Rapid formal hydrolysis of peptide-$^{\alpha}$thioesters

Supplementary Information

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1. 2-Mercaptoethanol-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*: A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (0.5 mg, 0.6 umol). 500 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher), 200 mM phosphate (Fisher), and 200 mM 2-mercaptoethanol (Aldrich) was added. The vial was capped, and the resulting solution was allowed to stand. At 30 min and 60 min timepoints, a 10 uL aliquot of the reaction mixture was diluted 20-fold into a solution of 1% buffer B (Buffer A = 0.1% (v/v) trifluoroacetic acid in H_2O; Buffer B = 0.08% (v/v) trifluoroacetic acid in acetonitrile) for LC-MS analysis. The t=0 aliquot was from the control reaction carried out in the absence of 2-mercaptoethanol (page 4).

Analytical separations were carried out on an Agilent 1100 equipped with an 1100 series LC/MSD trap, using a linear gradient of 1% - 61% buffer B over 15 min (3 min hold times at 1% B and 61% B). Analytical columns were either a self-packed Varian microsorb C_4 (2.1 x 50 mm, 3 um particle size, 300 Å pore size) or a commercial Phenomenex C_{18} (2.1 x 50 mm, 3.6 um particle size, 300 Å pore size). Separations were carried out at 40°C using a flow rate of 0.5 mL/min, and the eluent was monitored at 214 nm. Mass spectra were integrated over the principal HPLC peaks, unless otherwise indicated.

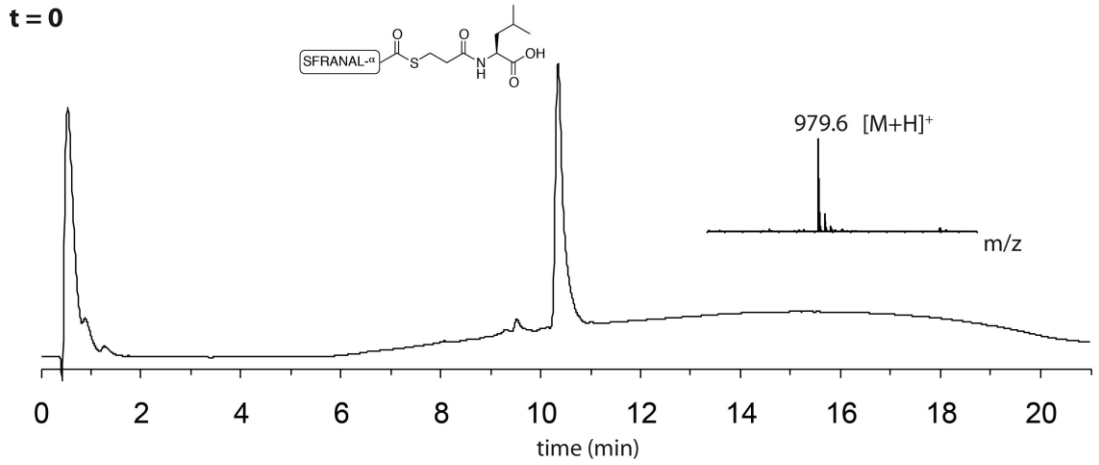

Figure S1. 2-Mercaptoethanol-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* (exact mass: 978.49 Da; found: 978.6 Da); t = 0.

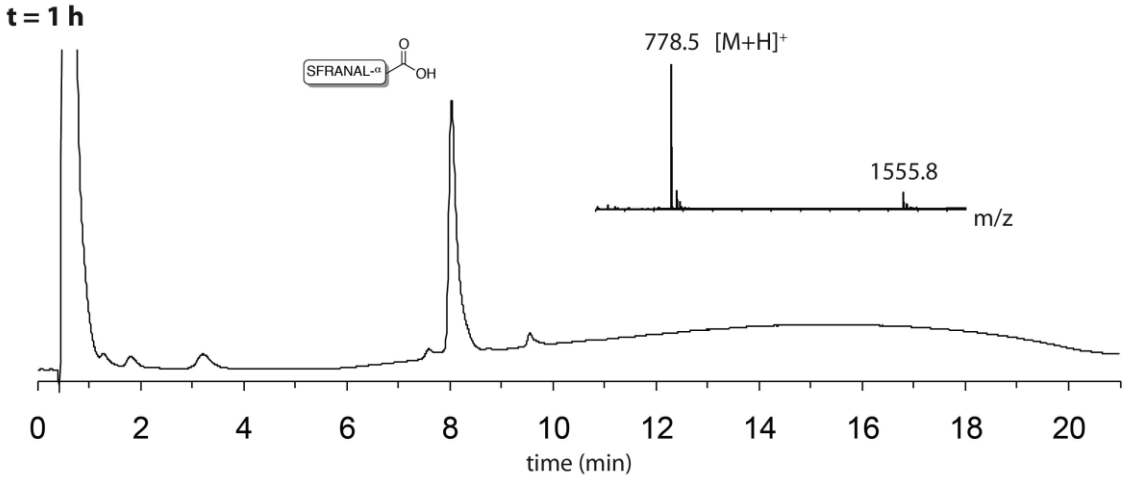

Figure S2. 2-Mercaptoethanol-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*; t = 1 h. The reaction product was SFRANAL-$^{\alpha}$*COOH* (exact mass: 777.41 Da; found: 777.5 Da).

2. Alkaline hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*: A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (0.3 mg, 0.4 umol). 250 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher) and 200 mM phosphate (Fisher) was added. Immediately, a 10 uL aliquot of the resulting solution was diluted 20-fold into 1% B for LC-MS analysis. The vial was then capped, and the reaction mixture allowed to stand. A second aliquot was taken after 7 h.

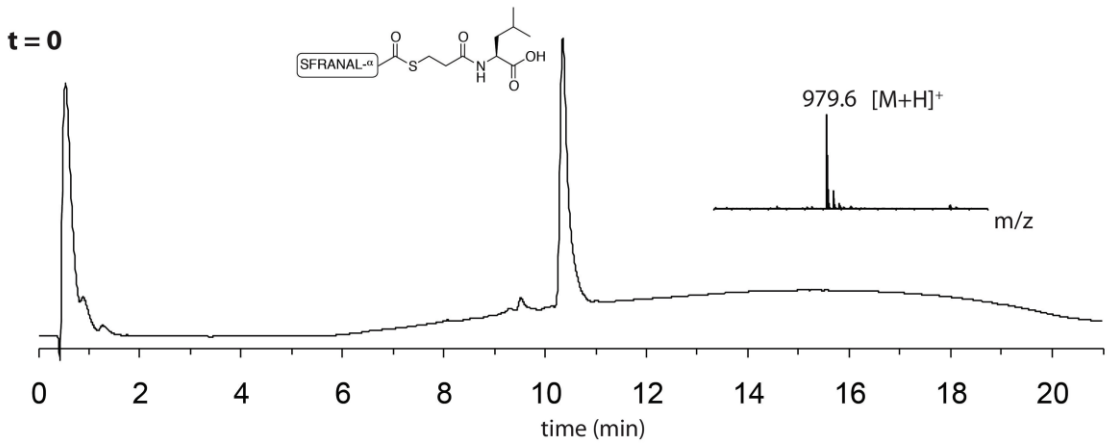

Figure S3. Alkaline hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* (exact mass: 978.49 Da; found: 978.6 Da); t = 0.

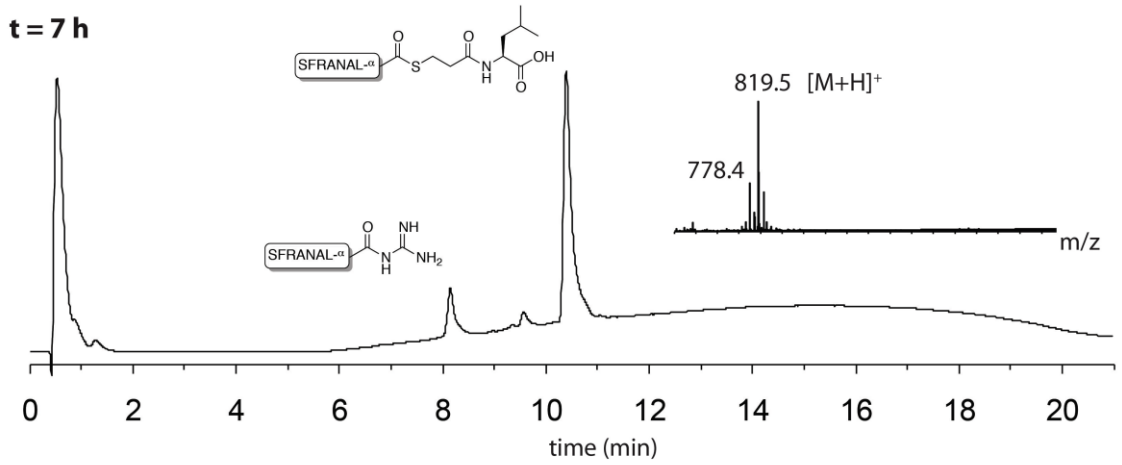

Figure S4. Alkaline hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*, t = 7 h. The inset MS was taken over the peak that corresponded to SFRANAL-$^{\alpha}$*acylguanidine* (exact mass: 818.44 Da; found: 818.5 Da). SFRANAL-$^{\alpha}$*COOH* (exact mass: 777.41 Da; found: 777.4 Da) co-eluted with SFRANAL-$^{\alpha}$*acylguanidine*, as seen in the MS.

3. 2-Mercaptoethanol-mediated hydrolysis of ThzAGGVGGAGGASGGTGVGG RGGKGGSGTPKGADGAPGAP-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*: A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (0.6 mg, 0.2 umol). 100 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher), 200 mM phosphate (Fisher), and 200 mM 2-mercaptoethanol (Aldrich) was added. The vial was capped, and the resulting solution was allowed to stand. After one hour, a 10 uL aliquot was diluted 20-fold into 1% B for LC-MS analysis. A second aliquot was taken after 7 h. The t = 0 timepoint was from the control reaction carried out in the absence of 2-mercaptoethanol (see page 8).

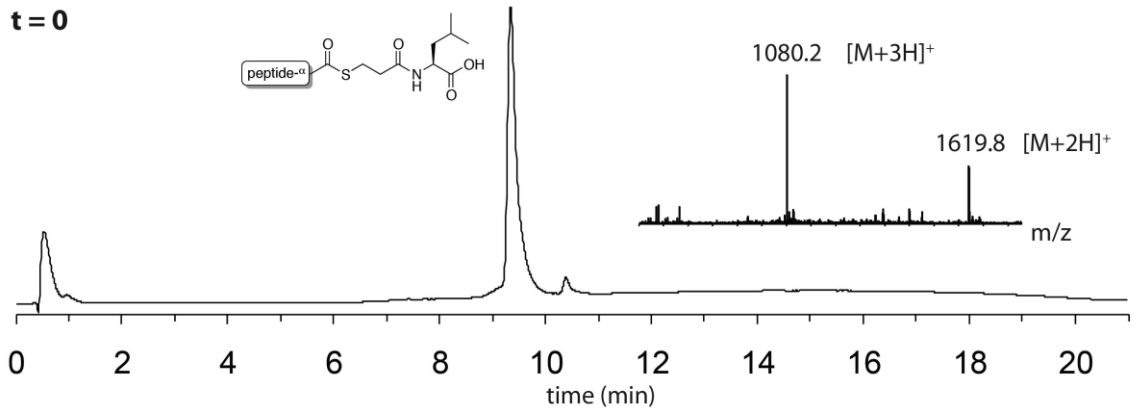

Figure S5. 2-Mercaptoethanol-mediated hydrolysis of snow flea antifreeze protein Thz^{43}-81-$^{\alpha}$*COSR* (exact mass: 3236.50 Da; average isotopes mass: 3238.51 Da; found: 3237.6 Da); t = 0.

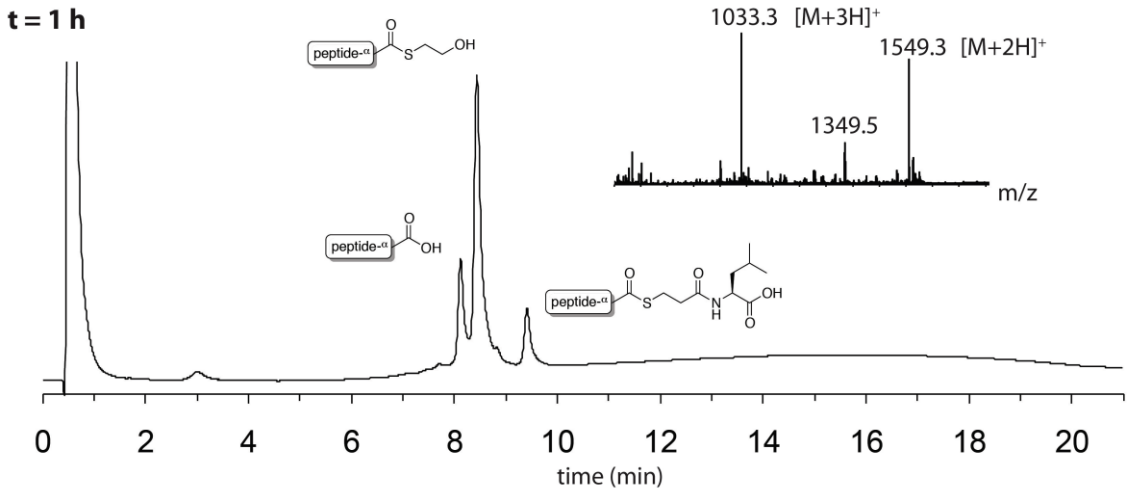

Figure S6. 2-Mercaptoethanol-mediated hydrolysis of snow flea antifreeze protein Thz^{43}-81-$^{\alpha}$*COSR*; t = 1 h. The inset MS was taken over the principle component, which was identified as the product of transthioesterification by mercaptoethanol (exact mass: 3095.42 Da; average isotopes mass: 3097.34 Da; found: 3096.6 Da).

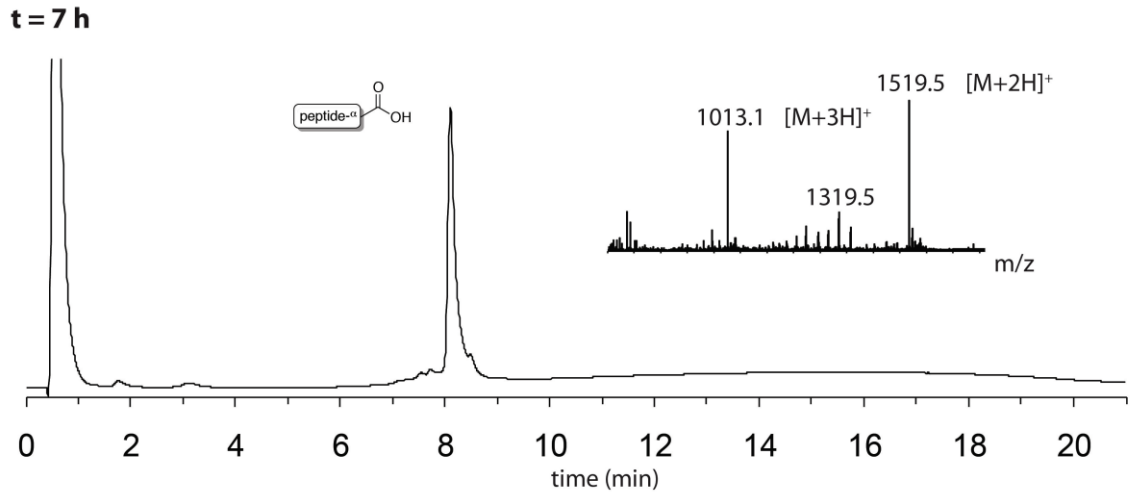

Figure S7. 2-Mercaptoethanol-mediated hydrolysis of snow flea antifreeze protein Thz^{43}-81-$^{\alpha}$*COSR*; t = 7 h. The reaction product was Thz^{43}-81-$^{\alpha}$*COOH* (exact mass: 3035.42 Da; average isotopes mass: 3037.23 Da; found: 3037.0 Da).

4. Alkaline hydrolysis of ThZAGGVGGAGGASGGTGVGGRGGKGGSGTP KGADGAPGAP-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*: A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (0.3 mg, 0.1 umol). 50 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher) and 200 mM phosphate (Fisher) was added. Immediately, a 10 uL aliquot of the resulting solution was diluted 20-fold into 1% B for LC-MS analysis. The vial was then capped, and the reaction mixture allowed to stand. A second aliquot was taken after 7 h.

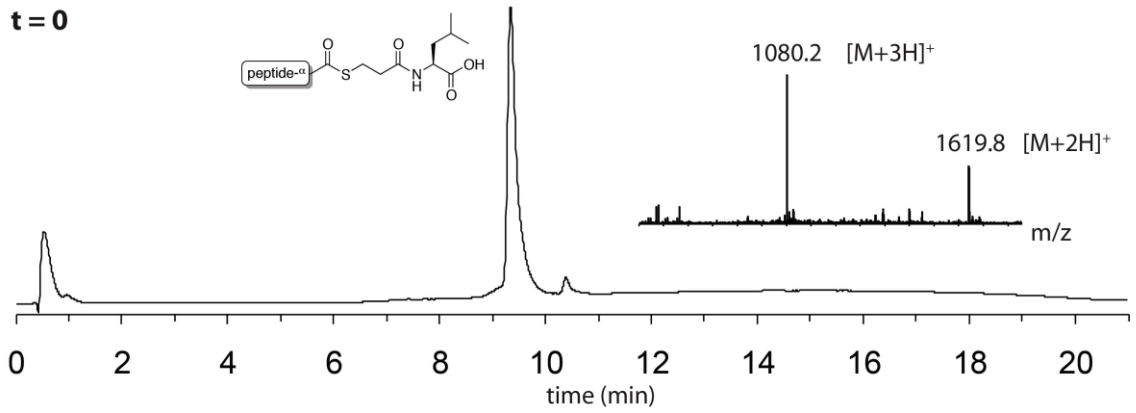

Figure S8. Alkaline hydrolysis of snow flea antifreeze protein Thz^{43}-81-$^{\alpha}$*COSR* (exact mass: 3236.50 Da; average isotopes mass: 3238.51 Da; found: 3237.6 Da); t = 0.

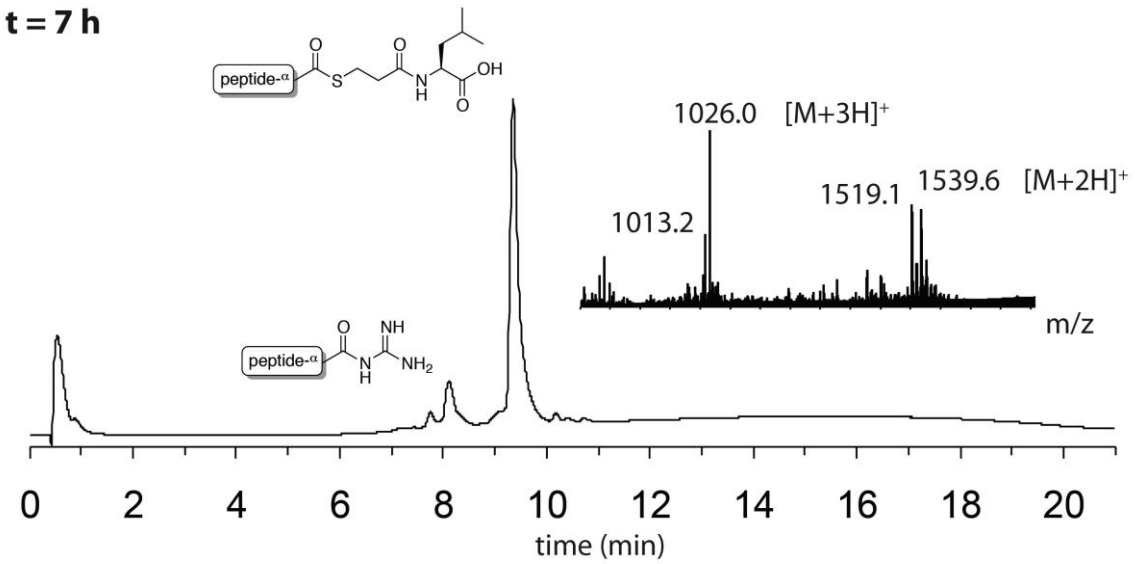

Figure S9. Alkaline hydrolysis of snow flea antifreeze protein Thz[43]-81-$^{\alpha}$*COSR*; t = 7 h. The inset MS was taken over the peak that corresponds to the peptide-$^{\alpha}$*acylguanidine* (exact mass: 3076.45 Da; average isotopes mass: 3078.28 Da; found: 3077.2 Da). The peptide-$^{\alpha}$*acylguanidine* co-eluted with the peptide-$^{\alpha}$*COOH* (exact mass: 3035.42 Da; average isotopes mass: 3037.23 Da; found: 3036.2 Da), as seen in the MS.

5. 2-Mercaptoethanol-mediated hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*: A 2 mL plastic centrifuge tube was charged with peptide-$^{\alpha}$thioester (0.2 mg, 0.2 umol). 100 uL of a buffer containing 6 M guanidine hydrochloride (Fisher), 200 mM phosphate (Fisher), and 200 mM 2-mercaptoethanol (Aldrich) was added. The vial was capped, and the resulting solution was allowed to stand. After one hour, a 10 uL aliquot was diluted 10-fold into 1% B for LC-MS analysis. A second aliquot was taken after 6 h. The t = 0 timepoint was from the control reaction carried out in the absence of 2-mercaptoethanol (see page 13).

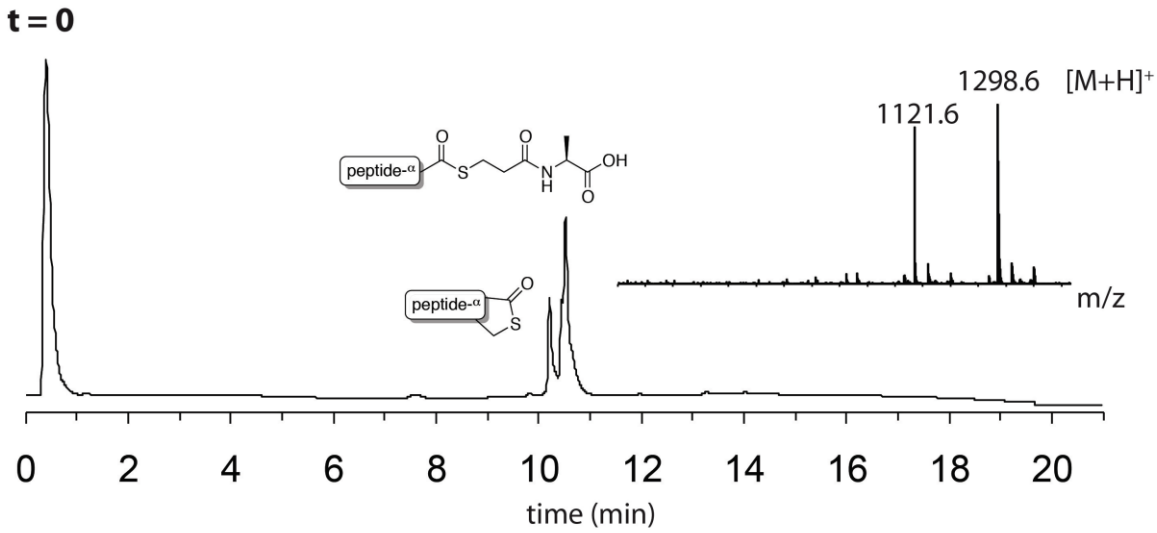

Figure S10. 2-Mercaptoethanol-mediated hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH* (exact mass: 1297.71 Da; average isotopes mass: 1298.48 Da; found: 1297.6 Da); t = 0. The earlier-eluting peak corresponded to the product of transthioesterification by an internal Cys residue (exact mass: 1120.40 Da; average isotopes mass: 1121.26 Da; found: 1120.6 Da). The inset MS was taken over the peak that corresponds to the starting material; a signal that corresponded to the thiolactone was also evident.

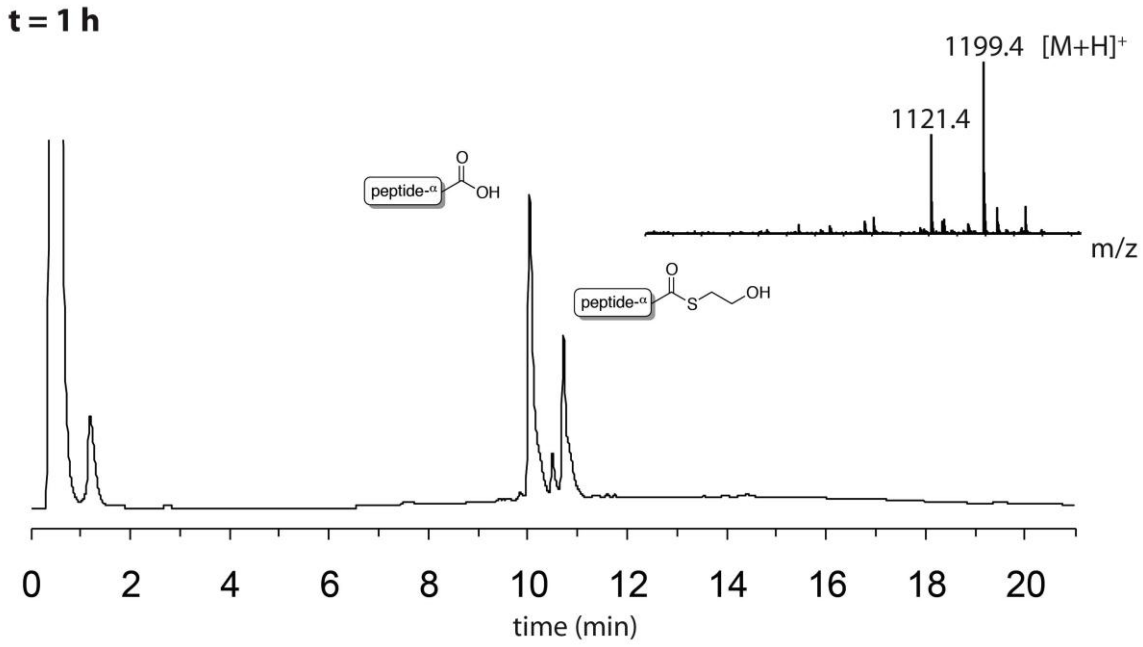

Figure S11. 2-Mercaptoethanol-mediated hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*; t = 1 h. The inset MS was taken over the peak that corresponds to transthioesterification by mercaptoethanol (exact mass: 1198.41 Da; average isotopes mass: 1199.39 Da; found: 1198.4 Da). As in Figure S10, a signal that corresponded to a thiolactone (not labeled in the HPLC trace; exact mass: 1120.40 Da; average isotopes mass: 1121.26 Da; found: 1120.7 Da) was also present.

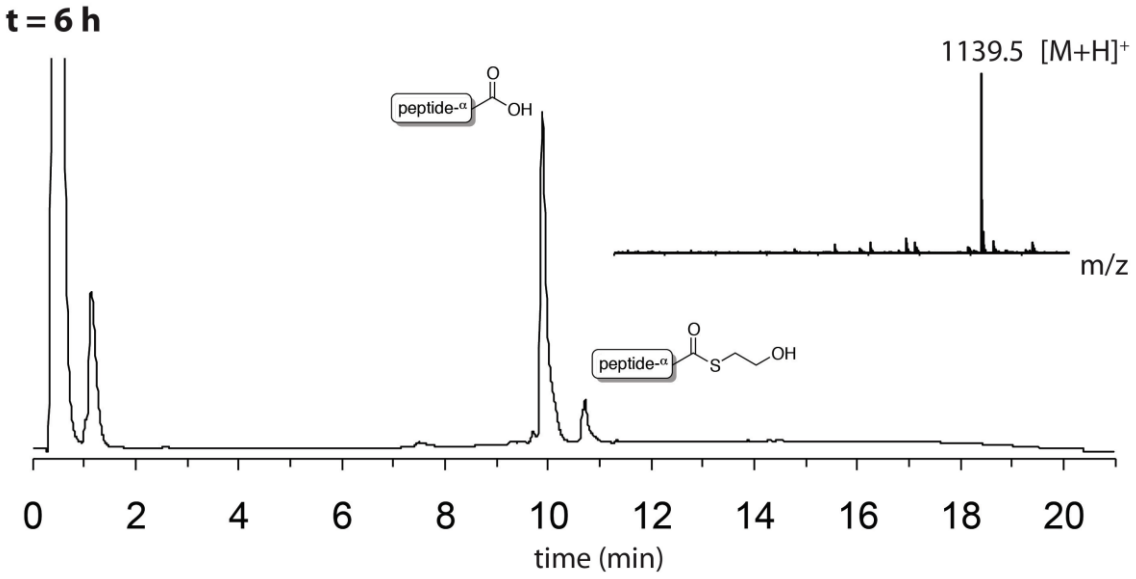

Figure S12. 2-Mercaptoethanol-mediated hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*; t = 6 h. The reaction product was ThzGGCCNEESLI-$^{\alpha}$*COOH* (exact mass: 1138.41 Da; average isotopes mass: 1139.28 Da; found: 1138.5 Da).

6. Alkaline hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*: A 2 mL plastic centrifuge tube was charged with peptide-$^{\alpha}$thioester (0.2 mg, 0.1 umol). 100 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher) and 200 mM phosphate (Fisher) was added. Immediately, a 10 uL aliquot of the resulting solution was diluted 20-fold into 1% B for LC-MS analysis. The vial was then capped, and the reaction mixture allowed to stand. A second aliquot was taken after 6 h.

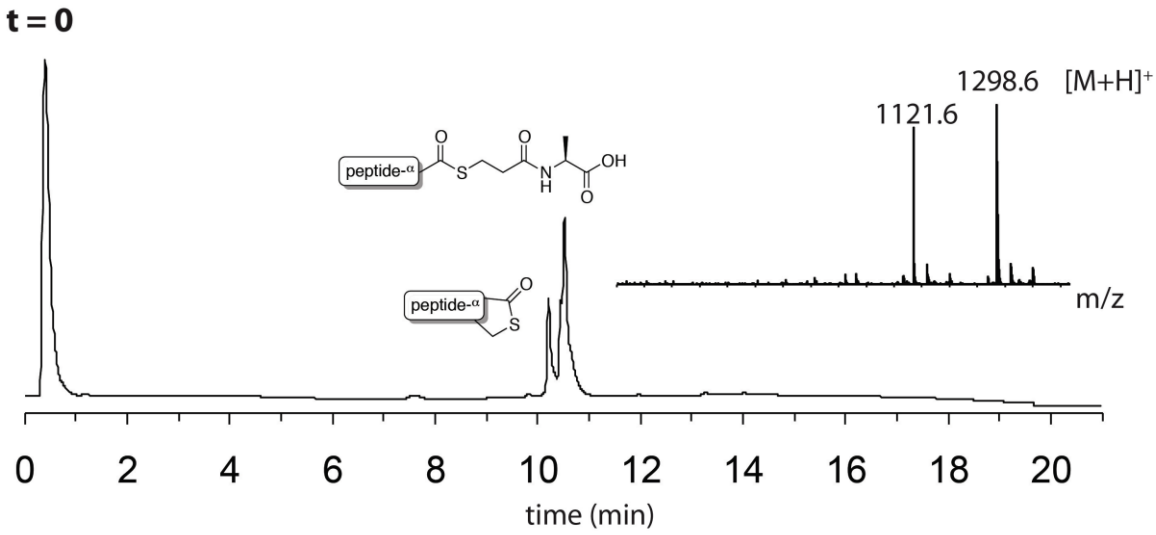

Figure S13. Alkaline hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH* (exact mass: 1297.71 Da; average isotopes mass: 1298.48 Da; found: 1297.6 Da); t = 0. The earlier-eluting peak corresponded to the product of transthioesterification by an internal Cys residue (exact mass: 1120.40 Da; average isotopes mass: 1121.26 Da; found: 1120.6 Da). The inset MS was taken over the peak that corresponded to the starting material; a signal that corresponded to the thiolactone was also evident.

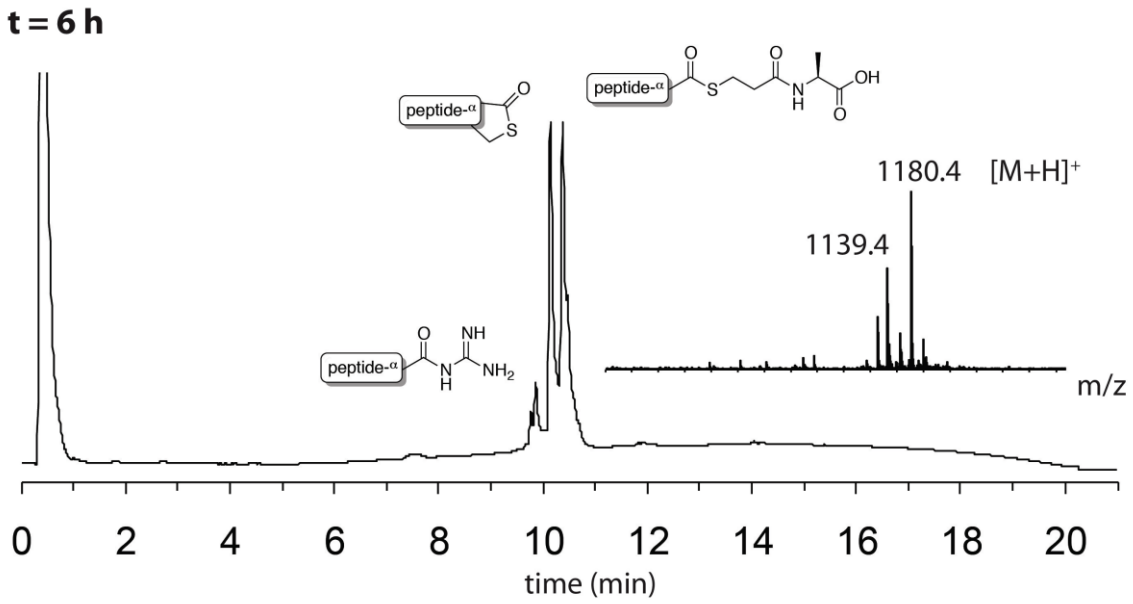

Figure S14. Alkaline hydrolysis of ThzGGCCNEESLI-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*; t = 6 h. The inset MS was taken over the peak the corresponded to the peptide-$^{\alpha}$*acylguanidine* (exact mass: 1179.44 Da; average isotopes mass: 1180.33; found: 1179.4 Da). The peptide-$^{\alpha}$*acylguanidine* co-eluted with the peptide-$^{\alpha}$*COOH* (exact mass: 1138.41 Da; average isotopes mass: 1139.28 Da; found: 1138.4 Da), as seen in the MS.

7. 2-Mercaptoethanol-mediated hydrolysis of FAATFYDIETLKVIDEE W(formyl)QRTQ-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*: A 2 mL plastic centrifuge tube was charged with peptide-$^{\alpha}$thioester (0.4 mg, 0.1 umol). 300 uL of a buffer containing 6 M guanidine hydrochloride (Fisher), 200 mM phosphate (Fisher), and 200 mM 2-mercaptoethanol (Aldrich) was added. The vial was capped, and the resulting solution was allowed to stand. After 30 min, a 10 uL aliquot was diluted 10-fold into 1% B for LC-MS analysis. The t = 0 timepoint was from the control reaction carried out in the absence of 2-mercaptoethanol (see page 17).

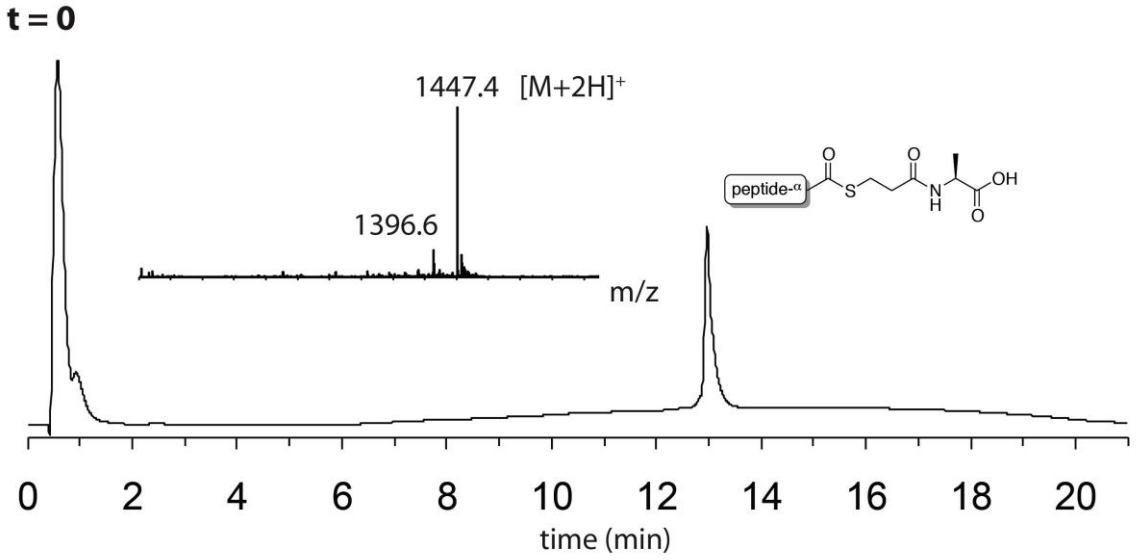

Figure S15. 2-Mercaptoethanol-mediated hydrolysis of FAATFYDIETLKVIDEE W(formyl)QRTQ-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH* (exact mass: 2889.35 Da; average isotopes mass: 2891.20 Da; found: 2892.8 Da); t = 0.

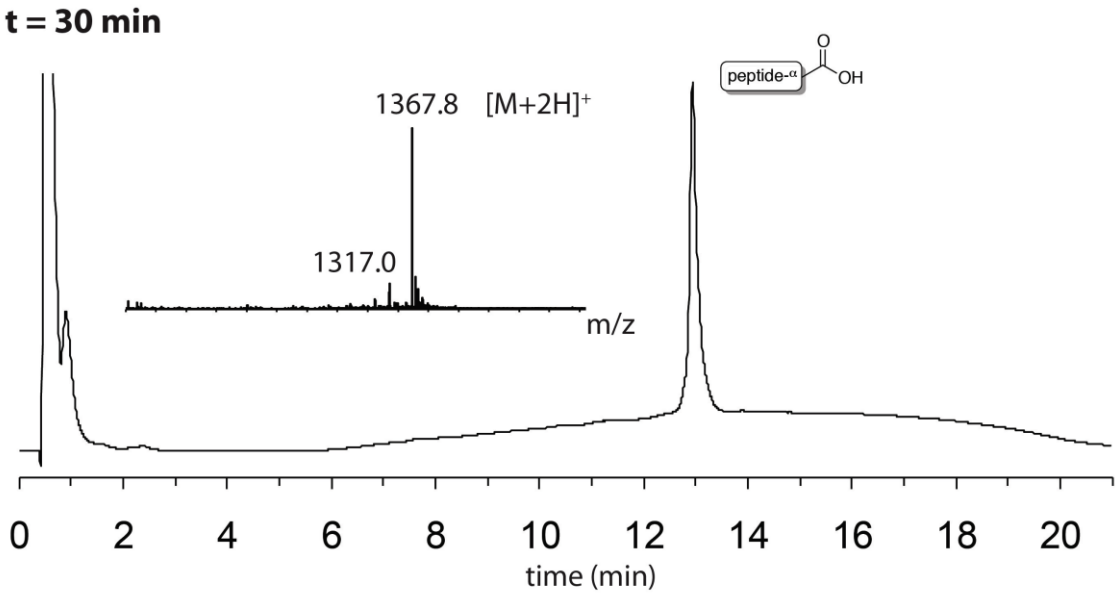

Figure S16. 2-Mercaptoethanol-mediated hydrolysis of FAATFYDIETLKVIDEE W(formyl)QRTQ-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*; t = 30 min. The reaction product was FAATFYDIETLKVIDEE W(formyl)QRTQ-$^{\alpha}$*COOH* (exact mass: 2730.32 Da; average isotopes mass: 2732.0 Da; found: 2733.6 Da).

8. Alkaline hydrolysis of FAATFYDIETLKVIDEEW(formyl)QRTQ-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*: A 2 mL plastic centrifuge tube was charged with peptide-$^{\alpha}$thioester (0.4 mg, 0.1 umol). 300 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher) and 200 mM phosphate (Fisher) was added. Immediately, a 10 uL aliquot of the resulting solution was diluted 20-fold into 1% B for LC-MS analysis. The vial was then capped, and the reaction mixture allowed to stand. A second aliquot was taken after 60 min.

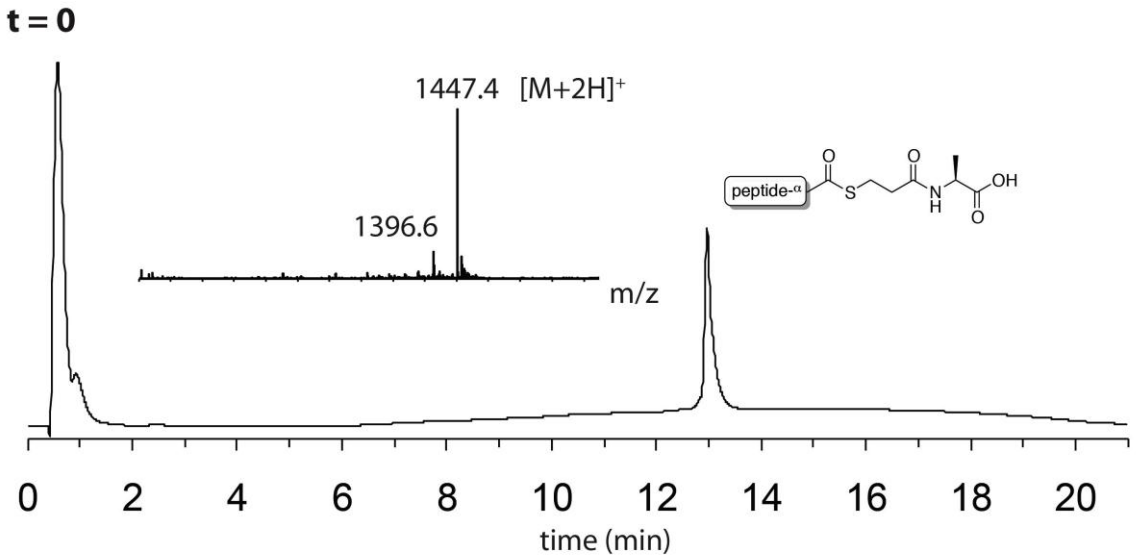

Figure S17. Alkaline hydrolysis of FAATFYDIETLKVIDEE W(formyl)QRTQ-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH* (exact mass: 2889.35 Da; average isotopes mass: 2891.20 Da; found: 2892.8 Da); t = 0.

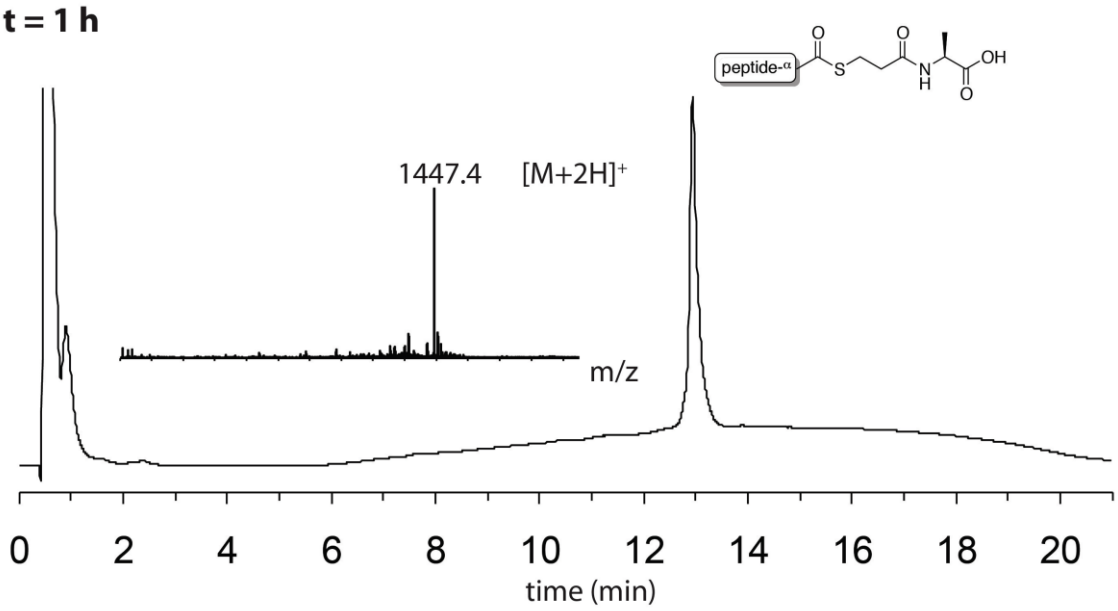

Figure S18. Alkaline hydrolysis of FAATFYDIETLKVIDEE W(formyl)QRTQ-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Ala-$^{\alpha}$*COOH*; t = 1 h. Only starting material was detected (exact mass: 2889.35 Da; average isotopes mass: 2891.20 Da; found: 2892.8 Da).

9. 2-mercaptoethanol-mediated hydrolysis of ThzGYGSTQTAQEESSLTAGYGSTQTAQEESSLT-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO-(Arg)$_6$*Leu-$^{\alpha}$*COOH*: A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (1.1 mg, 0.3 umol). 1.1 mL of a pH 5 buffer containing 6 M guanidine hydrochloride (Fisher) and 200 mM phosphate (Fisher) was added. 40 uL of the resulting solution was diluted 10-fold into Buffer A for LC-MS analysis. Finally, 2-mercaptoethanol (30 uL, Aldrich) was added to bring the concentration to 400 mM, and the pH was adjusted to 9.0. After one hour, a second aliquot was taken for LC-MS.

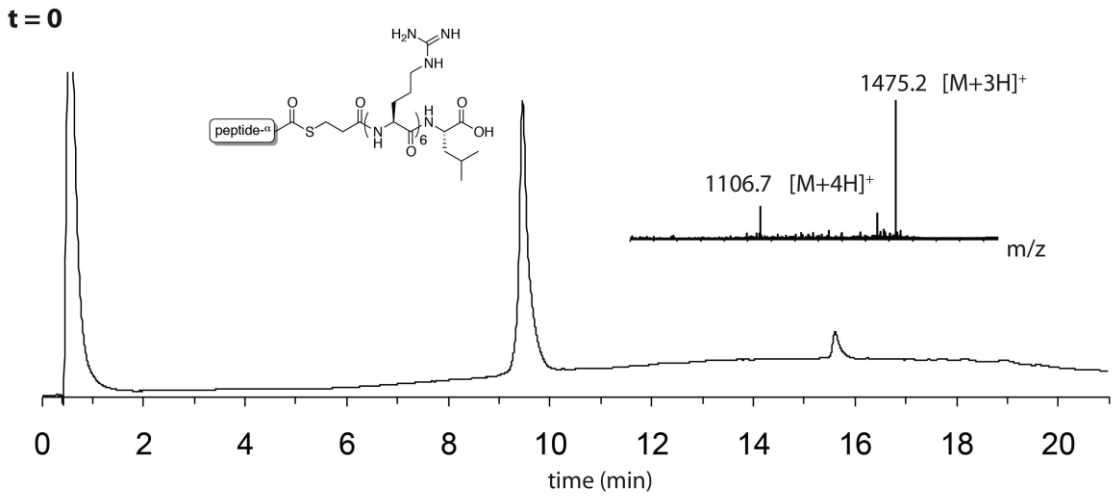

Figure S19. 2-Mercaptoethanol-mediated hydrolysis of ThzGYGSTQTAQEESSLTAGYGSTQTAQEESSLT-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO-(Arg)$_6$*Leu-$^{\alpha}$*COOH* (exact mass: 4421.08 Da; average isotopes mass: 4423.77 Da; found: 4422.6 Da); t = 0.

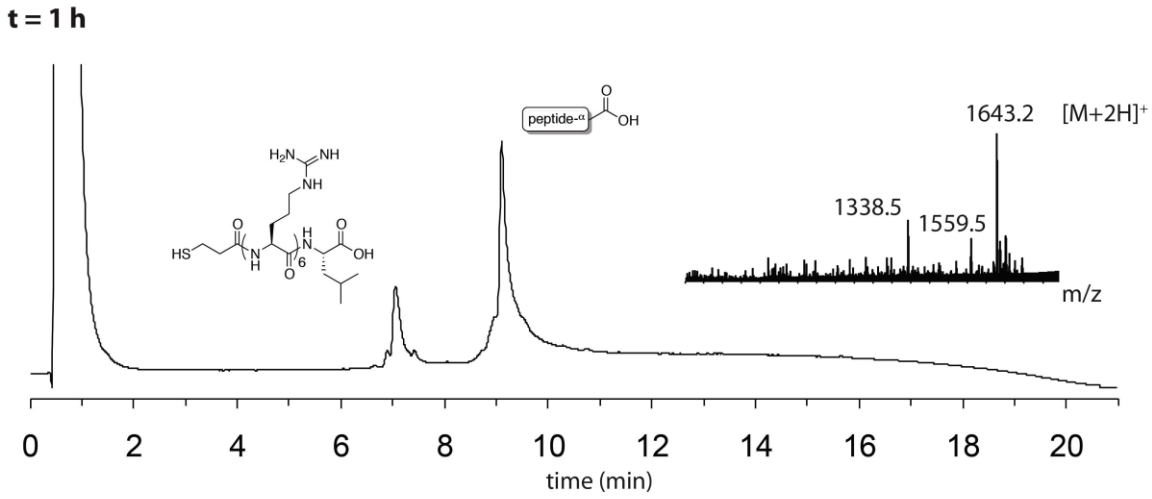

Figure S20. 2-Mercaptoethanol-mediated hydrolysis of ThzGYGSTQTAQEESSLT AGYGSTQTAQEESSLT-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO-(Arg)$_6$*Leu-$^{\alpha}$*COOH*; t = 1 h. The inset MS was taken over the reaction product, ThzGYGSTQTAQEESSLT AGYGSTQTAQEESSLT-$^{\alpha}$*COOH* (exact mass: 3283.39 Da; average isotopes mass: 3285.36 Da; found: 3284.4 Da).

10. Alkaline hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* in $H_2{}^{18}O$: A 6 M guanidine hydrochloride, 200 mM phosphate buffer was prepared by dissolving guanidine hydrochloride (Fisher, 141 mg) and dibasic sodium phosphate (Fisher, anhydrous, 9 mg) in 250 uL $H_2{}^{18}O$ (Aldrich, 97 atom %) in a 5 mL glass vial. The resulting solution was brought to pH 9.3 with concentrated NaOH solution. A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (0.4 mg, 0.4 umol). 125 uL of the above buffer was added, and the resulting solution was allowed to stand for 6 h before a 10 uL aliquot was taken and diluted 20-fold into 1% buffer B for LC-MS analysis.

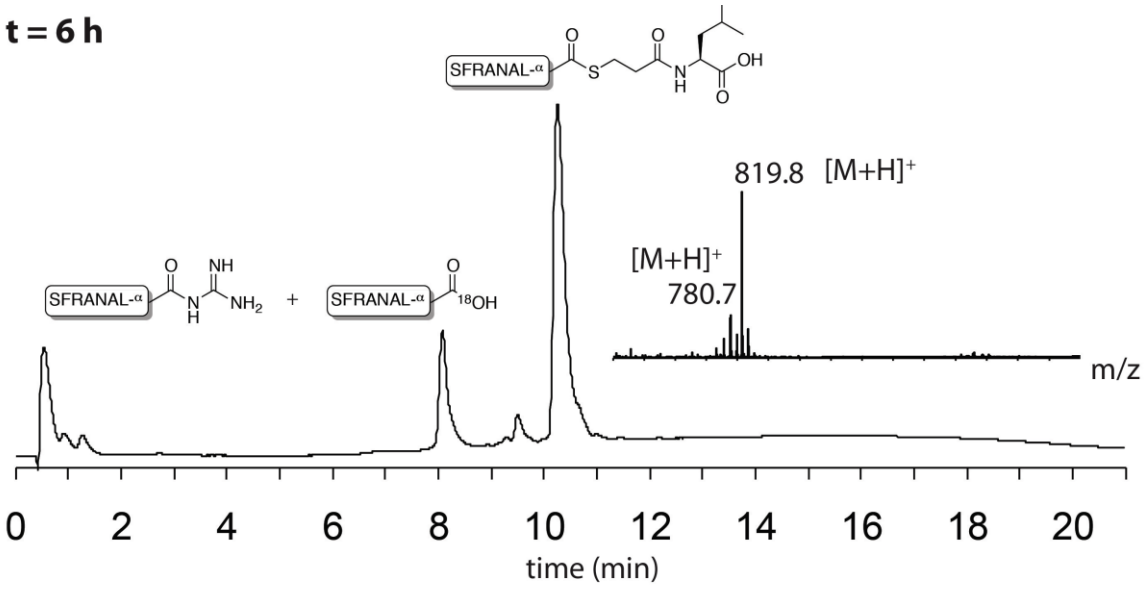

Figure S21. Alkaline hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* in $H_2{}^{18}0$; t = 6 h. The inset MS was taken over the earlier-eluting peak, which corresponded to a mixture of SFRANAL-$^{\alpha}$*acylguanidine* and SFRANAL-$^{\alpha}$*CO^{18}OH* (exact mass: 779.41 Da; found: 779.7 Da).

11. 2-Mercaptoethanol-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* in $H_2{}^{18}O$: A 5 mL glass vial was charged with peptide-$^{\alpha}$thioester (0.5 mg, 0.5 umol). 125 uL of the pH 9.3 buffer containing 6 M guanidine hydrochloride, 200 mM phosphate from the preceeding experiment (conducted in parallel) was added, followed by 2-mercaptoethanol (Aldrich, 4 uL). After 6 h, a 10 uL aliquot was diluted 20-fold into 1% B for LC-MS analysis.

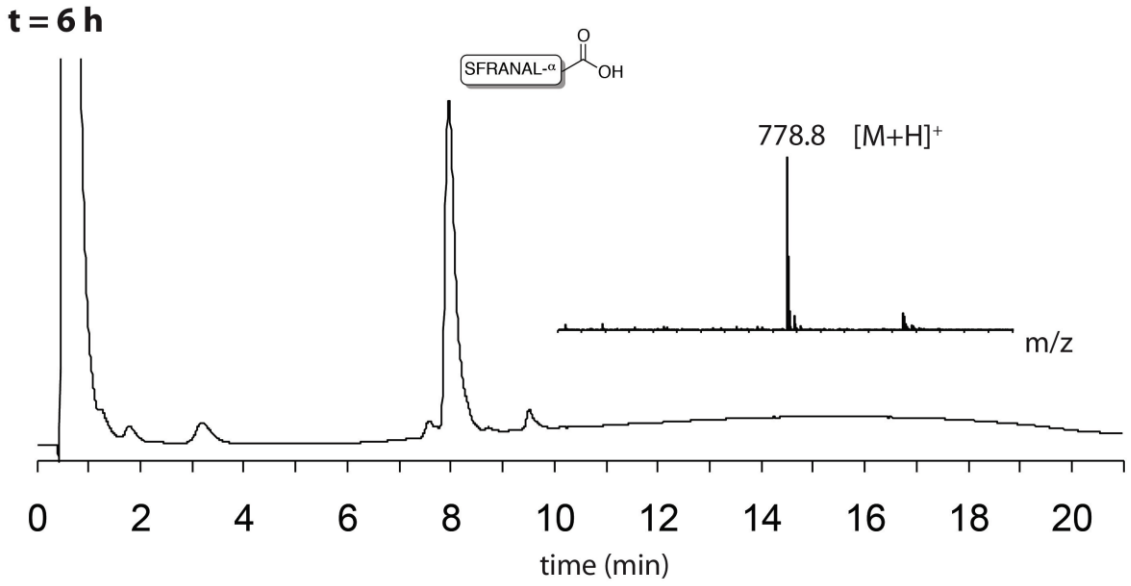

Figure S22. 2-mercaptoethanol-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* in $H_2{}^{18}0$; t = 6 h. The reaction product was SFRANAL-$^{\alpha}$*COOH* (exact mass: 777.41 Da; found: 777.8 Da).

12. DTT-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*: A 2 mL plastic centrifuge tube was charged with peptide-$^{\alpha}$thioester (0.8 mg, 0.8 umol). 800 uL of a pH 9.0 buffer containing 6 M guanidine hydrochloride (Fisher), 200 mM phosphate (Fisher), and 200 mM D,L-dithiothreitol (Aldrich) was added. 20 uL aliquots were taken after 30 min, 1 h, and 4 h, and diluted 10-fold into 1% B for LC-MS analysis. The sample used for t = 0 analysis was prepared by dissolving peptide-$^{\alpha}$thioester (0.8 mg, 0.8 umol) in 1% B (0.8 mL).

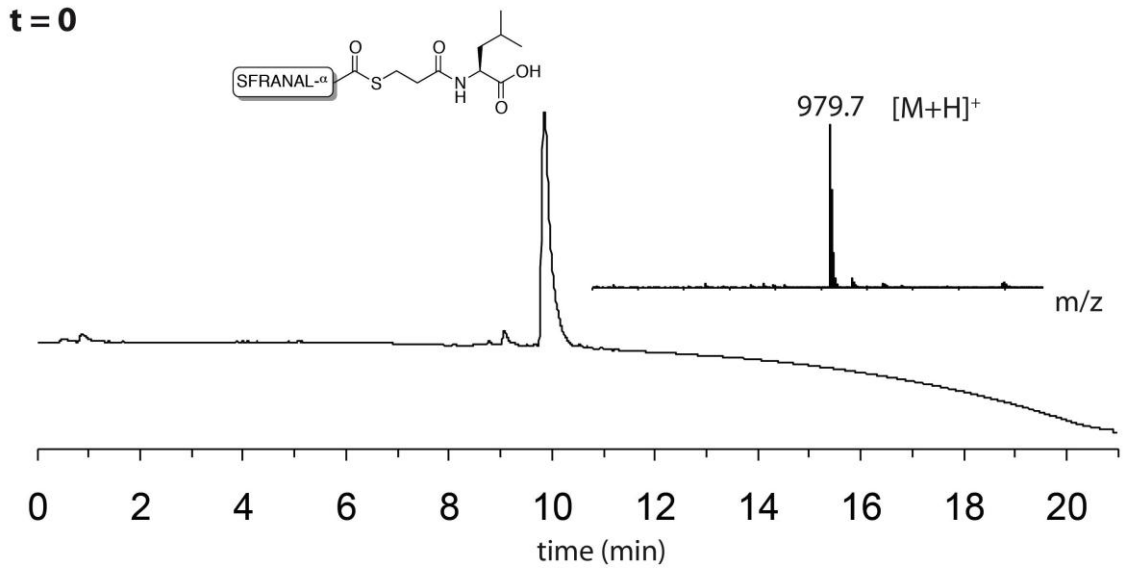

Figure S23. DTT-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH* (exact mass: 978.49 Da; found: 978.7 Da); t = 0.

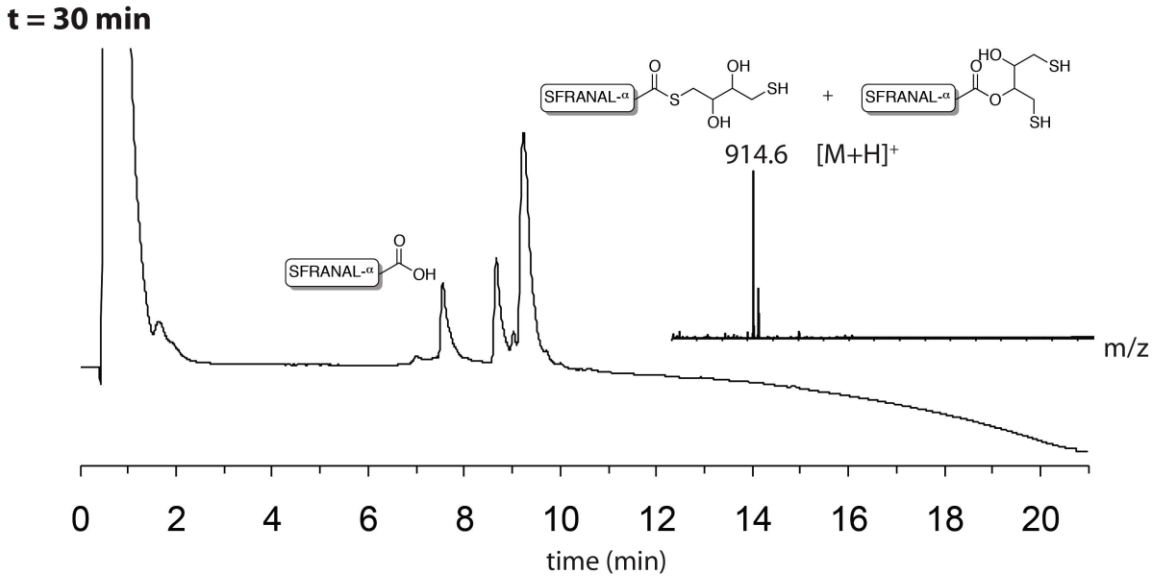

Figure S24. DTT-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*; t = 30 min. The inset MS was taken over the combined second and third peaks, which exhibit identical mass spectra. These compounds were presumed to be O and S-linked isomers of the product of transthioesterification by DTT (exact mass: 913.41 Da; found: 913.6 Da).

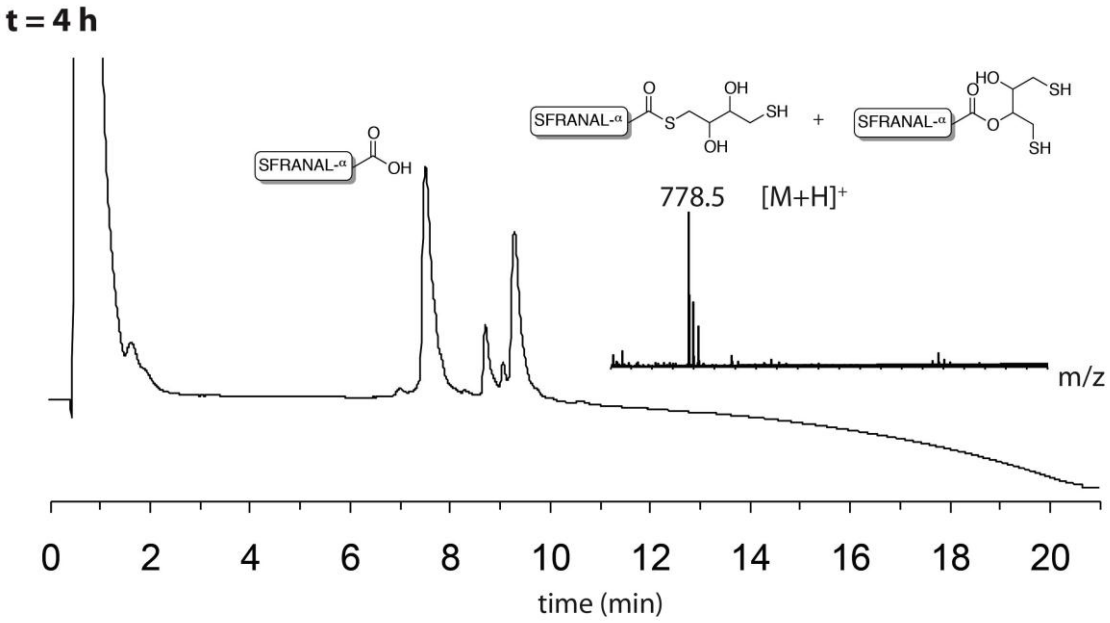

Figure S25. DTT-mediated hydrolysis of SFRANAL-$^{\alpha}$*CO-S-CH$_2$-CH$_2$-CO*-Leu-$^{\alpha}$*COOH*; t = 4 h. The inset MS was taken over the reaction product, SFRANAL-$^{\alpha}$*COOH* (exact mass: 777.41 Da; found: 777.5 Da).