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# Aminomethylene-Phosphonate Analogue as a Cu(II) Chelator: Characterization and Application as an Inhibitor of Oxidation Induced by the Cu(II)−Prion Peptide Complex

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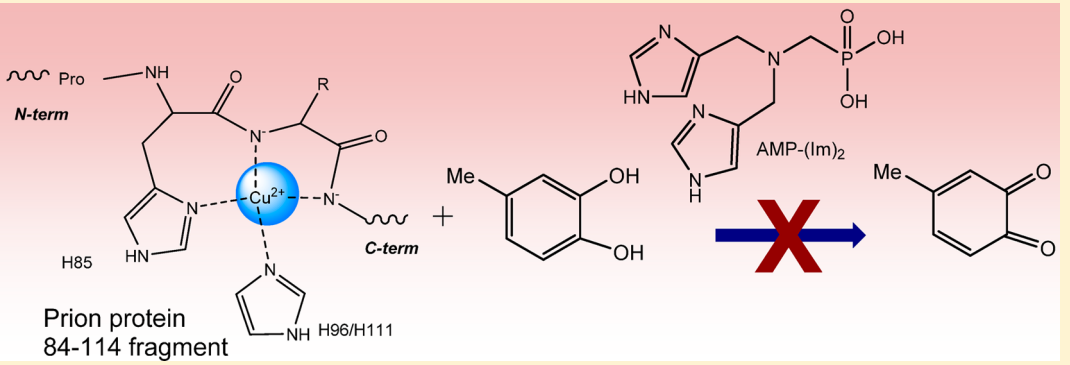
Supporting Information






**ABSTRACT:** Recently, we reported on a series of aminomethylene-phosphonate (AMP) analogues, bearing one or two heterocyclic groups on the aminomethylene moiety, as promising Zn(II) chelators. Given the strong Zn(II) binding properties of these compounds, they may find useful applications in metal chelation therapy. With a goal of inhibiting the devastating oxidative damage caused by prion protein in prion diseases, we explored the most promising ligand, {bis[(1*H*-imidazol-4-yl)methyl]amino}methylphosphonic acid, AMP-$(Im)_2$, **4**, as an inhibitor of the oxidative reactivity associated with the Cu(II) complex of prion peptide fragment 84−114. Specifically, we first characterized the Cu(II) complex with AMP-$(Im)_2$ by ultraviolet−visible spectroscopy and electrochemical measurements that indicated the high chemical and electrochemical stability of the complex. Potentiometric pH titration provided evidence of the formation of a stable 1:1 $[Cu(II)\text{-AMP-}(Im)_2]^+$ complex (ML), with successive binding of a second AMP-$(Im)_2$ molecule yielding $ML_2$ complex $[Cu(II)\text{-}(AMP\text{-}(Im)_2)_2]^+$ (log $K' = 15.55$), and log $\beta' = 19.84$ for $ML_2$ complex. The CuN3O1 ML complex was demonstrated by X-ray crystallography, indicating the thermodynamically stable square pyramidal complex. Chelation of Cu(II) by **4** significantly reduced the oxidation potential of the former. $CuCl_2$ and the 1:2 Cu:AMP-$(Im)_2$ complex showed one-electron redox of Cu(II)/Cu(I) at 0.13 and −0.35 V, respectively. Indeed, **4** was found to be a potent antioxidant that at a 1:1:1 AMP-$(Im)_2$:Cu(II)-$PrP_{84-114}$ molar ratio almost totally inhibited the oxidation reaction of 4-methylcatechol. Circular dichroism data suggest that this antioxidant activity is due to formation of a ternary, redox inactive Cu(II)-$Prp_{84-114}$-[AMP-$(Im)_2$] complex. Future studies in prion disease animal models are warranted to assess the potential of **4** to inhibit the devastating oxidative damage caused by PrP.

## INTRODUCTION

Aminomethylenephosphonic acid (AMPA), **1**, is similar to glycine, **2**, the simplest amino acid, where the carboxylic acid group is replaced by phosphonic acid, yet AMPA is dibasic compared to the monobasic glycine, thus allowing better binding for metal ions and increased water solubility. Therefore, for the synthesis of the new Zn(II)/Cu(II) chelators targeted for medicinal applications, we previously selected the aminomethylene-phosphonate scaffold.[1]

Recently, we reported on a series of aminomethylene-phosphonate (AMP) analogues, **3**−**9**, bearing one or two heterocyclic moieties (imidazolyl, pyridyl, and thiazolyl) on the aminomethylene group, as potential Zn(II) chelators (Figure 1).[1] Analogues **3**−**9** formed stable water-soluble 2:1 L:Zn(II) complexes. $ML_2$-type Zn(II) complexes of AMP, bearing either an imidazolyl or pyridyl moiety, **3**, **5**, and **6**, exhibited high log

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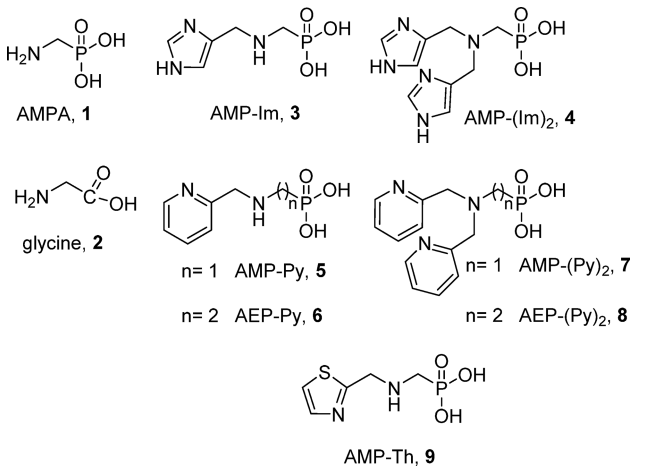


**Figure 1.** Structures of previously reported aminomethylene-phosphonate derivatives **3**–**9**.[1]

$\beta$ values (17.68, 16.92, and 16.65, respectively), while for the AMP-thiazolyl-Zn(II) complex, **9**, log $\beta$ = 12.53. Ligands **4**, **7**, and **8**, bearing two heterocyclic moieties, presented log $\beta$ values (22.25, 21.00, and 18.28, respectively) higher than those of analogues bearing one heterocyclic moiety.

Given the strong Zn(II) binding properties of these compounds, they may find useful applications in metal chelation therapy. As a preliminary assessment of this potential, we investigated the most promising of these ligands, AMP-$(Im)_2$ (**4**), as an inhibitor of the oxidative reactivity associated with the Cu(II) complex of prion peptide fragment 84–114 ($Prp_{84-114}$, Ac-PHGGGWGQGGGTHSQWNKPSK-PKTNMKHMAG-$NH_2$), which includes the high-affinity Cu(II) binding site.[2] Prion diseases make up a family of transmissible neurodegenerative disorders affecting humans and animals associated with the conformational conversion of the cellular prion protein (Prp, ~210 residues) into an oligomeric, $\beta$-sheet rich form.[3] A peculiar aspect of these proteins is their ability to bind several copper(II) ions,[4] although the physio-pathological implications of this interaction are not known.

With a goal of inhibiting the devastating oxidative damage caused by PrP in prion diseases, we explored AMP-$(Im)_2$, **4**, as an inhibitor of the oxidative reactivity associated with the Cu(II) complex of the $Prp_{84-114}$ fragment. Specifically, we report here on the characterization of a complex of Cu(II) with AMP-$(Im)_2$, including the stoichiometry, geometry, and

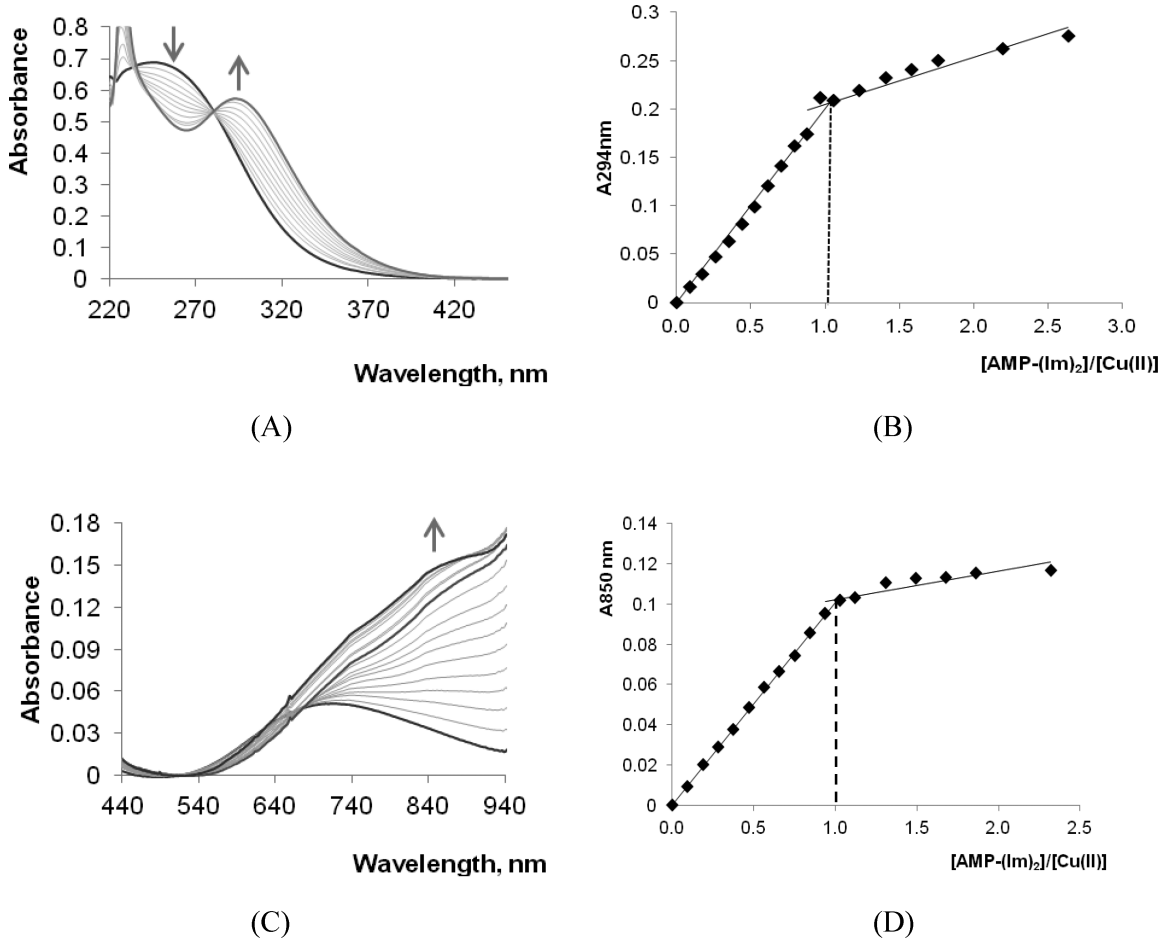


**Figure 2.** (A) Titration of a $4 \times 10^{-5}$ M solution of copper(II) perchlorate in 5 mM Hepes buffer (pH 7.4) with AMP-$(Im)_2$, from 0 to 1.0 equiv (cell path of 10 cm, room temperature). (B) Variation of absorbance data at 294 nm, corrected for background and dilution vs the AMP-$(Im)_2$:Cu(II) molar ratio from 0 to 2.5 equiv. (C) Titration of a $2 \times 10^{-4}$ M solution of copper(II) perchlorate in 5 mM Hepes buffer (pH 7.4) with AMP-$(Im)_2$ from 0 to 2.5 equiv (cell path of 10 cm, room temperature). (D) Variation of absorbance data at 850 nm, corrected for background and dilution vs the AMP-$(Im)_2$:Cu(II) molar ratio.



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coordination sphere. In addition, we describe the chemical and electrochemical stability of the complex and the effect of the chelator, AMP-$(Im)_2$, on the redox potential of copper ion. Furthermore, we explored the ability of AMP-$(Im)_2$ to inhibit the oxidation of a catecholamine model compound (4-methylcatechol) by Cu(II)-$PrP_{84-114}$ and to inhibit oxidation and chemical modifications of $PrP_{84-114}$ itself under these conditions. Finally, we studied the mode of action of AMP-$(Im)_2$ by circular dichroism (CD) and cyclic voltammetry.

## RESULTS AND DISCUSSION

**Characterization of the Cu(II) Complexes Formed with AMP-$(Im)_2$.** Replacement of the hydrogen atoms of the amino group in AMPA, **1**, with nitrogen-containing heterocyclic moieties, e.g., AMP-$(Im)_2$, **4**, makes the latter a potential copper and zinc ion chelator. Heterocyclic moieties such as the imidazolyl group mimic histidine, which is known as the best amino acid ligand for Cu(II) and Zn(II) ions in proteins and peptides.[5] Indeed, we recently reported that AMP-$(Im)_2$ forms a most stable complex with Zn(II) (log $\beta$ = 22).[1] AMP-$(Im)_2$ was synthesized by formation of an intermediate Schiff base from imidazolyl aldehyde and AMPA followed by Schiff base reduction by 2-picoline-borane. Various aminomethylene-phosphonate derivatives bearing heterocyclic substituents (i.e., imidazolyl, pyridyl, or thiazolyl) at the aminomethylene carbon atom were also reported as Cu(II) and Ni(II) ion chelators.[6]

It should be noted that under neutral conditions AMP-$(Im)_2$ is expected to coordinate to Cu(II) as a monoanionic ligand, due to the capability of copper(II) to reduce the acidity of the secondary amino group, and therefore, both a 1:1 complex $[Cu(II)\text{-AMP-}(Im)_2]^+$ and a 1:2 complex $[Cu(II)\text{-}(AMP\text{-}(Im)_2)_2]$ can be formed depending on the ligand concentration. To investigate the AMP-$(Im)_2$ binding properties toward Cu(II), we performed a spectrophotometric study monitoring the charge transfer band developing in the range between 250 and 400 nm, with an apparent maximum near 294 nm, upon addition of the ligand to a $4 \times 10^{-5}$ M solution of copper(II) perchlorate in 5 mM Hepes buffer (pH 7.4). The near-ultraviolet band contains contributions from $\pi$(imidazole) → Cu(II)[7] and $\sigma$(amine) → Cu(II) LMCT[8] and is more easily followed than the spectral changes occurring at higher energies, where an overlap between intraligand (imidazole) $\pi \rightarrow \pi^*$ and $\sigma$ (imidazole) → Cu(II) LMCT transitions occur (Figure 2A). An isosbestic point at 278 nm can be observed in the initial part of the titration, up to an ~1:1 ligand:Cu(II) molar ratio. Upon further addition of the ligand, the increase in absorbance is smaller, indicating that further coordination of AMP-$(Im)_2$ molecules occurs with reduced affinity (see Figure S1). The plot in Figure 2B shows the stepwise formation of a high-affinity 1:1 Cu(II)-AMP-$(Im)_2$ complex, $[Cu(II)\text{-AMP-}(Im)_2]^+$, and the successive binding of a second AMP-$(Im)_2$ molecule yielding $[Cu(II)\text{-}(AMP\text{-}(Im)_2)_2]$. This result is confirmed by performing the titration experiment in the low-energy spectral range, where the d−d transitions of the Cu(II) chromophore can be followed. As shown in Figure 2C, the addition of AMP-$(Im)_2$ to a $2 \times 10^{-4}$ M solution of Cu(II) under the same conditions produces a broad increase in absorption above 650 nm, extending to the near-infrared range. As described above, an isosbestic point can be noted in the initial part of the titration [up to the intermediate point outlined in Figure 2C (see selected spectra in Figure S2)]. The plot of the absorbance change taken at 850 nm confirms the formation of a high-affinity $[Cu(II)\text{-AMP-}(Im)_2]^+$ complex (Figure 2D).

**Determination of Stability Constants of Cu(II)-AMP-$(Im)_2$ Complexes.** Analyzing the stability of Cu(II) complexes with ligands often encounters problems due to the strong affinity of this divalent ion compared to those of other first row transition metals and in particular Zn(II). Moreover, ligands with soft binding sites (e.g., N-heterocycles, thiols, or those with $\pi$-bonds) usually form stronger complexes with Cu(II) than with Zn(II).[9] Although AMPA analogues **3**−**9** formed strong complexes with Zn(II) with log $K$ values from 6.69 to 12.68, AMP-Th formed the least stable Zn(II) complex, with the lowest log $K$ value, 6.69.[1] In this study, we determined the stability constants for Cu(II)-AMP-Th and Cu(II)-AMP-$(Im)_2$ complexes by potentiometric pH titration. All stability constants were calculated using Hyperquad software,[10] and the data are listed in Table 1.

**Table 1. Stability Constants for AMPA-Th and AMP-$(Im)_2$ with Zn(II) and Cu(II) for the 1:1 and 2:1 Complexes and Overall Log $\beta$ Values**

| M, ligand | log $K^{M}_{M(L)}$ | log $K^{L}_{M(L_2)}$ | log $\beta^{L}_{M(L_2)}$ |
|---|---|---|---|
| Zn, AMP-Th[a] | 6.69 ± 0.14 | 5.84 ± 0.11 | 12.53 ± 0.24 |
| Cu, AMP-Th[b] | 10.20 ± 0.14 | 6.24 ± 0.14 | 16.44 ± 0.28 |
| Zn, AMP-$(Im)_2$[a] | 12.02 ± 0.16 | 10.24 ± 0.12 | 22.25 ± 0.23 |
| Cu, AMP-$(Im)_2$[b] | 15.55 ± 0.14 | 4.29 ± 0.16 | 19.84 ± 0.30 |

[a]Data from ref 1. [b]In this study, stability constants determined by pH potentiometric titration.

The potentiometric pH titration (Figures S3 and S4) gives a high stability constant for the 1:1 complex of AMP-$(Im)_2$ with Cu(II) [log $K'_{(Cu/AMP\text{-}(Im)_2)}$ = 15.55 ± 0.14], whereas the log $\beta$ value [log $\beta'_{(Cu/(AMP\text{-}(Im)_2)_2)}$ = 19.84 ± 0.30] indicates that binding of the second ligand to form the $ML_2$ species is much weaker for Cu(II) than for Zn(II). The much higher stability of the ML complex with Cu(II) and the huge difference between the stability constants of the ML species for Cu(II) with respect to Zn(II) might be both explained by the different geometry of the complexes. While Zn(II) usually prefers an octahedral coordination sphere, Cu(II) forms stable complexes with four donor ligands in a square planar arrangement and weaker bonding in the axial position, due to strong Jahn−Teller effect.[11]

AMP-Th, **9**, has three coordination sites (*N*-thiazolyl, *N*-amine, and *O*-phosphonate), so that half of the coordination sites of Zn(II) are filled. When a second ligand chelates, too, the symmetry of the whole complex is quite high and the stability constants show that the contribution of the two ligands to the complex is almost equal [log $K'_{(Zn/AMP\text{-}Th)}$ = 6.69 ± 0.14, and log $\beta'_{(Zn/(AMP\text{-}Th)_2)}$ = 5.84 ± 0.11].

In the 1:1 Cu(II)-(AMP-Th) complex, the ligand can bind with three donors ($N_2O$ set) in the Cu(II) equatorial coordination plane. In the Cu(II)-$(AMP\text{-}Th)_2$ complex, it is likely that both ligands will act as bidentate ligands or, less likely, one AMP-Th will bind as a tridentate ligand and the second as a monodentate ligand. Therefore, the stability constant for binding of the second ligand in the 1:2 complex is much lower (~4 log units) as compared to that in the 1:1 complex.

AMP-$(Im)_2$, **4**, has four coordination sites (two *N*-imidazolyl, *N*-amine, and *O*-phosphonate), so the formation


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of the 1:1 complex, $[Cu(II)\text{-}AMP\text{-}(Im)_2]^+$, is particularly favored because the ligand binds with three strong N donors in a system of two five-membered chelate rings. The stability constant for 1:1 complex formation is 3.5 log units higher for copper(II) than for zinc(II). This is confirmed by the crystal structure of $Cu(II)\text{-}AMP(Im)_2$ described below. On the other hand, the stability constant for binding the second ligand to form the 1:2 complex $[Cu(II)\text{-}(AMP(Im)_2)_2]$ is much lower than that of the 1:1 complex, because only one equatorial position is available and binding of further donor groups could occur only opposite to, or displacing, the axial phosphonate ligand in a weak coordination position.

In contrast, zinc(II) can form complexes with higher coordination numbers, and for this reason, the formation of the $ML_2$ complex is particularly strong, with stability constants for the two successive binding equilibria that occur with almost similar values [log $K'_{(Zn/AMP\text{-}(Im)_2)}$ = 12.02 ± 0.16, and log $K''_{(Zn/(AMP\text{-}(Im)_2)_2)}$ = 10.24 ± 0.12].

**Crystal Structure of [Cu(II)-AMP-(Im)$_2$Cl].** A single crystal of the $Cu\text{-}AMP(Im)_2$ complex, crystallized in water under an acetone atmosphere, using a 1:2 Cu(II):ligand **4** ratio, was analyzed by X-ray diffraction. The crystal data are listed in Table S1. The Cu(II) ion was found to be coordinated by five atoms giving rise to a square pyramidal geometry, with the tridentate 3N moiety in the equatorial plane, bound in two fused five-membered chelate rings. This coordination mode represents a strong binding site and the best possible arrangement for a Cu(II) complex (Figure 3). The basal

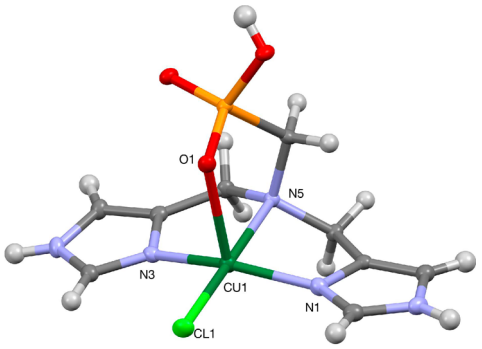


**Figure 3.** Crystal structure of the $[Cu(II)\text{-}AMP\text{-}(Im)_2Cl]$ complex.

coordination plane is completed by the chloride ion, while the apical position is occupied by the O atom of the phosphonate group. The Cu−O bond distance of the apical position is larger than the other copper bond distances of the basal plane, indicating the Jahn−Teller distortion in this complex.

We attempted to obtain a single crystal of the $ML_2$ complex using 2.5 equiv of ligand **4**. No crystals were obtained, but a precipitate was obtained. The latter was analyzed by HRMS as a mixture of $ML_2$ and ML complexes (see the Experimental Section).

The crystal structures of related copper(II) complexes of tripodal triimidazolyl methylamine ligands were previously reported [PIXPAN and PIXPER (Table 2)].[12] The cupric ion in these complexes is coordinated by four nitrogen atoms and a chloride ion in distorted trigonal bipyramidal structures, where three imidazole nitrogen atoms occupy equatorial positions of the trigonal bipyramid, while the amine nitrogen atom and chlorine atom occupy apical sites. Unlike these complexes, in the $[Cu(II)\text{-}AMP\text{-}(Im)_2Cl]$ complex the cupric ion is

**Table 2. Short List of Important Bonds and Angles in the Cu(II)-AMP-(Im)$_2$Cl Complex and Known Related Complexes**

| | $[Cu(II)\text{-}AMP\text{-}(Im)_2Cl]$ | PIXPAN[a] | PIXPER[a] |
|---|---|---|---|
| Cu−N1 (Å) | 1.943 | 2.018 | 2.043 |
| Cu−N3 (Å) | 1.921 | 2.050 | 2.003 |
| Cu−N5 (Å) | 2.124 | 2.144 | 2.124 |
| Cu−O (vertical position) (Å) | 2.442 | 2.078 (Cu−N) | 2.094 (Cu−N) |
| Cu−Cl (Å) | 2.241 | 2.245 | 2.226 |
| N1−Cu−N3 (deg) | 154.05 | 123.66 | 124.69 |
| Cl−Cu−N5 (deg) | 176.56 | 179.67 | 177.56 |
| Cl−Cu−O1 (deg) | 92.30 | 101.23 | 101.70 |

[a]From ref 12.

coordinated by three nitrogen atoms, one oxygen atom, and one chloride ion, resulting in a square pyramidal structure (equatorial positions are occupied by three nitrogen atoms and a chloride ion, while the apical position is occupied by the O atom of the phosphonate group).

**Chelation by AMP-(Im)$_2$ Significantly Reduces the Oxidation Potential of Cu(II).** Because free Cu(II) ion acts as a moderate oxidizing agent, we explored the effect of ligand **4** on the reduction potential of the copper ion. Cyclic voltammograms (CVs) were measured for $Cu(ClO_4)_2$- and $AMP\text{-}(Im)_2$-chelated copper ions.

Comparison between the voltammograms of the electrolyte solution [50 mM Hepes buffer (pH 7.4)] devoid of the metal ion and the ligand (black line in Figure 4) and the same

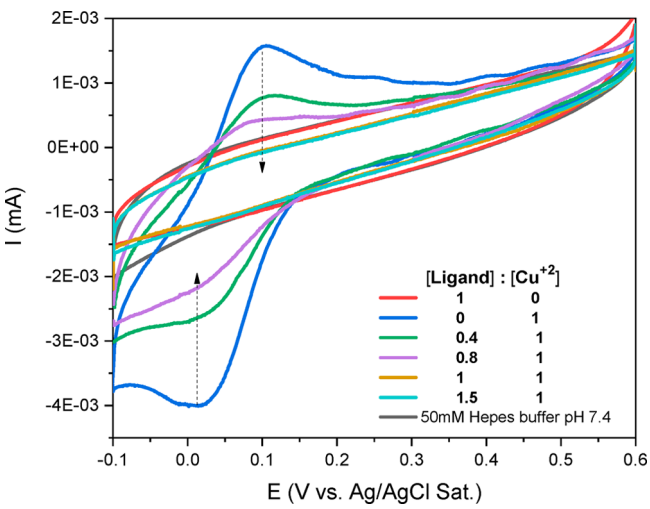


**Figure 4.** Cyclic voltammograms of 50 mM Hepes buffer (pH 7.4) without the ligand and copper ions (black), with 20 mM $AMP\text{-}(Im)_2$ (red), with 0.25 mM $Cu(ClO_4)_2$ in Hepes buffer (blue), and with a mixture of $AMP\text{-}(Im)_2$ and $Cu(ClO_4)_2$ in different ratios (green, purple, brown, and turquoise). All of the measurements were conducted in deaerated solutions at a scan rate of 50 mV/s.

solution after the addition of $AMP\text{-}(Im)_2$ (red line in Figure 4) clearly shows that the ligand is not electrochemically active in the voltage range from 0.6 to −0.1 V versus AglAgCl. The CV of the electrolyte solution containing 0.25 mM $Cu(ClO_4)_2$ without the ligand (blue line in Figure 4) disclosed a clear redox reaction at 0.06 V versus AglAgCl, which corresponds to the one-electron redox of Cu(II)/Cu(I) (note that the


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voltammogram shape suggests a high resistance of the system, due to the relatively low concentration of the electrolyte solution, specifically chosen to keep the experimental conditions of the different measurements, e.g., potentiometric titration, constant). The AMP-$(Im)_2$ ligand was gradually added to the solution until the characteristic copper peak completely disappeared at a 1:1 ligand:Cu(II) molar ratio. Upon further addition of the ligand, there was still no electrochemical response, indicating that the chelated copper ions are electrochemically inactive within the measured window. This confirms the formation of a 1:1 Cu(II) complex under these conditions.

**AMP-$(Im)_2$ Is Chemically Stable.** We have also analyzed the pH-dependent chemical stability of AMP-$(Im)_2$, **4**, by ultraviolet (UV) measurements at room temperature.

UV spectra (Figure S5) showed that the absorption maxima at pH 1, 7, and 13 remained at 225 and 275 nm. The band at 225 nm at pH 13 has a lower intensity, which can be explained by the deprotonation of the ammonium group.

The band at 275 nm has the highest intensity at pH 1. This band can be related to the AMP-$(Im)_2$ species that is protonated at all nitrogen donor groups. The UV spectra show that AMP-$(Im)_2$ did not decompose under alkaline (pH 13) or acidic (pH 1) conditions at room temperature.

**The Effect of AMP-$(Im)_2$ on Cu(II)/$Prp_{84-114}$ Induced Oxidative Activity.** Encouraged by the promising properties of AMP-$(Im)_2$ described above, we next explored its application to the inhibition of the devastating oxidative activity of the Cu(II)-PrP complex.[13] Specifically, AMP-$(Im)_2$ by acting as copper ion chelator may remove the metal ion from the copper-peptide complex or form a ternary complex that prevents reduction of the cupric ion. For these experiments, we used the $Prp_{84-114}$ fragment, which includes the high-affinity Cu(II) binding site of the protein, comprising His85 and two adjacent deprotonated amide groups within the octarepeat sequence (PHGGGWGQ) and His111 (or His96) as copper(II) ligands (Figure 5).[2] The copper(II) complexes

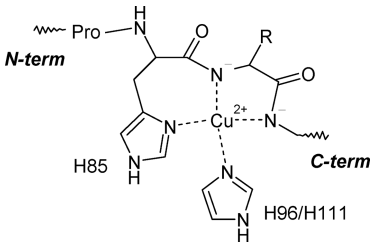


**Figure 5.** Proposed structure of the Cu(II) complex with prion fragment $Prp_{84-114}$.[2]

with this and related PrP peptides have been recently used to show the amplification of toxic effects associated with Cu redox cycling in the presence of catecholamines and related catechols.[14]

The inhibition experiments were carried out by assessing the effect of AMP-$(Im)_2$ in the aerobic oxidation of the model compound 4-methylcatechol (MC) promoted by the Cu(II)-$Prp_{84-114}$ complex. The reaction produces the corresponding 4-methylquinone (MQ) in the initial phase and more complex oligomeric products at longer times.[14] Given the low concentration range of the Cu-peptide complex and the competing binding properties of MC (used in large excess) toward Cu(II), we chose to investigate the kinetics of MC oxidation at a Cu(II):$Prp_{84-114}$ molar ratio of 1:2. Indeed, it was previously reported that under these conditions almost all copper(II) is bound to the peptide even in a dilute solution.[14] When catechol oxidation by Cu(II)-$Prp_{84-114}$ was studied in the presence of AMP-$(Im)_2$, a rapid decrease in the rate was observed as a function of the added ligand (Figure 6). Actually,

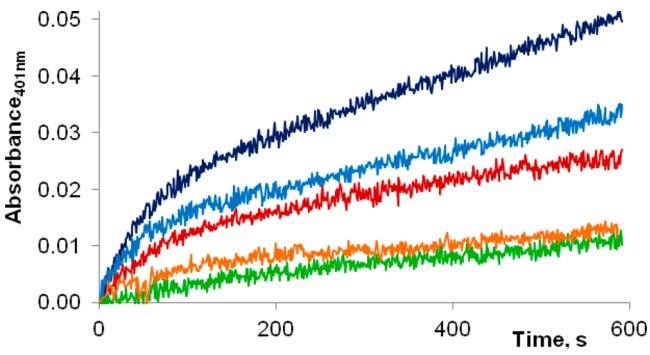


**Figure 6.** Kinetic profiles of oxidation of 4-methylcatechol (3 mM) in 50 mM Hepes buffer (pH 7.4) at 25 °C in the presence of (a) Cu(II) (25 $\mu$M) (red); (b) Cu(II) (25 $\mu$M) and $Prp_{84-114}$ (50 $\mu$M) (dark blue); (c) Cu(II) (25 $\mu$M), $Prp_{84-114}$ (50 $\mu$M), and AMP-$(Im)_2$ (10 $\mu$M) (light blue); (d) Cu(II) (25 $\mu$M), $Prp_{84-114}$ (50 $\mu$M), and AMP-$(Im)_2$ (25 $\mu$M) (orange); and (e) Cu(II) (25 $\mu$M), $Prp_{84-114}$ (50 $\mu$M), and AMP-$(Im)_2$ (50 $\mu$M) (green ).

at a 1:1 AMP-$(Im)_2$:$Prp_{84-114}$ molar ratio, the reaction was almost totally quenched. This suggests that AMP-$(Im)_2$ either acts as strong chelator of Cu(II), abstracting it from the $Prp_{84-114}$ complex, or is able to form a ternary, redox inactive Cu(II)-$Prp_{84-114}$-[AMP-$(Im)_2$] complex. Notably, the Cu(II)-AMP-$(Im)_2$ complex used as a control displayed no significant reactivity toward MC oxidation (Figure S6).

**Analysis of $Prp_{84-114}$ Oxidative Modifications.** As shown by our recent study, the copper redox activation promoted by coordination of PrP peptides not only affects MC, the external substrate, but also produces several types of modifications at the endogenous PrP peptide.[14] It is therefore important to establish whether the presence of the ligand AMP-$(Im)_2$ also inhibits the redox reactivity of copper toward the peptide. To this end, samples of the reaction mixtures of Cu(II)-$Prp_{84-114}$, MC, and AMP-$(Im)_2$ as used in the kinetic studies were analyzed at various times by liquid chromatography−mass spectrometry (LC−MS). The data collected (Table 3) show that indeed the presence of AMP-$(Im)_2$ protects the peptide from oxidation and modification, even though complete protection would require larger amounts of the ligand.

The types of modifications identified by LC−MS analysis include (a) insertion of an O atom into Met109, Met112, or His96 (which can be hardly differentiated) or His85 (+16 mass increment), (b) insertion of two atoms at the two Met residues or a Met and a His residue (+32), (c) addition of methyl quinone (MQ) to His96 or His111 (+120), and (d) a double modification consisting of addition of MQ to one His residue (+120) and insertion of an O atom into Met or His (+16), yielding a total mass increment of +136 (see Figure 7).

**CD Investigation of the Interaction of AMP-$(Im)_2$ with the Cu(II)/$Prp_{84-114}$ Complex.** The far-UV CD spectrum in neutral buffer of $Prp_{84-114}$ is characterized by a negative minimum near 198 nm, indicative of its unstructured nature in solution (Figure 8). The CD spectrum of the corresponding


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**Table 3. Modifications of $Prp_{84-114}$ Detected by LC−ESI-MS Analysis upon Oxidation of MC (3 mM) Promoted by Cu(II) (25 $\mu$M) and $Prp_{84-114}$ (50 $\mu$M), in the Absence and Presence of AMP-$(Im)_2$ (50 $\mu$M)**

| time (min) | $Prp_{84-114}$ (%) | +16 (%) | +32 (%) | +120 (%) | +136 (%) |
|---|---|---|---|---|---|
| | | Cu(II)-$Prp_{84-114}$ + MC | | | |
| 20 | 86.2 | 4.0 | 1.5 | 8.3 | 0.0 |
| 110 | 45.2 | 16.0 | 11.5 | 11.0 | 16.3 |
| 200 | 37.8 | 20.4 | 14.8 | 10.5 | 16.4 |
| | | Cu(II)-$Prp_{84-114}$ + MC + AMP-$(Im)_2$ | | | |
| 20 | 93.6 | 4.6 | 0.6 | 1.2 | 0.0 |
| 110 | 85.9 | 2.6 | 0.9 | 10.5 | 0.0 |
| 200 | 85.5 | 6.9 | 1.0 | 6.6 | 0.0 |

copper(II) complex can be almost superimposed with that of the free peptide, with only a slight decrease in the intensity of the 198 nm peak. Also, the addition of AMP-$(Im)_2$ has a negligible effect on the CD spectrum, indicating that the presence of this ligand does not affect the structure of $Prp_{84-114}$.

The visible portion of the CD spectrum of Cu(II)-$Prp_{84-114}$ is shown in Figure 9. The main features are the weak positive band around 525 nm and a stronger negative CD activity at a higher energy, between 300 and 350 nm.[15] Addition of 1 equiv of AMP-$(Im)_2$ to the Cu(II)-$Prp_{84-114}$ complex yields a red shift of the 525 nm band to ~600 nm and an inversion of the near-UV CD activity in the range of 300−350 nm. These changes suggest the formation of a Cu(II)/$Prp_{84-114}$/AMP-$(Im)_2$ ternary complex rather than copper abstraction by the ligand from the Cu(II)-$Prp_{84-114}$ complex, as the Cu(II)-AMP-$(Im)_2$ complex would give a flat CD curve because it is not optically active.

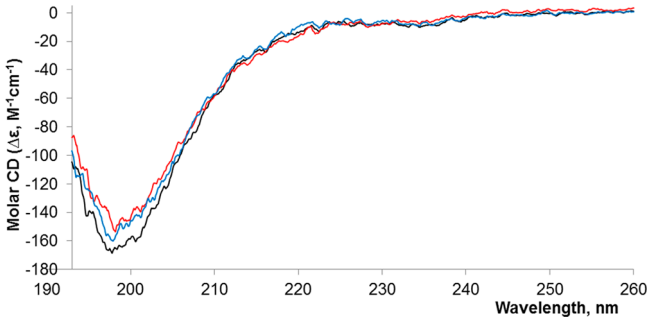


**Figure 8.** Far-UV CD spectra of $Prp_{84-114}$ (50 $\mu$M) in 5 mM phosphate buffer (pH 7.3) (black line) and after addition of 1 equiv of Cu(II) (red line) and after further addition of 1 equiv of AMP-$(Im)_2$ (blue line).

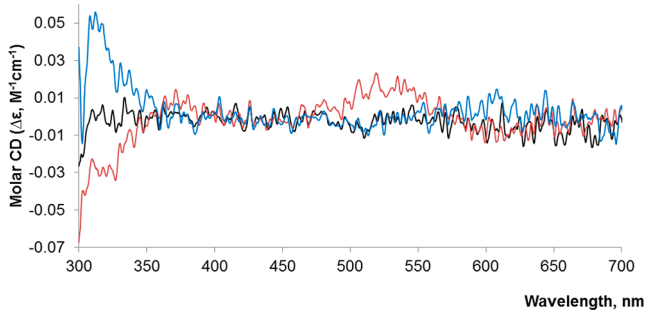


**Figure 9.** Visible CD trace of $Prp_{84-114}$ (740 $\mu$M) in 5 mM phosphate buffer (pH 7.3) (black line) and spectra after the addition of 1 equiv of Cu(II) (red line) and after further addition of 1 equiv of AMP-$(Im)_2$ (blue line).

## CONCLUSION

The design of AMP-$(Im)_2$, **4**, as a Cu(II)/Zn(II) chelator, was inspired by both EDTA and L-histidine, which are known to be excellent chelators of soft and borderline M(II) ions such as Cu(II) and Zn(II).

Indeed, AMP-$(Im)_2$, **4**, was shown in this study to form a highly chemically and electrochemically stable complex with Cu(II). This complex was proven to exist predominantly as a ML species, N3O1(Cl). X-ray crystallography indicated that the thermodynamically stable complex is square pyramidal.

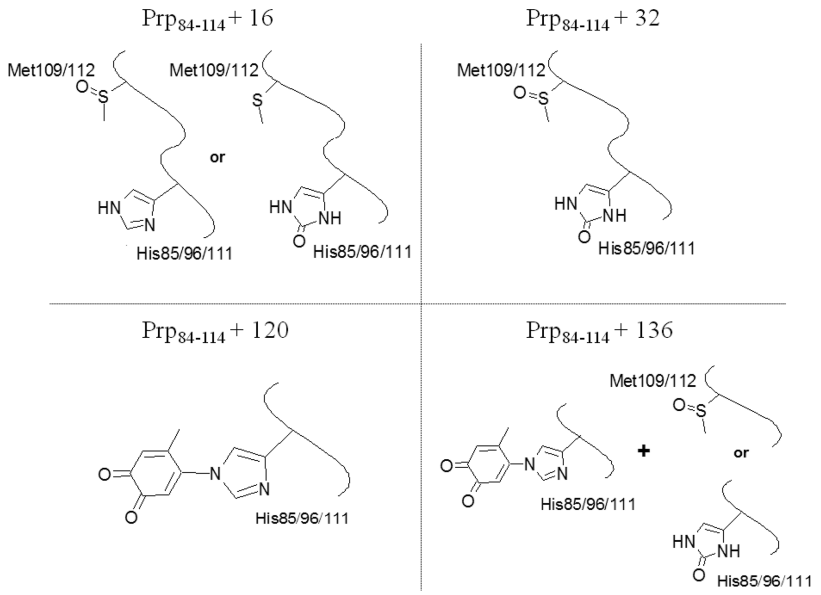


**Figure 7.** Schematic illustration of the type of chemical modifications undergone by the $Prp_{84-114}$ fragment upon catechol-induced Cu redox cycling.



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Furthermore, potentiometric pH titrations indicated a high stability constant for the 1:1 complex with Cu(II) [log $K'_{(Cu/AMP\text{-}(Im)_2)}$ = 15.55 ± 0.14, and log $\beta'_{(Cu/(AMP\text{-}(Im)_2)_2)}$ = 19.84 ± 0.30]. While $CuCl_2$ showed redox reaction at 0.13 V versus AgIAgCl, one-electron redox of Cu(II)/Cu(I), the 1:2 $CuCl_2$-AMP-$(Im)_2$ complex, showed an irreversible reduction process peaking at −0.35 V versus AgIAgCl. The observation of the high stability of Cu(II) complexes of **4**, together with the antioxidant nature of **4**, encouraged us to explore its potential as an inhibitor of oxidation induced by the Cu(II)−prion peptide complex.

Indeed, ligand **4** was found to be a potent antioxidant that at a 1:1 AMP-$(Im)_2$:Cu(II)-$Prp_{84-114}$ molar ratio almost totally inhibited the oxidation reaction of methylcatechol. CD data suggest that this antioxidant activity is due to the formation of a ternary, redox inactive Cu(II)-$Prp_{84-114}$-[AMP-$(Im)_2$] complex. Future studies in prion disease animal models are warranted to assess the potential of **4** for delaying the progression of signs of neurodegeneration by inhibiting the devastating oxidative damage caused by PrP.

## EXPERIMENTAL SECTION

**General.** CD spectra were recorded with a Jasco J-710 spectropolarimeter. Mass spectra and LC-MS/MS data were obtained with a LCQ ADV MAX ion-trap mass spectrometer, with an ESI ion source. The system was run in automated LC-MS/MS mode and using a surveyor high-performance liquid chromatography (HPLC) system (Thermo Finnigan, San Jose, CA) equipped with a Phenomenex Jupiter 4u Proteo column (4 μm, 150 mm × 2.0 mm). For the analysis of peptide fragments, Bioworks 3.1 and Xcalibur 2.0.7 SP1 software were used (Thermo Finnigan). UV−visible (UV−vis) spectra and kinetic data were recorded on an Agilent 8453 diode array spectrophotometer, equipped with a thermostated, magnetically stirred optical cell. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 spectrometer. Absorption data were measured with a Shimadzu UV−vis 2401PC spectrophotometer. Compounds were characterized by NMR spectroscopy with Bruker spectrometers. $^1$H, $^{31}$P, and $^{13}$C NMR spectra were measured with a Bruker DPX-300 spectrometer (300 and 75 MHz for $^1$H and $^{13}$C, respectively) and an AV-400 spectrometer (400, 162, and 100.6 MHz for $^1$H and $^{13}$C). All spectra were measured at room temperature with $D_2O$ samples. High-resolution mass spectra were recorded with an AutoSpec-E FISION VG (Waters) mass spectrometer by chemical ionization. Primary purification of ligand **4** was achieved on an LC (Isco UA-6) system using a column of Sephadex DEAE-A25, swollen in 1 M $NaHCO_3$ or 1 M TEAB at 4 °C for 24 h. The resin was washed with deionized water before use. LC separation was monitored by UV detection at 220 and 190 nm.

**Procedure for the Synthesis of AMP-$(Im)_2$, Compound 4.** AMP-$(Im)_2$, **4**, was synthesized as described previously.[1] Purification of compound **4** was performed by subjecting the reaction's residue to ion-exchange chromatography (on DEAE-Sephadex A-25 chloride form, swollen overnight in a 1 M $NaHCO_3$ solution at 4 °C). The product was eluted with a gradient from 0 to 0.5 M TEAB (triethylammonium bicarbonate) at pH 7.6 (adjusting the pH with a 5% HCl solution). The product was obtained at 0.05 M TEAB. Freeze-drying provided the product as a white solid (325 mg, 27% yield).

**Titration of Cu(II) with Compound 4 Monitored by UV−Vis Spectroscopy.** Titrations of Cu(II) with compound **4** were performed in a quartz optical cell with a path length of 10 cm. To monitor the spectral changes in the near-UV range (collecting data at 294 nm), a 4 × $10^{-5}$ M solution of copper(II) perchlorate in 5 mM Hepes buffer at pH 7.4 (22 mL) was titrated with small portions of a slightly acidic aqueous solution of AMP-$(Im)_2$ (0.03 M) at room temperature, with ligand additions of 0−2.5 equiv. The final pH of the titrated solution was measured to exclude any pH variation during the titration. The absorbance readings were corrected for background, at 510 nm, and for dilution. The titration experiment with AMP-$(Im)_2$ (0.03 M) was repeated on a 2 × $10^{-4}$ M Cu(II) solution in the same buffer following the absorbance changes in the visible and near-infrared range, with optical readings recorded at 850 nm. Also in this case, correction for background at 510 nm and for dilution was made.

**Potentiometric pH Titration of Cu-AMP-$(Im)_2$ Complexes.** Potentiometric titrations were performed in an aqueous solution with 0.1 M $NaNO_3$ as the supporting electrolyte in a thermostated cell at 25 °C under a nitrogen atmosphere. Protonation constants of AMP-$(Im)_2$ were determined at a constant ionic strength, carrying out the experiments on 10 mL of a 5 × $10^{-4}$ M solution of the ligand (containing $NaNO_3$ salt) to which an excess of $HNO_3$ was added. Titrations were performed by adding small aliquots of standard NaOH (0.1 M), from pH 2.5 to 12. Copper binding constants were determined under the same conditions, starting from a 3.5 × $10^{-4}$ M solution of [Cu-(AMP-$(Im_2)$)$_2$]. The standard electrochemical potential of the glass electrode was previously determined with a titration in accordance with the method of Gans.[16] The experimental data were processed with Hyperquad to determine equilibrium constants.[10]

**Crystallization of [Cu(II)-AMP-$(Im)_2$Cl].** Compound **4** (20 mg) was dissolved in HPLC grade water (1000 μL), and 0.11 M $CuCl_2$ (680 μL in water) was added. The mixture was mixed for 1 h and then freeze-dried for 2 days. The residue was dissolved in HPLC grade water (300 μL), and the vial was immersed in an acetone environment. After 2 days (at room temperature), blue single crystals were obtained. HRMS ESI (positive $m/z$): $[C_9H_{13}CuN_5O_3P]^{+\bullet}$ calcd 333.0047, found 333.0049; $[C_9H_{14}CuN_5O_3P]^+$ calcd 334.0125, found 334.0101.

**Electrochemical Measurements.** All electrochemical measurements were conducted in a three-electrode Teflon cell, where the working electrode was a glassy carbon disk electrode (0.196 cm$^2$), the counter electrode was a glassy carbon rod, and the reference electrode was a Ag/AgCl electrode. Throughout all measurements, Ar gas was purged into the cell to keep the system inert from oxygen.

**Peptide Synthesis.** The $Prp_{84-114}$ peptide (Ac-PHGGGWGQG-GGTHSQWNKPSKPKTNMKHMAG-$NH_2$, molecular weight of 3297.65) was synthesized using the standard fluorenyl methoxy carbonyl (Fmoc) solid phase method in dimethylformamide, following a literature procedure.[14] Purification was performed by HPLC, using a linear gradient of water, containing 0.1% trifluoroacetic acid (TFA), and acetonitrile, also containing 0.1% TFA, from 0 to 100%. The column was a Jupiter 4u proteo 90A 250 mm × 10 mm, 4 μm column; elution was carried out at flux rate of 5 mL/min. The retention time of the peptide was 12 min. The product was lyophilized, yielding a white solid, which was characterized by ESI-MS (direct injection, MeOH, positive ion mode, capillary temperature of 200 °C, $m/z$): +472.14 ($Prp_{84-114}H_7^{7+}$), +550.65 ($Prp_{84-114}H_6^{6+}$), +660.52 ($Prp_{84-114}H_5^{5+}$), +825.3 ($Prp_{84-114}H_4^{4+}$), +1099.99 ($Prp_{84-114}H_3^{3+}$).

**Kinetics of 4-Methylcatechol (MC) Oxidation by Cu(II)-$Prp_{84-114}$ in the Presence of AMP-$(Im)_2$.** The dependence of the rate of MC oxidation promoted by Cu(II)-Prp on AMP-$(Im)_2$ concentration was studied in 50 mM Hepes buffer (pH 7.4) and at 25 °C. The experiments were carried out spectrophotometrically, following the increase in the intensity of the band at 401 nm due to formation of 4-methyl quinone ($\varepsilon$ = 1550 $M^{-1}$ $cm^{-1}$). Initially, MC (3 mM) autoxidation, MC (3 mM) oxidation promoted by Cu(II) salt [25 μM copper(II) nitrate], and MC (3 mM) oxidation by a 2:1 $Prp_{84-114}$-Cu(II) complex [50 μM $Prp_{84-114}$ and 25 μM Cu(II)] were evaluated. Then, the dependence of the rate on AMP-$(Im)_2$ concentration was investigated by comparison of kinetic profiles at increasing AMP-$(Im)_2$ concentrations (from 10 to 50 μM), maintaining all other reagents unchanged.

**Characterization of Modified $Prp_{84-114}$ by HPLC−ESI-MS Analysis.** The competitive modification of $Prp_{84-114}$ during MC oxidation was investigated by HPLC−ESI-MS analysis, preparing samples of 25 μM copper(II) nitrate, 50 μM $Prp_{84-114}$, and 3 mM MC in the absence and presence of AMP-$(Im)_2$ (50 μM), in 50 mM


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Hepes buffer (pH 7.4). LC−ESI-MS analyses were performed at different reaction times. The elution of reaction mixtures was carried out with a linear gradient of $H_2O$ (with 0.1% HCOOH) and acetonitrile (with 0.1% HCOOH), at a flow rate of 0.2 mL/min. The identification of specific modifications was performed with a Bioworks database.

**CD Measurements.** The far-UV CD spectrum of a 50 $\mu$M solution of $Prp_{84-114}$ in 5 mM phosphate buffer (pH 7.3) was recorded in a cell with a path length of 0.1 cm. The spectral changes upon addition of 1 equiv of $Cu(NO_3)_2$ to the $Prp_{84-114}$ solution and the effect of further addition of 1 equiv of AMP-$(Im)_2$ were subsequently assessed. Spectra were recorded with a scanning rate of 100 nm/min with 10 accumulations.

The visible CD spectrum of a solution of $Prp_{84-114}$ (740 $\mu$M) in the same buffer was recorded in a cell with a path length of 1 cm. Spectra were then recorded after addition of 1 equiv of $Cu(NO_3)_2$ to the peptide solution and upon further addition of 1 equiv of AMP-$(Im)_2$ to the Cu(II)-$Prp_{84-114}$ solution. Spectra were recorded with a scanning rate of 100 nm/min with six accumulations.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.9b00287.

Tables S1−S6 and Figures S1−S6 (PDF)

### Accession Codes

CCDC 1893813 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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### Notes

The authors declare no competing financial interest.

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