

## ***Electronic Supplementary Information (ESI) for***

### **Peptide Ligation via Side-Chain Auxiliary**

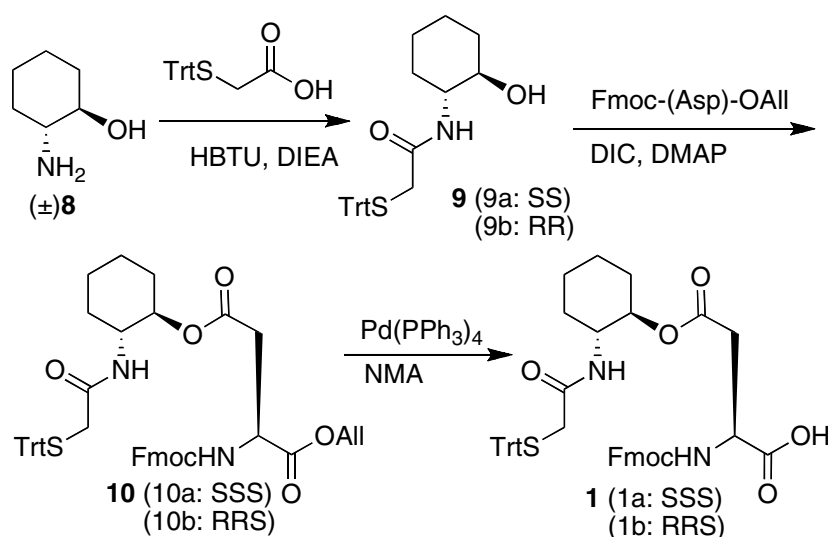
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**General:** All Chemical reagents were purchased from Aldrich or Acros and used without further purification. Amino acids and coupling reagents were purchased from Novabiochem. Tetrahydrofuran (THF) was distilled over sodium/ benzophenone, and methylene chloride ( $\text{CH}_2\text{Cl}_2$ ) was distilled over calcium hydride. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F<sub>254</sub> glass plates, compound spots were visualized by UV light (254 nm) and were stained with citric ammonium molybdate. Flash chromatography was performed on Silica- P flash silica gel.  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra were recorded on a Bruker AMX-500 MHz spectrometer.

### Synthesis of building block 1



**Synthesis of 9:** Compound **8** (1.0 g, 6.38 mmol) was added to a preactivated S-trityl-L-2-mercaptoacetic acid (2.13g, 6.38 mmol) with HBTU (2.53 g, 6.66 mmol) and DIEA (3.7 mL, 21.05 mmol) in dry DMF (15.0 mL). The reaction mixture was stirred at rt for overnight, diluted with 40 mL ethyl acetate and washed with water and brine. The organic layer was dried over  $\text{MgSO}_4$  and concentrated for flash column chromatography ( $\text{CHCl}_3/\text{MeOH}$  20:1), to give 1.3 g of **9** (50%).  $R_f$  0.73 [ $\text{CHCl}_3$ -MeOH (10:1)];  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ) 0.92-1.35 (4H, m,  $\text{CH}_2$ cyclohexane), 1.55-1.75 (3H, m,  $\text{CH}_2$  cyclohexane), 1.9 (1H, m,  $\text{CH}_2$ cyclohexane), 2.79 (2H, s,  $\text{CH}_2$  STrt), 3.09-3.2 (2H, m, NH  $\text{CH}$ cyclohexane, OH), 3.28-3.38 (1H, m, O  $\text{CH}$ cyclohexane), 6.07 (1H, d,  $J$  = 6.8 Hz NH), 7.18-7.35 (9H, m,  $\text{CH}$ Trt), 7.35-7.43 (6H, m,  $\text{CH}$ Trt);  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ ) 23.8, 24.36, 30.92, 34.05 ( $\text{CH}_2$ cyclohexane), 35.92 ( $\text{CH}_2$  STrt), 55.97 (NH

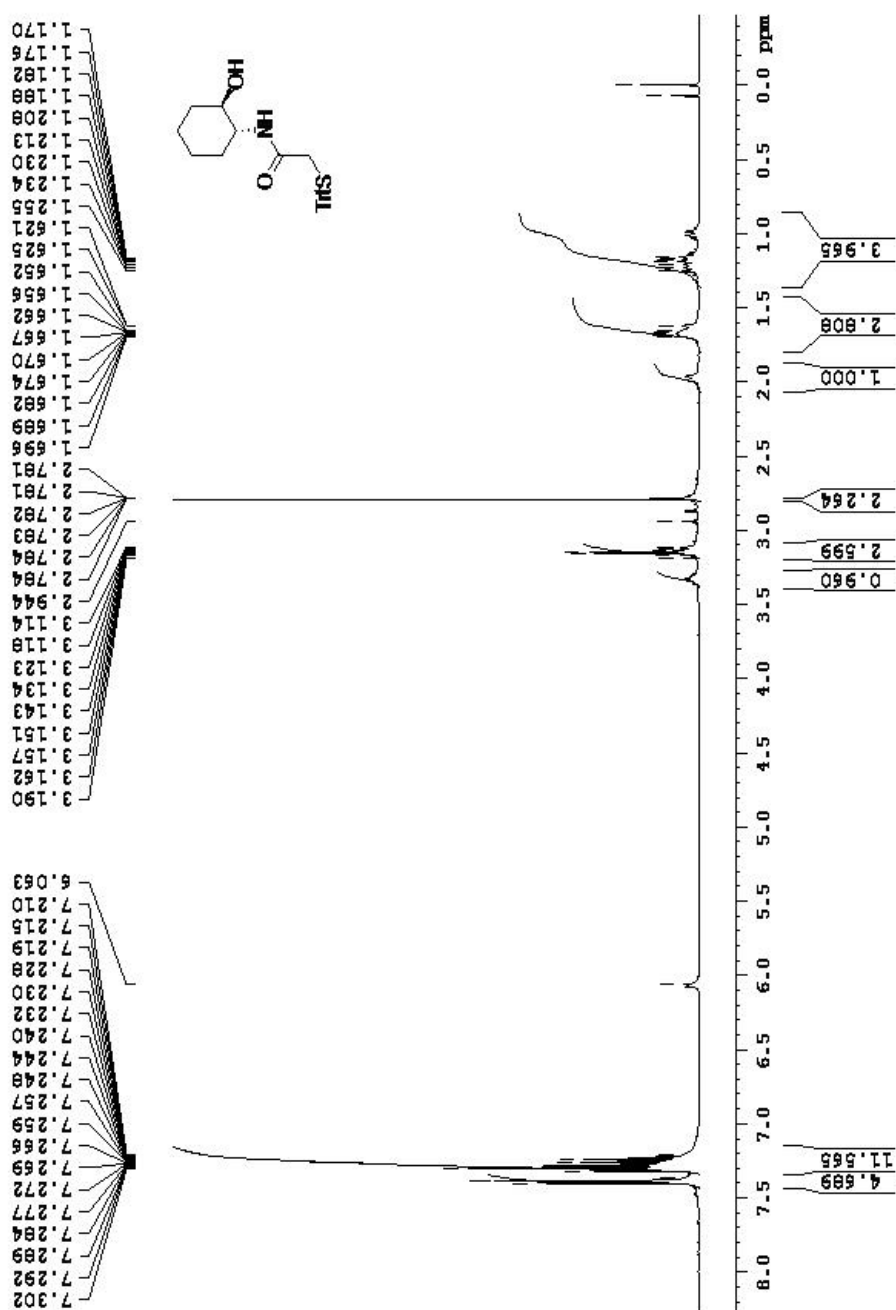
CHcyclohexane), 68 (CPh<sub>3</sub>), 74.89 (O CHcyclohexane), 127.12, 128.17, 129.42 (CHTrt), 143.92 (ipso Ph Trt), 170.02 (CO). MALDI-TOF: calcd. for C<sub>27</sub>H<sub>29</sub>NO<sub>2</sub>S.Na<sup>+</sup>, [M+Na]<sup>+</sup>: 454.18; C<sub>27</sub>H<sub>29</sub>NO<sub>2</sub>S.K<sup>+</sup> [M+K]<sup>+</sup>: 470.29, found: 454.20, 470.16.

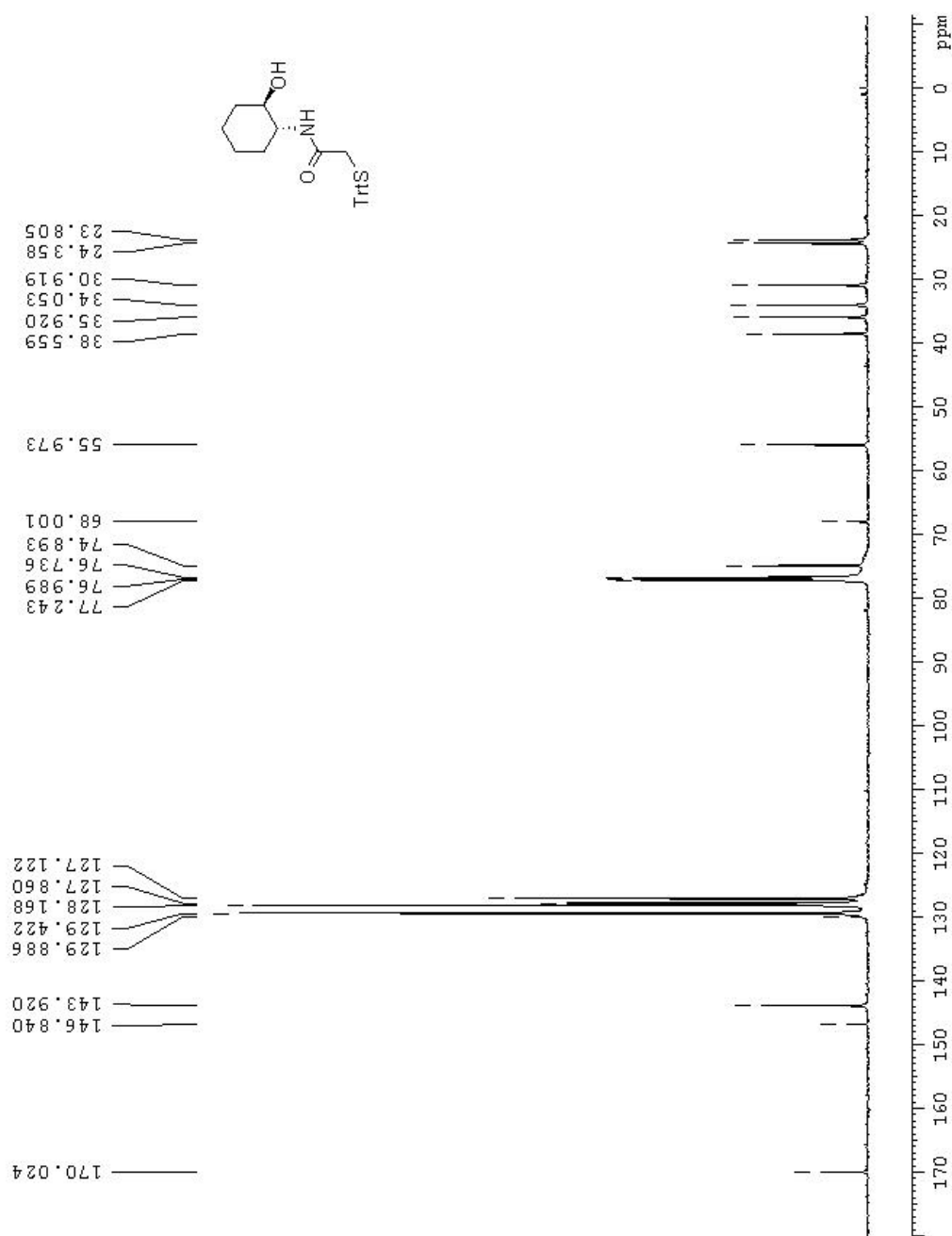
**Synthesis of 10:** Compound **9** (1.1 g, 2.55 mmol) was added to a preactivated Fmoc-Asp-OAll (0.8 g, 2.04 mmol) with DIC (0.35 mL, 2.3 mmol) and catalytic amount of DMAP in dry DCM (20 mL) at 0 °C. The reaction mixture was stirred at rt for 12 h. The organic solvent was concentrated for flash column chromatography (Hexane/Ethyl Acetate 3:1) to give 1.2 g of **10** (72%). *R<sub>f</sub>* 0.27 [EtOAc-Hex (1:2)]; δ<sub>H</sub> (CDCl<sub>3</sub>): 0.94-1.45 (4H, m, CH<sub>2</sub>cyclohexane), 1.52-1.99 (4H, m, CH<sub>2</sub>cyclohexane), 2.7-3.25 (4H, m, CH<sub>2</sub> COOcyclohexane, CH<sub>2</sub> STrt), 3.62-3.82 (1H, m, NH CHcyclohexane), 4.1-4.27 (2H, m, OCH<sub>2</sub>Allyl), 4.27-4.42 (1H, m, CHFmoc), 4.42-4.58 (1H, m, O CHcyclohexane), 4.58-4.75 (3H, m, CH<sub>2</sub> Fmoc, aCHAsp), 5.23 (1H, d, CH<sub>2</sub>allyl, *J* = 10.7 Hz), 5.31 (1H, d, CH<sub>2</sub>allyl, *J* = 17.1 Hz), 5.8-5.94 (1H, m, CHallyl), 7.06-7.64 (19H, m, CHFmoc Ph, CHTrt), 7.57-7.70 (2H, m, CHFmoc Ph), 7.76 (2H, d, CHFmoc Ph, *J* = 7.4 Hz); δ<sub>C</sub> (CDCl<sub>3</sub>) 23.77, 23.97, 24.08, 30.69, 30.71, 30.72-31.33 (CH<sub>2</sub>cyclohexane), 36.185, 36.23 (CH<sub>2</sub> STrt), 37.02, 37.09 (CH<sub>2</sub> COOcyclohexane), 47.02, 47.06 (CHFmoc), 50.26, 50.63 (aCHAsp), 51.77, 52.23 (NH CHcyclohexane), 66.24, 66.38 (OCH<sub>2</sub>Allyl), 67.27, 67.42 (CH<sub>2</sub>Fmoc), 67.85, 67.98 (CPh<sub>3</sub>), 75.46, 75.83 (O CHcyclohexane), 118.7, 118.85 (CH<sub>2</sub>allyl), 119.9-132 (CHFmoc Ph, CHTrt, CHallyl), 141.23, 143.7, 143.83 (C<sub>quat</sub>, Fmoc Ph), 144.03 (ipso Ph Trt), 155.96, 156.21 (CO Fmoc), 168.23, 168.33 (CO CH<sub>2</sub>STrt), 170.17, 170.29 (COO cyclohexane), 170.69 (COOAllyl). MALDI-TOF: calcd. for C<sub>49</sub>H<sub>48</sub>N<sub>2</sub>O<sub>7</sub>S.Na<sup>+</sup> [M+Na]<sup>+</sup>: 831.31; C<sub>49</sub>H<sub>48</sub>N<sub>2</sub>O<sub>7</sub>S.K<sup>+</sup> [M+K]<sup>+</sup>: 847.42, found: 831.45, 847.40.

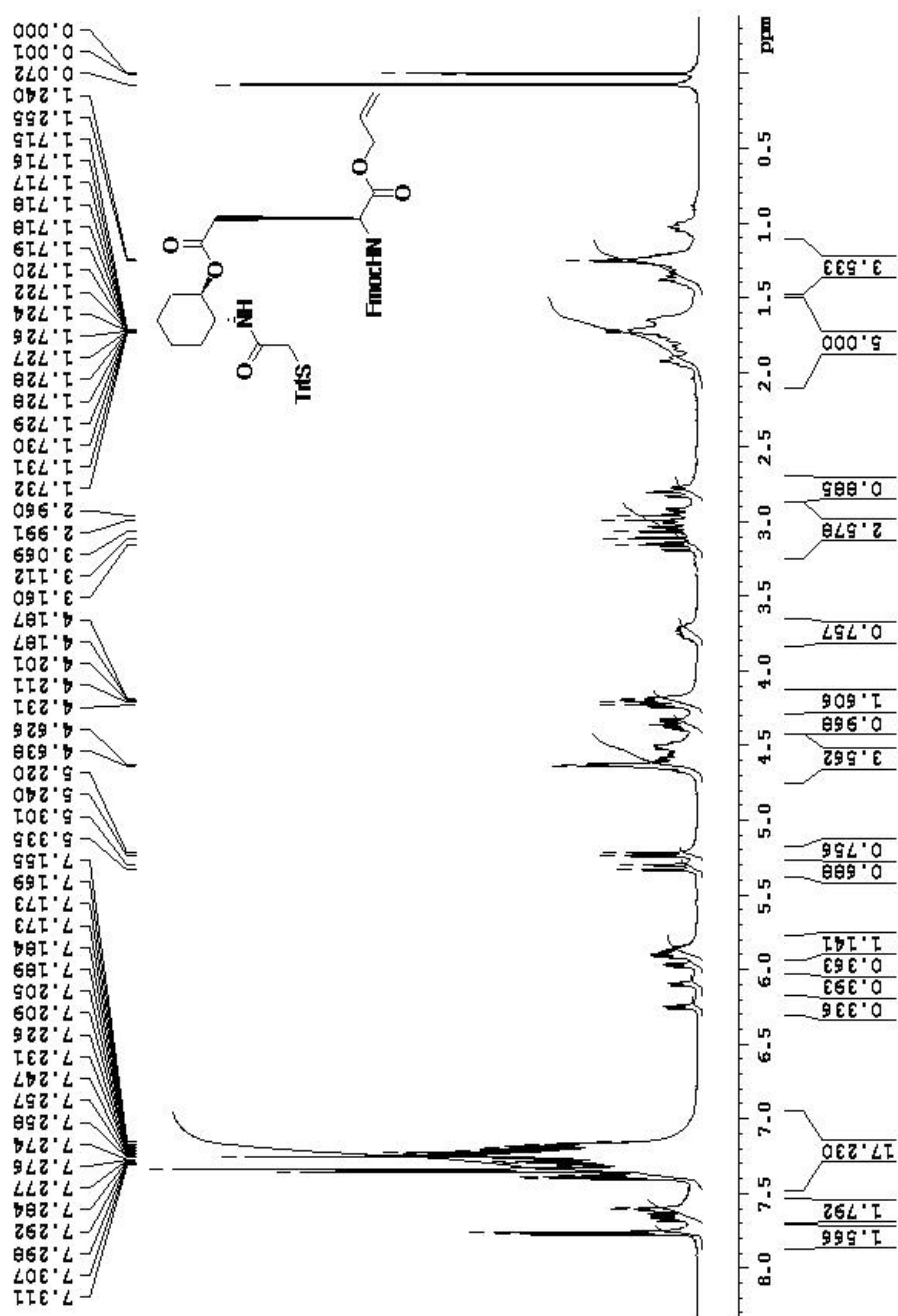
**Synthesis of 1:** Compound **10** (1.2 g, 1.5 mmol) was dissolved in THF (25 mL), *N*-methylaniline (1.58 mL, 15 mmol) and Pd(PPh<sub>3</sub>)<sub>4</sub> (170 mg, 0.15 mmol) were added subsequently. The reaction mixture was stirred at rt for 1 h. After removing the solvent under reduced pressure, the residue was subjected to column chromatography (MeOH/CH<sub>3</sub>Cl 20:1) to give the product in 90% yield. *R<sub>f</sub>* 0.55 [CHCl<sub>3</sub>-MeOH (10:1)]; δ<sub>H</sub> (CDCl<sub>3</sub>) 1.14-1.64 (8H, m, CH<sub>2</sub>cyclohexane), 2.76-3.24 (4H, m, CH<sub>2</sub> COO cyclohexane, CH<sub>2</sub> STrt), 3.79-3.95 (1H, m, NH CHcyclohexane), 4.12-4.24 (1H, m, CH Fmoc), 4.24-4.38 (2H, m, CH<sub>2</sub>Fmoc), 4.55-4.69 (1H, O CHcyclohexane), 4.8-4.97 (1H, m, aCHAsp), 5.96-6.14 (1 H, m, NH), 6.44-6.59 (1 H, m, NH), 7.02-7.4 (19H, m, CH Fmoc Ph, CHTrt), 7.66-7.70 (2H, m, CHFmoc Ph), 7.75 (2H, d,

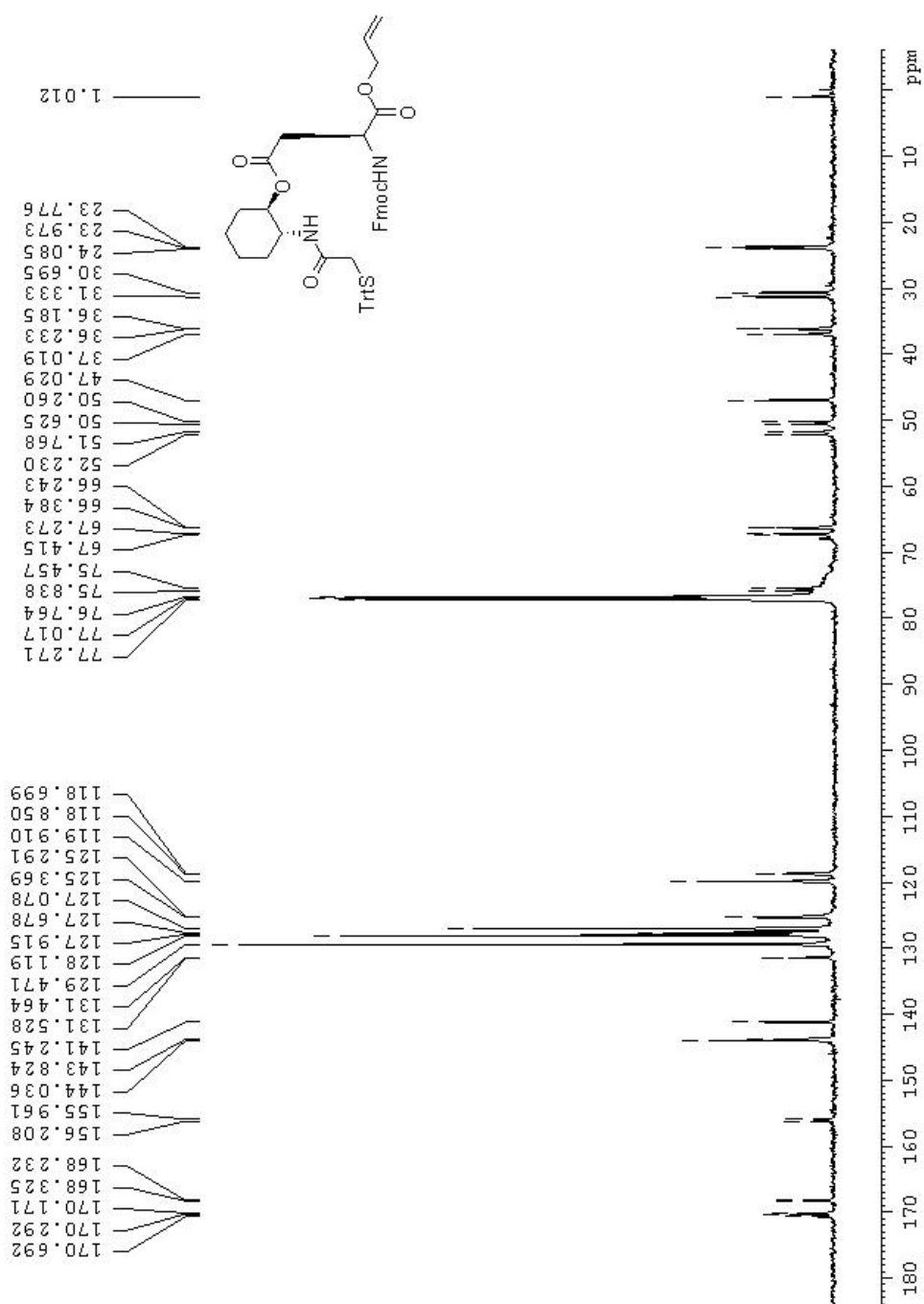
CHFmoc Ph,  $J = 7.4$  Hz);  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ ) 20.25, 20.53, 22.96, 23.33, 27.33, 27.38, 28.1, 28.34 ( $\text{CH}_2\text{cyclohexane}$ ), 36.1 ( $\text{CH}_2$  STrt), 37.05, 37.36 ( $\text{CH}_2$  COO $\text{cyclohexane}$ ), 47.06, 47.07 (CHFmoc), 48.96, 49.19 (aCHAsp), 50.52, 50.56 (NH CH $\text{cyclohexane}$ ), 67.33, 67.36 ( $\text{CH}_2\text{Fmoc}$ ), 67.87 ( $\text{CPh}_3$ ), 72.76, 72.82 (O CH $\text{cyclohexane}$ ), 119.9-132 (CHFmoc Ph, CHTrt), 141.25, 143.74, 143.77 ( $\text{C}_{\text{quat}}$ , Fmoc Ph), 144.01 (ipso Ph Trt), 156.1, 156.18 (CO Fmoc), 168.49, 168.52 (CO  $\text{CH}_2\text{STrt}$ ), 169.96 (COO  $\text{cyclohexane}$ ), 173.01, 173.15 (COOH). MALDI-TOF: calcd. for  $\text{C}_{46}\text{H}_{44}\text{N}_2\text{O}_7\text{S}.\text{Na}^+$   $[\text{M}+\text{Na}]^+$ : 791.28,  $\text{C}_{46}\text{H}_{44}\text{N}_2\text{O}_7\text{S}.\text{K}^+$   $[\text{M}+\text{K}]^+$ : 807.39, found: 791.39, 807.38.

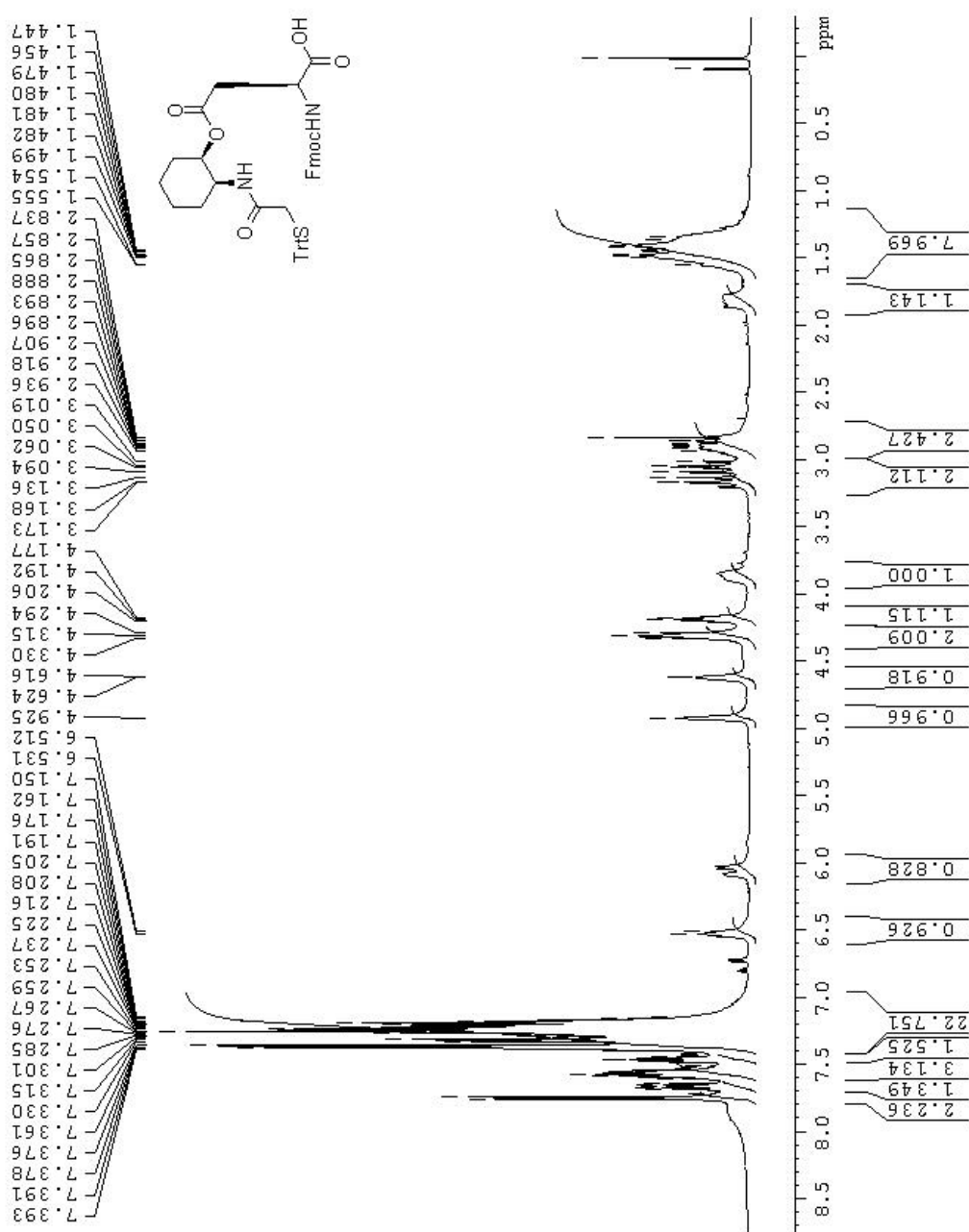
**$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR and MS spectra of compounds of the various building blocks**

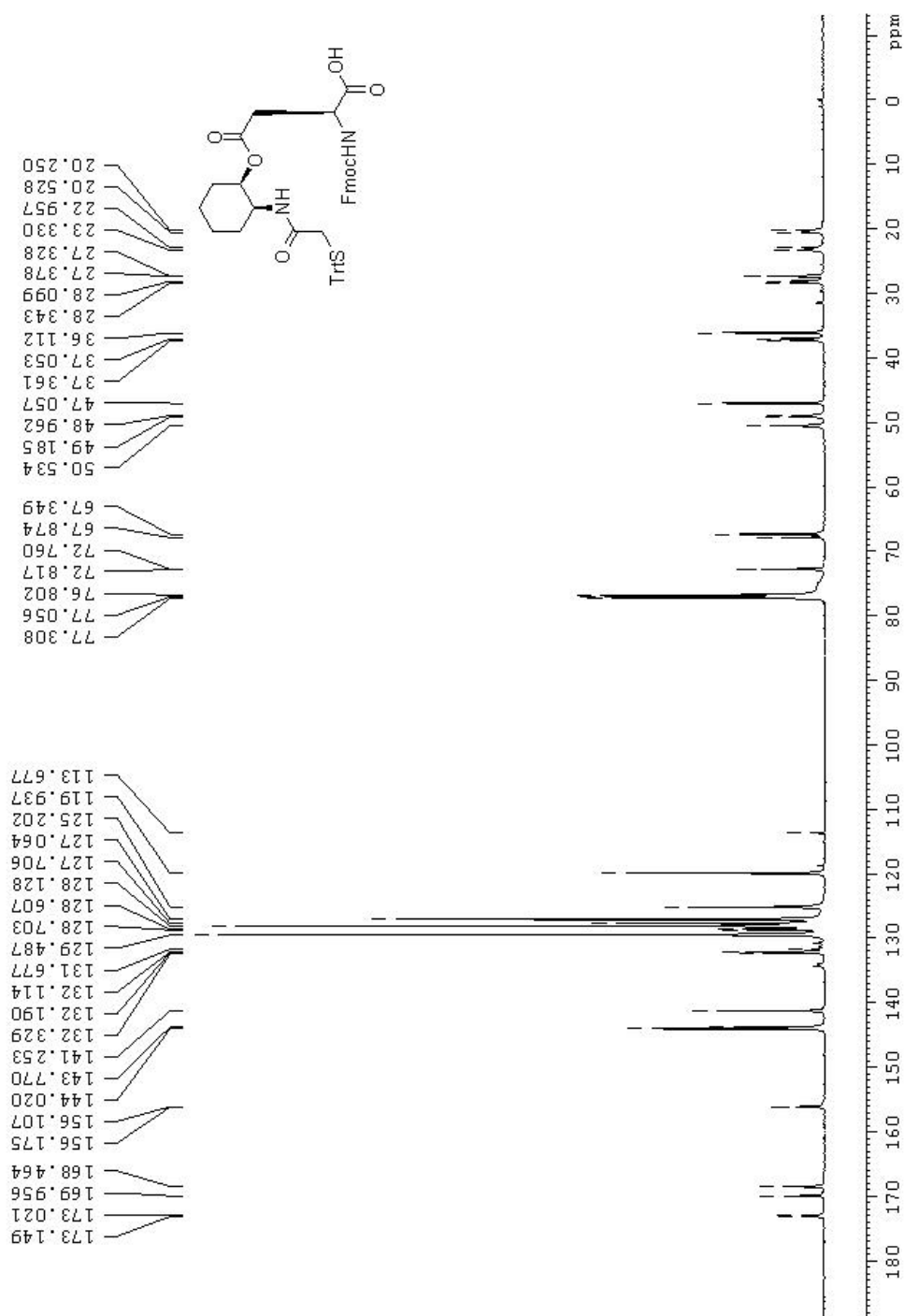


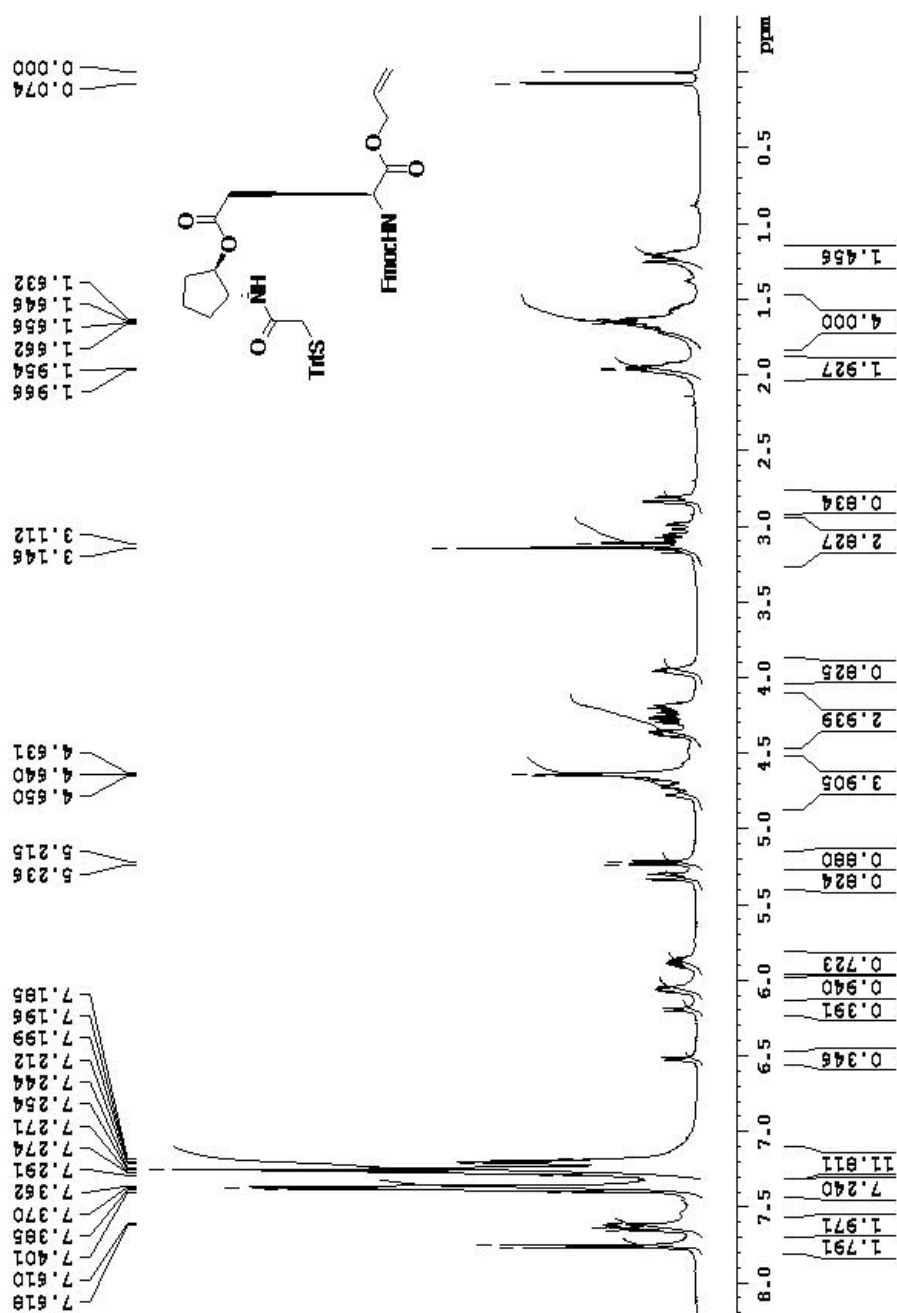


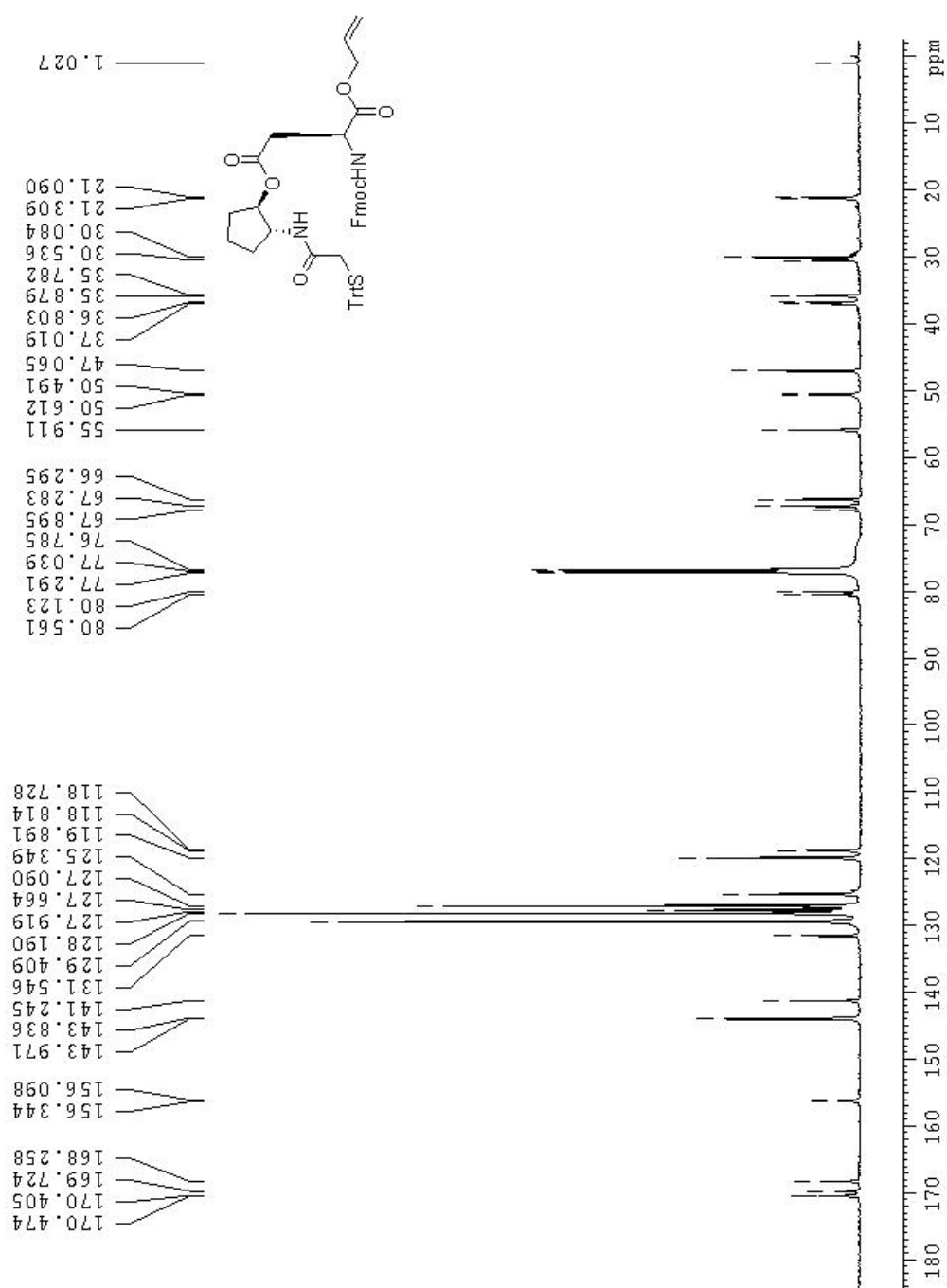


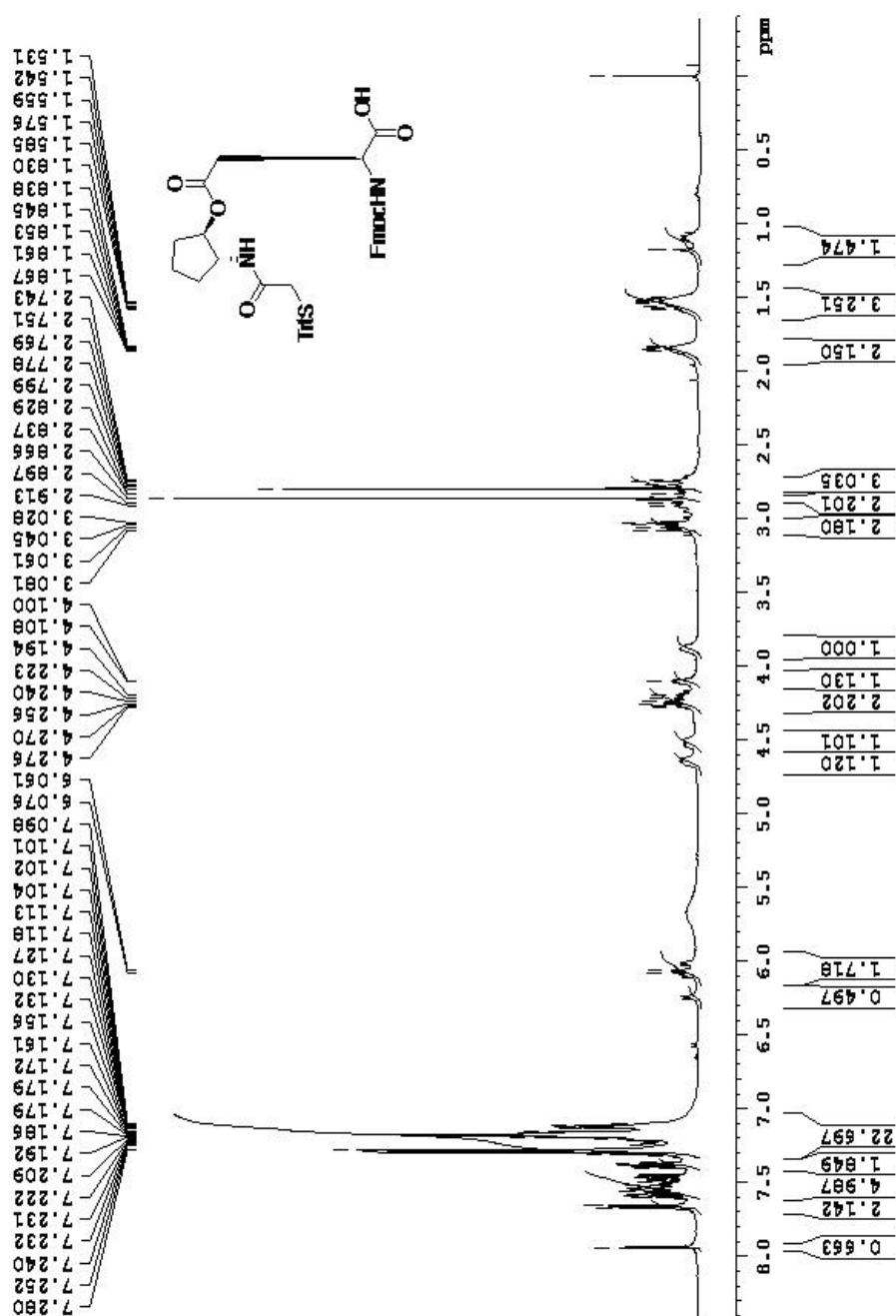


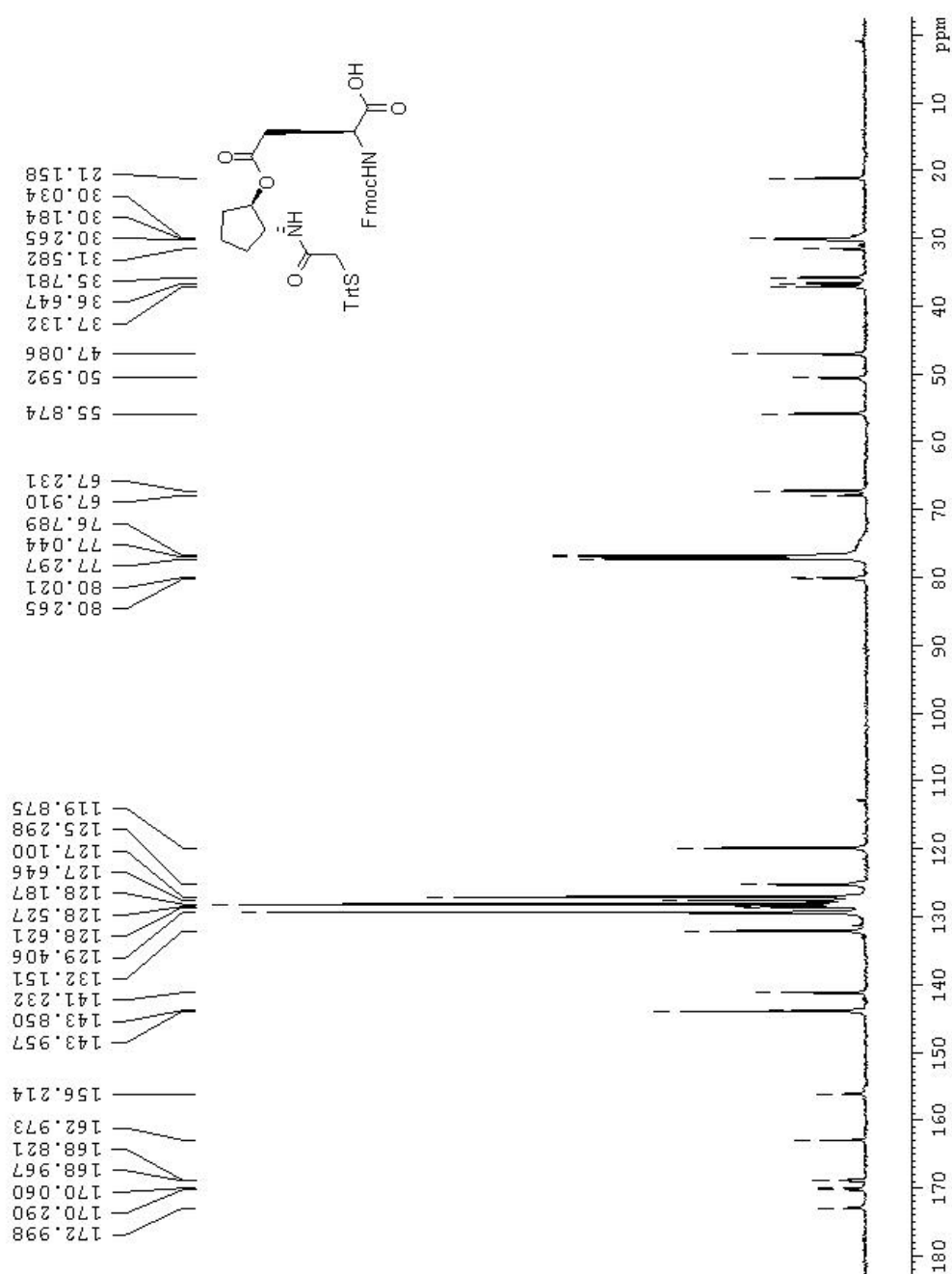


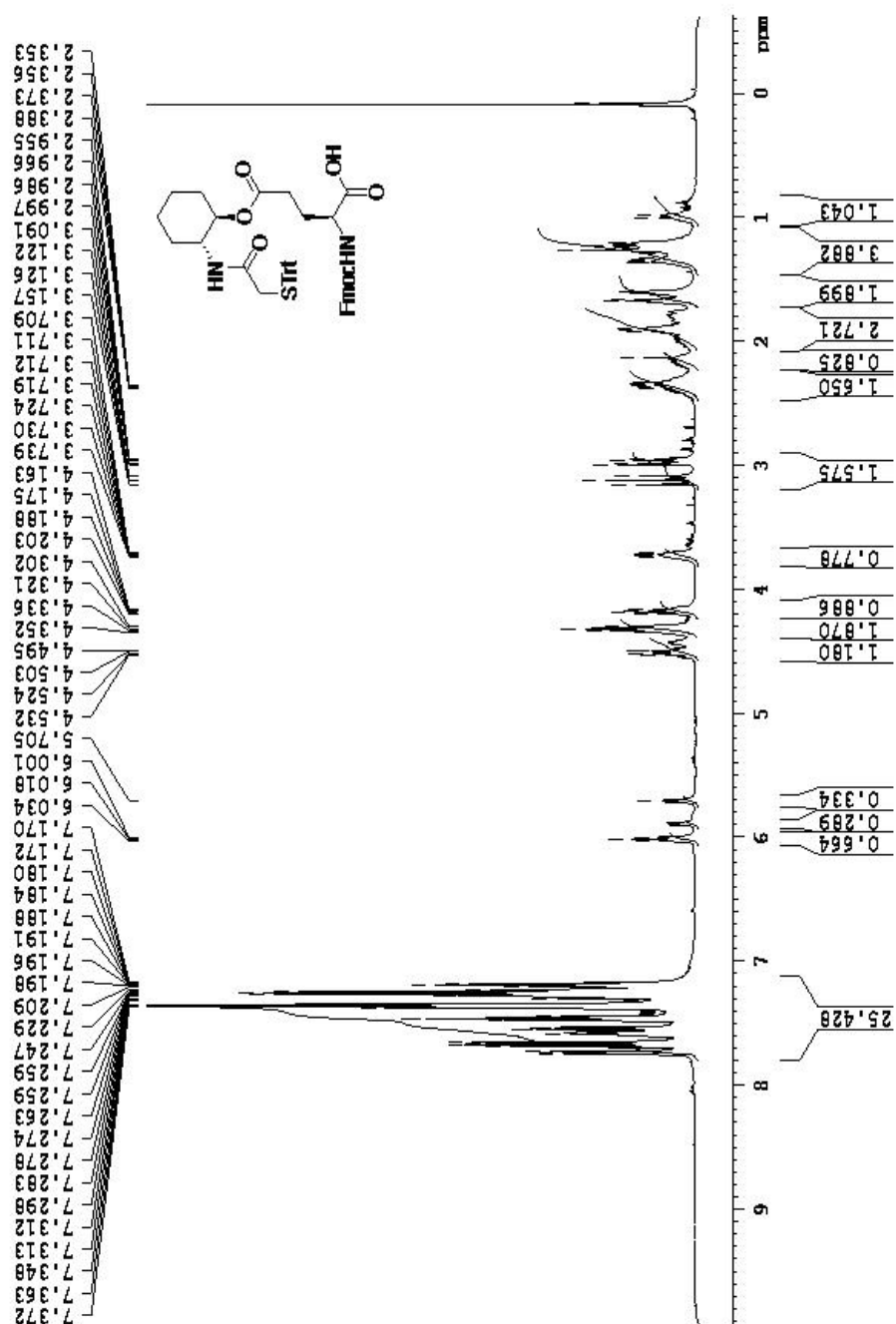


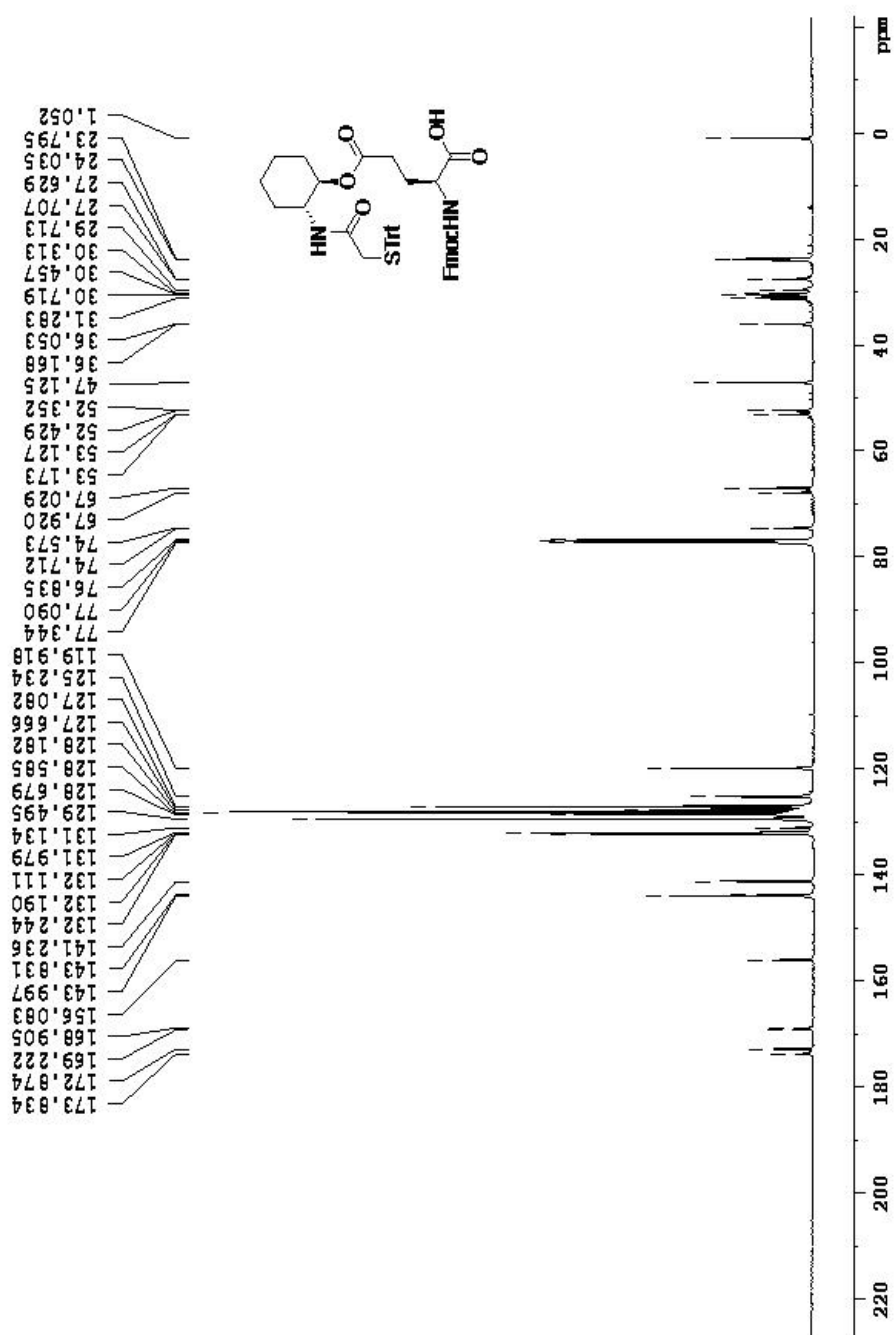




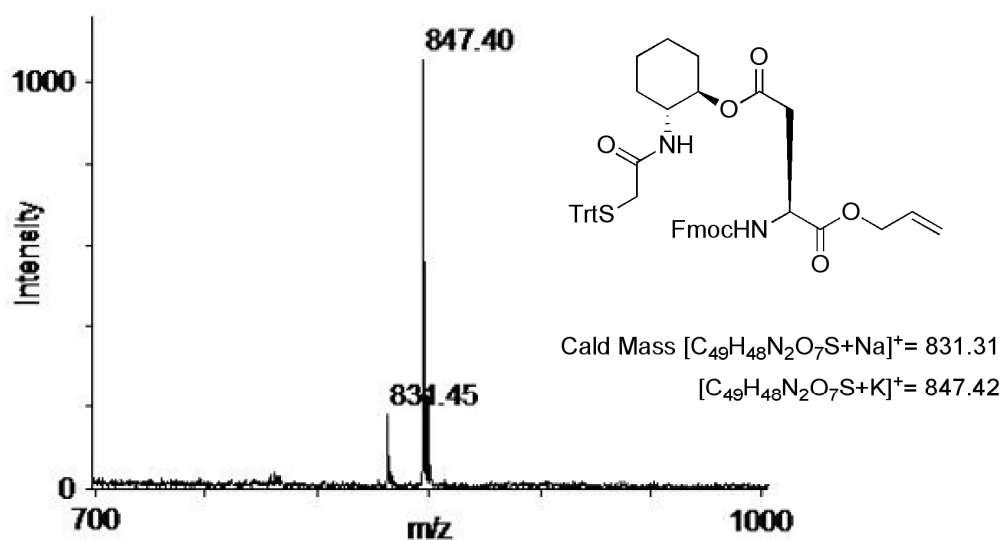
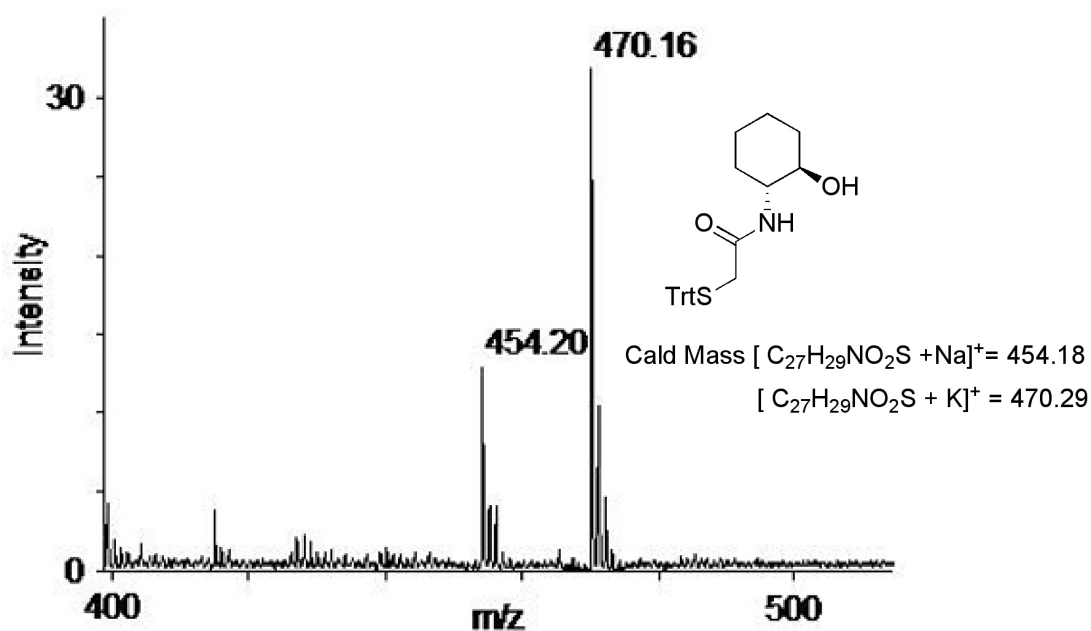


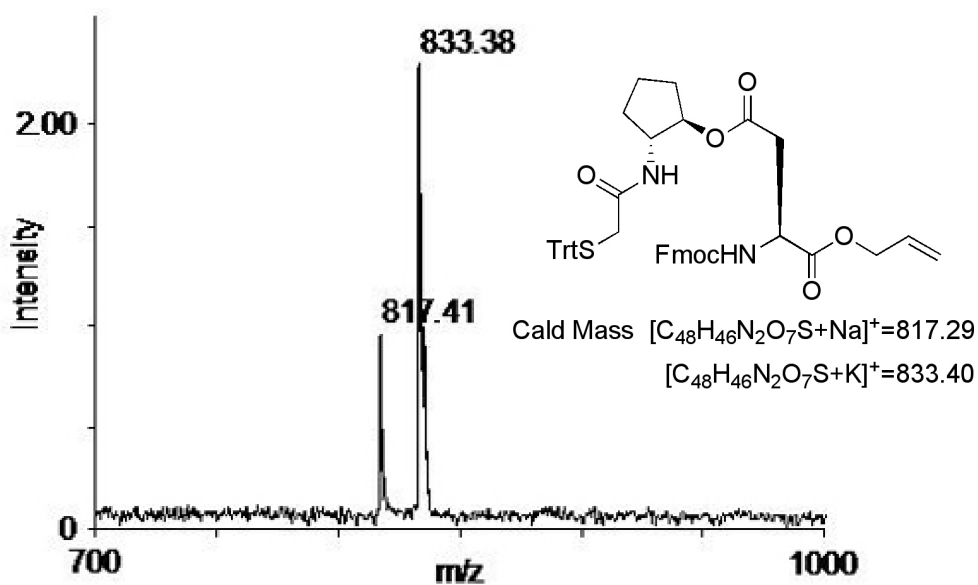
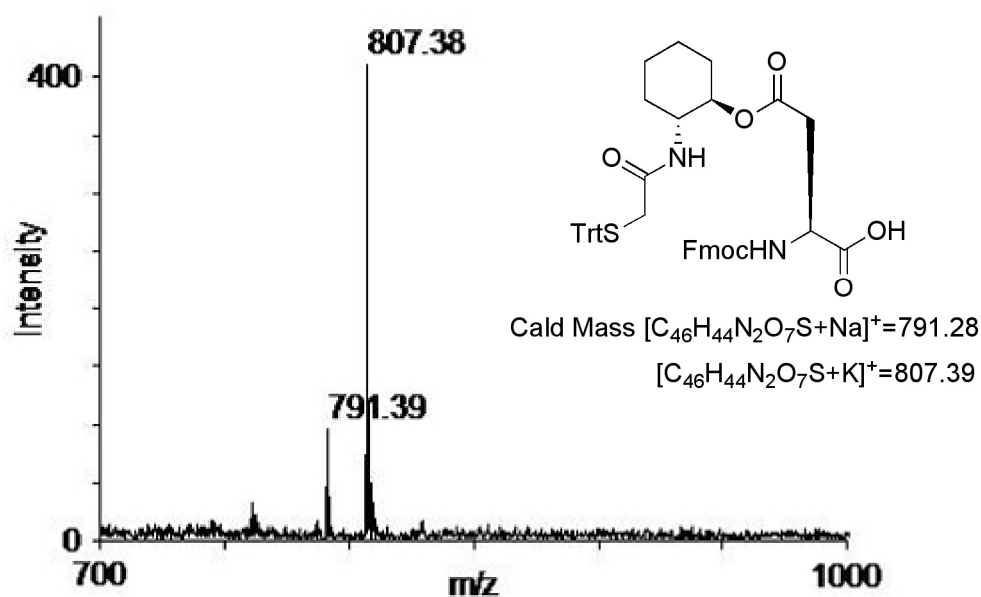


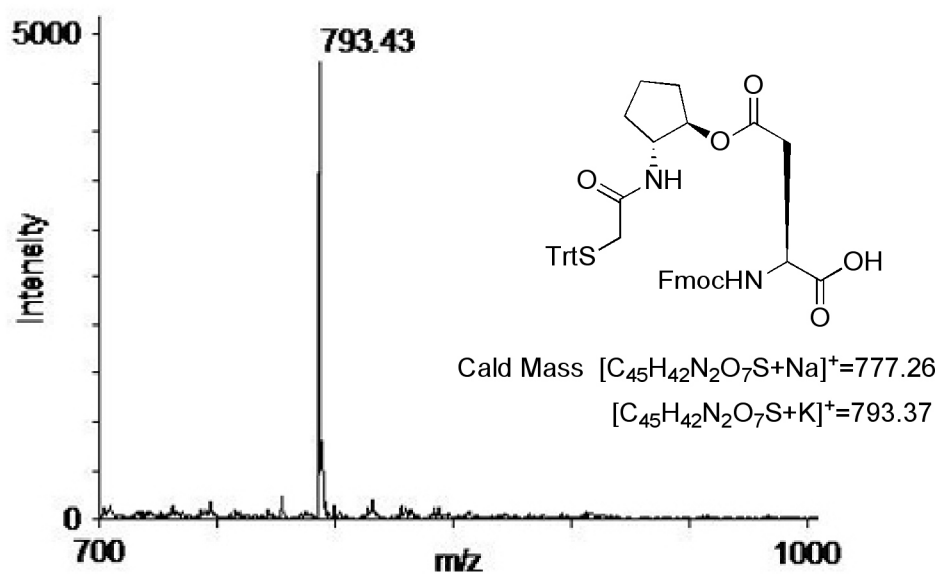




# -TOF analysis of auxiliaries and their precursors



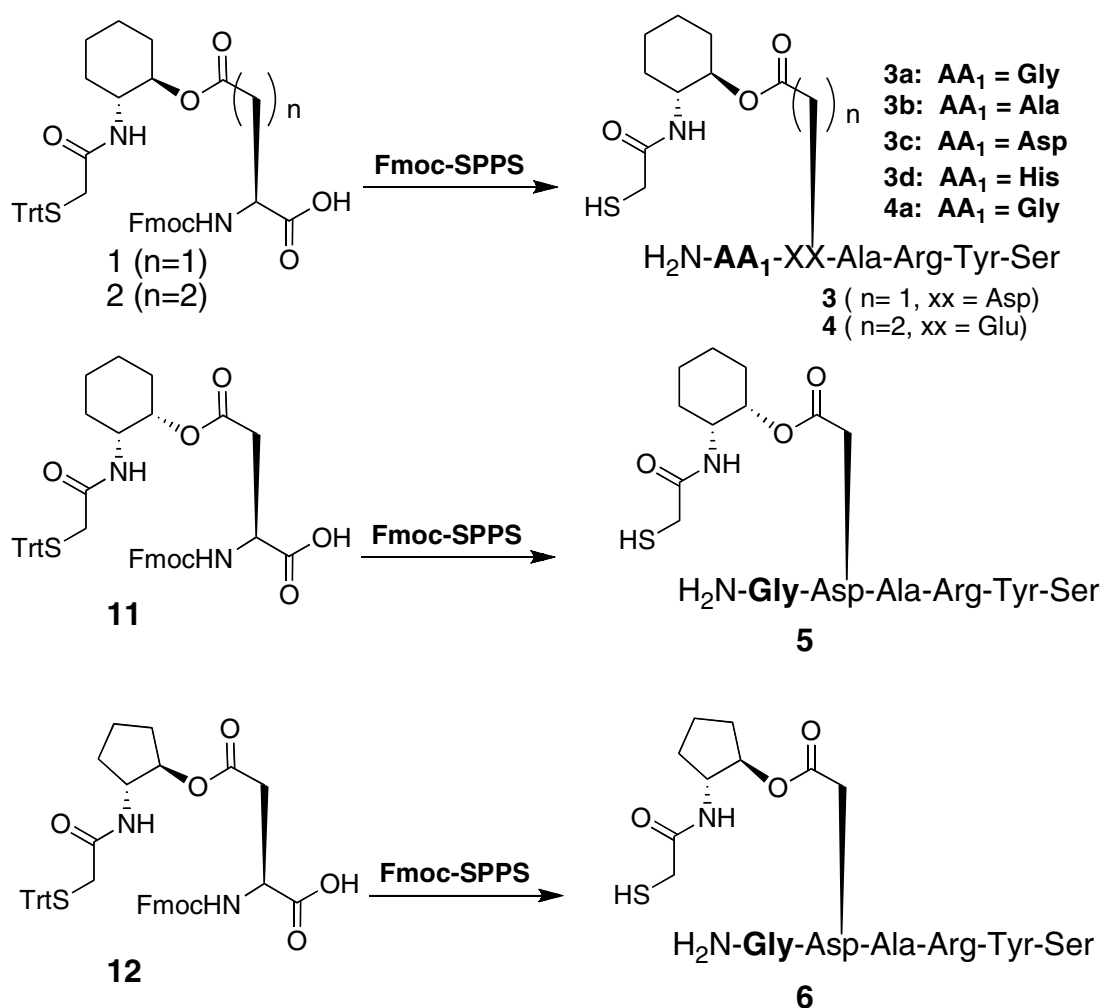




QuickTime™ and a  
TIFF (Uncompressed) decompressor  
are needed to see this picture.

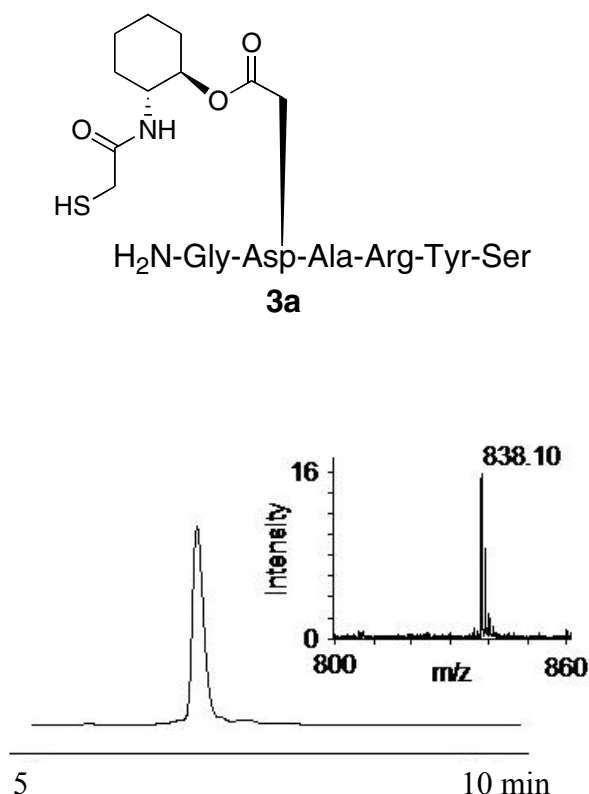
**Solid Phase Peptide Synthesis:** Solid-phase chemistry was carried out in syringes, equipped with teflon filters, purchased from Torviq. If not differently described, all reactions were carried out at room temperature. Analytical HPLC was performed on a Thermo instrument (Spectra System p4000) using an analytical Column (Jupiter 5 micron, C18, 300A 150 x 4.6 mm) and a flow rate of 1.2 mL/min. Preparative HPLC was performed on an ECOM instrument using a preparative Column (Jupiter 5 micron, C18, 300A, 250 x 10 mm) and a flow rate of 25mL/min. DMF was purchased in biotech grade. Peptide Commercial reagents were used without further purification. Resins, protected amino acids and HBTU were purchased from Novabiochem.

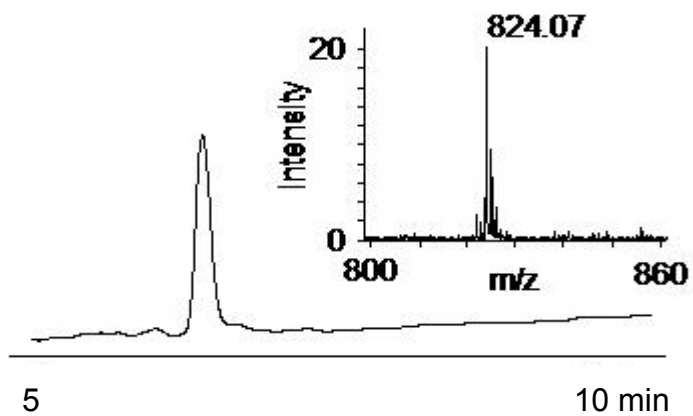
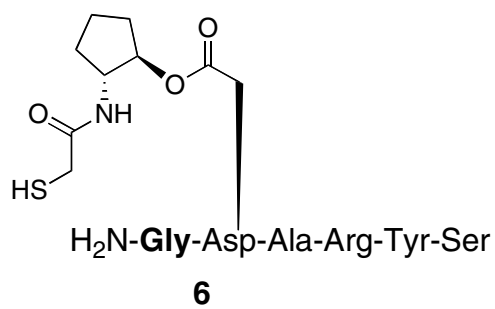
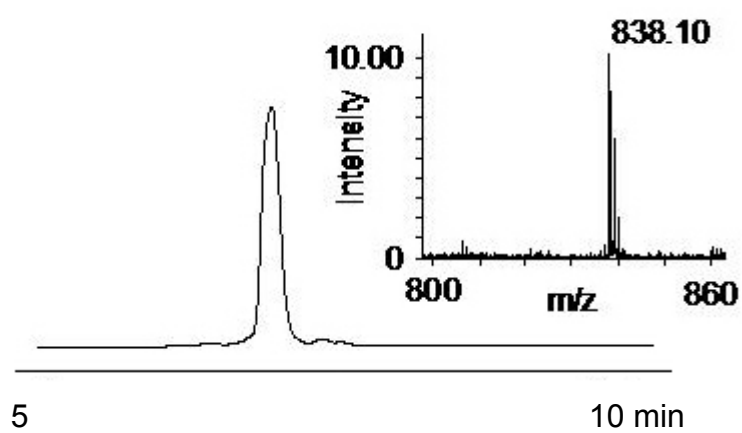
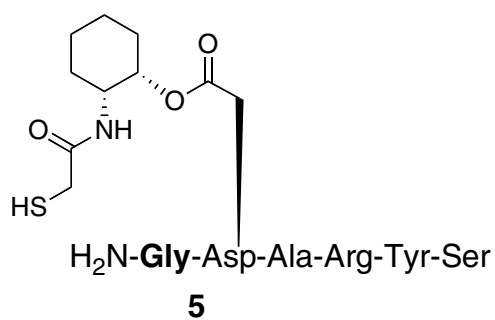
### Solid Phase Peptide Synthesis of auxiliary containing peptides

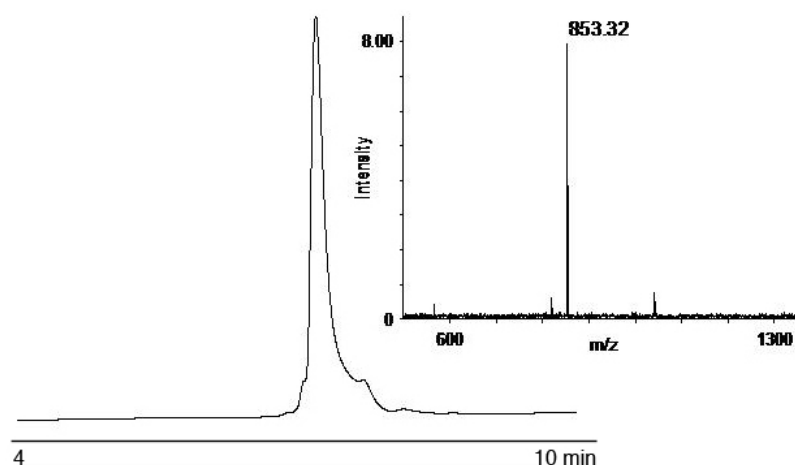
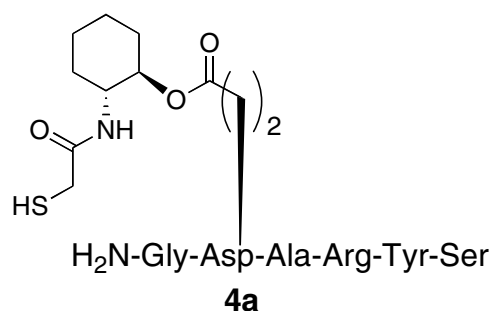


**SPPS of peptides 3a-4a.** H-Ser(tBu)-2-chlorotrityl resin (0.49 mmol/g) was used for the synthesis of peptides **3a-4a**, **5**, **6**. Amino acids (including the Fmoc-Asp(auxiliary)-OH and HBTU) were used in 5 folds excess of the initial loading of the resin. DIEA was used in 10 folds excess. Peptide coupling was performed for 30 min. Fmoc deprotection was achieved by treatment of the resin with 20% piperidine (3 x 3min). *Cleavage from the resin:* A mixture of TFA, triisopropylsilane and water (95:2.5:2.5) was added to the dried resin peptide. After 2 h, the resin was washed with TFA (4x 4 mL). *Work-up:* The combined solutions were concentrated in vacuo. The residue was dissolved in water, purified by preparative reverse-phase HPLC using a linear gradient of buffer A in buffer B over 20 min (buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile). The fractions were analyzed by MALDI-TOF/MS (RefleX IV, Bruker), (matrix:  $\alpha$ -Cyano-4-hydroxycinnamic acid). The desired fractions were collected, lyophilized, and stored at -4 °C.

### HPLC and MS analysis of peptides-auxiliary 3-7







**Peptide thioester synthesis:** MBHA resin (0.59 mmol/g) was washed (5x DMF, 10 min 10% DIEA/DMF, 5 x DMF). Preactivated 3-(tritylthio) propanoic acid with HBTU in DMF containing DIEA. After 5 min of preactivation, the mixture was added to the resin. After 1 h the resin was washed with DMF. The trityl was removed using mixture of TFA/triisopropylsilane/water (90/5/5 v/v). The *in situ* neutralization protocol developed by Kent and coworkers was applied for peptide assembly. **Cleavage:** A mixture of TFMSA/TFA/thioanisole (2/8/1 v/v) was added to the resin. After 1.5 h, the resin was washed with TFA (4 x 4 mL). **Work-up:** The combined solutions were concentrated in vacuo. The residue was dissolved in water, purified by preparative HPLC and analyzed by MALDI-TOF/MS (RefleX IV, Bruker), (matrix:  $\alpha$ -Cyano-4-hydroxycinnamic acid).

**General procedure for chemical ligation:** The ligation of unprotected peptide segments was performed as follows: 0.2 M phosphate buffer (pH 8.0) containing 6 M guanidine.HCl was degassed with Argon for 10 minutes before use. Peptides were

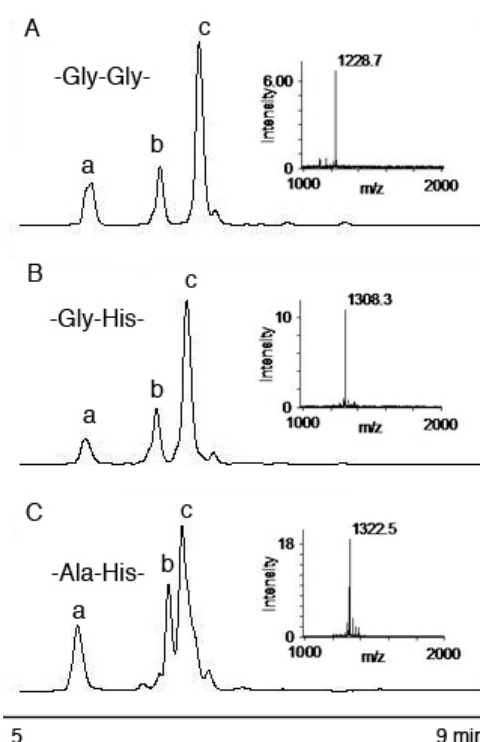
dissolved (3mg of peptide-auxiliary and 1.2 eq peptide thioester) at a final concentration of 5 mM and the ligation reaction was performed in a heating block at 37 °C. Before analysis, tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (50 mM) was added to reduce any disulfide bonds. The reaction was monitored using reverse-phase HPLC analysis on a C18 column using a linear gradient (100-10%A) of buffer A in buffer B over 15 min.

**General procedure for auxiliary hydrolysis:** Auxiliary hydrolysis was performed by adjusting the pH of the ligation mixture to pH 10 using 1N NaOH and left for 5 min at rt. The hydrolysis reaction was monitored by analytical HPLC as described above.

### Representative ligation from table 1

Representative analytical HPLC traces of ligation reactions and mass spectrometry analysis of the products (MALDI-TOF/MS).

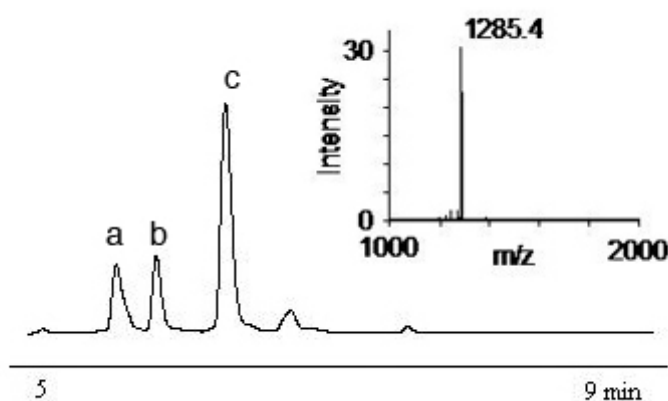
In all the examples, over 80% the peptide bearing the side-chain auxiliary was consumed. All reactions were analyzed after basic hydrolysis by adjusting the pH of the ligation mixture to pH 10. (A) Ligation reaction at 8 h, (Table 1, entry 1): peak a, an aspartamide by product of the peptide-(auxiliary) with the expected mass of  $650.2 \pm 0.2$  Da; mixed with the hydrolysis product of the unreacted peptide-(auxiliary) with the expected mass of  $668.2 \pm 0.2$  Da, peak b,



thioester hydrolysis (1.2 eq of peptide thioester was used in the ligation reaction) ; peak c, ligation product with the expected mass of  $1228.7 \pm 0.2$  Da. (B) Ligation reaction at 8 h, (Table 1, entry 3): peak a, an aspartamide by product of the peptide-(auxiliary) with the expected mass of  $730.3 \pm 0.2$  Da, mixed with the hydrolysis product of the unreacted peptide-(auxiliary) with the expected mass of  $748.2 \pm 0.2$  Da ; peak b, thioester hydrolysis; peak c, ligation product with the expected mass of

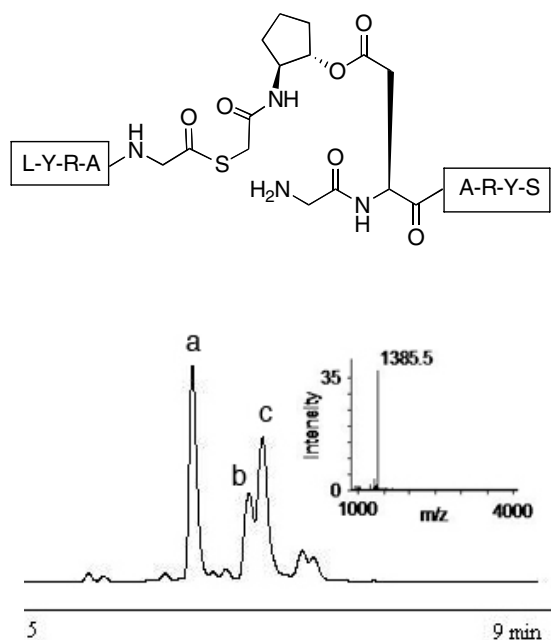
$1308.3 \pm 0.2$  Da. (C) Ligation reaction at 36 h, (Table 1, entry 9): peak a, an aspartamide by product from the peptide-(auxiliary) with the expected mass of  $730.3 \pm 0.2$  Da; mixed with the hydrolysis product of the unreacted peptide-(auxiliary) with the expected mass of  $748.2 \pm 0.2$  Da, peak b, thioester hydrolysis; peak c, ligation product with the expected mass of  $1322.5 \pm 0.2$  Da, the shoulder in peak c corresponds to the aspartamide by product that leads to isoapartyl residue with the expected mass of  $1322.5 \pm 0.2$  Da .

### HPLC/MS analysis of ligation of thioester containing Lysine (LYRKG-SR) and peptide-(auxiliary) 3a



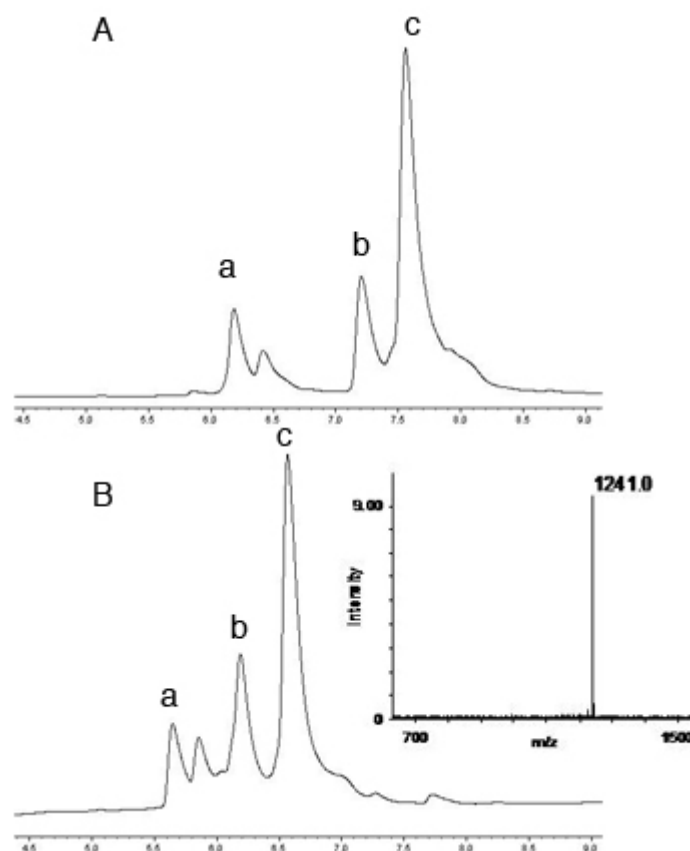
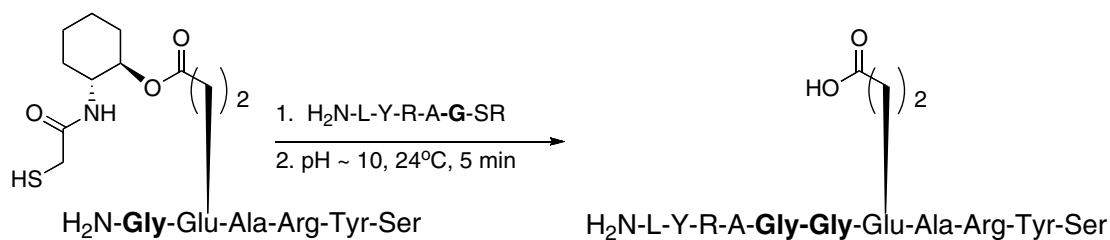
Ligation reaction at 8 h: peak a, an aspartamide by product from the peptide-(auxiliary) with the expected mass of  $650.3 \pm 0.2$  Da, mixed with the hydrolysis product of the unreacted peptide-(auxiliary) with the expected mass of  $668.3 \pm 0.2$  Da, peak b, thioester hydrolysis; peak c, ligation product with the expected mass of  $1285.4 \pm 0.2$  Da.

**S → N acyl transfer is a rate-determining step**



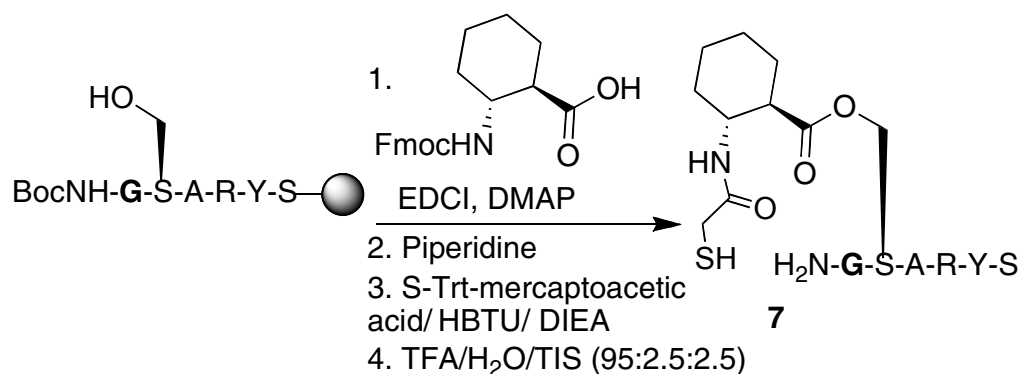
Analytical HPLC traces of ligation reaction (peptide **6** and LYRAG-SR) after 5 min and mass spectrometry analysis (MALDI-TOF/MS). Peak a, thioester substrate with the expected mass of  $665.56 \pm 0.2$  Da ; peak b, peptide-(auxiliary) with the expected mass of  $825.32 \pm 0.2$  Da ; *peak c, the intermediate, shown in top of this figure, with the expected mass of  $1385.5 \pm 0.2$  Da*. Peak c was isolated, lyophilized, and subjected to pH 10. Shortly , the peak was converted to two peaks that correspond by mass analysis to GDARYS peptide and LYRAG-COOH.

## Ligation at glutamic acid junction

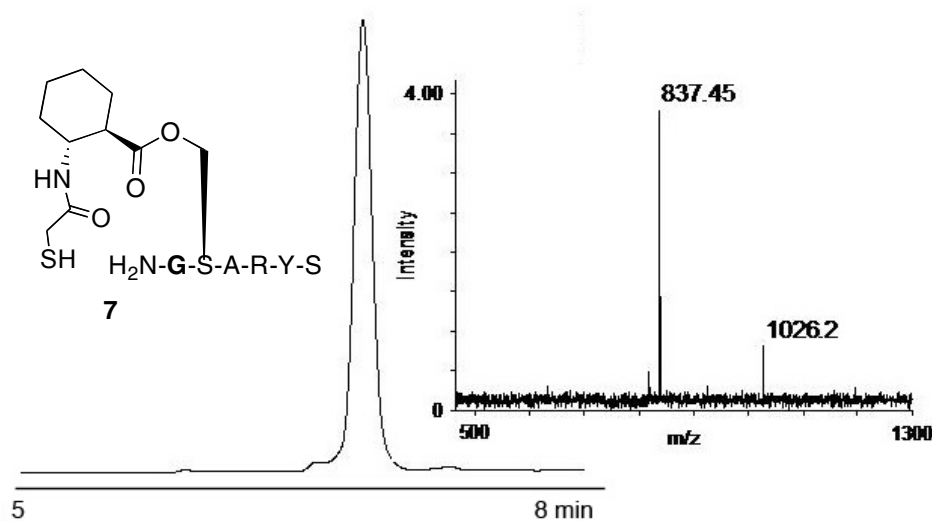


Analytical HPLC traces/(MALDI-TOF/MS) of ligation reaction at 14 h. A) peak a, thioester hydrolysis with the expected mass of  $579.27 \pm 0.2$  (1.2 eq of peptide thioester was used in the ligation reaction); peak b, peptide-(auxiliary) with the expected mass of  $853.32 \pm 0.2$ ; peak c ligation product with the expected mass of  $1412.64 \pm 0.2$  Da. B) Ligation reaction after auxiliary removal (pH 10, 5 min): peak a hydrolyzed peptide-(auxiliary) with the expected mass of  $681.51 \pm 0.2$ ; peak b hydrolyzed thioester; peak c ligation product with the expected mass of  $1241.0 \pm 0.2$  Da.

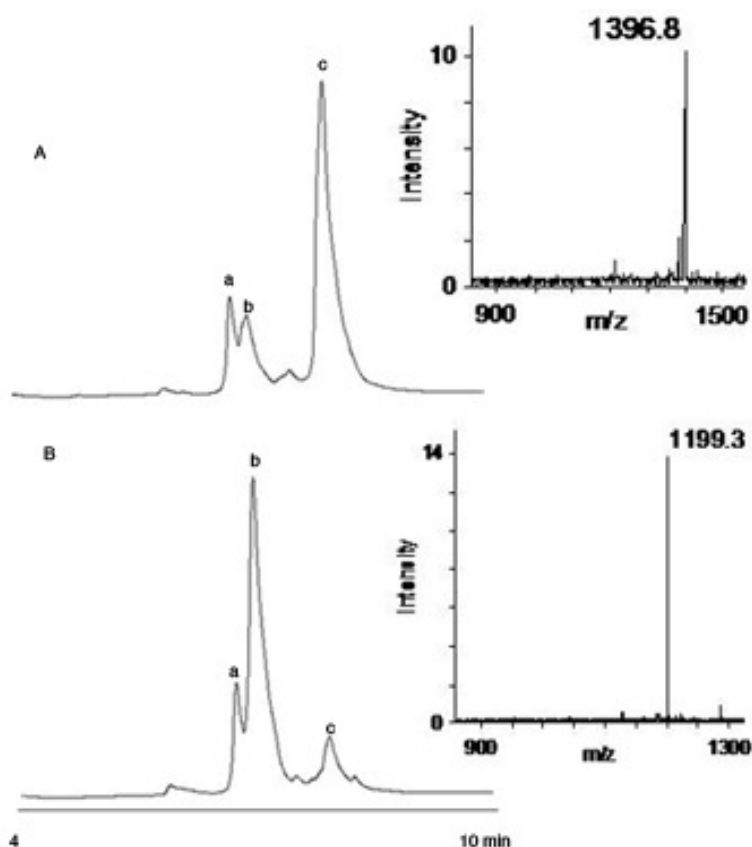
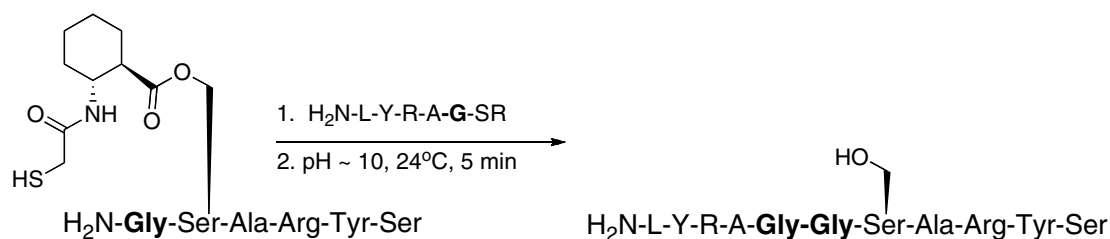
## Synthesis of peptide containing serine with a side-chain auxiliary



H-Ser(tBu)-2-chlorotrityl resin (0.49 mmol/g) was used for the synthesis of peptides in which amino acids and HBTU were used in 5 folds excess of the initial loading of the resin. DIEA was used in 10 folds excess. Peptide coupling was performed for 30 min. Fmoc deprotection was achieved by treatment of the resin with 20% piperidine (3 x 3min). Coupling of unprotected Fmoc-Ser-OH was achieved using DIC/HOBT. Boc-Gly-OH was used as the N-terminal amino acid. Peptide resin was treated with 10% N<sub>2</sub>H<sub>2</sub> in methanol (2X1.5h) to hydrolyze any undesired ester on the serine side chain. Next, commercially available Fmoc-trans-1,2-aminocyclohexane carboxylic was coupled to the side chain using EDCI in the presence of catalytic amount of DMAP in DCM. The Fmoc protecting group was then removed with 20% piperidine followed by coupling the S-Trt-mercaptoacetic acid using HBTU/DIEA coupling conditions. *Cleavage from the resin and work up was done as described previously.*



## Ligation at serine junction



Analytical HPLC traces/(MALDI-TOF/MS) of ligation reactions after 6 h. A) peak a, thioester hydrolysis with the expected mass of  $579.27 \pm 0.2$  (1.2 eq of peptide thioester was used in the ligation reaction); peak b, unreacting thioester with the expected mass of  $666.32 \pm 0.2$  Da, in addition to hydrolyzed ligation product with the expected mass of  $1199.3 \pm 0.2$  Da; peak c ligation product with the expected mass of  $1396.8 \pm 0.2$  Da. B) Ligation reaction after auxiliary removal (pH 10, 5 min): peak a, hydrolyzed thioester peak b, ligation product with the expected mass of  $1199.3 \pm 0.2$  Da. Peak c, remaining unhydrolyzed ligation product.