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Simplest Monodentate Imidazole Stabilization of the oxy-Tyrosinase Cu_2O_2 Core: Phenolate Hydroxylation through a Cu^{III} Intermediate

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Abstract: *Tyrosinases are ubiquitous binuclear copper enzymes that oxygenate to $Cu^{II}_2O_2$ cores bonded by three histidine Nτ-imidazoles per Cu center. Synthetic monodentate imidazole-bonded $Cu^{II}_2O_2$ species self-assemble in a near quantitative manner at −125 °C, but Nπ-ligation has been required. Herein, we disclose the syntheses and reactivity of three Nτ-imidazole bonded $Cu^{II}_2O_2$ species at solution temperatures of −145 °C, which was achieved using a eutectic mixture of THF and 2-MeTHF. The addition of anionic phenolates affords a $Cu^{III}_2O_2$ species, where the bonded phenolates hydroxylate to catecholates in high yields. Similar $Cu^{III}_2O_2$ intermediates are not observed using Nπ-bonded $Cu^{II}_2O_2$ species, hinting that Nτ-imidazole ligation, conserved in all characterized Ty, has functional advantage beyond active-site flexibility. Substrate accessibility to the oxygenated Cu_2O_2 core and stabilization of a high oxidation state of the copper centers are suggested from these minimalistic models.*

Tyrosinases (Ty) are ubiquitous binuclear Cu enzymes responsible for melanin biosynthesis through regioselective tyrosine aromatic hydroxylation to form catechols, and their subsequent oxidation to quinones. Spectroscopic and X-ray crystallographic analysis of the oxygenated form of tyrosinase (oxyTy) reveals a side-on μ-η^2:η^2 peroxide dicopper (II) (^{S}P) species ligated exclusively by six histidine residues through the τ-nitrogen (Nτ) of each imidazole (Figure 1).[1] This results in a highly accessible ^{S}P Cu_2O_2 core where the 4-substituents of the imidazoles are positioned remotely. This Nτ-ligated ^{S}P core is conserved in oxygenated hemocyanin (oxyHc) proteins,[2] an evolutionary descendant of Ty[3] responsible for reversible dioxygen (O_2) uptake and storage.[1b,4] The reactivity difference between oxyTy and oxyHc is attributed to substrate access to the oxygenated Cu_2O_2 core,[5] as partially denatured hemocyanin proteins can exhibit monooxygenase reactivity.[6] Alternatively, more sterically demanding and less-flexible π-nitrogen (Nπ) imidazole ligation is observed exclusively in electron-transfer blue-copper sites, suggesting Nτ-ligation may serve a functional purpose, such as providing flexibility for O_2 capture, allowing substrate access to the oxygenated ^{S}P core, and potentially stabilizing higher oxidation states of copper in the mono-oxygenase reaction.[1c,7]

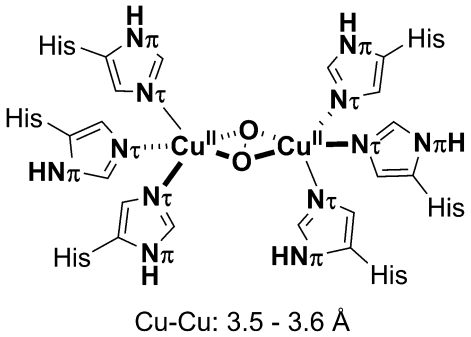

Figure 1. The conserved ligation structure of oxyTy.[1]

Synthetic ^{S}P analogues of oxyTy have received extensive attention over the last few decades.[4a,8] Stoichiometric phenolate oxidation to catecholates is possible at the low reaction temperatures required to stabilize most synthetic ^{S}P species, although several catalytic systems operating at elevated temperatures are now known.[9] These complexes are generally supported by multi-nucleating alkyl amines or heterocycles, such as pyridines, pyrazoles, or benzimidazoles. However, synthetic ^{S}P species exclusively ligated by imidazoles, especially by imidazoles bearing an N–H group,[10] are limited.[8b,c,11] Over 30 years ago, Zuberbühler reported that Cu^I bonded by monodentate imidazoles can react with O_2 under ambient aqueous conditions via a binuclear intermediate, as inferred from kinetic measurements.[12] In the early 1990s, Zuberbühler and Karlin subsequently investigated the oxygenation of $[Cu^I(1,2\text{-dimethylimidazole})_3]^+$ at lower temperatures using weakly coordinating anions in aprotic solvents,[13] but the nature of those intermediates could not be assigned definitively.

More recently, the near quantitative self-assembly of a series of imidazole-ligated ^{S}P species (^{S}P$_{RImd}$) by direct oxygenation of $[Cu^I(RImd)_3]^+$ at −125 °C in 2-methyltetrahydrofuran (2-MeTHF) was disclosed.[10] At these temperatures, deleterious reactions along the oxygenation and subsequent ^{S}P$_{RImd}$ formation pathways are presumably eliminated as yields greater than 90 % are realized. However, Nπ-ligation with 2- or 4,5-substitued imidazoles (e.g., 1,2-dimethylimidazole or 4,5-dimethylimidazole) was necessary for good formation yields; no significant formation of ^{S}P$_{RImd}$ species with Nτ-ligation (e.g., 1-methylimidazole) was observed. Herein, we present the synthesis and characterization of the first examples of ^{S}P$_{RImd}$ species bearing Nτ

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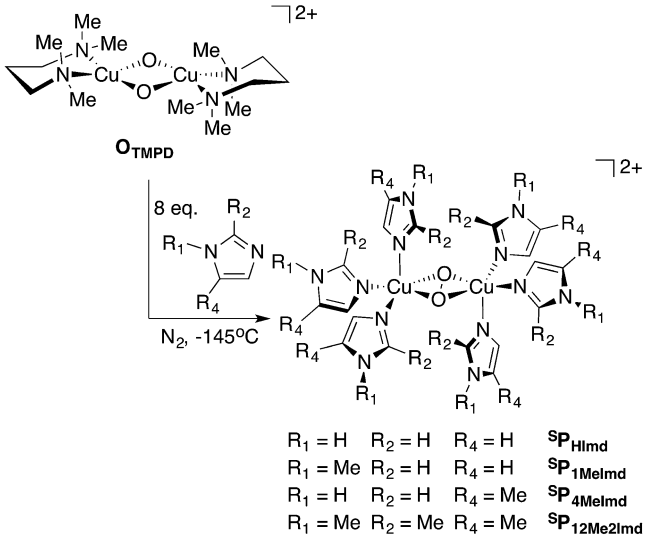
ligation by the simplest imidazoles (1-methyl, 4-methyl, and unsubstituted imidazole), and their reactivity with phenolates to form a transient but detectable Cu^{III} intermediate at −145 °C that subsequently yield their corresponding hydroxylated products.

While direct oxygenation of $[Cu^{I}(RImd)_3]^+$ to $^{S}P_{RImd}$ species with these simplest imidazoles at −145 °C is possible, the yields are improved significantly using an indirect ligand exchange pathway, designated as core-capture (Scheme 1).[14]



Scheme 1. Core-capture synthetic methodology to generate $^{S}P_{RImd}$ species.

The bis-μ-oxide dicopper (III) species of tetramethylpropylenediamine (O_{TMPD}) is a robust $Cu–O_2$ compound that forms fully at −80 °C.[15] At −125 °C, the addition of 8 equiv of 1,2-dimethylimidazole to O_{TMPD} yields an identical spectrum to that achieved by direction oxygenation of $[Cu^{I}$(1,2-dimethylimidazole$)_3]^+$ (Supporting Information, Figure S1); neither spectra appreciably changed over 30 minutes. By contrast, adding 8 equiv of one of the simplest imidazoles to O_{TMPD} at −125 °C only leads to transient formation of the target $^{S}P_{RImd}$ species. To slow thermal decay, lower solution temperatures are necessary. With the freezing point of 2-MeTHF at −136 °C, only viscous, unworkable slurries were possible below −125 °C in our experimental setup. A eutectic mixture of 2-MeTHF:THF (4:1), cooled externally by a slurry of pentane:2-methylbutane (4:3) with liquid N_2, yields stable solution temperatures of −145 °C while maintaining millimolar concentrations of charged copper complexes. Adding 8 equiv of each of the simplest imidazoles to O_{TMPD} at −145 °C leads to full formation of their respective Nτ-ligated $^{S}P_{RImd}$ species in approximately 5 minutes (Figure 2). While $^{S}P_{1MeImd}$ is stable indefinitely at −145 °C, $^{S}P_{4MeImd}$ and $^{S}P_{HImd}$, containing N–H imidazoles, decay with half-lives ($t_{1/2}$) of 30 and 15 minutes, respectively.

The yield of $^{S}P_{1MeImd}$ is greater than 95 %, assessed through a subsequent core-capture experiment using tris[(2-dimethylamino)ethyl]amine (Me_6Tren) to yield an end-on *trans*-μ-η^1:η^1 peroxide dicopper (II) ($^{T}P_{Me6Tren}$) complex (Figure S4).[10] The

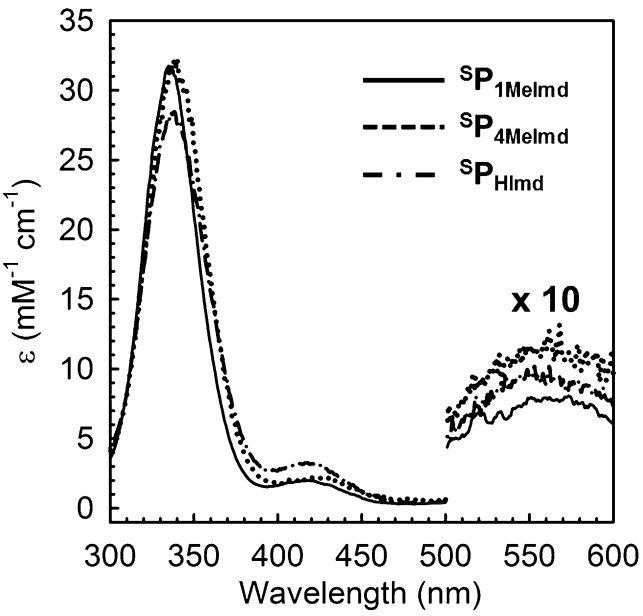


Figure 2. UV/Vis absorption spectra of $^{S}P_{1MeImd}$ (solid, λ_{max} = 335, 417, 560 nm), $^{S}P_{4MeImd}$ (dotted, λ_{max} = 338, 423, 560 nm), and $^{S}P_{HImd}$ (dash-dotted, λ_{max} = 337, 417, 550 nm) generated from O_{TMPD} at −145 °C. [Cu] = 0.9 mM, 0.1 cm path, 4:1 2-MeTHF/THF, −145 °C.

comparable extinction coefficients (340 nm) of the ligand-to-metal charge transfer (LMCT) band for $^{S}P_{4MeImd}$ and $^{S}P_{HImd}$ suggests comparably good formation yields (Table 1).

Solution Cu K-edge X-ray absorption spectroscopy (XAS) of $^{S}P_{1MeImd}$, $^{S}P_{4MeImd}$, and $^{S}P_{HImd}$ exhibit pre-edge energies of near 8979 eV, which are characteristic of the Cu^{II} oxidation state (Figure S5).[10,16] The extended X-ray absorption fine structure (EXAFS) analyses of $^{S}P_{1MeImd}$ and $^{S}P_{4MeImd}$ provide Cu–Cu distances in the 3.55–3.57 Å range, which are similar to other characterized $^{S}P_{RImd}$ species (Table 1, Figures S6–S7). EXAFS fitting of $^{S}P_{HImd}$ was complicated by its thermal instability.

Broken symmetry density functional theory (DFT) calculations at a B3LYP/6-31g(d)/SMD^{THF} level in C_i symmetry optimize $^{S}P_{1MeImd}$, $^{S}P_{4MeImd}$, and $^{S}P_{HImd}$ to planar $Cu^{II}_2O_2$ cores with Cu–Cu distances of approximately 3.63 Å, in reasonable agreement with their experimental EXAFS metrical parameters (Table 1).[10] In addition, shorter Cu–ligand bond distances ($^{S}P_{1MeImd}$: Cu–N_{eq} = 1.99 Å, Cu–N_{ax} = 2.14 Å, Cu–O = 1.96 Å; $^{S}P_{12Me2Imd}$: Cu–N_{eq} = 2.00 Å, Cu–N_{ax} = 2.18 Å, Cu–O = 1.97 Å) and slight lengthening of the O–O bond is predicted ($^{S}P_{1MeImd}$: O–O = 1.48 Å; $^{S}P_{4MeImd}$: O–O = 1.47 Å; $^{S}P_{HImd}$: O–O = 1.48 Å; $^{S}P_{12Me2Imd}$: O–O = 1.47 Å). An elongated O–O bond is consistent with greater back-bonding from the Cu center into the peroxide σ* orbital afforded by stronger bonds by the imidazole ligands.[17]

The UV/Vis absorption spectra of $^{S}P_{1MeImd}$, $^{S}P_{4MeImd}$, and $^{S}P_{HImd}$ all exhibit an intense UV feature, along with two weak and broad visible features (Figure 2, Table 1).[10] The LMCT UV feature[18] is blue-shifted markedly from the 344 nm feature of $^{S}P_{12Me2Imd}$ and $^{S}P_{2MeImd}$, both bonded by Nπ-ligating imidazoles (Table 1, Entries C and D). This is consistent with stronger imidazole bonding to the $Cu^{II}_2O_2$ core relative to other $^{S}P_{RImd}$ species.

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Table 1: Physical properties of $^{S}P_{RImd}$ species formed by core-capture at −145 °C.

Entry	Compound[a]	UV/Vis: λ_{max}, nm (ε, mM^{-1} cm^{-1})[a,b]	Metrical parameters: EXAFS, Å;[c] (DFT, Å[d])				
			Cu–Cu	Cu–N_{eq}/O	Cu–O	Cu–N_{ax}	O–O
A	$^{S}P_{1MeImd}$	335 (32.2), 417 (2.1), 560 (1.0)	3.55 (3.63)	1.95 (1.99)	(1.96)	2.24 (2.15)	(1.48)
B	$^{S}P_{4MeImd}$	338 (31.9), 423 (2.2), 560 (1.2)	3.57 (3.63)	1.96 (1.99)	(1.96)	2.20 (2.14)	(1.47)
C	$^{S}P_{12Me2Imd}$[b]	344 (30.6), 428 (1.9), 560 (1.0)	3.57 (3.66)	1.95 (2.00)	(1.97)	2.21 (2.18)	(1.47)
D	$^{S}P_{2MeImd}$[b]	344 (29.6), 425 (1.6), 560 (1.1)	3.58 (3.66)	1.96 (2.00)	(1.97)	2.23 (2.17)	(1.47)

[a] SbF_6^-, 4:1 2-MeTHF/THF. [b] See Ref. [10] for further characterization. [c] Resolution ±0.01 Å. [d] Optimized at B3LYP/6-31g(d)/SMD^{THF} level of theory in C_i symmetry.

Time-dependant DFT spectra of $^{S}P_{1MeImd}$, $^{S}P_{4MeImd}$, and $^{S}P_{HImd}$ are similar to their experimental spectra (Figure S8), as well as other $^{S}P_{RImd}$ species. Three significant transitions are predicted that are blue-shifted from other $^{S}P_{RImd}$ species. Natural Transition Orbital (NTO) analysis[19] reveals that the most intense experimental absorbance at 335 nm is an in-plane peroxide $\pi_\sigma^*\rightarrow d_{xy}$ transition, while the broad transition near 550 nm is an out-of-plane peroxide $\pi_v^*\rightarrow d_{xy}$, both consistent with previous assignments (Figure S9).[18] The 417 nm feature, which was also observed and predicted for $^{S}P_{12Me2Imd}$ and $^{S}P_{2MeImd}$, is assigned as an imidazole $\pi^*\rightarrow d_{xy}$ from the NTO analyses (Figure S9).[10]

The origin of blue-shifting for the $\pi_\sigma^*\rightarrow d_{xy}$ transition of $^{S}P_{1MeImd}$ in comparison to $^{S}P_{12Me2Imd}$ was probed by TD-DFT. $^{S}P_{12Me2Imd}$ optimized in C_i symmetry exhibits a predicted transition at 349 nm. Removal of the 2-methyl substituent without further optimization results only in a 1 nm blue-shifted absorbance at 348 nm. Subsequent C_i symmetric optimization results in a slight contraction of coordinative bond distances ($\Delta Cu–N_{avg} = 0.02$ Å, $\Delta Cu–Cu = 0.03$ Å), affording $^{S}P_{1MeImd}$ with a 338 nm transition, a 10 nm blue-shift. This supports strongly that the observed blue-shifting is associated with stronger imidazole bonding to the metal center, corresponding from a reduction in ligand steric demands from Nπ to Nτ ligation. The increased experimental intensity of this LMCT feature for $^{S}P_{1MeImd}$ compared to $^{S}P_{12Me2Imd}$ further supports enhanced covalency in these contracted bonds.

While $^{S}P_{12Me2Imd}$ is favored thermodynamically over $^{S}P_{1MeImd}$ with respect to ligand substitution as established by ligand competition experiments (Figures S10 and S11), the increased steric demands of the ligating imidazoles consequently preclude substrate access to the ^{S}P core. Indeed, $^{S}P_{1MeImd}$ reacts rapidly and stoichiometrically with 0.5 equiv of 5,6-isopropylidene-L-ascorbic acid (H_2Asc), a net two-electron, two-proton reductive substrate, upon mixing at −145 °C (Figure S12).[20] $^{S}P_{12Me2Imd}$ exhibits essentially no reactivity towards H_2Asc under the same conditions, and only reacts in a pseudo-first-order manner in the presence of 20 equiv of H_2Asc ($t_{1/2} = 2$ min) at −145 °C (Figure S13). This highlights that $Cu^{II}_2O_2$ core exposure, by minimizing ligand steric demands, facilitates substrate access and rapid reaction with exogenous substrates.

The effects of increasing substrate access to the Cu_2O_2 core is further demonstrated through phenolate reactivity, in which $^{S}P_{1MeImd}$ reacts with 4 equiv of sodium 15-crown-5,2-*tert*-butyl-4-cyano phenolate at −145 °C to form an intermediate species in 1 minute (thermal decay $t_{1/2} = 10$ min, Figure 3). Formation of a similar intermediate is not observed for $^{S}P_{12Me2Imd}$, although comparable yields of the catechol product are detected.

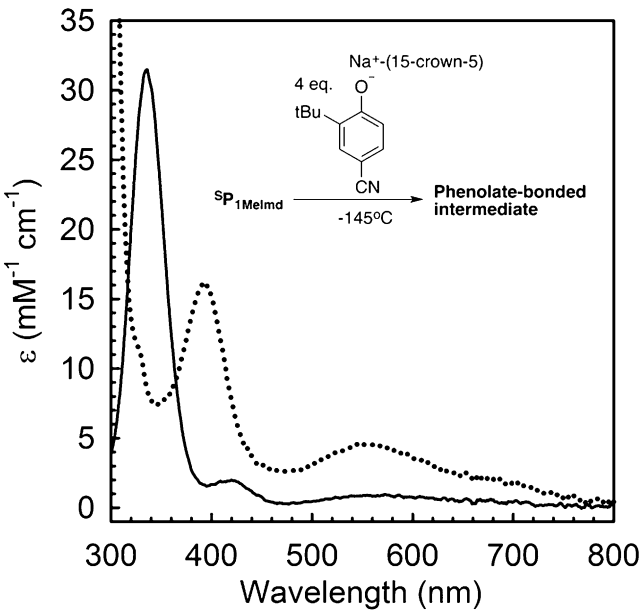

Figure 3. UV/Vis absorption spectra of a phenolate-bonded Cu^{III} intermediate (dotted, $\lambda_{max} = 394$, 555, 650 nm) generated from $^{S}P_{1MeImd}$ (solid) at −145 °C. [Cu] = 0.9 mM, 0.1 cm path, 4:1 2-MeTHF/THF, −145 °C.

Cu K-edge XAS analysis supports this intermediate as a bis-μ-oxide dicopper (III) species based on its pre-edge energy of 8980.2 eV and a characteristic 2.8 Å Cu–Cu distance (Figures S14 and S15). While its identity is currently under investigation, this intermediate is ligated exclusively with imidazoles, phenolates, and O_2-based ligands.[21] A Cu^{III}-containing species coordinated by such biologically relevant ligands is unprecedented, and its characterization may provide insight into the active hydroxylating oxidant of tyrosinases.[4a,22]

Demonstrated here is the formation and stabilization of three previously unobserved $^{S}P_{RImd}$ species supported by the simplest imidazoles at −145 °C. These imidazoles contain no substituent adjacent to the ligating nitrogen atom, allowing for Nτ-ligation that is conserved in all of the oxyTy and oxyHc structures.[1a,b,2,23] $^{S}P_{4MeImd}$ represents the most structurally

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faithful model to oxyTy, where 4-methylimidazole acts as a truncated histidine mimic that is expected to exhibit Nτ-ligation to the ^{S}P core based on its optical spectrum, while $^{S}P_{HImd}$ is the simplest imidazole-ligated ^{S}P species possible. The effect of $Cu^{II}_2O_2$ core exposure by minimizing imidazole steric demands and the importance of this work is realized in their reactivity towards H_2Asc at −145 °C; $^{S}P_{1MeImd}$ reacts stoichiometrically as an overall two H-atom acceptor, while $^{S}P_{12Me2Imd}$ only reacts in the presence of an excess of H_2Asc. This demonstrates that exposed $^{S}P_{RImd}$ cores facilitate substrate access to enhance reaction rates. Further evidence comes from the reaction of $^{S}P_{1MeImd}$ with phenolates to afford a transient phenolate-bonded bis-μ-oxide dicopper (III) $Cu^{III}_2O_2$ intermediate, the stabilization of which is enhanced by the lesser steric demands of Nτ-ligated imidazoles. The evolutionary selection of exclusive Nτ-ligated imidazole ligation in such dioxygen-activating copper enzymes may indeed be tied to accessing, albeit transiently, the higher Cu^{III} oxidation state, a non-intuitive functional advantage.

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