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Binding of 2-Hydroxypyridine-*N*-oxide on Dicopper(II) Centers: Insights into Tyrosinase Inhibition Mechanism by Transition-State Analogs

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2-Hydroxypyridine-*N*-oxide (HOPNO) is described as a new and efficient transition-state analog (TS-analog) inhibitor for the mushroom tyrosinase with an IC_{50} = 1.16 μM and a K_I = 1.8 μM. Using the binuclear copper(II) complex $[Cu_2(BPMP)(\mu-OH)](ClO_4)_2$ (**2**) known as a functional model for the tyrosinase catecholase activity, we isolated and fully characterized a 1:1 (**2**)/OPNO adduct in which the HOPNO is deprotonated and chelates only one Cu-atom of the binuclear site in a bidentate mode. On the basis of these results, a structural model for the tyrosinase inhibition by HOPNO is proposed.

Tyrosinases (Ty, EC 1.14.18.1) are copper-containing metalloenzymes widely distributed throughout microorganisms, plants, and animals, where they catalyze the oxidation of phenolic compounds into catechols (phenolase activity) and catechol into *o*-quinone (catecholase activity), successively (Scheme 1).[1] In mammals, Ty is involved in the two-step oxidation of L-tyrosine into dopaquinone, which is the key product for melanin pigment biosynthesis. Melanin-related disorders are known to cause serious skin lesions,[2] Parkinson's disease,[3] and melanoma.[4] Because Ty inhibition is now a well-known approach against increased production and accumulation of melanin,[5,6] the development of Ty inhibitors has a huge economical and industrial impact.[5,7,8]

In spite of a large number of Ty sources, only the X-ray structure of *Streptomyces castaneoglobisporus* Ty has been solved recently.[9] This X-ray structure confirms that Ty belongs to the type-3 copper-containing enzyme family with an active site composed by a dicopper core where both copper ions are surrounded by three N-atoms from histidine residues. Along the catalytic cycle three forms have been identified: (i) a native met state with an aqua(hydroxo)-bridging ligand that provides antiferromagnetic coupling between the copper ions, leading to an EPR-silent behavior; (ii) a reduced deoxy state; and (iii) an oxy state where dioxygen is bound to the dicopper center as a $\mu-\eta^2:\eta^2$ peroxo ligand (Scheme 1).

Among the large number of Ty inhibitors described in the literature, kojic acid (IC_{50} = 30.61;[10] 16.67 μM[11]) and L-mimosin (IC_{50} = 3.68 μM[11]) exhibit the best inhibition features. These compounds belong to the transition-state analog (TS-analog) inhibitor family because of their structural analogy to the quinone product as well as to the catechol substrates, while occurring in an oxidation state that is not suitable for reaction. Because these molecules target the binuclear copper site, they represent the best strategy to achieve Ty specificity. In this regard, small molecules interactions on the dicopper catalytic center in relation with the inhibition behavior is fundamental to establish binding properties/inhibition activity relationship.[12] This report investigates 2-hydroxypyridine-*N*-oxide (HOPNO) as a new TS-analog inhibitor for the mushroom Ty and studies its

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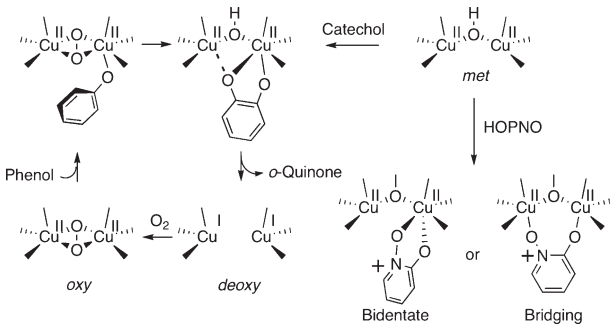
Scheme 1. Ty-Catalyzed Oxidations and Possible Binding Modes for HOPNO Inhibitor

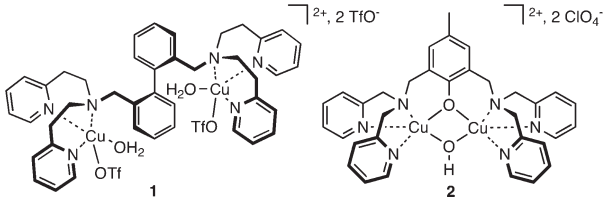


binding to the Ty dicopper(II) centers using model compounds.

The effect of HOPNO on the oxidation of L-DOPA by mushroom Ty was first studied. Ty inhibition by HOPNO was concentration-dependent with an IC_{50} estimated to 1.16 μM. In these conditions, HOPNO exhibited kinetic features of a pure competitive inhibitor with K_I of 1.8 μM, what places HOPNO among the best Ty inhibitors (Supporting Information). In order to elucidate interaction of HOPNO with Ty, we next studied the binding of HOPNO to Ty model complexes. To the best of our knowledge, only one study on the influence of inhibitors on Ty model complexes has been published.[13] This study reports the inhibiting effect of kojic acid on the oxidation of 3,5-di-*tert*-butyl catechol by dicopper(II) model complexes, leading to the proposal that kojic acid acts as a bridging ligand between the two copper(II) centers of the Ty model compound (Scheme 1).[14] This proposal was supported by two reports in which tetrachloro-*o*-catecholate-bridged dicopper(II) complexes were isolated and fully characterized.[15,16] Later reports argued for an asymmetric binding of catechol to only one of the two available copper(II) ions in a η^2 fashion.[17,18] From model complexes studies, alternative binding mode associated with semiquinone intermediate has been proposed.[19,20] So, a nonbridging, bidentate coordination to one copper center as unreactive transition state could be considered in relation with inhibition mechanism.

To investigate the binding mode of HOPNO, we have performed experiments with dicopper(II) model complexes for Ty. We studied two binuclear copper(II) complexes known to reproduce the Ty catecholase activity: (i) complex **1** $[Cu_2(BiPhNPY_{22})(H_2O)_2(CF_3SO_3)_2](CF_3SO_3)_2$ which bears two nearby but independent copper(II) ions[21] and

Scheme 2. Copper(II) Complexes Used in This Study



(ii) complex **2** $[Cu_2(BPMP)(\mu-OH)](ClO_4)_2$ which possesses two copper(II) ions bridged by one hydroxo and one phenoxo anion[22] (see Scheme 2).

Reaction of complex **1** with 1 equiv. of HOPNO in acetone/water medium and subsequent layering with toluene afforded compound **3** as a crystalline material. The X-ray structure of this complex revealed the presence of a 1:2 adduct of the complex **1** with the deprotoned form of HOPNO (OPNO, Figure 1). The geometry around both copper ions is a square-base pyramid (τ = 0.26 and 0.04 for Cu1 and Cu2 respectively).[23] One OPNO is coordinated to each metal in a bidentate mode. For Cu1 the basis of the pyramid is occupied by the two O-atoms of the OPNO and two N-atoms, one amine (N1) and one pyridine (N2). The axial coordination is completed by the second pyridine (N3). For Cu2 the axial position is occupied by the amine (N5) while the equatorial ligands are the two O-atoms of the OPNO and the N-atoms N6 and N7 of the remaining pyridines. The out-of-plane distances from the plane defined by the four equatorial ligands to the metals are equal to 0.18 Å and 0.29 Å for Cu1 and Cu2, respectively. The torsion angle of the biphenyl moiety is 64.8°, and the distance between the copper ions is 7.05 Å.

The biphenyl spacer appears too flexible to accommodate one OPNO bridging ligand, and the two copper(II) centers act independently accepting one OPNO ligand each. We then turned our attention to the less-flexible dicopper(II) complex **2**. The interaction of HOPNO with complex **2** was spectrophotometrically monitored (see Figure 3 in the Supporting Information). The absorption band at 410 nm assigned to LMCT transition between the bridging phenoxo and the copper ions is shifted up to 420 nm and the molar extinction coefficient (ε) is raised from 480 to 1584 M^{-1} cm^{-1}. The d−d transition band at 785 nm (ε = 280 M^{-1} cm^{-1}) of **2** is also affected with a shift to 640 nm (ε = 400 M^{-1} cm^{-1}). Further addition of HOPNO did not induce additional changes in the

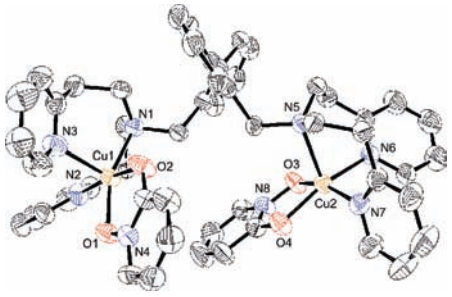


Figure 1. ORTEP drawing of the dication $[Cu_2(BiPhNPY_{22})(OPNO)_2]^{2+}$ (**3**). H-atoms and the two $CF_3SO_3^-$ counteranions were omitted for clarity.

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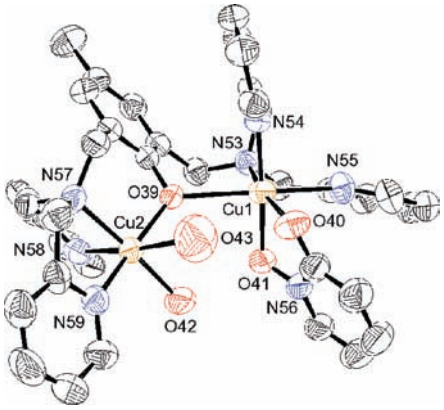

Figure 2. ORTEP drawing of the dication $[Cu_2(BPMP)(OPNO)(H_2O)_2]^{2+}$ (**4**). H-atoms and ClO_4^- counteranions were omitted for clarity.

spectrum, indicating that only one equivalent binds the dicopper center.

Alternatively, reaction of complex **2** with an excess of HOPNO in acetone/water medium and subsequent layering with toluene afforded pure compound **4** as a crystalline material (Figure 2). Spectroscopic and analytic data in the solid state proved this material to be identical to the former one prepared in solution. The X-ray structure of complex **4** revealed the presence of a 1:1 (**2**)/OPNO adduct. OPNO is coordinated to copper Cu1 in a bidentate mode and forms the basis of an elongated octahedral geometry with the N-atoms of one amine (N53) and one pyridine (N54) of the ligand. The axial coordinations are occupied by a second pyridine (N55) and the phenoxo moiety (O39). This latter bridges the two metals but belongs to the equatorial Cu2 coordinations which are completed by one water molecule (O42) and two N-atoms from the second amine (N57) and a third pyridine (N59). The axial positions of the octahedral Cu2 coordination are completed by one water molecule (O43) and the N-atom (N58) of the fourth pyridine. OPNO is not coplanar with the basis of the octahedron as the angle between the plane defining this latter and the one defined by OPNO is close to 31°. The longer distances of the axial coordinations (~2.4 Å) are relevant of a strong Jahn−Teller effect. This effect is also important for Cu2 (Cu2−O43 = 2.586 Å and Cu2−N58 = 2.29 Å). Finally, in addition to these important changes in the coordination of the two copper ions, OPNO binding increases the copper−copper distance from 2.966 Å in **2**[22] to 3.927 Å in **4**.

The magnetic properties and the NMR spectrum of complex **2** have been reported earlier.[22] It exhibits antiferromagnetic coupling between the copper ions ($J = -112 \pm 2\ cm^{-1}$, with $H = -2JS_1S_2$; $S_1 = S_2 = 1/2$). The EPR spectra of **2** and **4** in acetone/toluene frozen solutions (120K) are displayed in Figure 4 of the Supporting Information. Complex **2** is EPR-silent, which is in accordance with the relatively strong antiferromagnetic coupling between the copper ions and consistent with the doubly bridged structure reported previously.[22] Upon addition of 1 equiv. of HOPNO, the spectrum reveals a broad signal from 200 to 400 mT, which is characteristic of slightly or nonmagnetically coupled copper(II) ions. Structural data show that the copper(II) ions have distorted octahedral coordination geometries and are bridged by a phenoxo group in an axial−equatorial mode. The bridging phenoxo oxygen is indeed in the axial position relative to one copper(II) ion (Cu1) and equatorial to the other one (Cu2). The absence of strong magnetic exchange interaction in compound **4**, as evidenced by the observed EPR spectrum, could thus be connected to the relative orientation of the magnetic orbitals (i.e., the orbitals that contain the unpaired electrons). The NMR resonances of **2** broaden upon addition of HOPNO, and their intensity decreases without any new resonances appearing in the spectrum (see Figure 2 in the Supporting Information). Altogether, these results show that binding of 1 equiv. of HOPNO on dicopper center occurs with the subsequent dissociation of the hydroxo bridge, leading to the decrease in the antiferromagnetic coupling between the copper centers.

In summary, our results demonstrate that HOPNO is an efficient competitive inhibitor for mushroom Ty with one of the best features reported in literature ($IC_{50} = 1.16\ \mu M$; $K_I = 1.8\ \mu M$). Moreover, our results describe the first example of a dicopper(II) complex model for Ty with a TS-analog inhibitor as bidentate ligand to one copper center. Thus, we proposed that HOPNO targets the Ty met form to produce a bidentate unreactive adduct. Scheme 1 represents a further contribution in the description of the reaction mechanism of Ty, because HOPNO should resemble the expected coordination mode of the catechol substrate on its way to product formation. These results, which are of biological relevance in the context of Ty mechanism, has to be placed in the perspective of the work of Woolery et al. with the L-mimosine as adduct of *Neurospora crassa* Ty[24] and the more recent one of Bubacco et al. with kojic acid as adduct of *Streptomyces antibioticus* Ty.[25] In this later study, on the basis of an X-ray absorption analysis,[25] ESEEM,[26] and HYSCORE,[27] kojic acid was proposed to be a bidentate ligand to CuB of Ty with one O-atom axially coordinated and the second probably bridging the two copper ions in a $\eta^2{:}\eta^1$ bidentate mode. These finding are of great interest in relation with new Ty inhibitor design.

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Supporting Information Available: Experimental details for the synthesis and spectroscopic data of **3** and **4** and enzymatic data for the mushroom Ty inhibition by HOPNO (PDF); CIF files for complexes **3** (CCDC 713849) and **4** (CCDC 713850). This material is available free of charge via the Internet at http://pubs.acs.org.

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