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RESEARCH ARTICLE

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Ergothioneine in a peptide: Substitution of histidine with 2-thiohistidine in bioactive peptides

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Ergothioneine (EGT) is the betaine of 2-thiohistidine (2-thioHis) and may be the last undiscovered vitamin. EGT cannot be incorporated into a peptide because the α-nitrogen is trimethylated, although this would be advantageous as an EGT-like moiety in a peptide would impart unique antioxidant and metal chelation properties. The amino acid 2-thioHis is an analogue of EGT and can be incorporated into a peptide, although there is only one reported occurrence of this in the literature. A likely reason is the harsh conditions reported for protection of the thione, with similarly harsh conditions used in order to achieve deprotection after synthesis. Here, we report a novel strategy for the incorporation of 2-thioHis into peptides in which we decided to leave the thione unprotected. This decision was based upon the reported low reactivity of EGT with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a very electrophilic disulfide. This strategy was successful, and we report here the synthesis of 2-thioHis analogues of carnosine (βAH), GHK-tripeptide, and HGPLGPL. Each of these peptides contain a histidine (His) residue and possesses biological activity. Our results show that substitution of His with 2-thioHis imparts strong antioxidant, radical scavenging, and copper binding properties to the peptide. Notably, we found that the 2-thioHis analogue of GHK-tripeptide was able to completely quench the hydroxyl and ABTS radicals in our assays, and its antioxidant capacity was significantly greater than would be expected based on the antioxidant capacity of free 2-thioHis. Our work makes possible greater future use of 2-thioHis in peptides.

KEYWORDS
2-thio, histidine, antioxidant, antioxidant peptide, carnosine, copper binding, ergothioneine, GHK-tripeptide, histidine

Abbreviations: 2-thioHis, 2-thiohistidine; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Cu (II), copper (II); Cys, cysteine; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGT, Ergothioneine; EPR, electron paramagnetic resonance; EtCA, ethyl (hydroxyimino)cyanoacetate; Fmoc, fluorenylmethoxycarbonyl; Fmoc-OSu, Fmoc *N*-hydroxysuccinimide ester; HATU, 1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; His, histidine; HPLC, high-pressure liquid chromatography; K_A, equilibrium association constant; K_D, equilibrium dissociation constant; Meb, 4-methylbenzyl; MS, mass spectrometry or mass spectrometric; NMM, *N*-methylmorpholine; OH, hydroxyl radical; ROS, reactive oxygen species; RSA, radical scavenging activity; SPPS, solid-phase peptide synthesis; TEA, triethylamine; TEAC, Trolox equivalent antioxidant capacity; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TLC, thin-layer chromatography; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UV–Vis, ultraviolet–visible; Asc, ascorbate; βA, β-alanine; H-βAH-OH or βAH, carnosine.

1 | INTRODUCTION

Ergothioneine (EGT) is the trimethylated betaine of 2-thiohistidine (2-thioHis) and may be the last undiscovered vitamin.[1–7] EGT is produced by fungi and mycobacteria and has been shown to be a potent antioxidant compound in vitro, but its precise biological function is unknown.[1–7] Humans and other animals obtain EGT by ingesting food and accumulate it in target tissues such as red blood cells, by the use of a specific cation transporter.[1–8] EGT also chelates metals, especially Cu (II), and prevents the metal from undergoing redox cycling reactions that generate reactive oxygen species (ROS) that damage biological macromolecules.[1,2,6,9]

EGT cannot be incorporated into a peptide because the α-nitrogen is trimethylated (Figure 1), although incorporation of an EGT-like moiety into a peptide would impart unique antioxidant and metal chelation properties to the peptide. This goal could be achieved by incorporating 2-thioHis, an EGT analogue, into a peptide and is possible because 2-thioHis has both α-amino and α-carboxylic acid groups that enable coupling to other amino acids. An advantage of such an approach is that peptides enable precise targeting to specific tissues and organelles and have defined hydrophobicity.

We are aware of only one other reported instance of incorporating 2-thioHis into a peptide.[10] The previous work used a 4-methylbenzyl (Meb) protecting group to protect the thione using harsh conditions and then similarly harsh conditions to affect deprotection.[10] Due to the reported low reactivity of the thione of EGT towards thiol/disulfide exchange, we decided to take the novel approach of not using any protecting group for the thione.[3,11] Such an approach easily enables the advantageous incorporation of 2-thioHis in place of His. Examples of bioactive His-containing peptides include carnosine, GHK-tripeptide, GHTD-amide, histatins, and collagen-like peptides, some of which are depicted in Figure 2.[12–22]

In this report, we synthesized the 2-thioHis analogs of bioactive peptides H-HGPLGPL-OH, H-GHK-OH, and H-βAH-OH (carnosine). These His-containing peptides were chosen as targets because it has been reported that they each have antioxidant and/or metal chelating properties that could be enhanced by substitution with 2-thioHis.[14–22]

We measured their antioxidant capacity by measuring their ability to scavenge hydroxyl radicals and various organic radicals. We also qualitatively determined their ability to bind Cu (II) by using low-temperature electron paramagnetic resonance (EPR) spectroscopy

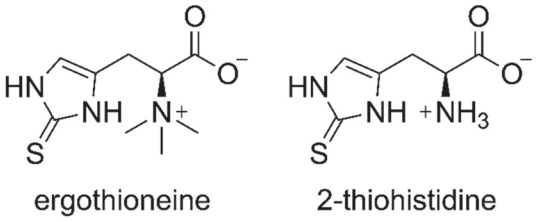

FIGURE 1 Structure of ergothioneine and 2-thiohistidine. Ergothioneine (EGT) is the betaine (contains a trimethylated amine) of 2-thioHis

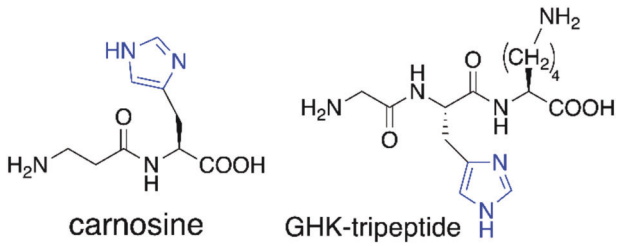

FIGURE 2 Naturally occurring His-containing peptides. Carnosine is a His-containing dipeptide found in the muscle of mammals where it has important antioxidant, metal chelation, and buffering properties. GHK-tripeptide is a matrikine from the extracellular matrix. It has a high affinity for Cu (II) and helps to prevent oxidative stress and is important for wound healing. Each has a His that can be replaced with 2-thioHis

and ultraviolet–visible (UV–Vis) spectroscopy. Our results support our contention that the thione of 2-thioHis does not need to be protected in solid-phase peptide synthesis (SPPS) and that the resulting 2-thioHis-containing peptides have enhanced antioxidant and metal chelation properties.

2 | MATERIALS AND METHODS

2.1 | Materials

Solvents for peptide synthesis were purchased from Fisher Scientific (Pittsburgh, PA). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from TCI America (Portland, Oregon). *N*-Fmoc amino acids were purchased from RSsynthesis (Louisville, KY, USA). 2-chlorotritylchloride resin (100–200 mesh, 1% DVB) was purchased from Novabiochem (St. Louis, MO, USA). L-Carnosine was purchased from Acros Organics (Pittsburgh, PA, USA). 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) was purchased from Oakwood Chemical (Estill, SC, USA). All other chemicals were purchased from either Sigma-Aldrich (Milwaukee, WI), Acros Organics (Pittsburgh, PA, USA), or Fisher Scientific (Pittsburgh, PA, USA). Samples were ionized by electrospray ionization (ESI) on a Thermo Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA). ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded with a Bruker Advance III HD 500 MHz NMR spectrometer. All UV–Vis assays were performed on a Cary 50 UV–Vis spectrophotometer (Varian, Walnut Creek, CA, USA). All EPR experiments were performed on a Bruker EMXplus EPR spectrometer (Billerica, MA). High-pressure liquid chromatography (HPLC) analysis of all samples was performed using a Shimadzu HPLC system with a Symmetry® C18 5-μm column from Waters Corp (Milford, MA, USA) (4.6 × 150 mm). For HPLC analysis, peptides were dissolved in water/HPLC-grade acetonitrile (5:1) to a concentration of 0.5 mM following lyophilization. The aqueous and organic phases were 0.1% trifluoroacetic acid (TFA) in distilled, deionized water (Buffer A) and 0.1% TFA in HPLC-grade acetonitrile (Buffer B), respectively. Beginning with 100% Buffer A,

Buffer B was increased by 1% up to 50% over 50 min with a 1.4 ml/min gradient elution. Buffer B was then increased from 50% to 100% over 10 min. This method was used for analysis of each sample. Peptide elution was monitored via absorbance at both 214 and 254 nm.

2.2 | Synthesis of L-2-thio-histidine

L-2-Thiohistidine was synthesized according to the procedure from Erdelmeier et al.[23] This reaction works best when performed on 10 g or higher scale. Histidine (14 g, 66.8 mmol, 1.0 eq.) was dissolved in 134 ml of deionized water. After the His was fully dissolved, this solution was cooled in an ice bath at 0°C. Once the reaction was cooled, bromine (4.45 ml, 86.8 mmol, 1.3 eq.) was added resulting in a bright orange solution. After 6 min, Cys (24.3 g, 200.4 mmol, 3.0 eq.) was added to the reaction. The solution was stirred at 0°C for 1 h. An oil bath was preheated to 95°C. After 1 h, 3-mercaptopropionic acid (34.9 ml, 400.7 mmol, 6.0 eq.) was added to the reaction, and the reaction was transferred to the oil bath at 95°C. A condenser was attached to the reaction, and the reaction was stirred for 18 h at 95°C, after which the reaction had turned dark brown. The reaction was removed from the oil bath and condenser and allowed to cool to room temperature. The aqueous solution was then extracted with ethyl acetate. The aqueous layer remained dark brown after extraction. The aqueous layer was transferred to a clean flask and placed in an oil bath preheated to 40°C. The pH of the solution was adjusted to 6.5 with 30% ammonia hydroxide to precipitate 2-thioHis. The reaction was chilled to allow complete precipitation. The off-white precipitate was filtered out of the reaction and washed with cold deionized water and ethanol. The precipitate was dried under high vacuum to give 5.04 g (26.9 mmol) of an off-white powder. The percent yield of this reaction was 40% which is consistent with the findings of Erdelmeier et al.[23] Mass spectrometric (MS) analysis revealed a peak at 188.1 *m/z*. ^{1}H-NMR (D_2O/DCl): δ 3.06–3.20 (2H, (3.06 dd), (3.20 dd)), 4.21 (1H, dd), 6.79 (1H, s); ^{13}C-NMR (D_2O/DCl): δ 25.32, 51.82, 115.96, 123.23, 156.49, 170.38.

2.3 | Addition of Fmoc protecting group to L-2-thioHis

N-Fmoc-L-2-thioHis was prepared using a standard procedure for the addition of fluorenylmethoxycarbonyl (Fmoc) protecting groups to amino acids. In a 250-ml round-bottom flask, 2-thioHis (1.0 g) was added to 5–10 ml of deionized water to create a slurry. Triethylamine (TEA) (750 μl, 5.35 mmol, 1.0 eq.) was added to the amino acid slurry, and the reaction was stirred at room temperature. Fmoc *N*-hydroxysuccinimide ester (Fmoc-OSu) (1.99 g, 5.89 mmol, 1.1 eq.) was dissolved in 20–30 ml of acetonitrile and added to the amino acid slurry. A second eq. of TEA (750 μl) was added to the reaction along with acetonitrile and water to completely dissolve the 2-thioHis. The reaction was stirred for 2 h at room temperature and monitored by thin-layer chromatography (TLC). The reaction was quenched by acidifying with 20 ml of 1 *N* HCl. The reaction was extracted three times with ethyl acetate followed by a back extraction of the ethyl acetate layer with water, 1 *N* HCl, and brine (1:1:1). The ethyl acetate solution was then dried with $MgSO_4$, filtered with a Büchner funnel, and roto-evaporated to dryness. The oil was dissolved in 10–20 ml of ethyl acetate with 1–2 ml of methanol. The addition of hexanes precipitated the *N*-Fmoc-2-thioHis derivative as a cream colored solid. The solid was purified by redissolving it in 10–20 ml of warm ethyl acetate and 1–2 ml of methanol, filtering the solution through a Büchner funnel, and then reprecipitating the product with cold hexanes. The product was dried under high vacuum and used without further purification. MS analysis showed a dominant peak at 410 *m/z* for the product as well as smaller peaks for the Na^+ adduct (M + 23) at *m/z* 432 and the K^+ adduct (M + 39) at *m/z* 448. ^{1}H-NMR (MeOD): δ 2.88 (dd, 1H), 3.08 (dd, 1H), 4.22 (t, 1H), 4.35 (d, 2H), 4.43 (dd, 1H), 6.60 (s, 1H), 7.32 (t, 2H), 7.40 (t, 2H), 7.65 (d, 2H), 7.80 (d, 2H); ^{13}C-NMR (MeOD) δ 26.77, 53.06, 66.61, 119.49, 124.81, 126.78, 127.38, 141.16, 143.76, 143.83, 157.00, and 172.87. An average yield was 82.5%. We note that that the MS of one of the reactions showed either a mixed disulfide between Fmoc-2-thioHis and 2-thioHis or a symmetrical disulfide with Fmoc-2-thioHis (Figure S1). This disulfide could be eliminated by redissolving the product in ethyl acetate and extracting with 10-mM ascorbate (Asc), pH 4.25 (Figure S1). If this step was necessary, the yield was reduced significantly. The disulfide was only observed when addition of the Fmoc group to 2-thioHis did not go to completion.

2.4 | Peptide synthesis

All His-containing peptides (H-βAH-OH, H-HGPLGPL-OH, and H-GHK-OH) were synthesized according to standard SPPS on a 0.1-mmol scale using a glass vessel shaken with a model 75 Burrell wrist action shaker. We used 300 mg of 2-chlorotrityl chloride resin (100–200 mesh, Chem-Impex) for each peptide. This resin was swelled in dichloromethane (DCM) for 20 min. The first amino acid was directly coupled to the resin using *N*-methylmorpholine (NMM)/DCM (2:98), shaking for 1 h. The resin was then capped using DCM/methanol/NMM (8:1:1). Subsequent amino acids were coupled using 0.2 mmol of Fmoc-protected amino acid, 0.2 mmol of HATU, and 1.8 mmol NMM in dimethylformamide (DMF), shaking for 1 h. Between amino acid couplings, the Fmoc-protecting group was removed by two 10-min washes with piperidine/DMF (2:8). The success of Fmoc removal and amino acid couplings were monitored qualitatively using a ninhydrin test.[24] Peptides were cleaved from the resin with a cleavage cocktail consisting of TFA/triisopropylsilane (TIS)/water (96:2:2) for 1.5 h. Following cleavage, the resin was washed with DCM, and the volume of the cleavage solution was reduced by evaporation with argon gas. Each peptide was precipitated with cold, anhydrous ether. Centrifugation at 3000 rpm on a clinical centrifuge (International Equipment Co, Boston, MA, USA) for 5 min pelleted the peptide. Peptides were dried under argon gas, then dissolved in a minimal amount of water/HPLC-grade acetonitrile (5:1), lyophilized, and used without further purification.

The 2-thioHis-containing peptides (H-βAthioH-OH [thioHcarnosine], H-thioHGPLGPL-OH, and H-GthioHK-OH) were synthesized with a similar protocol prior to coupling of 2-thioHis. For the coupling of 2-thioHis, 0.12 mmol of Fmoc-2-thioHis, 0.12 mmol of ethyl (hydroxyimino)cyanoacetate (EtCA), and 0.36 mmol of diisopropylcarbodiimide (DIC) was dissolved in DMF, added to the resin and shaken for 2 h. Double couplings were typically performed. When 2-thioHis was the first amino acid coupled to the resin, as was the case for H-βAthioH-OH, coupling was performed with 0.11 mmol of Fmoc-2-thioHis and 1.8 mmol NMM in DCM for 1 h. All subsequent couplings after 2-thioHis were performed with 0.2 mmol of Fmoc-protected amino acid, 0.2 mmol ETCA, and 0.6 mmol DIC in DMF for 1 h. The rest of the procedure is identical to the one used for the His-containing peptides. We note that the peptides containing 2-thioHis had a yellow color upon precipitation in ether and after lyophilization, while the His-containing peptides were white.

2.5 | Measurement of 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) activity assays were conducted according to the procedures outlined by Liu et al. and You et al. with some modifications.[25,26] A 1-mM stock solution of DPPH was prepared by dissolving DPPH in 95% ethanol. Peptide stock solutions were prepared at a concentration of 5 mM in 100 mM potassium phosphate buffer, pH 7.0 for each of the His- and 2-thioHis-containing peptides. 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was used as a standard for antioxidant activity and a positive control. A 5-mM solution of Trolox in 100-mM potassium phosphate, pH 7.0 was prepared. A blank was prepared by mixing 600 μl of 95% ethanol with 400 μl of 100-mM potassium phosphate, pH 7.0 in a cuvette. This solution was used to blank the spectrophotometer from 200–800 nm. In each sample, a 60:40 ratio of ethanol:buffer was maintained. To test the ability of Trolox and the different peptides to reduce DPPH, 50-μM DPPH was combined with different concentrations of peptide or Trolox ranging from 5 to 500 μM and the total volume of each sample was adjusted to 1 ml with buffer and ethanol. The samples were allowed to incubate at room temperature in the dark for 15 min, after which each sample was scanned and changes in the absorbance at 517 nm were monitored. Trials were run in triplicate for each of the peptides and Trolox. For every trial, one sample was prepared with only 50-μM DPPH (no peptide or Trolox) to serve as a control. The percentage of inhibition of DPPH oxidation was calculated using Equation 1:

$$DPPH\ scavenging\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the control (DPPH and buffer only) and A_{sample} is the absorbance at 517 nm of the test peptide/standard. A Trolox calibration curve was prepared with concentrations ranging from 5–50 μM (Figure S2). Similar curves were prepared for each of the peptides (Figure S2). These curves were used to calculate Trolox equivalent antioxidant capacity (TEAC) values for each of the peptides by calculating the ratio of peptide to Trolox to achieve the same percent reduction of DPPH.

2.6 | Measurement of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)] radical scavenging activity

2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate(6)] (ABTS) scavenging assays were performed according to previously published protocols with some modifications.[25,27,28] A 10-mM stock solution of potassium persulfate was prepared in deionized water. A stock solution of ABTS was prepared by dissolving 7-mM ABTS and 2.45-mM potassium persulfate in deionized water. This solution was allowed to incubate at room temperature in the dark for 12–16 h to allow potassium persulfate to oxidize the ABTS. Following oxidation, the solution was a dark blue/green color. This solution was stable in the dark at room temperature for at least 48 h. This solution was diluted with 100-mM phosphate buffered saline, pH 7.2 so that the final absorbance was 0.70 ± 0.02 at 734 nm. Stock solutions for all peptides and for Trolox were prepared in a range from 0.1 mM to 3.2 mM in 100-mM phosphate buffered saline, pH 7.2. To test the ability of Trolox and peptides to reduce the ABTS radical cation, samples were prepared with 990 μl of the diluted ABTS solution and 10 μl of each of the peptide or Trolox stock solutions. For every trial, one sample was prepared with 990-μl ABTS and 10-μl of buffer. The samples were allowed to incubate at room temperature in the dark for 10 min. The spectrophotometer was blanked with 1 ml of 100-mM phosphate buffered saline, pH 7.2 from 200–800 nm. Scans were taken for each of the samples and the change in absorbance at 734 nm was monitored. Every sample was run in triplicate and the results were averaged. The percentage of inhibition of ABTS oxidation was calculated using Equation 2.

$$ABTS\ scavenging\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

where $A_{control}$ is the absorbance of the control (ABTS and buffer only) and A_{sample} is the absorbance at 734 nm of the test peptide/standard. A Trolox calibration curve was prepared with concentrations ranging from 1–32 μM (Figure S3). Similar curves were prepared for each of the peptides (Figure S3). These curves were used to calculate TEAC values for each of the peptides by calculating the ratio of peptide to Trolox to achieve the same percent reduction of ABTS.

2.7 | Measurement of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity (RSA) of peptides was measured with room temperature EPR spectroscopy according to the procedure of Nazeer et al. with some modifications.[29] Hydroxyl radicals were

generated by the iron-catalyzed Fenton Haber–Weiss reaction, and the generated hydroxyl radicals were reacted with the nitrone spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO).[30] The resultant DMPO-OH adducts were detectable with EPR spectroscopy. The peptide (1-mM final concentration) was added to a solution containing: 24mM DMPO, 0.8-mM $FeSO_4$, and 0.8-mM H_2O_2 in 100-mM potassium phosphate buffer, pH 7.4 in a final reaction volume of 250 μl. This solution was immediately transferred into a 50-μl quartz capillary tube. After 5 min, the EPR spectrum was recorded using a Bruker X-band EPR spectrometer operating at 9.42 GHz. The experimental conditions employed were as follows: magnetic field, 336.5 ± 10 mT; power, 2 mW; modulation amplitude, 2.00 G; time constant, 327.68 ms, modulation frequency, 100 kHz; receiver gain, 30-dB sweep time, 60 s. Trials were performed in triplicate for each peptide. The double integral of the EPR spectra was obtained and used to determine the signal intensity for each sample. The hydroxyl radical scavenging ability was calculated using Equation 3, in which H and H_0 are relative peak height of radical signals with and without sample, respectively.

$$\textit{Hydroxyl radical scavenging}\ (\%) = \frac{1-H}{H_0} \times 100 \qquad (3)$$

2.8 | Spectrophotometric determination of Cu (II) binding constants (K_D)

We attempted to quantify the Cu (II) binding constants for carnosine and thioHcarnosine by titrating each peptide with Cu (II) and monitoring changes in the absorbance spectra. To detect formation of the Cu (II) complex, 10-mM carnosine in 50-mM MOPS buffer, pH 7.4 was titrated with $CuCl_2$ at concentrations ranging from 1–25mM, whereas 100-μM thioHcarnosine in 50-mM MOPS buffer, pH 7.4 was titrated with $CuCl_2$ at concentrations ranging from 5–250 μM. The thioHcarnosine/Cu (II) solution was allowed to incubate at room temperature for 40 min before absorbance spectra were recorded to ensure complete formation of the Cu (II) complex. Both peptides had a unique wavelength of maximum absorbance for the Cu (II) complex: thioHcarnosine at 260 nm and carnosine at 630 nm. The K_A values were calculated by fitting the resulting titration data to the appropriate equation describing the association constant.[31] The K_D values were calculated from the reciprocal of the K_A value for each peptide.

2.9 | Low-temperature EPR studies

The ability of each peptide to bind Cu (II) was determined by low-temperature (95°K) EPR spectroscopy using the methods from Zhu et al. and Motohashi et al. with some modifications.[9,32] Samples of 200- and 400-μM peptide were incubated for 10 min at room temperature with 200-μM $CuCl_2$ in 100-mM potassium phosphate, pH 7.4. Following incubation, aliquots of each sample were transferred into 4-mm inner diameter quartz EPR tubes (Wilmad-LabGlass). The samples were frozen in liquid nitrogen and stored in liquid nitrogen prior to use. Control samples were prepared with only buffer and with 200-μM $CuCl_2$ in potassium phosphate buffer. Low temperature EPR spectra were gathered with a Bruker EPR spectrometer operating at 9.42 GHz with the following parameters: scan range, 6000 G; field set, 3200 G; time constant, 164 ms; scan time, 60 s; modulation amplitude, 7.000 G; modulation frequency, 100 kHz; receiver gain, 30 dB; microwave power, 20 mW; and number of scans, 5.

3 | RESULTS AND DISCUSSION

3.1 | Past use of 2-thioHis in peptides

As mentioned in the Introduction, 2-thioHis has only been incorporated into a peptide one time in the entire history of peptide science. While lack of commercial availability is certainly one cause for its dearth of use in peptides, it can be readily synthesized from histidine (His) and cysteine (Cys) via a labile His-bromolactone intermediate on a gram scale.[23] We believe the problem of protection/deprotection of the thione reported by Maggiora et al. has created a barrier to entry for many peptide chemists such that 2-thioHis has not been incorporated into a peptide since their initial report (31 years).[10]

In order to achieve protection of the thione, Maggiora et al. used harsh conditions consisting of sodium dissolved in liquid ammonia followed by addition of α-bromo-*p*-xylene to install the Meb group on the sulfur.[10] The Boc-2-thioHis (Meb) derivative was then incorporated into a peptide using Merrifield SPPS. After synthesis of the peptide was complete, deprotection was achieved using an excess of monohalogenated alkanes such as bromomethane or excess bis-halogenated alkanes in sodium/liquid ammonia to yield various mono-alkylated and bisalkylated products (Figure 3).[10] Of note, they were able to create a unique disulfide mimic by connecting two 2-thioHis residues with an alkyl linker by addition of 1,6-dibromohexane in the sodium/liquid ammonia reaction.[10]

We decided to attempt incorporation of 2-thioHis into a peptide without protecting the thione due to the reported low reactivity of EGT with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a highly reactive disulfide.[3,11] The thione of 2-thioHis (like EGT) is in equilibrium with the thiol form, with the thione being the dominant form in solution.[1–7] We reasoned that the thione form is even more favored in DMF compared to water and that the low 2-electron nucleophilicity of the thione would render it unreactive with the carbodiimide of DIC given its lack of reactivity with DTNB. If true, this would remove a large barrier to using 2-thioHis in peptide synthesis.

3.2 | Synthesis and analysis of 2-thioHis-containing peptides

Our results show that we were able to successfully insert 2-thioHis into the three target peptides in place of His using standard Fmoc SPPS techniques with carbodiimide-mediated coupling. The three

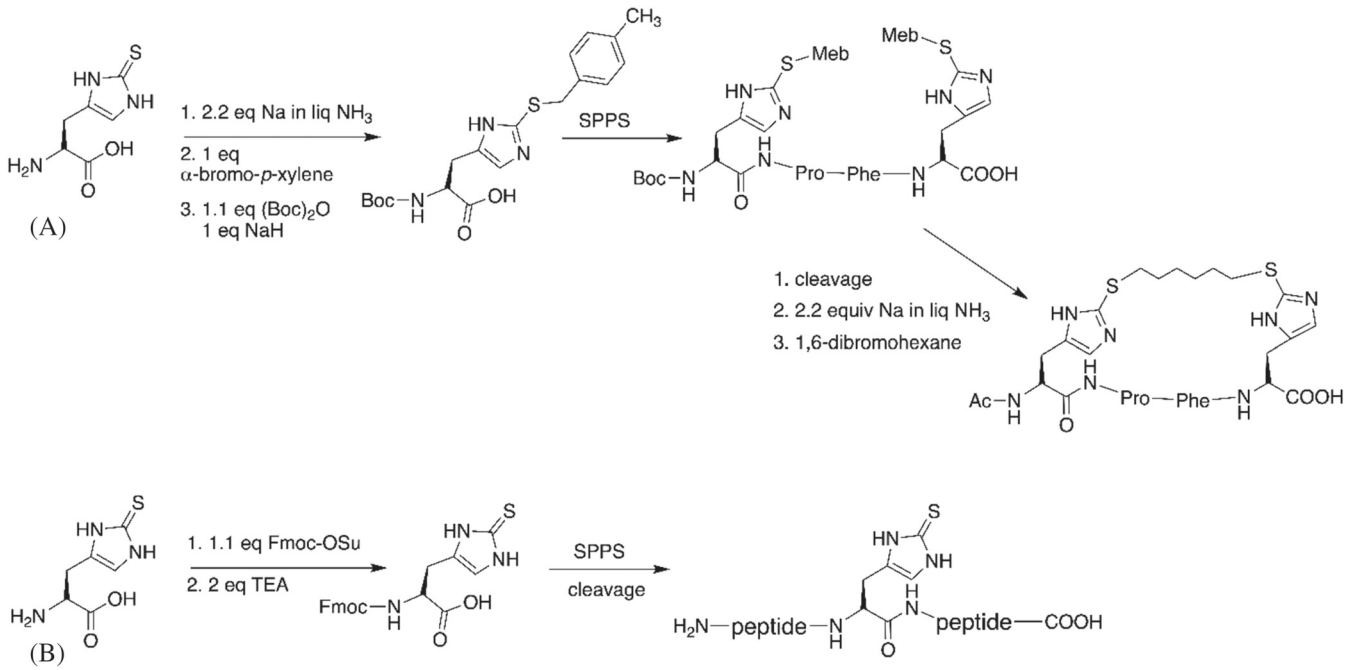

FIGURE 3 Inserting 2-thioHis into a peptide. (A) Method used by Maggiora et al.[10] to protect 2-thioHis before insertion into a peptide, followed by deprotection in the presence of linker. (B) Our simplified method eliminates protection of 2-thioHis prior to insertion into a peptide

peptides we chose place 2-thioHis as the first amino acid coupled (thioHcarnosine), the middle amino acid coupled (GthioHK), or the last amino acid coupled (thioHGPLGPL). The yield of each crude peptide after precipitation into ether and lyophilization was 90% or greater.

HPLC analysis of the 2-thioHis-containing peptides shows that the purity of the GthioHK and thioHcarnosine peptides were nearly identical to their His-containing counterparts (Figure 4A–D). We note that 2-thioHis-containing peptides are readily identified in the analytical HPLC due to their strong absorbance at 254 nm. The imidazothione of 2-thioHis has a λ_{max} at 257 nm with an extinction coefficient 14,000 M^{-1} cm^{-1}.[5] In the case of peptide thioHGPLGPL, we observed an unidentified peak that eluted very late in the gradient. The main peak corresponding to thioHGPLGPL is clearly identified in the chromatogram due to the very strong absorbance at 254 nm (Figure 4F). The unidentified peak has a much less intense absorbance at 254 nm. It is possible that this unidentified peak is a significant impurity due to a side-reaction or incomplete coupling. However, another possibility is that this peak is the disulfide form of the peptide. A disulfide can form between two 2-thioHis residues as we observed during the synthesis of Fmoc-2-thioHis as we indicated in the Methods section. We synthesized this peptide several times, and this peak was always present. To investigate the possibility that this was the disulfide, we incubated the sample with 1-mM Asc at pH 4.5 for 1 h and then injected the reaction onto the column. The result showed that the unidentified peak disappeared (Figure S4). This is strong evidence that this peptide formed a disulfide between two monomers following precipitation into ether and dissolution into water prior to lyophilization. In fact, the MS analysis of each peptide shows evidence for disulfide formation. The MS analysis of each peptide in Figure 4 is given in Figure S5. The disulfide bond between two 2-thioHis residues must be very weak because Asc was able to reduce it. We did not utilize the fact that disulfide bond formation is possible between 2-thioHis residues, but this should make for an interesting future application.

3.3 | Antioxidant activity of 2-thioHis-containing peptides

The His- and 2-thioHis containing peptides, as well as 2-thioHis, were tested for their ability to scavenge DPPH and ABTS. Trolox, a water-soluble vitamin E analogue, was used as a standard for the ABTS and DPPH assays. The reduction of DPPH and ABTS was determined by monitoring the decrease in absorbance at 517 and 734 nm, respectively, after the addition of peptide or standard. The His-containing peptides all showed little or no ability to scavenge DPPH or ABTS radicals at the concentration of peptides chosen here. We note that carnosine and HGPLGPL have been reported to scavenge the DPPH radical, but at higher concentrations than we tested.[14,33] Peptide GHK was the strongest antioxidant peptide tested of the His-containing peptides and caused only a slight decrease in the DPPH absorbance peak at 517 nm and no decrease in the ABTS absorbance peak at 734 nm (Figure 5). In contrast to the His-containing peptides, all of the 2-thioHis-containing peptides showed strong RSA with both DPPH and ABTS radicals. Peptide GthioHK was the strongest antioxidant peptide tested overall with the ability to completely reduce the DPPH and ABTS radicals (Figure 5). The same absorbance spectra for the other peptides in this study are given in Figures S6 and S7.

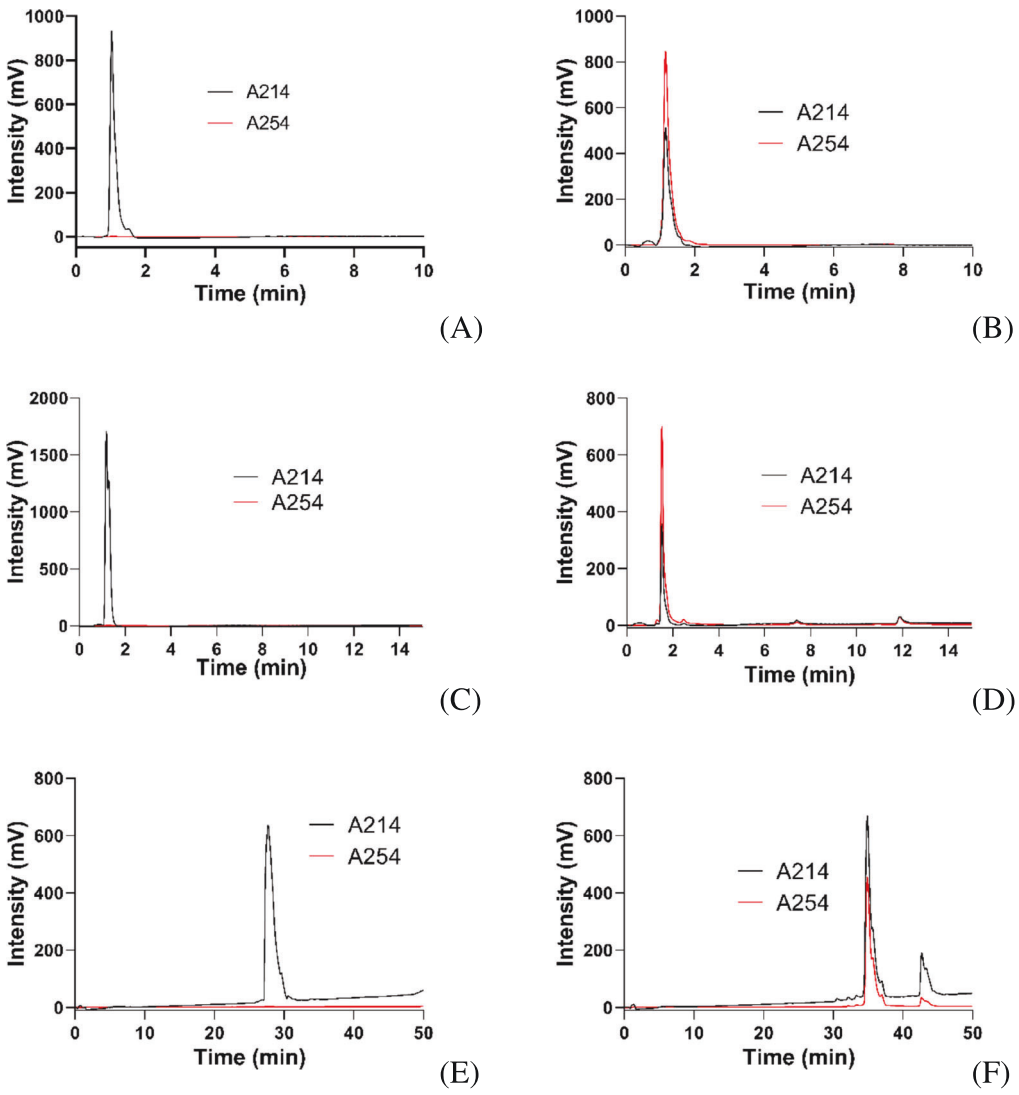

FIGURE 4 Analytical high-pressure liquid chromatography (HPLC) analysis of His- and thioHis-containing peptides in this study. (A) GHK. (B) GthioHK. (C) Carnosine. (D) ThioHcarnosine. (E) HGPLGPL. (F) ThioHGPLGPL

Next, the His- and 2-thioHis containing peptides, as well as 2-thioHis, were tested for their ability to scavenge •OH. Asc was used as a standard for •OH scavenging activity, with 1-mM Asc completely reducing the hydroxyl radical. The reduction of •OH was measured using EPR spectroscopy. The Fenton Haber–Weiss reaction was used to produce •OH in the presence of peptide and the spin-trapping reagent DMPO.[30] The RSA was calculated by measuring the decrease in peak height upon addition of 1-mM peptide (final concentration) compared with the control with no peptide.[29] All RSA values represent the average of three or more trials. Each of the peptides tested showed ability to scavenge •OH, although the 2-thioHis-containing peptides showed improved activity over their His-containing counterparts. GthioHK had the highest RSA of all compounds tested. (Figure 6, Table 1).

In comparing the •OH scavenging ability of His with 2-thioHis, we found an increase of 7.5% (Table 1). We could therefore expect a similar increasing in •OH scavenging ability when substituting 2-thioHis for His in various peptides. However, we found that this substitution produced a synergistic effect for all of the peptides studied here, with the increase in •OH scavenging ability ranging from a 12% to 13% increase for peptides thioHGPLGPL and thioHcarnosine, respectively, to a dramatic 31.6% increase for GthioHK (Table 1). It is interesting to note that in the case of thioHGPLGPL, the •OH scavenging ability is equivalent to 2-thioHis itself, while for thioHcarnosine, the •OH scavenging ability is less than 2-thioHis. Carnosine itself was not a good •OH scavenger in comparison to the other peptides tested.

Substitution of His with 2-thioHis in peptide GHK produced the most dramatic and interesting result as is clearly evidenced by the EPR spectra shown in Figure 6. The GthioHK peptide showed a similar RSA as Asc, with 1-mM GthioHK nearly completely abolishing the signal. Clearly, the 2-thioHis residue, in the context of the amino acid sequence of the peptide, produces an antioxidant that is much better than expected in comparison with the difference between the

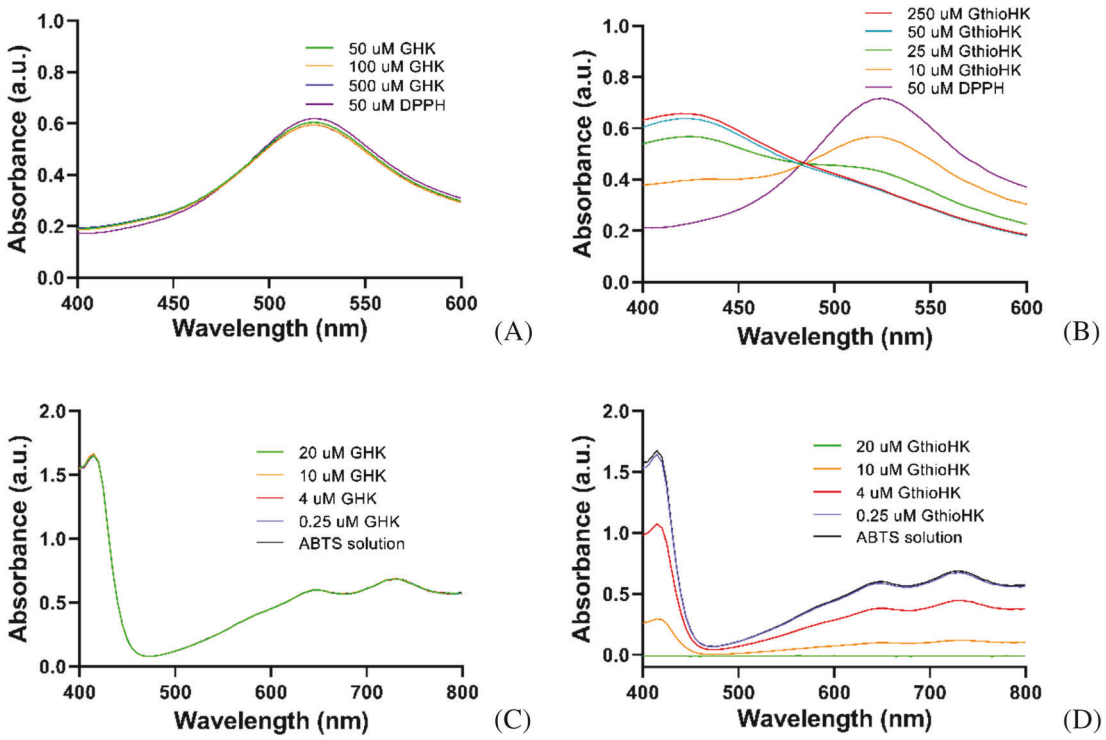

FIGURE 5 Radical scavenging ability of peptides against DPPH and ABTS. (A) Absorbance spectrum for 50-μM DPPH with different concentrations of GHK peptide. (B) Absorbance spectrum for 50-μM DPPH with different concentrations of GthioHK peptide. (C) Absorbance spectrum for ABTS solution with different concentrations of GHK peptide. (D) Absorbance spectrum for ABTS solution with different concentrations of GthioHK peptide

ability of His and 2-thioHis to scavenge •OH. Our data demonstrate that GHK itself is a good antioxidant, which agrees with other studies.[18,20,21] The naturally occurring *matrikine* peptide GHK and its Cu (II) complex are commonly used in skin care products and cosmeceuticals. The GHK tripeptide has well-studied wound healing and anti-aging properties stemming in part from its ability to block the formation of reactive oxygen and carbonyl species as well as its ability to readily chelate redox active metals such as Cu (II) and Fe (II).[17–21] Studies of the antioxidant capabilities of GHK have demonstrated its strong ability to scavenge •OH in vitro suggesting it may play a role in managing oxidative stress in cells.[21] Our GthioHK analogue could also be potentially used as a therapeutic or in cosmetics due to its enhanced antioxidant properties.

3.4 | Summary of antioxidant activities of 2-thioHis-containing peptides

The antioxidant capacities of all peptides tested were compared by calculating TEAC values for both the ABTS and DPPH assays (Table 1). All TEAC values represent the average from three or more trials. The curves used to determine TEAC values for 2-thioHis peptides are shown in Figures S2 and S3. The His-containing peptides all had TEAC values of approximately 0 and showed no ability to scavenge the ABTS radical at the concentrations tested (Table 1). The 2-thioHis-containing peptides possessed high ABTS RSA, with GthioHK having a slightly higher TEAC value than the other 2-thioHis peptides. All three 2-thioHis-containing peptides were stronger scavengers of the ABTS radical compared to both Trolox and 2-thioHis on its own (Table 1). 2-thioHis had the same ability as Trolox to scavenge the DPPH radical. The His-containing peptides showed no significant ability to scavenge the DPPH radical even at 10:1 peptide: DPPH (Table 1 and Figure S6). In contrast, the 2-thioHis-containing peptides showed significant ability to scavenge the DPPH radical; however, they were less efficient than Trolox and took longer to react with the radical, likely due to steric hindrance (Table 1).[34] Although we did not study the kinetics of the reaction specifically, we noticed an immediate color change of the DPPH solution upon addition of Trolox (change from purple to orange color), while the 2-thioHis-containing peptides took several minutes to achieve the same color change. The ability of the 2-thioHis-containing peptides to scavenge the DPPH radical and ABTS radical indicates that the presence of 2-thioHis imparts strong antioxidant activity to these peptides. In comparison, the His-containing peptides had very weak antioxidant activity in the same assays.

3.5 | Copper binding ability of 2-thioHis-containing peptides

Next, we investigated the stoichiometry and affinity of copper binding of the 2-thioHis-containing peptides in comparison to the

His-containing peptides. The ability of the test peptides to chelate Cu (II) was determined with low temperature (95°K) EPR spectroscopy. The EPR experiments showed that the 2-thioHis-containing peptides could all bind Cu (II) as evidenced by the disappearance of the Cu (II) signature in the EPR spectra and the change in peak shape and position (Figure 7). Comparing the EPR spectra of the 2-thioHis-containing peptides to that of 2-thioHis indicates that the 2-thioHis containing peptides bind Cu (II) in a similar configuration as 2-thioHis (Figure 7). The 2-thioHis-Cu (II) complex peak is almost identical to the peaks for the three 2-thioHis containing peptides indicating that these peptides are binding Cu (II) through the sulfur (Figure 7).[35] In contrast, all of the His-containing peptides have unique EPR spectra *compared to each other*, which indicates differences in Cu (II) binding geometry between the three of them or differences in Cu (II) binding affinity. (Figure 7).

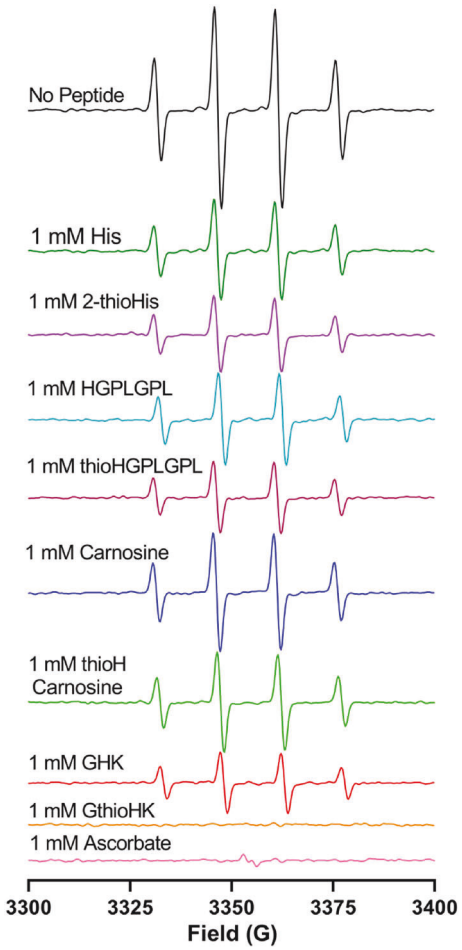

FIGURE 6 Hydroxyl radical scavenging activity of peptides measured by electron paramagnetic resonance (EPR). Hydroxyl radical was generated by a reaction of 0.8-mM $FeSO_4$, with 0.8-mM H_2O_2, pH 7.0 (250 μl). The hydroxyl radicals were trapped with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) prior to obtaining spectra

In addition to Cu (II) binding ability, the EPR data in Figure 8 offers some insight into the equilibrium dissociation constants (K_D) and binding stoichiometry for some of the peptides. For peptide GHK, the K_D for Cu (II) has previously been determined to be 7.0×10^{-14} M using isothermal titration calorimetry with competitive chelators (such as glycine) in different buffer systems.[36] Because GHK has a very high affinity for Cu (II), it can be concluded that in the conditions used for EPR, all of the Cu (II) in solution would be bound to GHK. Based on this information, we can conclude from the EPR spectra that GHK binds to Cu (II) in a 2:1 ratio because at 1:1 GHK:Cu (II) the Cu (II) trace is still visible in the EPR spectrum, but with 2:1 GHK:Cu (II) the Cu (II) trace is almost completely gone (Figure 8). The EPR spectra for GthioHK shows a similar trend indicating that GthioHK, like 2-thioHis and GHK, likely also binds Cu (II) in a 2:1 ratio (Figure 8).

However, the affinity for Cu (II) and binding stoichiometry appears to be significantly different for carnosine in comparison to thioHcarnosine upon comparison of their EPR spectra (Figure 8). The K_A value reported in the literature for carnosine is 1.1 M, which indicates a weak affinity for Cu (II).[37] Carnosine has been reported to bind Cu (II) in a 1:1 ratio which is confirmed in the EPR spectra as the Cu (II) trace is still highly visible at both 1:1 and 2:1 peptide: Cu (II) (Figure 8).[37] Although we cannot make any quantitative determinations of K_A for thioHcarnosine from the EPR spectra, we can

TABLE 1 Antioxidant capacity of His- and 2-thioHis-containing peptides compared with Trolox or ascorbate and 2-thioHis

Analyte or peptide	ABTS radical TEAC	DPPH radical TEAC	Hydroxyl radical RSA[a] (%)
Trolox	1.00	1.00	NA[b]
Asc	NA[b]	NA[b]	100
His	NA[b]	NA[b]	48.5 ± 7.3
2-thioHis	1.50 ± 0.03	1.00 ± 0.02	56.0 ± 2.1
HGPLGPL	ND[c]	ND[c]	46.8 ± 1.3
thioHGPLGPL	2.14 ± 0.02	0.95 ± 0.01	59.7 ± 3.0
GHK	ND[c]	ND[c]	65.4 ± 0.008
GthioHK	2.61 ± 0.01	0.95 ± 0.02	97.0 ± 0.3
Carnosine	ND[c]	ND[c]	31.4 ± 4.2
thioHCarnosine	2.25 ± 0.05	0.80 ± 0.01	43.5 ± 1.1

[a]All analytes were tested at a concentration of 1 mM.
[b]Not applicable because it was not used in the assay.
[c]The activity was too low to be determined, these peptides have TEAC values close to zero and are not comparable to Trolox.

reasonably conclude that it has a higher affinity for Cu (II) than carnosine due to the lack of a strong Cu (II) signal at 1:1 thioHcarnosine:Cu (II) (Figure 8). In addition, we can conclude that unlike carnosine, thioHcarnosine binds Cu (II) with a 2:1 stoichiometry as indicated by the change in the EPR signal as the peptide concentration is increased from 200 to 400 μM while keeping the Cu (II) concentration constant at 200 μM (Figure 8).

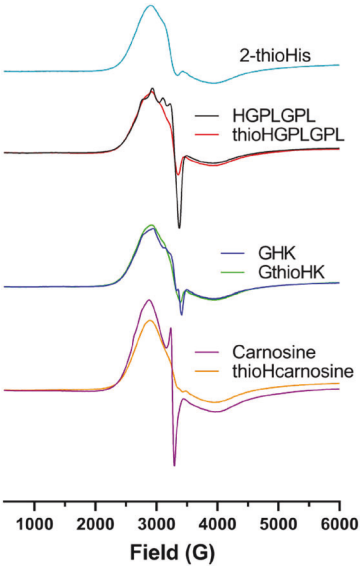

FIGURE 7 Low temperature electron paramagnetic resonance (EPR) spectra of peptide-Cu (II) complexes in potassium phosphate buffer, pH 7.4. All peptides and 2-thioHis are at a concentration of 400- with 200-μM $CuCl_2$

Based on the comparison of EPR spectra of the His and 2-thioHis-containing peptides, we can conclude that substitution of 2-thioHis for His in peptides can change the Cu (II) binding geometry, binding affinity, and binding stoichiometry. The comparison of the 2-thioHis-containing peptides to their His counterparts and free 2-thioHis indicates that these peptides are binding Cu (II) through the sulfur with a geometry similar to 2-thioHis.[35] Using known K_A values for GHK and carnosine, it can also be concluded that insertion of 2-thioHis in place of His resulted in a 2:1 peptide: Cu (II) binding geometry, and in the case of thioHcarnosine, also increased the Cu (II) binding affinity.

We then attempted to quantify K_D for Cu (II) for carnosine and thioHcarnosine via titration of Cu (II) using UV–Vis spectroscopy. As has been reported in the literature, carnosine forms a blue complex when binding Cu (II), which can be detected at 630 nm.[37] We also detected this blue complex upon addition of Cu (II) to carnosine as is shown by the plot of absorbance versus wavelength at various Cu (II) concentrations as shown in Figure 9A. We then made a plot of the change in absorbance at 630 nm versus Cu (II) concentration (Figure S8). The resulting curve was fit to the appropriate equation for a 1:1 association constant.[31] This yields an association constant (K_A)

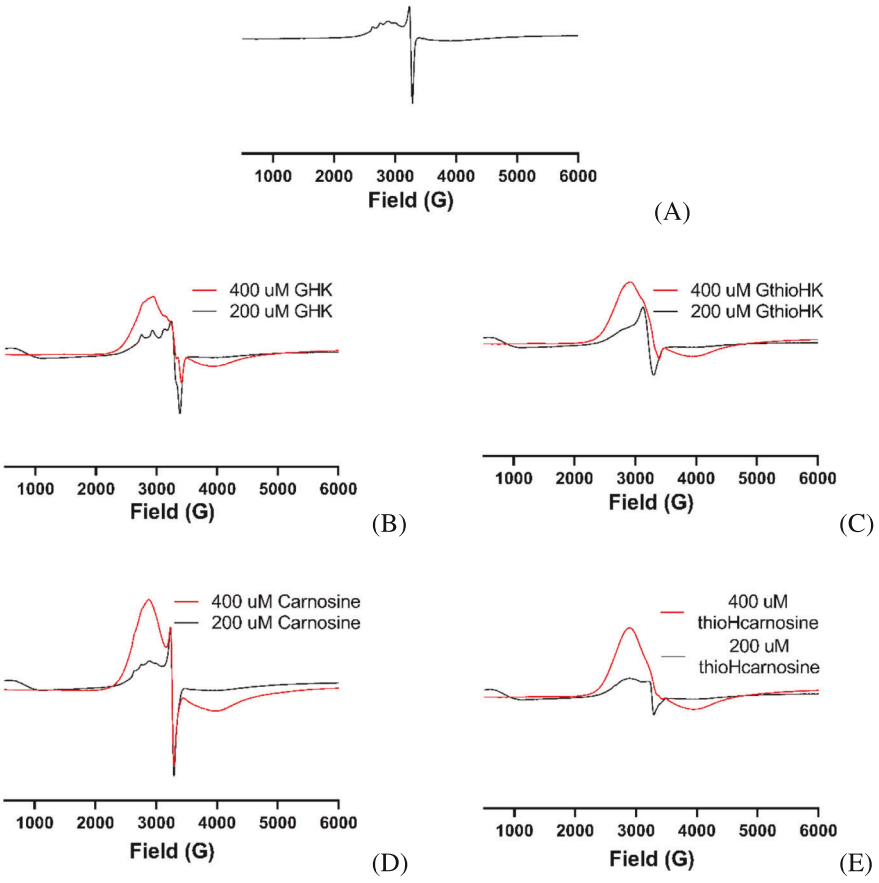

FIGURE 8 Low temperature electron paramagnetic resonance (EPR) spectra of peptide-Cu (II) complexes in potassium phosphate buffer, pH 7.4. (A) 200-μM $CuCl_2$, (B) GHK with 200-μM $CuCl_2$, (C) GthioHK with 200-μM $CuCl_2$, (D) carnosine with 200-μM $CuCl_2$, and (E) thioHcarnosine with 200-μM $CuCl_2$

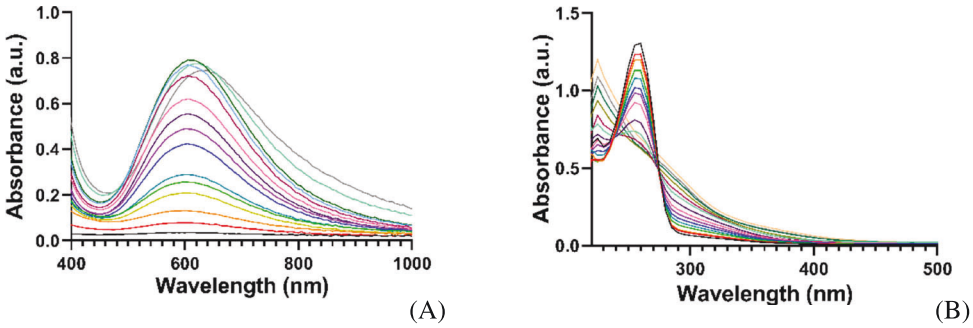

FIGURE 9 Absorbance spectral scan of carnosine and thioHcarnosine as a function of $CuCl_2$ concentration. (A) Absorbance scan of carnosine (10 mM) as $CuCl_2$ is titrated from 0 mM (black line) to 30 mM (light grey line). The absorbance at 610 nm increases as $CuCl_2$ is added to the peptide and then levels off. (B) Absorbance scan of thioHcarnosine (100 μM) as $CuCl_2$ is titrated from 0 μM (black line) to 250 μM (light beige line). The absorbance at 255 nm decreases as $CuCl_2$ is added to the peptide, whereas the absorbance at 220 nm increases. The tailing absorbance at 300 nm also increases as $CuCl_2$ is titrated

of 0.228 M ($K_D = 4.39$ M). This compares to a reported value of 1.1 M.[37] We note that we made our measurement in MOPS buffer at pH 7.4, while the literature value was determined in pure water.[37]

We then performed the same analysis for thioHcarnosine as shown by the absorbance scan shown in Figure 9B. However, we were unable to do the same analysis because of the strong absorbance of the 2-thioHis residue at 255 nm, which limited the concentration of peptide we could analyze by UV–Vis spectroscopy. This led us to perform the experiment with thioHcarnosine on a different scale from carnosine, rendering comparison impossible using this method. However, the EPR data in Figure 8 lead us to believe that thioHcarnosine binds Cu (II) more tightly than carnosine, but we have not quantified this parameter.

4 | CONCLUSIONS

Our decision to leave the thione of 2-thioHis unprotected during SPPS while seemingly unconventional proved to be sound and enabled the facile synthesis of multiple biologically relevant peptides. Our study has shown that the substitution of 2-thioHis for His in bioactive peptides greatly enhances their existing antioxidant properties, equal to that of 2-thioHis, or in some cases much more. An example of this greater enhancement is substitution of 2-thioHis for His in the matrikine GHK-tripeptide, which very strongly quenched both the ABTS and hydroxyl radicals. The therapeutic potential for the GthioHK analogue is high and not yet fully explored. Substitution of His with 2-thioHis also confers the ability to bind Cu (II) to the peptide. Overall, the newfound ability to easily insert 2-thioHis into a peptide opens up a new and unexplored realm of possibilities for peptide chemistry and therapeutics.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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