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Copper monooxygenase models. Aromatic hydroxylation by a dinuclear copper(I) complex containing methionine sulfur ligands

Gloria Alzuet,[a] Luigi Casella,*[,a] Maria Laura Villa,[a] Oliviero Carugo[a] and Michele Gullotti[b]

[a] *Dipartimento di Chimica Generale, Università di Pavia, Via Taramelli 12, 27100 Pavia, Italy*
[b] *Dipartimento di Chimica Inorganica, Metallorganica e Analitica, Università di Milano, Centro CNR, Milano, Italy*

A dinuclear copper(I) complex **1** containing a bis(imine) ligand derived from the condensation between benzene-1,3-dicarbaldehyde and two molecules of L-methionine has been prepared. When this compound reacts with dioxygen a partial aromatic hydroxylation of the ligand occurs, giving a dinuclear μ-phenoxo-μ-hydroxo-dicopper(II) complex **2**, together with simple copper oxidation products. Definitive evidence of the monooxygenase activity of the present sulfur-containing model system results from the crystallographic characterisation of the dinuclear copper(II) complex **3** of the hydroxylated dicarbaldehyde, $[Cu_2\{C_6H_3(CHO)_2O\}(ClO_4)_2]$, which forms upon hydrolysis of the imine groups of **2**. In this complex two deprotonated 1,3-diformylphenoxide ligands bind two copper(II) ions, with di-μ-phenoxo bridges. Each copper is essentially square pyramidal, with a basal O_4 donor set, including two phenoxide and two carbonyl oxygen atoms from two 2-hydroxybenzene-1,3-dicarbaldehyde ligands. Two perchlorate oxygen atoms are bound in axial positions on opposite sides of the Cu_2O_6 plane. A minor fraction (15–20%) of **2** contains *S*-oxygenated methionine residues. However, oxygenation at sulfur is a secondary process, resulting from the reaction of H_2O_2, formed according to the simple copper(I) oxidation pathway, and the dinuclear copper(II) complex **2**.

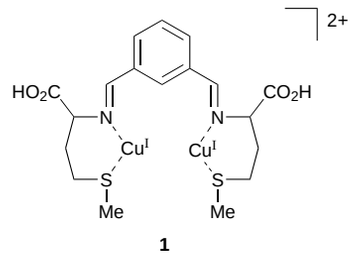
Dinuclear copper complexes with ligands of biological relevance and containing metal centres in close proximity have been extensively studied[1–3] since this structural unit is present in the active sites of several copper proteins involved in dioxygen transport and activation, *e.g.* hemocyanin and tyrosinase.[4] The recent characterisation of several dinuclear peroxocopper(II) complexes represents a topical achievement that has provided valuable information about the binding mode of dioxygen to the dicopper sites of the proteins. The redox chemistry associated with such characterised or putative peroxodicopper(II) complexes has produced a number of systems in which activation of dioxygen results in ligand oxygenation reactions. The systems where an aromatic hydroxylation of the ligand occurs[5–7] can be considered as functional models of tyrosinase, while those performing an aliphatic hydroxylation in the ligand[8] mimic the activity of dopamine β-hydroxylase[9] or peptidylglycine α-amidating monooxygenase.[10]

In a communication[7e] we briefly reported the ligand hydroxylation reaction undergone by a series of dicopper(I) complexes with the bis(imines) derived from benzene-1,3-dicarbaldehyde and substituted amines carrying various types of donor groups in the alkyl chain in the presence of dioxygen. Among these complexes the most interesting was certainly that derived from L-methionine (**1**, see Scheme 1) because of the presence of thioether sulfur donors. The relevance of this feature is related to the identification of a methionine ligand in the co-ordination sphere of the dioxygen-binding, Cu_B site of dopamine β-hydroxylase[9e] and peptidylglycine α-amidating enzyme.[10c] Since the monooxygenase activity promoted by the Cu–S centres of **1** is unprecedented in copper biomimetic chemistry a more complete characterisation of this system, for instance with respect to the possible oxidation of the sulfur donor, is a necessary step to assess the catalytic significance of the methionine–copper bonding. Here we report the characterisation of the oxygenation product of the model monooxygenase reaction, **2**, the X-ray structural characterisation of the dinuclear copper(II) complex **3** resulting from hydrolytic cleavage of **2**, and show that a small fraction of the complex undergoing aromatic hydroxylation also undergoes subsequent sulfur oxidation at the thioether group, according to a secondary pathway involving hydrogen peroxide.

Results and Discussion

Complex **1** belongs to a family of relatively simple dicopper(I) model compounds of general structure **4** where the ligands are derived from the condensation between benzene-1,3-dicarbaldehyde and substituted amines.[7] These ligands provide two co-ordinating atoms to each copper(I) centre, but related macrocyclic complexes reported by Martell and co-workers[7c] contain triamine residues and hence three-co-ordinated metal centres. All these complexes react with dioxygen performing, at least to some extent, an aromatic hydroxylation reaction which leads to the corresponding μ-phenoxo-μ-hydroxo-dicopper(II) complexes, as shown by structure **2** in Scheme 1. The ligand oxygenation is accompanied by simple copper(I) oxidation, and both the ratio between oxygenation and oxidation products and the rate of oxygenation depend on the nature of the X donor group and the reaction solvent.[7e,11] The idea to introduce a methionine sulfur donor in the copper(I) complex was stimulated by the possibility that such a ligand could be present in the co-ordination sphere of the copper monooxygenase dopamine β-hydroxylase.[12] This feature has been subsequently confirmed by more detailed studies.[9c–f] Other recent studies disclose strong structural similarities between dopamine β-hydroxylase and peptidylglycine α-amidating monooxygenase, and hence the possibility that such a methionine–copper bond can be present also in the latter enzyme.[10c] In our model studies we also performed several attempts to extend the investigation to analogues of **1** containing L-cysteine or *S*-methyl-L-cysteine residues in place of L-methionine, but all these were unsuccessful, probably because the resulting copper(I) compounds would contain less sterically favourable five-membered chelate rings.

For the reaction of complex **1** with dioxygen, at least in dilute methanol solution, the ratio between the ligand oxygenation product (**2**) and copper oxidation product is about 1:1.[7e] This



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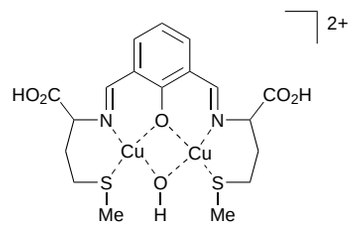

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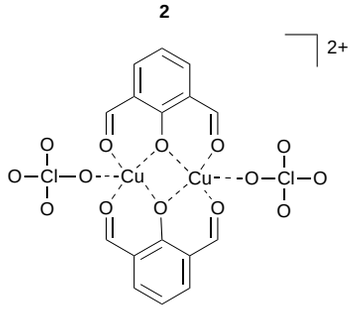

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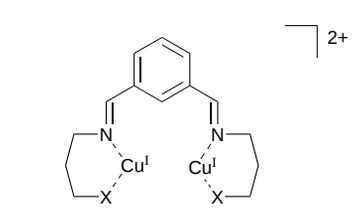
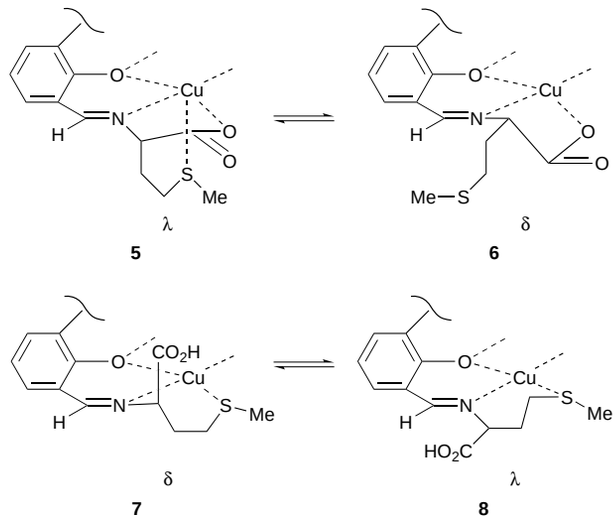


4

Scheme 1

ratio decreases when, as it has been done here, the reaction is performed on a larger scale, and operating under heterogeneous conditions. The two products can be separated by fractional precipitation or, better, chromatography on Sephadex LH-20, but both suffer the inconvenience of being somewhat unstable to hydrolysis of the imine groups in solution by the presence of even trace amounts of water. This reaction has been often observed for imine complexes (see *e.g.* ref. 13) and in the present case it may be favoured by the weak binding properties of the thioether sulfur donors towards Cu^{II}. The characterisation of **2** as the ligand oxygenation product is based on the moderately intense UV band near 360 nm, typical for the 2-hydroxyphenylimino chromophore,[7a] and the medium-intensity IR band near 1560 cm^{-1}, due to the ν(C–O) vibration of the phenolate group, which assumes a partial double-bond character by conjugation with the imine group.[14] The CD spectrum of **2** in the UV region shows several resolved features. It is dominated by a negative band at 375 nm, which corresponds to the optical absorption at 362 nm, and is essentially due to the low-energy conjugated imine $\pi \longrightarrow \pi^*$ transition. The apparent red shift in the CD peak with respect to the optical absorption is due to partial cancellation with a weaker CD band of opposite sign near 330 nm, which is probably contributed both by the thioether $S(\sigma) \rightarrow Cu^{II}$ ligand-to-metal charge transfer (LMCT)[15] and the higher-energy component of the phenolate to Cu^{II} LMCT, or metal-to-ligand charge transfer (MLCT),[16] the lower-energy component of this charge transfer being responsible for the weak CD activity above 400 nm. The other prominent CD band of negative sign at 290 nm is due to a benzenoid $\pi \longrightarrow \pi^*$ transition of the ligand.



5 **6** **7** **8**

Scheme 2

It is interesting that the sign of the optical activity of the 2-hydroxyphenylimino CD band at 375 nm depends on the chirality of the amino acid chelate ring.[17] For imine complexes containing L-amino acid residues, negative CD activity within this band correlates with a preferred conformation chirality of sign λ.[17] In the case of **2** this indicates that the L-methionine residue is either bound as a substituted glycine, with an anionic carboxylate group in the co-ordination plane and an axial thioether chain (**5**), or as a substituted γ-aminopropyl thioether, with an unbound, protonated carboxyl group in equatorial disposition (**8**) (Scheme 2). The latter agreement is to be preferred because, as indicated by the analytical data and the IR spectrum, the carboxyl groups in **2** are protonated.

As stated above, solutions of complex **2** are unstable to hydrolysis of the imine bonds by traces of water. This process can be followed easily in solution by UV spectroscopy, where a progressive shift of the 362 nm band to higher energy, 344 nm, typical of the hydroxydialdehyde, can be observed. Upon standing, methanolic solutions of **2** deposit blue crystals of the dinuclear copper(II) complex of the anion of 2-hydroxybenzene-1,3-dicarbaldehyde, **3**. The IR spectrum of **3** exhibits strong bands corresponding to vibrations of the carbonyl (1640 cm^{-1}) and the phenolate groups (1560 cm^{-1}) and the characteristic splitting of the ≈1100 cm^{-1} band of co-ordinated perchlorate ions. The structure of this compound definitively confirms the monooxygenase reaction undergone by **1** (Fig. 1). The geometry around each copper is essentially undistorted from a tetragonal pyramid with a basal O_4 donor set which includes two phenolate oxygen atoms from two 2-hydroxybenzene-1,3-dicarbaldehyde ligands. Two perchlorate oxygen atoms occupy the axial positions on opposite sides of the Cu_2O_6 plane. The distances and bond angles in the co-ordination spheres (Table 1) are normal for copper(II).[16e,18]

The presence of a sulfur donor in the ligand of complex **1** raises the problem of the possible oxidation at this site, in parallel or alternatively to the aromatic hydroxylation reaction. Although co-ordination to a metal ion is expected to make the thioether group less easily oxidisable, by reducing the electron density at the sulfur atom, the necessity to establish the integrity of this residue in a system, like **1**, capable of performing a carbon oxygenation reaction is certainly important in the context of dopamine β-hydroxylase chemistry. The presence of methionine sulfoxide was examined by isolation of the amino acids upon decomposition of the various fractions resulting from chromatography or fractional precipitation of the products of oxygenation of several samples of **1**. Somewhat surpris-

Table 1 Selected bond distances (Å) and angles (°) for $[Cu_2\{C_6H_3(CHO)_2O\}_2(ClO_4)_2]$

Cu–O(1)	1.951(1)	Cu–O(3^I)	1.928(4)
Cu–O(1^I)	1.958(3)	Cu–O(12)	2.504(5)
Cu–O(2)	1.923(4)		
O(1)–Cu–O(1^I)	78.3(1)	O(1^I)–Cu–O(3^I)	92.0(2)
O(1)–Cu–O(2)	92.8(2)	O(1^I)–Cu–O(12)	94.8(1)
O(1)–Cu–O(3^I)	169.4(2)	O(2)–Cu–O(3^I)	96.6(1)
O(1)–Cu–O(12)	90.9(1)	O(2)–Cu–O(12)	88.4(2)
O(1^I)–Cu–O(2)	170.5(2)	O(3^I)–Cu–O(12)	94.1(2)

Symmetry relation: I $-x, -y, -z$.

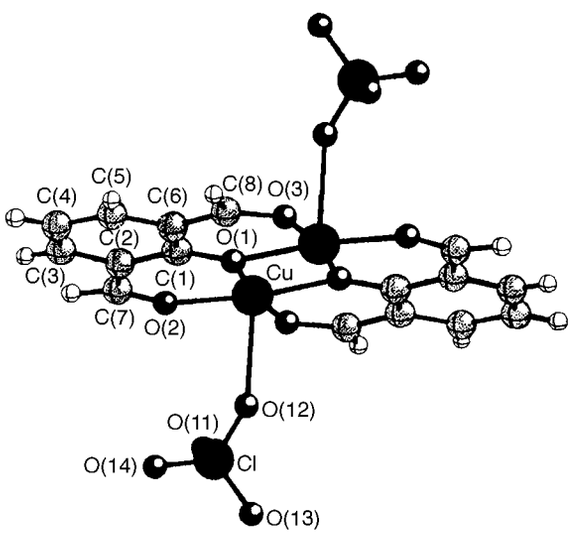


Fig. 1 View of the complex $[Cu_2\{C_6H_3(CHO)_2O\}_2(ClO_4)_2]$ **3**, showing the structure of the copper(II) centres and the numbering scheme of the ligands atoms

ingly, we consistently found methionine sulfoxide, in an amount of 15–20% with respect to methionine, only in the fraction of **2** undergoing carbon hydroxylation, while only methionine was present in the fractions containing simple copper oxidation product. Since a simultaneous dioxygenation reaction at such separated carbon and sulfur centres of **1** is unlikely to occur, we investigated the possibility that oxidation at sulfur could involve reactivity of **2** in a step subsequent to the monooxygenase reaction. As **2** is stable to dioxygen, such reactivity may depend on the presence of hydrogen peroxide. This can be formed by two-electron reduction of dioxygen by the fraction of **1** undergoing simple oxidation at copper(I). In Scheme 3 the two oxidative pathways undergone by **1**, leading to ligand hydroxylation and simple copper oxidation, are separated. The latter pathway may be complex, and involve both partial reduction of dioxygen to hydrogen peroxide (stoichiometry 2 Cu^I : 1 O_2) and complete reduction of dioxygen to water (4 Cu^I : 1 O_2).

In order to check the possible involvement of H_2O_2 in the sulfur oxygenation, the reaction between complex **2** and H_2O_2 was studied. When **2** was treated with hydrogen peroxide, a rapid oxidation of the methionine ligand to methionine sulfoxide occurred, through the probable intermediacy of the hydroperoxide complex **12** (Scheme 4). The increase in the content of methionine sulfoxide in the amino acid mixture, after decomposition of the product complex, corresponds to the amount of hydrogen peroxide used in the reaction. Blank experiments using copper(II) salt and methionine showed that no sulfoxide is formed by hydrogen peroxide through non-specific reactions under the same (mild) conditions.

A final comment is deserved by the preliminary step in the monooxygenase reaction, *i.e.* dioxygen binding to the copper(I) complex, since so far this step has been thoroughly characterised only for a binuclear copper(I) complex by Karlin *et al.*[6c] and the copper(I)–triazacyclononane complexes of Tolman and co-workers.[8h,i] We performed several low-temperature oxygenation experiments (down to −60 °C) with complex **1** to try to characterise an analogous dioxygen complex, but under these conditions we either observed no reaction or an extremely slow reaction to give **2**, according to the temperature and solvent used. This indicates that either the oxygenation equilibrium lies almost totally to the side of the dicopper(I) complex even at low temperature, or the reaction of **1** with dioxygen is extremely slow, while the subsequent reaction to **2** is faster. The latter possibility was recently proposed[19] on the basis of kinetic studies for the reaction between a dicopper(I) complex with a macrocyclic tetraimine reported by Menif *et al.*[7c] and dioxygen. We can add that the difficulty to build up dioxygen adducts for bis(imine) complexes of type **4** is not surprising in view of the sluggish reactivity these systems exhibit towards carbon monoxide.[7a] Regarding the different routes that the putative dioxygen intermediate(s) can take, our proposal is summarised in Scheme 3. The regiospecific aromatic hydroxylation yielding **2** can only be accounted for by an intramolecularly bound dioxygen complex **10**, formally a peroxodicopper(II) species. This species may be preceded by an initially formed 1 : 1 Cu : O_2 complex **9**, formally a mixed-valence copper(I)–copper(II) superoxo species. The latter species can alternatively form an intermolecular Cu_2O_2 complex (not shown in Scheme 3) which is responsible for the path leading to copper(I) oxidation, but is unable to perform any oxygenase chemistry on the ligand. Oxygenation at sulfur is a secondary process involving the hydrogen peroxide formed upon copper(I) oxidation and specifically activated by the dinuclear complex **2**, where it can bind intramolecularly as a bridging hydroperoxo ligand in **12**. Similar μ-phenoxo, μ-1,1-hydroperoxo, or acylperoxo dicopper(II) complexes reported by Karlin *et al.*[20] have been shown to be able quantitatively to transfer an oxygen atom to exogenous phosphines or sulfides through analogous reactions.

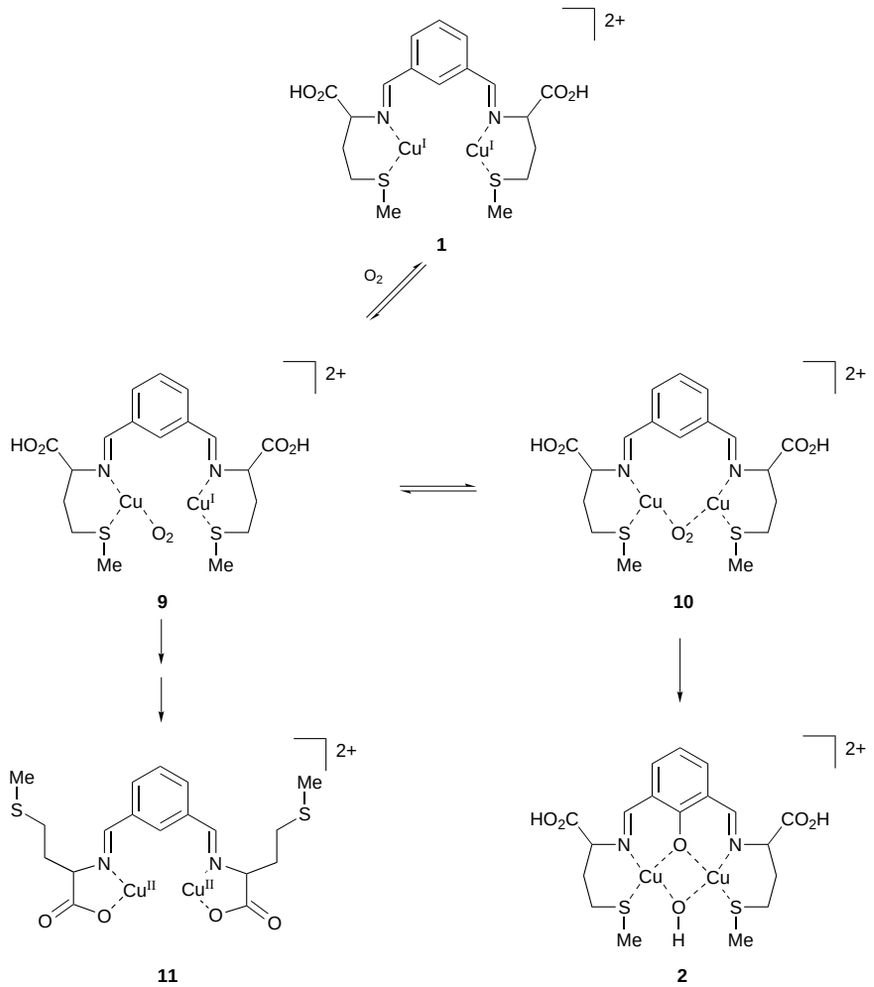
Experimental

Materials and methods

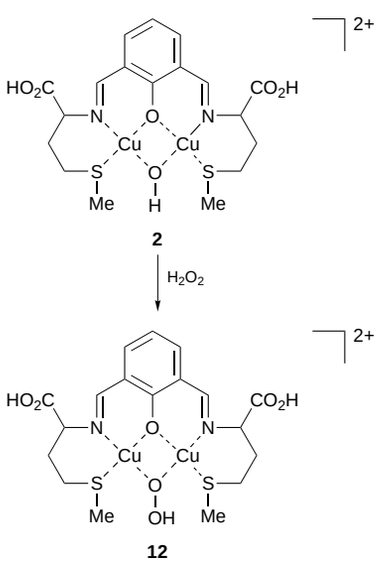
Tetrakis(acetonitrile)copper(I) perchlorate was prepared according to a literature method.[21] Methanol was dried with molecular sieves and degassed by repeated vacuum/purge cycles or bubbling argon directly through the solvent. Elemental analyses were performed by the microanalytical laboratory of the University of Milano. Infrared spectra of solid compounds were obtained as Nujol mulls with a Mattson Galaxy model 5020 FT-IR instrument, optical spectra on a HP-8452 diode-array spectrophotometer, ^1H NMR spectra at 200 MHz on a Bruker spectrometer and circular dichroism spectra on a JASCO 710 spectrometer.

Preparation of complex 1·0.5MeCN

The synthesis of this copper(I) complex was carried out in Schlenk glassware under an atmosphere of purified argon. L-Methionine (373 mg, 2.5 mmol) was added to a solution of benzene-1,3-dicarbaldehyde (166 mg, 1.25 mmol) in methanol–water (2 : 1 v/v, 30 cm^3) and heated to reflux temperature. The mixture was stirred for 0.5 h and then, after cooling, a slight excess of solid $Cu(MeCN)_4ClO_4$ (845 mg, 2.58 mmol) was quickly added. The resulting light yellow solution was stirred for 1 h at room temperature and subsequently concentrated to a small volume under reduced pressure, until a yellow precipitate appeared. Then degassed methanol was added and the solid present was filtered off, washed with a small amount of degassed methanol, and dried under vacuum (yield 65%) (Found: C, 30.41; H, 3.68; Cu, 17.6; N, 4.50. $C_{18}H_{24}Cl_2Cu_2N_2O_{12}S_2 \cdot 0.5CH_3CN$ requires C, 30.71; H, 3.46; Cu, 17.1; N, 4.71%). $\tilde{\nu}_{max}/cm^{-1}$ (Nujol) 1700 (CO_2H), 1634 (C=N) and 1097 (ClO_4).



Scheme 3



Scheme 4

Isolation of complex 2

A stirred suspension of complex **1** (70 mg) in degassed methanol (100 cm^3) was exposed to dioxygen (1 atm, *ca.* 101 325 Pa) and left to stir overnight at room temperature. The resulting green solution was filtered, to discard the small amounts of blue solid present [identified as copper(II) methioninate], and concentrated to a small volume. Then it was chromatographed on a Sephadex LH-20 column (1 × 20 cm) using methanol as eluent. The fractions collected were controlled by optical spectroscopy, by measuring the A_{260}:A_{362} ratio. The oxygenated product was precipitated by addition of diethyl ether to the fraction that showed the minimum value of A_{260}:A_{362} (about 5:1). The product was filtered off and dried under vacuum (Found: C, 28.44; H, 3.31; N, 3.54. $C_{18}H_{24}Cl_2Cu_2N_2O_{14}S_2$ requires C, 28.66; H, 3.21; N, 3.71%). $\tilde{\nu}_{max}/cm^{-1}$ (Nujol) 3285 (OH), 1728 (CO_2H), 1640 (C=N), 1562, 1237 (Ph–O), 1098 and 624 (ClO_4). UV/VIS: λ_{max}/nm ($\varepsilon/dm^3\ mol^{-1}\ cm^{-1}$) (MeOH) 260 (30 000), 362 (6000) and 670 (230). CD: λ_{max}/nm ($\Delta\varepsilon/dm^3\ mol^{-1}\ cm^{-1}$) (MeOH) 270 (+1.3), 290 (−4.0), 330 (+0.3), 375 (−4.8), 420 (+0.2) and 650 (−0.9).

Isolation of the amino acids from the oxygenation/oxidation products

Complex **2**, isolated as described before, or other fractions separated by chromatography or fractional precipitation from the mixture obtained upon oxygenation of **1** (about 10 mg) were treated with dilute HCl and the dialdehyde was extracted six times with dichloromethane. The aqueous phase was concentrated and chromatographed on Chelex 100 (sodium form, 1 × 8.0 cm), using water as eluent, in order to remove the copper(II) ions. The recovered solution was evaporated to dryness to give a white solid residue. The presence of methionine sulfoxide can be detected by TLC, but the amino acid composition was established more conveniently by ^1H NMR spectroscopy in D_2O, *e.g.* from the relative intensity of the signals of the S-methyl groups (δ 2.13 for the methyl group of methionine, 2.76 for that of methionine sulfoxide).

Table 2 Crystallographic details for $[Cu_2\{C_6H_3(CHO)_2O\}_2(ClO_4)_2]$

Formula	$C_8H_5ClCuO_7$
M_r	312.12
System	Monoclinic
Space group	$P2_1/c$
a/Å	8.206(1)
b/Å	6.890(2)
c/Å	18.707(3)
β/°	97.52(1)
U/Å^3	1048.7(4)
Z	4
D_c/g cm^{-3}	1.977
Radiation (λ/Å)	Cu-Kα (1.541 84 Å), grapite monochromated
μ/cm^{-1}	55.7
T/K	293(2)
Crystal size/mm	0.1 × 0.1 × 0.4
Scan type	ω–2θ
Scan speed/° min^{-1}	3.3
Scan width/°	0.7 + 0.14 tan θ
Reflections measured	hkl, $hk-l$; $0 < h < 10$, $0 < k < 8$, $-22 < l < 22$
Unique reflections	2085
Observed reflections [$I > 1.5\sigma(I)$]	1407
Refined parameters	175
$R = \Sigma\|\|F_o\| - \|F_c\|\|/\Sigma\|F_o\|$	0.045
$R' = \Sigma w(\|F_o\| - \|F_c\|)^2/\Sigma w(F_o)^2$	0.041
Weighting scheme	$w = 1.0$
Final Fourier map features/e Å^{-3}	0.21, −0.14
$(\text{shift/e.s.d.})_{max}$	0.08

Reactions with hydrogen peroxide

Complex **2** (68 mg), obtained upon oxygenation of **1**, was diluted in MeOH–water (1 : 1, 20 cm^3) and then an equimolar amount of aqueous H_2O_2 was added. The solution was stirred for 10 min and subsequently evaporated to dryness. The product was treated with dilute HCl (50 cm^3) and the dialdehyde was extracted six times with dichloromethane (100 cm^3). The aqueous phase was then chromatographed on Chelex 100 (1 × 10 cm) using water as eluent, in order to remove the copper(II) ions. The recovered solution was evaporated to dryness to give a white solid residue, which was analysed by ^{1}H NMR spectroscopy as described above.

A blank experiment was carried out as follows. A solution of L-methionine (1 mmol) in water (30 cm^3) was treated at room temperature with an equimolar amount of $Cu(ClO_4)_2 \cdot 6H_2O$ and stirred for 1 h. Then aqueous H_2O_2 (0.5 mmol) was added with stirring. After 10 min of reaction the mixture was concentrated to about 20 cm^3 under vacuum, acidified with HCl, and chromatographed on Chelex 100 (1 × 10 cm) using water as eluent. The recovered solution was then evaporated to dryness to give a white solid residue, which was identified as methionine.

CAUTION: Although the compounds reported in this paper seem to be stable to shock and heat, care should be used in handling them because of the potentially explosive nature of perchlorate salts.

X-ray crystallography

Green prismatic crystals of compound **3** were obtained by slow evaporation of a concentrated oxygenated solution of **1** in methanol [spectral data for the crystals: UV λ_{max}/nm (MeOH) 344 nm; $\tilde{\nu}_{max}$/cm^{-1} 3160 (CH), 1640 (C=O), 1560 (Ph–O) and 1080, 1115, 1140 and 623 (ClO_4)]. Unit-cell parameters and intensity data were obtained on a Enraf-Nonius CAD-4 diffractometer. Calculations were performed with the SDP[22a] and MOLEN[22b] software on a MicroVax-3100 computer.

The cell dimensions were determined by least-squares fitting of 25 centred reflections monitored in the range $30 < \theta < 40°$. No damage of the crystal was observed during the data collection (maximum decay = 1.0%). The space group was obtained by systematic extinctions. Lorentz-polarisation corrections were applied. The structure was solved by direct methods.[23] All the atoms were refined by full-matrix least squares (the non-hydrogen ones anisotropically, the hydrogen ones isotropically). Secondary extinctions[24] were applied. Atomic scattering factors were taken from ref. 25. Empirical absorption corrections were applied according of ref. 26. Pertinent experimental details are given in Table 2.

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