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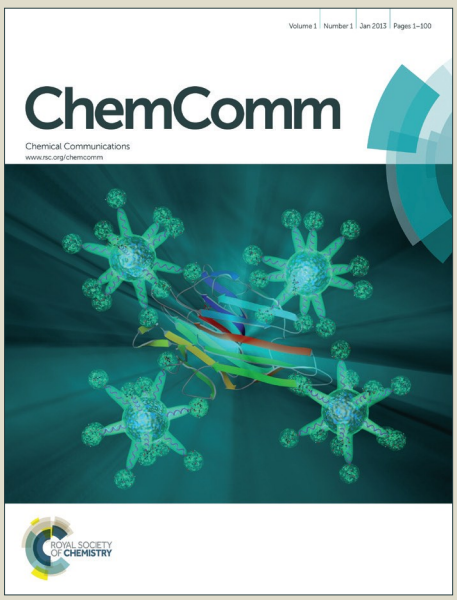

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A Versatile Water-Soluble Chelating and Radical Scavenging Platform

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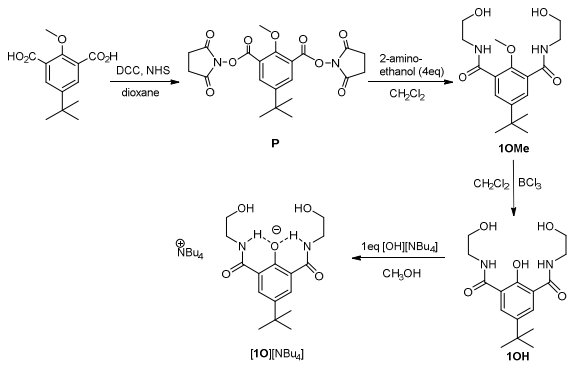
The phenol-diamide compound, 5-(tert-butyl)-2-hydroxy-N1,N3-bis(2-hydroxyethyl)isophthalamide (1OH), is water-soluble, non-cytotoxic, and capable of both, trapping ROS species and chelating Cu(II) and Fe(III) ions; these combined properties confer a protective effect against ROS induced cell death.

Oxidative stress is associated with many pathologies including aging, cancer, diabetes, arthritis, lung diseases, cardiovascular diseases, as well as neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Wilson's disease, and Menke's disease.[1] Under oxidative stress, the uncontrolled production of reactive oxygen species (ROS; *e.g.* hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), peroxyl radicals) can lead to the damage of known biomolecules (e.g. lipids, proteins, or DNA) with subsequent tissue damage.[2] The excessive production of ROS is directly linked with abnormal levels of free iron or copper ions in cells; which catalyse ROS production through Fenton and Haber-Weiss reactions.[3] Therefore, therapeutic strategies curtailing excess free radical production include the use of either ROS-scavengers (antioxidants) or iron/copper chelators.[4] Natural and pharmaceutical antioxidants, such as vitamin E, polyphenols and others have long been promoted to prevent or delay the onset and progress of most types of cancers[5-7] and their potential use as therapeutics for neurodegenerative diseases has also been suggested.[6] Chelating drugs are also widely explored for the treatment of many diseases,[8] and can be more effective than antioxidant, by neutralizing the ability of free copper and iron ions to catalyze ROS species production. Efficient Cu/Fe-chelators include O_6 hexadentate (deferoxamine), O_2-bidentate hydroxypyridinonate (HOPO), phenol O_2-bidentate (deferiprone), NO-bidentate 8-hydroxyquinolin (cloquinol, VK28, M30) and poly-quinoline, and *bis*-phenol NO_2-tridentate (deferasirox).[8] Recently, Green and co-workers have reported a new N_4 Cu-chelator bearing a free phenol group, that possesses antioxidant properties and enhanced protective effects against ROS-induced cell death in neurodegenerative disorders.[9] The development of phenol compounds that can exist in a persistent radical state as well as being able to chelate transition metal ions, is thus thought to be a promising approach to novel therapeutics. Suitably designed phenol-Schiff base N,O- ligands have been reported to permit the production of relatively stable phenoxyl radical.[10] In that regards, we have recently shown, that the O,O-salicylamide chelating unit, when suitably protected with tert-Butyl groups at the ortho and para positions, can assume a persistent phenoxyl radical oxidised state in organic solvents.[11] Herein, we investigate the much more polar 2-hydroxyisopthalamide (phenol diamide) molecular platform; **1OH**, which possesses a central para-tert-Bu-protected phenol and two adjacent ethanol amide side arms (Scheme 1). We show that **1OH** is water-soluble, non-cytotoxic, and capable of trapping ROS species as well as chelating Cu(II) and Fe(III) ions. These combined properties confer protective effect against ROS induced cell death.



Scheme 1. Synthetic route to **1OH** and **1O**$^{-}$.

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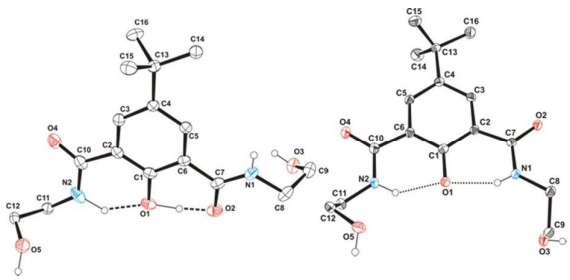

Fig. 1 ORTEP representation of the molecular structures of **1OH** (left) and **1O**$^-$ (right).(**1OH**: O(1)–H···O(2) : O···O 2.449(2) Å, <O–H···O> 163(4)°; N(2)-H···O(1): N···O 2.637(2) Å, <N–H···O> 137(4) °; **1O**$^-$: N(1)-H···O(1)$^-$···H-N(2): O···N 2.606 and 2.617 Å, <O(1)$^-$···H-N(1)> 146 °, <O(1)$^-$···H-N(2)> and 142 °).

The phenol diamide compound **1OH** was synthesized in three steps from 5-(tert-butyl)-2-methoxyisophthalic acid[12] as described in Scheme 1 (ESI†). The corresponding phenolate salt, [**1O**][NBu_4], is readily obtained upon reaction with an equimolar amount of [OH][NBu_4] in dry MeOH (ESI†). The structures of **1OH** and [**1O**][NBu_4] have been then successfully determined by X-ray crystallography (Fig. 1, Table SI1); both reveal a planar phenol-diamide moiety that appears to be stabilized through intramolecular H-bonding between the phenol oxygen atom and the two adjacent amide groups.[11] In **1OH** the two amide groups show different orientation as O···H-N(amide) and O–H···O(amide) H-bonds are established. In contrast, in **1O**$^-$, both the two N-H hydrogen atoms are directed towards the phenol-O atom in a N-H···O···H-N fashion (Figure 1).

The cyclic voltammogram (CV) of **1OH** in CH_3CN at room temperature exhibits an irreversible oxidation process at *ca.* 1 V *vs.* Fc^+/Fc. In contrast, that of [**1O**][NBu_4] displays a one-electron reversible oxidation process at $E_{1/2}$ = 0.137 V *vs.* Fc^+/Fc ($E_p{}^a$ = 184 mV, ΔE = 97 mV at 100 mVs^{-1}) (Figure 2) attributed to the formation the phenoxyl radical **1O**$^\bullet$ that is stable on the time scale of the CV scan. In water, the *in-situ* generated **1O**$^-$ displays a quasi-reversible oxidation at 0.423 V (Fig. 2), showing again the production of a transient radical. In an effort to characterise the radical **1O**$^\bullet$, bulk electrolysis of [**1O**][NBu_4] in CH_2Cl_2 at 273 K was performed. Though the electrolysis shows a typical Q/t curve with a plateau reached upon transfer of 0.91 e^- per **1O**$^-$, confirming a single electron transfer (see Fig. SI1); the resulting oxidized solution remained colourless and displays neither visible bands nor an EPR signal, as one would expect for a stable phenoxyl radical species.[11] The only observed change upon electrolysis was a blue-shift of the π-π* transition from 371 nm to 321 nm. In addition, no sign of oxidative breakdown were identified by mass spectrometry. We tentatively suggest the formation of a short-lived phenoxyl radical which may dimerize by O-O coupling to the corresponding peroxo compound **1O-O1**.

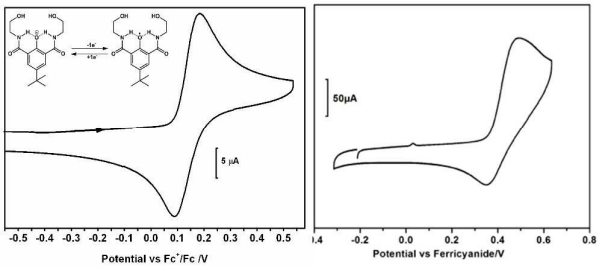

Fig. 2 Cyclic voltammogram of (left) [**1O**][NBu_4] (*ca.*1 mM) in CH_3CN at 298 K containing [NBu_4][PF_6] (0.2 M), and (right) **1OH** (*ca.*1 mM) + 1 equiv. of NaOH in water containing Li[ClO_4] (0.2 M). Scan rate of 100 $mV.s^{-1}$.

Chelating properties. **1OH** possesses a phenol-amide O,O-bidentate acac-type binding unit, suitable for the stabilization of Fe(III) and Cu(II) ions.[13] **1OH** readily reacts with either $Fe(ClO_4)_3{\cdot}H_2O$ or $Cu(acetate)_2{\cdot}H_2O$ in MeOH in a 3:1 or 2:1 ratio in the presence of Et_3N to produce the stable *tris*-Fe(III) and *bis*-Cu(II) complexes, $Fe(1O)_3$ (**1O-Fe**) and $Cu(1O)_2(H_2O)$ (**1O-Cu**) respectively; as confirmed by MS, elemental analysis, UV/vis, EPR, and X-ray crystallography (for **1O-Cu**) (ESI†, Fig SI2-3). Thus, the deep red compound **1O-Fe** possesses typical characteristics of an Fe^{III}, d^5, HS centre with an O_6 coordination sphere as the result of the binding of three O,O-ligands;[13b,c] *i.e.* a X-band EPR signal at 4.2 (Fig. SI2) and phenolate-Fe(III) LMCT band at ca. 460 nm (Fig. SI3). The pale green compound **1O-Cu** exhibits a typical Cu^{II} ion (d^9, S = 1/2) axial EPR signal (in CH_3OH at 120 K) with g_{zz}(2.31) >> g_{xx} (2.08) ~ g_{yy} (2.05) and A_{zz} (^{63,65}Cu) of 167 G (Fig. SI2). The g_z and A_z values match with a Cu(II) ion in a square-pyramidal geometry.[14]

The molecular structure of **1O-Cu** has been determined by X-ray crystallography as $[Cu(1O)_2(H_2O)]{\cdot}0.5\ H_2O$ (Figure 3,4, Table SI1, ESI†). The X-ray structure reveals two independent molecules of **1O-Cu** (A (Cu1) and B (Cu2)) with virtually identical coordination geometry. In each molecule, the copper ions displays a slightly distorted square pyramidal O_5 coordination. The base of the pyramid is formed by the O,O-binding of two ligands through the O-phenolate and one amide O-carbonyl donor atoms (average Cu-O distances of 1.92 Å and 1.98 Å, respectively), with a water molecule occupying the axial position (Cu-O distances of 2.171(2) Å (A) and 2.222(2) Å (B)). Interestingly, the Cu-OH_2 bond length of 2.17 Å is amongst the shortest apical bond in square pyramidal complexes of Cu. The two coordinated ligands are oriented in a "butterfly wings" fashion as indicated by the angle between the two phenol-diamide planes of 74.34° (A) and 76.89° (B). Each ligand binds the copper ions in a unsymmetrical manner; i.e., one amide arm is coordinated though O-carbonyl, while the other non-coordinated amide side arm is oriented so that a intramolecular N–H···O H-bonding is established between the amide N-H and the central coordinated phenolate-O atom (N···O distance 2.673 (3) Å and N–H···O angle 133.2°). Thus, the phenolate groups are both coordinated and H-bonded in **1O-Cu**; as in very few other cases.[15]

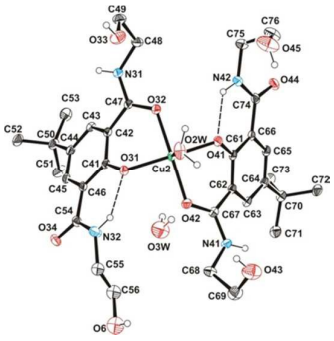

Fig. 3 ORTEP representation of one of the two independent molecules of **1O-Cu** in a single crystal of $[Cu(1O)_2(H_2O)]\cdot 0.5\ H_2O$.

As displayed in Figure SI3, each **1O-Cu** molecule (A or B) strongly interacts with its symmetrically related counterpart (A' or B') through multiple hydrogen bonds involving both the water molecules and the four alcohol pendant arms of each complex; yielding A/A' or B/B' dimers. The latter dimers further H-bond to each other (through NH-amide/C=O amide), to produce $\{A/A' - B/B'\}_n$ 1D-chains.

In solution, the UV/vis spectra of **1O-Cu** and **1O-Fe** are characterized by a phenolate-to-metal LMCT band at 425(sh) nm (ε = 764 M^{-1} cm^{-1}) and 458 nm (ε = 4230 M^{-1} cm^{-1}) respectively (Fig. SI4), and a weak d-d transition at 696 nm (ε = 80 M^{-1} cm^{-1}) for **1O-Cu**. The LMCT band show no solvent-dependency and appear identical in acetonitrile, ethanol or water (see Fig. SI4). This suggests that **1O-Cu** and **1O-Fe** do not dissociate in aqueous medium. To further support this, binding experiments were performed. The ligand **1OH** and $Fe(ClO_4)_3$ or $Cu(SO_4)_2$ salts were mixed in water in various molar fractions (from 0 to 1), and the absorption at the LMCT band was recorded (Fig. SI5-6). The resulting Job's plots[16] (Fig. SI7) show a maximum absorbance reached for metal ion molar fractions of 0.30 (Fe) and 0.35 (Cu), close to the values expected for *tris*-Fe(III) and *bis*-Cu(II) complexes. Other maxima are observed at higher copper molar fractions, suggesting the possible formation of dinuclear species (Fig. SI7). These findings suggest that **1OH** chelates Cu(II) and Fe(III) ions in aqueous medium yielding **1O-Cu** and **1O-Fe** as the main species.

Protective effect against oxidative stress. The water solubility, the ease of oxidation, and the chelating properties of **1OH**, may induce protective effects against oxidative stress.[17]

The ability of **1OMe** and **1OH** to scavenge HO radicals was examined by EPR, under H_2O_2 photolysis conditions, using DMPO spin trapping.[18] DMPO reacts rapidly with $HO^{\bullet}$ to form the stable spin adduct $[DMPO\text{-}HO]^{\bullet}$, characterized by a four line (1:2:2:1) EPR signal with intensity proportional to the concentration of $HO^{\bullet}$. As displayed in Figure SI8, under these conditions, the IC_{50} of **1OH** at 1.4 µM indicates that **1OH** is an excellent OH radical scavenger, considerably better than either **1OMe** (IC_{50} 200 µM) or Trolox (IC_{50} 180 µM) measured under the same conditions. These results correlate well with the ability of **1OH** to be oxidized easily to its phenoxyl radical, presumably via H-atom abstraction.

In addition, the reaction between metmyoglobin and H_2O_2 has been used to reproduce oxidative stress conditions in which $HO^{\bullet}$, $^1O_2$, $O_2^{\bullet-}$ are formed.[19] The assay measures the $ABTS^{\bullet+}$ radical cation formed by oxidation of ABTS during this process. As displayed in Fig. 4, **1OH** acts as a good antioxidant with an IC_{50} (80 µM) similar to that of Trolox. In contrast, **1OMe** has no significant inhibiting effect, emphasizing the importance of the presence of the phenol group for ROS scavenging.

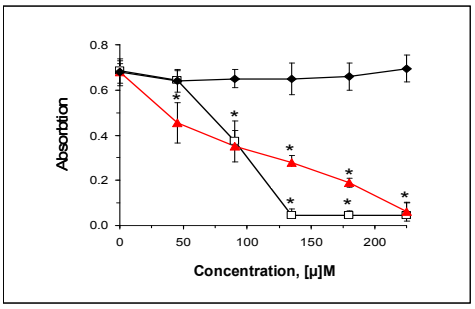

Fig. 4 Dose response effect of **1OH**, **1OMe**, and Trolox on $ABTS^{\bullet+}$ formation. Experimental conditions: Metmyoglobin (0.047 µM), ABTS (0.22 mM), H2O2 (20 µM) in potassium phosphate buffer (pH 7.5), and tested compounds 1OH (▲), 1OMe (♦) or Trolox (□). The $ABTS^{\bullet+}$ was monitored by UV/Vis measurements (λ=750 nm) after 30 minutes incubation time at room temperature. *, p<0.05 in comparison with an absorption level of blank sample.

Both **1OMe** and **1OH** possess no significant cytotoxicity effect on PC12 rat pheochromocytoma cells, L6 myotubes, or ^{93A}hSOD1-NSC-34 with concentration up to 0.1 mM (ESI†).

MTT cell viability assays[20] were performed using ^{G93A}hSOD1-NSC-34 cells. This cell line represents an *in vitro* model for Amyotrophic Lateral Sclerosis (ALS), in which oxidative stress is involved in its pathogenesis.[21] Oxidative stress conditions were induced by supplying glucose oxidase (GO, 50 mU/ml) to the growing cell medium containing a high level of glucose (23.5 mM, see SI), resulting in an elevated H_2O_2 concentration in the medium (reaching 29.0 ± 9.6 µM in 4 h of incubation).[22]

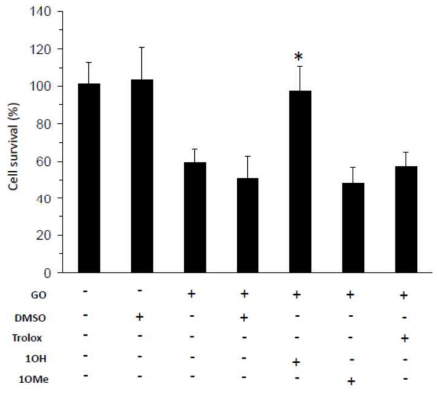

Fig. 5 Effect of the tested compounds on GO-mediated cell toxicity. ^{G93A}hSOD1-NSC-34 cells were grown in high glucose medium and treated with GO in presence or absence of tested compounds (100 µM). Cell viability was then determined by MTT assay *, p<0.05 in comparison with sample incubated without GO.

Figure 5 displays the effect of **1OMe**, **1OH,** and Trolox on GO-mediated cell toxicity. As expected, the GO- treated cells show a nearly 40% decrease in cell viability due to oxidative stress. In the presence of either **1OMe** or Trolox, cell viability was

poor, indicating that these compounds have little or no protective effect. In contrast, in the presence of **1OH**, cell viability remains at nearly the same level as untreated cells, clearly demonstrating that **1OH** prevents cell apoptosis induced by oxidative stress.

Finally, to further test the protective effect against oxidative stress of **1OH**, anti-lipid peroxidation (MDA assay) and total antioxidant capacity assays (TEAC assay) have been performed on 93AhSOD1-NSC-34 cells, and the results are shown in Figures SI9 and SI10, respectively. Both assays (see ESI) clearly show that, unlike **1OMe** which has no significant effect, **1OH** both significantly inhibit the lipid peroxidation and increase the total antioxidative capacity in the cells, and so to a greater extent than Trolox.

Herein, we have reported the synthesis and characterization of the first representative, **1OH**, of a newly designed family of phenol compounds that incorporate a para-*tert*-Bu-protected phenol-di-amide framework. Remarkably, **1OH** is a water-soluble, non-cytotoxic compound that has been demonstrated to support a phenoxyl radical state. We show that **1OH** traps ROS species and chelates Cu(II) and Fe(III) ions. Our results suggest that these combined properties have a protective effect against ROS-induced cell death. The synthetic versatility and the unique properties of this phenol-diamine platform allow promising new developments in chelation and antioxidant therapy.

Notes and references

† Experimental, instrumental, crystallographic, together with supplementary results are presented in the Supporting Information. The structures of **1OH**, [**1O**][NBu4] and **1-Cu**·0.5 H_2O are available at the Cambridge Crystallographic Data Centre as supplementary publications CCDC-829301, 829302 and 995321 respectively.

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