



# Supplementary material: Reduced Schiff-base derivatives to stop reactive oxygen species production by the Cu(A $\beta$ ) species: a structure–activity relationship

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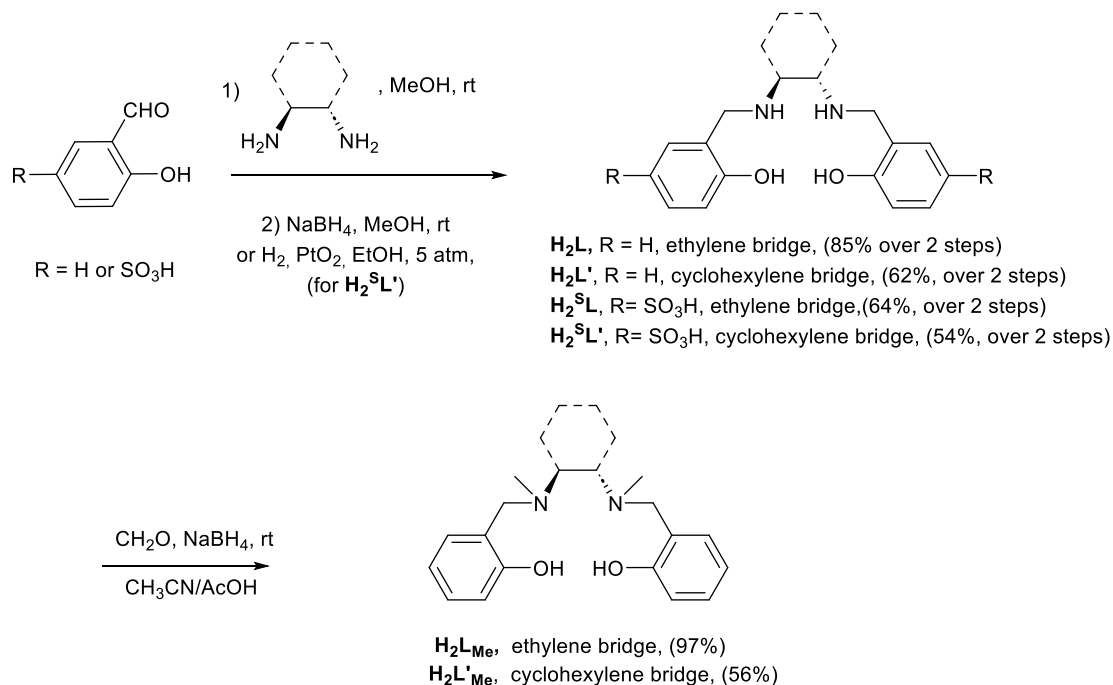
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## 1. Synthesis



### Supplementary Scheme S1. Synthetic routes to the ligands $\mathcal{L}$ .

## 2. Experimental section

### 2.1. Chemicals

All the chemicals were purchased from Sigma-Aldrich unless otherwise specified. All of the solutions were prepared using ultrapure water (18.2 M $\Omega$ ). Stock solutions of metallic salts and peptides were prepared by dissolving the salts or the peptides in ultrapure water; the concentrations were determined using UV-vis absorption spectroscopy. A stock solution of Zn(II) ions was prepared at 100 mM using monohydrated  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$  salt. A stock solution of Cu(II) was prepared at 100 mM using hydrated  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  salt. The concentration of the Cu(II) stock solution was determined using UV-vis ( $\lambda = 800$  nm corrected at 400 nm) while considering a molar extinction coefficient of 12 M $^{-1}\cdot\text{cm}^{-1}$ . A stock solution of HEPES buffer

(sodium salt of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) was prepared at 500 mM and the pH was adjusted to 7.4 using concentrated sodium hydroxide. All pH values are given with a  $\pm 0.1$  pH unit error. The stock solution of sodium ascorbate were prepared at 5 mM every day due to its auto-oxidation in solution. The  $\text{A}\beta_{1-16}$  (DAEFRHDSGYEVHHQK) peptide was purchased from Genecust (purity > 95%). A stock solution and its concentration was determined using UV-vis absorption spectroscopy with Tyr10 absorption considered as free tyrosine (in acidic condition,  $\epsilon_{276} - \epsilon_{360} = 1410$  M $^{-1}\cdot\text{cm}^{-1}$ ). Stock solutions of ligands were prepared and concentration was determined by a Cu(II) titration followed by UV-vis absorption spectroscopy (see Figure S1 as a matter of example). The stock solutions of ligands and peptide were stored at  $-20$  °C.

## 2.2. Synthesis of the ligands

All experiments were carried out in methanol or ethanol purchased from VWR chemicals without further purifications. NMR spectra were recorded on Bruker Avance 300 MHz spectrometers.  $^1\text{H}$  NMR spectra were referenced to residual protonated solvents ( $\delta\text{H} = 4.79$  ppm for  $\text{D}_2\text{O}$ ). Reactions were monitored by TLC on silica gel Alugram<sup>®</sup> Xtra SIL G/UV254.

All the ligands but  $^s\text{L}'$  were obtained according to reported protocols. Using similar procedures to those reported previously, the ligands **L** [1], **L'** [2] on the one hand and ligands  $^s\text{L}$  [3] and  $^s\text{L}'$  [4] on the other hand were synthesized in two steps from 2-hydroxybenzaldehyde or 4-formyl-3-hydroxybenzenesulfonic acid [5], respectively. To that end, the required aldehydes were condensed on ethylene diamine or *rac*(1,2)-cyclohexanediamine to yield the corresponding diimine intermediates. Diamines **L**, **L'** and  $^s\text{L}$  were readily obtained using  $\text{NaBH}_4$  as reductive agent. However,  $\text{PtO}_2$  catalytic hydrogenation was required to obtain pure ligand  $^s\text{L}'$ . Subsequent *N*-methylation of compounds **L** and **L'** was achieved by reductive amination with an excess of formaldehyde to form the intermediate imine, followed by reduction in the presence of  $\text{NaBH}_4$  to give diimines **L**<sub>Me</sub> [1] and **L'**<sub>Me</sub> [6].

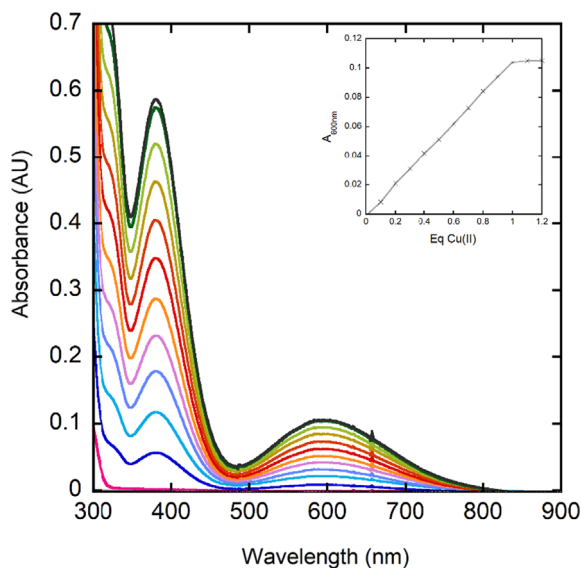
Their characterization by  $^1\text{H}$  NMR spectroscopy correspond to the one described in the literature.

Ligand  $^s\text{L}'$  was obtained according to an alternative protocol:

A mixture of 4-formyl-3-hydroxybenzenesulfonic acid (424 mg, 2.1 mmol) and ( $\pm$ )-*trans*-1,2-diaminocyclohexane (115 mg, 1 mmol) was refluxed in EtOH for 20 h. The reaction mixture is cooled to room temperature, and the precipitated diimine was collected by filtration (322 mg, 68% yield) and used in the next step without further purification.

The latter diimine (70 mg, 0.14 mmol) was suspended in absolute EtOH (11 mL),  $\text{PtO}_2$  was added (10 mg) and the reaction mixture was subjected to catalytic hydrogenation at 5 atm for 24 h and room temperature. Filtration and concentration under vacuum yielded the expected ligand  $^s\text{L}'$  (55 mg, 80% yield) as a white solid [4].

$^1\text{H}$  NMR (300 MHz,  $\text{D}_2\text{O}$ ):  $\delta = 7.60$  (m; 4H); 6.81 (d,  $J' = 9.2$  Hz; 2H); 4.11 (d;  $J = 13.4$  Hz; 2H); 3.94



**Supplementary Figure S1.** UV-vis spectra corresponding to the titration of  $\text{Cu}^{\text{II}}$  into a solution of ligand **L**. Inset: Absorbance at 600 nm versus number of  $\text{Cu}^{\text{II}}$  equiv. added. [hepes] 50 mM, pH 7.4,  $T = 25$  °C, [**L**] is approx. 500  $\mu\text{M}$ ; 1 equiv. of  $\text{Cu}^{\text{II}}$  = 500  $\mu\text{M}$ .

(d;  $J = 13.4$  Hz; 2H;  $\text{H}_{6'}$ ); 2.70 (m; 2H); 2.24 (m; 2H); 1.77 (m; 2H); 1.28 (m; 4H).

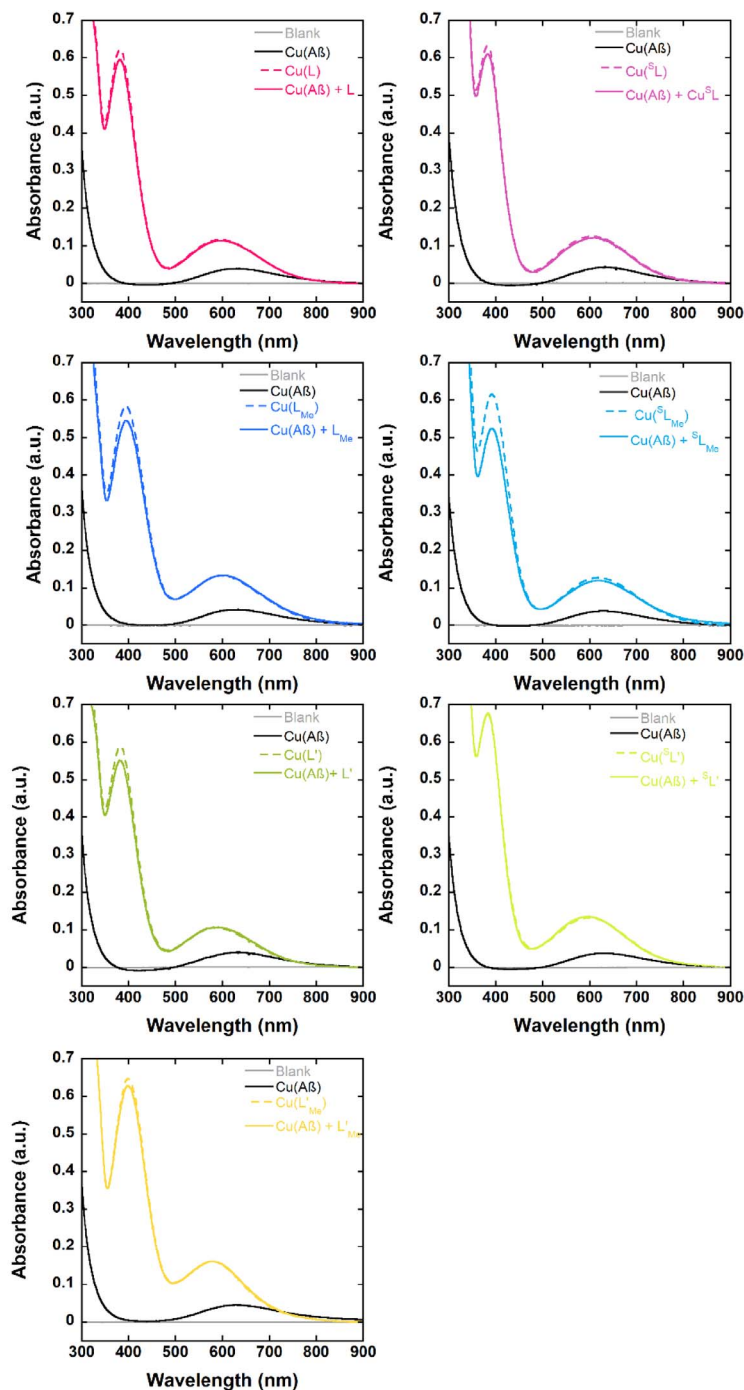
*HR-MS*: (DCI- $\text{CH}_4$ ) calculated for  $\text{C}_{20}\text{H}_{27}\text{N}_2\text{O}_8\text{S}_2$  [ $\text{MH}$ ]<sup>+</sup> = 487.1209; found: 487.1206 m/z.

### 2.2.1. UV-visible spectroscopy

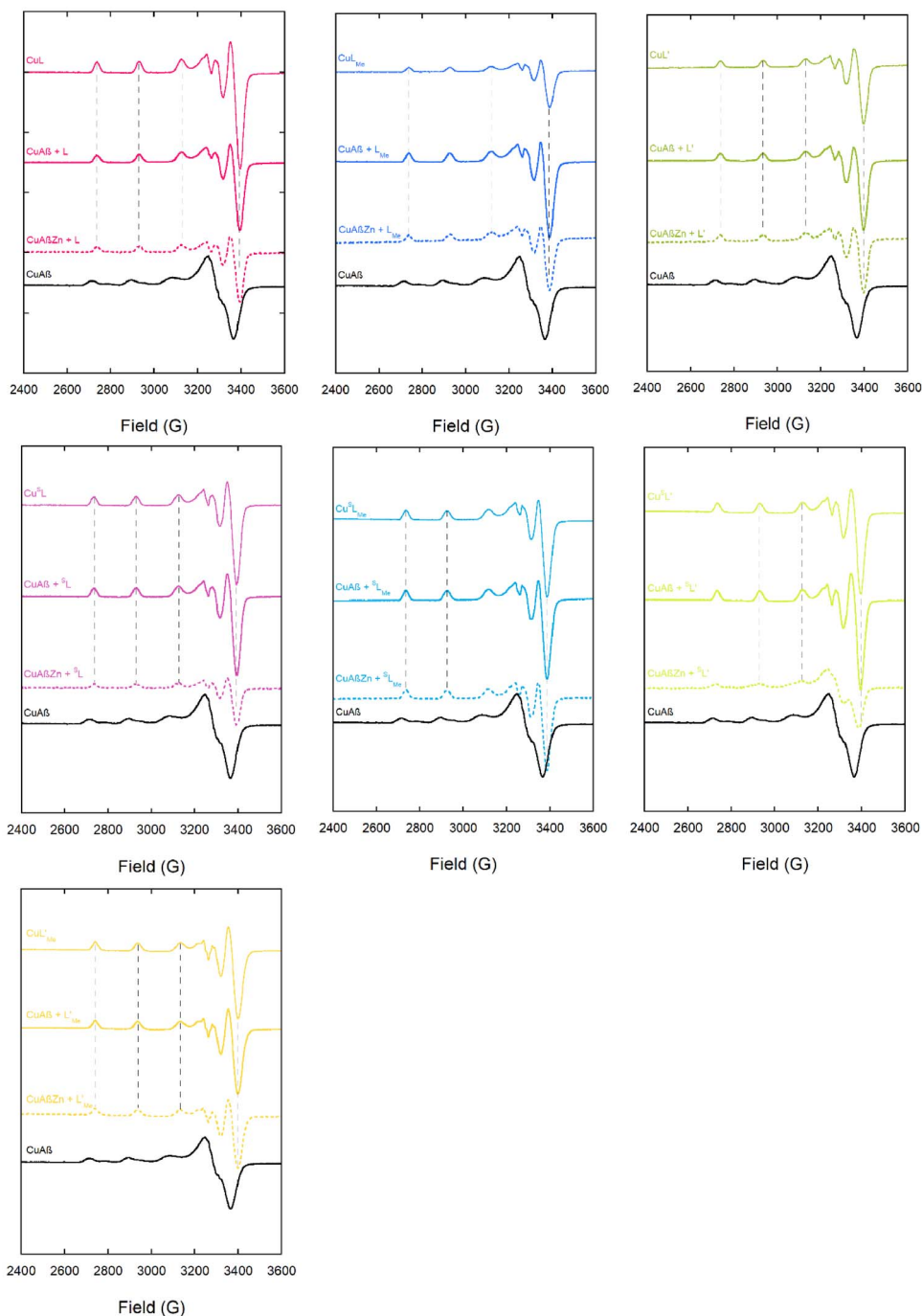
Titration and UV-vis kinetic data from the ascorbate consumption experiments were recorded with a Hewlett Packard Agilent 8453 or 8454 spectrophotometer at a controlled temperature of 25 °C in a 1 cm path length quartz cuvette with 800 rpm stirring.

### 2.2.2. $\text{Cu}^{\text{II}}$ titration

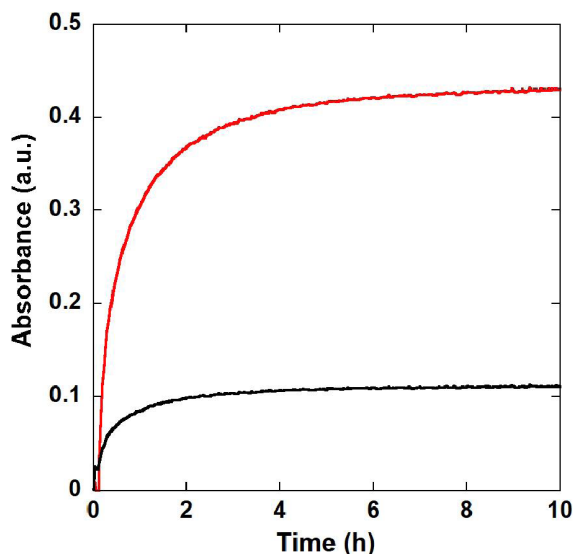
The concentrations of ligands stock solutions were determined via  $\text{Cu}^{\text{II}}$  titration with a solution of known concentration using the d-d and LMCT transition absorption of the formed complex (see for example Figure S1 for the titration of ligand **L**). All the ligands were titrated at about 500  $\mu\text{M}$  (based on weighting) with the addition of an increasing amount of a  $\text{Cu}^{\text{II}}$  stock solution. For competition experiment,  $\text{Cu}^{\text{II}}$  and  $\text{A}\beta$  were added to the cuvette to preform  $\text{Cu}^{\text{II}}(\text{A}\beta)$  complex and **L** was added after.



**Supplementary Figure S2.**  $\text{Cu}^{\text{II}}$  removal from  $\text{Cu}^{\text{II}}(\text{A}\beta)$  by  $\mathcal{L}$  followed by UV-vis. Each panel contains spectra of  $\text{Cu}^{\text{II}}(\text{A}\beta)$  (black curves),  $\text{Cu}^{\text{II}}$  (L) (L in pink,  $\text{L}_{\text{Me}}$  in blue,  $\text{L}'$  in green,  $^{\text{S}}\text{L}$  in light pink,  $^{\text{S}}\text{L}'$  in light green and  $^{\text{S}}\text{L}_{\text{Me}}$  in light green), and spectra of the addition of L to  $\text{Cu}^{\text{II}}(\text{A}\beta)$  with mixing for 5 min (thick curves). Experimental conditions:  $[\text{Cu}^{\text{II}}] = 500 \mu\text{M}$ ,  $[\text{L}] = [\text{A}\beta] = 500 \mu\text{M}$ ,  $[\text{HEPES}] = 100 \text{ mM}$ ,  $\text{pH } 7.4$ ,  $T = 25 \text{ }^{\circ}\text{C}$ .



**Supplementary Figure S3.**  $\text{Cu}^{\text{II}}$  removal from  $\text{Cu}^{\text{II}}$  ( $\text{A}\beta$ ) and  $\text{Cu}^{\text{II}}$   $\text{Zn}^{\text{II}}$  ( $\text{A}\beta$ ) by  $\mathcal{L}$  followed by X-band EPR. Each panel contains spectra of  $\text{Cu}^{\text{II}}$  ( $\text{A}\beta$ ) (black curves),  $\text{Cu}^{\text{II}}$  ( $\mathcal{L}$ ) and of the addition of  $\mathcal{L}$  to  $\text{Cu}^{\text{II}}$  ( $\text{A}\beta$ ) (thick curve) and  $\text{Cu}^{\text{II}}$   $\text{Zn}^{\text{II}}$  ( $\text{A}\beta$ ) (dotted curve) and mixing for 5 min. Experimental conditions:  $[\text{Cu}^{\text{II}}] = 480 \mu\text{M}$ ,  $[\mathcal{L}, \text{A}\beta] = 500 \mu\text{M}$ , or  $[\text{Cu}^{\text{II}}] = [\text{Zn}] = 180 \mu\text{M}$ ,  $[\mathcal{L}, \text{A}\beta] = 200 \mu\text{M}$  [HEPES] = 50 mM, pH 7.4, 10% of glycerol as cryoprotectant,  $T = 120 \text{ K}$ ,  $\nu \approx 9.5 \text{ GHz}$ , mod. ampl. = 5 G, microwave power: 5 mW.

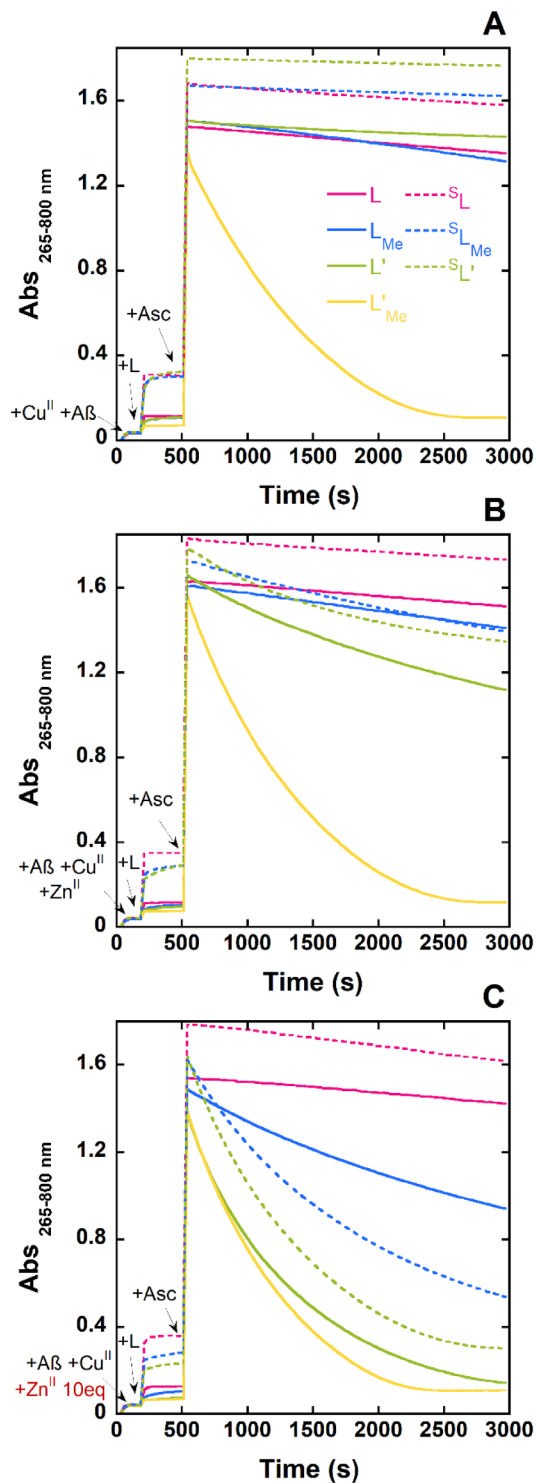


**Supplementary Figure S4.**  $\text{Cu}^{\text{II}}$  removal from  $\text{Cu}^{\text{II}}(\text{A}\beta)$  and  $\text{Cu}^{\text{II}}$  by  $\text{L}'_{\text{Me}}$  monitored by UV-vis. Experimental conditions:  $[\text{L}'_{\text{Me}}] = [\text{Cu}(\text{II})] = [\text{Zn}(\text{II})] = [\text{A}\beta] = 500 \mu\text{M}$ ,  $[\text{HEPES}] = 100 \text{ mM}$ ,  $\text{pH} 7.4$ ,  $T = 25 \text{ }^\circ\text{C}$ .  $\text{Cu}(\text{II})\text{A}\beta$  was pre-formed in the cuvette and then,  $\text{L}'_{\text{Me}}$  was added at 500 s in red: absorbance at 392 nm and in black at 620 nm.

### 2.2.3. ROS experiment

The ROS production was determined using an ascorbate consumption assay monitored via UV-vis spectroscopy. The decrease in the absorption band at  $\lambda_{\text{max}} = 265 \text{ nm}$  of the Asc ( $\epsilon = 14,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ , corrected at 800 nm for the background absorption) was plotted as a function of time.

The samples were prepared from stock solutions at 1 mM and mixed in situ in the UV-vis cuvette at a final concentration of 12  $\mu\text{M}$  for  $\mathcal{L}$ ,  $\text{A}\beta_{1-16}$ , and  $\text{Zn}^{\text{II}}$  and 10  $\mu\text{M}$  for  $\text{Cu}^{\text{II}}$  in HEPES at  $\text{pH} 7.4$ . Ascorbate was added to obtain 100  $\mu\text{M}$  as the final concentration. The final volume was adjusted with ultrapure water to 2 mL. The ROS experiments were performed following three different procedures: (i) starting from  $\text{Cu}(\text{II})$  with 300 s incubation time where  $\text{Cu}^{\text{II}}/\text{Zn}^{\text{II}}$ ,  $\text{A}\beta$  and  $\mathcal{L}$  were added at 30, 60 and 120 s respectively; (ii) with long enough incubation time to reach the thermodynamic equilibrium where  $\text{Cu}^{\text{II}} + \text{A}\beta + \mathcal{L}$  or  $\text{Cu}^{\text{II}} + \text{A}\beta + \text{Zn}^{\text{II}} + \mathcal{L}$  mixtures were prepared out of the cuvette, in Eppendorf tubes, and incubated at 25  $^\circ\text{C}$  with stirring. These experiments are



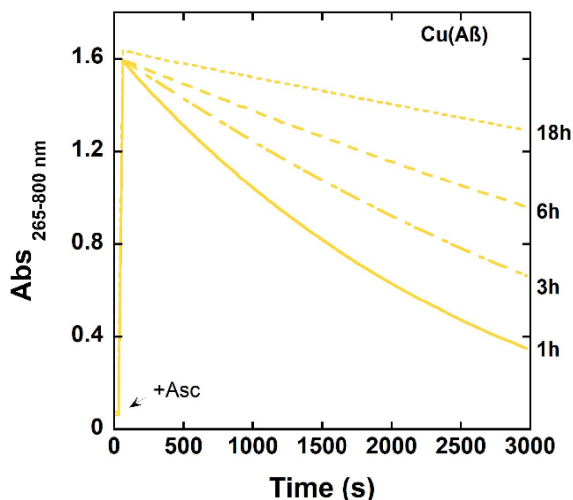
**Supplementary Figure S5.** Continued on next page.

**Supplementary Figure S5. (cont.)** Ascorbate consumption induced by Cu(A $\beta$ ). Starting from Cu(II) (in presence of increasing Zn stoichiometry, A, B and C) and with Ascorbate added 300 s after mixing of the others chemicals. [ $\mathcal{L}$ ] = [A $\beta$ ] = [Zn<sup>II</sup>] = 0, 12 and 120  $\mu$ M, [Cu<sup>II</sup>] = 10  $\mu$ M, [Asc] = 100  $\mu$ M, [HEPES] = 100 mM, pH 7.4,  $T$  = 25  $^{\circ}$ C. The arrows indicate the time at which the different components are added into the cuvette.

called “A” in the main text. Third, (iii) with the ligand added in the course of Asc consumption (experiment called “B”) where ascorbate was first introduced into the cuvette, then, either Cu<sup>II</sup> + A $\beta$  +  $\mathcal{L}$  or Cu<sup>II</sup> + A $\beta$  + Zn<sup>II</sup> +  $\mathcal{L}$  was added. Ascorbate, A $\beta$ , and/or Zn<sup>II</sup> and Cu<sup>II</sup> were added at 30, 120, and 240 s, respectively. When the absorbance reached about 1.1 in O.D.,  $\mathcal{L}$  was added. These experiments were run under aerobic conditions.

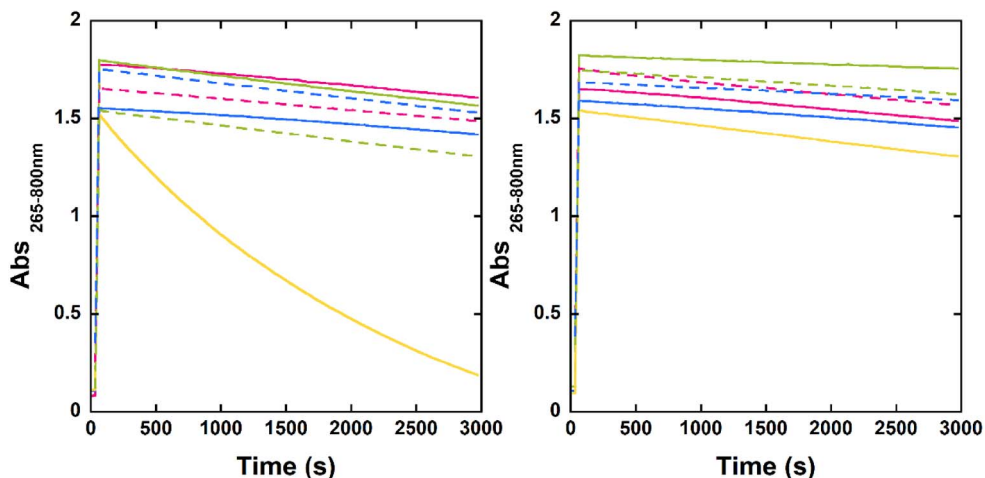
#### 2.2.4. EPR

Electron paramagnetic resonance (EPR) spectra were recorded using an Elexsys E-500 Bruker spectrometer operating at a microwave frequency of approximately 9.5 GHz. The spectra were recorded using a microwave power of 5 mW, a magnetic field range of 2400 to 3700 G, and a modulation amplitude of 5 G. The experiments were carried out at 120 K using a liquid nitrogen cryostat. EPR samples were prepared in Eppendorf tubes from a 10 mM <sup>65</sup>Cu<sup>II</sup> and A $\beta$  stock solution and diluted to 480  $\mu$ M and 500  $\mu$ M respectively in 50 mM of HEPES buffer (pH 7.4) with

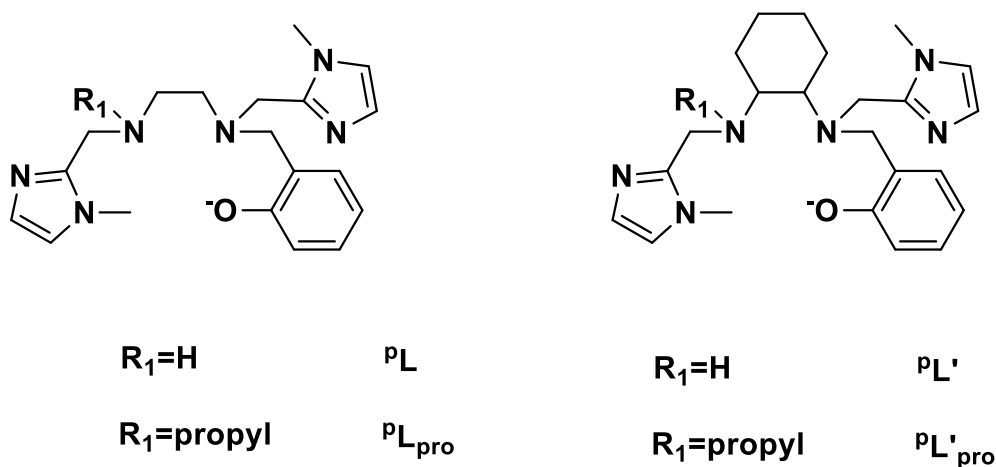


**Supplementary Figure S6.** Ascorbate consumption induced by Cu(A $\beta$ ) and effect of pre-incubation of L'<sub>Me</sub> (times are given in the figure). [L'<sub>Me</sub>] = [A $\beta$ ] = 12  $\mu$ M, [Cu<sup>II</sup>] = 10  $\mu$ M, [Asc] = 100  $\mu$ M, [HEPES] = 100 mM, pH 7.4,  $T$  = 25  $^{\circ}$ C. Ascorbate was added at 30 s (after having taken the starting spectrum corresponding to the incubated mixture).

the addition of 500  $\mu$ M of ligands. Experiment with Zn<sup>II</sup> were conducted at 200  $\mu$ M to limit precipitation. As a cryoprotectant, 10% glycerol was added. The final volume was adjusted to 200  $\mu$ L using ultrapure water. The mixture was then transferred into a EPR quartz tube and frozen in liquid nitrogen.



**Supplementary Figure S7.** Ascorbate consumption induced by Cu,Zn(A $\beta$ ) with Zn<sup>II</sup> 10 eq and effect of pre-incubation of  $\mathcal{L}$  (left: 1 h of CuZn(A $\beta$ ) +  $\mathcal{L}$  incubation and right: 24 h of CuZn(A $\beta$ ) +  $\mathcal{L}$  incubation). [ $\mathcal{L}$ ] = [A $\beta$ ] = 12  $\mu$ M, [Zn<sup>II</sup>] = 120  $\mu$ M, [Cu<sup>II</sup>] = 10  $\mu$ M, [Asc] = 100  $\mu$ M, [HEPES] = 100 mM, pH 7.4,  $T$  = 25  $^{\circ}$ C. Ascorbate was added at 30 s (after having taken the starting spectrum corresponding to the incubated mixture). Solutions of ligands  $\mathcal{L}$  and Cu, Zn(A $\beta$ ) were pre-incubated at room temperature, with agitation and at experimental concentrations.



**Supplementary Scheme S2.** Scheme of the ligands from Ref. [7]. Charges are omitted for simplicity.

## References

- [1] X. Zhao, D. Zhang, R. Yu, S. Chen, D. Zhao, *Eur. J. Inorg. Chem.*, 2018, **2018**, 1185-1191.
- [2] V. J. Lillo, J. Mansilla, J. M. Saá, *Angew. Chem. Int. Ed.*, 2016, **55**, 4312-4316.
- [3] I. Correia, J. C. Pessoa, M. T. Duarte, M. F. M. da Piedade, T. Jackush, T. Kiss, M. M. C. A. Castro, C. F. G. C. Geraldes, F. Avecilla, *Eur. J. Inorg. Chem.*, 2005, **2005**, 732-744.
- [4] K. Voronova, L. Homolya, A. Udvardy, A. C. Bényei, F. Joó, *ChemSusChem*, 2014, **7**, 2230-2239.
- [5] Y. Yong-Kang, Y. Cai-Xia, H. Fang-Jun, *J. Coord. Chem.*, 2014, **67**, 2039-2047.
- [6] H. Du, A. H. Velders, P. J. Dijkstra, J. Sun, Z. Zhong, X. Chen, J. Feijen, *Chem. Eur. J.*, 2009, **15**, 9836-9845.
- [7] G. Schanne, M. Zoumpoulaki, G. Gazzah, A. Vincent, H. Preud'homme, R. Lobinski, S. Demignot, P. Saksik, N. Del-suc, C. Policar, *Oxid. Med. Cell. Longev.*, 2022, **2022**, article no. 3858122.