Rapid formal hydrolysis of peptide-α-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\(_2\)O; 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-\(\text{S-CH}_2\text{-CH}_2\text{-CO-}\)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.

![Alkaline hydrolysis of SFRANAL](image)

**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 ThzAGGVGGAGGASGGTG-GGG
RGGKGGSGTPKGADGAPGAP-$\alpha^{co-s-CH_2-CH_2-CO-Leu-c-\alpha\ COOH}$: 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](image)

**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\textsuperscript{43-81} \textsuperscript{\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\textsuperscript{43-81} \textsuperscript{\alpha}COSR; t = 7 h. The reaction product was Thz\textsuperscript{43-81} \textsuperscript{\alpha}COOH (exact mass: 3035.42 Da; average isotopes mass: 3037.23 Da; found: 3037.0 Da).
4. Alkaline hydrolysis of ThZAGVGGAGGASGGTGVGGRGGKGGSGTP 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](image.png)

**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 ThzGGCNEESLI-\(\alpha\)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 ThzGGCNEESLI-\(\alpha\)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 ThzGGCNEESLI$^{\alpha}$CO-S-CH$_2$-CO-Ala$^{\alpha}$COOH; t = 1 h. The inset MS was taken over the peak that corresponds to transthoesterification 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-αCO-S-CH₂-CH₂-CO-Ala-αCOOH; t = 6 h. The reaction product was ThzGGCCNEESLI-αCOOH (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-αCO-S-CH₂-CH₂-Co-Ala-αCOOH: A 2 mL plastic centrifuge tube was charged with peptide-α-thioester (0.4 mg, 0.1 umol). 300 μL 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 μL 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-αCO-S-CH₂-CH₂-Co-Ala-αCOOH (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-α-CO-S-CH₂-CH₂-CO-Ala-α-COOH; t = 30 min. The reaction product was FAATFYDIETLKVIDEE W(formyl)QRTQ-α-COOH (exact mass: 2730.32 Da; average isotopes mass: 2732.0 Da; found: 2733.6 Da).
8. Alkaline hydrolysis of FAATFYDIETLKVIDEEW(formyl)QRTQ-\textsuperscript{\( \alpha \)}CO-S-\( \text{CH}_2\)-\( \text{CH}_2\)-CO-Ala-\textsuperscript{\( \alpha \)}COOH: A 2 mL plastic centrifuge tube was charged with peptide-\textsuperscript{\( \alpha \)}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 FAATFYDIETLKVIDEEW(formyl)QRTQ-\textsuperscript{\( \alpha \)}CO-S-\( \text{CH}_2\)-\( \text{CH}_2\)-CO-Ala-\textsuperscript{\( \alpha \)}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 ThzGYGSTQTAQESSLTAGYGSTQTAQESSLT-αCO-S-CH₂-CH₂-CO-(Arg)₆Leu-αCOOH: A 5 mL glass vial was charged with peptide-α 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 μL of the resulting solution was diluted 10-fold into Buffer A for LC-MS analysis. Finally, 2-mercaptoethanol (30 μL, 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 ThzGYGSTQTAQESSLTAGYGSTQTAQESSLT-αCO-S-CH₂-CH₂-CO-(Arg)₆Leu-αCOOH (exact mass: 4421.08 Da; average isotopes mass: 4423.77 Da; found: 4422.6 Da); t = 0.
**Figure S20.** 2-Mercaptoethanol-mediated hydrolysis of ThzGYGSTQTAQESSLT\textsubscript{AGYGSTQTAQESSLT-}\textsubscript{\textalpha}CO\textsubscript{S}\textsubscript{CH\textsubscript{2}}\textsubscript{CH\textsubscript{2}}\textsubscript{CO}(Arg\textsubscript{6})Leu-\textsubscript{\textalpha}COOH; \textit{t} = 1\text{ h}. The inset MS was taken over the reaction product, ThzGYGSTQTAQESSLT\textsubscript{AGYGSTQTAQESSLT-}\textsubscript{\textalpha}COOH (exact mass: 3283.39 Da; average isotopes mass: 3285.36 Da; found: 3284.4 Da).
10. Alkaline hydrolysis of SFRANAL-$\alpha_{CO-S-CCH_{2}-CCH_{2}-CO-Leu-\alpha-COOH}$ 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-$\alpha_{CO-S-CCH_{2}-CCH_{2}-CO-Leu-\alpha-COOH}$ in H$_2^{18}$O; $t = 6$ 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$_2$O$^{18}$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$_2$O$^{18}$O; 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-α-S-CH₂-CH₂-CO-Leu-α-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-αCO-S-CH₂-CH₂-CO-Leu-αCOOH; t = 4 h. The inset MS was taken over the reaction product, SFRANAL-αCOOH (exact mass: 777.41 Da; found: 777.5 Da).