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Letter

Prebiotic Environments with Mg^{2+} and Thiophilic Metal Ions Increase the Thermal Stability of Cysteine and Non-cysteine Peptides

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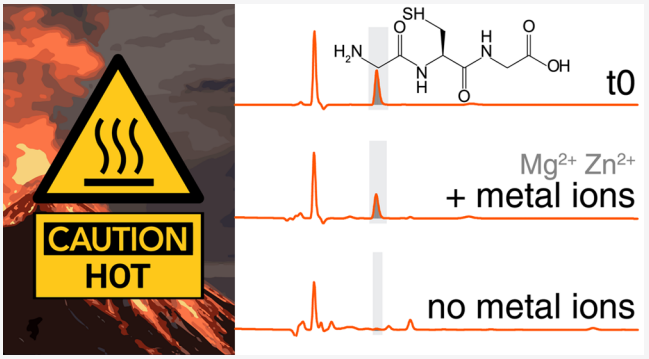
ABSTRACT: Wet−dry cycles driven by heating to high temperatures are frequently invoked for the prebiotic synthesis of peptides. Similarly, iron−sulfur clusters are often cited as an example of an ancient catalyst that helped prune early chemical systems into metabolic-like pathways. Because extant iron−sulfur clusters are metallocofactors of protein enzymes and nearly ubiquitous across biology, a reasonable hypothesis is that prebiotic iron−sulfur peptides formed on the early Earth. However, iron−sulfur clusters are coordinated by multiple cysteine residues, and the stability of cysteines to the heat steps of wet−dry cycles has not been determined. It, therefore, has remained unclear if the peptides needed to stabilize the formation of iron−sulfur clusters could have formed. If not, then iron−sulfur-dependent activity may have emerged later, when milder, more biological-like peptide synthesis machinery took hold. Here, we report the thermal stability of cysteine-containing peptides. We show that temperatures of 150 °C lead to the rapid degradation of cysteinyl peptides. However, the presence of Mg^{2+} at environmentally reasonable concentrations leads to significant protection. Thiophilic metal ions also protect against degradation at 150 °C but require concentrations not frequently observed in the environment. Nevertheless, cysteine-containing peptides are stable at lower, prebiotically plausible temperatures in seawater, carbonate lake, and ferrous lake conditions. The data are consistent with the persistence of cysteine-containing peptides on the early Earth in environments rich in metal ions. High concentrations of Mg^{2+} are common intra- and extra-cellularly, suggesting that the protection afforded by Mg^{2+} may reflect conditions that were present on the prebiotic Earth.



KEYWORDS: *prebiotic chemistry, origins of life, cysteine, iron−sulfur cluster, metallopeptides*

Iron−sulfur clusters are believed to be ancient cofactors that were present on the prebiotic Earth.[1] While there are several theories for how iron−sulfur clusters could have impacted prebiotic chemistry, few experiments have been geared toward identifying plausible pathways from non-enzymatic to enzymatic iron−sulfur-dependent chemistry. One proposal is that small metallopeptides grew through a process of accretion into the types of structures found today in iron−sulfur proteins.[2−5] Such a scenario requires the non-enzymatic formation of peptide bonds through, e.g., aminonitrile coupling,[6] diamidophosphate-mediated activation,[7] or wet−dry cycling of solutions containing amino acids and/or α-hydroxy acids,[8] perhaps facilitated by air−water interfaces[9] or clay surfaces.[10] Of the investigated pathways for the formation of peptide bonds, condensation driven by high temperatures is most frequently invoked.[11]

While heat drives condensation in the absence of water, heat also promotes the degradation of amino acid side chains and peptidyl termini.[12] Such an effect has not been frequently encountered, because previous studies typically focused on peptide bond formation through wet−dry cycling between glycines in the absence of other amino acids, which minimizes side reactions. There is good reason to focus on glycine, because this amino acid is easily synthesized. However, peptides consisting of only glycine are unlikely to make functionally useful peptides. Because iron−sulfur clusters are typically coordinated by cysteines and the prebiotic synthesis of cysteine was recently uncovered,[13] we sought to determine if cysteinyl peptides could survive the high temperatures needed for condensation into larger peptides.

To determine if peptidyl cysteines survive heating, we monitored the stability of a peptide with a single cysteine

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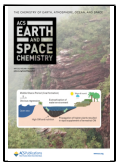

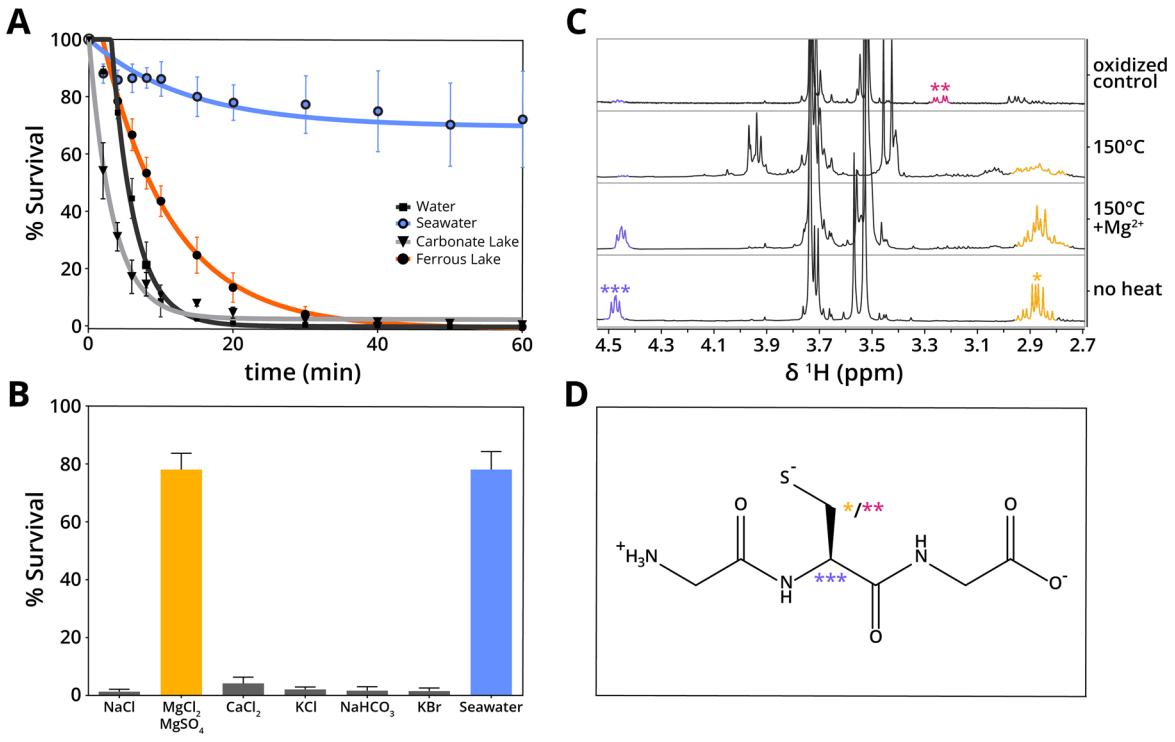

Figure 1. (A) Degradation of GCG upon heating at 150 °C to dryness for 120 min in buffered water (20 mM glycylglycine at pH 8.7), seawater (pH 8.0), carbonate lake (pH 9.0), and ferrous lake (pH 6.3) conditions. Data are the mean ± standard deviation (SD); $n = 3$. (B) Contribution of individual salts to peptide survival during a single wet−dry cycle at 150 °C and pH 8.0. The concentration of each salt was equivalent to that component in seawater. Incubation at 150 °C was for 20 min. Data are the mean ± SD; $n = 3$. (C) Effect of Mg^{2+} on peptide survival at 150 °C and pH 8.7 for 20 min by ^{1}H NMR spectroscopy. Spectra are of GCG before heating, heating in the presence of 40 mM Mg^{2+}, heating in the absence of Mg^{2+}, and an oxidized control. (D) For reference, the chemical structure of GCG is provided. Asterisks indicate protons that gave rise to the observed resonances: (∗) reduced cysteine, (∗∗) oxidized cysteine, and (∗∗∗) chiral center.

residue Gly-Cys-Gly (GCG). GCG was previously shown to stabilize the formation of mononuclear centers and [2Fe−2S] and [4Fe−4S] clusters.[14] GCG was placed in aqueous solution at 150 °C and pH 8.7 for 2 h in an open vial under anaerobic conditions. Within the first 3 min, the water evaporated. After 2 h, the dry sample was rehydrated and analyzed by liquid chromatography−mass spectrometry (LC−MS). GCG progressively degraded over time with a $t_{1/2}$ = 2.3 ± 0.2 min (Figure S1 of the Supporting Information). The main degradation products were GC/CG (m/z 178.9), GA/AG (m/z 147.0), GAG (m/z 204.1), and Gly-Dha-Gly (m/z 202.0), where Dha indicates dehydroalanine. GC and CG were products of hydrolysis of the starting GCG. GAG may have been produced by radical desulfurization,[15] while Gly-Dha-Gly was likely the result of β-elimination of thiol (Scheme S1 of the Supporting Information). GA and Gly-Dha were produced by a combination of desulfurization and β-elimination followed by hydrolysis. Only small amounts of GAG and no Gly-DhA-Gly was detected by nuclear magnetic resonance (NMR) spectroscopy. Therefore, most of the degradation of the starting GCG occurred via hydrolysis. Glycylglycine was used as a buffer in these reactions, which could be observed to both degrade and form longer peptides. Glycylglycine degraded to 2,5-diketopiperazine (m/z 115.0) and additionally formed GGG (m/z 190.0), GGGG (m/z 247.0), and GCGGG (m/z 350.1). As expected, the extent of degradation was dependent upon pH. Reactions performed at pH 5.0, 7.0, and 8.7 revealed that the half-life of the peptide increased with decreasing pH (Figure S2 of the Supporting Information), which was consistent with an alkaline-driven β-elimination mechanism for the loss of the sulfhydryl group. Additionally, the dehydroalanine-containing peptide, i.e., Gly-Dha-Gly, was only detected at pH 7.0 and 8.7 but not at pH 5.0, where only GAG was observed. At low pH, radical desulfurization prevailed (Figure S3 of the Supporting Information). Lanthionine was not detected under any of the conditions tested.

After determination of the intrinsic lability of cysteine-containing peptides, we next probed whether plausible environmental conditions could alter stability. To do so, we heated GCG to 150 °C in an open vial under anaerobic conditions for up to 2 h in aqueous solutions that represented seawater, a ferrous lake, or a carbonate lake.[16,17] Strikingly, seawater significantly increased the survival of GCG, with ∼70% still present after 2 h (Figure 1A and Figure S4 of the Supporting Information). For comparison, incubation in buffered water at pH 8.7 without additional components resulted in complete degradation of GCG within 20 min, with a $t_{1/2}$ of 2.3 ± 0.2 min. Ferrous lake conditions (pH 6.3) also increased the stability of GCG ($t_{1/2}$ = 6.7 ± 0.6 min) in comparison to water buffered at pH 8.7 but still led to complete degradation within 60 min. However, the amount of peptide that survived was similar to reactions at pH 5.0 (Figure S6 of the Supporting Information), suggesting that the increased survival in ferrous lake conditions was at least partly due to pH. Control experiments did not identify an additional stabilizing factor (Figure S7 of the Supporting Information). Carbonate lake conditions did not detectably increase stability at 150 °C ($t_{1/2}$ = 2.3 ± 0.2 min).

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To determine which component of seawater was responsible for the increased survival of the peptide, the effect of solutions consisting of a single component of seawater on the stability of GCG was evaluated. Contemporary seawater contains ca. 50 mM Mg^{2+}, with estimates for the early sea ranging from 30 to 50 mM.[18] Neither 400 mM NaCl, 10 mM $CaCl_2$, 10 mM KCl, 2 mM $NaHCO_3$, nor 1.7 mM KBr alone significantly improved the stability of GCG. However, the presence of 40 mM $MgCl_2$ was sufficient to confer the same level of protection as seawater (Figure 1B). The LC−MS data were confirmed by NMR spectroscopy (panels C and D of Figure 1). Lower concentrations (5 mM) of Mg^{2+} did not protect as well (Figure S8 of the Supporting Information).

Mg^{2+} does not interact with thiolates; therefore, the observed effect must have been due to interactions with other regions of the peptide, e.g., backbone carbonyls or the C-terminal carboxylate. The larger alkaline Earth metal Ca^{2+} protected less efficiently when present at the same concentration. A value of 60 ± 7.6% of the peptide survived at 150 °C for 20 min in the presence of 40 mM Ca^{2+}, in comparison to a value of 78 ± 5.6% with Mg^{2+}. The transition metal Mn^{2+}, which is capable of binding both oxygen and sulfur ligands, protected as well as Mg^{2+} (Figures S4 and S9 of the Supporting Information).

Because cysteine-containing tripeptides readily bind thiophilic metal ions[19] and none of the major components of seawater are thiophilic, we tested the impact of Zn^{2+}, Fe^{2+}, and Fe^{3+} on the stability of GCG and GAG at 150 °C. Reactions were run at pH 8.7 to ensure the deprotonation of thiol and, thus, coordination to the metal ions. After 20 min, 44.7 ± 2.7% of GCG survived in the presence of 5 mM Zn^{2+}, whereas only 1.3 ± 1.4% of the starting concentration of GCG was detectable in the absence of metal ions (Figure 2). Therefore, thiophilic metal ions were able to protect GCG. Zn^{2+} (5 mM) protected 19% more GCG than Fe^{2+} (5 mM), as would be expected if binding affinity for the cysteinyl side chain correlated with protection. Both Zn^{2+} and Fe^{2+} protected GCG more than Fe^{3+}, consistent with the decreased affinity of Fe^{3+} for thiolates and the ability of Fe^{3+} to oxidize cysteines. As noted above, 5 mM Mg^{2+} did not protect GCG well, with only 13.3 ± 11.5% surviving after 20 min at 150 °C. The tested concentrations (5 mM) of Zn^{2+}, Fe^{2+}, and Fe^{3+} were well above the typical conditions found on the Earth today or in the past. These concentrations were chosen to probe the effect of metal binding to the cysteine residue of GCG and not to indicate prebiotic relevance. Conversely, 5 mM Mg^{2+} is lower than what is encountered in the environment today and in the past. Environmental and physiological concentrations of Mg^{2+} are high, and thus, low-affinity complexes with Mg^{2+} are relevant. At a more relevant concentration (40 mM), 78 ± 5.6% of GCG survived after 20 min at 150 °C.

To confirm that the increased stability afforded by Mg^{2+} was not due to the binding of the cysteine residue of GCG, the impact of Mg^{2+} on the stability of GAG was tested. In the absence of metal ions, 43 ± 9.4% of GAG survived after 20 min at 150 °C (Figure 2). GAG was much more stable than GCG (1.3 ± 1.4% survival) in the absence of metal ions. Nevertheless, 40 mM Mg^{2+} increased the stability of GAG to 91 ± 5.2%, a value similar to that observed for GCG in the presence of the same concentration of Mg^{2+}. Therefore, the effect of Mg^{2+} on the stability of the peptides was independent of the presence of cysteine. The stability of GAG in the presence of Zn^{2+} was within error of the stability of GAG in the absence of Zn^{2+}, consistent with the lack of a Zn^{2+}-binding site.

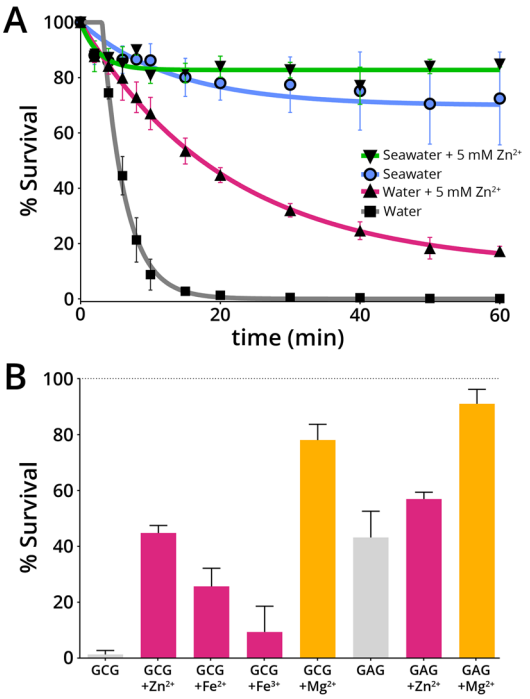

Figure 2. Survival of GCG upon heating in the presence of thiophilic metal ions. (A) GCG in the presence or absence of 5 mM Zn^{2+} in buffered water (20 mM glycylglycine at pH 8.7) or seawater (pH 8.0) conditions. (B) GCG (5 mM) or GAG (5 mM) in buffered water was heated at 150 °C for 20 min in the presence of 5 mM Zn^{2+}, Fe^{2+}, or Fe^{3+} (magenta) or 40 mM Mg^{2+} (yellow). Data are the mean ± SD; n = 3.

Estimates of the temperature of the early Earth vary greatly. One recent calculation indicated that temperatures were likely between 0 and 50 °C,[20] much lower than the 150 °C tested here. To obtain a better picture as to the plausibility of cysteine stability, we next measured the stability of GCG at lower temperatures and in solution; i.e., samples were not allowed to evaporate. GCG was completely stable for 24 h at both 25 and 40 °C at seawater, alkaline lake, and ferrous lake conditions. Higher temperatures led to increased degradation under all conditions, with seawater typically providing the most stabilizing conditions (Figure 3). For example, at 100 °C, GCG was ∼3-fold more stable in comparison to ferrous lake conditions. To ensure that stability at 40 °C was retained when dry, fresh samples were lyophilized and then incubated at 40 °C for 24 h prior to analysis by LC−MS, GCG lyophilized from seawater, ferrous lake, and carbonate lake conditions was completely stable at 40 °C (Figure S10 of the Supporting Information). We additionally tested the impact of wet−dry cycling under seawater conditions. A value of 78.1 ± 3.6% of the starting GCG peptide survived one cycle of heating for 20 min at 150 °C followed by rehydration with deionized water. Two and three cycles showed 70.5 ± 3.9 and 57.7 ± 7.1% survival of GCG, respectively (Figure S11 of the Supporting Information).

Clearly, environmental conditions impacted the stability of peptides. High temperatures in the absence of metal ions would have likely led to degradation on the prebiotic Earth. Although Mg^{2+} was not alone in increasing stability, the abundance of Mg^{2+} strongly argues for a role of this metal ion

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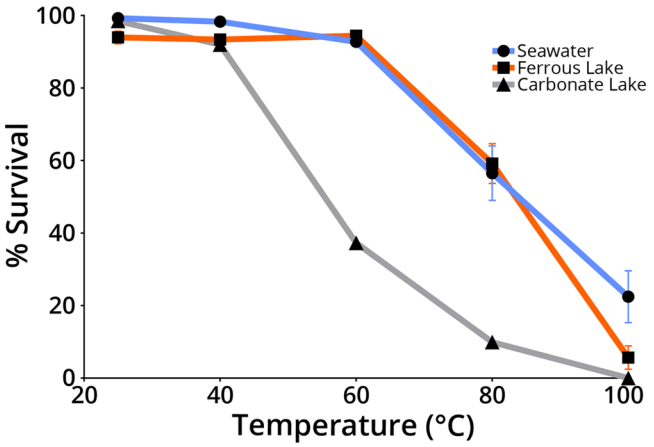

Figure 3. Survival of GCG in solutions mimicking prebiotic geological conditions (seawater, pH 8.0; carbonate lake, pH 9.0; and ferrous lake, pH 6.3). Solutions were heated in a sealed glass vial for 24 h. Data are the mean ± SD; n = 3.

in maintaining the integrity of small peptides that were incapable of folding. Larger peptides that could chelate transition metals with cysteinyl ligands may have been able to bind with high enough affinity to protect cysteines from degradation. Such a scenario would have presumably selected for metal binding motifs over cysteine-containing sequences that bound metal ions with low affinity. Perhaps such motifs emerged from previously described wet−dry cycles that relied on high salt conditions and that promoted the formation of peptide bonds.[8,21]

It is possible that the presence of other types of molecules could have increased stability further. For example, nucleobases and amino acids stabilize fatty acid membranes.[22,23] The benefit of metal ions to peptides is in contrast to the degradative effects observed with fatty acid vesicles. Either synergies between lipids and peptides (or amino acids[24]) yielded a system stable under the same conditions or the membranes were not solely composed of fatty acids.[25] Because lipids help to promote the formation of peptides from amino acids during wet−dry cycling,[26,27] a deeper investigation of the role of metal ions on such mixtures is likely to be insightful. If such cycles gave rise to metal-binding motifs, then more stable peptides could have concomitantly led to more stable vesicles.

The conditions tested were meant to mimic surface environments and not deep-sea hydrothermal vents. Much higher temperatures than tested here can be found at hydrothermal vents. For example, black smokers can reach temperatures of 300−400 °C.[28] However, milder conditions exist, as seen with alkaline vents (e.g., the Lost City), which fall in the range of 40−75 °C.[28] To probe the compatibility of such conditions with the integrity of peptides, it would be necessary to evaluate the effect of pressure in addition to temperature. Hydrothermal regions can be found close to the surface and as deep as 5800 m below sea level, where pressures can reach 580 MPa.[29] Alanine, leucine, phenylalanine, serine, and aspartic acid degrade rapidly at high temperatures and pressures.[30] Only by comparing data collected under different conditions can we begin to systematically narrow the environments compatible with the prebiotic chemistry amenable to the emergence of life-like chemical systems. Here, we show the benefit of metal ions on the stability of peptides at a few conditions meant to mimic environments at or near the surface of the early Earth.

MATERIALS AND METHODS

All reagents were purchased from Merck (Darmstadt, Germany) or Fisher Scientific (Nepean, Canada) and used without further purification. GCG was synthesized via solid-phase peptide synthesis, as previously described.[14] GCG was dissolved under anoxic conditions in aqueous 20 mM glycylglycine to a final concentration of 5 mM, and the pH was adjusted to 8.7 or at different pH values, as indicated. Seawater was prepared as in the study by Marcos et al.[31] with 400 mM NaCl, 20 mM $MgSO_4$, 20 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM KCl, 2 mM $NaHCO_3$, and 1.7 mM KBr at pH 8.0. The carbonate lake conditions were adapted from Toner and Catling[16] and Ranjan et al.[17] and included 0.1 M NaH_2PO_4, 10 mM KBr, 1.0 M Na_2SO_4, 0.24 mM Na_2SO_3, 10 mM lithium acetoacetate, 1.0 M H_3BO_3, 3.0 M NaCl, 10 mM $CaCO_3$, 10 mM $CaCl_2$, 2.2 mM Na_2SiO_3, 100 mM $NaHCO_3$, 7.0 mM Na_2CO_3, 7.0 mM $NaNO_3$, 0.6 mM KI, and 0.2 mM Na_2SO_3 at pH 9.0. The ferrous lake conditions were adapted from Ranjan et al.[17] and Hao et al.[32] and included 0.1 mM $K_4[Fe(CN)_6]$, 0.15 mM KBr, 0.021 mM Na_2SO_4, 0.2 mM NaCl, 0.23 mM $CaCO_3$, 0.19 mM $MgCl_2$, 0.19 mM Na_2SiO_3, 1.0 mM $NaHCO_3$, and 100 nM Na_2CO_3 at pH 6.3. For the heat degradation experiments, 200 μL of 5 mM peptide solution was placed in glass vials that were preheated at 150 °C in a Corning LSE digital dry bath heater and incubated. For reactions in solution at 40, 60, 80, and 100 °C, the same conditions as per the 150 °C experiments were used, but the glass vials were capped and sealed to avoid evaporation. The glass vials were then removed from the heat block and cooled to room temperature. All steps were carried-out under anoxic conditions inside a Genesis 2P glovebox system (Vacuum Atmospheres Company) with O_2 < 1 ppm. Lyophilized samples were obtained by flash freezing the solutions in liquid nitrogen followed by freeze drying in a 2.5 L Labconco FreeZone Freeze Dryer at −84 °C. Before chromatographic analysis, dried samples were thoroughly resuspended in 200 μL of degassed ultrapure water (Synergy UV water purification system, Merck, Darmstadt, Germany). Only for the carbonate lake samples, 25 μL of 12 M HCl was also added before chromatographic analysis. Aliquots (2.5 μL) were either analyzed with a Shimadzu high-performance liquid chromatography (HPLC) system (CBM-20 A, binary pump LC-20AB) or an Agilent 1260II HPLC with an Infinity iQ mass selective detector. Runs on the Shimadzu system used a Kinetex C18 column (100 Å pore size, 4.6 mm inner diameter, 2.6 μm particle size, and 25 cm length, Phenomenex). The mobile phase was composed of solvent A [0.1% trifluoroacetic acid (TFA) in water] and solvent B (0.1% TFA in acetonitrile) and a gradient from 1 to 20% (v/v) solvent B in 20 min. Data were acquired with LabSolutions LC software. Runs on the Agilent system used a Zorbax SB-Aq column (80 Å pore size, 4.6 mm inner diameter, 1.8 μm particle size, and 15 cm length, Agilent) and a gradient from 100% solvent A for 6 min to 100% solvent B in 20 min. Data were acquired with OpenLab 2.5 software. MS measurements were carried out in electrospray ionization (ESI) positive ion mode. Half-life values were obtained by fitting the data to a delayed one-phase decay model [equation: $y = (y_0 - p)e^{(-k(x - x_0))} + p$] with GraphPad Prism, version 6.00 (GraphPad Software, La Jolla, CA, U.S.A.), where y_0 is the survival at t_0, p is the survival at infinite time, x_0 is the time at

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which the decay begins, and k is the rate constant. All experiments were in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.2c00042.

LC−MS of GCG degradation, effect of pH, degradation in solution, control experiments, and schematic of β-elimination of the thiol moiety (PDF)

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Notes

The authors declare no competing financial interest.

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