Supporting Information Material

Materials and methods

Reagents and solvents:

N-α-Fmoc-protected amino acids for peptide synthesis and coupling reagents were obtained from Novabiochem or IrisBiotech. Cu(II) ion source, $Cu(Cl)_2 \cdot 2H_2O$, sodium ascorbate, hydrogen peroxide solution 30 % (w/w) in H_2O, Bathocuproinedisulfonic acid disodium salt (BCS), 3-coumarin carboxylic acid (3-CCA), L-Histidine, POBN spin strap, were purchased from Sigma Aldrich and used without further purification. KH_2PO_4, K_2HPO_4, HEPES (buffer preparation) were purchased from Alfa Aesar. 5,5′-Dimethyl-2,2′dipyridyl (5,5′-DmBipy) and 1,10-Phenantroline (Phen) were a gift from Dr. Romain Ruppert (UMR7177- Institut de Chimie, Strasbourg).

Peptides:

H-DAHK-OH and H-FRHD-OH were purchased from Genecust (Dudelange, Luxemburg). The peptide H-KGHK-NH_2 was synthesized according to the Fmoc/*t*Bu strategy, purified by RP-HPLC and controlled by ESI-MS: observed monoisotopic m/z: $[M+H^+]$= 468.40; calculated monoisotopic m/z: $[M+H^+]$=468.30 .

Stock solutions preparation:

Stock solutions of the peptides KGHK, DAHK, FRHD were prepared by dissolving the powder in to Milli-Q water (resulting pH ~ 2). The concentration of the peptides was estimated by Cu(II) titration in phosphate buffer 50 mM, pH 7.4, monitoring by UV-Vis Spectroscopy the formation of the d-d band for the 1:1 Cu(II)-XZH complex at 525 nm (Fig. S1) ([KGHK] = 12.4 mM, [DAHK] = 7.2 mM, [FRHD] = 10.4 mM).
A stock solution of Histidine (100 mM) was prepared in Milli-Q water; a stock solution of 5,5′-DmBipy (100 mM) and Phen (100 mM) was prepared in 100 % DMSO and then further diluted.
A stock solution of Cu(II) (100 mM) was prepared in Milli-Q water from $CuCl_2 \cdot 2H_2O$ and then further diluted for the different experiments. Its concentration was verified by UV-Vis Spectroscopy from the Cu(II) d-d band at 780 nm (ε = 12 $M^{-1}cm^{-1}$). A stock solution of phosphate buffer (500 mM, pH 7.4) was prepared by mixing potassium dihydrogen phosphate 99 % (KH_2PO_4) with potassium hydrogen phosphate 98 % (K_2HPO_4) in Milli-Q water, adjusting the pH with a 5M solution of NaOH. A stock solution of HEPES buffer (500 mM, pH 7.4) was prepared by dissolving HEPES (free acid) in Milli-Q water, adjusting the pH with a 5 M solution of NaOH. A stock solution of sodium ascorbate (500 mM) and H_2O_2 (500 mM) were freshly prepared daily in Milli-Q water. A stock solution of BCS (50 mM) was prepared in Milli-Q water. A stock solution of the spin-trap POBN (500 mM) was prepared in 500 mM PB, pH 7.4.

Ascorbate Oxidation:

Ascorbate oxidation ($[AscH^-]$ = 100 μM) was monitored by UV-Vis Spectroscopy at λ = 265 nm in 50 mM phosphate buffer, pH 7.4 with or without H_2O_2. After monitoring ascorbate oxidation for 5-10 min, free Cu(II) or the preformed Cu(II) complex was added ([Cu(II)] = 10 μM) and the reaction monitored over time. Kinetics of ascorbate oxidation were performed 3 times at different days with different solutions. Representative measurements are shown in the figures. The molar ascorbate oxidation rate (μM/min) was obtained by dividing the slope of the variation in $AscH^-$ concentration by the extinction coefficient of $AscH^-$ (ε = 14500 $M^{-1}cm^{-1}$).[2] The average values of r_{obs} (μM/min) with standard deviations are reported in Table 1.

Fluorescence detection of •OH with CCA Assay:

Coumarin-3-carboxylic acid (CCA) was used to detect hydroxyl radicals (HO•). HO• generated reacts with CCA to form 7-hydrioxy-coumarin-3-carboxylic acid (7-OH-CCA), which upon excitation at λ = 390 nm emits at λ = 450 nm. To a solution containing 0.5 mM CCA, Cu(II) 25 µM and/or peptide 30 µM (ratio Cu(II):peptide, 1:1.2), and/or H_2O_2 250 µM in phosphate buffer 50 mM, pH 7.4, $AscH^-$ was added to trigger the reaction. The final concentration of $AscH^-$ in the wells was 250 µM (stock solution 25 mM).
Kinetics of HO•generation via fluorescence of 7-OH-CCA were performed at least 3 times at different days with different solutions. No significant differences were observed and hence representative measurements are shown in the figures.

UV-Vis Spectroscopy:

UV-Vis measurements were performed on a Cary 60 spectrophotometer at room temperature (~25 °C) or on a Clario Star Plate reader (ascorbate oxidation, Fig. 2A). Stock solutions of all the reactants were mixed directly inside quartz cuvettes or inside a 96 wells transparent microplate with a final volume of 100 µl.

Fluorescence Spectroscopy:

Fluorescence measurements with 3-CCA were performed on a Clario Star Plate reader, inside a 384 wells black microplate.

EPR Spectroscopy:

Both low temperature (100K) and room temperature (294±1K) spin trapping investigations were performed on an EMX X-band spectrometer (EMXplus from Bruker Biopsin GmbH, Germany), equipped with a high sensitivity resonator (4119HS-W1, Bruker). The g factor was calibrated in the experimental conditions using the Bruker strong pitch (g = 2.0028). Principal spectrometer settings for spin-trapping: Center Field: 3510 G, Sweep Width: 80 G, Microwave power: 4.5 mW, Modulation Amplitude: 1 G, Gain: 50 dB, Conversion Time: ca 250 ms, Time Constant: ca. 80 ms, 1 scan/720 pts/180sec. Low temperature was achieved using continuous flow liquid nitrogen cryostat. Principal spectrometer settings for low temperature: Center Field: 3100 G, Sweep Width: 1500 G, Microwave power: 0.1 mW, Modulation Amplitude: 5 G, Gain: 30 dB, Conversion Time: *ca*. 200 ms, Time Constant: ca. *80* ms, 3 scans/1500 pts/300sec each.

The room temperature spin trapping investigations were carried out with α-(4-Pyridyl N-oxide)-N-tert-butylnitrone (POBN), used as primary spin trap, and EtOH (5% v/v), added as hydroxyl scavenger to enhance the detection of the HO• produced.
BCS (bathocuproinedisulfonate) was added into the reaction mixture to confirm the relation of Cu(I) formation to the HO• production and used as specific Cu(I)-chelator. The overall association constant log β_2 for the $[Cu(I)(BCS)^2]^{3-}$ complex, quantified *via* the Nernst equation, was estimated to be 19.8.[3]

1 K. H. S. Schwab, J. Shearer, S. E. Conklin, B. Alies, *J. Inorg. Biochem.*, 2015, **25**, 368.

2 E. Atrián-Blasco, M. Del Barrio, P. Faller and C. Hureau, *Anal. Chem.*, 2018, **90**, 5909.

3 G.A. Lappin, M. P. Youngblood, D. W. Margerum, *Inorg. Chem.*, 1980, **19**, 407.

I) Titration of the ATCUN peptides with Cu(II):

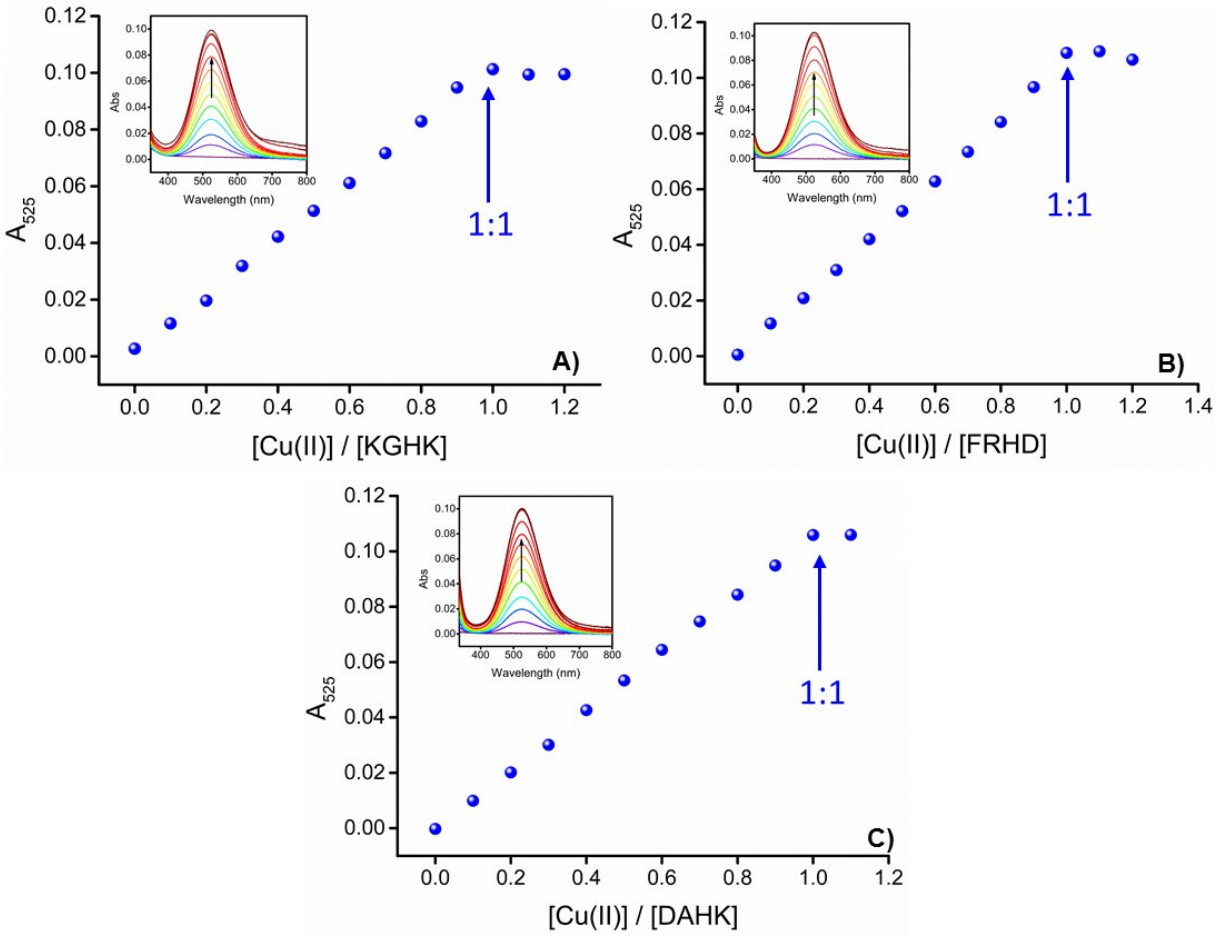

Fig. S1 Cu(II)-titration experiments of the three XZH ATCUN peptides studied in this work, KGHK (A), FRHD (B), DAHK (C) carried out to determine their the concentration of the peptides.

Titrations were monitored by UV-Vis Spectroscopy through the characteristic d-d band for the 1:1 complex Cu(II)-ATCUN at λ_{max} = 525 nm. Above one equivalent of Cu(II) the Cu(II)-phosphate precipitation started to be observed, shown by the increase in the background absorption. These titrations confirm the stoichiometry of the Cu to peptide complexes and all show the typical d-d bands around 525 nm for the Cu-binding to the XZH motif (ATCUN).

Experimental conditions: 1 mM peptide in phosphate buffer 50 mM, pH 7.4. Titrations were made adding 1 μl aliquots of a 10 mM Cu(II) stock solution.

II) HO• production catalysed by Cu(II)-XZH ATCUN complexes in the presence of ascorbate alone (A) or H_2O_2 alone (B), measured with CCA by fluorescence spectroscopy:

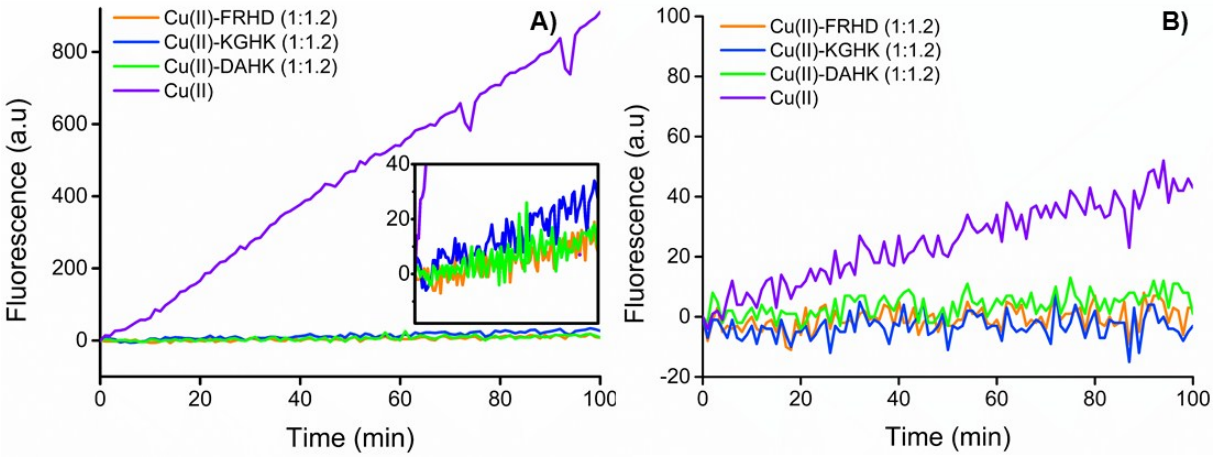

Fig. S2 Evolution of fluorescence of the HO• adduct of 3-CCA (HO-CCA) as a function of time in the presence of A) $AscH^-$, B) H_2O_2.

Experimental conditions: the concentration of Cu(II) and peptide (DAHK, KGHK, FRHD) used were 25 µM and 30 µM respectively. Cu(II)-XZH complexes were pre-formed at the desired ratio (Cu(II):peptide, 1:1.2). The concentration of $AscH^-$, H_2O_2 and CCA used were 250 µM, 250 µM and 0.5 mM respectively. Phosphate buffer (50 mM, pH 7.4) was used in all the experiments and the reactions monitored over 100 min.

III) Spin trapping investigation for different ratios of the Cu(II)-KGHK complex in the presence of $AscH^-$ and H_2O_2:

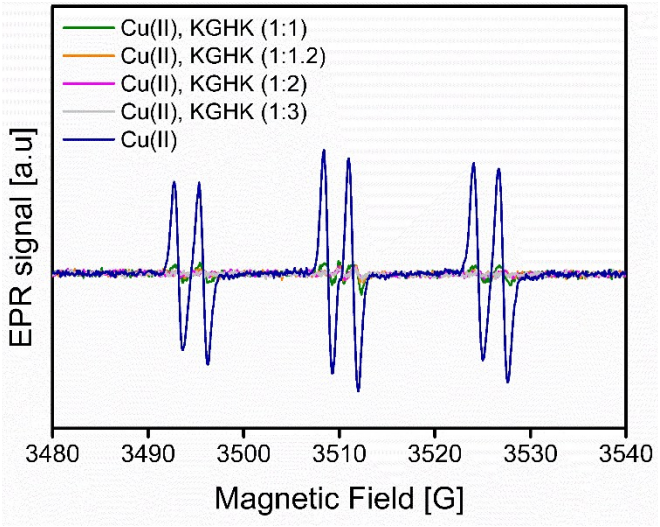

Fig. S3 Indirect evidence of $HO^\bullet$ generation by Cu(II)-KGHK complexes: EPR spectra of the POBN-CH_3 spin-adduct (g = 2.0056, A_H = 2.7 G, A_N = 16 G) collected for the different Cu:KGHK ratios: 1:1, 1:1.2, 1:2, 1:3 after triggering the reaction with ascorbate.

Experimental conditions: the POBN spin adduct results from the reaction between the POBN spin trap and a carbon centred radical originating from the decomposition of EtOH with $HO^\bullet$. EtOH was added here as a secondary spin trap (5% v/v) to enhance the EPR signal S/N. The concentrations of Cu(II) and KGHK were respectively 100 µM and 100 µM (1:1), 120 µM (1:1.2), 200 µM (1:2), 300 µM (1:3). The concentration of $AscH^-$, H_2O_2 and POBN were 1 mM, 1 mM and 50 mM. Reaction mixtures were prepared in 50 mM phosphate buffer, pH 7.4 and experiments performed at RT.

IV) Consumption of the substrate ascorbate with and without H_2O_2 by Cu(II)XZH complexes:

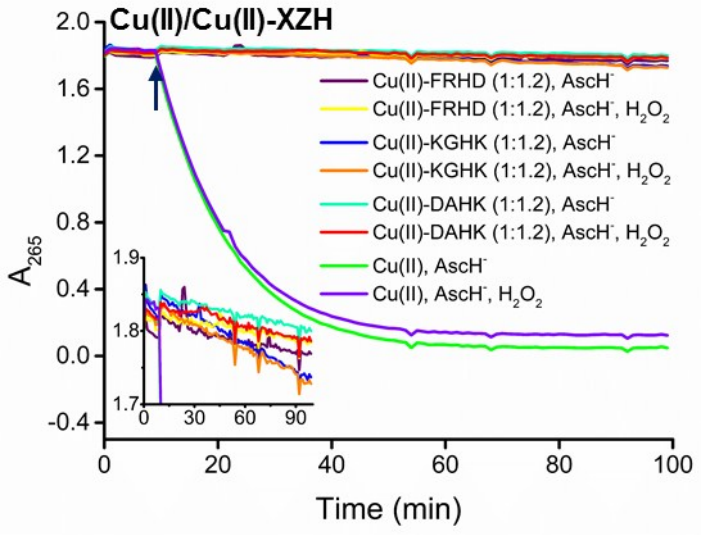

Fig. S4 Evolution of the ascorbate absorption (λ_{max} = 265 nm) as a function of time after exposure to the three Cu(II)-XZH ATCUN complexes (ratio Cu(II):peptide, 1:1.2) and free Cu(II) with and without H_2O_2.

Experimental conditions: ascorbate oxidation was triggered by the addiction of free Cu(II) or the preformed Cu(II)-XZH complexes after 10 min. The final concentrations of Cu(II), XZH peptide, $AscH^-$ and H_2O_2 were respectively 10 µM, 12 µM, 100 µM and 100 µM. The reactions were performed in 50 mM phosphate buffer, pH 7.4. Inset on the right bottom: zoom of the ascorbate oxidation profile for Cu(II)-XHZ complexes.

V) Changes in the d-d band of Cu(II)-KGHK in the presence of $AscH^-$ and/or H_2O_2 :

To evaluate the reactivity of Cu-XZH with $AscH^-$ or H_2O_2, we monitored the Cu(II) d-d bands of Cu(II)-KGHK, Cu(II)-DAHK and Cu(II)-FRHD by Absorbance over 60 min after the addiction of high concentrations (5 mM) of $AscH^-$ and/or H_2O_2 . In case of a high reactivity with $AscH^-$ or H_2O_2 one could expect a disappearance of the d-d bands, either due to reduction to Cu(I) by $AscH^-$ or oxidation to Cu(III) by H_2O_2. For Cu(III) appearance of new d-d bands could also be expected. Although one could expect a small decrease of the Cu(II) d-d band in the presence of $AscH^-$, as Cu(I) is implicated in the mechanism (see main text), this was not observed, due to two reasons: i) the reduction of Cu(II)-XZH to Cu(I) is slow (in line with the inefficient ROS production) and ii) the slowly formed Cu(I) is rapidly reoxidized by H_2O_2 to Cu(II), and hence the steady state Cu(I) concentration is below the detection limit even at the high concentrations (10 mM) $AscH^-$ /H_2O_2 we used.

Moreover under the conditions with ascorbate i.e. $AscH^-$ only (B) and $AscH^-$ and H_2O_2 (A) a new band around 370 nm appeared in line with the literature.[1]

After a longer incubation up to 15h, the band around 370 nm steadily increase and then decrease (Fig. S6A). Thus the tale of the band started to overlap with the d-d band of Cu(II)-KGHK and it seemed that the d-d band started to decrease. However, as shown by EPR (Fig. S6B) this apparent decrease in the d-d band at λ = 525 nm is not due to a reduction or oxidation of Cu(II), but rather to a degradation of the peptide and hence a change in the Cu(II)-coordination site.

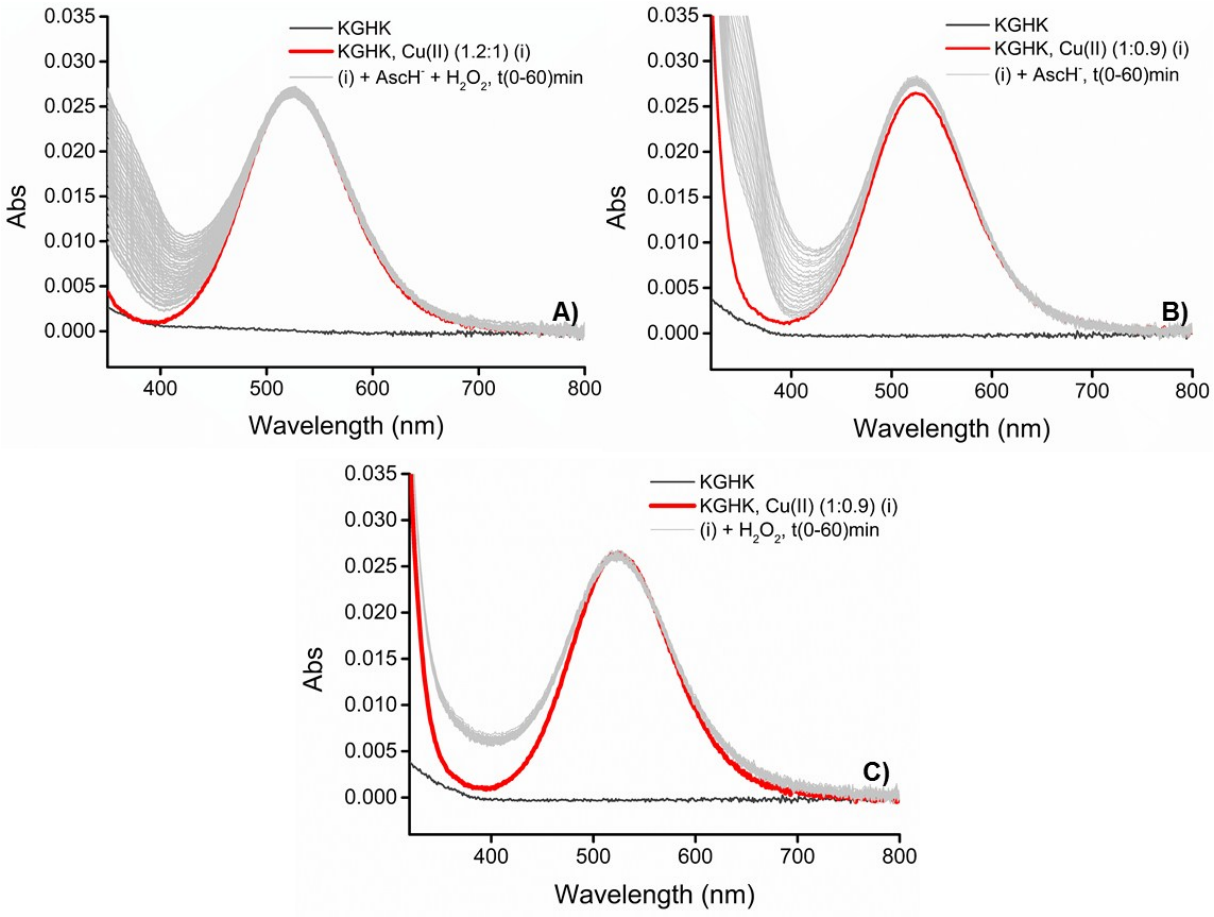

Fig. S5 Uv-Vis spectra for the reaction of Cu(II)-KGHK (ratio 1:1.2, Cu(II):KGHK) with $AscH^-$ and H_2O_2 (A), $AscH^-$ (B) and H_2O_2 (C).

Experimental conditions: the concentration of Cu(II), KGHK, $AscH^-$ and H_2O_2 were respectively 100 µM, 120 µM, 5 mM and 5 mM. The reaction were performed in 50 mM phosphate buffer, pH 7.4 and monitored over time over a period of 1h, collecting each intermediate spectrum at 2 min intervals

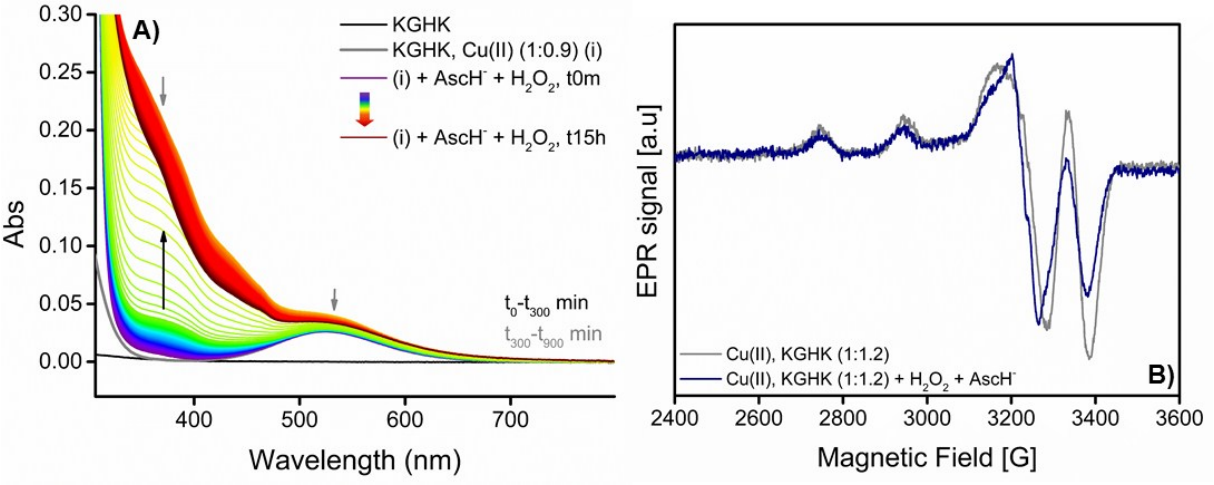

Fig. S6 Partial degradation of the peptide shown by UV-Vis Spectroscopy (A) and EPR Spectroscopy at low temperature (100K) (B) for the reaction of Cu(II)-KGHK (ratio 1:1.2, Cu(II):KGHK) with $AscH^-$ and H_2O_2.

Experimental conditions for UV-Vis Spectroscopy: the concentration of Cu(II), KGHK, $AscH^-$ and H_2O_2 were respectively 100 µM, 120 µM, 5 mM and 5 mM. The reaction was performed in 50 mM phosphate buffer, pH 7.4 and monitored over a period of 15h, collecting each intermediate spectrum at 10 min intervals.

Experimental conditions for EPR Spectroscopy: the concentration of Cu(II), KGHK, $AscH^-$ and H_2O_2 were respectively 200 µM, 240 µM, 4 mM and 4 mM (B). The reaction was performed in 80 mM phosphate buffer, pH 7.4. The reaction mixtures were incubated for 24h under aerobic conditions and then transferred into a 4 mm outer diameter quartz tubes (Wilmad-Labglass) and freeze-quenched with liquid nitrogen before their introduction into the precooled cavity.

VI) Cu(I) binding to BCS of Cu(II)-KGHK in the presence of $AscH^-$ or $AscH^-$ and H_2O_2:

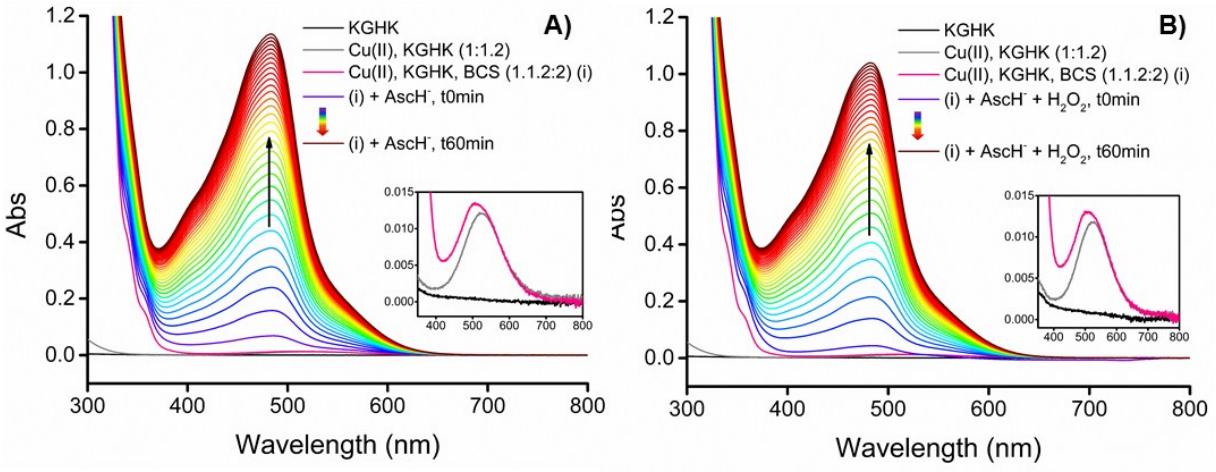

Fig. S7 Uv-Vis spectra for the reaction of Cu(II)-KGHK (ratio 1:1.2, Cu(II):KGHK) with $AscH^-$ (A), $AscH^-$ and H_2O_2 (B) in the presence of BCS.

Experimental conditions: the concentrations of Cu(II), KGHK, $AscH^-$, H_2O_2 and BCS were respectively 100 µM, 120 µM, 10 mM, 10 mM and 200 µM. The reactions were performed in 50 mM phosphate buffer, pH 7.4, and monitored over a period of 1h, collecting each intermediate spectrum at 2 min intervals.

VII) Ascorbate oxidation of Cu(II) at different Cu:KGHK ratios in phosphate and HEPES buffer:

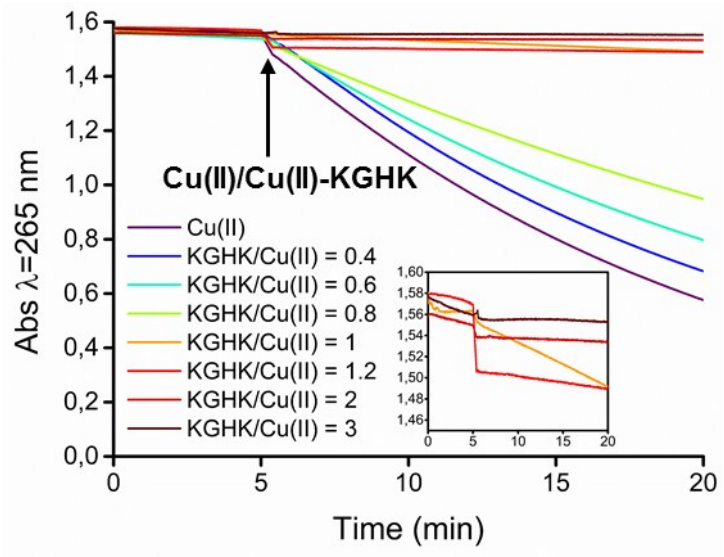

Fig. S8 Kinetics of ascorbate oxidation by Cu(II)-KGHK at different ratios Cu(II):KGHK (1:0, 1:0.4, 1:0.6, 1:0.8, 1:1, 1:1.2, 1:2, 1:3) monitored by UV-Vis Spectroscopy at λ_{max} = 265 nm as a function of time in phosphate buffer, pH 7.4. Ascorbate oxidation was started by the addiction of the preformed Cu(II)-KGHK complex at t(5min).

Experimental conditions: the concentration of $AscH^-$ was 100 µM. The final concentration of Cu(II) was 10 µM and that of KGHK ranging from 0-30 µM. The reactions were performed in 50 mM phosphate buffer, pH 7.4 and monitored over a period of 20 min.

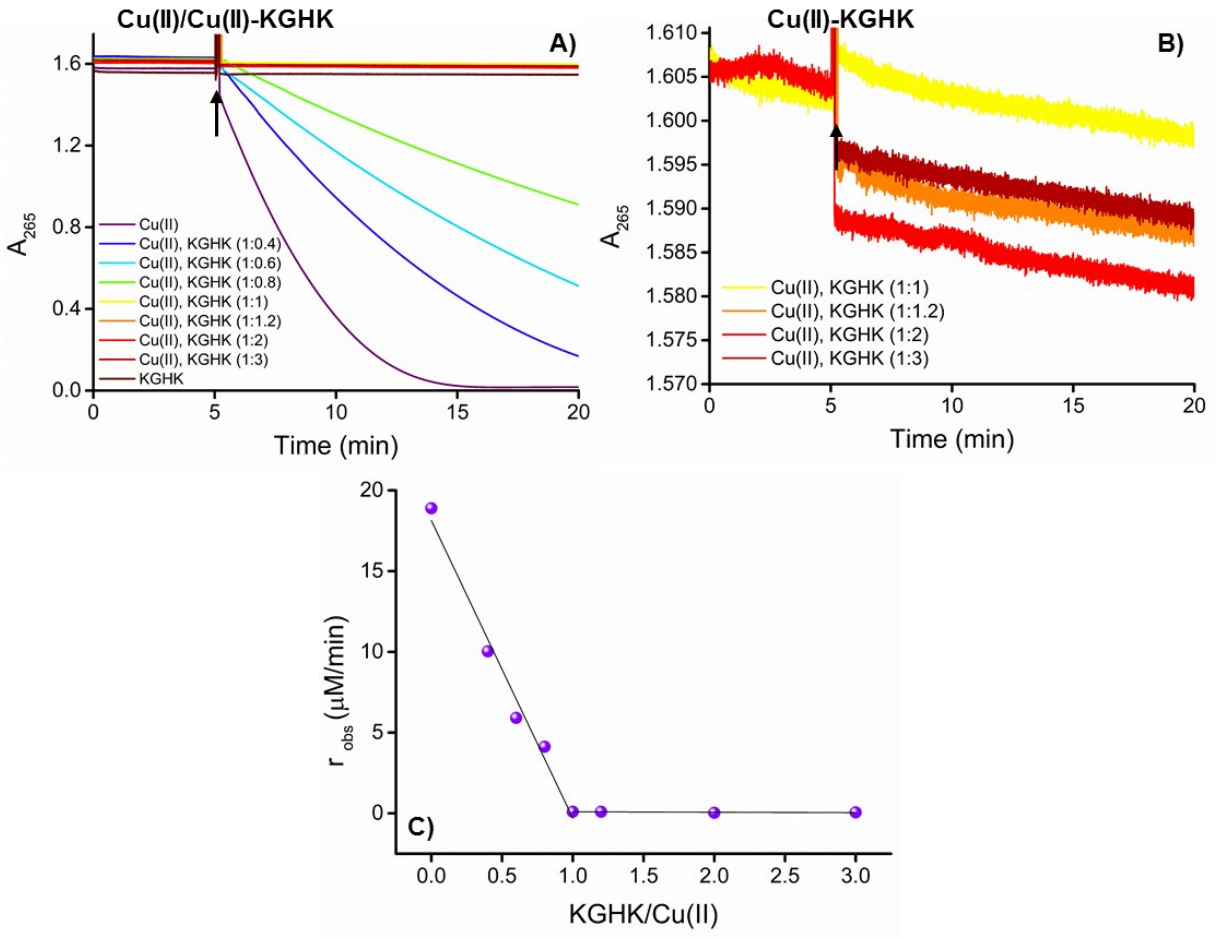

Fig. S9 A) Kinetics of ascorbate oxidation by Cu(II)-KGHK at different ratios Cu(II):KGHK (1:0, 1:0.4, 1:0.6, 1:0.8, 1:1, 1:1.2, 1:2, 1:3) monitored by UV-Vis Spectroscopy at λ_{max} = 265 nm as a function of time in HEPES Buffer, pH 7.4. Ascorbate oxidation was started by the addiction of the preformed Cu(II)-KGHK complex at t(5min). B) Corresponding kinetics for the ratios Cu:KGHK, 1:1, 1:1.2, 1:2, 1:3. C) Molar ascorbate oxidation rate (µM/min) as a function of KGHK to Cu(II) ratio.

Experimental conditions: the concentration of $AscH^-$ was 100 µM. The final concentration of Cu(II) was 10 µM and that of KGHK ranging from 0-30 µM. The reactions were performed in 50 mM HEPES buffer, pH 7.4, and monitored over a period of 20 min.

The same behaviour in terms of ascorbate oxidation was observed in case of the two different buffers tested, phosphate and HEPES. KGHK bound to Cu(II) (Cu(II)-KGHK) was very inefficient in ascorbate oxidation and hence almost completely stopped the catalytic production of $HO^\bullet$ at 1:1 ratio, Cu(II):KGHK. In case of HEPES buffer the rate of ascorbate oxidation for the ratios Cu(II):peptide, 1:1, 1:1.2, 1:2, 1:3 was the same whereas in phosphate buffer Cu(II)-KGHK at 1:1 ratio was slightly more active (0.28 µM/min) compared to the ratios 1:1.2, 1:2 and 1:3 (~0.11 µM/min). This we assign to a slight competition of phosphate for Cu at ratio 1:1 Cu(II):KGHK during the redox cycling Cu(II)/Cu(I). This is also the reason why in case of the ratios Cu(II):KGHK 1:0.4, 1:0.6, 1:0.8 a proportional decrease in the rate of ascorbate oxidation was not observed (Fig S5). Nevertheless phosphate buffer has been used through all the experiments to be consistent with the conditions of all the assays. Indeed measurements of $HO^\bullet$ production are preferred in such buffer, considering that HEPES itself can trap the small amount of $HO^\bullet$ produced.