Oxo-ester Mediated Native Chemical Ligation:

Concept and Applications

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Materials and Methods

All commercial materials (Aldrich, Fluka, Nova) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). Anhydrous THF, diethyl ether, CH_2Cl_2, toluene, and benzene were obtained from a dry solvent system (passed through column of alumina) and used without further drying. All reactions were performed under an atmosphere of pre-purified dry Ar(g). NMR spectra (^1H and ^{13}C) were recorded on a Bruker AMX-400 MHz or Bruker Advance DRX-500 MHz, referenced to TMS or residual solvent. Low-resolution mass spectral analyses were performed with a JOEL JMS-DX-303-HF mass spectrometer or Waters Micromass ZQ mass spectrometer. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and flash column chromatography was performed on E. Merck silica gel 60 (40–63 mm). Yields refer to chromatographically pure compounds.

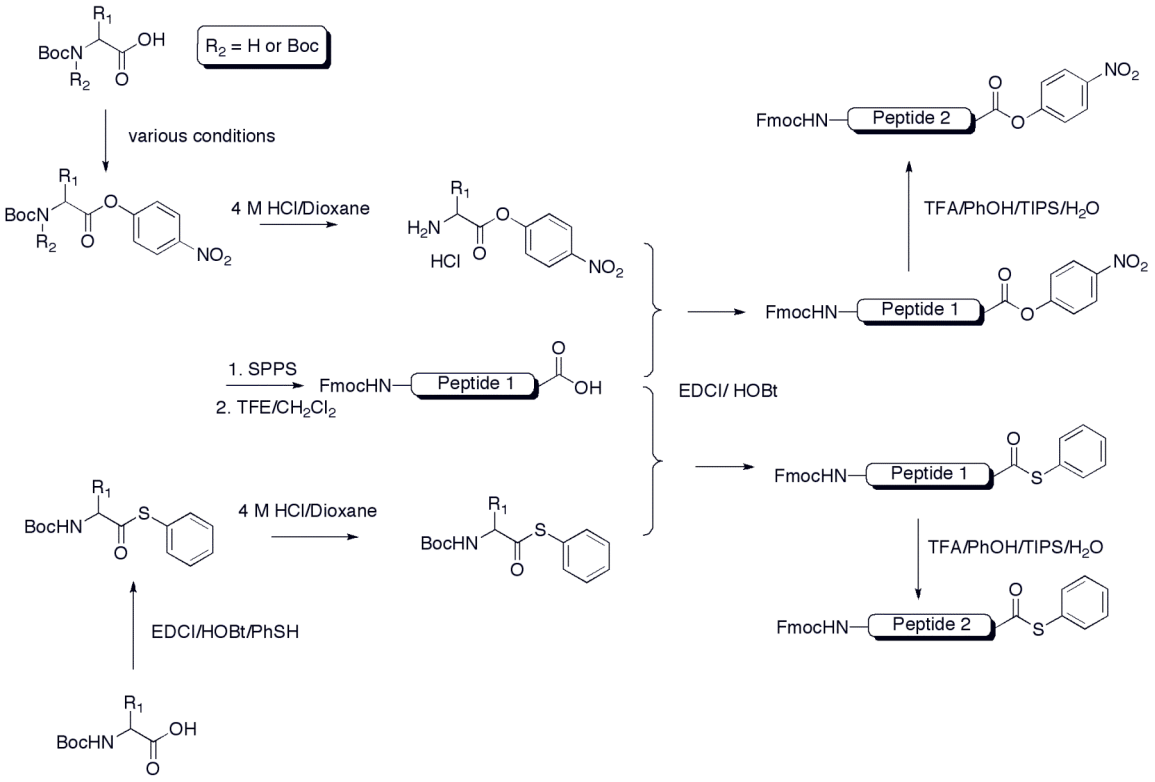
HPLC:

All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A) /0.04% TFA in acetonitrile (solvent B). Preparative and analytical HPLC separations were performed using a Rainin HPXL solvent delivery system equipped with a Rainin UV-1 detector. LC-MS chromatographic separations were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with XBridge™ C18 column (5.0 μm, 2.1 x 150 mm), X-Terra™ MS C18 column (3.5 μm, 2.1 x 100.0 mm) or Varian Microsorb C18 column (2 x 150 mm) at a flow rate of 0.2 mL/min**. HPLC** separations were performed using: X-Bridge™ Prep C18 column OBD™ (5.0 μm, 19 x 150 mm), a flow rate of 16 mL/min. Microsorb 100-5 C18 column at a flow rate of 16.0 mL/min or Microsorb 300-5 C4 column at a flow rate of 16.0 mL/min.

Solid-phase Peptide Synthesis According to Fmoc-strategy

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc/t-Bu protocols. The deblock mixture was a mixture of 100/5/5 of DMF/piperidine/DBU. The following Fmoc amino acids from NovaBiochem were employed: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cys(*t*Buthio)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide synthesis vessel with DCM. The resin was subjected to a cleavage cocktail (60.0 mg of phenol, 0.2 ml of water, 0.15 ml of triisopropylsilane, and 3.0 ml TFA) for 2.0 hours. The resin was removed by filtration, and the resulting solution was concentrated. The oily residue was triturated with diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The resulting solid was ready for HPLC purification.

General Procedure for the synthesis of Peptide-Oxo/Thiol-ester:[1]



General Procedure for *p*-Nitrophenyl ester (Oxo-ester) Mediates Native Chemical Ligation:

The cysteinyl peptide (2.0 eq) and oxo-ester (1.0 eq) were dissolved in 0.5 mL of Guanidine buffer*. To the solution, were added 40.0 μL of 0.5 M bond-breaker® TCEP solution (Pierce). The reaction mixture was stirred at room temperature. The reactions were monitored by LC-MS and purified directly by HPLC upon consumption of the starting material.

* **Guanidine buffer**: To 1.0 mL of 6.0 M guanidine buffer, were added 26.8 mg of Na_2HPO_4 and 1.0 mg of *p*-NO_2Phenol. The pH value of resulting solvent was nearly 6.3-6.5.

[1] Wan, Q.; Danishefsky, S. J. "Free Radical Based, Specific Desulfurization of Cysteine: A Powerful Advance in the Synthesis of Polypeptides and Glycopolypeptides" *Angew. Chem.* **2007**, *119*, 9408; *Angew. Chem. Int. Ed.* **2007**, *46*, 9248.

Compound 1: Cys-GDRYTFRWG-OH

Fmoc-Gly-NovaSyn® TGT resin, Boc-Cys(S*t*Bu)-OH and Fmoc-Tyr(OtBu)-Thr($\psi^{Me,Me}$pro)-OH were used following the general SPPS procedure. Semiprep HPLC purification (10-40% CH_3CN/H_2O over 30 min, XBridge column, 226 nm, 16 mL/min) followed by concentration at reduced pressure and lyophilization afforded compound **1** as a white powder.

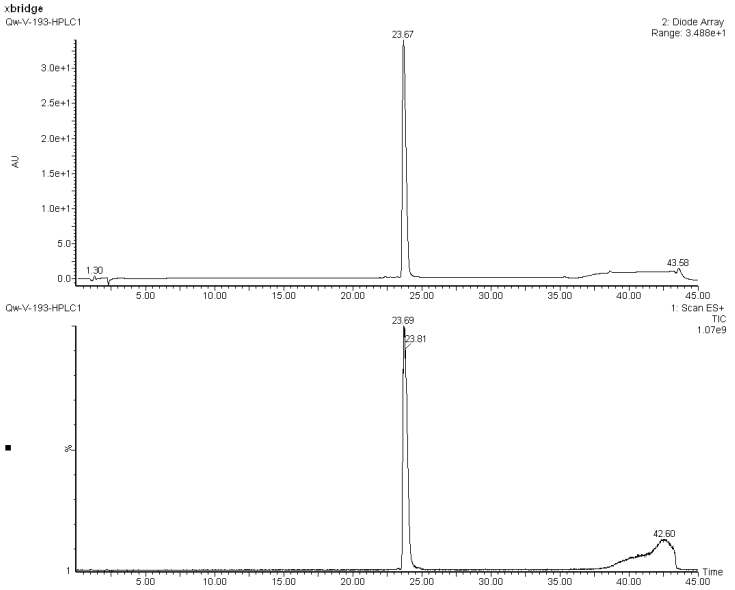


Figure 1: UV and MS traces from LC-MS analysis of compound **1**: gradient 10-40% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

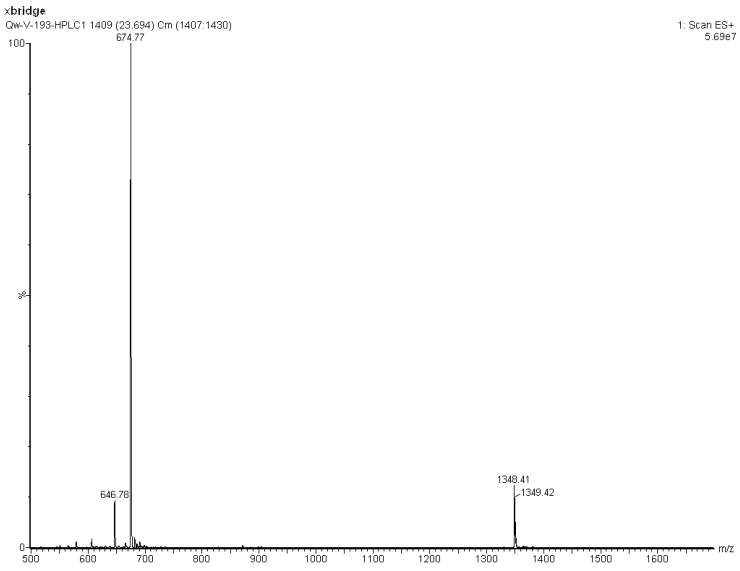


Figure 2: ESI-MS of compound **1**
ESI calcd for $C_{60}H_{85}N_{17}O_{15}S_2$ $[M+H]^+$ *m/z* = 1348.59 $[M+2H]^{2+}$ *m/z* = 675.29, found: 1348.41, 674.77.

Compound 2: Fmoc-RTGDSAG-Thr-*p*-NO_2Ph

Boc-Thr(*t*Bu)-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (20-45% CH_3CN/H_2O over 30 min, xbridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **2** as a white powder.

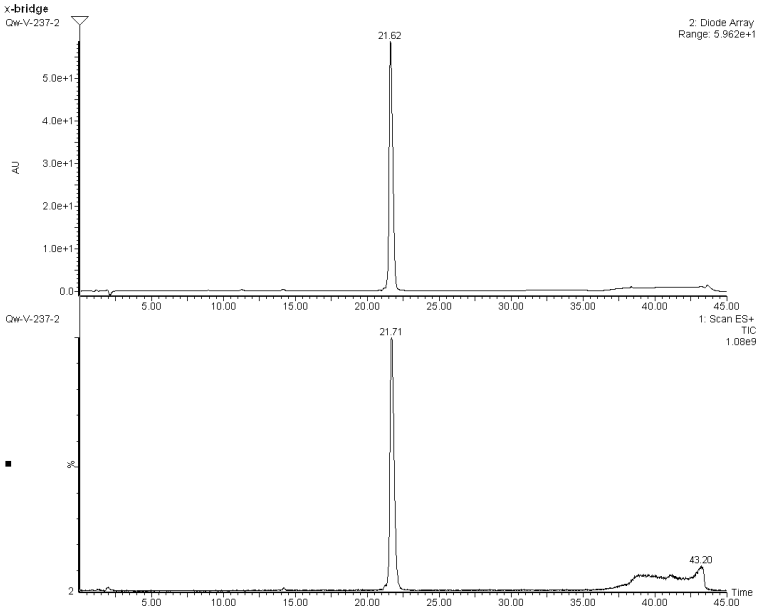


Figure 3: UV and MS traces from LC-MS analysis of compound **2**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

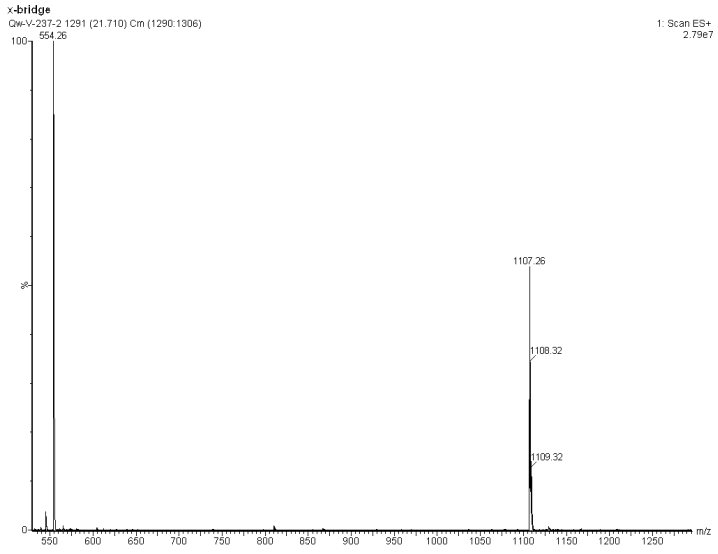


Figure 4: ESI-MS of compound **2**
ESI calcd for $C_{49}H_{62}N_{12}O_{18}$ $[M+H]^+$ *m/z* = 1107.43 $[M+2H]^{2+}$ *m/z* = 554.22, found: 1107.26, 554.26.

Compound 3: Fmoc-RTGDSAG-Val-*p*-NO_2Ph

Boc-Val-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (30-50% CH_3CN/H_2O over 30 min, C-18 column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **3** as a white powder.

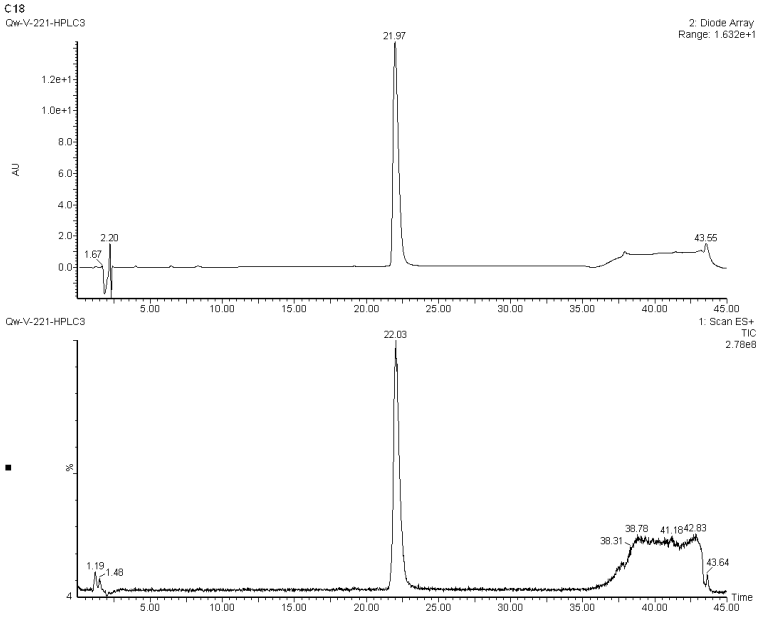


Figure 5: UV and MS traces from LC-MS analysis of compound **3**: gradient 30-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C-18 column.

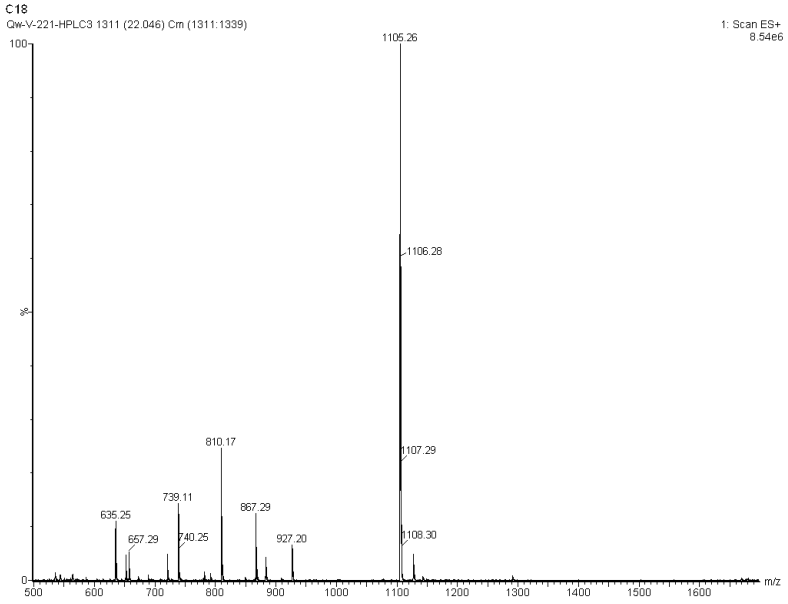


Figure 6: ESI-MS of compound **3**
ESI calcd for $C_{50}H_{64}N_{12}O_{17}$ $[M+H]^+$ *m/z* = 1105.45; found: 1105.26.

Compound 4: Fmoc-RTGDSAG-Ile-*p*-NO_2Ph

Boc-Ile-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (20-50% CH_3CN/H_2O over 30 min, x-bridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **4** as a white powder.

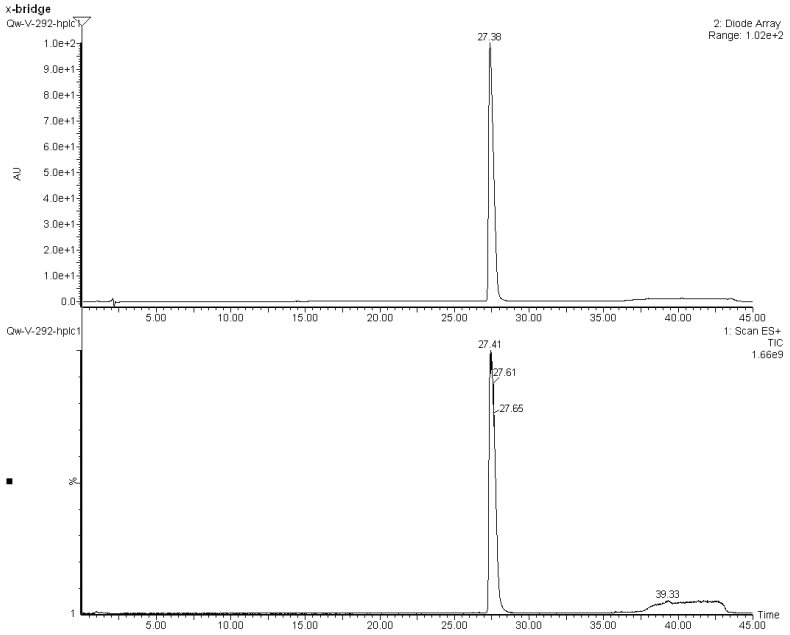


Figure 7: UV and MS traces from LC-MS analysis of compound **4**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, xbridge column.

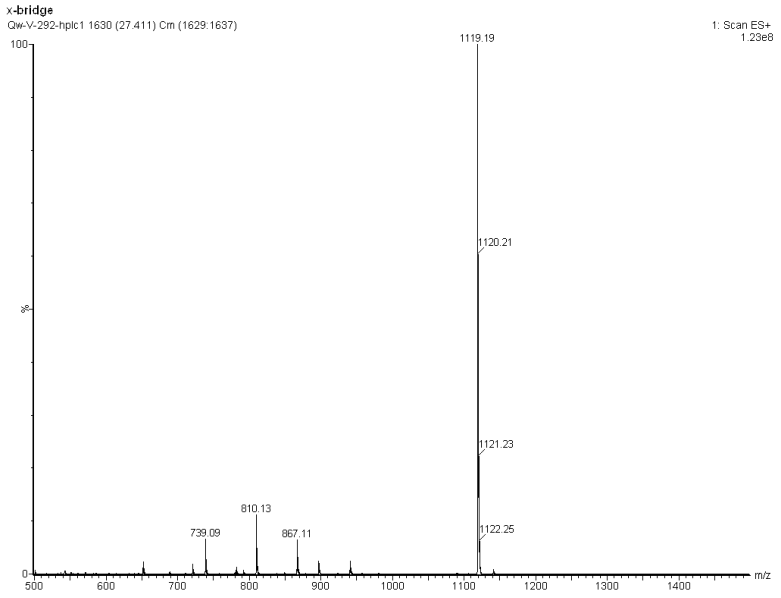


Figure 8: ESI-MS of compound **4**
ESI calcd for $C_{51}H_{66}N_{12}O_{17}$ $[M+H]^+$ *m/z* = 1119.47; found: 1119.19.

Compound 5: Fmoc-RTGDSAG-D-*allo*-Ile-*p*-NO_2Ph

Boc-D-*allo*-Ile-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (20-50% CH_3CN/H_2O over 30 min, x-bridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **5** as a white powder.

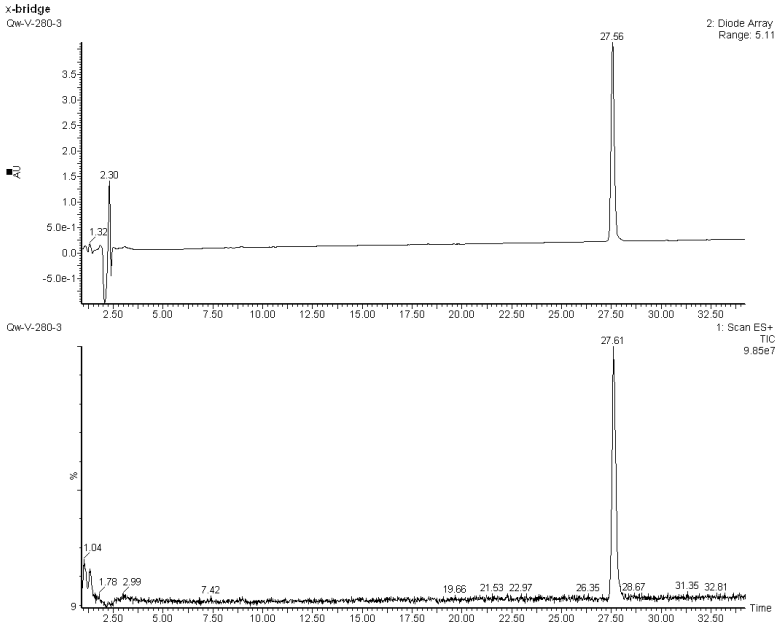


Figure 9: UV and MS traces from LC-MS analysis of compound **5**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, xbridge column.

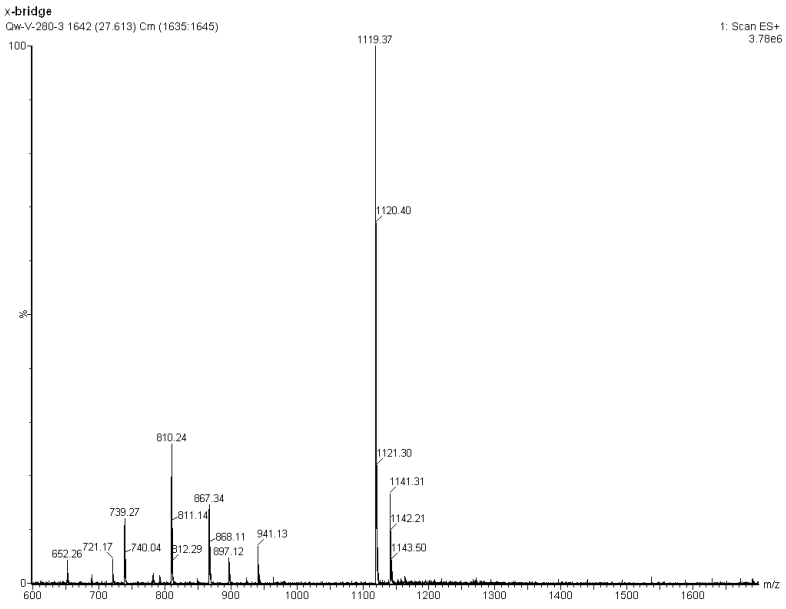


Figure 10: ESI-MS of compound **5**
ESI calcd for $C_{51}H_{66}N_{12}O_{17}$ $[M+H]^+$ *m/z* = 1119.47, $[M+Na]^+$ *m/z* = 1141.47; found: 1119.37, 1141.31.

Compound 6: Fmoc-RTGDSAG-Pro-*p*-NO_2Ph

Boc-Pro-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (30-50% CH_3CN/H_2O over 30 min, C-18 column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **6** as a white powder

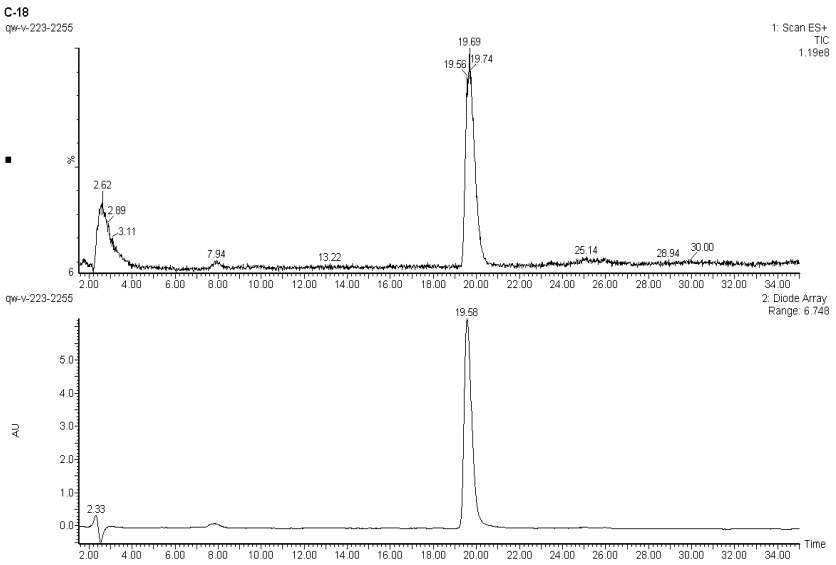


Figure 11: UV and MS traces from LC-MS analysis of compound **6**: gradient 30-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, C-18 column.

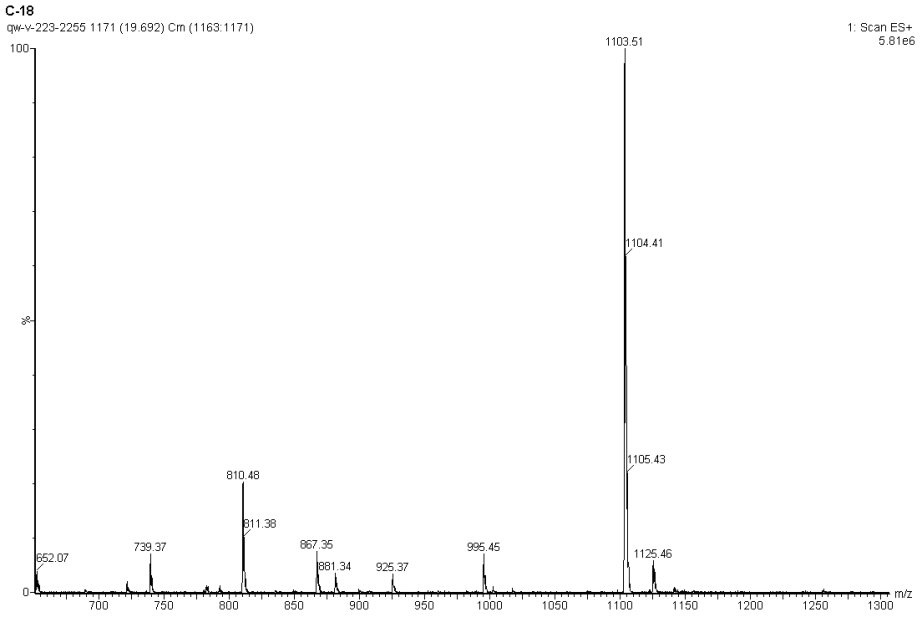


Figure 12: ESI-MS of compound **6**
ESI calcd for $C_{50}H_{62}N_{12}O_{17}$ $[M+H]^+$ *m/z* = 1103.44, $[M+Na]^+$ *m/z* = 1125.44; found: 1103.51, 1125.46.

Compound 7: Fmoc-RTGDSAG-**Thr**-CGDRYTFRWG-OH

0.5 mg of compound **2** and 1.3 mg of compound **1** were subjected to the ligation condition as described in the general procedure. HPLC purification (20-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.8 mg of compound **7** as a white powder, 79% yield.

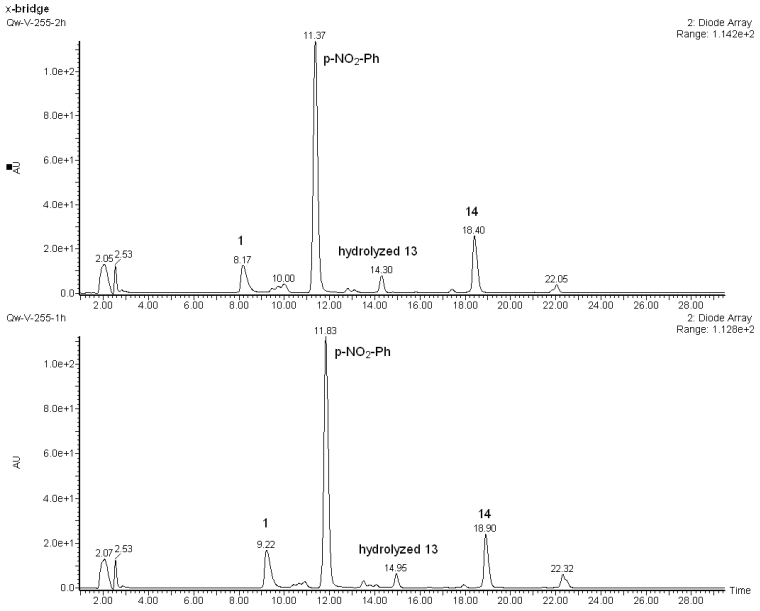


Figure 13: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

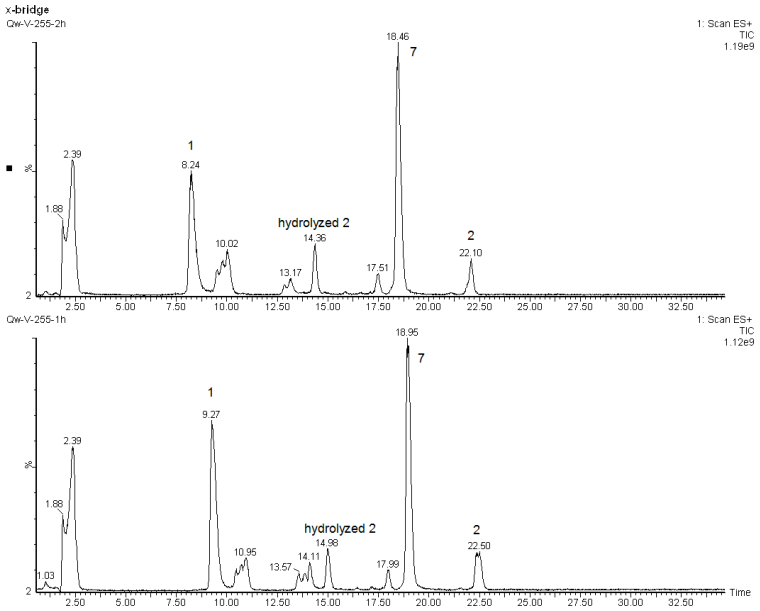


Figure 14: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

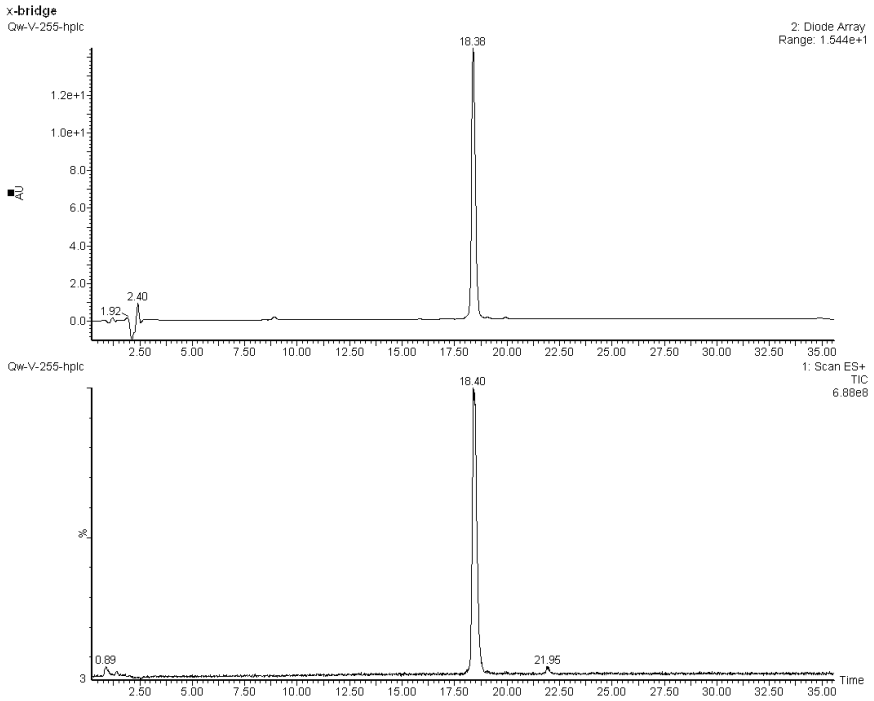


Figure 15: UV and MS traces from LC-MS analysis of compound **7** after purification: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

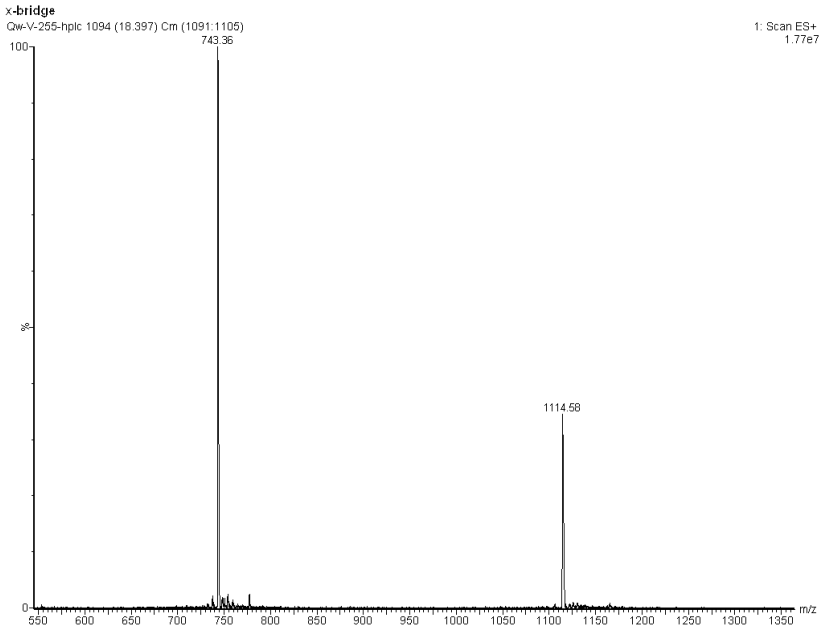


Figure 16: ESI-MS of compound **7**
ESI calcd for $C_{99}H_{134}N_{28}O_{30}S$ $[M+2H]^{2+}$ *m/z* = 1114.48, $[M+3H]^{3+}$ *m/z* = 743.32, found: 1114.58, 743.36.

Compound 8: Fmoc-RTGDSAG-**Val**-CGDRYTFRWG-OH

0.5 mg of compound **3** and 1.3 mg of compound **1** were subjected to the ligation condition as described in the general procedure. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.7 mg of compound **8** as a white powder, 69% yield.

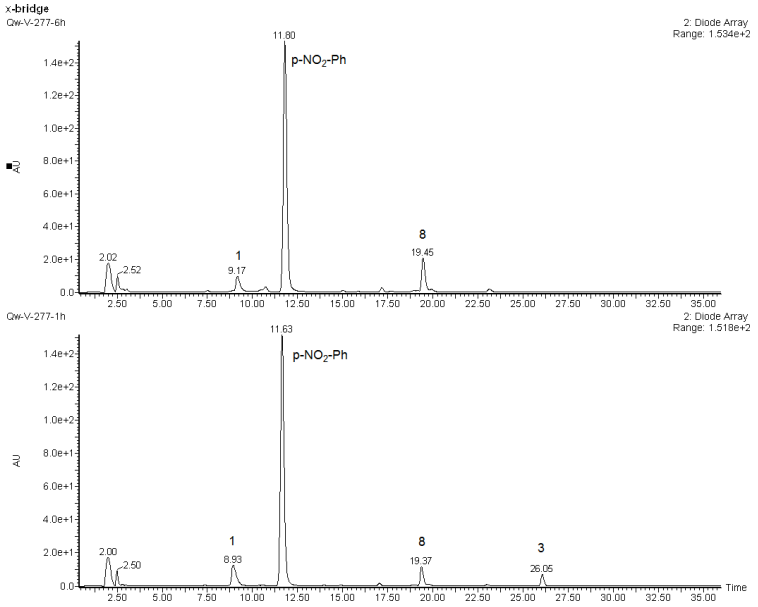


Figure 17: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

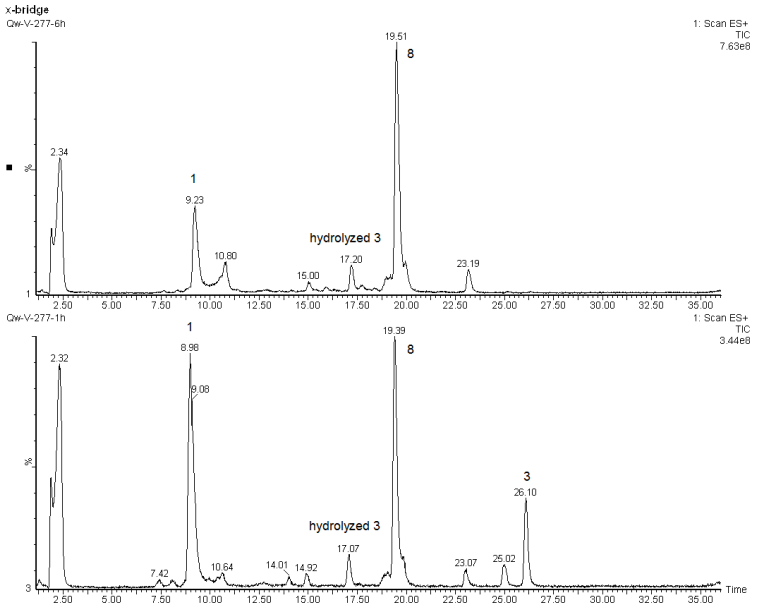


Figure 18: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

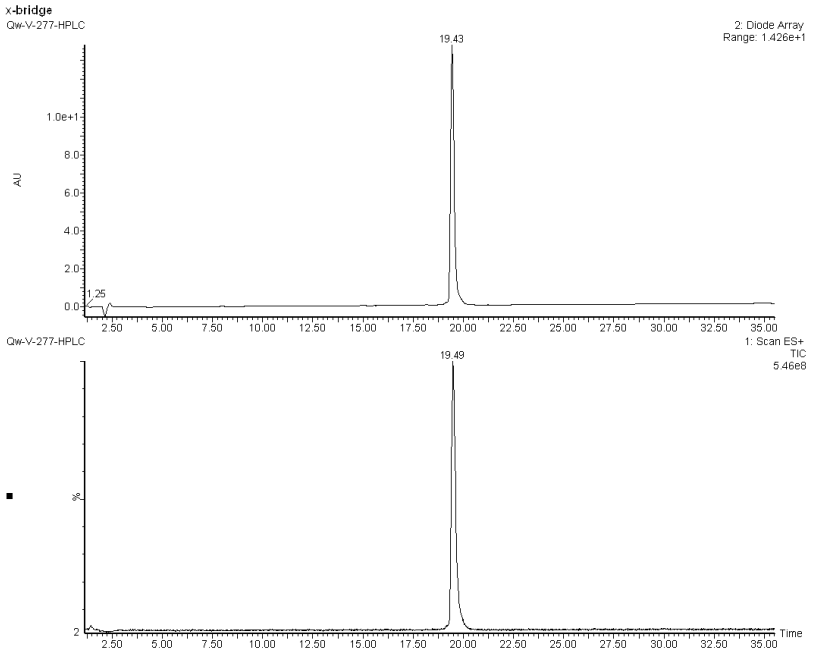


Figure 19: UV and MS traces from LC-MS analysis of compound **8** after purification: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

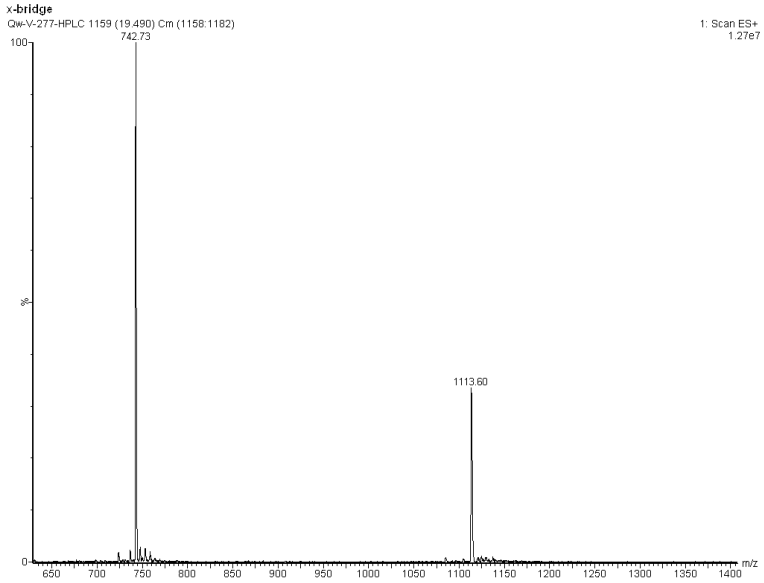


Figure 20: ESI-MS of compound **8**
ESI calcd for $C_{100}H_{136}N_{28}O_{29}S$ $[M+2H]^{2+}$ *m/z* = 1113.48, $[M+3H]^{3+}$ *m/z* = 742.65, found: 1113.60, 742.73.

Compound 9: Fmoc-RTGDSAG-**Ile**-CGDRYTFRWG-OH

0.5 mg of compound **4** and 1.3 mg of compound **1** were subjected to the ligation condition as described in the general procedure at 30 ^{o}C. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.7 mg of compound **9** as a white powder, 70% yield.

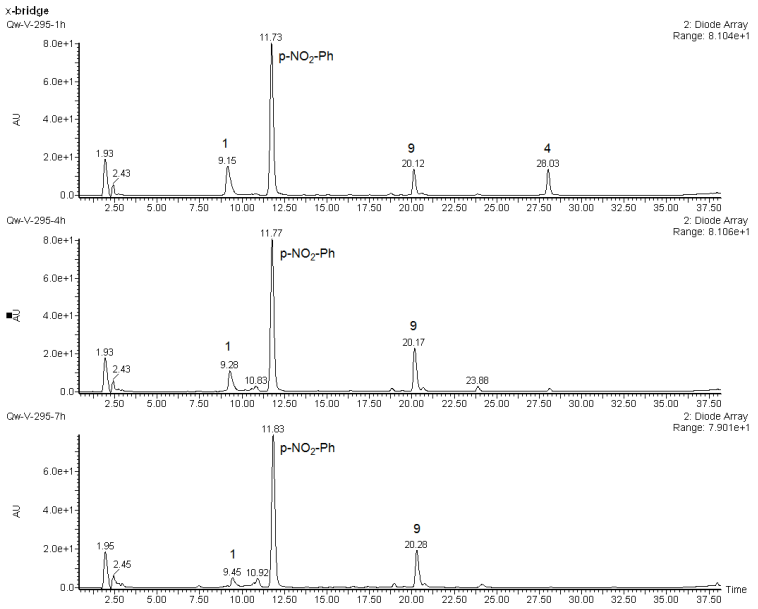


Figure 21: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

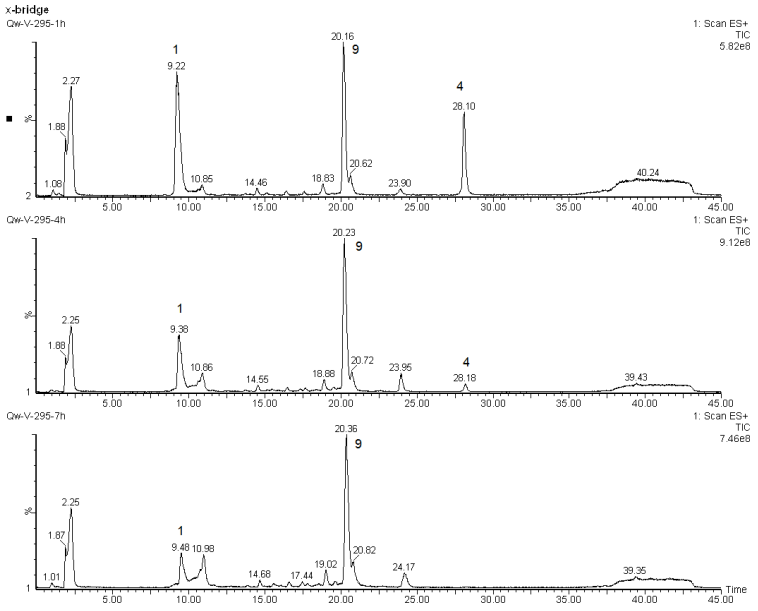


Figure 22: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

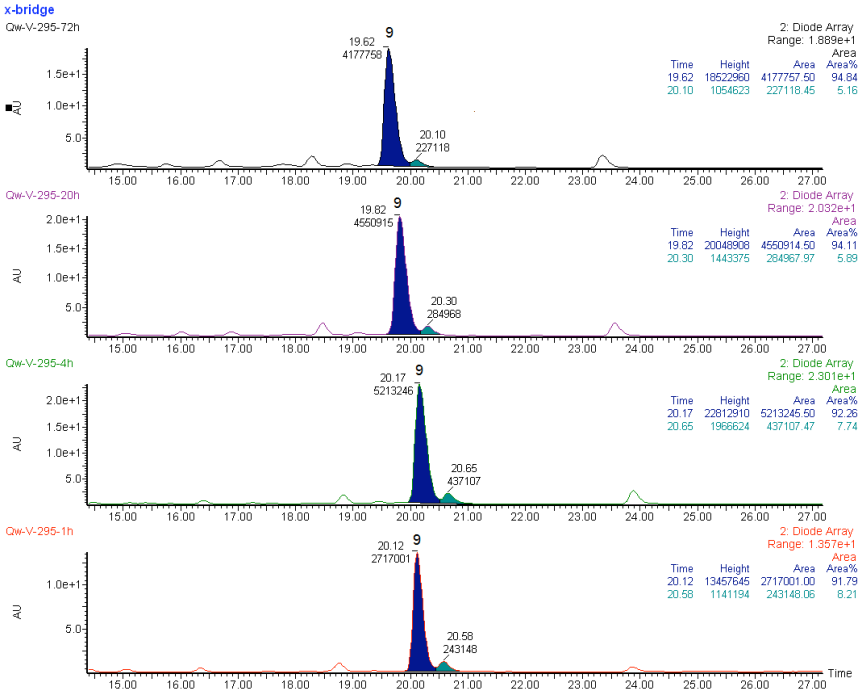


Figure 23: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

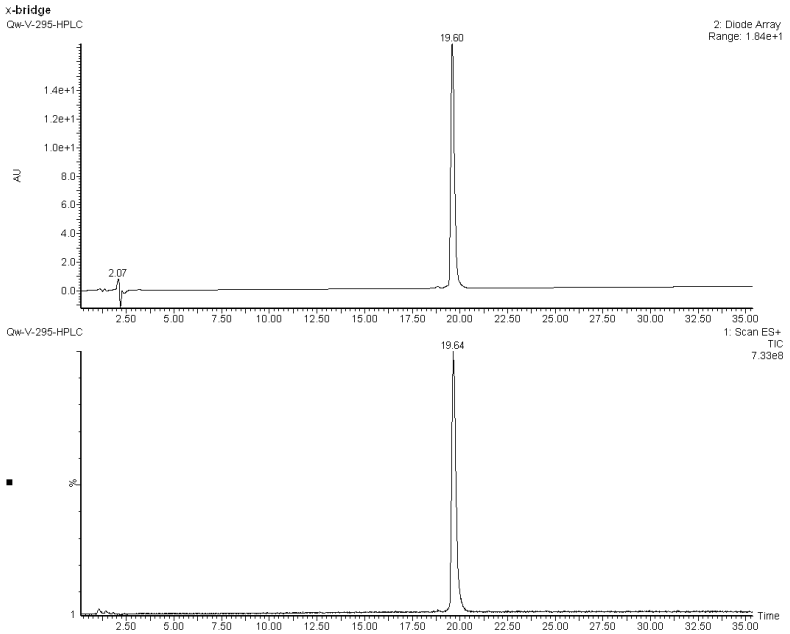


Figure 24: UV and MS traces from LC-MS analysis of compound **9** after purification: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, xbridge column

Figure 25: ESI-MS of compound **9**
ESI calcd for $C_{101}H_{138}N_{28}O_{29}S$ $[M+2H]^{2+}$ *m/z* = 1120.49, $[M+3H]^{3+}$ *m/z* = 747.33, found: 1120.53, 747.35.

Compound 10: Fmoc-RTGDSAG-*allo*-**Ile**-CGDRYTFRWG-OH

0.5 mg of compound **5** and 1.3 mg of compound **1** were subjected to the ligation condition as described in the general procedure. HPLC purification (15-35% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.6 mg of compound **10** as a white powder, 60% yield.

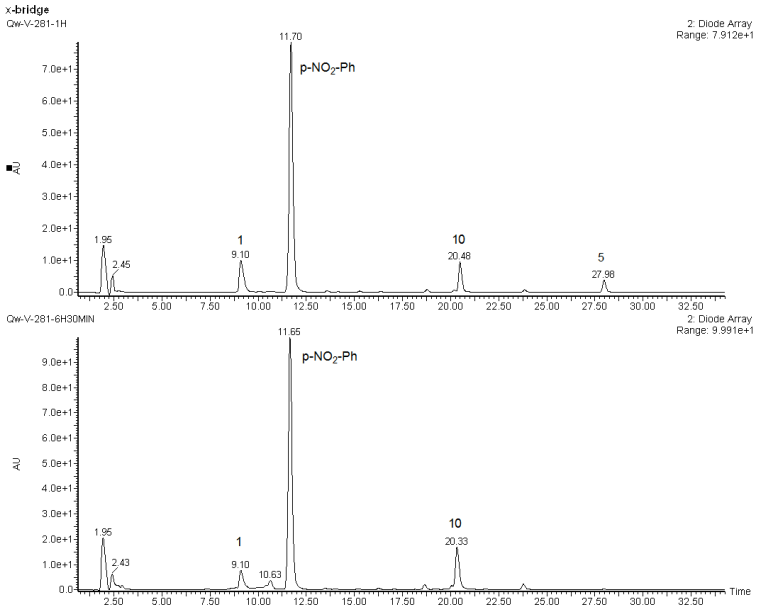


Figure 26: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

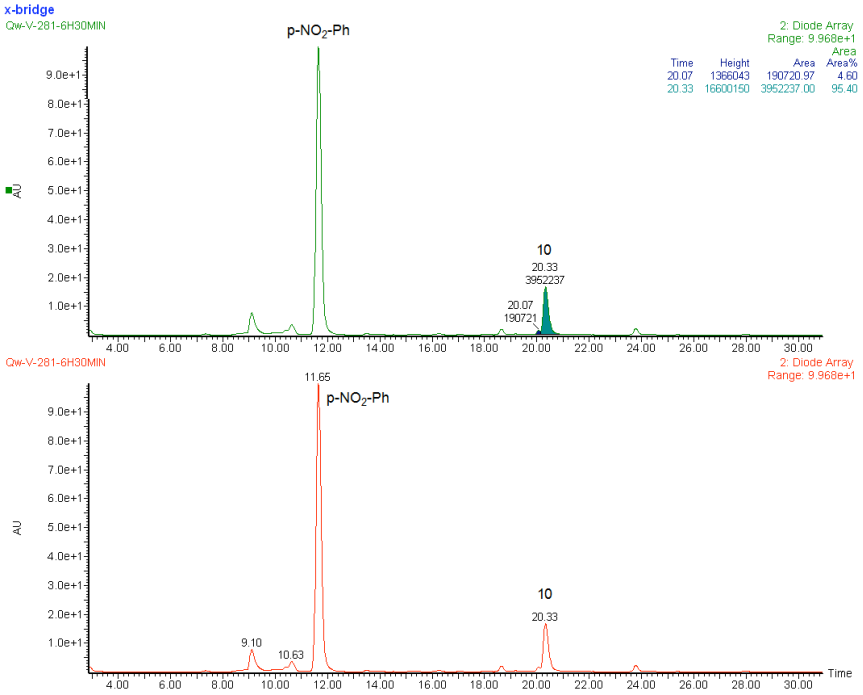


Figure 27: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge colum.

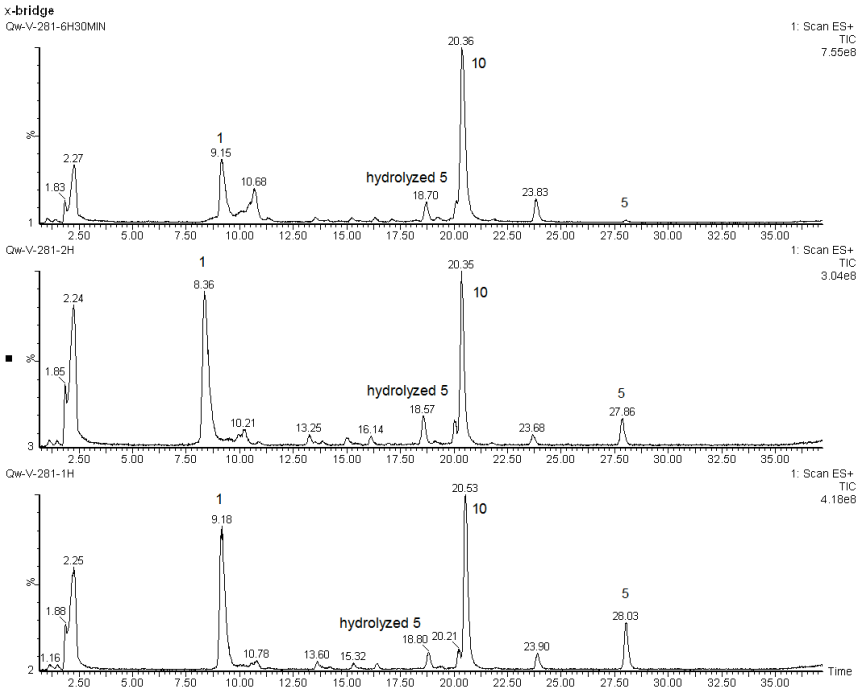


Figure 28: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

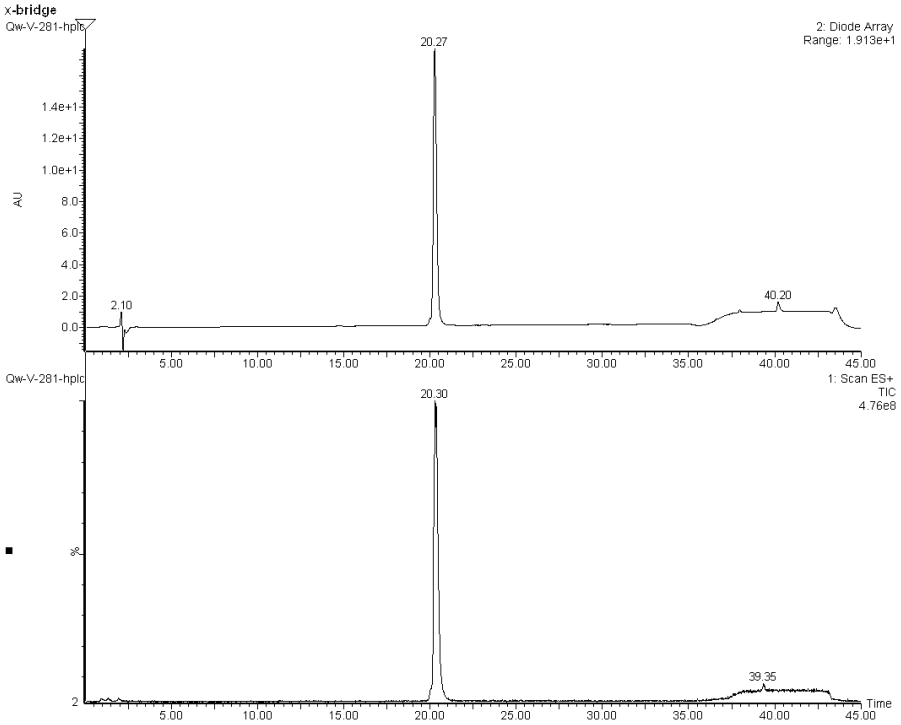


Figure 29: UV and MS traces from LC-MS analysis of compound **10** after purification: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

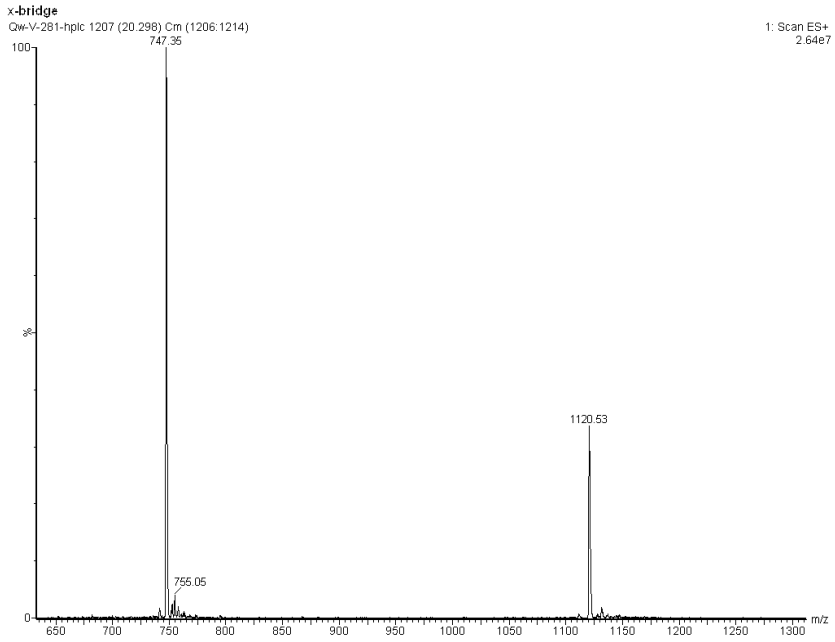


Figure 30: ESI-MS of compound **10**
ESI calcd for $C_{101}H_{138}N_{28}O_{29}S$ $[M+2H]^{2+}$ *m/z* = 1120.49, $[M+3H]^{3+}$ *m/z* = 747.33, found: 1120.53, 747.35.

Compound 11: Fmoc-RTGDSAG-**Pro**-CGDRYTFRWG-OH

0.5 mg of compound **6** and 1.3 mg of compound **1** were subjected to the ligation condition as described in the general procedure. HPLC purification (20-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.5 mg of compound **11** as a white powder, 50% yield.

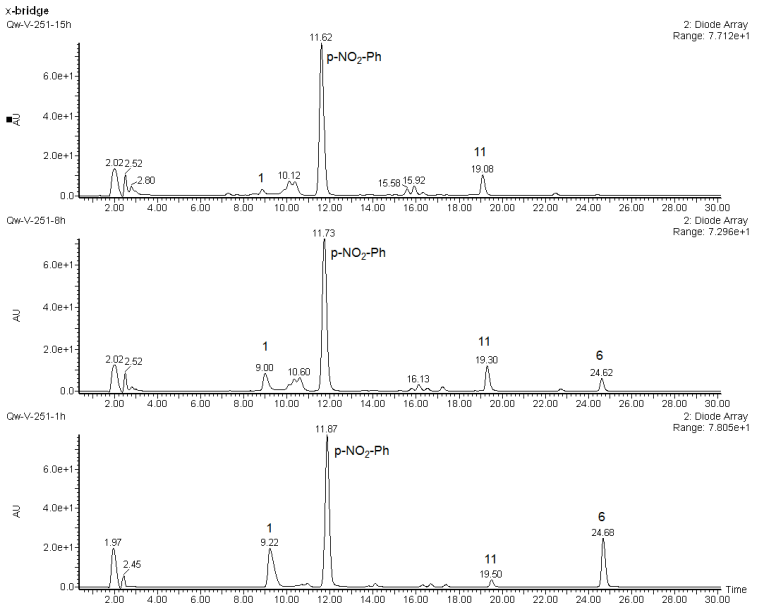


Figure 31: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

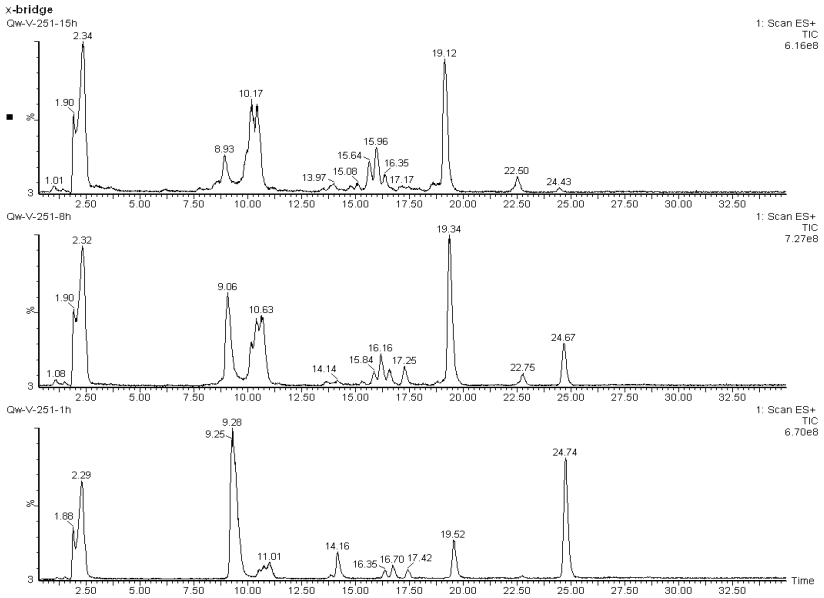


Figure 32: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

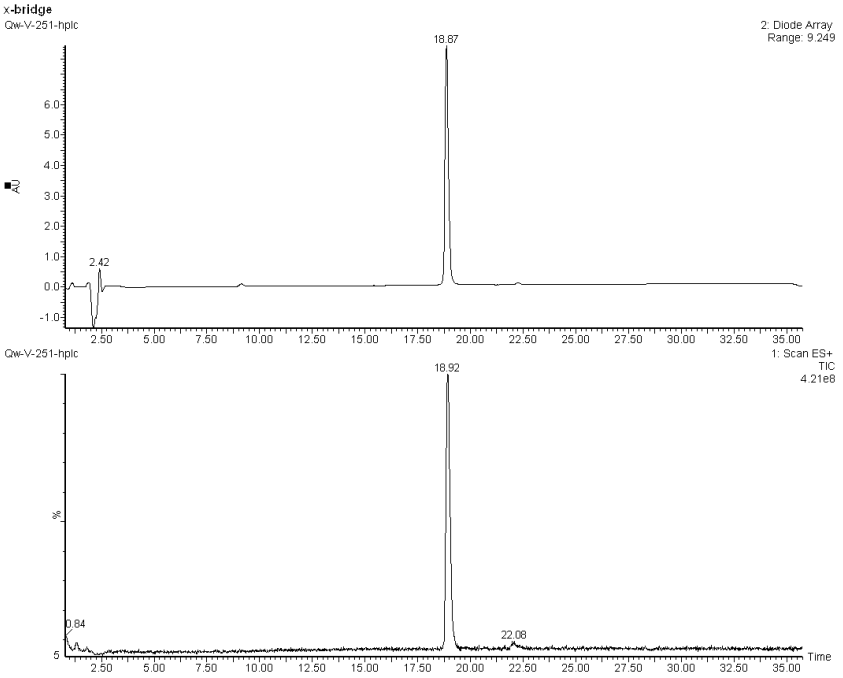


Figure 33: UV and MS traces from LC-MS analysis of compound **11** after purification: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

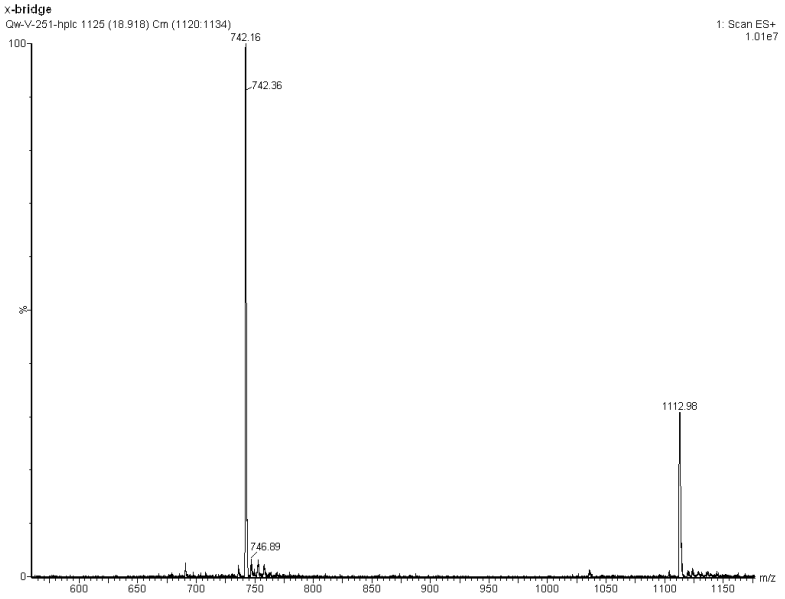


Figure 34: ESI-MS of compound **11**
ESI calcd for $C_{100}H_{134}N_{28}O_{29}S$ $[M+2H]^{2+}$ *m/z* = 1112.48, $[M+3H]^{3+}$ *m/z* = 741.99, found: 1112.96, 742.16.

Compound 12: Fmoc-RTGDSAG-Ile-SPh

Boc-Ile-OH was used following the general Thiol-ester synthesis procedure. Semiprep HPLC purification (20-50% CH_3CN/H_2O over 30 min, x-bridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **12** as a white powder

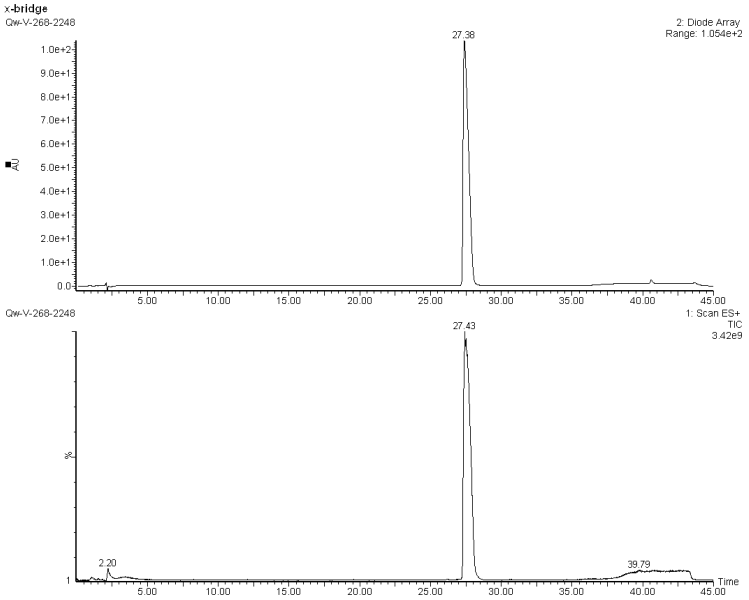


Figure 35: UV and MS traces from LC-MS analysis of compound **12**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, x-bridge column.

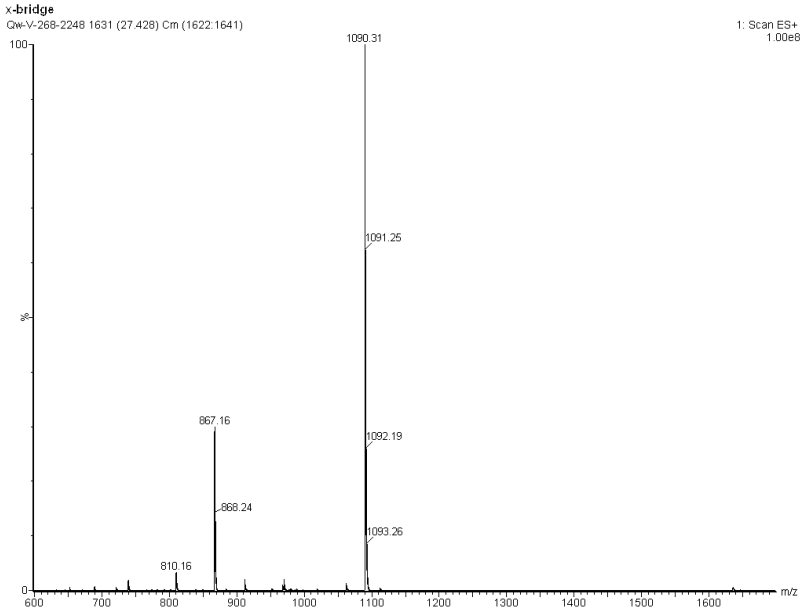


Figure 36: ESI-MS of compound **12**
ESI calcd for $C_{51}H_{67}N_{11}O_{14}S$ $[M+H]^+$ *m/z* = 1090.46; found: 1090.31.

Compound 9: Fmoc-RTGDSAG-**Ile**-CGDRYTFRWG-OH

0.5 mg of compound **12** and 1.3 mg of compound **1** were dissolved in 0.5 mL of Guanidine buffer*. To the solution, were added 1.0 µL of PhSH and 40.0 µL of 0.5 M bond-breaker® TCEP solution (Pierce). The reaction mixture was stirred at 30 °C for 72h. The reactions were monitored by LC-MS and purified directly by HPLC. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.6 mg of compound **9** as a white powder, 58% yield.

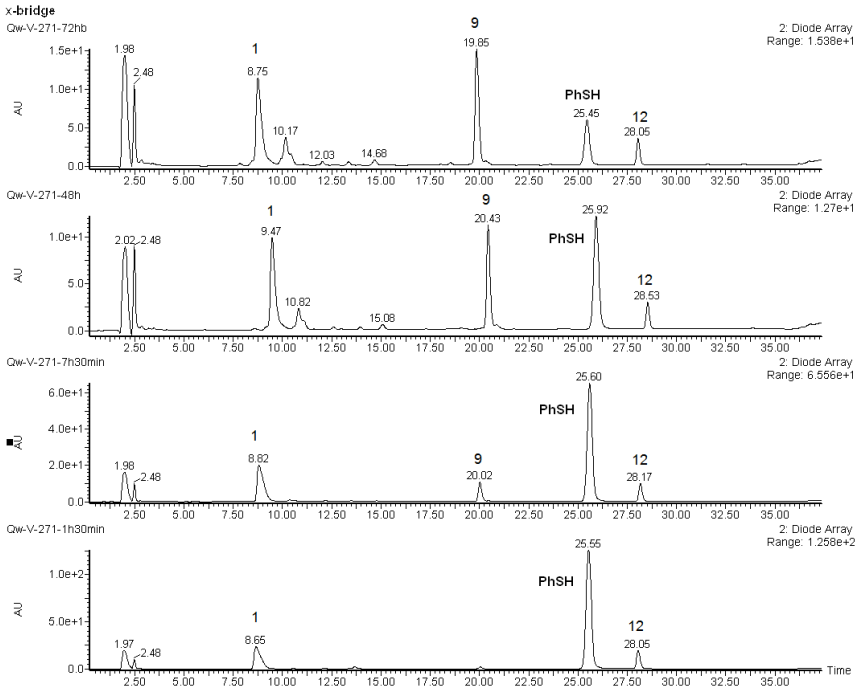


Figure 37: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column. According to the UV trace, the thioester shows about 51% conversion at 7.5 hours and 83% conversion at 72 hours.

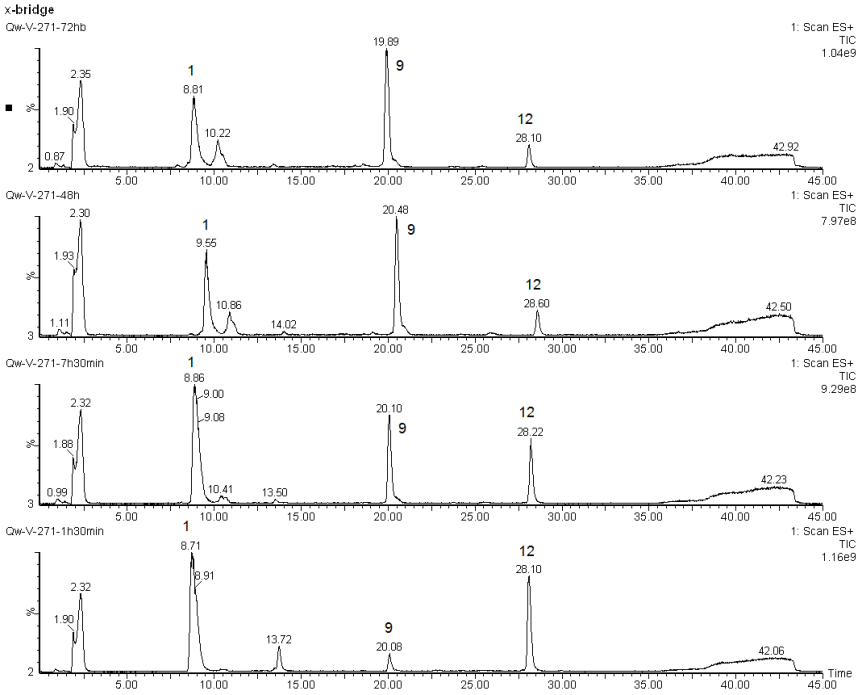


Figure 38: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column.

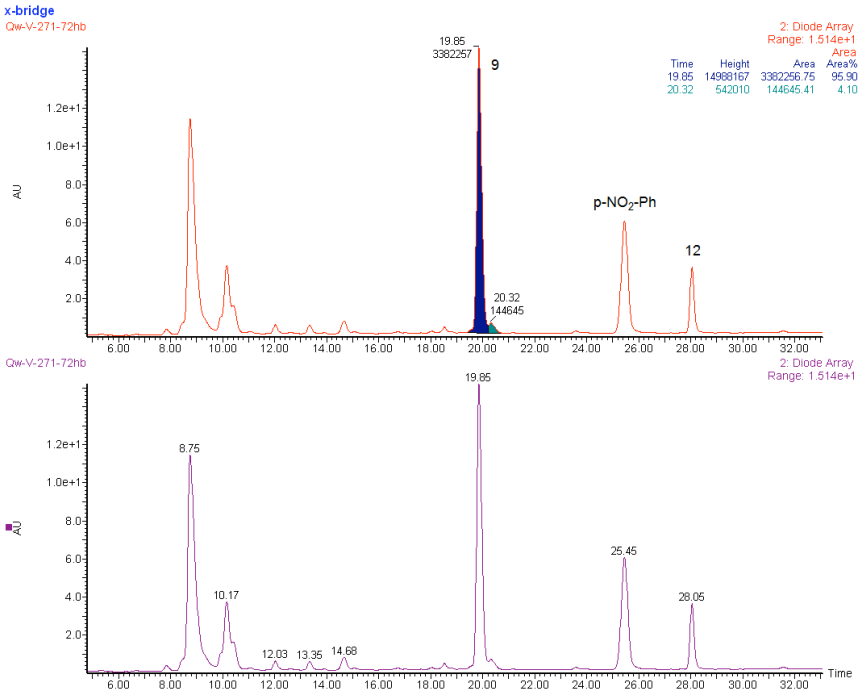


Figure 39: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

Compound 13: Fmoc-LysYDSRG-Leu-*p*-NO_2Ph

Boc-Leu-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (20-50% CH_3CN/H_2O over 30 min, xbridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **13** as a white powder.

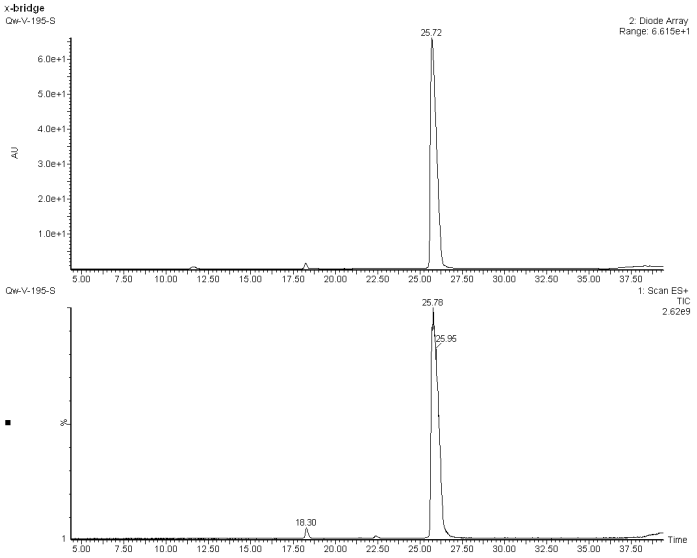


Figure 40: UV and MS traces from LC-MS analysis of compound **13**: gradient 10-40% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

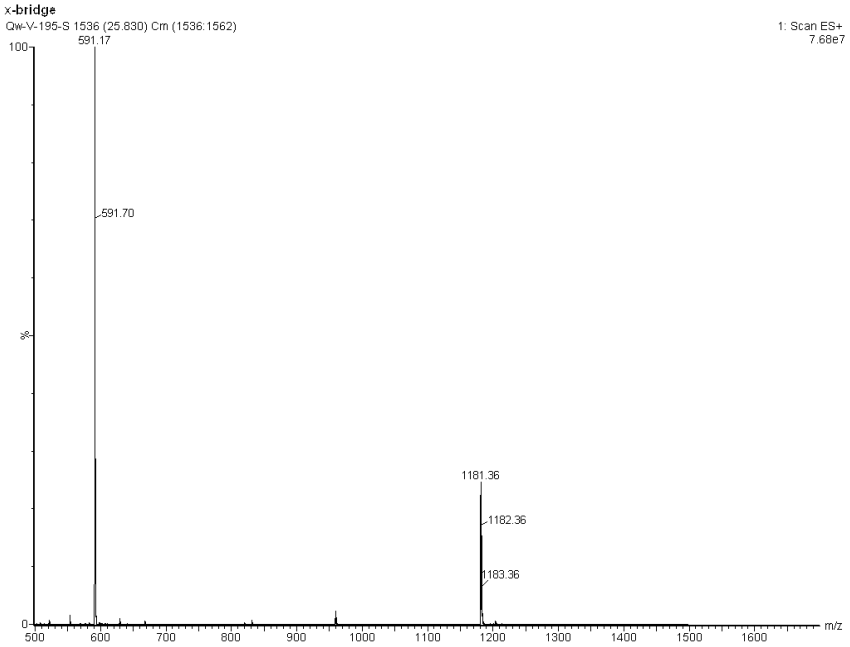


Figure 41: ESI-MS of compound **13**
ESI calcd for $C_{57}H_{72}N_{12}O_{16}$ $[M+H]^+$ *m/z* = 1181.52, $[M+2H]^{2+}$ *m/z* = 591.26; found: 1181.36, 591.17.

Compound 14: Fmoc-LysYDSRG-**Leu**-CGDRYTFRWG-OH

0.5 mg of compound **13** and 1.3 mg of compound **1** were subjected to the ligation condition as described in the general procedure. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.6 mg of compound **14** as a white powder, 61% yield.

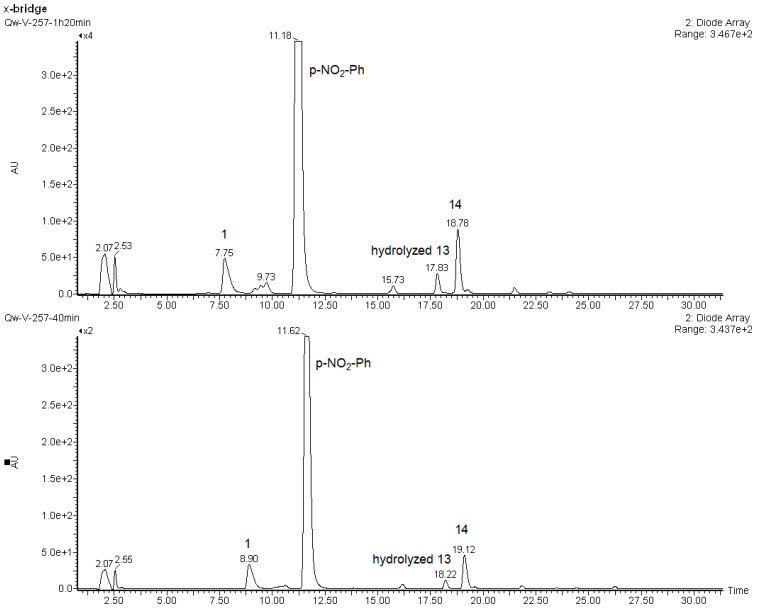


Figure 42: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

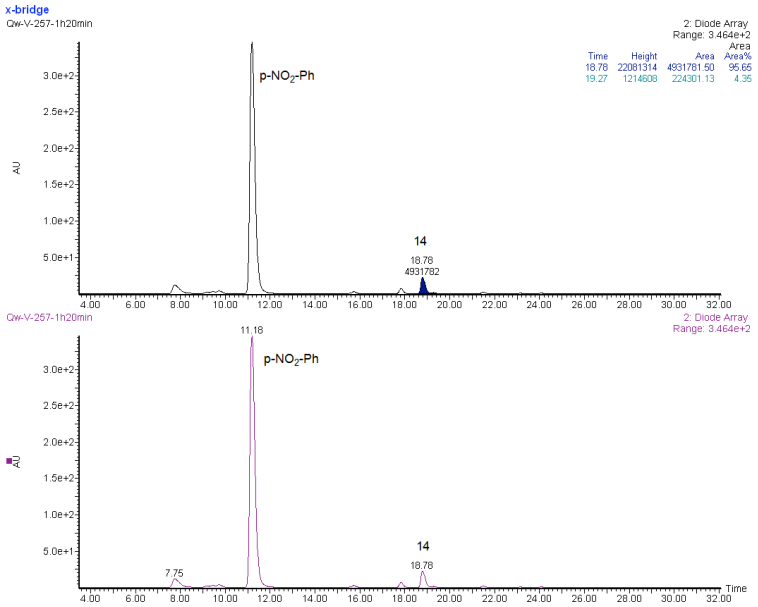


Figure 43: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

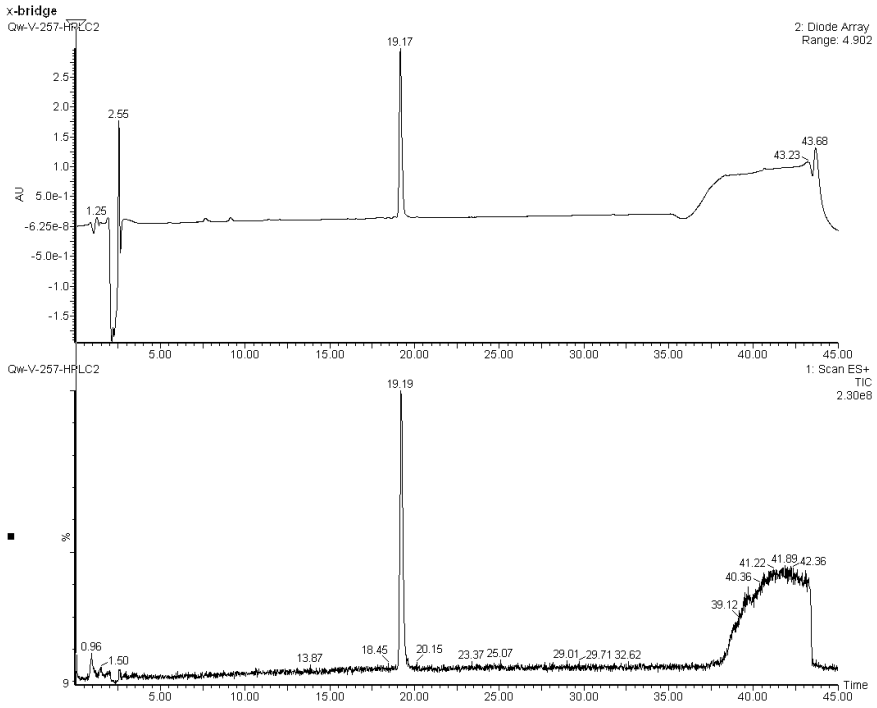


Figure 44: UV and MS traces from LC-MS analysis of compound **14** after purification: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, xbridge column

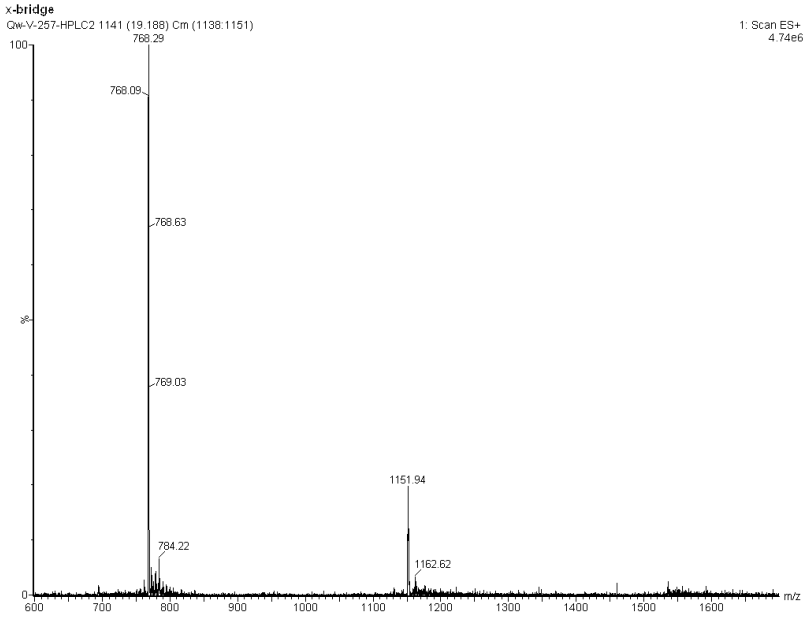


Figure 45: ESI-MS of compound **14**
ESI calcd for $C_{107}H_{144}N_{28}O_{28}S$ $[M+2H]^{2+}$ *m/z* = 1151.52, $[M+2H]^{2+}$ *m/z* = 768.01; found: 1151.94, 768.29.

Compound 16: Fmoc-KYDSRG-Phe-*p*-NO_2Ph

Boc-Phe-OH was used following the general oxo-ester synthesis procedure. Semiprep HPLC purification (30-50% CH_3CN/H_2O over 30 min, xbridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **16** as a white powder.

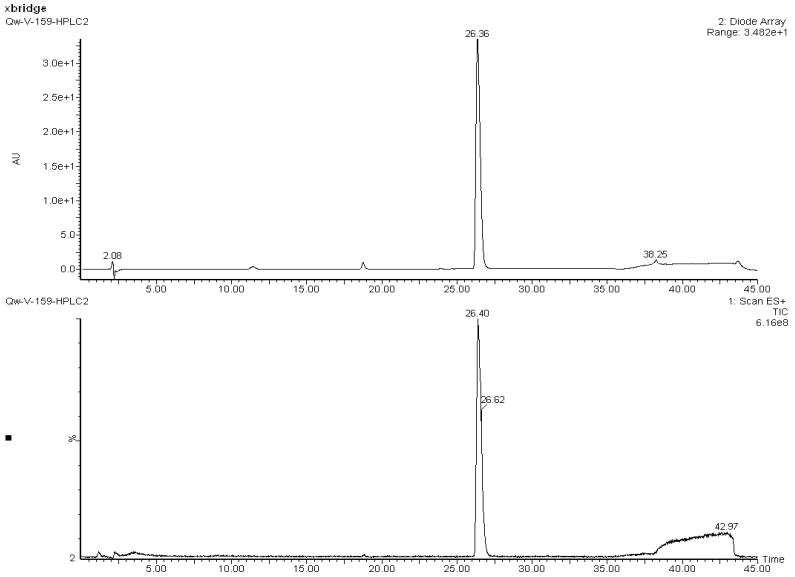


Figure 46: UV and MS traces from LC-MS analysis of compound **16**: gradient 30-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

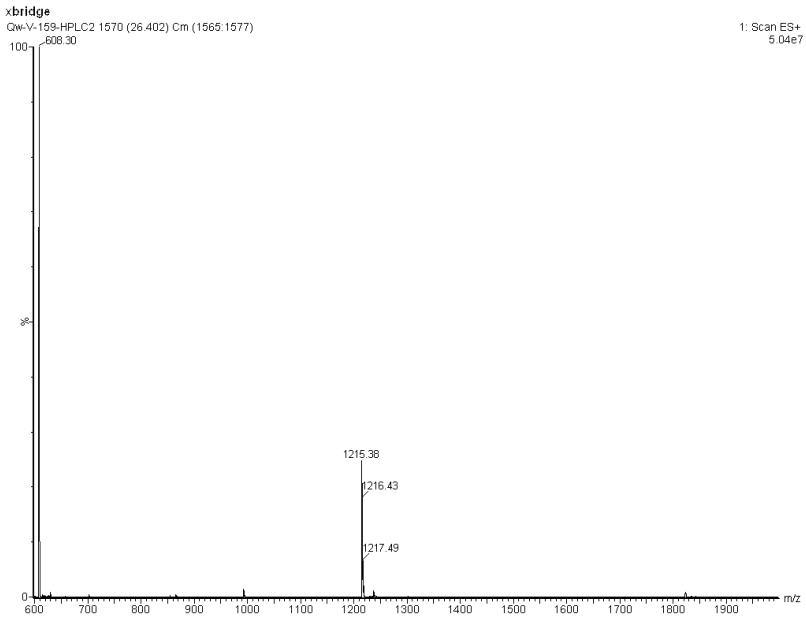


Figure 47: ESI-MS of compound **16**
ESI calcd for $C_{60}H_{70}N_{12}O_{16}$ $[M+H]^+$ *m/z* = 1215.50 $[M+2H]^{2+}$ *m/z* = 608.25, found: 1215.38, 608.30.

Compound 17: Fmoc-KYDSRGF-**Pen**-OH

1.0 mg of compound **16** and 1.0 mg of compound **15**, penicilamine, were dissolved in 0.5 mL of Guanidine buffer*. To the solution, were added and 40.0 µL of 0.5 M bond-breaker® TCEP solution (Pierce). The reaction mixture was stirred at room temperature for 2h. The reactions were monitored by LC-MS and purified directly by HPLC. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.4 mg of compound **17** as a white powder, 40% yield.

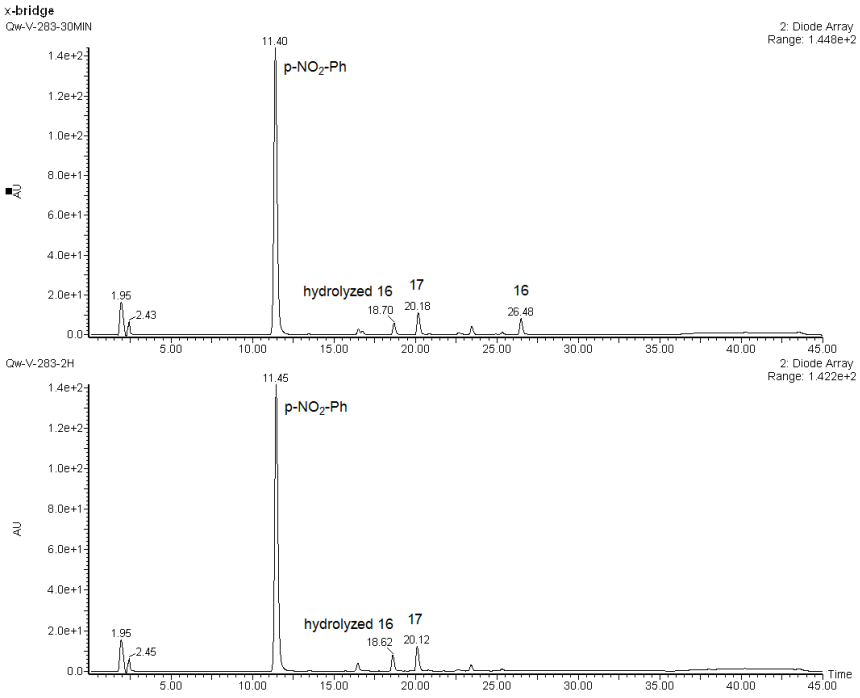


Figure 48: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

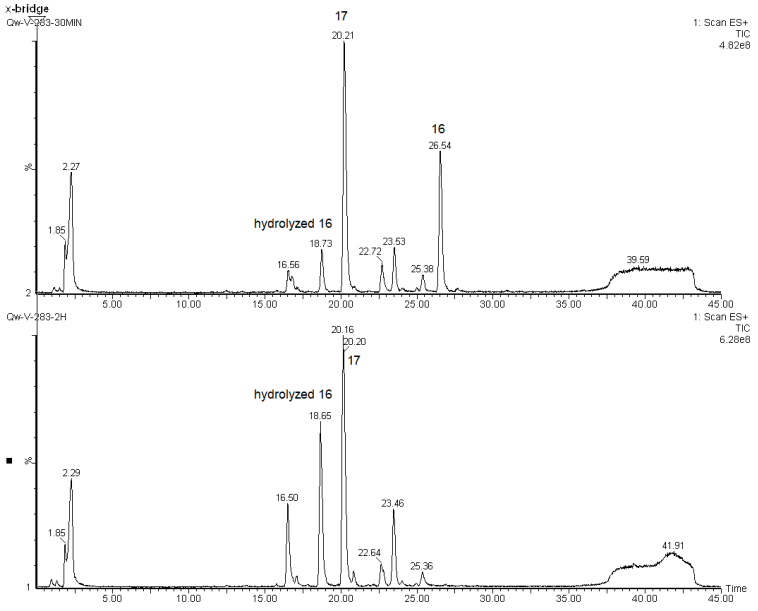


Figure 49: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

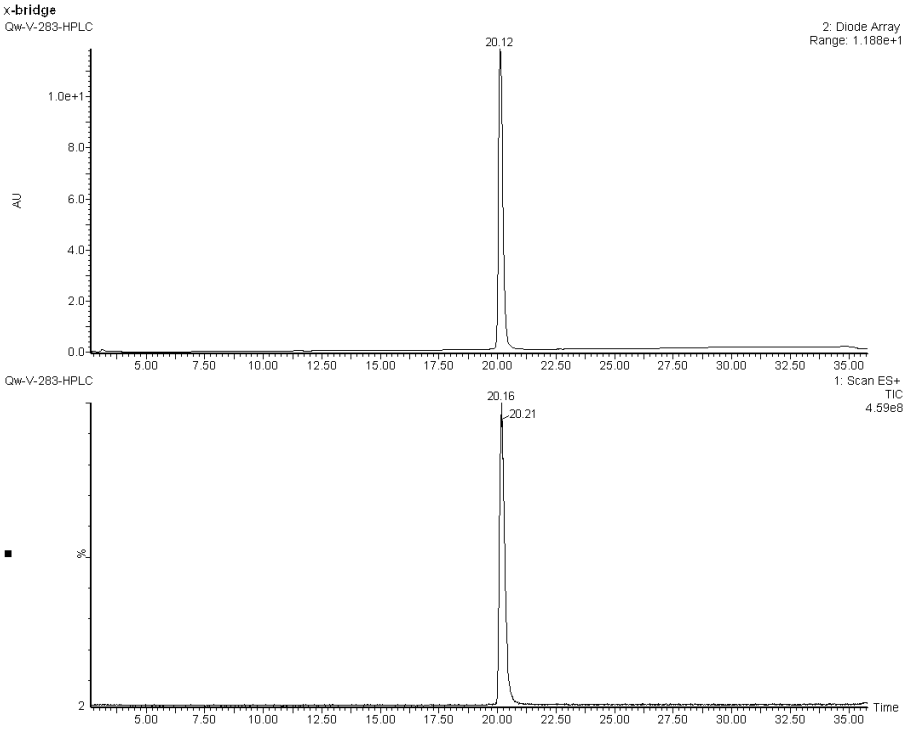


Figure 50: UV and MS traces from LC-MS analysis of compound **17**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

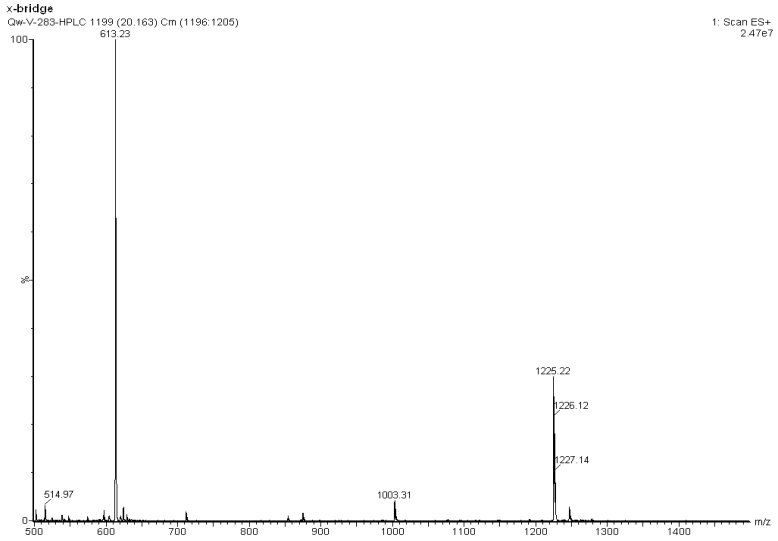


Figure 51: ESI-MS of compound **17**
ESI calcd for $C_{59}H_{76}N_{12}O_{15}S$ $[M+H]^+$ *m/z* = 1225.53 $[M+2H]^{2+}$ *m/z* = 613.27, found: 1225.22, 613.23.

Compound 18: Fmoc-KYDSRGF-**Val**-OH

The 0.4 mg of cysteinyl peptide, **Compound 17**, was dissolved in 200.0 μL of water and 100.0 μL of CH_3CN under argon. To the solution, were added 200.0 μL of 0.5 M bond-breaker® TCEP solution (Pierce), 20.0 μL of 2-methyl-2-propanethiol and 20.0 μL of radical initiator VA-044 (0.1 M in water). The reaction mixture was stirred at 37 ^oC for 2h. The reactions were monitored by LC-MS and purified directly by HPLC upon consumption of the starting material. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.3 mg of compound **18** as a white powder, 77% yield.

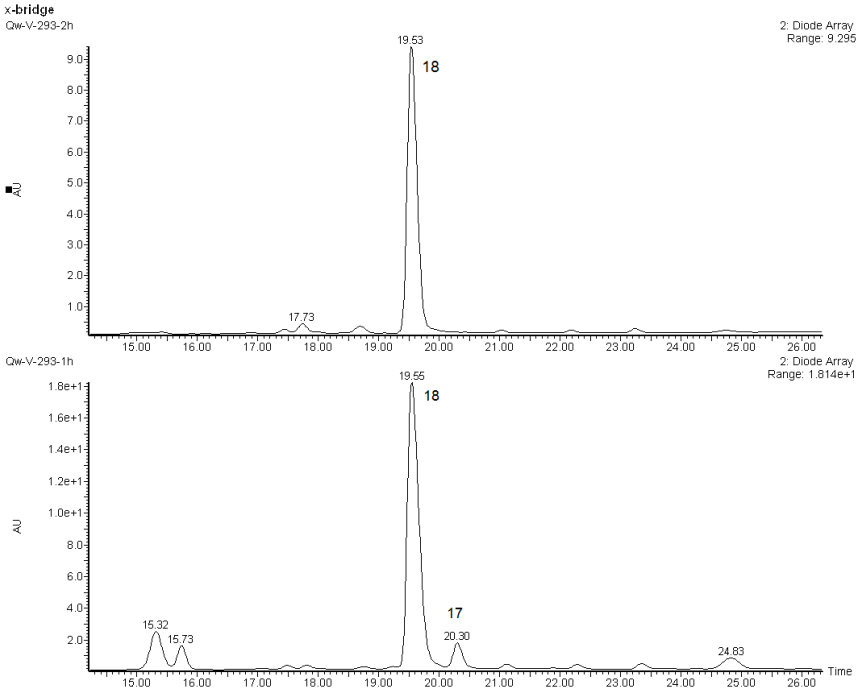


Figure 52: UV traces from LC-MS analysis of desulfurization: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

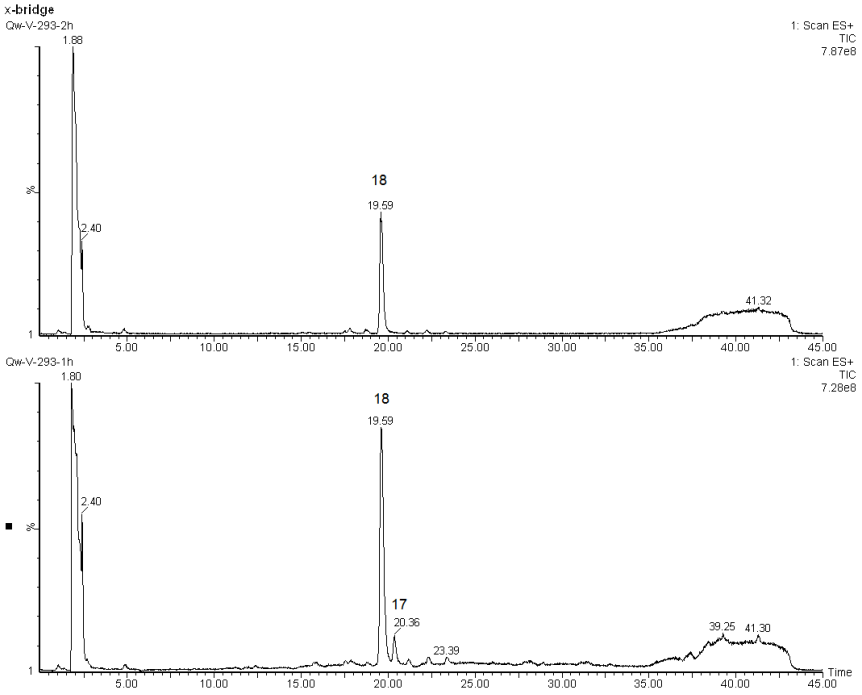


Figure 53: MS traces from LC-MS analysis of desulfurization: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

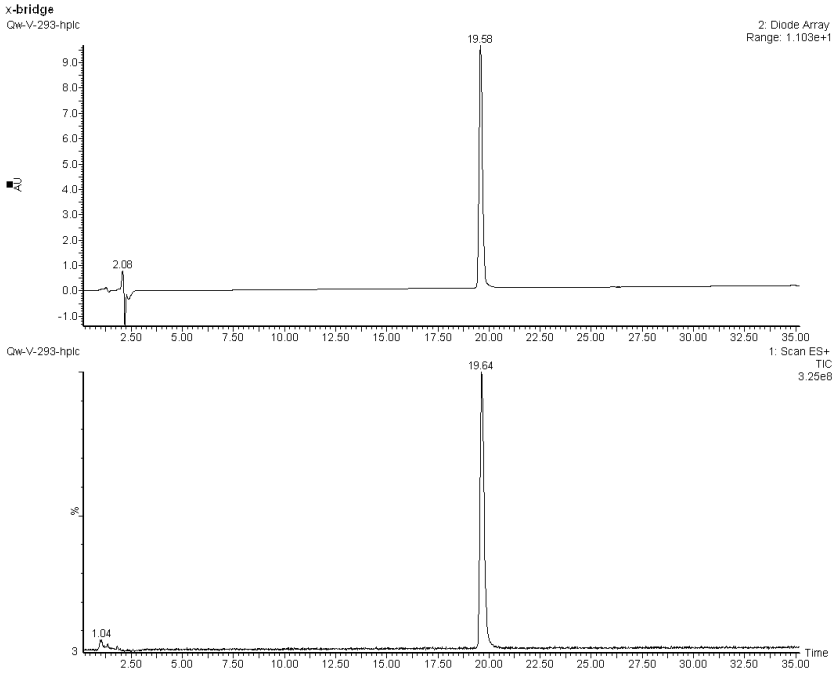


Figure 54: UV and MS traces from LC-MS analysis of compound **18**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

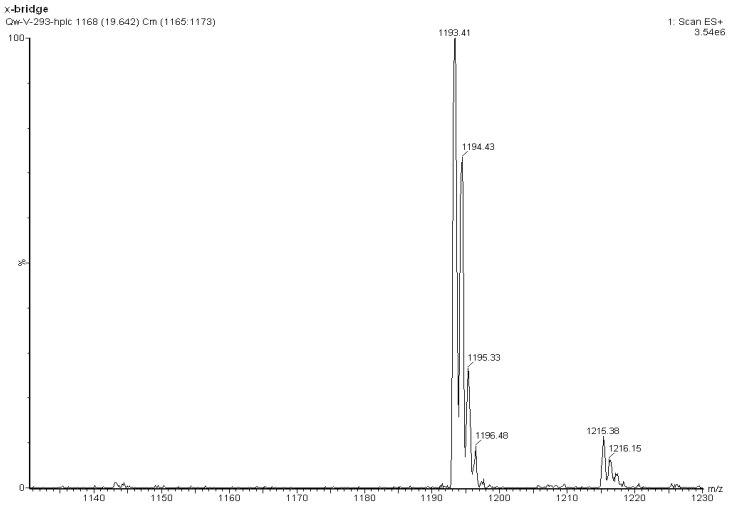


Figure 55: ESI-MS of compound **18**
ESI calcd for $C_{59}H_{76}N_{12}O_{15}$ $[M+H]^+$ *m/z* = 1193.56 $[M+Na]^+$ *m/z* = 1215.56, found: 1193.41, 1215.38.

Compound 19: Fmoc-RTGDSAGT-**Pen**-OH

1.0 mg of compound **2** and 1.0 mg of compound **15**, penicilamine, were dissolved in 0.5 mL of Guanidine buffer*. To the solution, were added and 40.0 µL of 0.5 M bond-breaker® TCEP solution (Pierce). The reaction mixture was stirred at 30 ^oC for 3 h. The reactions were monitored by LC-MS and purified directly by HPLC. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by

concentration at reduced pressure and lyophilization afforded 0.6 mg of compound **19** as a white powder, 54% yield.

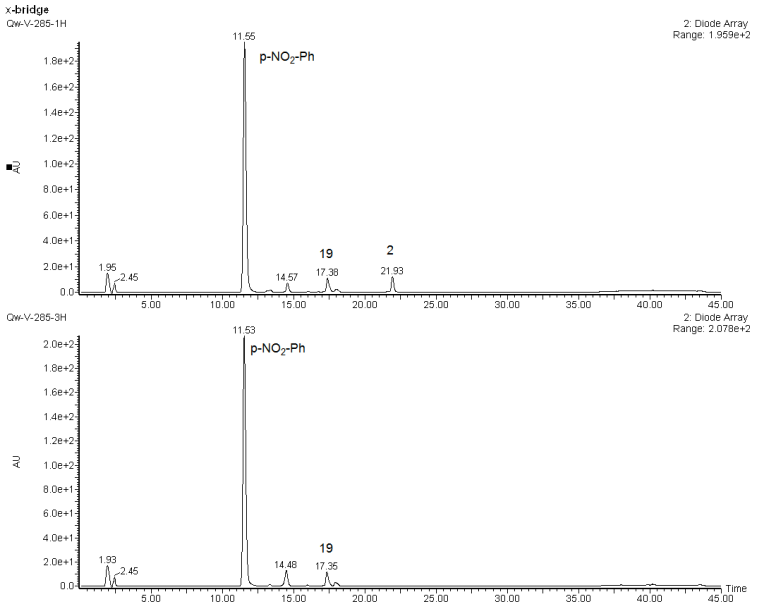


Figure 56: UV traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

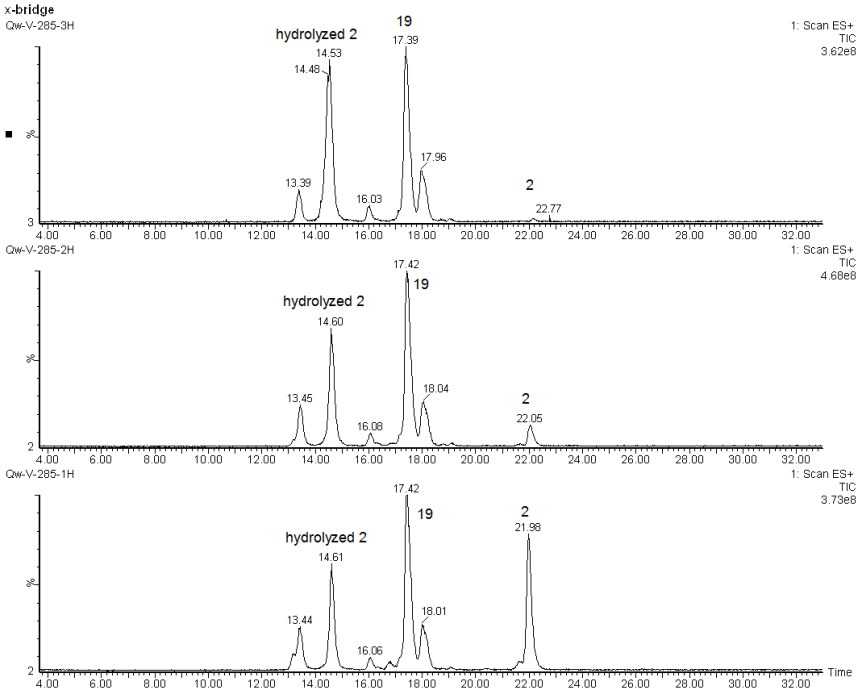


Figure 57: MS traces from LC-MS analysis of the ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

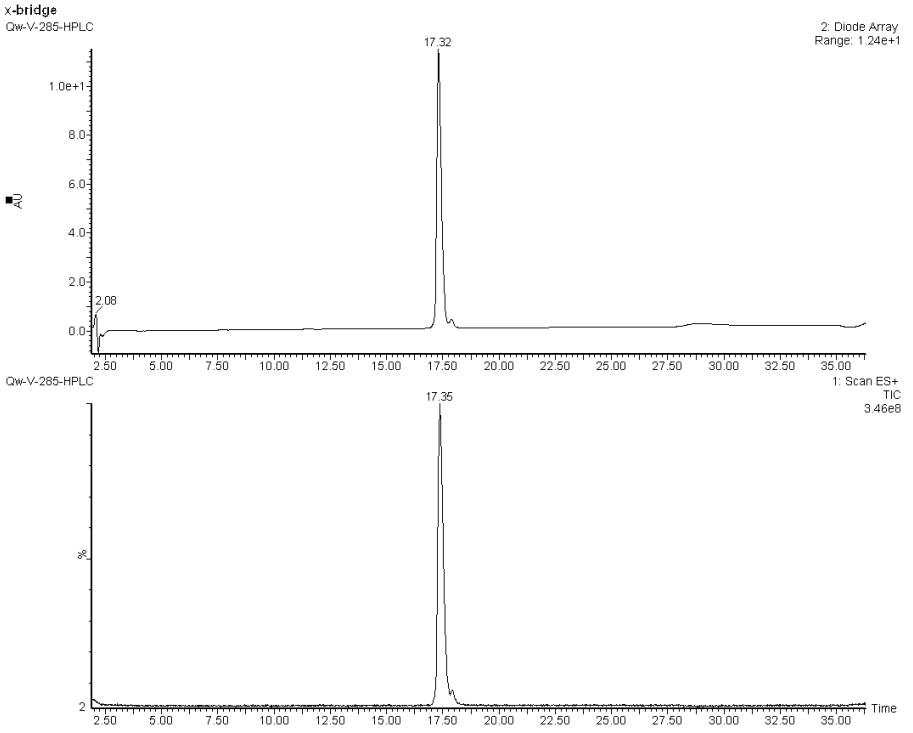


Figure 58: UV and MS traces from LC-MS analysis of compound **19**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

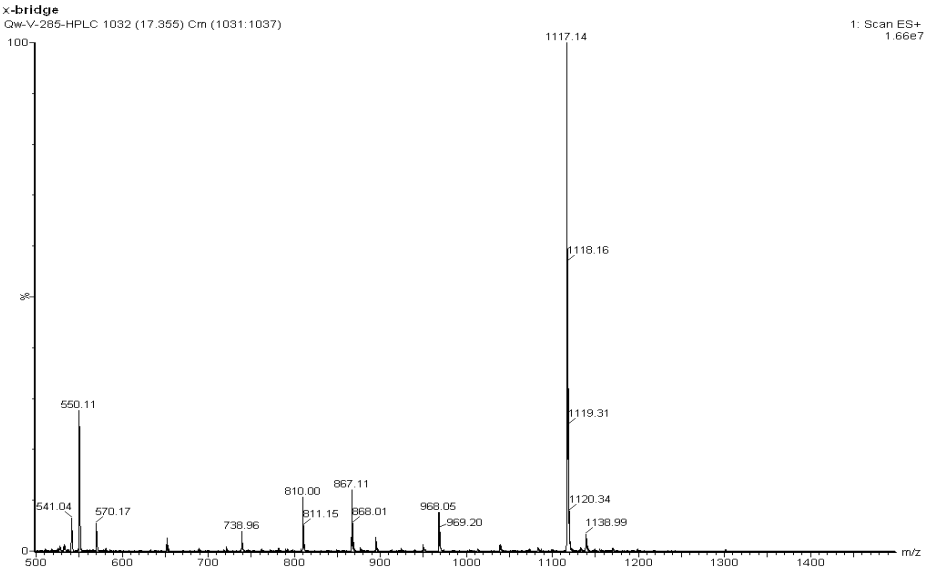


Figure 59: ESI-MS of compound **19**
ESI calcd for $C_{48}H_{68}N_{12}O_{17}S$ $[M+H]^+$ *m/z* = 1117.45, found: 1117.14.

Compound 20: Fmoc-RTGDSAGT-**Val**-OH

The 0.6 mg of cysteinyl peptide, **Compound 19**, was dissolved in 200.0 μL of water and 100.0 μL of CH_3CN under argon. To the solution, were added 200.0 μL of 0.5 M bond-breaker® TCEP solution (Pierce), 20.0 μL of 2-methyl-2-propanethiol and 20.0 μL of radical initiator VA-044 (0.1 M in water). The reaction mixture was stirred at 37 ^oC for 3h. The reactions were monitored by LC-MS and purified directly by HPLC upon consumption of the starting material. HPLC purification (15-45% CH_3CN/H_2O over 30 min, X-bridge, 265 nm, 16.0 mL/min) followed by concentration at reduced pressure and lyophilization afforded 0.3 mg of compound **20** as a white powder, 83% yield.

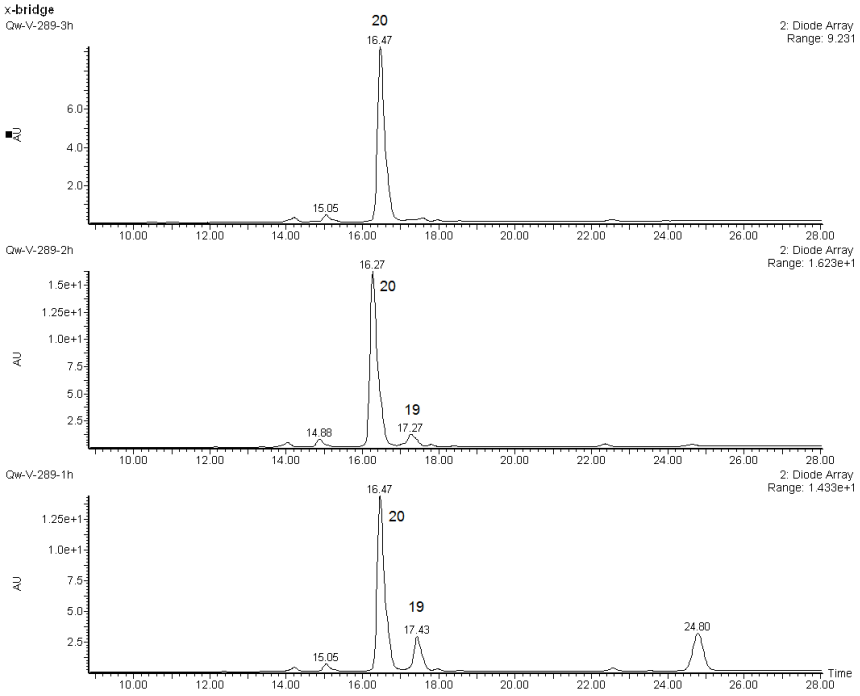


Figure 60: UV traces from LC-MS analysis of desulfurization: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

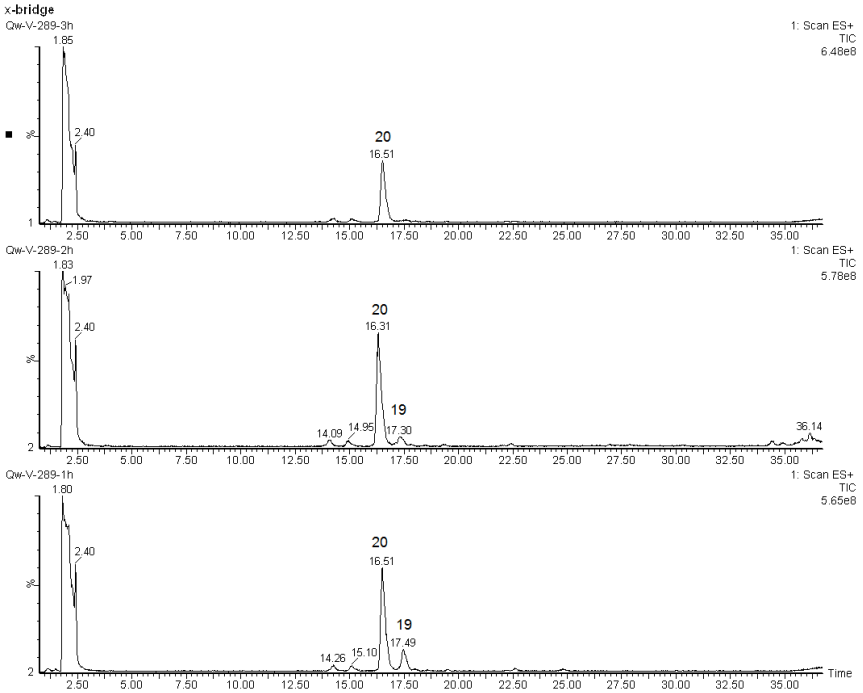


Figure 61: MS traces from LC-MS analysis of desulfurization: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

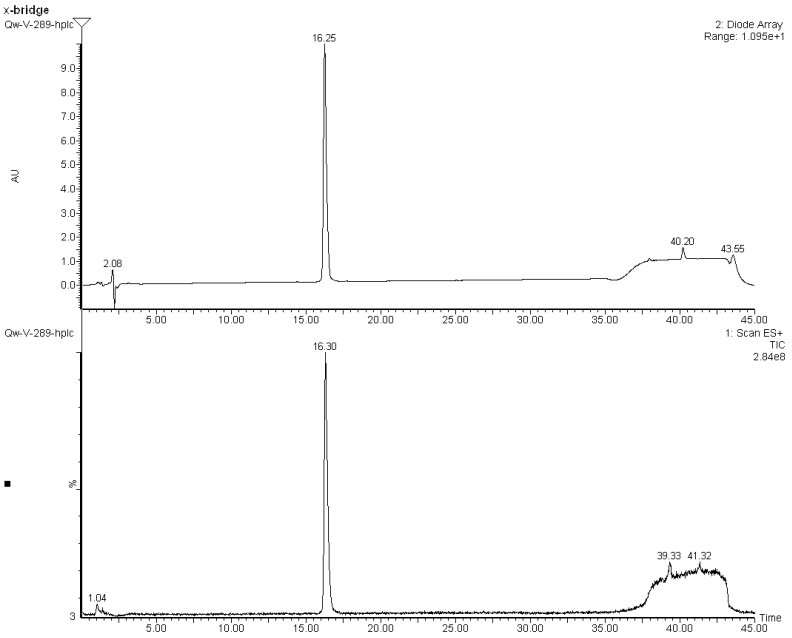


Figure 62: UV and MS traces from LC-MS analysis of compound **20**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

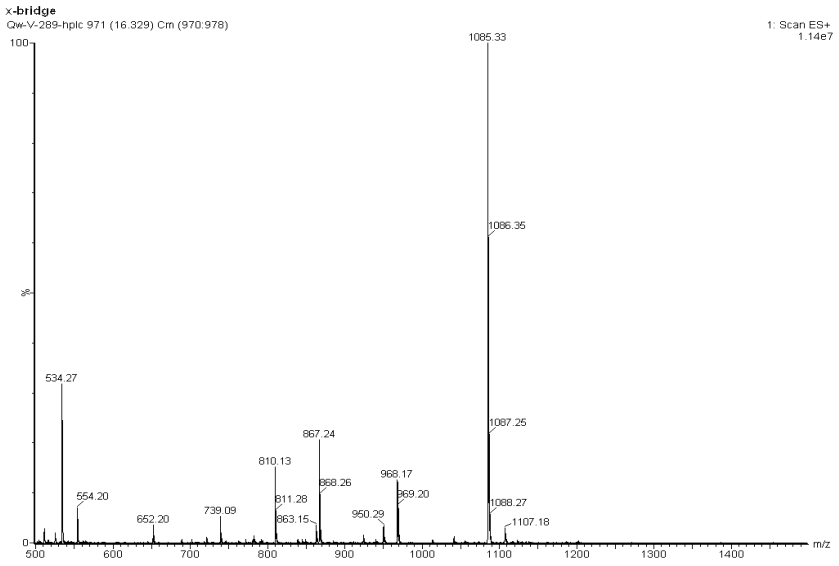


Figure 63: ESI-MS of compound **20**
ESI calcd for $C_{48}H_{68}N_{12}O_{17}$ $[M+H]^+$ *m/z* = 1085.48, found: 1085.33.

Compound 21: Fmoc-RTGDSAG-Thr-SPh

Boc-Thr(*t*Bu)-OH was used following the general thiol-ester synthesis procedure. Semiprep HPLC purification (20-50% CH_3CN/H_2O over 30 min, x-bridge column, 16.0 mL/min, 265 nm) followed by concentration at reduced pressure and lyophilization afforded compound **21** as a white powder.

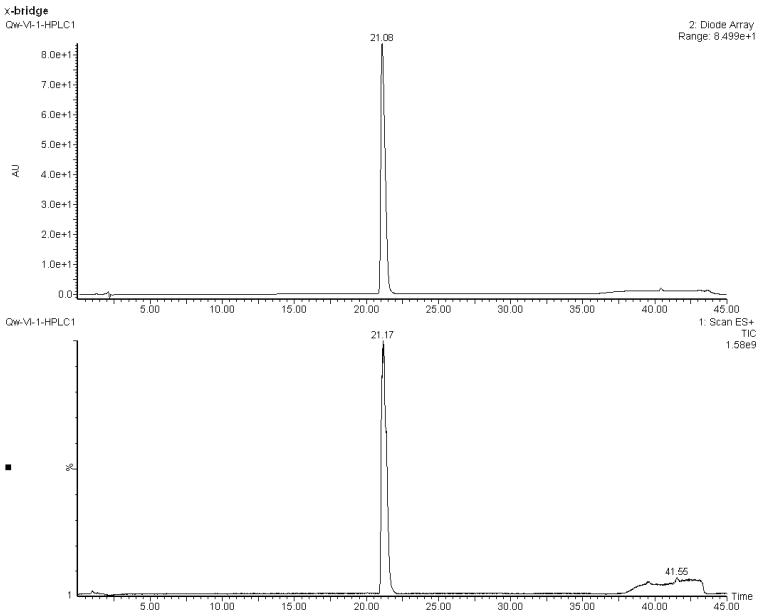


Figure 64: UV and MS traces from LC-MS analysis of compound **21**: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column

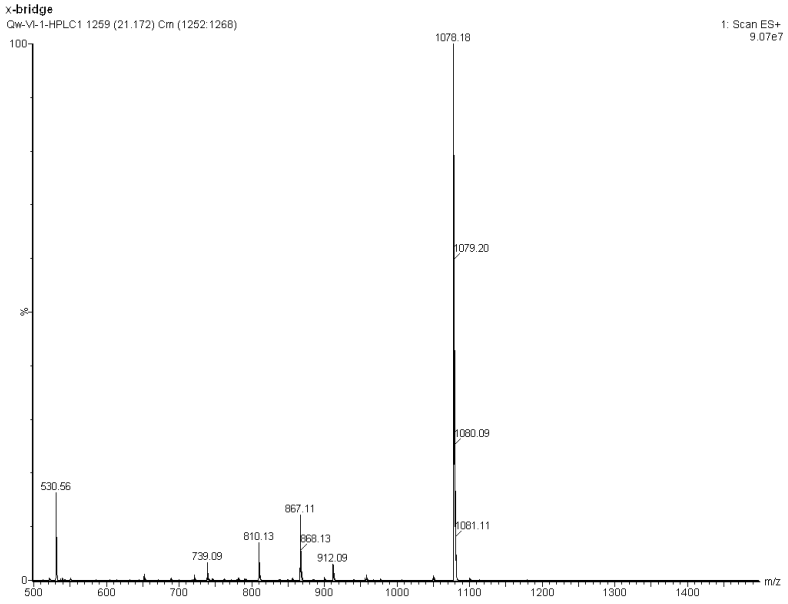
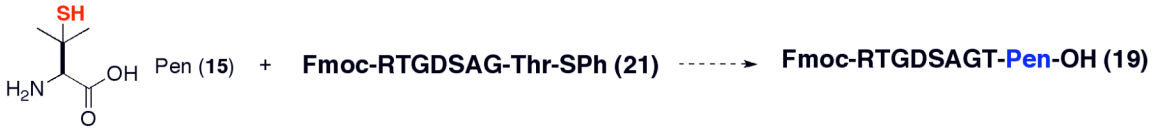


Figure 65: ESI-MS of compound **21**
ESI calcd for $C_{49}H_{63}N_{11}O_{15}S$ $[M+H]^+$ *m/z* = 1078.42; found: 1078.18.

Compound 19: Fmoc-RTGDSAGT-Pen-OH



1.0 mg of compound **21** (t = 21.58 min) and 1.0 mg of compound **15**, penicilamine, were dissolved in 0.5 mL of Guanidine buffer (6.0 M Gn·HCl, 188.8 mM Na_2HPO_4, 18.8 mM TCEP·HCl). To the solution, were added and 40.0 µL of 0.5 M bond-breaker® TCEP solution (Pierce). The reaction mixture was stirred at 30 °C for 48 h. The reaction was monitored by LC-MS. Only trace amount of compound **19** (t= 17.65 min) was observed. Hydrolysis of compound **21** was the major side reaction (t = 14.73 min).

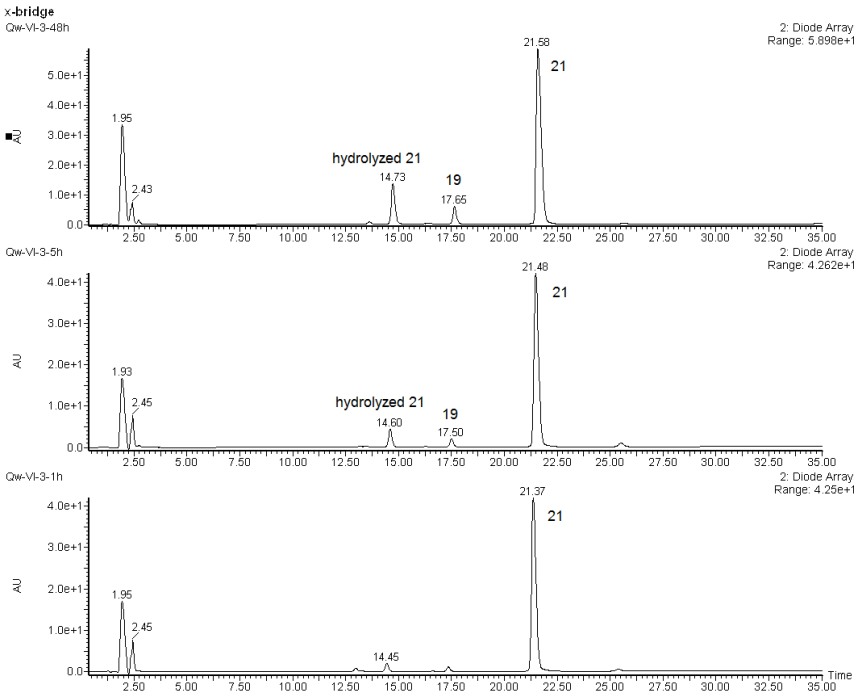


Figure 65: UV traces from LC-MS analysis of Pen-ligation: gradient 20-50% CH_3CN/H_2O over 30 min at a flow rate of 0.2 mL/min, X-bridge column