Stepwise Formation of a Nonsymmetric Dinuclear Copper Complex of Ochratoxin A

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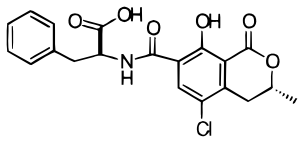
Introduction

Ochratoxin A (OTA, **1**) is a fungal toxin produced by species of *Aspergillus* and *Penicillium*.[1] It contaminates a wide range of agricultural products and is implicated in human kidney carcinogenesis.[2] The toxin facilitates DNA cleavage[3] and DNA adduction when metabolically activated,[4] features that establish a basis for its cancer-causing properties. Ingestion of **1** also leads to mitochondrial dysfunction in rats,[5] apparently through the inhibition of metalloenzymes responsible for electron transfer.[6]



OTA, **1**

While the mechanism(s) underlying the deleterious activities of **1** are unclear, the ability of **1** to coordinate metals, such as iron(III),[7] has been well established. Examination of the toxin shows that both the dihydroisocoumarin (lactone) and the amide-attached L-phenylalanine could facilitate metal binding by the phenolic oxygen. In this regard, we have recently shown that **1** binds copper(II) to form a 1:1 Cu(II)·OTA complex (equilibrium binding affinity (log $K_{1:1}$) = 6.40 M^{-1} in aqueous buffered media).[8] Structure−activity relationships employing synthetic analogues of **1** established that the phenolic oxygen and carboxylic acid of the phenylalanine moiety participate in copper(II) coordination.[8] We now describe the ability of **1** to coordinate copper(II) in a stepwise fashion to form a nonsymmetric dinuclear copper complex (**2**).[9] This ability is an intriguing coincidence given that **1** inhibits that activity of metalloenzymes in the mitochondria that depend on the juxtaposition of copper and iron centers to transfer electrons during enzyme turnover.[10] The resulting complex **2** is also unique in that it represents an nonsymmetric dinuclear copper complex[9] with an oxygen-rich coordination environment.[11] Under physiological conditions, copper binding by **1** also provides a rationale for the production of the opened lactone form **3**, which has been implicated in the in vivo toxicity of OTA.[7b]

Experimental Section

Materials and Methods. Ochratoxin A (OTA, **1**) was purchased from Dr. Ronald Marquardt, Department of Animal Sciences, University of Manitoba, and was used without further purification. **Caution!** *OTA is a mutagen and carcinogen. Protective clothing should be worn and appropriate safety procedures followed when working with this compound.* All other reagents were obtained commercially (Aldrich) and were used without further purification. Distilled, deionized water from a Milli-Q system was used for all aqueous solutions and manipulations. Absorption measurements were made on a Hitachi U-2001 UV−vis spectrophotometer. High-pressure liquid chromatography (HPLC) was carried out on a Hitachi L-7000 series interfaced to a Waters 991 photodiode array detector. Mass spectra were acquired using a Micromass Quattro II operating in the negative ion spray mode (ES^-). The system acquired signal over a *m*/*z* range of 200−1000 at 8 s/scan. Samples (0.1 mg/mL) were prepared in methanol and injected via syringe into a fixed loop injector port (100 μL volume) interfaced to the ionspray source.

NMR Titration Experiment. The ^{1}H NMR titration experiment was carried out on a Varian VXR 200-MHz instrument in CD_3OD. Initially, a CD_3OD solution of OTA (27 mM in 630 μL, concentration determined by UV−vis, ϵ_{330} = 5500 M^{-1} cm^{-1}) was placed in a septum-capped NMR tube. Injection of 1 equiv of $CuCl_2$ (10 μL from a 1.7 M stock solution in D_2O) followed by 2 equiv of NaOD (32 μL from a 1 M stock solution in D_2O) afforded a sample of the 1:1 Cu·OTA complex (verified by UV−vis, ϵ_{365} = 6750 M^{-1} cm^{-1}). A second equivalent of $CuCl_2$ was then added to the 1:1 Cu·OTA solution. ^{1}H NMR spectra of both the 1:1 and 2:1 Cu·OTA solutions were recorded.

X-ray Structure and Determination. The dinuclear copper complex **2** was generated by first dissolving 20 mg (0.05 mmol) of **1** in 1 mL of methanol followed by the addition of 2 equiv of copper acetate ($Cu(OAc)_2$) and 3 equiv of potassium *tert*-butoxide (t-BuOK dissolved in methanol). The solvent was allowed to evaporate, and X-ray-quality crystals were grown from a mixture of methanol and ethyl acetate over several days at ambient temperature. X-ray measurements, using a full hemisphere of diffracted intensities (omega scan width of 0.25°), were carried out on a Siemens/Bruker SMART CCD single-crystal diffraction system. Solution and refinement of **2** were accomplished with the SHELXTL-V5.0 set of programs. Crystallographic parameters for **2** are listed in Table 1.

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Table 1. Crystal Data and Structure Refinement for $Cu_2(C_{20}H_{15}NO_6Cl)(OCH_3)(CH_3OH)$[a]

empirical formula	$C_{22}H_{22}ClCu_2NO_8$
fw	590.94
cryst syst	orthorhombic
cryst size	0.09 × 0.10 × 0.13 mm
space group	$P2_12_12_1-D_2^4$ (No. 19)
	a = 12.8919(2) Å
	b = 12.9977(3) Å
	c = 13.4006(3) Å
volume, Z	2245.47(8) Å^3, 4
density (calcd)	1.748 g cm^{-3}
abs coeff, range of transm factors	2.064 mm^{-1}, 0.180−0.214
F(000)	1200
radiation type (wavelength)	Mo K$\bar{\alpha}$ (0.710 73 Å)
temperature	198(2) K
2θ range for data collcn	4.36 to 56.54°
limiting indices	$-17 \le h \le 7$, $-17 \le k \le 17$, $-17 \le l \le 16$
no. of reflcns collcd	14 477
no. of indep reflcns	5369 [R_{int} = 0.065]
refinement method	full-matrix least-squares on F^2
data/parameters	5058/314
goodness-of-fit on F^2	1.098
final R indices	
4140 data, $I > 2\sigma(I)$	R1 = 0.0573
5058 data, $F_o^2 > 0$	wR2 = 0.1111
all 5369 data	R1 = 0.0912, wR2 = 0.1221
absolute structure param	0.00(2)
largest diff peak and hole	0.604 and −0.700 e$^-$/Å^3

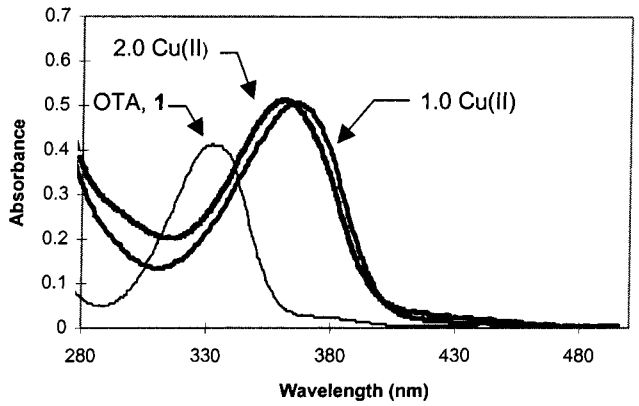

Figure 1. UV−vis titration of **1** with $Cu(OAc)_2$ in methanol at room temperature.

Reaction of OTA with Excess Copper(II) in Aqueous Buffered Media. Concentrated stock solutions of **1** and $Cu(OAc)_2$ were added to 100 mM 4-morpholinepropanesulfonic acid (MOPS) buffer, pH 7.8, to give a final concentration of 1 mM **1** and 6 mM $Cu(OAc)_2$. The reaction vessel was placed into a 37 °C water bath. Aliquots (100 μL) were removed at timed intervals and quenched with excess ethylenediaminetetraacetic acid (EDTA). The samples were subsequently analyzed by HPLC using a linear gradient of 30−70% buffer B over 25 min on a reversed-phase C-18 column with a flow rate of 1 mL/min: buffer A = (80% buffer, 20% MeOH), buffer B = (20% buffer, 80% MeOH); buffer = 20 mM phosphate, pH 6.5. The half-life ($t_{1/2}$ = 0.693/k, where ln(A) = $-kt$ ln(A°): A° = initial [**1**]; A is [**1**] at time t, k = first-order rate constant) for the hydrolysis of **1** into the opened lactone form **3**[7b] was determined by measuring the time course of the change in the peak area for **1** at 330 nm. An authentic sample of **3** prepared from the reaction of **1** with 0.5 N NaOH[7b] served as an HPLC standard. The ENZFITTER program was used to determine k and $t_{1/2}$.

Results and Discussion

The ability of **1** to bind copper(II) was initially studied in methanol (Figure 1). Addition of 1 equiv of $Cu(OAc)_2$ to a methanolic solution of **1** yields a complex of 1:1 stoichiometry

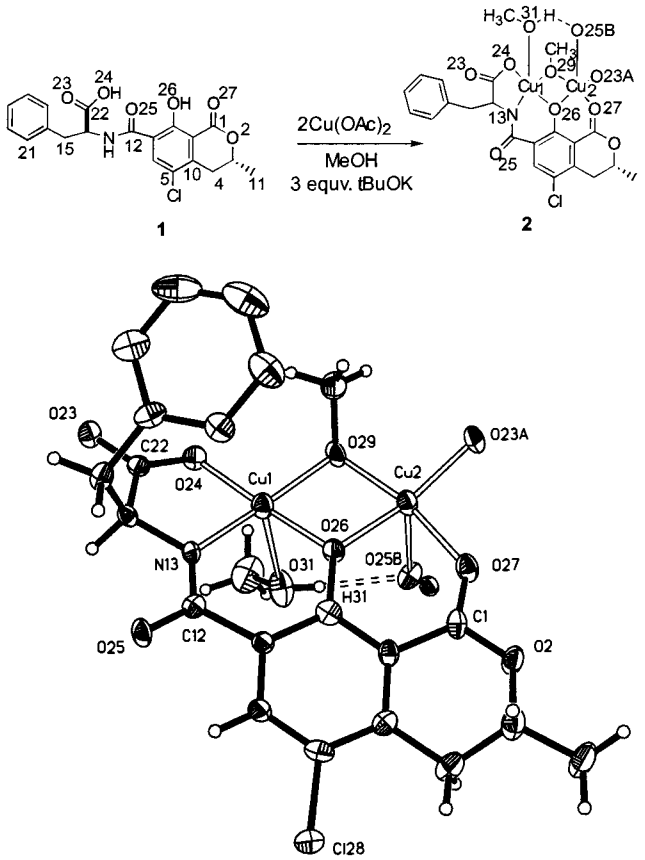

Figure 2. Synthesis of **2** and a perspective drawing of its solid-state structure. Nonhydrogen atoms are represented by thermal vibration ellipsoids drawn to encompass 50% of their electron density. Selected hydrogen atoms are omitted for purposes of clarity.

with λ_{max} = 365 nm (ϵ = 6750 M^{-1} cm^{-1}) and 608 nm ($\epsilon \sim$ 60 M^{-1} cm^{-1}). Electrospray mass spectrometry provided evidence for a complex with molecular formula $CuC_{20}H_{16}NO_6Cl$ ([M − H]$^-$ = 463).[8] Addition of a second equivalent of $Cu(OAc)_2$ yielded a new species with λ_{max} = 360 nm (ϵ = 6870 M^{-1} cm^{-1}) and 634 nm ($\epsilon \sim$ 30 M^{-1} cm^{-1}). The electrospray mass spectrum of the 2:1 (Cu·OTA) mixture using negative ionization (ES$^-$) revealed that the major complex had [M − H]$^-$ = 615.9 (Supporting data). Collision induced dissociation of this species indicated loss of m/z 153 mass units to afford the 1:1 Cu·OTA species with [M−H]$^-$ = 463.1. This result suggested the possibility that a second copper atom coupled with an exogenous acetate group (m/z 59) and a methoxide ion (63 + 59 + 31 = 153) had been added to the 1:1 Cu·OTA complex.

Further evidence for stepwise complexation of Cu(II) by **1** was provided by ^{1}H NMR spectroscopy. Addition of 1 equiv of $CuCl_2$ to a CD_3OD solution of **1** led to a spectrum with broad unresolved ^{1}H resonances (Supporting Information). However, upon addition of the second equivalent of copper, the resonances in the diamagnetic region sharpened considerably and new broad resonances at 19.3, 14.1, and 8.0 ppm were observed (Supporting Information). These results were fully consistent with initial formation of a mononuclear copper complex were the paramagnetic copper (S = $^1/_2$) leads to broad unresolved ^{1}H NMR resonances,[12] followed by formation of a dinuclear copper complex were coupling of the two paramagnetic copper centers leads to relatively sharp signals.[13]

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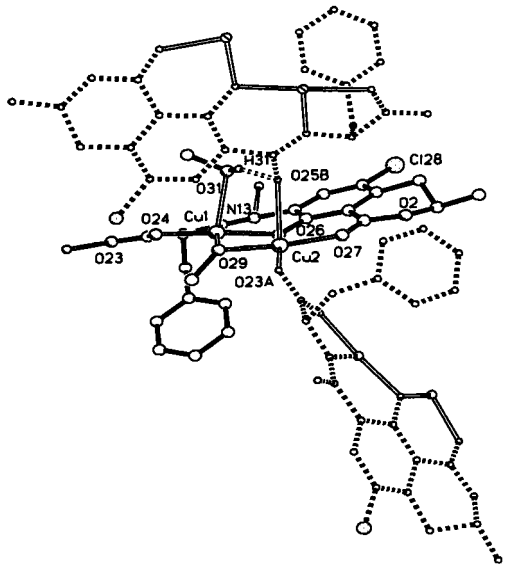


Figure 3. Perspective drawing of the solid-state structure of $Cu_2(C_{20}H_{15}NO_6Cl)(OCH_3)(CH_3OH)$, **2**, highlighting the polymeric linkage for three asymmetric units.

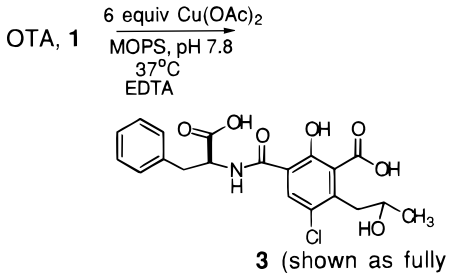
Unambiguous support for the ability of **1** to bind two copper ions was obtained from X-ray crystallography. The asymmetric unit of the resulting polymeric structure **2** (Figures 2 and 3) consists of one OTA^{3-} moiety, two Cu(II) ions, a methoxide anion, and a methanol molecule. The Cu ions adopt edge-shared square-pyramidal geometry in the crystal by bonding to five nitrogen or oxygen atoms.[14] The common (basal) edge of their coordination polyhedra are occupied by μ_2-phenoxy (O26) and μ_2-methoxy (O29) oxygens from the OTA^{3-} and methoxide ligands, respectively.[15] The remaining basal coordination sites for Cu1 are occupied by O24 and N13 of the bridging OTA^{3-} ligand. The remaining basal sites for Cu2 are occupied by O27 of this same OTA^{3-} ligand and the carboxylic oxygen (O23A) of a symmetry-related OTA^{3-} ligand. The fifth (apical) coordination site of Cu1 is occupied by the oxygen atom (O31) of the methanol molecule; the hydrogen atom (H31) of this moiety is hydrogen-bonded to an amide oxygen (O25B) of another symmetry-related OTA^{3-} ligand that occupies the fifth (apical) coordination site of Cu2. Cu1 and Cu2 are displaced by 0.13 and 0.11 Å from their respective basal planes toward their apical ligands.

Selected bond lengths and angles for **2** are listed in Table 2 and are consistent with complexation of two Cu(II) ions to an OTA^{3-} ligand. The Cu−L−Cu separation of 2.9504(9) Å is in the range observed for doubly bridged μ-phenoxo dinuclear copper complexes, and on the basis of magnetic data for dinuclear copper complexes of this type, the two copper atoms are expected to exhibit antiferromagnetic coupling.[13,15−17] With respect to structural changes in **1** upon copper coordination, comparison of **2** to the X-ray structure of fully protonated **1**[18] indicates lengthening of the C1−O27 bond (1.230(7) vs. 1.190 Å). The amide carbonyl bond C12−O25 also lengthens (1.271(7) vs 1.221 Å) and the C12−N13−C14 dihedral angle widens (117.9 to 119.9(4)°); these features are consistent with amide nitrogen deprotonation.[17] Other changes include the equivalency of the carboxylic acid carbon−oxygen bonds (C22−O23 and C22−O24 = 1.264(7) Å), as both oxygens participate in copper binding.

Table 2. Selected Bond Lengths (Å) and Angles (deg) for **2**

Cu1···Cu2	2.9504(9)	Cu2−O27	1.923(4)
Cu1−O29	1.920(4)	Cu2−O29	1.910(4)
Cu1−O24	1.955(4)	Cu2−O23A	1.965(4)
Cu1−O26	1.960(4)	Cu2−O26	1.964(4)
Cu1−N13	1.908(4)	Cu2−O25B	2.487(5)
Cu1−O31	2.399(5)	C1−O27	1.230(7)
C22−O23	1.264(7)	C1−O2	1.313(7)
C22−O24	1.264(7)	C12−O25	1.271(7)
C5−Cl28	1.751(6)	O31−H31	0.85(7)
O31···O25B	2.777(7)	O25B···H31	1.96(7)
Cu1−O26−Cu2	97.5(2)	O29−Cu2−O27	167.4(2)
Cu2−O29−Cu1	100.8(2)	O29−Cu2−O26	80.33(15)
N13−Cu1−O29	167.8(2)	O27−Cu2−O26	87.6(2)
N13−Cu1−O24	85.8(2)	O29−Cu2−O23A	96.0(2)
N13−Cu1−O31	95.3(2)	O27−Cu2−O23A	96.6(2)
N13−Cu1−O26	93.9(2)	O26−Cu2−O23A	165.1(2)
O29−Cu1−O24	99.2(2)	O27−Cu2−O25B	81.9(2)
O24−Cu1−O26	175.4(2)	O26−Cu2−O25B	95.1(2)
O29−Cu1−O31	94.6(2)	O29−Cu2−O25B	95.5(2)
O24−Cu1−O31	101.1(2)	O23A−Cu2−O25B	99.6(2)
O29−Cu1−O26	80.2(2)	C12−N13−C14	119.9(4)
O26−Cu1−O31	83.5(2)	O31−H31···O25B	159(7)

The interaction of **1** with $Cu(OAc)_2$ in aqueous MOPS buffer pH 7.8 was also examined. Upon incubation of **1** with 6 equiv of $Cu(OAc)_2$ at 37 °C, **1** was transformed into the opened lactone form **3** (eq 1) with a half-life ($t_{1/2}$) of 14 h (Supporting Information). This species has also been detected in the bile of rats injected with **1**, and is formed in high yield when **1** is treated with 0.5 N NaOH.[7b] However, $t_{1/2}$ = 72 h for production of **3** in 0.5 M Na_2CO_3 (pH 10−11)[7b] and virtually no **3** is produced upon incubation of free **1** at 37 °C, pH 7.8 (eq 1).



3 (shown as fully protonated) (1)

The results presented here, together with our previous studies,[8] have allowed us to propose the stepwise formation of **2** outlined in Scheme 1. While we have yet to obtain X-ray structural data for the 1:1 Cu·OTA complex, our mass spec data and structure−activity relationships[8] indicate that **1** binds 1 equiv of Cu(II) by its phenolic oxygen and carboxylic oxygen of the phenylalanine moiety. Thus, possible binding arrangements illustrated by **4a/b** are envisioned in initial copper complexation by **1**. Addition of base and a second equivalent of Cu(II) would

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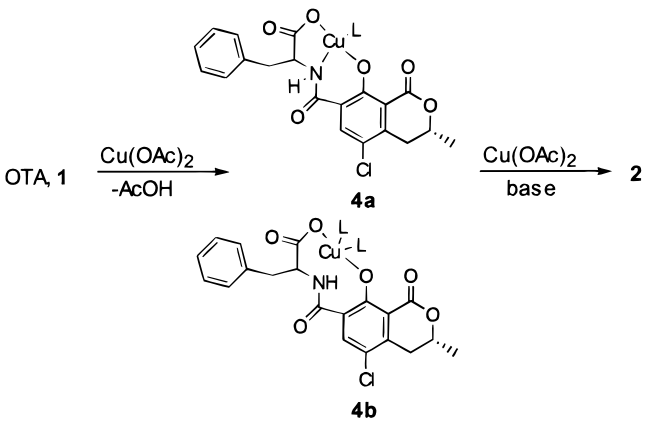
Scheme 1. Proposed Model for the Stepwise Complexation of $Cu(OAc)_2$ by **1**, Where L Is an Exogenous Ligand



facilitate amide nitrogen deprotonation and attachment of Cu(II) to the lactone carbonyl oxygen of **4a/b** to yield a nonsymmetric dinuclear complex, such as **2**. This unique property provides a rationale for formation of **3** and may well lead to an understanding of some of the mechanisms underlying the toxic features of **1**.

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Supporting Information Available: Mass spectra of both the 1:1 and 2:1 Cu·OTA mixtures in methanol, ^1H NMR spectra of 1:1 and 2:1 Cu·OTA in CD_3OD, kinetic data for hydrolysis of **1** (formation of **3**), and X-ray structural information for **2** (32 pages). Ordering information is given on any current masthead page.

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