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Tripodal scaffolds with three appended imidazole thiones for Cu(I) chelation and protection from Cu-mediated oxidative stress

Ranajit Das [b,1], Michael Claron [a,1], Colette Lebrun [a], Jacques Pécaut [a], Srinivas Ambala [b], Christelle Gateau [a], Gouriprasanna Roy [c,*], Pascale Delangle [a,*]

[a] Univ. Grenoble Alpes, CEA, CNRS, IRIG, SyMMES, F-38000 Grenoble, France
[b] Department of Chemistry, School of Natural Sciences, Shiv Nadar University, NH91, Dadri, Gautam Budha Nagar, UP 201314, India
[c] Department of Chemistry, Indian Institute of Technology Tirupati, Tirupati, AP 517 506, India

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ABSTRACT

Imidazole thiones appear as interesting building blocks for Cu(I) chelation and protection against Cu-mediated oxidative stress. Therefore, a series of tripodal molecules derived from nitrilotriacetic acid appended with three imidazole thiones belonging either to histamine-like or histidine-like moieties were synthesized. These tripods demonstrate intermediate affinity between that previously measured for tripodal analogues bearing three thiol moieties such as cysteine and those grafted with three thioethers, like methionines, consistently with the thione group in the imidazole thione moiety existing as a tautomer between a thiol and a thione. The two non-alkylated tripods derived from thioimidazole, $\mathbf{T^H}$ and $\mathbf{T^H}$* demonstrated three orders of magnitude larger affinity for Cu(I) ($\log K^{pH\ 7.4} = 14.3$) than their analogues derived from N,N′-dialkylated thioimidazole $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ ($\log K^{pH\ 7.4} =$ 11–11.6). Their efficiency to inhibit Cu-mediated oxidative stress is demonstrated by several assays involving ascorbate consumption or biomolecule damages and correlates with their ability to chelate Cu(I), related to their conditional complexation constants at pH 7.4. The two non-alkylated tripods derived from thioimidazole, $\mathbf{T^H}$ and $\mathbf{T^H}$* are significantly more powerful in reducing Cu-mediated oxidative stress than their analogues derived from N,N′-dialkylated thioimidazole $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$.

1. Introduction

Copper (Cu), is an essential micronutrient to all human beings and it is involved in many biological processes essential to Life. Nonetheless, excess Cu is equally adverse and toxic as it can produce highly toxic hydroxyl radical ($HO^{\bullet}$) from hydrogen peroxide (H_2O_2) via Fenton-like reactions and thereby can cause oxidative damage to biomolecules including proteins, lipids and nucleic acids. The intracellular Cu concentration is thus strictly controlled and highly regulated by a pool of proteins of which metallothioneins (MT), which are Cu storage proteins, Cu exporting proteins such as the two ATPases, ATP7A and ATP7B, and intracellular Cu trafficking chaperones such as the antioxidant protein 1 (ATOX1) and the copper chaperone for superoxide dismutase (CCS) [1,2]. Most of the cytosolic Cu is bound to the abundant endogenous thiol-containing tripeptide glutathione (GSH), which is known to be the most important contributor to Cu exchangeable pool in the cytosol. The main contributors to Cu homeostasis are presented in Fig. 1. Furthermore, mutations of the *ATP7B* gene result in malfunctioning of the ATP7B protein causing Cu overload in tissues including brain, liver, eyes and kidneys of patients, which eventually leads to Wilson's disease (WD) [3,4]. Cu is also known to be involved in the progression of numerous neurodegenerative disorders including Alzheimer's (AD) and

Abbreviations: AD, Alzheimer's disease; Asc, ascorbate; Atox1, antioxidant protein 1; BCA, bicinchoninic acid; Boc, N-tert-butoxycarbonyl; BSA, bovine serum albumin; CCS, copper chaperone for superoxide dismutase; Cys, cysteine; DIPEA, diisopropylethyl amine; DNA, deoxyribonucleic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ESI-MS, electrospray ionization mass spectrometry; FZ, ferrozine; GSH, glutathione; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; Met, methionine; NHS, N-hydroxy succinimide; NTA, nitrilotriacetic acid; PD, Parkinson's disease; ROS, reactive oxygen species; RP18 HPLC, C-18 reverse phase high performance liquid chromatography; Tpm, tris(3,5-dimethylpyrazolyl)-methane; WD, Wilson disease.

* Corresponding authors.
E-mail addresses: gproy@iittp.ac.in (G. Roy), pascale.delangle@cea.fr (P. Delangle).
[1] contributed equally to this work.

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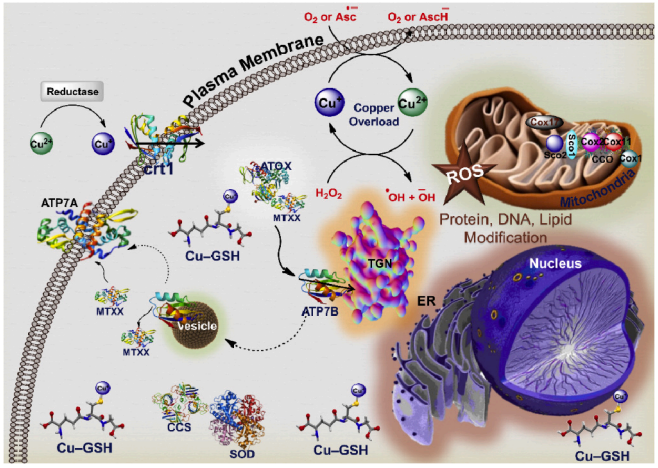


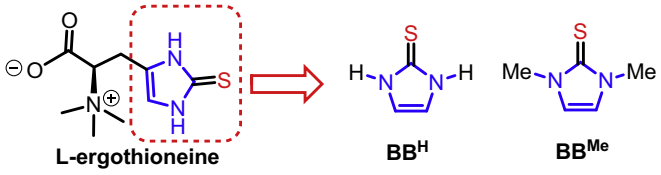
Fig. 1. Schematic representation of Cu homeostasis and Cu overload that leads to oxidative stress in the cellular environment. In case of overload, Cu is mostly bound to GSH or other small endogenous ligands.

Parkinson's diseases (PD) [5].

Treatments in Wilson's disease include lifelong medication with Cu chelators such as D-penicillamine and trientine or zinc therapy, which induce Cu binding in blood or tissues and facilitates its excretion [6,7]. However, commonly known chelators are not always efficient for symptomatic neurological patients and have harmful side effects. Thus, efforts were made to design organ and tissue-specific Cu chelators with high specificity for Cu(I) [8–12]. Indeed, Cu is found in our body in both Cu(I) and Cu(II) oxidation states. Although dietary Cu is mostly found in the Cu(II) state, intracellular Cu is found in the reduced state Cu(I), due to the large concentration of GSH reaching up to the millimolar range in cells. The soft Lewis-acid character of Cu(I) favors its binding to proteins in either linear, trigonal, or tetrahedral environments with 2, 3 or 4 sulfur atoms of cysteine (Cys) or methionine (Met) residues [6]. For instance, metallothioneins are small cysteine-rich proteins that store Cu when it is in excess of physiological requirements by the formation of Cu (I) clusters [13,14]. No free Cu is available in the cytosolic environment, as all Cu ions are coordinated to proteins or ligands of low molecular mass such as reduced GSH.

Considering that in the cytosolic reducing environment Cu mostly remains in the +1 oxidation state, it is extremely relevant to target the Cu(I) oxidation state to tackle intracellular Cu overload. Therefore, considering the soft character of Cu(I), thiolate based ligands appeared as candidates of choice for efficient intracellular Cu(I) chelation [15,16]. Moreover, the trigonal CuS_3 coordination found in Cu(I)-Metallothioneins complexes gives access to a larger stability than the digonal CuS_2 coordination mainly found in metallochaperones [12,17]. Therefore, we have turned to the design of tripodal pseudopeptides, which are chelating molecules where three sulfur amino acids are grafted to a nitrilotriacetic scaffold via peptide bonds to get water-soluble chelating agents favoring a CuS_3 coordination [17,18]. As expected, tripods grafted with three negatively charged thiolate functions of cysteines [18] display larger affinities for Cu(I) than tripods grafted with three neutral thioethers groups of methionines [19]. Besides, the comparison between the chiral scaffold derived from cysteines and the non-chiral analogues based on cysteamine, demonstrated that chirality was an asset in these architectures. Indeed the chiral scaffold allows a controlled speciation of the Cu(I)-thiolate species with a larger affinity than the non-chiral tripod [20]. The nitrogen-donor trishistidine pseudopeptide was also investigated and showed a Cu(I) complexing ability in between that of the thiolate and the thioether tripod with a CuN_3X tetrahedral coordination [21].

On the other hand, the naturally occurring amino acid L-ergothioneine (2-mercaptohistidine trimethylbetaine, see Scheme 1) that



Scheme 1. From L-ergothioneine to the two building blocks $\mathbf{BB^H}$ and $\mathbf{BB^{Me}}$ used in this study.

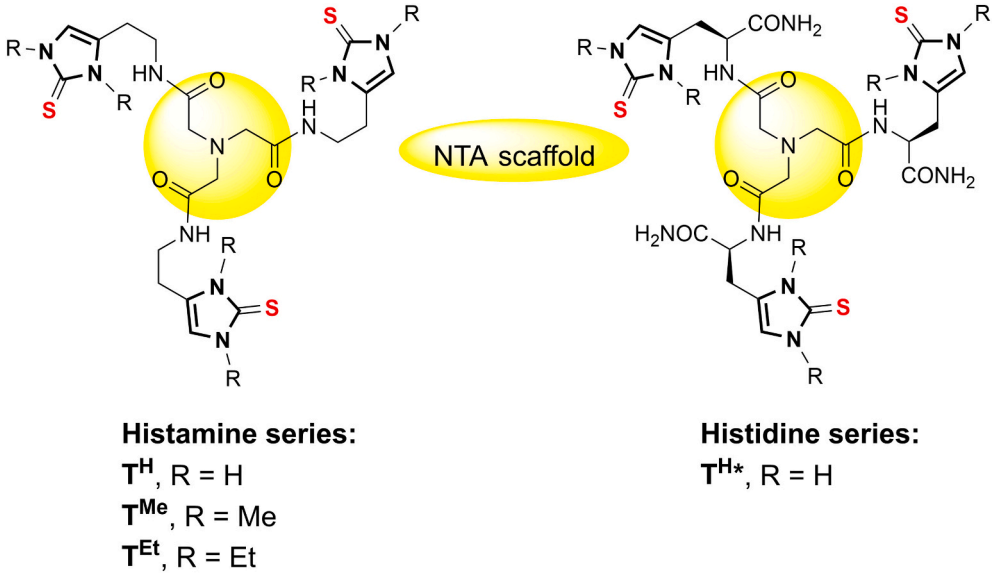
contains an imidazole-2-thione moiety has several unique properties like antioxidant and cytoprotective capabilities against a wide range of cellular stress [22,23]. The thione group of ergothioneine exists as a tautomer between thiol and thione in which the thione is the predominant form at pH 7.4. This makes ergothioneine specific among biological thiol compounds. Moreover, ergothioneine has been found to prevent Cu-induced oxidative damage to DNA and proteins by forming a redox-inactive ergothioneine-Cu complex, which makes ergothioneine of prime interest for its protective effects against Cu-induced cytotoxicity [24]. Kimani *et al.* have previously shown that the *N, N′*-dimethyl imidazole thione, $\mathbf{BB^{Me}}$, binds to the redox-active Cu(I) center of tris (3,5-dimethylpyrazolyl)-methane (Tpm)Cu(I) complex, thereby forming a redox inactive complex, and thus preventing Cu from oxidation and providing targeted sacrificial antioxidant activity [25]. In recent times, Rai *el al.* have also shown that the *N*-substituted imidazole and benzimidazole based thiones 1-methyl-1*H*-imidazole-2(3H)-thione or 1-methyl-1*H*-benzo[*d*]imidazole-2(3*H*)-thione had a favourable effect in protecting biomolecules from Cu(I)-mediated oxidative stress through Cu(I) coordination [26]. The copper-bound *N*-substituted imidazole and benzimidazole-based thiones are more reactive towards hydrogen peroxide than the copper-bound *N,N′*-disubstituted thiones and the corresponding free ligands (thiones). Upon H_2O_2 treatment, the Cu(I)-bound *N*-methyl substituted thiones react readily with H_2O_2, and thus, prevent the oxidation of Cu(I) to Cu(II) [25]. DFT calculations suggest that the higher activity of N-substituted thione compared to the *N,N′*-disubstituted series can be attributed due to the presence of a free N − H group, which can participate in the hydrogen bonding with H_2O_2 and greatly stabilizes the thione-H_2O_2 adduct by the formation of intermolecular hydrogen bonding between the N − H group of the copper-bound *N*-substituted thione and H_2O_2 [26].

Hence, imidazole thiones appear as interesting building blocks for Cu (I) chelation and protection against Cu-mediated oxidative stress. Therefore, the objective of the present work was to combine the two building blocks, $\mathbf{BB^H}$ and $\mathbf{BB^{Me}}$, shown in Scheme 1, with the tripodal approach that enhances the metal-binding affinity, to design efficient Cu (I) chelating agents. In a first series of ligands, NTA (nitrilotriacetic acid) was appended with imidazole thiones belonging to histamine-like moieties to afford the three tripods $\mathbf{T^H}$, $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$. Three chiral histidine-amide-like moieties were grafted to NTA to give $\mathbf{T^{H*}}$ and to evaluate the effect of chirality on the Cu(I) complexes' formation (Scheme 2). In vitro complexation studies with the series of tripodal ligands presented in Scheme 2 demonstrated that these molecules bind Cu(I) in water at pH 7.4 and significantly decrease Cu-mediated oxidative stress.

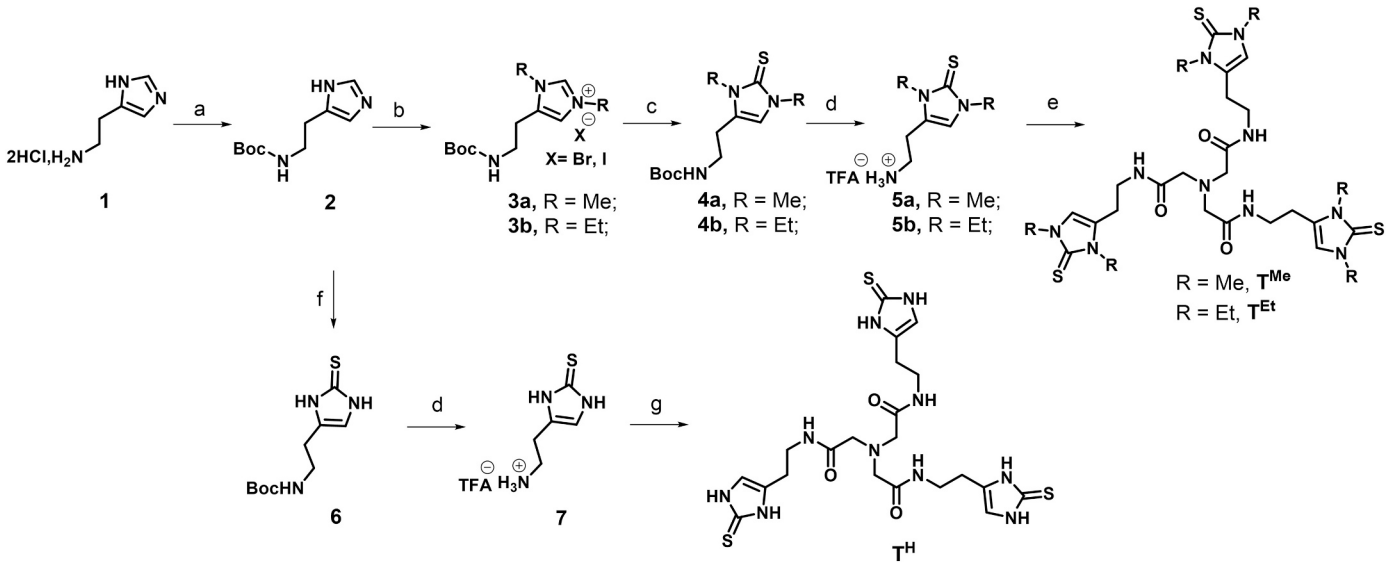
2. Results and discussion

2.1. Synthesis and characterization of the tripodal ligands

The synthesis of the three histamine based tripodal ligands $\mathbf{T^H}$, $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ is described in Scheme 3. Histamine was first selectively protected at the amine function with a Boc (N-tert-butoxycarbonyl) group to afford compound **2**. A two-step process involving first the protection of the two nitrogen atoms of the primary amine and of the imidazole was followed by a selective mild deprotection of the aromatic amine [27]. Introduction of sulfur was then successfully performed in mild conditions reacting *O*-phenyl chlorothionoformate with protected histamine **2**



Scheme 2. Chemical structures of the series of imidazole thione tripodal ligands.

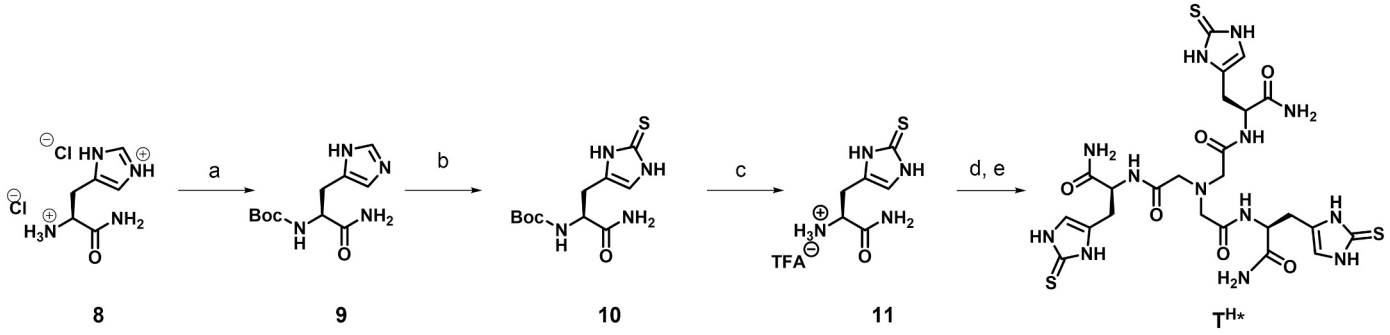


Scheme 3. Reagents and conditions: (a) (i) Et_3N, $(Boc)_2O$, MeOH, 0 °C-RT, 8 h; (ii) K_2CO_3, MeOH reflux, 4 h; 54%; (b) MeI/EtI in acetone, Reflux, 12 h, 90%; (c) K_2CO_3, sulfur powder, MeOH, reflux, 16–24 h, 84–88%; (d) TFA/DCM, quant. Yield; (e) T^{Me}: NTA, HATU, DIPEA in dry DMF, RT, 36 h, 12%; T^{Et}: NTA, HOBt, EDC, DIPEA in dry DMF, RT, 24 h, 35%; (f) (i) *O*-phenyl chlorothionoformate in H_2O/Et_2O, 5 h; (ii) Et_3N in MeOH, 16 h; 63%; (g) NTA, NHS, DIPEA, in DMF, RT 3 h, 25%.

to lead compound **6**, which was then deprotected in acidic conditions to afford compound **7**. The final coupling was performed by using a NHS (N-hydroxy succinimide) ester promoted activation of the NTA template and reacting with 5 equiv. of the thioimidazole precursor **7** in the presence of DIPEA (diisopropylethyl amine) with 25% yield in T^H. To get the alkylated tripods, the dialkylated precursors **3a** and **3b** were first obtained with 90% yield from protected histamine **2** by reacting either methyl or ethyl iodide. Sulfur was then introduced using solid S_8 at reflux during several hours [28,29] to give the two thione derivatives **4a** and **4b**. The two thione precursors **5a** and **5b**, bearing two methyl or ethyl groups, were isolated after deprotection of the amine function of **4a** and **4b** in acidic conditions. The tripodal scaffolds T^{Et} was then synthesized by the acid amine coupling of 3 equiv. of the corresponding histamine building block **5b** with NTA using HOBt (1-hydroxybenzotriazole), EDC-HCl (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) as a coupling reagent with an overall yield of 35%. Similar coupling conditions were tested for the synthesis of T^{Me}. However, the coupling was not efficient even using higher temperature (up to 60 °C) and longer reaction times (Table S1). Finally, T^{Me} was obtained from NTA and 3 equiv. of compound **5a** in 12% yield using HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate) as a coupling reagent.

The synthesis of the chiral histidine-based tripodal ligand T^{H*} is described in Scheme 4, with procedures similar to the ones used for the synthesis of the histamine T^H tripod. Commercial compound **8** was first Boc protected on the amine function to get compound **9**, which gave the thioimidazole derivative **10** after sulfur introduction with *O*-phenyl chlorothionoformate in mild conditions. The latter was successfully coupled to NTA after Boc deprotection in acidic conditions to afford the tripod T^{H*} with three unsubstituted thioimidazole moieties with a yield of 84% (Scheme 4).

The four tripodal ligands were obtained as pure white powders after RP18 HPLC purification. Their full characterization is given in the Supplementary Data.



Scheme 4. Reagents and conditions: (a) Et_3N, $(Boc)_2O$, MeOH, RT, 2 h; (ii) K_2CO_3, MeOH reflux, 4 h; 84% (b) (i) *O*-phenylchlorothionoformate H_2O/Et_2O, 5 h; (ii) Et_3N in MeOH, 16 h; 38%; (c) TFA/DCM, quant. Yield; (d) NTA, NHS, DIPEA in dry DMF, RT, 24 h, yield: $\mathbf{T^{H*}}$, 845%.

2.2. Cu(I) binding experiments

The evaluation of Cu(I) binding with the four thioimidazole tripodal ligands was performed thanks to competition experiments, which were implemented with Cu(I) competing ligands previously reported in the literature. A series of Cu(I) ligands of known stabilities and having characteristic absorption properties for their 1:2 Cu(I) complexes have been obtained over the years [30–32]. Four competitors have been described for the determination of affinity constants at pH 7.4 for Cu(I), each being adapted to the determination of an appropriate range of constants (Table S3). Considering the partial thiolate character of thioimidazole moieties, their affinity is expected to be in between that of thiolates and thioethers. Previous studies with thiolate tripodal ligands grafted with three cysteines or three methionines indicated affinities at pH 7.4 for Cu(I) of ca 10^{19} and 10^{10}, respectively [17–19]. Therefore, we first chose BCA, bicinchoninic acid, which forms the $Cu(I)(BCA)_2$ complex with a $\log\beta_{12} = 17.2$, and allows to measure affinities for Cu(I) in competition experiments for Cu(I) ligands displaying an affinity in the range $\log K \approx 12–16$. In brief, competition experiments were conducted in water at pH 7.4 in anaerobic conditions, in a glove box, to avoid any oxidation reaction. Furthermore, 10% acetonitrile in volume was added to guaranty the absence of Cu(I) disproportionation. In these experiments, the Cu(I) tripod complex was prepared in the solution (ca 50 μM), and BCA was added progressively from 2 equiv. per tripod until the full formation of the $Cu(BCA)_2$ complex detected by its absorption at 582 nm with an absorption coefficient of 7900 $mol^{-1}Lcm^{-1}$. The following reaction, where **T** stands for any tripodal ligand,

$$Cu(I)\mathbf{T} + 2\ BCA \leftrightarrows Cu(I)(BCA)_2 + \mathbf{T} \quad (1)$$

was followed by measuring the UV–visible spectrum obtained after each addition of BCA. A first qualitative analysis of the data can be performed by comparing the percentage of Cu(I) withdrawn from the tripod by addition of 2 equiv. of BCA. This allows a classification of the tripodal ligands according to their Cu(I) binding ability. Fig. 2A shows clearly that the two non-alkylated compounds $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ complex more efficiently Cu(I) than the alkylated ones $\mathbf{T^{Me}}$, $\mathbf{T^{Et}}$, which loose almost all the Cu metal ion upon addition of only 2 equiv. of BCA. The affinity of the two most efficient molecules $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ could thus be calculated from the competition data as shown in Fig. 2B for the tripod $\mathbf{T^H}$, as an example. Stability constants of $10^{14.3}$, calculated for a 1:1 complex, are obtained for both Cu(I) complexes with molecules $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ at pH 7.4. As seen in the following, Cu(I) clusters are formed with these non-alkylated tripods. Although the calculated constant considering the formation of a Cu**T** complex does not fully describe the system, it allows an appropriate comparison with other ligands forming complexes with various stoichiometry.

Another Cu(I) competitor was used to assess the stability constants with the two alkylated tripods. Indeed Fz, Ferrozine, displays a lower affinity for Cu(I), and forms the $Cu(I)(Fz)_2$ complex with a $\log\beta_{12} = 15.1$,

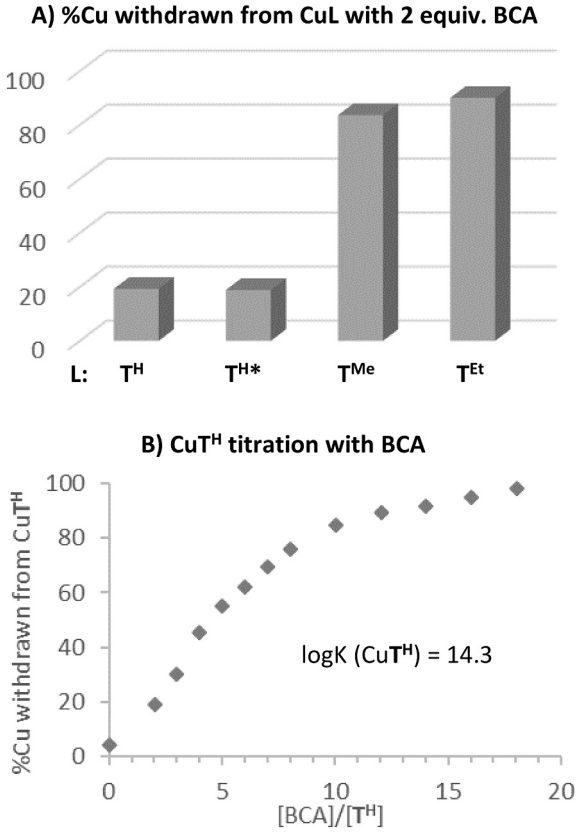


Fig. 2. Competition experiments with BCA, [Cu] = 46 μM, [**L**] = 48 μM in phosphate buffer (20 mM, pH 7.4) / acetonitrile 9/1 *v*/v. **A)** Percentage of Cu withdrawn from the CuL complex by addition of 97 μM BCA (2 equiv. with respect to L) for the 4 tripodal ligands. B) CuT^H titration with BCA in the same experimental conditions.

which allows to measure affinities for Cu(I) in competition experiments in the range $\log K \approx 10–14$ [30,31]. The competition assays with Fz show no Cu(I) displacement from $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ when 2 equiv. of Fz were added, whereas a significant amount of the $Cu(I)(Fz)_2$ complex that absorbs at 470 nm with an absorption coefficient of 4320 $mol^{-1}Lcm^{-1}$ is detected for methylated and ethylated compounds (Fig. S1). These results are consistent with the larger affinity of $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ for Cu(I) in comparison to the alkylated compounds. The data derived from the competition assays with Fz could be fitted for the alkylated compounds to afford the stability constants at pH 7.4, which are reported in Table 1. The series of imidazole thione tripodal ligands developed in this study can thus be classified according to their decreasing Cu(I) affinity: $\mathbf{T^H} \approx \mathbf{T^{H*}} >> \mathbf{T^{Et}} > \mathbf{T^{Me}}$.

Table 1
Conditional Cu(I) complexation constants (log unit), calculated for the formation of Cu**T** complexes, obtained in competition experiments performed in phosphate buffer (pH 7.4, 20 mM) / CH_3CN, 90/10 *v*/v either with BCA or Fz. The number in bracket indicates the error on the last figure.

Tripodal ligand	T^H	T^{Me}	T^{Et}	T^{H*}
logK (Cu**T**)	14.3(2)[§]	11.0(1)[†]	11.6(1)[†]	14.3(1)[§]

[§]Determined with BCA assay. [†]Determined with the Fz assay.

ESI-MS spectra were recorded for different Cu(I) to ligand ratios in aqueous ammonium acetate (pH 6.9) and show striking differences between the alkylated and non-alkylated tripods. Indeed, alkylated tripods, namely $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ show the formation of CuT complexes only, as seen in Fig. 3A for the methyl derivative. By contrast, the non-alkylated versions $\mathbf{T^{H}}$ and $\mathbf{T^{H*}}$ show the presence of several polymetallic species and poor desorption of these complexes leading to low-intensity ESI-MS spectra (Fig. 3B). As observed before, the latter behavior is characteristic of Cu(I) cluster formation, which are more difficult to detect in the ESI-MS spectra, because of their poor desorption and their propensity to fragmentation [33–35]. These data point to different speciation of the Cu(I) complexes depending on the substitution of the nitrogen atoms of the thioimidazole moieties.

The thioimidazole tripodal ligands developed in this study demonstrate significant affinities for Cu(I) in water at pH 7.4 with logK values from 11 to 14.3, K being the Cu(I) conditional complexation constant at pH 7.4. These constants indicate an intermediate affinity between that previously measured for tripodal analogues bearing three thiol moieties such as cysteine [18] or D-penicillamine [36] and those grafted with three thioethers, like methionines [19]. This is consistent with the thione group in the imidazole thione moiety existing as a tautomer between a thiol and a non prototropic neutral thione function. The two tripods $\mathbf{T^{H}}$ and $\mathbf{T^{H}}$* demonstrated three orders of magnitude larger affinity for Cu(I) ($\log K^{pH\ 7.4} = 14.3$) than their bisalkylated analogues $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ ($\log K^{pH\ 7.4} = 11–11.6$). Besides the introduction of chiral thiohistidine moieties in $\mathbf{T^{H}}$* showed no beneficial effect of the ligand's chirality onto Cu(I) chelation, by contrast to tripods derived from cysteine previously investigated [20]. Considering the significant affinity for Cu(I) of the thioimidazole tripodal ligands developed here, we investigated their ability to quench Cu-induced oxidative stress in the following.

2.3. Protection against Cu(I) mediated oxidation of ascorbic acid

It is a well-proven fact that redox-active free Cu facilitates the oxidation of vitamin C (ascorbic acid) to dehydroascorbic acid, which is inhibited by endogenous thiols (cysteine, GSH) and proteins including antioxidant enzymes such as ceruloplasmin, superoxide dismutase, and catalase [37,38]. So, we first checked the ability of the tripods to protect ascorbate (Asc) from Cu-catalyzed oxidation. The disappearance of ascorbate through Cu-catalyzed oxidation was measured experimentally by UV at 265 nm. The Cu(I) complexes were formed previous to oxygen exposure of the samples as reported previously [21]. Fig. 4a shows a very rapid consumption of ascorbate (≈ 600 s) exposed to Cu(I) alone, whereas the reaction is significantly slowed when a tripodal ligand is added in excess with respect to Cu. Interestingly, the thioimidazole based tripods $\mathbf{T^{H}}$ and $\mathbf{T^{H*}}$ are more efficient in protecting Cu-mediated oxidation of ascorbate compared to the *N,N′*-disubstituted thione functionalized tripods $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ (see also Fig. S2). For instance, $\mathbf{T^{H}}$ and $\mathbf{T^{H*}}$ inhibit the oxidation of ascorbate with a protection (after 1600 s) of about 96%, whereas $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ inhibition is 82 and 83%, respectively, under identical reaction conditions.

Cu-mediated ascorbate oxidation inhibition due to the tripods was then compared with that measured with the independent building blocks $\mathbf{BB^{H}}$ and $\mathbf{BB^{Me}}$ (Fig. S3), in the same experimental conditions. Increasing concentrations of the two building blocks were used from 15 μM to 45 μM, the latter concentration value mimicking the three building blocks in the tripodal architectures. We found that addition of the building blocks $\mathbf{BB^{H}}$ and $\mathbf{BB^{Me}}$ in the Cu/Asc solution was significantly less efficient for inhibiting the oxidation of ascorbate (Fig. 4) as compared to their tripodal counterparts $\mathbf{T^{H}}$, $\mathbf{T^{H}}$*, $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$. For instance, the protection provided by 45 μM $\mathbf{BB^{H}}$ and $\mathbf{BB^{Me}}$ are 62% and 13%, respectively whereas the same building blocks assembled in the corresponding tripodal ligands afford protections of 96 and 82%, for $\mathbf{T^{H}}$ and $\mathbf{T^{Me}}$, respectively. Again, these data measured on the independent building blocks demonstrate a larger efficacy of the non-substituted residues.

The Cu/Asc couple is known to produce hydroxyl radicals ($HO^{\bullet}$) via Fenton-type reactions [39]. The production of $HO^{\bullet}$ radicals can be followed using a fluorescent probe, such as terephthalic acid [40]. Typically, the non-fluorescent terephthalic acid reacts with $HO^{\bullet}$ radicals to form 2-hydroxy terephthalic acid, which is highly fluorescent, with excitation and emission wavelength of 315 nm and 425 nm, respectively. As expected, a significant amount of $HO^{\bullet}$ radical formation is measured in the Cu/Asc system. Most importantly, the addition of the tripods $\mathbf{T^{Me}}$, $\mathbf{T^{Et}}$, $\mathbf{T^{H}}$, and $\mathbf{T^{H*}}$ significantly reduced the amount of $HO^{\bullet}$ radicals (Fig. S4) with nearly no radical detection for the two non-substituted molecules $\mathbf{T^{H}}$ and T^{H}*. So, the addition of the efficient imidazole thione tripodal Cu(I) chelators, in the system Cu/Asc remarkably inhibit the oxidation of ascorbate and the production of $HO^{\bullet}$ radical; with a efficacy significantly improved by the tripodal architecture with respect to the individual building blocks. Therefore, their

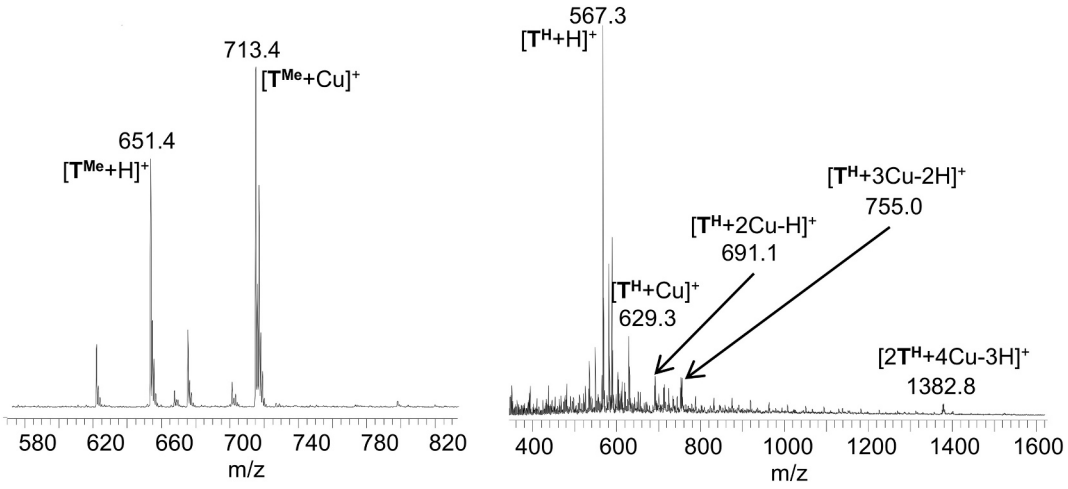


Fig. 3. (+) ESI-MS spectra of **A)** Cu(*I*)$\mathbf{T^{Me}}$ and **B)** Cu(*I*)$\mathbf{T^{H}}$ (100 μM) in ammonium acetate buffer (20 mM, pH 6.9) / acetonitrile 9/1 *v*/v.

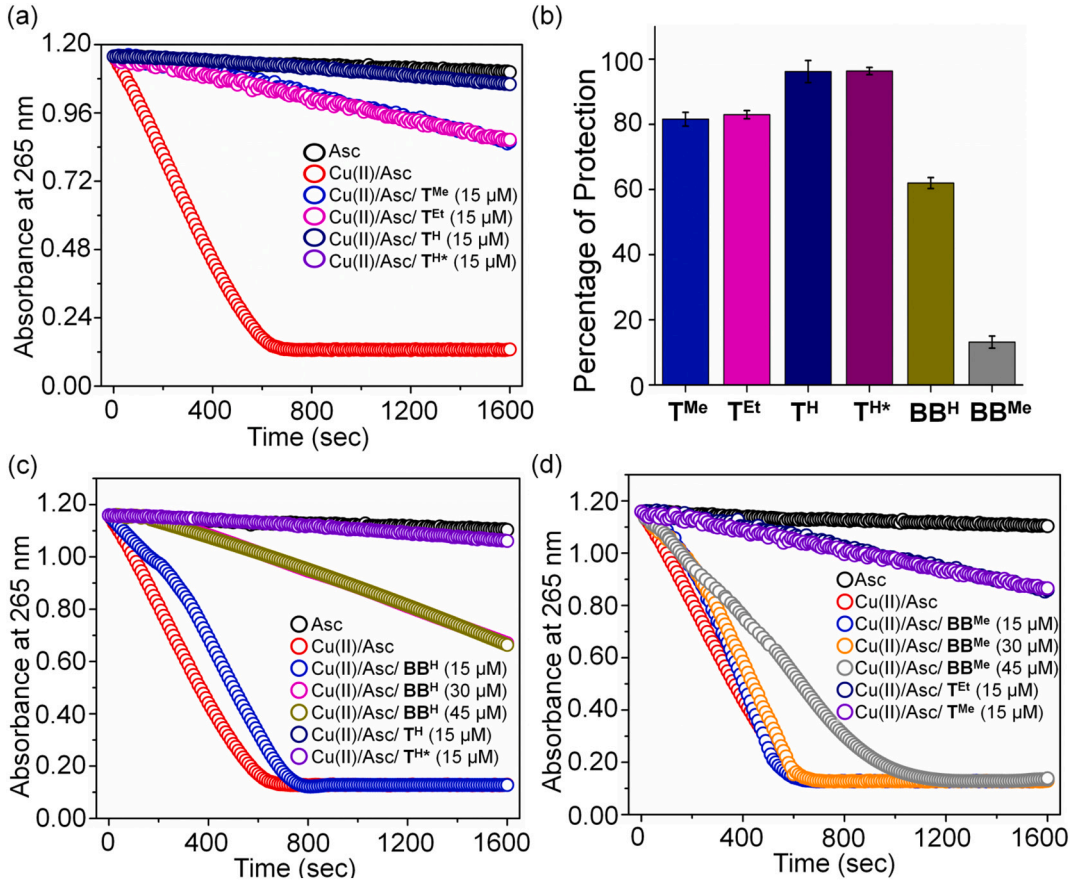


Fig. 4. Inhibition of Cu-catalyzed oxidation of ascorbate by the imidazole thione ligands, [Cu] = 10 μM. (a) Absorbance of Asc at 265 nm with time in the presence of the 4 ligands (15 μM) (b) Percentage of protection from ascorbate oxidation after 1600 s by the series of tripods (15 μM) and the two building blocks (45 μM). (c) Absorbance of Asc at 265 nm with time in the presence of $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ compared to $\mathbf{BB^H}$ (15–45 μM). (d) Absorbance of Asc at 265 nm with time in the presence of $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ compared to $\mathbf{BB^{Me}}$ (15–45 μM).

ability to protect biomolecules from Cu-mediated oxidative damage was studied through protein carbonylation and DNA cleavage assays in the following.

2.4. Protection against Cu-mediated protein carbonylation

Since the series of imidazole thione tripods reduces the production of Cu-mediated free radical concentrations, it prompted us to investigate their protective effect against Cu-mediated oxidative damage to biomolecules. Indeed, reactive oxygen species (ROS) can convert some amino groups of proteins to carbonyl moieties [41,42]. Oxidation of proteins results in the formation of carbonyl groups, which reflect the extent of oxidative damage to the protein. This can be measured spectrophotometrically at 370 nm by treating the carbonylated proteins with 2,4-dinitrophenyl hydrazine (DNPH) [43]. In brief, incubation of bovine serum albumin (BSA) for 1 h with Cu^{2+}/Asc or Cu^{2+}/Asc/H_2O_2 results in the formation of 40.8 and 52.7 nmol/mg of BSA, protein carbonyls, respectively, at pH 7.4. Fig. 5 and Table S4 present the results of the

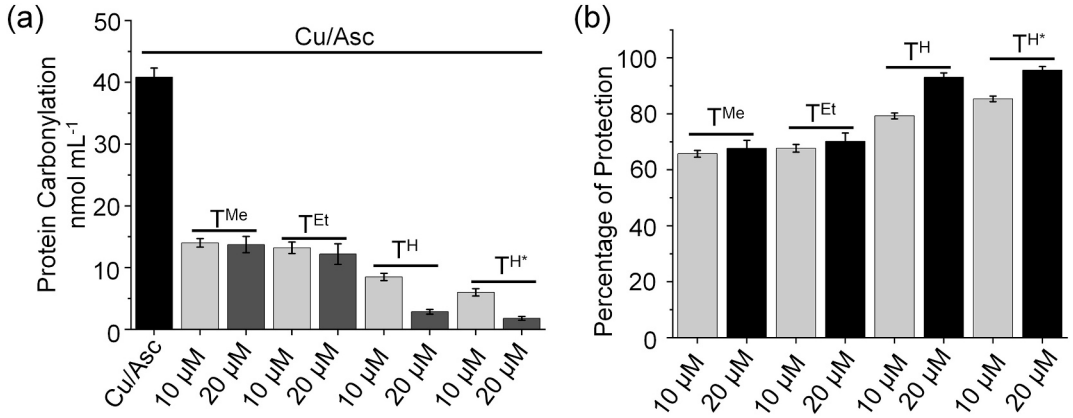


Fig. 5. Inhibition of protein carbonylation induced by the Cu/Asc system by the tripods (a) Bar diagram showing the amount of protein carbonylation, (b) Bar diagram showing the percentage of protection. [Condition: BSA = 0.25 mg mL^{-1}; [Cu] = 25 μM; [Asc] = 250 μM; [H_2O_2] = 250 μM; [DNPH] = 1.3 mM in PBS Buffer 0.1 mM pH = 7.4; where DNPH stands for 2,4-dinitrophenylhydrazine].

inhibition of protein carbonylation induced by the Cu/Asc system by the tripodal ligands. Once again, we found that the unsubstituted thioimidazole based tripods $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ are more effective compared to the *N,N′*-disubstituted thione analogues $\mathbf{T^{Me}}$, and $\mathbf{T^{Et}}$ in inhibiting Cu-mediated protein carbonylation. Interestingly, a similar effect although lower was observed when BSA was co-treated with H_2O_2 along with Cu(II)/Asc: the formation of protein carbonylation was also very efficiently inhibited by the tripods. For instance, the inhibition of BSA carbonylation by the Asc/Cu system, promoted by the tripod $\mathbf{T^H}$ drops only from 93% to 81% when H_2O_2 is added in the experiment. Data obtained in the presence of H_2O_2 are shown in Fig. S5 and Table S5 in the Supplementary Data.

So, the protein carbonylation assay demonstrated that the thioimidazole tripods are efficiently protecting protein from oxidation caused by reactive oxygen species (ROS) generated from the redox-active Cu system.

2.5. Plasmid cleavage assay

Finally, the protective effect of the tripods on DNA cleavage induced by Cu-mediated oxidative damage was investigated by DNA gel electrophoresis [44]. The generation of radicals from Cu/Asc and Cu/Asc/H_2O_2, results in the normal supercoiled plasmid DNA to unwind at pH 7.4. The degree of DNA damage was assessed using electrophoresis to separate the nicked (Form II) and supercoiled (Form I) conformation [45]. Fig. 6 and Table S6 show the results for the system containing H_2O_2, i.e. Cu^{2+} = 10 μM, ascorbate = 10 μM, H_2O_2 = 10 μM. Again, the unsubstituted tripods $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ show a very high inhibition, namely 90% and 91% inhibition of DNA damage at 20 μM concentration, respectively. The *N,N′*-disubstituted thiones $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ although less efficient also show a significant inhibition of 59% and 47%, respectively. Similar conclusions are drawn from the data obtained without H_2O_2 (Cu^{2+} = 25 μM, ascorbate = 50 μM), which are reported in Fig. S6 and Table S7.

3. Conclusion

In conclusion, four tripodal Cu(I) ligands derived from nitrilotriacetic acid grafted with three thioimidazole moieties were synthesized and demonstrated their ability to chelate Cu(I) in water at pH 7.4. Alkylated imidazole thiones are expected to behave as neutral sulfur donors. Consistently the N,N′-alkylated tripods $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ ($\log K^{pH\ 7.4}$ = 11–11.6) display affinities for Cu(I) in a similar range as the one reported previously with analogs bearing three neutral thioether functions [19]. By contrast, the imidazole thione function can exist as a tautomer between a thiol and a neutral thione, if non-alkylated due to the presence of the NH groups. Therefore, it may be seen as a hidden thiol function, with an affinity for the soft Cu(I) cation in between that expected with a thiol and a neutral sulfur donor. Consistently, the affinities of the two tripods $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ for Cu(I) are lower than those measured previously with similar ligands grafted with three thiol moieties [18,36] and larger than those evaluated with analogs bearing three neutral thioether functions [19]. The speciation of their Cu(I) complexes is also more complicated with the formation of Cu(I) clusters, a feature often observed with thiolate ligands. They also showed a significantly larger affinity for Cu(I) ($\log K^{pH\ 7.4}$ = 14.3) than their N,N′-alkylated analogues $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$ ($\log K^{pH\ 7.4}$ = 11–11.6).

The affinity of the novel Cu(I) chelating molecules reported here is probably too low to compete in the cytoplasm with abundant simple endogenous thiols such as glutathione, which has been demonstrated to form a tetranuclear cluster that sets a firm upper limit of ca 1 fM on the thermodynamic availability of intracellular copper [46]. However, the two imidazole thione tripods $\mathbf{T^H}$ and $\mathbf{T^{H*}}$ bear three hidden thiol functions that represent an interesting compromise between resistance to

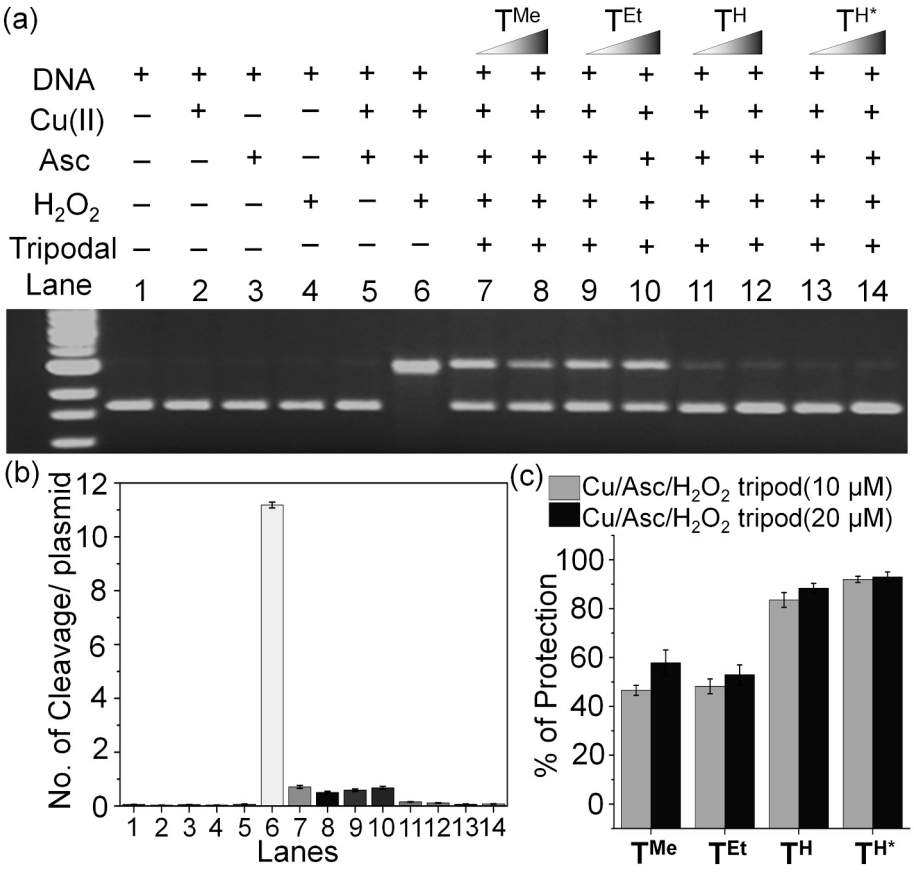


Fig. 6. (a) Agarose gel showing the copper mediated oxidative (Cu^{2+}/Asc/H_2O_2) DNA damage in various conditions and the inhibition resulting from the addition of the tripods. Lane 1: DNA, Lane 2 DNA + Cu^{2+}, Lane 3: DNA + Asc, Lane 4: DNA + H_2O_2, Lane 5: DNA + Cu^{2+} + Asc, Lane 6: DNA + Cu^{2+} + Asc + H_2O_2, Lane 7: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^{Me}}$ (10 μM), Lane 8: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^{Me}}$ (20 μM); Lane 9: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^{Et}}$ (10 μM), Lane 10: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^{Et}}$ (20 μM), Lane 11: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^H}$ (10 μM), Lane 12: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^H}$ (20 μM), Lane 13: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^H}$ (10 μM), Lane 14: DNA + Asc + Cu^{2+}+ H_2O_2 + $\mathbf{T^{H*}}$ (20 μM), (b) No. of cleavage/ per plasmid from (S) = $-lnf_1$ where f1 is the fraction of form I (supercoiled) for tripodal chelators $\mathbf{T^{Me}}$, $\mathbf{T^{Et}}$, $\mathbf{T^H}$ and $\mathbf{T^{H*}}$. (c) Bar diagram showing percentage of protection by tripodal chelators $\mathbf{T^{Me}}$, $\mathbf{T^{Et}}$, $\mathbf{T^H}$, and $\mathbf{T^{H*}}$ form Cu/Asc/H_2O_2 mediated DNA damage.

oxidation and high Cu(I) affinity. Therefore they may be of interest for Cu(I) chelation in more oxidizing compartments such as the endoplasmic reticulum or even in the extracellular environment.

Interestingly, all the four ligands demonstrated ability to inhibit Cu-mediated oxidative stress as shown in various assays, involving ascorbate consumption associated with H_2O_2 or $HO^{\bullet}$ production, the protein carbonylation assay or DNA cleavage assay. Their efficacy in limiting Cu-induced oxidative stress is correlated to their Cu(I) complexation constants implying the formation of redox inactive Cu(I)-thione complexes with the tripods. $\mathbf{T^H}$ and $\mathbf{T^{H^*}}$ show a nearly total inhibition of Cu-induced oxidative stress, which is significantly better than with their *N, N′*–alkylated thione counterparts $\mathbf{T^{Me}}$ and $\mathbf{T^{Et}}$. Importantly, the tripodal architecture revealed beneficial in the ascorbate assay, with larger inhibition of Cu-mediated oxidation with the tripods than the imidazole thione building blocks at a similar concentration.

To conclude, the thioimidazole tripodal derivatives $\mathbf{T^H}$ and $\mathbf{T^{H}}$* display the largest affinity for Cu(I) and the best ability to quench Cu-mediated oxidative stress in the series of ligands presented in this paper and may have therapeutic interest either as antioxidant agents or Cu chelating agents.

Author contributions

• Ranajit Das	• Investigation, Writing - original draft
• Michael Claron	• Investigation, Writing - original draft
• Colette Lebrun	• Investigation
• Jacques Pécaut	• Investigation
• Srinivas Ambala	• Investigation.
• Christelle Gateau	• Supervision
• Gouriprasanna Roy	• Funding acquisition, Supervision, Writing - review & editing.
• Pascale Delangle	• Funding acquisition, Supervision, Writing - original draft.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2021.111518.

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