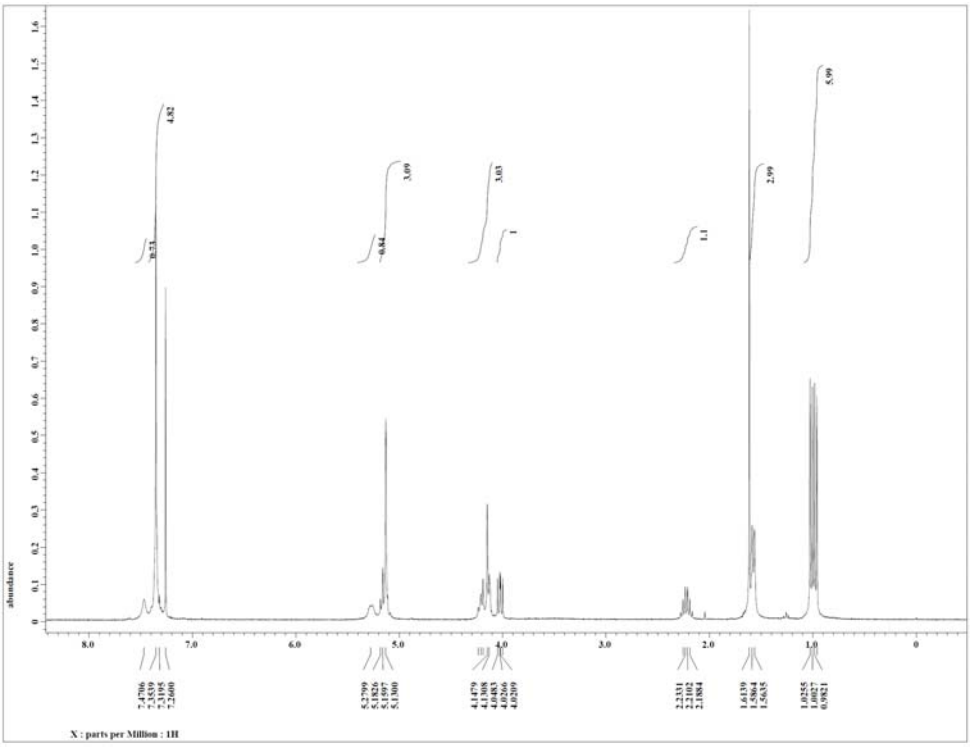

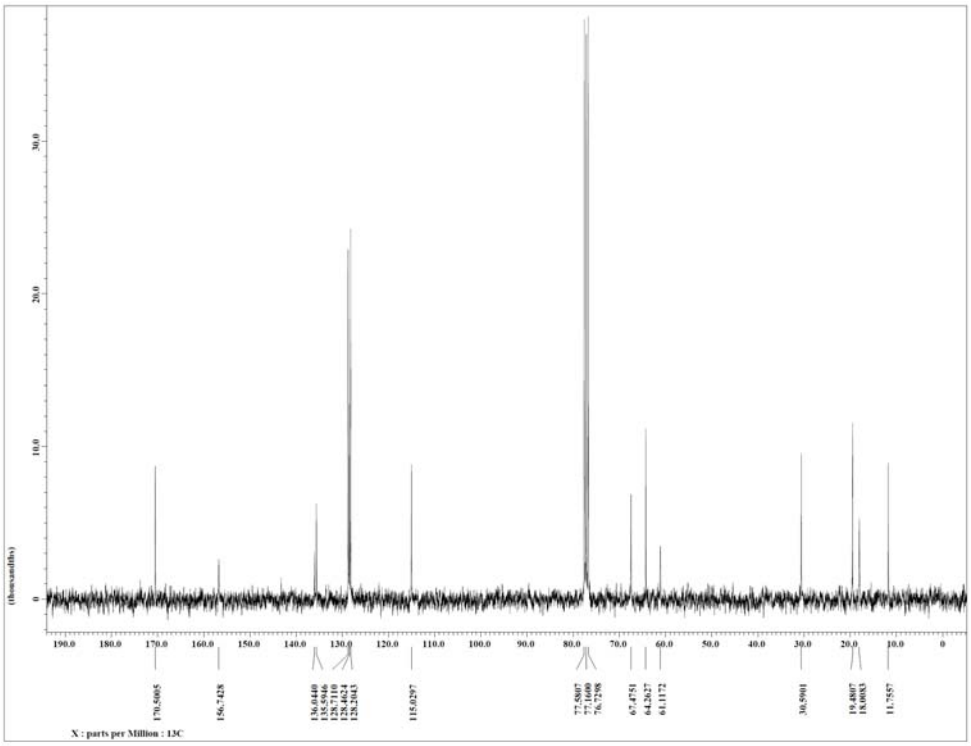

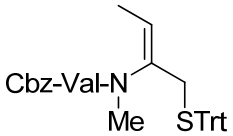

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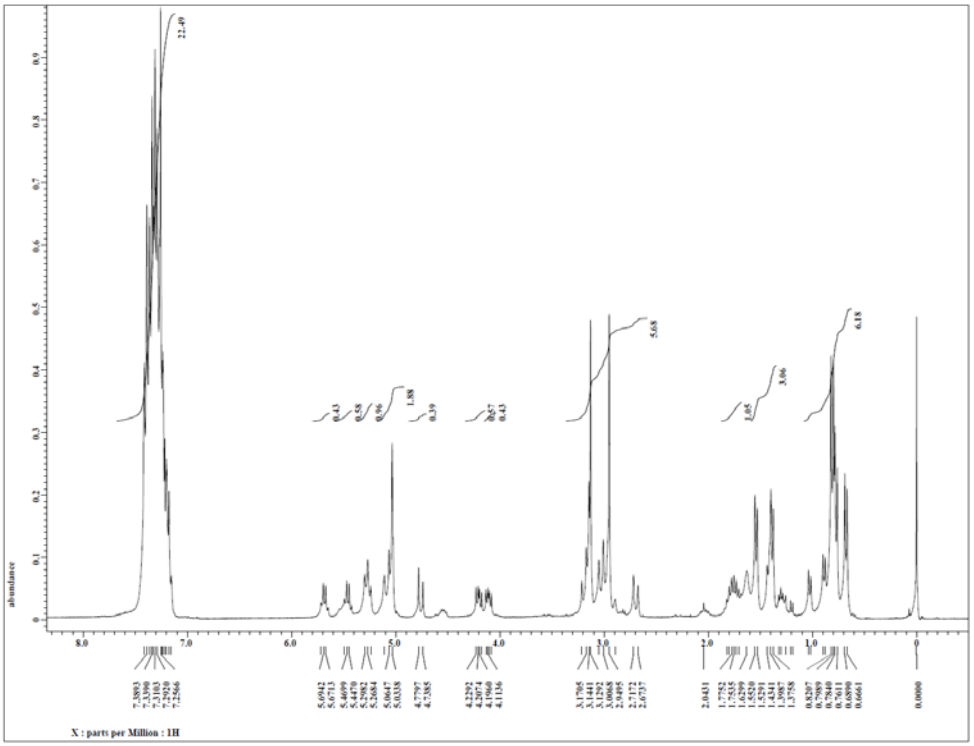

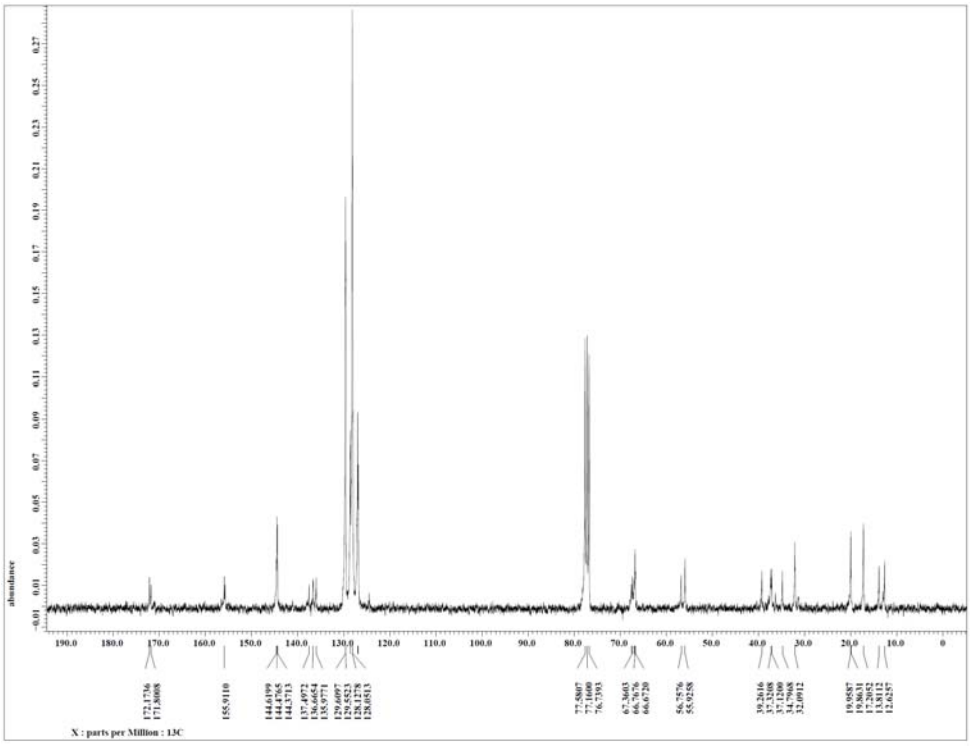

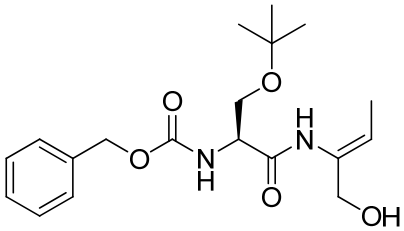

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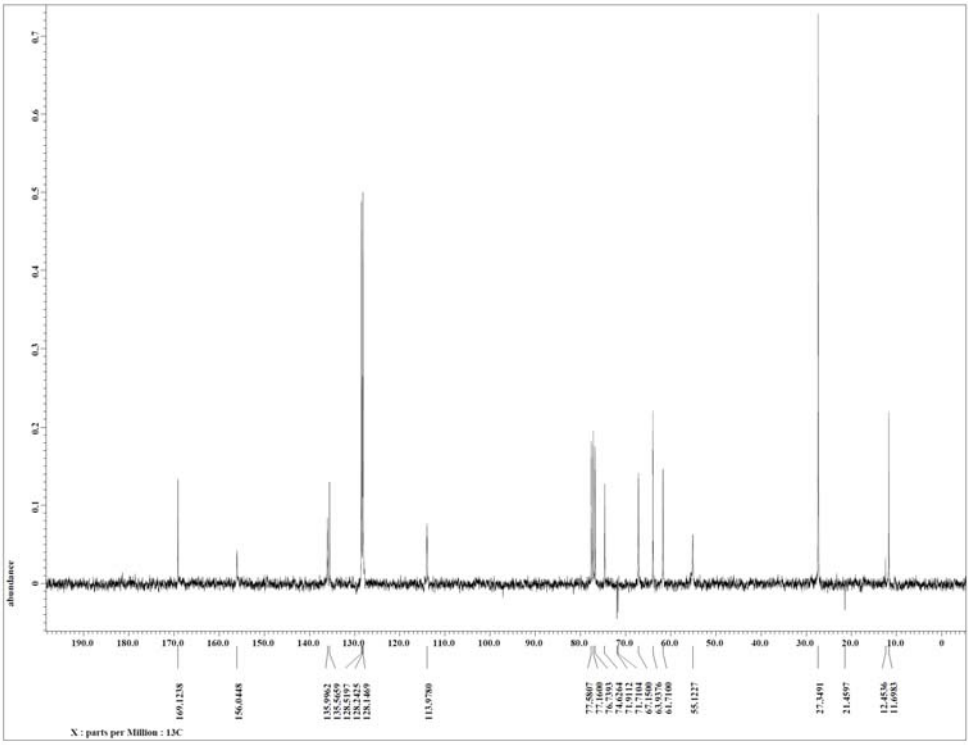

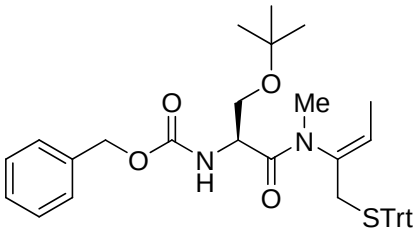

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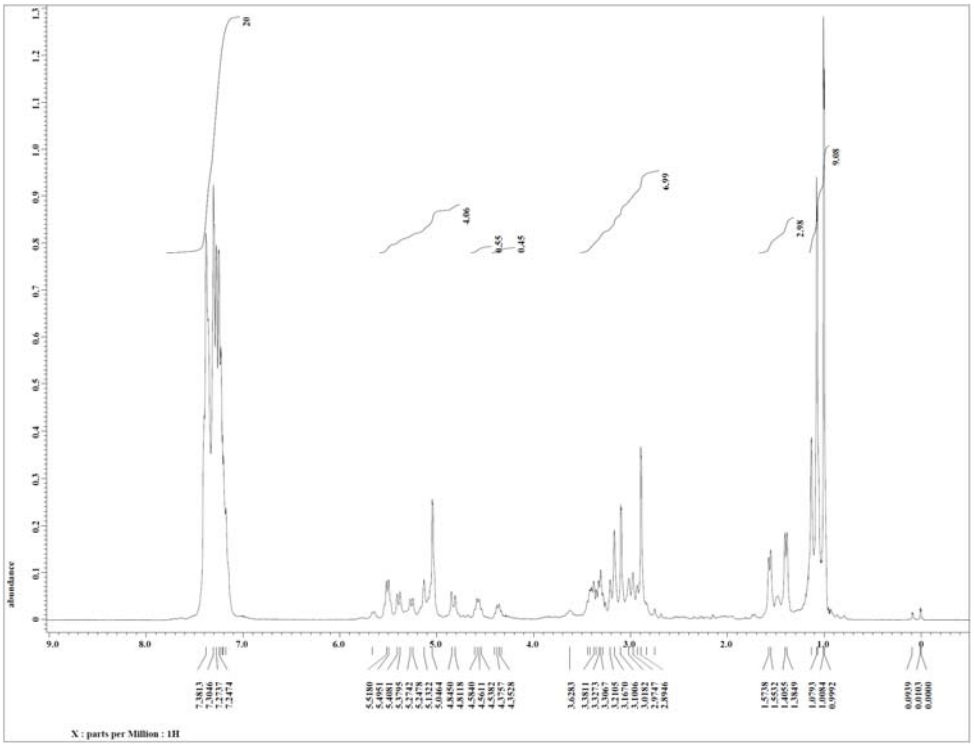

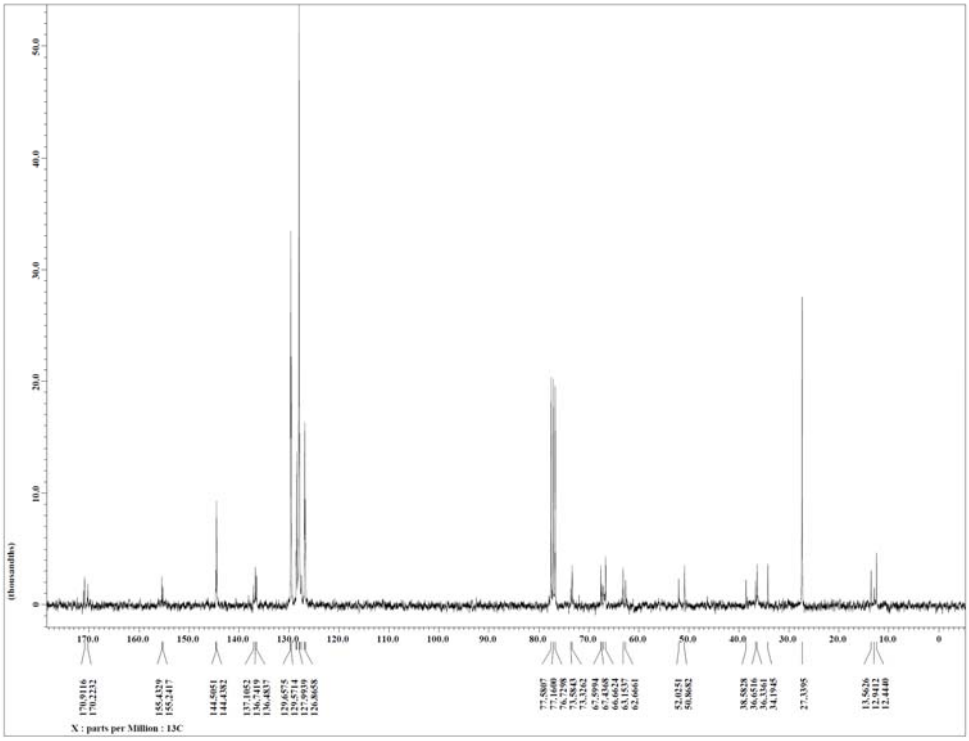

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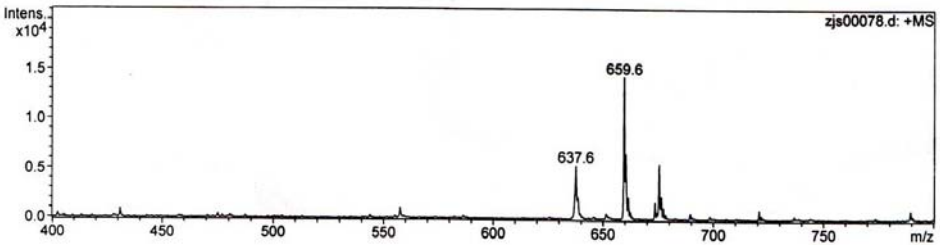

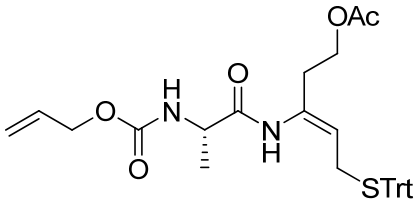

(*S*,*Z*)-3-(2-(allyloxycarbonylamino)propanamido)-5-(tritylthio)pent-3-enyl acetate

Mass Spectrum List Report

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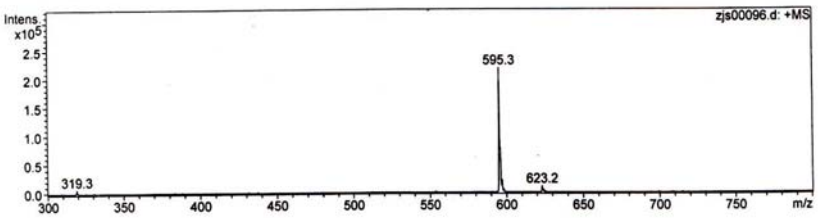

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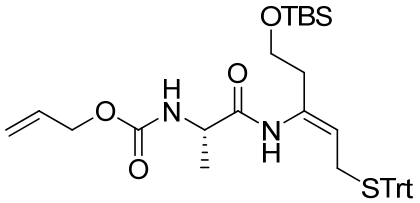
4	597.0	22187	0.5	309.4
5	598.2	5373	0.4	74.9
6	623.2	10608	0.6	147.9
7	624.2	4894	0.4	68.2



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Mass Spectrum List Report

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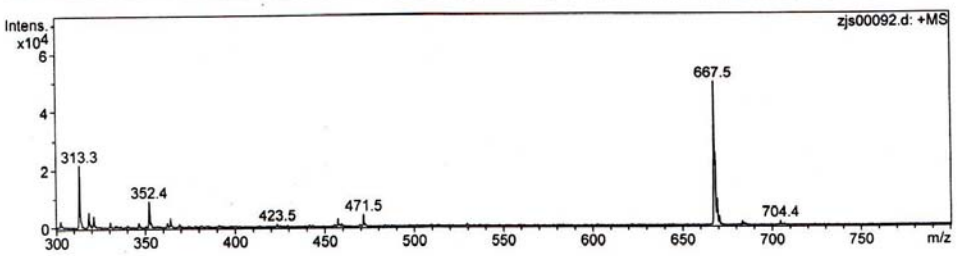

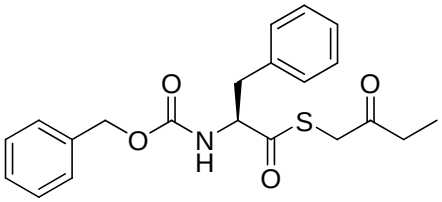

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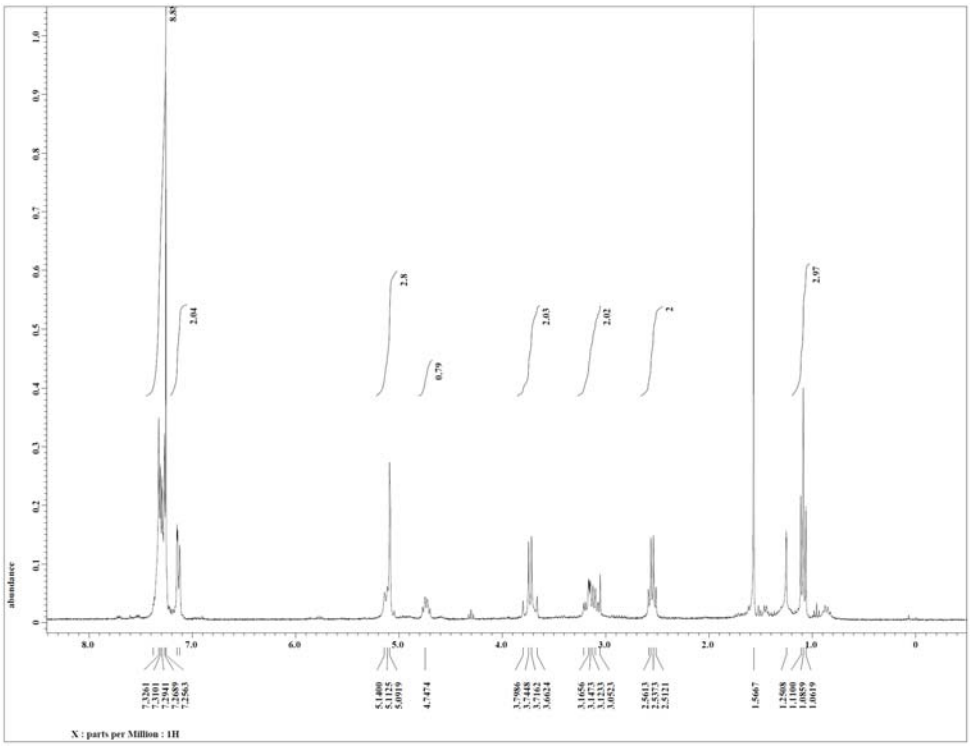
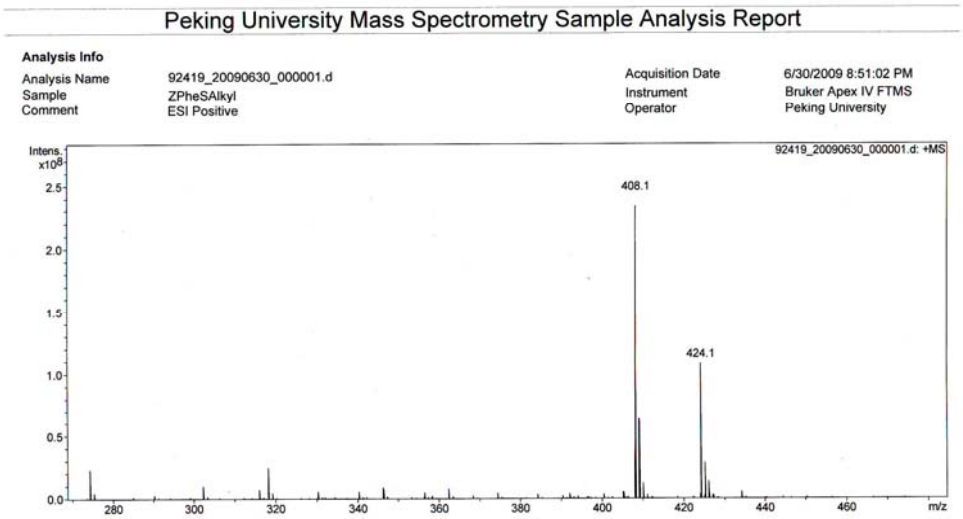
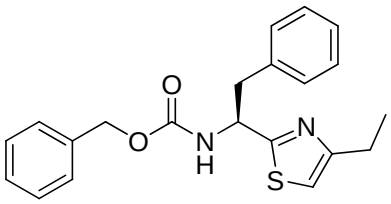
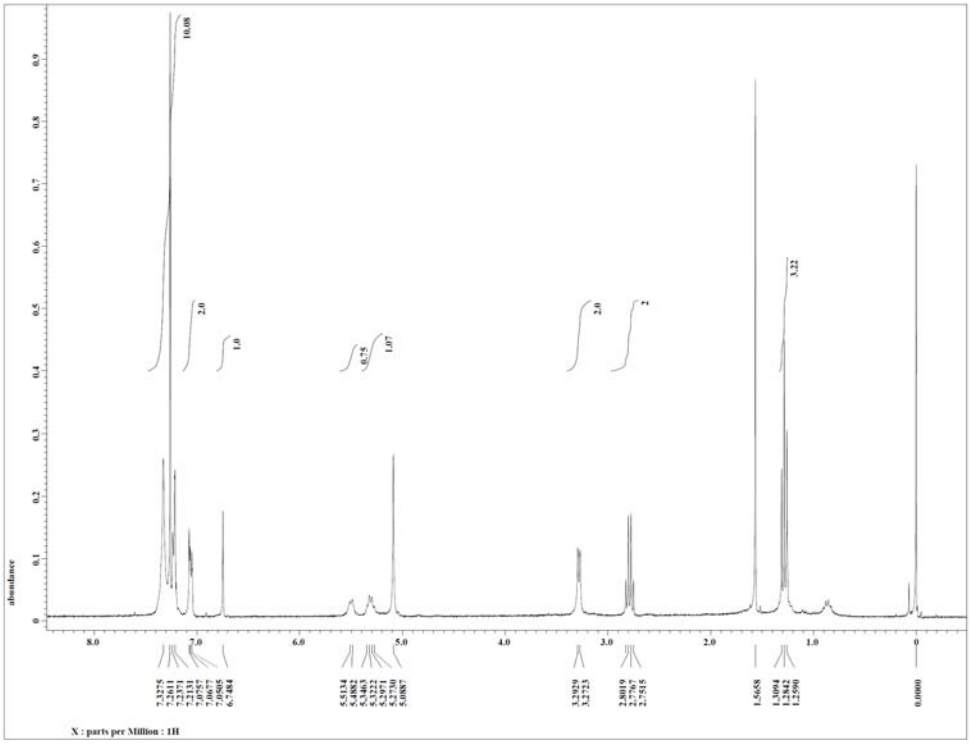






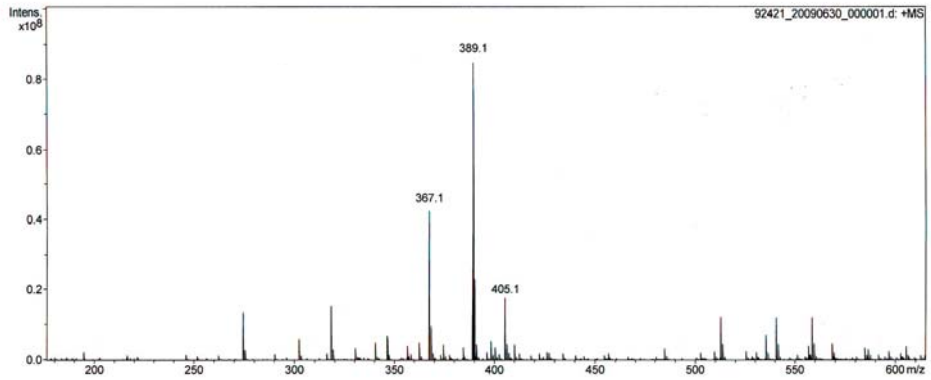
(*S*)-benzyl 1-(4-ethylthiazol-2-yl)-2-phenylethylcarbamate

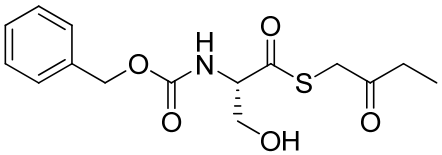


Peking University Mass Spectrometry Sample Analysis Report

Analysis Info

Analysis Name	92421_20090630_000001.d	Acquisition Date	6/30/2009 8:54:06 PM
Sample	ZPheSAlkyl-1	Instrument	Bruker Apex IV FTMS
Comment	ESI Positive	Operator	Peking University





(*S*)-*S*-2-oxobutyl 2-(benzyloxycarbonylamino)-3-hydroxypropanethioate

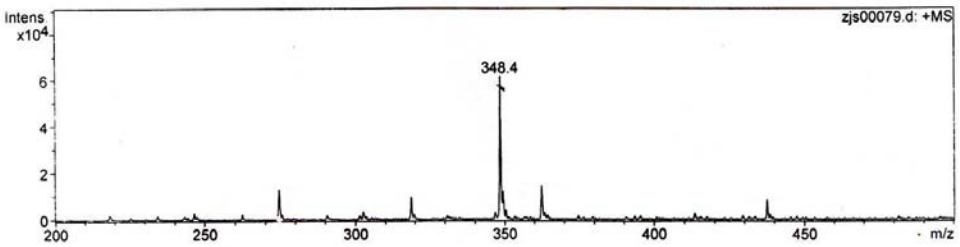
Mass Spectrum List Report

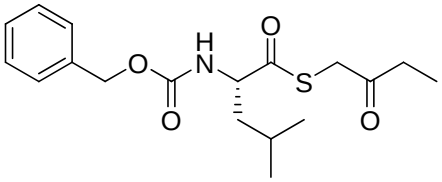
Analysis Info

Analysis Name	zjs00079.d	Acquisition Date	04/07/10 09:53:33
Method	XQ Default.ms	Operator	Administrator
Sample Name	Default	Instrument	Esquire-LC_00136
Comment	353		

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	n/a
Mass Range Mode	Std/Normal	Scan Begin	200.00 m/z	Scan End	500.00 m/z
Capillary Exit	103.5 Volt	Skim 1	31.6 Volt	Trap Drive	34.1
Accumulation Time	13213 µs	Averages	8 Spectra	Auto MS/MS	Off





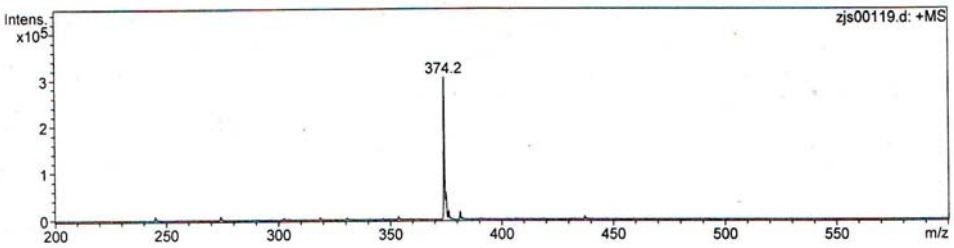
(*S*)-*S*-2-oxobutyl 2-(benzyloxycarbonylamino)-4-methylpentanethioate

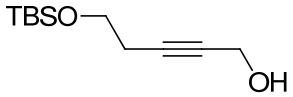
Analysis Info

Analysis Name	zjs00119.d	Acquisition Date	06/10/10 11:11:37
Method	XQ Default.ms	Operator	Administrator
Sample Name	Default	Instrument	Esquire-LC_00136
Comment	351xia		

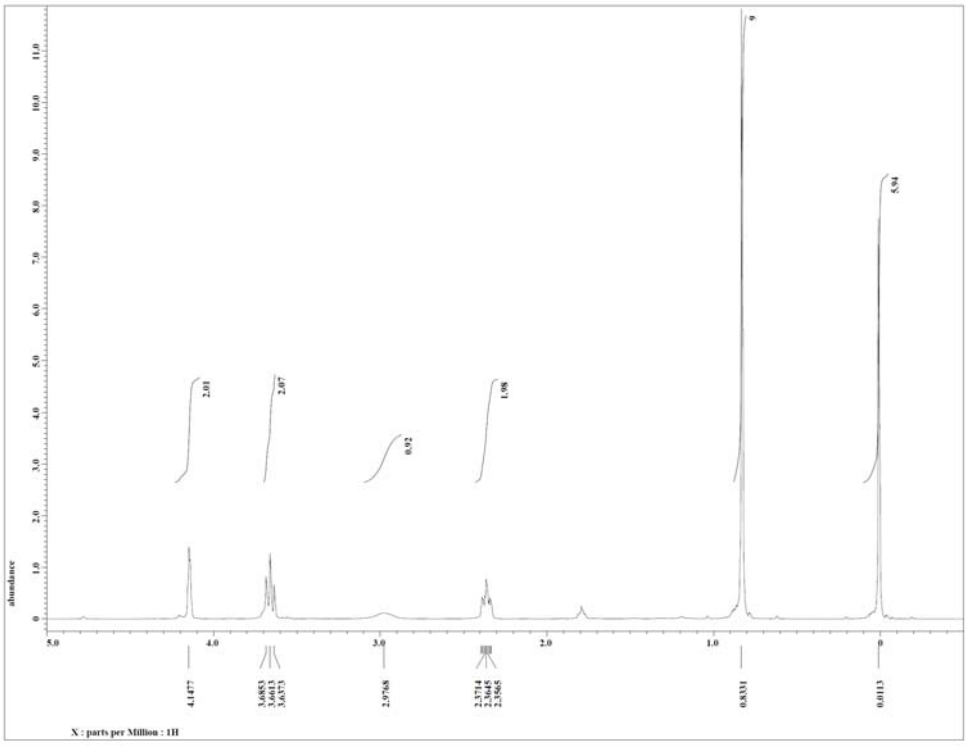
Acquisition Parameter

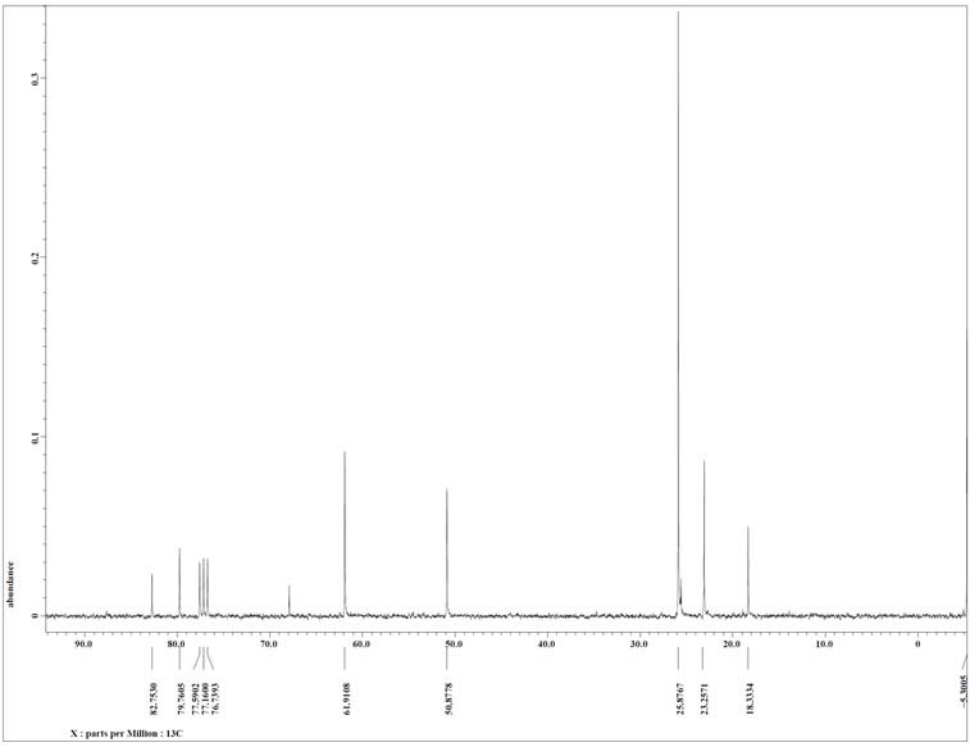
Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	n/a
Mass Range Mode	Std/Normal	Scan Begin	200.00 m/z	Scan End	600.00 m/z
Capillary Exit	109.2 Volt	Skim 1	35.7 Volt	Trap Drive	34.0
Accumulation Time	6791 µs	Averages	10 Spectra	Auto MS/MS	Off

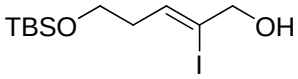




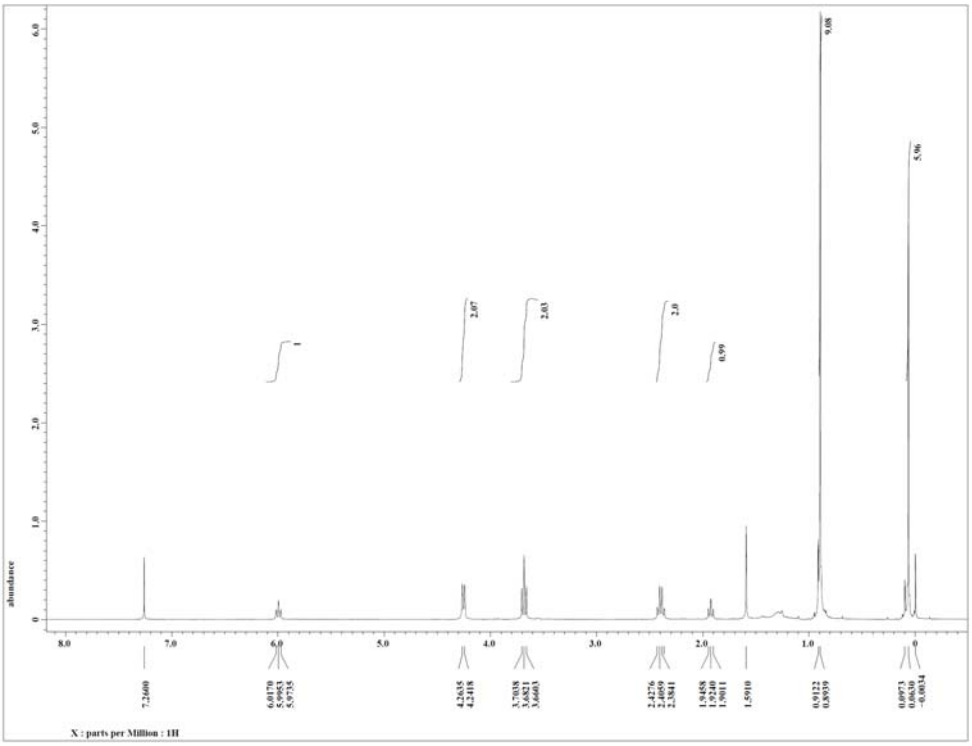
5-((*tert*-butyldimethylsilyl)oxy)pent-2-yn-1-ol

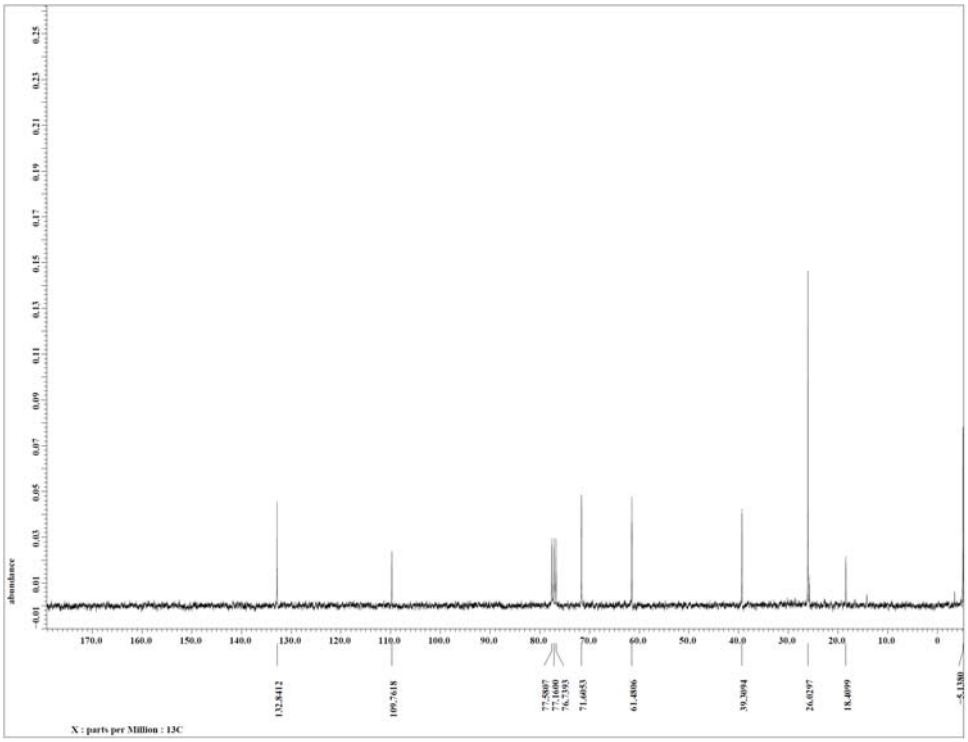


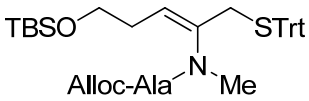




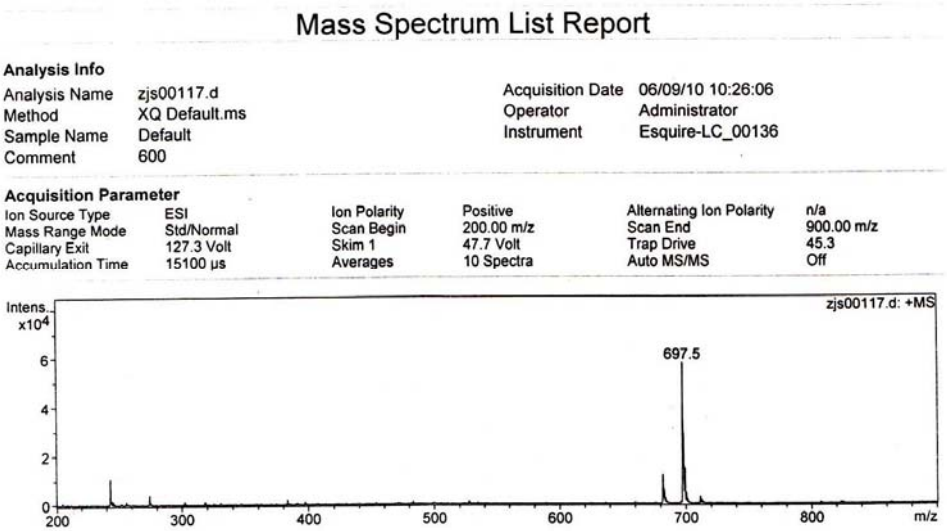
(*Z*)-5-(*tert*-butyldimethylsilyloxy)-2-iodopent-2-en-1-ol







(*Z*)-allyl 1-((5-(*tert*-butyldimethylsilyloxy)-1-(tritylthio)pent-2-en-2-yl)(methyl)amino)-1-oxopropan-2-ylcarbamate



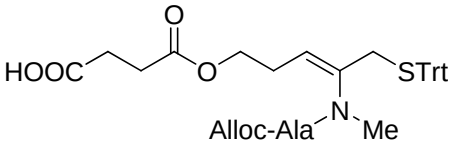
Mass Spectrum List Report

Analysis Info

Analysis Name	zjs00117.d	Acquisition Date	06/09/10 10:26:06
Method	XQ Default.ms	Operator	Administrator
Sample Name	Default	Instrument	Esquire-LC_00136
Comment	600		

Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	n/a
Mass Range Mode	Std/Normal	Scan Begin	200.00 m/z	Scan End	900.00 m/z
Capillary Exit	127.3 Volt	Skim 1	47.7 Volt	Trap Drive	45.3
Accumulation Time	15100 µs	Averages	10 Spectra	Auto MS/MS	Off



(*Z*)-7,9-dimethyl-5,8,15-trioxo-10-(tritylthiomethyl)-4,14-dioxa-6,9-diazaoctadeca-1,10-dien-18-oic acid

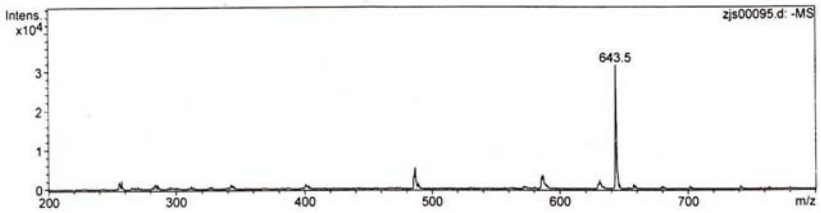
Mass Spectrum List Report

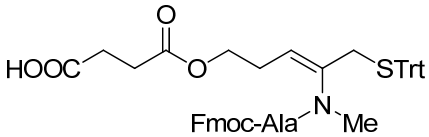
Analysis Info

Analysis Name	zjs00095.d	Acquisition Date	05/11/10 10:59:45
Method	XQ Default.ms	Operator	Administrator
Sample Name	Default	Instrument	Esquire-LC_00136
Comment	1		

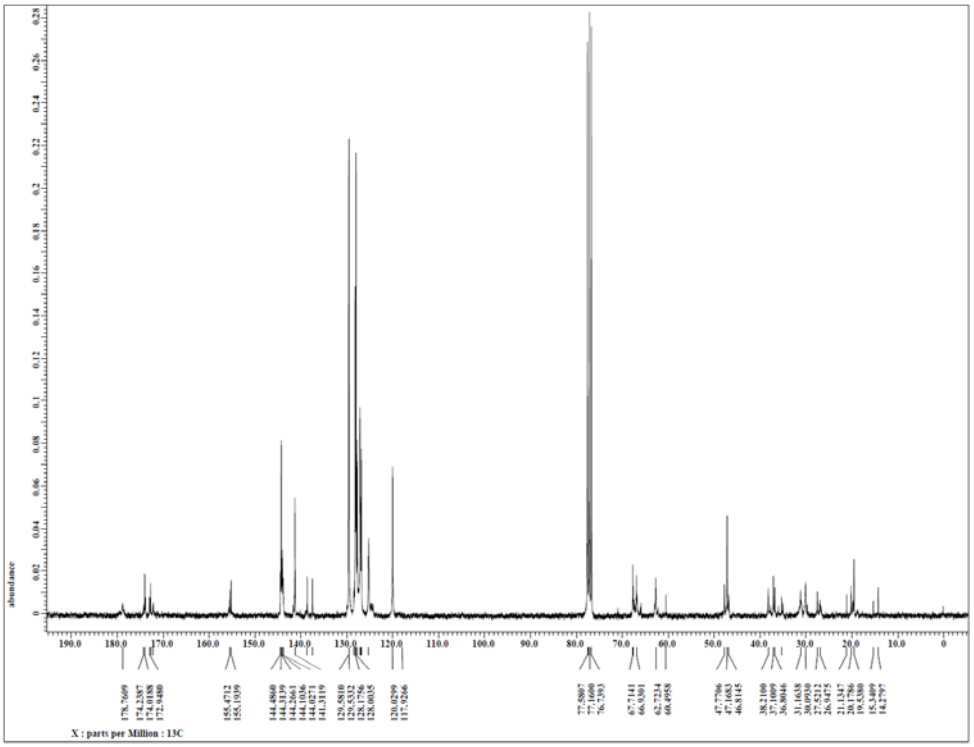
Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Negative	Alternating Ion Polarity	n/a
Mass Range Mode	Std/Normal	Scan Begin	200.00 m/z	Scan End	800.00 m/z
Capillary Exit	-123.5 Volt	Skim 1	-45.2 Volt	Trap Drive	44.6
Accumulation Time	17053 µs	Averages	5 Spectra	Auto MS/MS	Off





(*Z*)-4-(4-(2-(4-((9*H*-fluoren-9-yl)methoxy)-3,4-dioxobutan-2-yl)-1-methylhydrazinyl)-5-(tritylthio)pent-3-enyloxy)-4-oxobutanoic acid



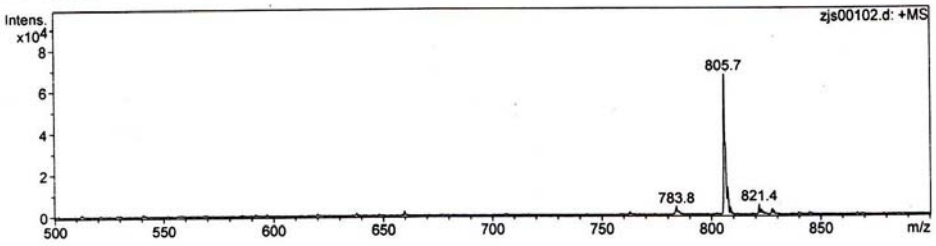
Mass Spectrum List Report

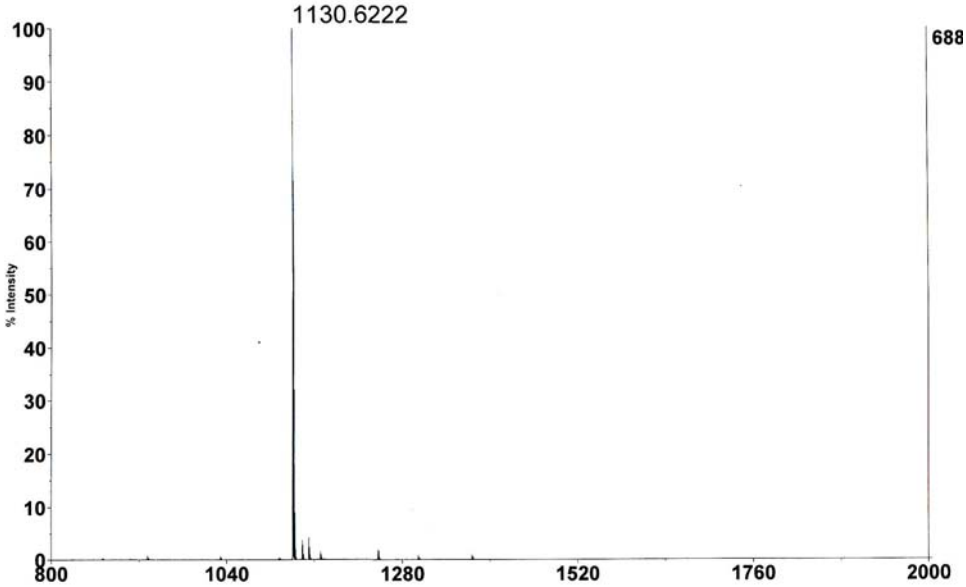
Analysis Info

Analysis Name	zjs00102.d	Acquisition Date	05/21/10 11:17:18
Method	XQ Default.ms	Operator	Administrator
Sample Name	Default	Instrument	Esquire-LC_00136
Comment	1		

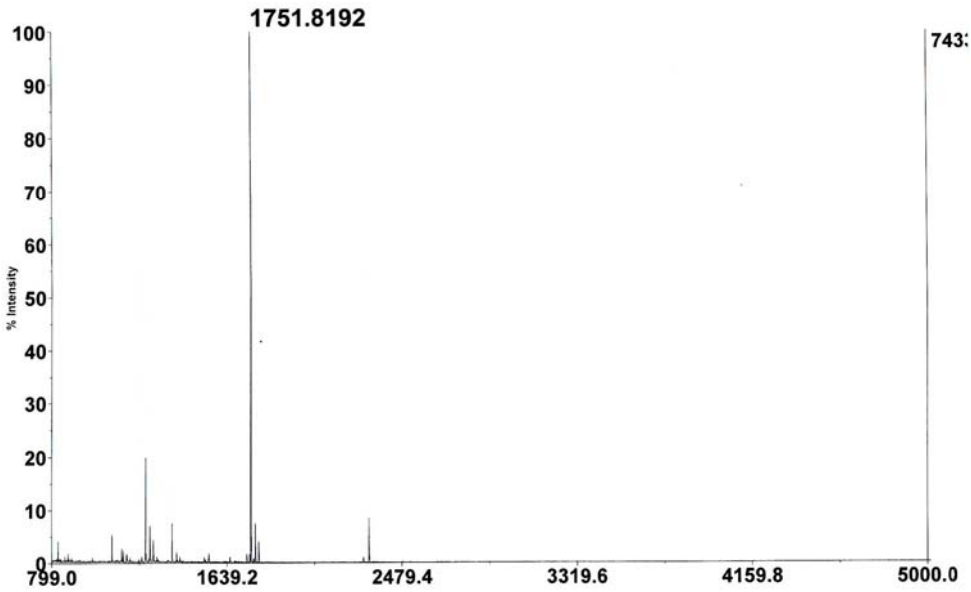
Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	n/a
Mass Range Mode	Std/Normal	Scan Begin	500.00 m/z	Scan End	900.00 m/z
Capillary Exit	133.8 Volt	Skim 1	51.8 Volt	Trap Drive	49.8
Accumulation Time	14564 µs	Averages	5 Spectra	Auto MS/MS	Off

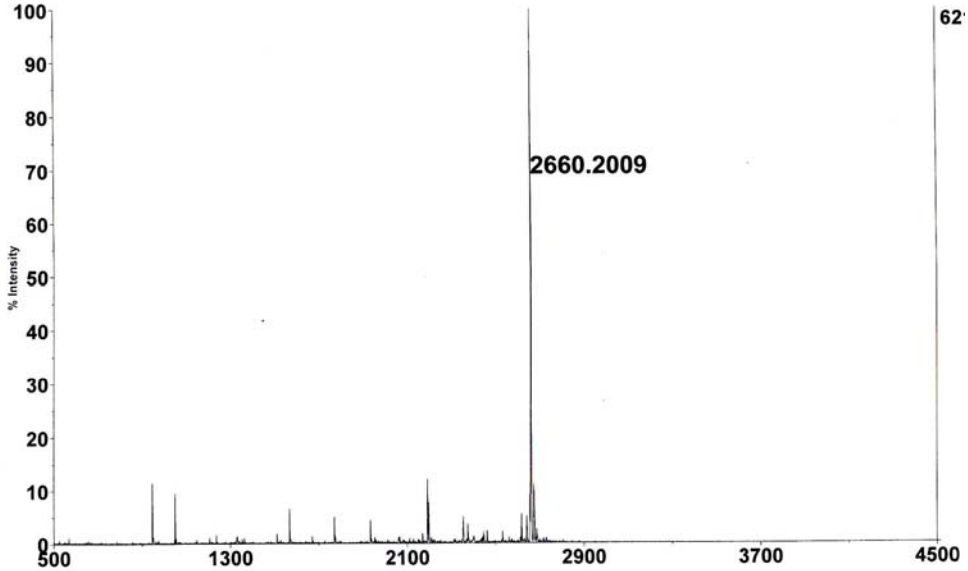




The MALDI-TOF/MS of of peptide thioesters **11**

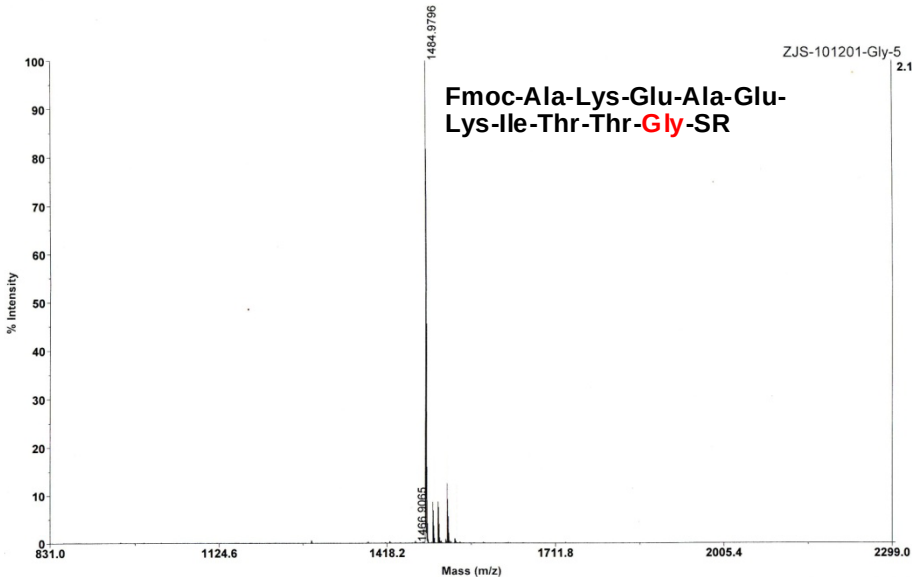


The MALDI-TOF/MS of of crude glycopeptide thioesters **13**

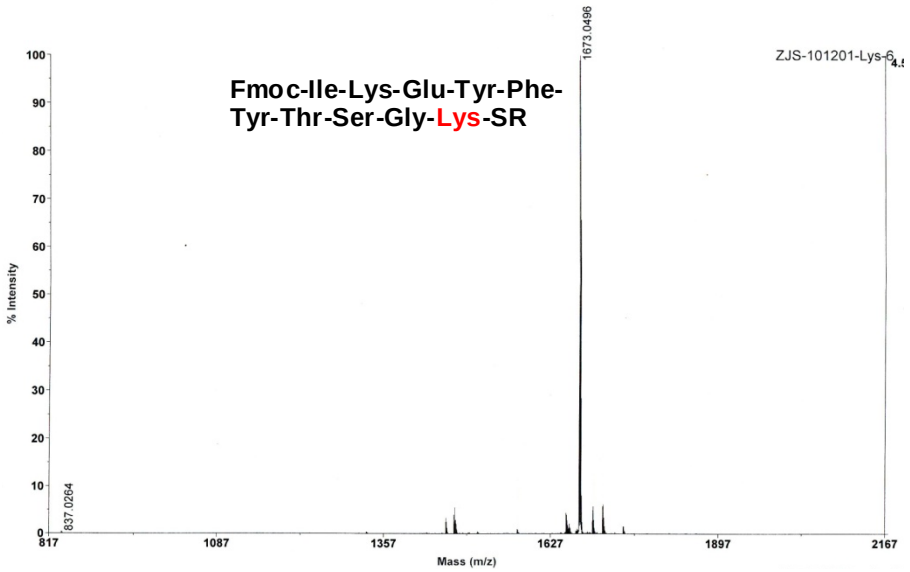


The MALDI-TOF/MS of of glycopeptide thioesters **14**

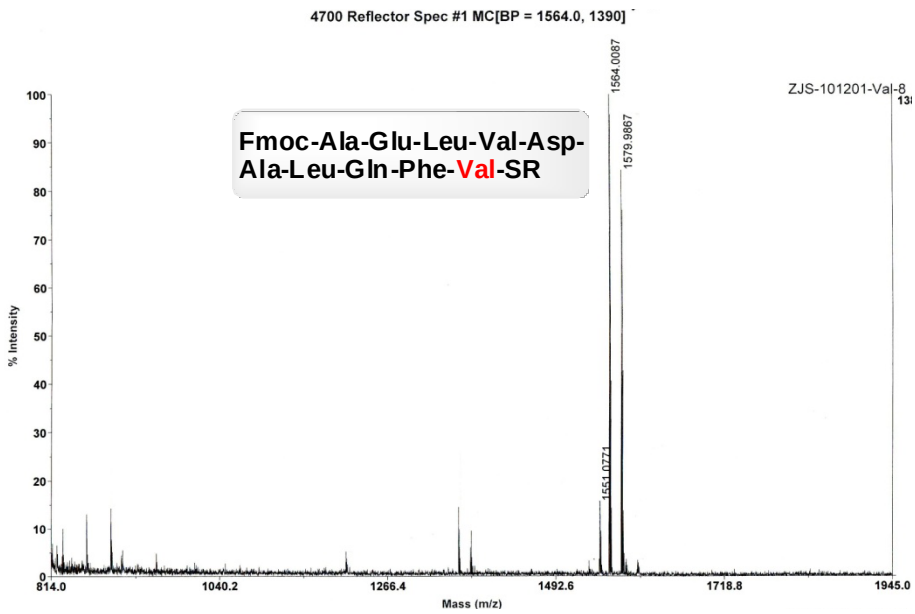
Fmoc-**Met**-Phe-Val-Phe-Ala-Val-
Arg-Thr-Thr-Gly-Ile-**Phe**-SR



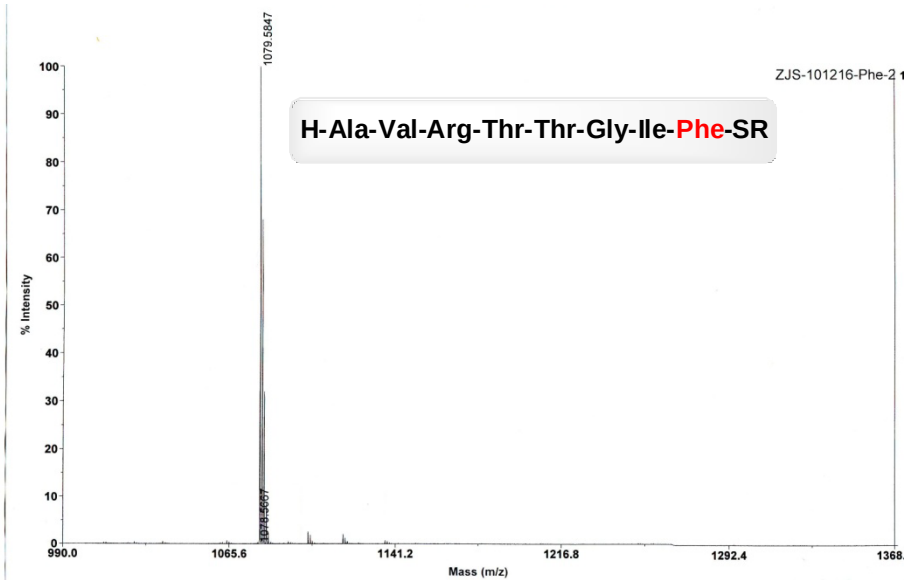
The MALDI-TOF/MS of of peptide Gly-thioesters



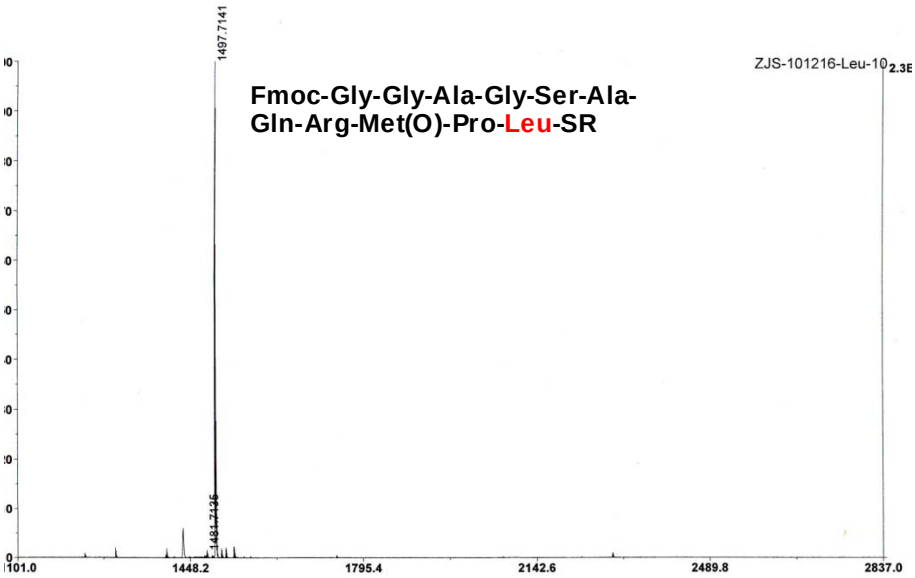
The MALDI-TOF/MS of of peptide Lys-thioesters



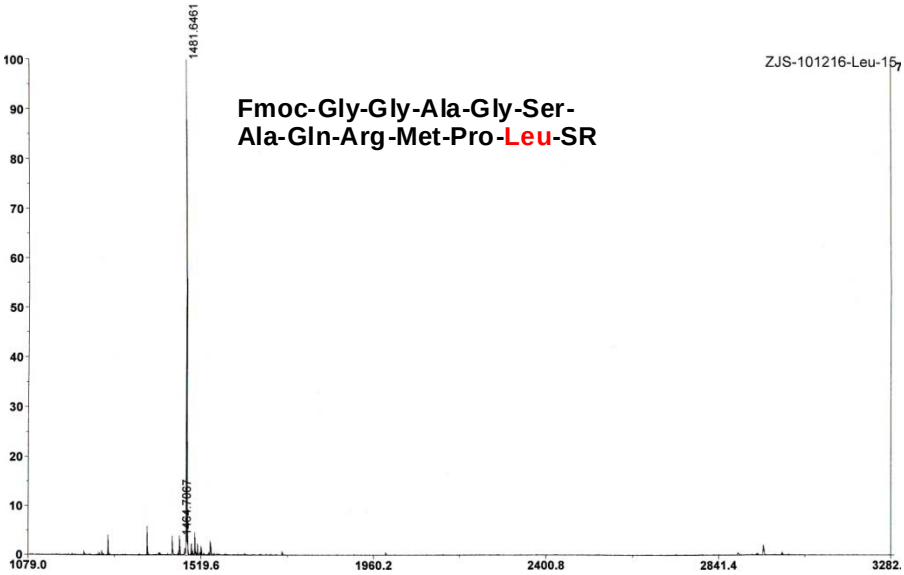
The MALDI-TOF/MS of of peptide Val-thioesters



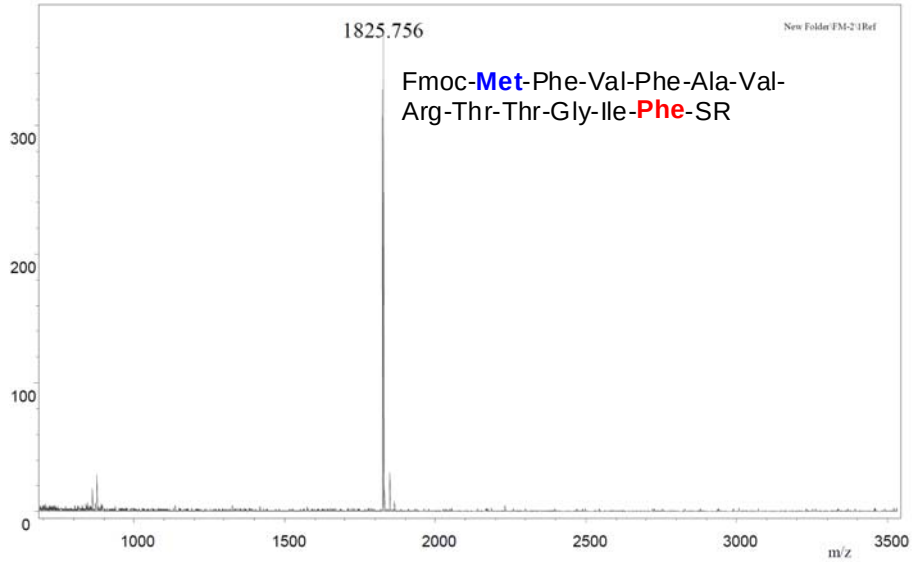
The MALDI-TOF/MS of of peptide Phe-thioesters



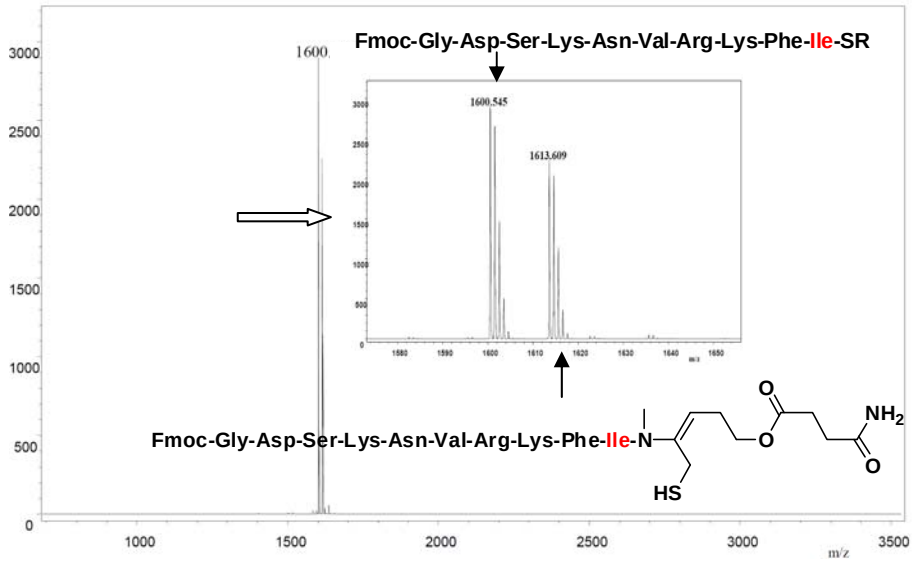
The MALDI-TOF/MS of of peptide Leu-thioesters



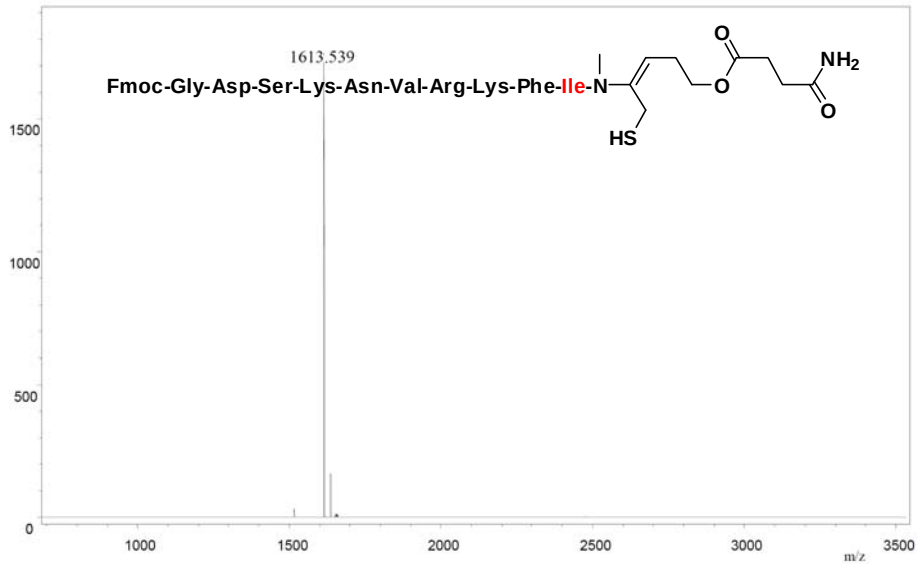
The MALDI-TOF/MS of of peptide Leu-thioesters



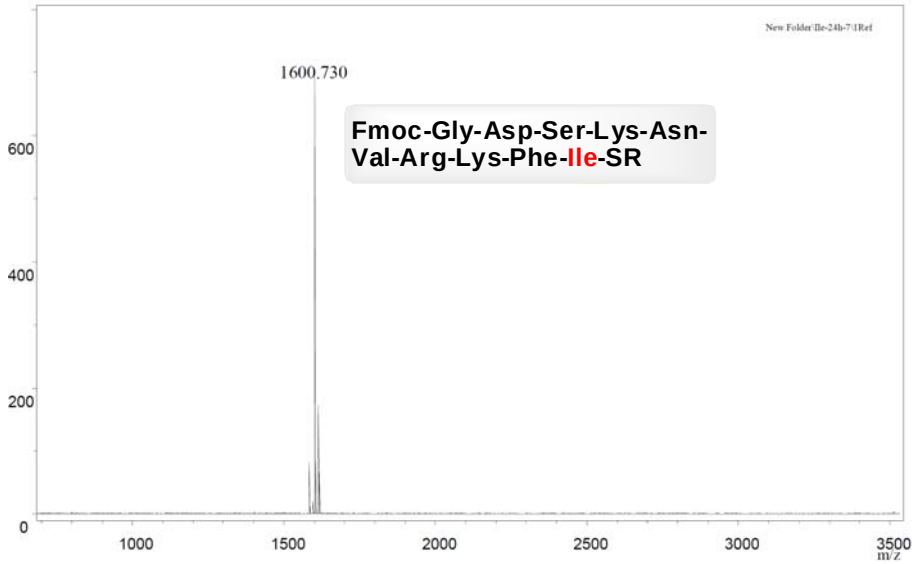
The MALDI-TOF/MS of of peptide Phe-thioesters



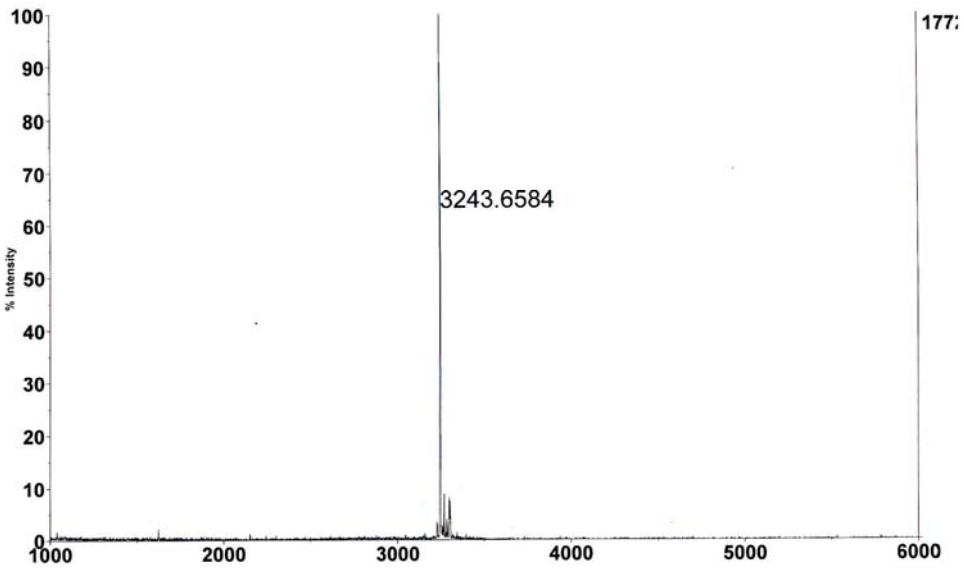
The MALDI-TOF/MS of of peptide Ile-thioesters and its precursors.



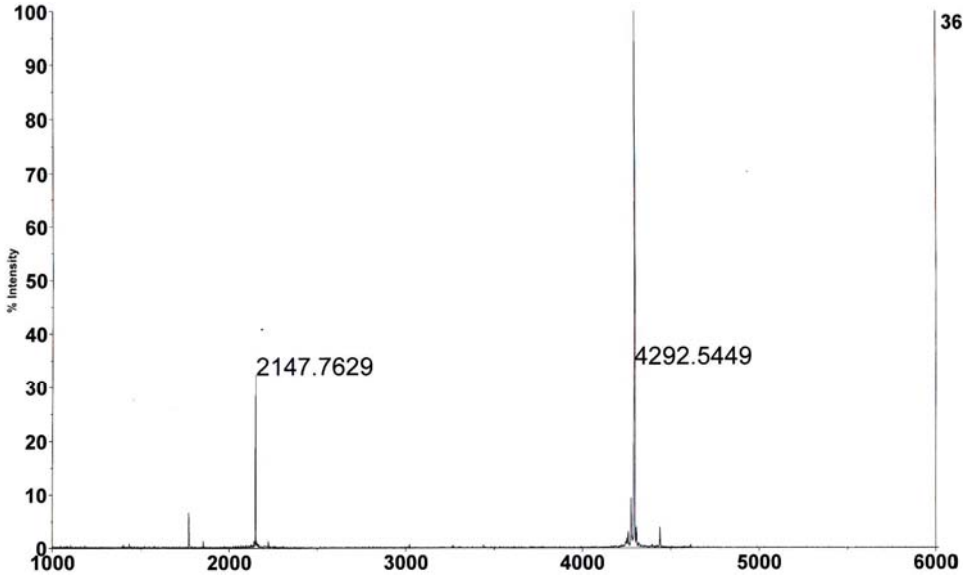
The MALDI-TOF/MS of of peptide Ile-thioester precursors



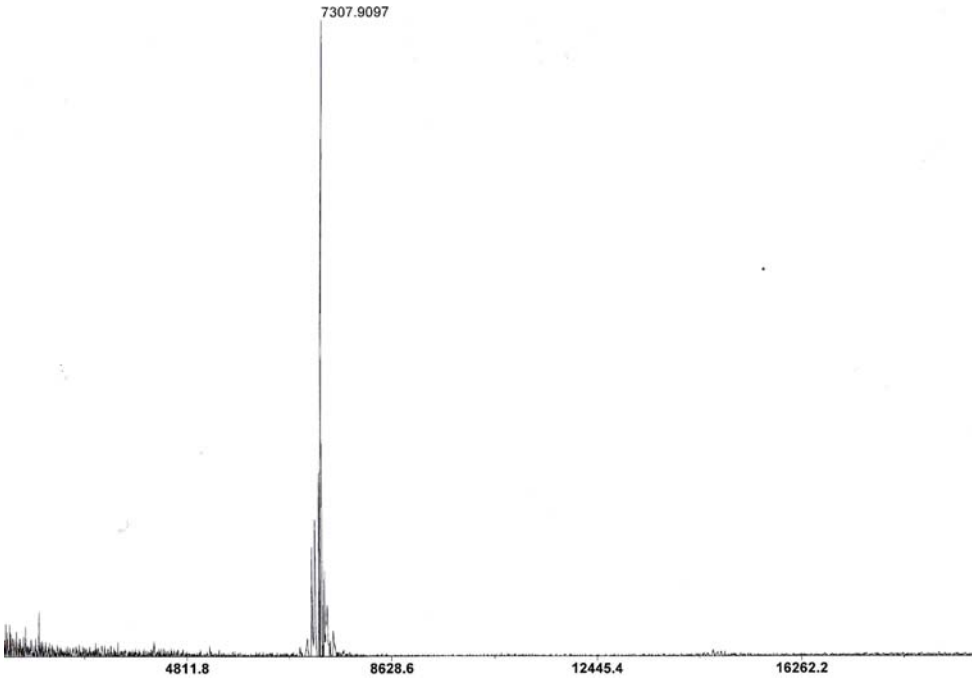
The MALDI-TOF/MS of of peptide Ile-thioesters



The MALDI-TOF/MS of peptide thioesters **15**



The MALDI-TOF/MS of the Cys-peptide **16**



MALDI-TOF/MS of the The Human Cox17 protein **17**