

Solid-phase synthesis of a bicyclic peptide OL-CTOP containing two disulfide bridges and assessment of its *in vivo* μ -opioid receptor antagonism after nasal administration

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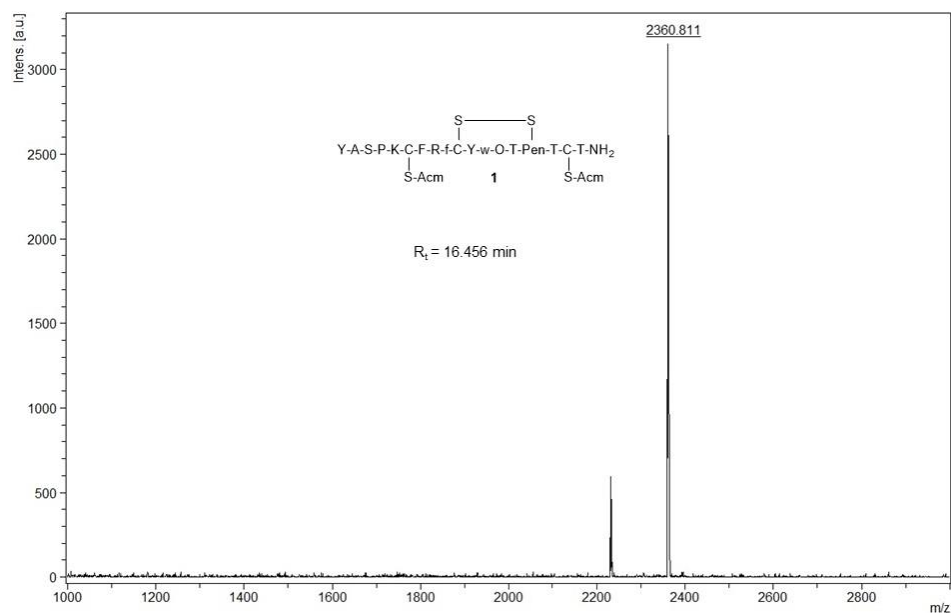
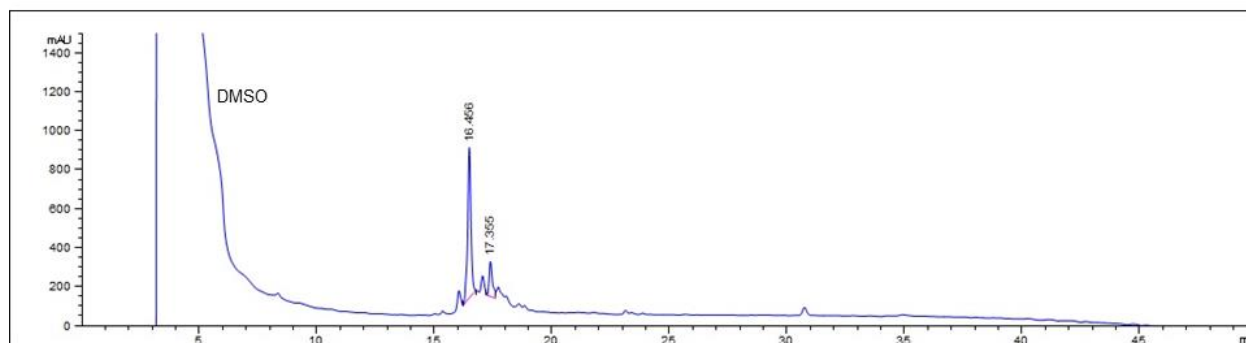
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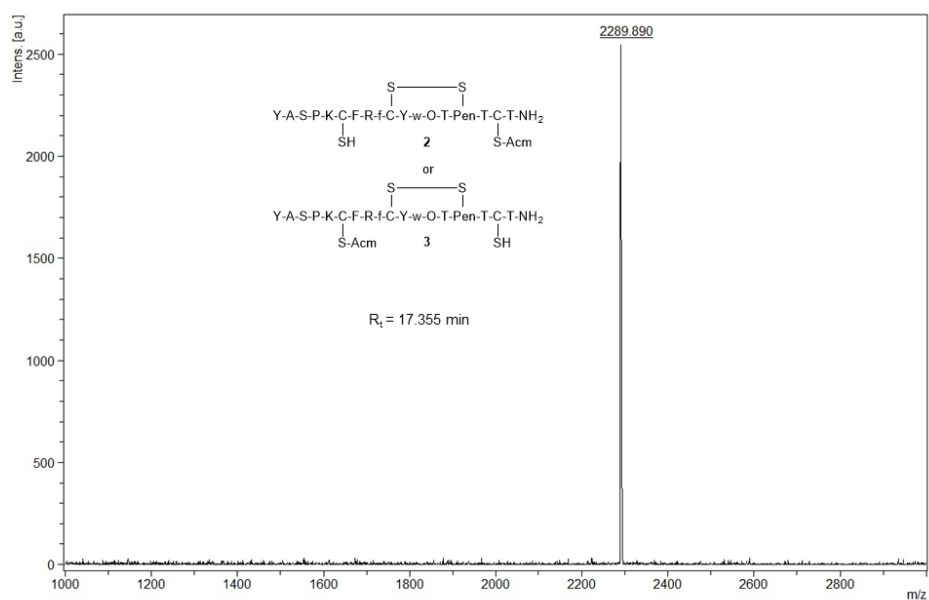
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RP-HPLC analysis: C18 monomeric column (Grace Vydac, 250 × 4.6 mm, 5 mm, 120 Å), 1 ml/min flow rate, and elution method with a linear gradient of 2 → 100% B over 45 min, where A is 0.1% TFA in H₂O, and B is 0.08% TFA in CH₃CN. Because of limited solubility in water, all peptides were dissolved in DMSO and DMSO solutions were used for RP-HPLC analysis.

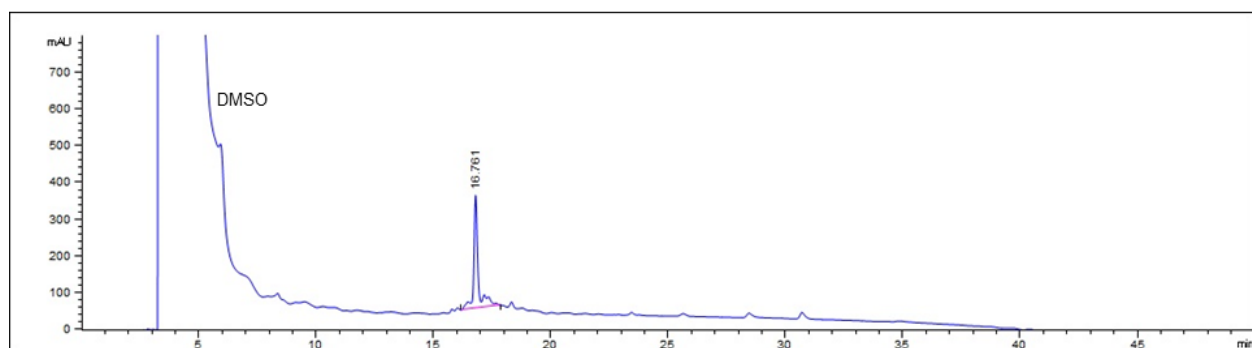


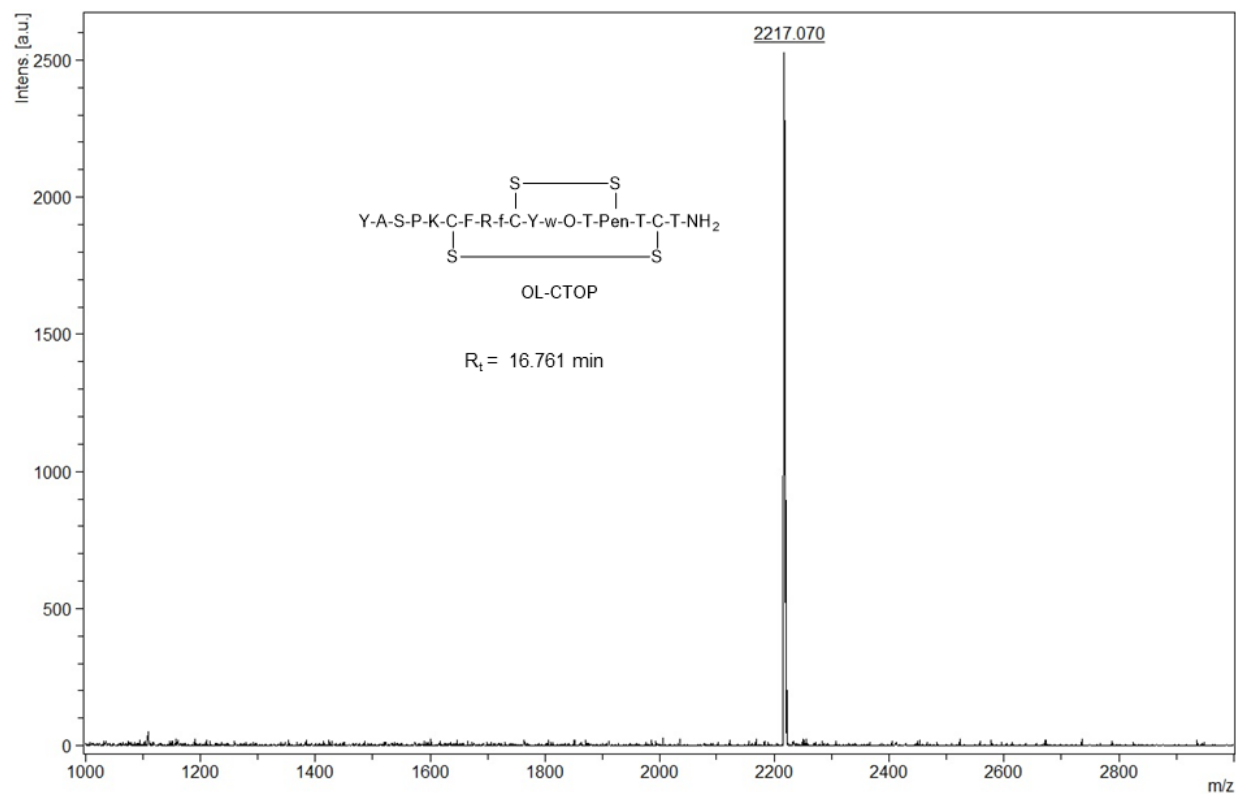
Expected [M+H]⁺ = 2360.036 Da, observed [M+H]⁺ = 2360.811 Da



Expected $[M+H]^+ = 2288.99 \text{ Da}$, observed $[M+H]^+ = 2289.89 \text{ Da}$

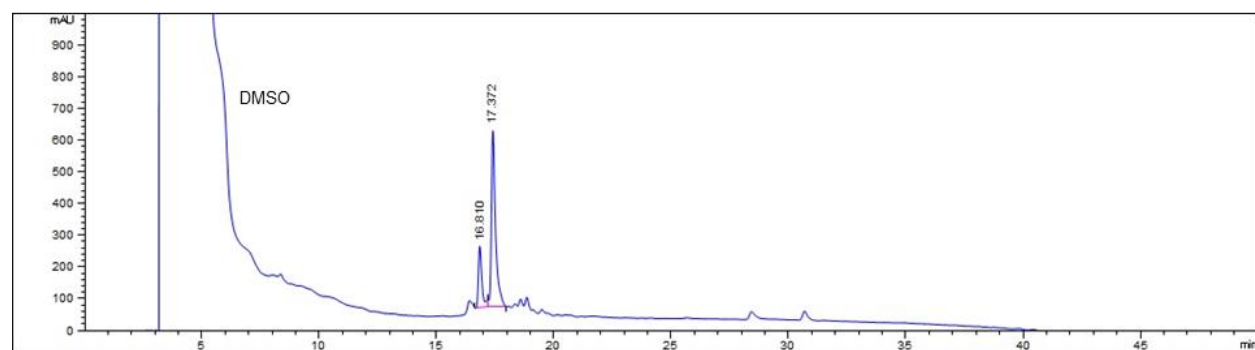
Figure S1. HPLC traces and MALDI ToF MS spectra of synthetic precursors following Cys¹⁰ and Pen¹⁵ oxidation.

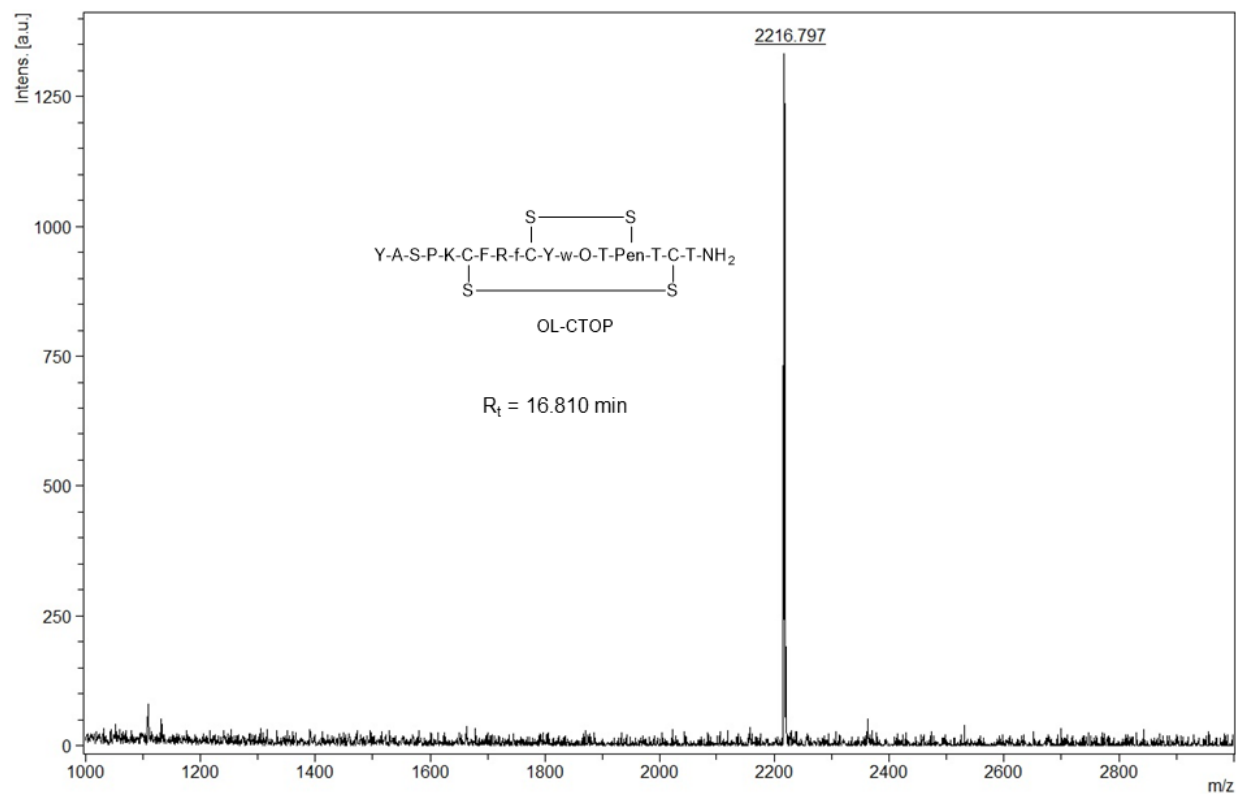




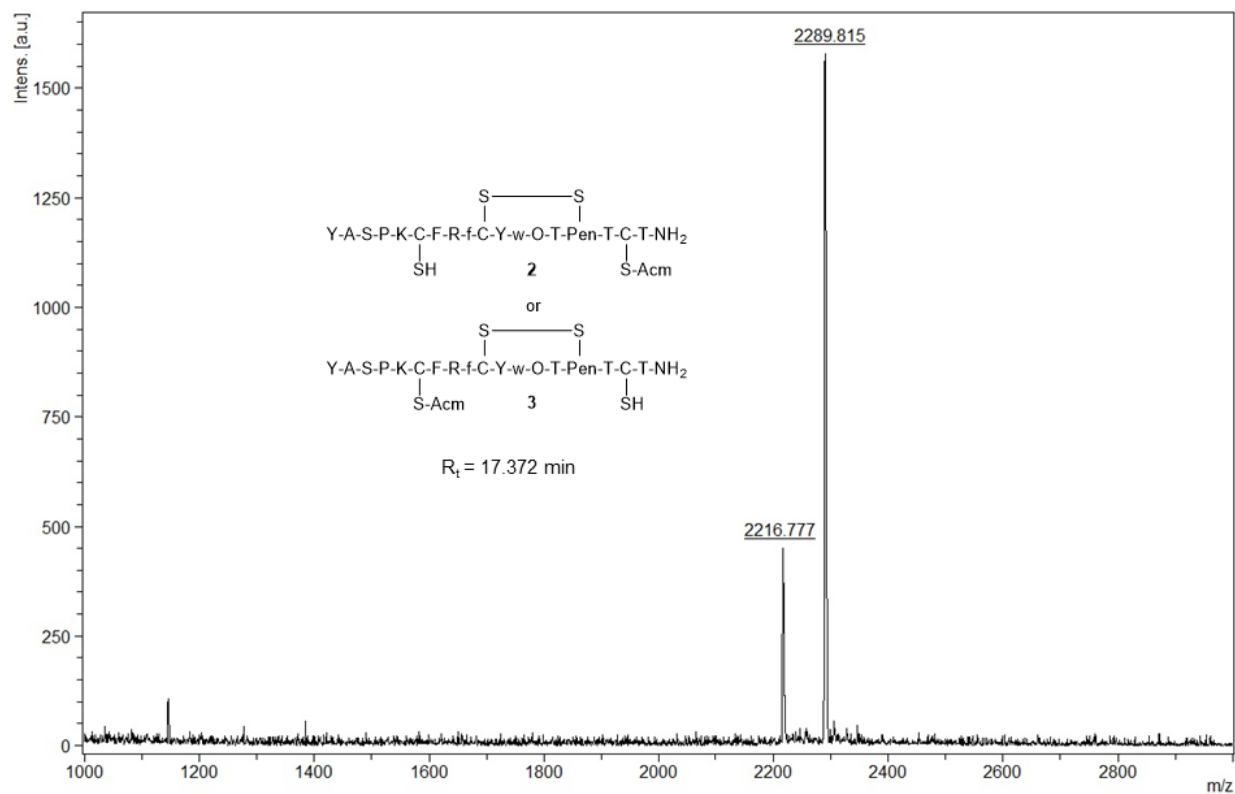
Expected [M+H]⁺ = 2215.946 Da, observed [M+H]⁺ = 2217.070 Da

Figure S2. HPLC traces and MALDI ToF MS spectra of synthetic precursors following Cys⁶ and Cys¹⁷ oxidation.





Expected $[M+H]^+ = 2215.946$ Da, observed $[M+H]^+ = 2216.797$ Da



Expected [M+H]⁺ = 2288.999 Da, observed [M+H]⁺ = 2289.815 Da

Figure S3. HPLC traces and MALDI ToF MS spectra of OL-CTOP following single-step oxidation.

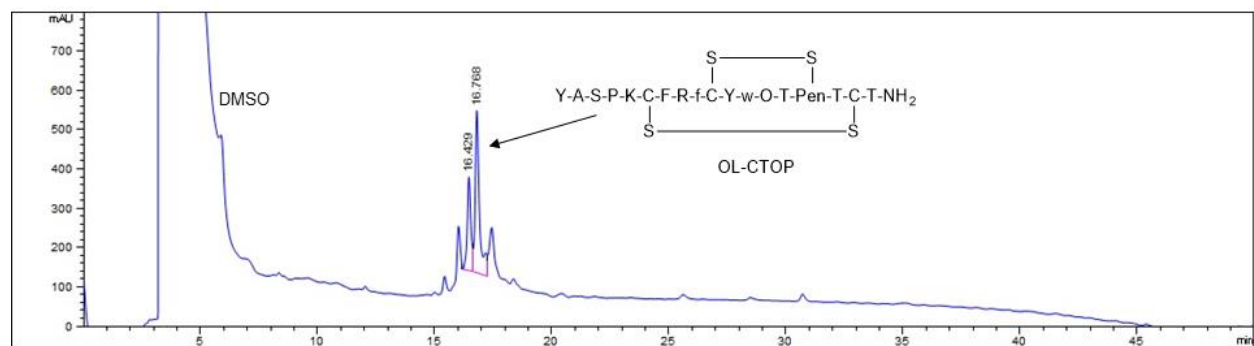


Figure S4. Representative HPLC trace of crude OL-CTOP following single-step oxidation with 30 eq of DMSO as a co-solvent.

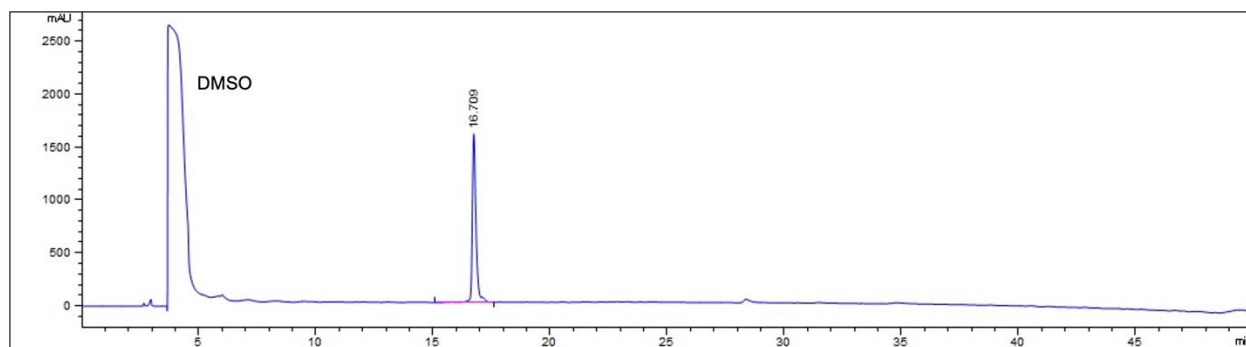
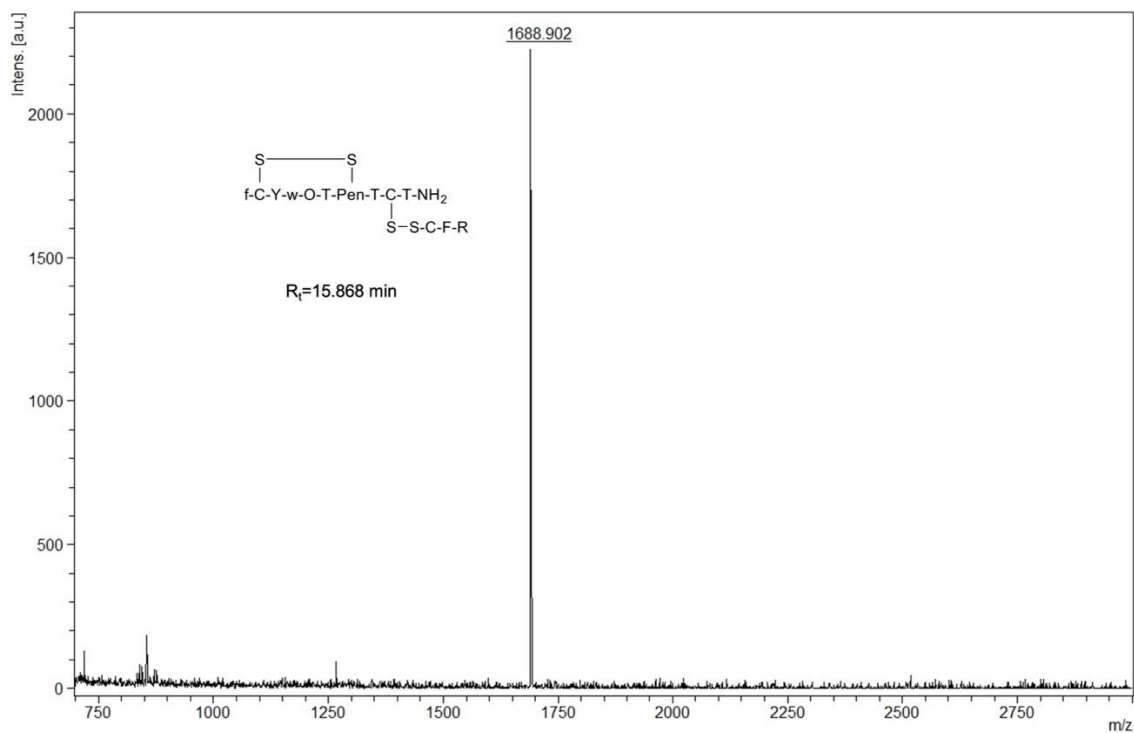
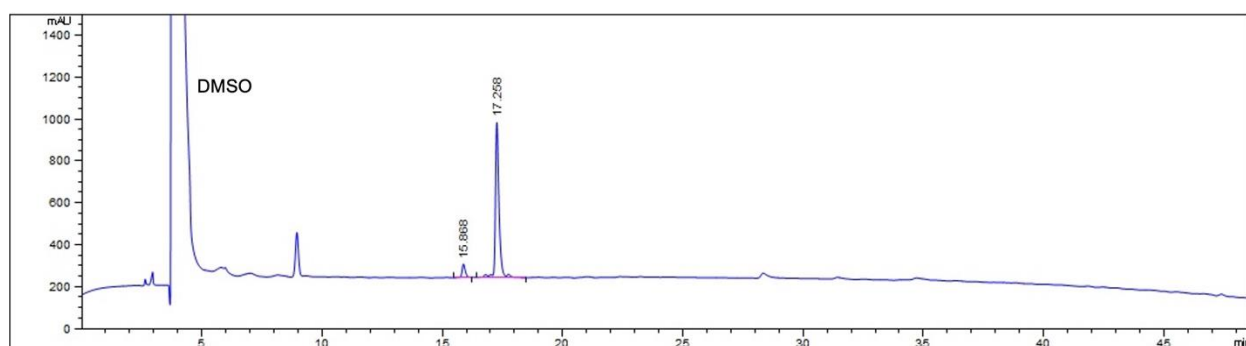
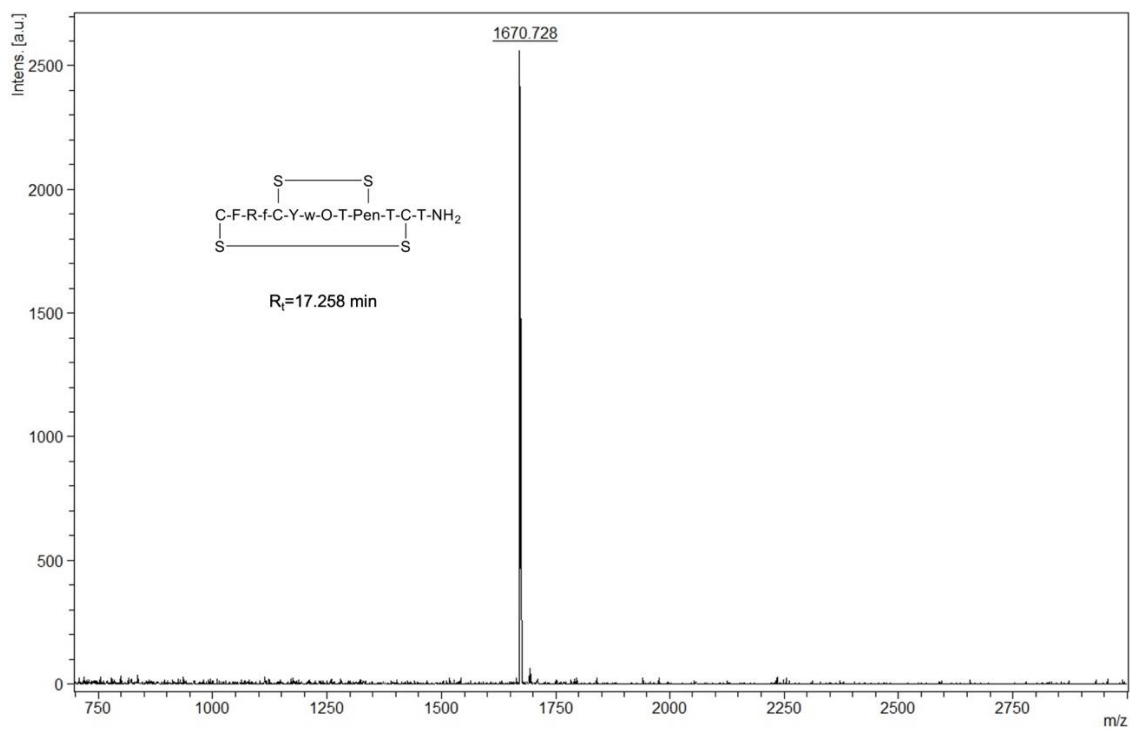


Figure S5. HPLC trace of OL-CTOP following trypsin digestion, 0 min time interval.

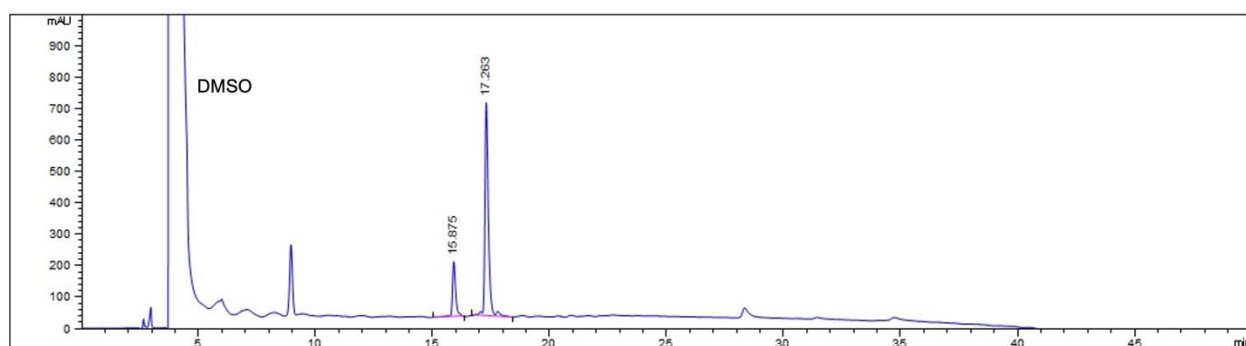


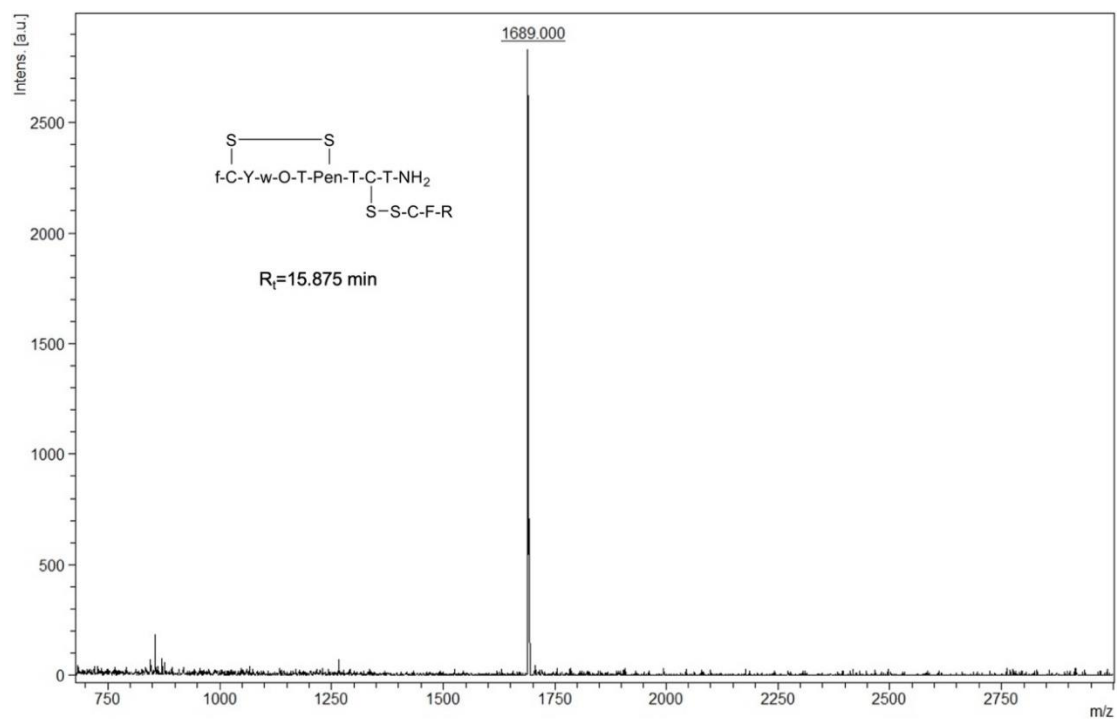
Expected $[M+H]^+ = 1687.68 \text{ Da}$, observed $[M+H]^+ = 1688.902 \text{ Da}$



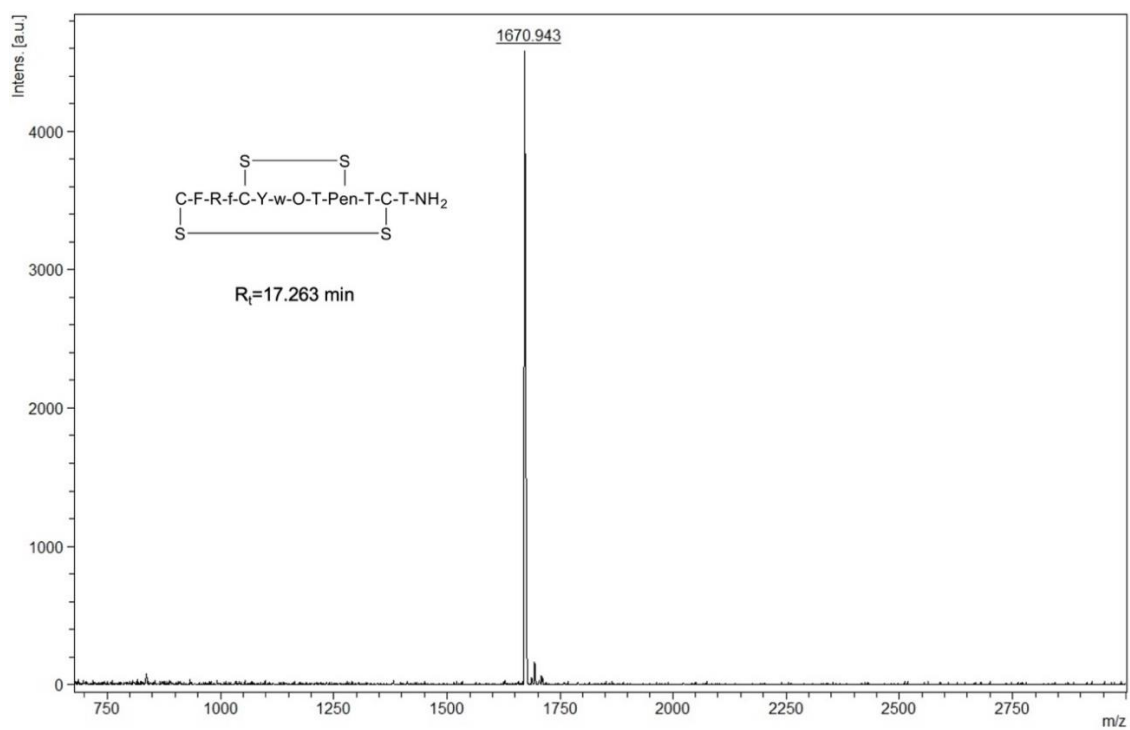
Expected $[M+H]^+ = 1671.01$ Da, observed $[M+H]^+ = 1670.728$ Da

Figure S6. HPLC trace and MALDI ToF MS spectra of OL-CTOP following trypsin digestion, 10 min time interval.



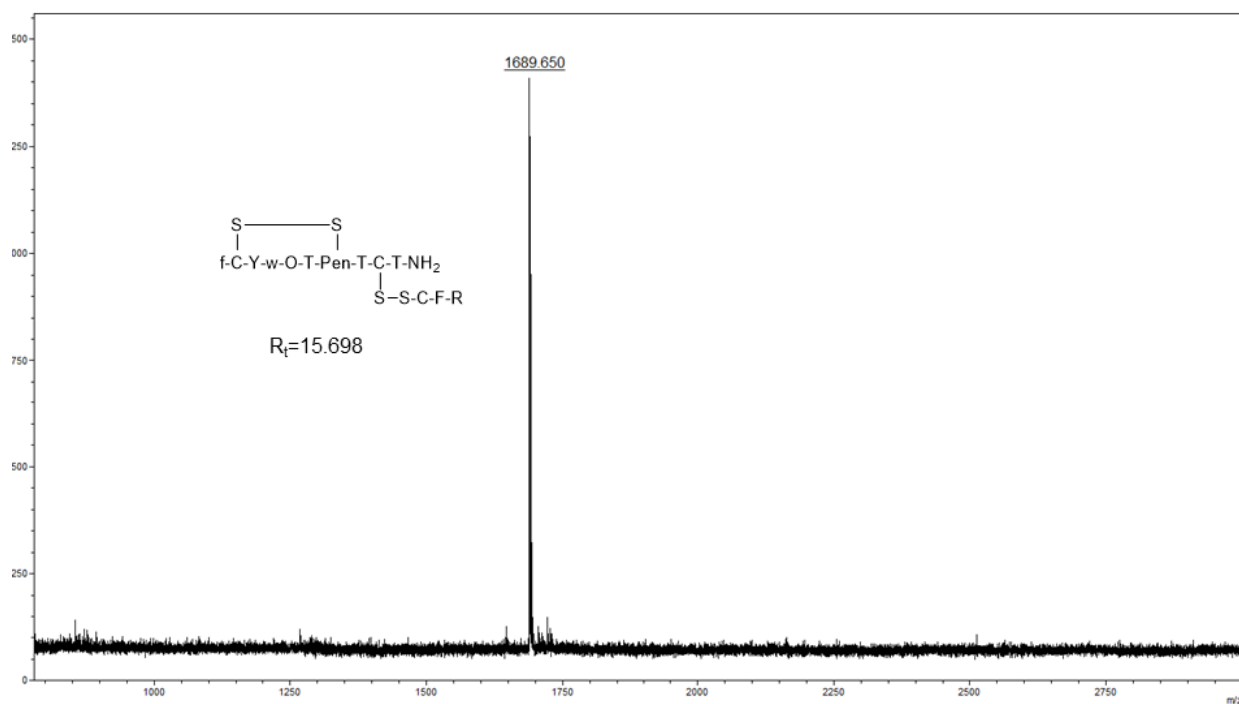
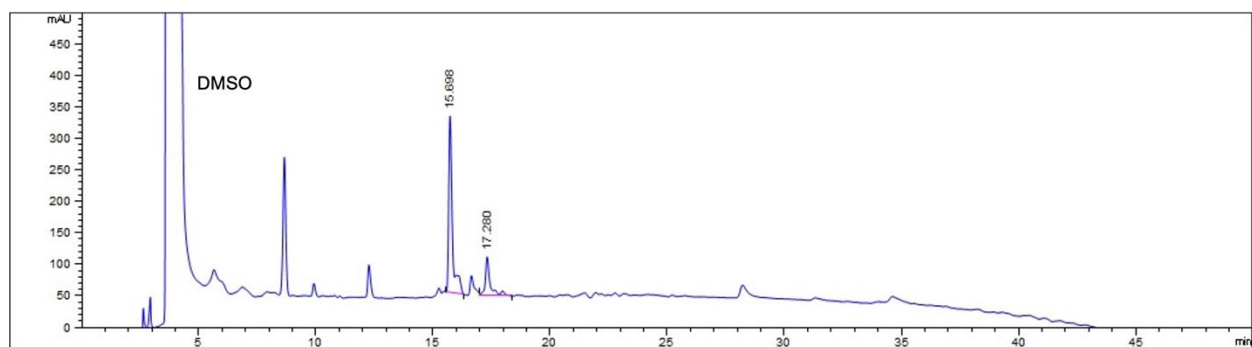


Expected $[M+H]^+ = 1687.68$ Da, observed $[M+H]^+ = 1689.000$ Da

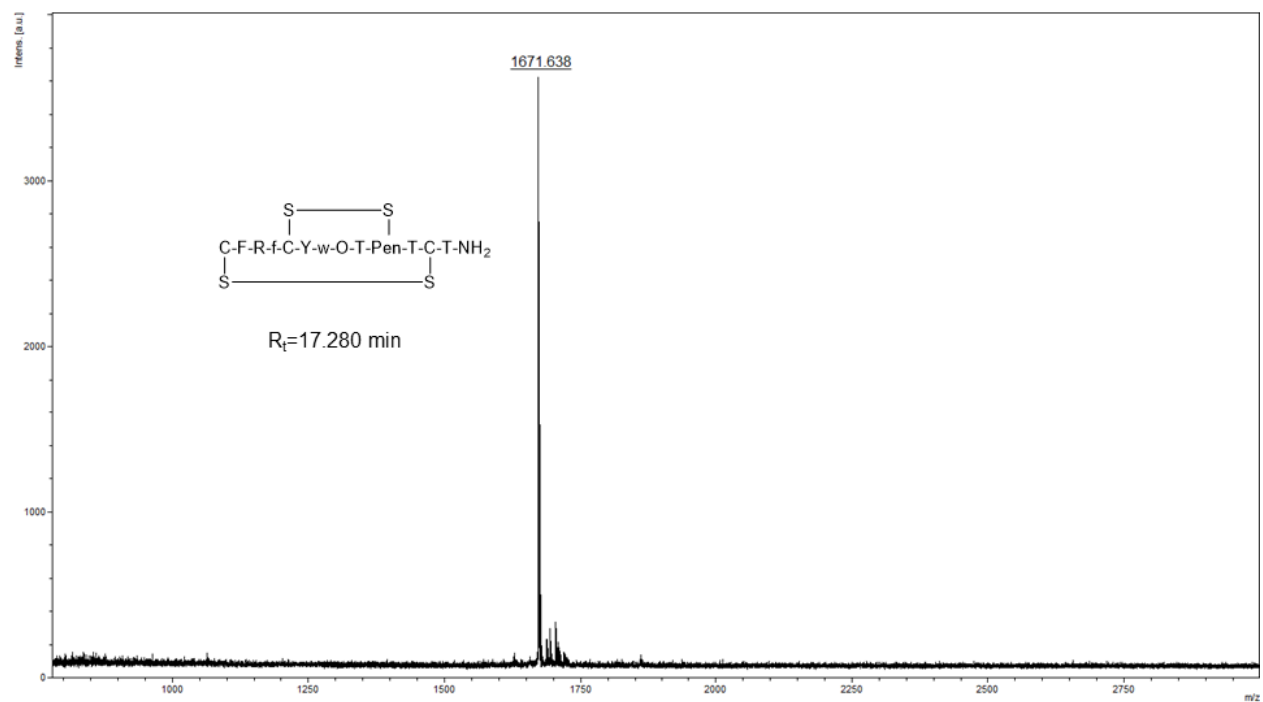


Expected $[M+H]^+ = 1671.01$ Da, observed $[M+H]^+ = 1670.943$ Da

Figure S7. HPLC trace and MALDI ToF MS spectra of OL-CTOP following trypsin digestion, 1h time interval.



Expected $[M+H]^+ = 1687.68$ Da, observed $[M+H]^+ = 1689.650$ Da



Expected $[M+H]^+ = 1669.67$ Da, observed $[M+H]^+ = 1671.638$ Da

Figure S8. HPLC trace and MALDI ToF MS spectra of OL-CTOP following trypsin digestion, 24h time interval.