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Inorganica Chimica Acta 300-302 (2000) 622-632

Inorganica Chimica Acta

# Active site models for galactose oxidase containing two different phenol groups

Masayasu Taki<sup>a</sup>, Hideyuki Kumei<sup>a</sup>, Shigenori Nagatomo<sup>c</sup>, Teizo Kitagawa<sup>c,\*1</sup>, Shinobu Itoh<sup>b,\*2</sup>, Shunichi Fukuzumi<sup>a,\*3</sup>

<sup>a</sup> Department of Materials and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka,

Suita, Osaka 565-0871, Japan

<sup>b</sup> Department of Chemistry, Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan <sup>c</sup> Institute for Molecular Science, Myodaiji, Okazaki 444-8585, Japan

Received 22 September 1999; accepted 2 December 1999

#### Abstract

Model complexes of the active site of galactose oxidase (GAO) have been developed using a new ligand carrying two different phenol groups, N-[(2-hydroxy-3-methylthio-5-*tert*-butylphenyl)methyl]-N-[(2-hydroxy-3,5-di-*tert*-butylphenyl)methyl]-2-(2-pyridyl)ethylamine (L1H<sub>2</sub>). Deprotonated ligand L1<sup>2-</sup> forms a dimeric Cu(II) complex, [Cu(II)<sub>2</sub>(L1<sup>2-</sup>)<sub>2</sub>], in the solid state, the structure of which has been determined by X-ray crystallographic analysis. The dimeric Cu(II)-diphenolate complex can be converted into the monomeric complex, [Cu(II)(L1<sup>2-</sup>)(X)] (X = py, AcO, and PhCH<sub>2</sub>OH), in solution by adding exogenous ligands such as pyridine (py), acetate (AcO<sup>-</sup>), or benzyl alcohol (PhCH<sub>2</sub>OH). The structure and physicochemical properties (UV–Vis, ESR, redox potential) of [Cu(II)(L1<sup>2-</sup>)(X)] have been explored as a model for the resting state of the enzyme. One-electron oxidation of [Cu(II)(L1<sup>2-</sup>)(py)] and [Zn(II)(L1<sup>2-</sup>)(py)] by (NH<sub>4</sub>)<sub>2</sub>[Ce<sup>IV</sup>(NO<sub>3</sub>)<sub>6</sub>] (CAN) yielded the corresponding phenoxyl radical/phenolate complexes, Cu(II)(L1<sup>•-</sup>) and Zn(II)(L1<sup>•-</sup>), respectively, which have also been characterized by UV–Vis, resonance Raman, and ESR. The structure, physicochemical properties and reactivities of the diphenolate and phenoxyl radical/phenolate complexes of L1H<sub>2</sub> are compared to those of the corresponding monophenolate and monophenoxyl radical complexes in order to obtain further insight into the role of Tyr 495 in the native enzyme. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Galactose oxidase; Copper complexes; Zinc complexes; Phenoxyl radical; Cofactor

#### 1. Introduction

Galactose oxidase (GAO) is a mononuclear copper enzyme that catalyzes two-electron oxidation of primary alcohols to the corresponding aldehydes coupled with the reduction of  $O_2$  to  $H_2O_2$  (Eq. (1)) [1–4]. Structural details of the enzyme active site of GAO have been disclosed recently by X-ray crystallographic analysis [2], showing that the equatorial tyrosine (Tyr 272) is modified to make a covalent bond with the adjacent cysteine (Cys 228) as illustrated in Scheme 1. Mechanistic studies on the enzymatic reaction have shown that the Cu(II) complex of the phenoxyl radical derived from Tyr 272 is the active species that oxidizes primary alcohols to the corresponding aldehydes by the following mechanism: (i) deprotonation of the alcohol substrate by the axial tyrosinate ligand (Tyr 495); (ii) inner-sphere electron transfer from the deprotonated substrate to Cu(II); and (iii) hydrogen atom abstraction of the resulting ketyl radical by the phenoxyl radical of Tyr 272 (the ordering of electron transfer and hydrogen atom abstraction steps could be reversed) [3–5].

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \tag{1}$$

Model studies of the active site of GAO have recently been performed to provide insight into the structure and physicochemical properties of the inactive form

<sup>&</sup>lt;sup>1</sup>\*Corresponding author.

<sup>&</sup>lt;sup>2</sup>\*Corresponding author.

<sup>&</sup>lt;sup>3</sup>\*Corresponding author. Tel.: + 81-6-6879 7368; fax: + 81-6-6879 7370



Scheme 1.

[Cu(II)-phenolate state] and the active form [Cu(II)phenoxyl radical state] of the enzyme [6-12]. Efficient catalytic oxidation of alcohols by Cu(II)-phenoxyl radical complexes has also been achieved by mimicking the enzymatic functions in model systems [9,13]. Furthermore, detailed kinetic analysis of the oxidation of benzyl alcohols by Cu(II)-phenoxyl radical complexes has shown that the redox cycle between Cu(I) and Cu(II) as well as the interconversion between the phenol and phenoxyl radical states are very important for the efficient two-electron oxidation of alcohols at a mononuclear copper active site of GAO [6c,d]. However, little attention has been focused on the catalytic role of the axial tyrosinate ligand (Tyr 495) in model systems, although some Cu(II) complexes with diphenol ligands have been reported so far [7b,8,9,13]. In order to address this issue, we have developed a new ligand  $L1H_2^4$ carrying two different phenol groups as shown in Chart (1). Ligand  $L1H_2$  is a hybrid of ligands L2H and L3H, which we have developed in previous studies [6c,d]. Thus, a comparison of the structure and physicochemical properties of the Cu(II) and Zn(II) complexes of L1H<sub>2</sub> with those of the Cu(II) and Zn(II) complexes of L2H and L3H, in both the phenolate and the phenoxyl radical states, will give us valuable insight into the different roles of the tyrosine residues (Tyr 272 and Tyr 495) in enzymatic systems. In this study, we have examined the structure and physicochemical properties of the diphenolate complexes as well as the stability and spectroscopic features of the Cu(II) and Zn(II) complexes of the phenoxyl radical/phenolate species derived from L1H<sub>2</sub>.



#### Chart (1)

#### 2. Experimental

#### 2.1. General

Reagents and solvents used in this study, except for the ligand and the complexes, were commercial products of the highest available purity and were further purified by standard methods, if necessary [14]. IR spectra were recorded on a Shimadzu FTIR-8200PC and UV-Vis spectra on a Hewlett Packard 8452A or a Hewlett Packard 8453 photo diode array spectrophotometer. <sup>1</sup>H NMR spectra were recorded on a Jeol FT NMR GSX-400 or a Jeol FT NMR EX-270 spectrometer. ESR spectra were recorded on a Jeol JES-ME-2X spectrometer. The g values were determined using a Mn(II) marker as a reference. Computer simulation of the ESR spectra was carried out using ESRAII version 1.01 (Calleo Scientific) on a Macintosh personal computer. Mass spectra were recorded on a Jeol JNX-DX303 HF mass spectrometer or a Shimadzu GCMS-QP2000 gas chromatograph-mass spectrometer.

### 2.2. Electrochemical measurement

The cyclic voltammetry and second harmonic ac voltammetry (SHACV) measurements were performed on a BAS 100B electrochemical analyzer or an ALS electrochemical analyzer (model 600) in deaerated CH<sub>3</sub>CN containing 0.10 M NBu<sub>4</sub>PF<sub>6</sub> as supporting electrolyte. The Pt and Au working electrodes (BAS) were polished with BAS polishing alumina suspension and rinsed with acetone before use. The counter electrode was a platinum wire. The measured potentials were recorded with respect to an Ag/AgNO<sub>3</sub> (0.01 M) reference electrode. The  $E_{1/2}$  values (versus Ag/AgNO<sub>3</sub>)

<sup>&</sup>lt;sup>4</sup> In order to distinguish the different states of the cofactor moiety of the ligands more clearly, the symbols  $L1H_2$ ,  $L1^{2-}$ , and  $L1^{-}$  are used to denote the diphenol, diphenolate, and phenoxyl radical/phenolate forms, respectively.

are converted to values versus SCE by adding 0.29 V [15]. All electrochemical measurements were carried out under nitrogen at atmospheric pressure.

#### 2.3. Visible resonance Raman measurement

The 413.1 nm line of a Kr<sup>+</sup> laser (model 2060 Spectra Physics) was used as the exciting source. Visible resonance Raman scattering was detected with a CCD detector (model CCD3200, Astromed) attached to a 1 m single polychromator (model MC-100DG, Ritsu Oyo Kogaku). The slit width and slit height were set at 200  $\mu$ m and 10 mm, respectively. Wavenumber ranges per channel were 0.9 (Kr<sup>+</sup> laser) and 0.5 cm<sup>-1</sup> (dye laser). The laser power used was 2.5 mW at the sample point. All measurements were carried out at  $-60^{\circ}$ C with a spinning cell (1000 rpm). Raman shifts were calibrated with indene and the accuracy of the peak positions of the Raman bands was  $\pm 1$  cm<sup>-1</sup>.

#### 2.4. X-ray structure determination

X-ray diffraction data were collected with a Rigaku RAXIS-CS imaging plate two-dimensional area detector using graphite-monochromated Mo K $\alpha$  radiation ( $\lambda = 0.71070$  Å) to  $2\theta_{max}$  of 60.0°. The intensity was measured by sealing samples in a glass capillary tube containing the mother liquid. All the crystallographic calculations were performed using the TEXSAN software package form the Molecular Structure Corporation [TEXSAN: crystal structure analysis package, Molecular Structure Corp. (1985 & 1999)]. The crystal structure was solved by direct methods and refined by full-matrix least-squares. All non-hydrogen atoms and hydrogen atoms were refined anisotropically and isotropically.

#### 2.5. Synthesis

### 2.5.1. N-[(2-Hydroxy-3-methylthio-5-tertbutylphenyl)methyl]-2-(2-pyridyl)ethylamine (1)

To a solution of 2-(2-pyridyl)ethylamine (2). To a solution of 2-(2-pyridyl)ethylamine (2.44 g, 20 mmol) in CH<sub>3</sub>OH (10 ml) was added a suspension of paraformaldehyde (0.9 g, 30 mmol) in CH<sub>3</sub>OH (5 ml). The mixture was refluxed for 1 h under N<sub>2</sub>. A solution of 2-methylthio-4-*tert*-butylphenol [6d] (3.92 g, 20 mmol) in CH<sub>3</sub>OH (5 ml) was then added to the mixture, and the resulting solution was further refluxed for 24 h. After evaporation of the solvent, the expected product 1 was obtained as a yellow powder by SiO<sub>2</sub> column chromatography (CHCl<sub>3</sub>-ethyl acetate) in 39% yield. IR (KBr disk): 3400 cm<sup>-1</sup> (OH and NH). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  1.28 (s, 9H), 2.43 (s, 3H), 3.06 (t, J = 7.6 Hz, 2H), 3.21 (t, J = 7.6 Hz, 2H), 4.03 (s, 2H), 6.78 (d, J = 2.4 Hz, 1H), 7.08 (d, J = 2.4 Hz, 1H), 7.10 (ddd, J = 0.8, 4.8, 7.6 Hz, 1H), 7.17 (d, J = 7.6 Hz, 1H), 7.58 (td, J = 1.6, 7.6 Hz, 1H), 8.52 (ddd, J = 0.8, 1.6, 4.8 Hz, 1H). HRMS m/z: 330.1758 [ $M^+$ ]. Calc. for C<sub>19</sub>H<sub>26</sub>N<sub>2</sub>OS: 330.1766.

## 2.5.2. N-[(2-Hydroxy-3-methylthio-5-tertbutylphenyl)methyl]-N-[(2-hydroxy-3,5-di-tert-butylphenyl)methyl]-2-(2-pyridyl)ethylamine (L1H<sub>2</sub>)

To a solution of 1 (1.47 g, 4.45 mmol) in CH<sub>3</sub>OH (5 ml) was added a suspension of paraformaldehyde (0.30 g, 10 mmol) in CH<sub>3</sub>OH (5 ml). The mixture was refluxed for 1 h under N<sub>2</sub>. A methanol solution of 2,4-di-tertbutylphenol (2.06 g, 10 mmol) was then added to the mixture, and the resulting solution was further refluxed for 16 h. After evaporation of the solvent, ligand L1H<sub>2</sub> was obtained as a white powder by SiO<sub>2</sub> column chromatography (CHCl<sub>3</sub>) in 30% yield. IR (KBr disk): 3390 cm<sup>-1</sup> (OH). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 270 MHz): δ 1.26 (s, 9H), 1.27 (s, 9H), 1.33 (s, 9H), 2.36 (s, 3H), 2.88 (t, J = 7.3Hz, 2H), 3.13 (t, J = 7.3 Hz, 2H), 3.80 (s, 2H), 3.82 (s, 2H), 6.85 (d, J = 2.2 Hz, 1H), 7.06–7.17 (m, 4H), 7.29 (d, J = 2.2 Hz, 1H), 7.58 (td, J = 1.7, 7.8 Hz, 1H), 8.58 (d, J = 0.7, 5.0 Hz, 1H). EI MS (pos). m/z 548 [ $M^+$ ]. Anal. Calc. for C<sub>34</sub>H<sub>48</sub>N<sub>2</sub>O<sub>2</sub>S<sub>1</sub>: C, 74.41; H, 8.82; N, 5.10. Found: C, 74.30; H, 8.75; N, 4.94%.

# 2.5.3. $[Cu(II)_2(L1^{2-})_2]$

To a solution of ligand L1H<sub>2</sub> (252 mg, 0.46 mmol) and triethylamine (132 µl, 0.92 mmol) in CH<sub>3</sub>OH (50 ml) was added Cu(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O (170 mg, 0.46 mmol), and the mixture was refluxed for 3 h. The resulting solution was poured into excess water to provide a dark-violet solid that was collected by filtration, washed with ether, and dried in vacuo to give  $[Cu(II)_2(L1^{2-})_2]$  in 87% yield. Single crystals (dark-brown prism) for the X-ray structure determination were obtained by recrystallization from toluene-CH<sub>3</sub>OH-H<sub>2</sub>O. FAB MS: m/z 1221.6  $[M^+ + 1]$ . A set of prominent peaks showing an isotope distribution pattern consistent with the dimeric Cu(II)diphenolate complex was obtained. Anal. Calc. for  $[\operatorname{Cu}(\operatorname{II})_2(\operatorname{L1}^{2-})_2] \cdot \operatorname{C_7H_8} \cdot \operatorname{H_2O},$  $C_{75}H_{102}N_4O_5S_2Cu_2$ : С, 67.69; H, 7.73; N, 4.21. Found: C, 67.83; H, 7.63; N, 4.41%.

# 2.5.4. $[Zn(II)_2(L1^{2-})_2]$

This complex was prepared in a similar manner as used for the preparation of the copper complex, with Zn(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O instead of Cu(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, in 95% yield. FAB MS: m/z 1223.6 [ $M^+$  + 1]. A set of prominent peaks showing an isotope distribution pattern consistent with the dimeric Zn(II)–diphenolate complex was obtained. *Anal.* Calc. for [Zn(II)<sub>2</sub>(L1<sup>2-</sup>)<sub>2</sub>], C<sub>68</sub>H<sub>92</sub>N<sub>4</sub>O<sub>4</sub>S<sub>2</sub>-Zn<sub>2</sub>: C, 66.91; H, 7.60; N, 4.59. Found: C, 66.78, H, 7.54, N, 4.63%.

#### 2.5.5. Generation of phenoxyl radical species

The phenoxyl radical species of the Cu(II) and the Zn(II) complexes were generated in situ by adding a

CH<sub>3</sub>CN solution of an equimolar amount of  $(NH_4)_2[Ce^{IV}(NO_3)_6]$  (CAN,  $5.0 \times 10^{-4}$  M) into a deaerated 9:1 THF-CH<sub>3</sub>CN solution of  $[M(II)(L1^{2-})(py)]$  ( $5.0 \times 10^{-4}$  M) (M = Cu or Zn) in a UV cell (1 cm path length, sealed tightly with a silicon rubber cap) at  $-60^{\circ}$ C.

### 3. Results and discussion

#### 3.1. Ligand synthesis

The new ligand  $L1H_2$ , which has two different phenol groups, was prepared by the double Mannich reaction on 2-(2-pyridyl)ethylamine with 2-methylthio-4-*tert*butylphenol followed with 2,4-di-*tert*-butylphenol as indicated in Scheme 2. Thus, the reaction of 2-(2pyridyl)ethylamine and 2-methylthio-4-*tert*-butylphenol in the presence of paraformaldehyde in refluxing CH<sub>3</sub>OH for 24 h gave the monophenol derivative 1, to which another phenol group was introduced by the second Mannich reaction with 2,4-di-*tert*-butylphenol and paraformaldehyde in a similar manner.

### 3.2. Synthesis of Cu(II) and Zn(II) complexes

The addition of  $Cu(ClO_4)_2 \cdot 6H_2O$  to the deprotonated ligand  $L1^{2-}$  in  $CH_3OH$  led to the formation of a copper(II) complex that crystallized immediately as a dimeric form, as in the case of ligand L2H [6c,d]. A single crystal (dark-violet prism) for X-ray structure determination was obtained by recrystallization from a mixed solvent system of toluene- $CH_3OH-H_2O$ . Crystallographic data and selected bond distances and angles for the X-ray structure are summarized in Tables 1



Scheme 2.

Table 1 Summary of X-ray crystallographic data

Compound	$[\mathrm{Cu}(\mathrm{II})_2(1^{2-})_2] \cdot \mathrm{C}_6\mathrm{H}_5\mathrm{CH}_3$
Empirical formula	$C_{75}H_{100}N_4O_4S_2Cu_2$
Formula weight	1312.85
Crystal system	monoclinic
Space group	$P2_1/a$ (no. 14)
a (Å)	18.169(4)
b (Å)	22.295(5)
c (Å)	19.594(10)
β (°)	105.29(3)
$V(\text{\AA}^3)$	7656(8)
Ζ	4
<i>F</i> (000)	2800.00
$D_{\rm calc} \ ({\rm g} \ {\rm cm}^{-3})$	1.139
<i>T</i> (°C)	-50
Crystal size (mm)	$0.5 \times 0.5 \times 0.5$
$\mu$ (Mo K $\alpha$ ) (cm <sup>-1</sup> )	6.56
Diffractometer	RAXIS-CS
Radiation	Mo Kα (0.71070 Å)
$2\theta_{\rm max}$ (°)	60.0
No. of reflections measured	11 867
No. of reflections observed	11 714
$[I > 3\sigma(I)]$	
No. of variables	1149
R <sup>a</sup>	0.079
$R_{ m w}^{ m \ b}$	0.107
Goodness of fit <sup>c</sup>	2.61

<sup>a</sup>  $R = \Sigma ||F_{o}| - |F_{c}|| / \Sigma |F_{o}|.$ 

<sup>b</sup> 
$$[\Sigma w(|F_{o}| - |F_{c}|)^{2} / \Sigma wF_{o}^{2}]^{1/2}; w = 1/\sigma^{2}(F_{o}).$$

<sup>c</sup>  $[\Sigma w(|F_o| - |F_c|)^2 / (N_o - N_v)]^{1/2}$ .

and 2, with an ORTEP drawing shown in Fig. 1. The dimeric Cu(II) complex can be converted into the corresponding monomeric complex by adding an external ligand such as pyridine in solution (vide infra). The Zn(II) complex of  $L1^{2-}$  was also isolated as a dimeric

Table 2								
Selected	bond	lengths	(Å) and	l angles	(°) o	f [Cu(II) <sub>2</sub>	$(1^{2-})_{2}$	C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub>

-			
Cu(1)–O(1)	1.970(4)	Cu(1)–O(2)	2.035(5)
Cu(1)–O(4)	1.881(5)	Cu(1)–N(3)	2.190(7)
Cu(1)–N(4)	2.123(6)	Cu(2)–O(1)	2.048(5)
Cu(2)–O(2)	1.962(4)	Cu(2)–O(3)	1.880(4)
Cu(2)–N(1)	2.102(6)	Cu(2)–N(2)	2.197(7)
S(1)-C(28)	1.760(9)	S(1)-C(75)	1.77(1)
S(2)-C(2)	1.761(8)	S(2)-C(32)	1.771(9)
O(1)-Cu(1)-O(2)	74.8(2)	O(1)-Cu(1)-O(4)	161.4(2)
O(1)-Cu(1)-N(3)	89.8(2)	O(1)-Cu(1)-N(4)	93.1(2)
O(2)–Cu(1)–O(4)	89.3(2)	O(2)-Cu(1)-N(3)	117.5(2)
O(2)–Cu(1)–N(4)	146.1(2)	O(4)-Cu(1)-N(3)	106.3(2)
O(4)-Cu(1)-N(4)	94.9(2)	N(3)-Cu(1)-N(4)	93.6(2)
O(1)–Cu(2)–O(2)	74.7(2)	O(1)-Cu(2)-O(3)	89.8(2)
O(1)-Cu(2)-N(1)	146.6(2)	O(1)-Cu(2)-N(2)	118.5(2)
O(2)–Cu(2)–O(3)	162.6(2)	O(2)-Cu(2)-N(1)	93.9(2)
O(2)-Cu(2)-N(2)	92.2(2)	O(3)-Cu(2)-N(1)	94.9(2)
O(3)-Cu(2)-N(2)	102.3(2)	N(1)-Cu(2)-N(2)	92.8(2)
Cu(1)-O(1)-Cu(2)	92.4(2)	Cu(1)–O(2)–Cu(2)	93.0(2)
C(28)–S(1)–C(75)	102.8(6)	C(2)–S(2)–C(32)	102.5(5)



Fig. 1. ORTEP drawing of  $[Cu(II)_2(L1^{2-})_2] \cdot C_6H_5CH_3$ . The solvate molecule and hydrogen atoms are omitted for clarity.

form,  $[Zn(II)_2(L1^{2-})_2]$ , in a similar manner using  $Zn(ClO_4)_2$ ·6H<sub>2</sub>O instead of Cu(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O.

# 3.3. Crystal structure of the dimeric Cu(II)-diphenolate complex

The crystal lattice of the Cu(II) complex consists of a dinuclear copper(II) complex and one toluene molecule from the recrystallization solvent. The crystal structure of the dimeric copper(II) complex of  $L1^{2-}$  shown in Fig. 1 resembles the structures of the dimeric Cu(II) complex of duncamine (dnc) ligand {(2-pyridylmethyl)[(2 - hydroxy - 3,5 - dimethylphenyl)methyl][(2hydroxy-5-methyl-3-methylthiophenyl)methyl]amine} reported by Whittaker et al. [7b] and of the dimeric Cu(II) complex of the monophenol ligand L2H,  $[Cu(II)_2(L2^-)_2](PF_6)_2$  [6d]. Both Cu(II) centers of  $[Cu(II)_2(L1^{2-})_2]$  have a distorted square pyramidal structure with basal planes consisting of the tertiary amine nitrogen  $\{N(1) \text{ and } N(3)\}$  and three phenolate oxygen atoms  $\{O(1), O(3), and O(2) \text{ or } O(4)\}$ , two of which  $\{O(1) \text{ and } O(3)\}$  in the phenolate ring with the 2-methylthio group act as the bridges for both copper ions. The pyridine nitrogens  $\{N(2) \text{ or } N(4)\}$  are the axial ligands for both copper ions; the  $\tau$  values at the Cu(1) and Cu(2) centers are 0.27 and 0.26, respectively.

The copper–copper separation is 2.90 Å. There is no coordinative interaction between Cu(II) and sulfur atoms of the methylthio groups, as is the case for the enzymatic system (the distances Cu(1)–S(1), Cu(1)–S(2), Cu(2)–S(1), and Cu(2)–S(2) are 4.57, 4.04, 3.92, and 4.57 Å, respectively). The dihedral angles of the methylthio substituents and the phenol rings defined by C(7)–S(1)–C(6)–C(5) and C(41)–S(2)–C(40)–C(39) are 10.5 and 9.3°, respectively.

# 3.4. Characterization of the diphenolate complex (model complex for the resting state of GAO)

## 3.4.1. UV-Vis

The dimeric copper(II) complex,  $[Cu(II)_2(L1^{2-})_2]$ , exhibits a strong absorption band at 453 nm ( $\varepsilon = 2000$  M<sup>-1</sup> cm<sup>-1</sup> per Cu ion) together with a small band at 680 nm ( $\varepsilon = 200$  M<sup>-1</sup> cm<sup>-1</sup> per Cu ion) in CH<sub>3</sub>CN. Those absorption bands have been assigned to the phenolate-to-metal charge transfer (LMCT band) and the ligand field excitation (Cu(II) d-d band), respectively [6–8]. It is interesting to note that  $\lambda_{max}$  of the LMCT band of  $[Cu(II)_2(L1^{2-})_2]$  (453 nm) is very close to that of the inactive form of GAO (451 nm) [3e], but is considerably smaller than those of the monophenolate complexes  $[Cu(II)_2(L2^{-})_2]^{2+}$  (522 nm) and

 $[Cu(II)(L3^-)]^+$  (559 nm) [6c,d]. The higher energy of the LMCT feature in the diphenolate complexes compared with the monophenolate compounds may be due to a greater electron density at the Cu(II) ion induced by the second phenolate donor in the diphenolate complexes. A similar tendency has been reported in Tolman's TACN (triazacyclononane) ligand system [8b]. The Zn(II) complex, on the other hand, does not show any characteristic absorption band in the visible region (see, Fig. 5(B)).

The dimer complex can be converted into the corresponding monomeric Cu(II) complex,  $[Cu(II)(L1^{2-})]$ (X)]  $(X = py, AcO, PhCH_2OH)$ , by adding an exogenous ligand such as pyridine (py), acetate (AcO<sup>-</sup>), or benzyl alcohol (PhCH<sub>2</sub>OH) into a CH<sub>3</sub>CN solution of the dimer. As a representative example, the spectral change observed upon addition of pyridine (0-0.02 M)into a CH<sub>3</sub>CN solution of  $[Cu(II)_2(L1^{2-})_2]$  (2.5 × 10<sup>-4</sup> M) is shown in Fig. 2. The LMCT band of the dimer at 453 nm shifted to 466 nm ( $\varepsilon = 1360 \text{ M}^{-1} \text{ cm}^{-1}$ ) by the dissociation into the monomer complex  $[Cu(II)(L1^{2-})-$ (py)], while the d-d band at 660 nm shifted to 710 nm  $(\varepsilon = 100 \text{ M}^{-1} \text{ cm}^{-1})$ . In the monomer complex as well, the energy of the LMCT feature (466 nm) is significantly higher than that of the monophenolate complex  $[Cu(II)(L2^{-})(py)]^{+}$  (533 nm) [6d]. From the spectral change, the dissociation constant  $(K_a = [monomer]_2/$  $[dimer][py]_2$ ) was calculated as 27.4 M<sup>-1</sup> using the equation  $(A - A_0)_2/(A - A_\infty) = (1/4)K_a(\varepsilon_d - \varepsilon_m)[py]^2,$ where  $\varepsilon_d$  and  $\varepsilon_m$  are molar absorption coefficients of the dimer and monomer, respectively. A plot of  $(A - A_0)_2/$  $(A - A_{\infty})$  versus  $[py]^2$  gave a straight line passing through the origin, as shown in the inset of Fig. 2, from which  $(1/4)K_a(\varepsilon_d - \varepsilon_m)$  was obtained as the slope. The



Fig. 2. Spectral change of the titration of  $[Cu(II)_2(L1^{2-})_2]$  (2.5 × 10<sup>-4</sup> M) by pyridine (0 – 0.02 M) in CH<sub>3</sub>CN. Inset: plot of  $(A - A_0)^2/(A - A_\infty)$  vs. [pyridine]<sup>2</sup> for the titration.

 $K_{\rm a}$  value for the titration of  $[Cu(II)_2(L2^{-})_2]^{2+}$  with pyridine has been determined as 0.13 M<sup>-1</sup> in a similar manner [6d]. The significantly larger dissociation constant of  $[Cu(II)_2(L1^{2-})_2]$  compared with  $[Cu(II)_2 (L2^{-})_{2}^{]^{2+}}$  can be attributed to the steric hindrance of the tert-butyl group in  $L1^{2-}$ , destabilizing the dimeric form of the Cu(II) complex. The  $K_a$  values for the titration of  $[Cu(II)_2(L1^{2-})_2]$  with acetate ions and PhCH<sub>2</sub>OH have also been determined as 36.4 M<sup>-1</sup> and  $2.7 \times 10^{-5}$  M<sup>-1</sup>, respectively. It is noteworthy that even the neutral weak ligand PhCH<sub>2</sub>OH can coordinate to the metal center of the Cu(II) complex of ligand L1<sup>2-</sup>, since such coordinative interaction of PhCH<sub>2</sub>OH has never been observed in the Cu(II) complexes of ligands  $L2^-$  and  $L3^-$ . The coordination of PhCH<sub>2</sub>OH to the copper center is reminiscent of the substrate binding process in the enzymatic system.

#### 3.4.2. ESR

The dimeric Cu(II) complex of  $L1^{2-}$  in a frozen CH<sub>3</sub>CN solution (77 K) is ESR silent (Fig. 3(A)) owing to a strong antiferromagnetic coupling interaction between the two cupric ions in the  $\mu$ -bridged Cu<sub>2</sub>O<sub>2</sub> core as in the case of [Cu(II)<sub>2</sub>( $L2^{-}$ )<sub>2</sub>]<sup>2+</sup> and [Cu(II)<sub>2</sub>(**dnc**)<sub>2</sub>] [6d,7b]. The addition of pyridine into a CH<sub>3</sub>CN solu-



Fig. 3. (A) ESR spectrum of  $[Cu(II)_2(L1^{2-})_2]$  (2.5 × 10<sup>-4</sup> M) in CH<sub>3</sub>CN at 77 K; microwave frequency 9.180 GHz, modulation frequency 100 kHz, modulation amplitude 10 G, microwave power 3 mW. (B) ESR spectrum of  $[Cu(II)(L1^{2-})(py)]$  generated from the dimer (2.5 × 10<sup>-4</sup> M) by adding pyridine (2.0 × 10<sup>-2</sup> M) in CH<sub>3</sub>CN at 77 K; microwave frequency 9.193 GHz, modulation frequency 100 kHz, modulation amplitude 10 G, microwave power 3 mW. (C) The computer simulation spectrum with the parameters  $g_1 = 2.262$ ,  $g_2 = 2.071$ ,  $g_3 = 2.028$ ,  $A_1 = 186$ ,  $A_2 = 25$ , and  $A_3 = 18$  G.



Fig. 4. Cyclic voltammogram of  $[Cu(II)(L1^{2-})(py)]$  generated from  $[Cu(II)_2(L1^{2-})_2] (1.0 \times 10^{-3} \text{ M})$  by adding pyridine  $(8.0 \times 10^{-2} \text{ M})$  in CH<sub>3</sub>CN containing 0.1 M NBu<sub>4</sub>PF<sub>6</sub> at  $-40^{\circ}$ C; working electrode Pt, counter electrode Pt wire, reference electrode Ag/0.01 M AgNO<sub>3</sub>, scan rate 0.1 V s<sup>-1</sup>.

tion of  $[Cu(II)_2(L1^{2-})_2]$  resulted in a drastic increase in the ESR signal intensity (Fig. 3(B)). This indicates the formation of the monomer Cu(II) complex  $[Cu(II)(L1^{2-})(py)]$  as demonstrated by the UV-Vis titration shown in Fig. 2. Computer simulation of the spectrum (Fig. 3(C)) provided the ESR parameters  $g_1 = 2.262$ ,  $g_2 = 2.071$ ,  $g_3 = 2.028$ ,  $A_1 = 186$ ,  $A_2 = 25$ and  $A_3 = 18$  G, which are consistent with a  $d_{x^2-y^2}$ ground state with a minor rhombic perturbation.

### 3.4.3. Redox potential

Two quasi-reversible redox couples of  $[Cu(II)(L1^{2-})(py)]$  were only obtained at a low temperature (- 40°C) at  $E_{1/2}^{1} = 0.19$  V versus Ag/AgNO<sub>3</sub> (0.48 V versus SCE) and  $E_{1/2}^{2} = 0.42$  V versus Ag/AgNO<sub>3</sub> (0.71 V versus SCE) in CH<sub>3</sub>CN <sup>5</sup> (see Fig. 4). These redox couples may correspond to the sequential one-electron oxidation of the diphenolate (L1<sup>2-</sup>) complex to the phenoxyl radical/phenolate (L1<sup>2-</sup>) complex and that of L1<sup>•-</sup> to the diphenoxyl radical (L1<sup>2•</sup>) complex, respectively. [Zn(II)(L1<sup>2-</sup>)(py)], however, gave only an irreversible oxidation peak at around 0.50 V versus Ag/AgNO<sub>3</sub> (0.79 V versus SCE) even at the low temperature (-40°C)<sup>6</sup>. Thus, the phenoxyl radical complexes

turned out not to be very stable at ambient temperature, which is in contrast with the fact that the Cu(II) and Zn(II) complexes of monophenoxyl radicals L2<sup>•</sup> and L3<sup>•</sup> are much more stable at room temperature [6c,d]. The redox potentials of  $[Cu(II)(L1^{2-})(py)]$  are fairly similar to those of [Cu(II)(dnc)(py)]  $(E_{1/2}^1 = 0.12 \text{ V})$ and  $E_{1/2}^2 = 0.46$  V versus Ag/AgNO<sub>3</sub> in CH<sub>3</sub>CN) [7b] and of Tolman's diphenolate complex  $[Cu(II)(L^{tBu_4})]$  $(L^{^{t}Bu_{4}} = diphenolate of 1,4-bis(2-hydroxy-3,5-di-tert$ butylbenzyl) - 7 - isopropyl - 1,4,7 - triazacyclononane)  $(E_{1/2}^{1} = 0.50 \text{ V and } E_{1/2}^{2} = 0.78 \text{ V versus SCE in CH}_{2}Cl_{2}$ thus,  $E_{1/2}^{1} = 0.21 \text{ V and } E_{1/2}^{2} = 0.49 \text{ V versus Ag}/$ AgNO<sub>3</sub>) [8b]. The  $E_{1/2}^1$  value of [Cu(II)(L1<sup>2-</sup>)(py)] is also close to that of [Cu(II)(L2<sup>-</sup>)(AcO)] (0.14 V versus Ag/AgNO<sub>3</sub>) in CH<sub>3</sub>CN [6d]. The slight differences in the redox potentials among these complexes may reflect the differences in the geometry at the copper center, but there seems to be no relation between the radical stability and the redox potential.

# 3.5. Spectroscopic characterization of the phenoxyl radical complexes (model complex for the active form of GAO)

As suggested by the electrochemical studies of  $[M(II)(L1^{2-})(py)]$ , phenoxyl radical complexes of the new ligand are unexpectedly unstable at ambient temperature. Thus, the oxidation of the diphenolate complexes by (NH<sub>4</sub>)<sub>2</sub>[Ce(IV)(NO<sub>3</sub>)<sub>6</sub>] (CAN) was examined at a lower temperature ( $-60^{\circ}$ C). The addition of an equimolar amount of a CH<sub>3</sub>CN solution of CAN into a 9:1 THF-CH<sub>3</sub>CN solution of  $[Cu(II)(L1^{2-})(py)]$  $(5.0 \times 10^{-4} \text{ M})$  at  $-60^{\circ}\text{C}$  resulted in an immediate increase of a strong absorption band at 418 nm ( $\varepsilon =$ 2100 M<sup>-1</sup> cm<sup>-1</sup>) and a small one at 720 nm ( $\varepsilon = 100$  $M^{-1}$  cm<sup>-1</sup>) together with a decrease in the absorption band of the diphenolate complex at 466 nm (Fig. 5(A)). A characteristic absorption band at 398 nm ( $\varepsilon = 1500$ M<sup>-1</sup> cm<sup>-1</sup>) was obtained in the oxidation of  $[Zn(II)(L1^{2-})(pv)]$  by CAN under the same experimental conditions (Fig. 5(B)). The resulting absorption bands at around 400 nm are the characteristic feature of phenoxyl radical species commonly observed both in enzymatic systems and in model complexes [3,6-10,12,13], indicating formation of the phenoxyl radical complexes as in the case of the CAN oxidation of  $[M(II)_2(L2^-)_2]^{2+}$  and  $[M(II)(L3^-)(CH_3CN)]^+$  (M = Cu or Zn) [6c,d]. There is, however, a significant difference in the absorption band in the NIR (near IR) region (above 800 nm) among the phenoxyl radical complexes so far reported. For example,  $[M(II)(L2^{\bullet})(NO_3)]^+$ (M = Cu or Zn) [6c,d] and Halcolm's model complexes [12] exhibit strong and broad absorption bands above 800 nm, which is closely related to that of the active form of GAO [3]. However, most of the other phenoxyl radical complexes show a relatively weak absorption

<sup>&</sup>lt;sup>5</sup> At a higher temperature, only irreversible oxidation peaks were observed under otherwise identical experimental conditions.

<sup>&</sup>lt;sup>6</sup> The  $E_{1/2}^{1}$  of [Zn(II)( $L1^{2-}$ )(py)] was determined by the second harmonic ac voltammetric (SHACV) method as 0.23 V versus Ag/ AgNO<sub>3</sub> (0.52 V versus SCE). It has been well demonstrated that the SHACV method provides a better approach to direct evaluation of one-electron redox potentials in the presence of a follow-up chemical reaction [16]. Well-defined symmetrical SHACV traces were obtained for the one-electron oxidation of [Zn(II)( $L1^{2-}$ )(py)] in CH<sub>3</sub>CN as shown in Supporting Information (S1), in which the intersection with the dc potential axis corresponds to the one-electron oxidation potential.



Fig. 5. (A) UV–Vis spectra of [Cu(II)(L1<sup>2–</sup>)(py)] (dotted line) (5.0 ×  $10^{-4}$  M) and of the corresponding phenoxyl radical/phenolate complex, Cu(II)(L1<sup>•–</sup>) (solid line) generated by the CAN oxidation (5.0 ×  $10^{-4}$  M) in 9:1 THF–CH<sub>3</sub>CN at  $-60^{\circ}$ C. (B) UV–Vis spectra of [Zn(II)(L1<sup>2–</sup>)(py)] (dotted line) (5.0 ×  $10^{-4}$  M) and of the corresponding phenoxyl radical/phenolate complex, Zn(II)(L1<sup>•–</sup>) (solid line) generated by CAN oxidation (5.0 ×  $10^{-4}$  M) in 9:1 THF–CH<sub>3</sub>CN at  $-60^{\circ}$ C.

band in the shorter wavelength region (600-700 nm) [8-10,13]. Thus, the present model complexes belong to the latter category, although the reason for such a difference in the NIR features among the phenoxyl radical complexes has yet to be clarified.

The formation of the phenoxyl radical species is also supported by the resonance Raman spectra of the solutions resulting from the CAN oxidation of  $[M(II)(L1^{2-})(py)]$  at  $-60^{\circ}$ C. The Cu(II)-phenoxyl radical complexes display characteristic peaks at ~1543 cm<sup>-1</sup> together with small peaks at ~1638 and ~1662 cm<sup>-1</sup> as shown in Fig. 6. The Zn(II) complex



Fig. 6. Resonance Raman spectra of the Cu(II)( $L1^{\bullet-}$ ) (bottom) and Zn(II)( $L1^{\bullet-}$ ) (top) generated by CAN oxidation of [M(II)( $L1^{2-}$ )(py)] (M = Cu or Zn) in 9:1 THF-CH<sub>3</sub>CN at -60°C; excitation wavelength: 413.1 nm.

also shows Raman bands at nearly the same positions (Fig. 6). These Raman bands disappeared completely when the solution temperature was raised to room temperature. This is due to decomposition of the phenoxyl radical complexes at the higher temperature, as suggested by the irreversibility in the electrochemical measurements. Although detailed assignment of these peaks has yet to be accomplished, the  $\sim 1545$  cm<sup>-1</sup> and  $\sim 1640/\sim 1660$  cm<sup>-1</sup> features in the resonance Raman spectra may be due to the modes  $v_{7a'}$  and  $v_{8a'}$ , which predominantly include the C-O stretching and the Cortho-Cmeta stretching, respectively [8b,10]. If so, the frequencies of these bands are somewhat higher than those for the other metal-coordinated phenoxyl radical species [6d,8b,10,13a], suggesting a larger contribution of the quinonoid canonical form in this particular case [10b].

The oxidation of  $[Cu(II)(L1^{2-})(py)]$  by CAN at the low temperature caused a significant decrease in the ESR signal of Cu(II) (more than 90% decrease in peak area), consistent with the formation of the Cu(II)– monophenoxyl radical/monophenolate species. A magnetic coupling between the S = 1/2 Cu(II) ion and the S = 1/2 phenoxyl radical will make the compound ESR silent, although the type of coupling (antiferromagnetic or ferromagnetic) is unclear at present [8b,13b]. On the other hand, the solution ESR spectrum of the Zn(II) complex measured at  $- 80^{\circ}$ C in CH<sub>3</sub>OH (Fig. 7(A)) exhibits an isotropic signal at  $g = 2.0046^{-7}$ . The g value

 $<sup>^{7}</sup>$  Judging from the peak area of Fig. 7(A), the yield of radical formation is relatively low ( ~ 1%). Since the phenoxyl radical complex is highly unstable even at low temperature, a large portion of the radical species decomposes during the sampling for ESR measurement.

of this signal is slightly smaller than those of the cofactor radical (2.0055) of the apo-enzyme and of the phenoxyl radicals derived from our cofactor model compounds (2.0052–2.0060) [3c,6b]. Although resolution of the spectrum is not sufficient owing to the instability of the compound, the shape of the ESR spectrum in Fig. 7(A) is very similar to that of  $[Cu(II)(L2^{\bullet})(NO_3)]^+$  [6d] but rather different from that of  $[Cu(II)(L3^{\bullet})(NO_3)]^+$  [6d]. This indicates that the odd electron of the radical species exists mainly on the phenol ring having the methylthio substituent as shown



Fig. 7. Solution ESR spectrum (A) of  $Zn(II)(L1^{\bullet-})$ , generated by the CAN oxidation of  $[Zn(II)(L1^{2-})(CH_3OH)]$  in CH<sub>3</sub>OH at  $-80^{\circ}$ C; microwave frequency 9.210 GHz, modulation frequency 100 kHz, modulation amplitude 0.5 G, microwave power 5 mW, and its computer simulation spectrum (B) using the parameters (g and hfc values) presented in Scheme 3.



Scheme 3.

in Scheme 3, where the hyperfine coupling constants (hfc) determined by the computer simulation are also indicated. It is obvious that the spin density at the benzylic methylene group in  $L1^{\bullet-}$   $(a_{\rm H} = 1.38 \text{ G})^8$  is significantly smaller than that of L3<sup>•</sup> ( $a_{\rm H} = 7.19$  G) but rather similar to that of L2<sup>•</sup> ( $a_{\rm H} = 2.43$  G) in the Zn(II) complexes [6d]. It has been suggested that the smaller spin density at the benzylic position is due to the spin delocalization into the alkylthio group in L2<sup>•</sup> [6d]. This can also be applied to the case of L1<sup>•-</sup>. The smaller *hfc* value of the methylthio group in L1<sup>•-</sup> (1.86 G) and the smaller g value of  $L1^{\bullet-}$  (2.0046) compared with  $L2^{\bullet-}$  $(a_{\rm SMe} = 3.72 \text{ G} \text{ and } g = 2.0055)$  [6d], however, indicate that the spin delocalization into the methylthio group in L1<sup>•-</sup> is not as large as in L2<sup>•</sup>. This may be a reason in part for the instability of L1<sup>•-</sup>.

#### 3.6. Summary

In this study, we have developed model complexes of the active site of galactose oxidase (GAO) using a new ligand carrying two different phenol groups (L1H<sub>2</sub>) in order to examine the effect of the second phenol group on the physicochemical properties as well as the redox reactivity of the Cu(II)-phenoxyl radical complexes. The nature of the ligand to ligand charge transfer between Tyr 272° (tyrosyl radical) and Tyr 495 (tyrosinate) in the active form of GAO and the acid-base catalysis by Tyr 495 in the catalytic cycle of GAO are of particular interest, since these issues have yet to be disclosed at a molecular level. Unfortunately, however, the phenoxyl radical/phenolate species  $(L1^{\bullet})$  in the Cu(II) and Zn(II) complexes were too unstable to examine their reactivity in the alcohol-oxidation reactions. This instability of the phenoxyl radical complexes is an unexpected result, since the phenoxyl radical complexes of the corresponding monophenol ligands (L2H and L3H) are fairly stable [6c,d]. The reason for such a difference in stability remains to be understood.

The low temperature experiments below  $-60^{\circ}$ C allowed us to examine the spectroscopic features (UV–Vis, resonance Raman, and ESR) of the Cu(II) and Zn(II) complexes of phenoxyl radical/phenolate species

<sup>&</sup>lt;sup>8</sup> It has been reported that *hfc* of benzylic methylene protons of phenoxyl radicals can be estimated by the angle-dependent Mc-Connell-type relationship:  $a_{C-H} = \rho_{C1}B\cos^2\theta$ , where  $a_{C-H}$  is the *hfc* of the methylene proton,  $\rho_{C1}$  is the spin density at the C<sub>1</sub> position, *B* is a constant equal to 162 Hz, and  $\theta$  is the dihedral angle defined in figure 8 in reference [17]. Thus, the ratio of the *hfc* values of two methylene protons ( $a_{H1}/a_{H2}$ ) is proportional to  $\cos^2 \theta_1/\cos^2 \theta_2$ . If one accepts the assumption that the dihedral angles of benzylic methylene protons do not change upon one-electron oxidation, the  $a_{H1}/a_{H2}$  ratio can be calculated as 75 by using the dihedral angles obtained in the X-ray structure of  $[Cu_2^{IL}(L1^{2-})_2] (\theta_{1(\alpha m)} = 30.5, \theta_{2(\alpha m)} = 84.3)$ . Such a large value of  $a_{H1}/a_{H2}$  is consistent with the large difference between  $a_{H1}$  and  $a_{H2}$  (1.38 G versus ~ 0 G) determined by computer simulation of the ESR spectrum (Scheme 3).

(L1<sup>•-</sup>). To our surprise, the NIR features observed in [Cu(II)(L2<sup>•</sup>)(NO<sub>3</sub>)] and [Zn(II)(L2<sup>•</sup>)(NO<sub>3</sub>)] [6c,d] disappeared when the second phenol group was introduced into the ligand. This result indicates that the electronic structure of the excited state is greatly altered by the second phenolate group. The NIR features of  $[Cu(II)(L2^{\bullet})(NO_3)]$  and  $[Zn(II)(L2^{\bullet})(NO_3)]$  have been assigned to the intramolecular charge transfer from the benzene ring to the methylthio groups in the phenoxyl radical group [6]. Thus, neither intramolecular charge transfer nor ligand-to-ligand charge transfer occurs in  $Cu(II)(L1^{\bullet-})$  and  $Zn(II)(L1^{\bullet-})$  complexes. This phenomenon of the present model complexes is rather similar to that observed in Stack's diphenolate system, although their model complexes of the phenoxyl radical/phenolate species are very stable [9]. It is interesting to note that the odd electron of the phenoxyl radical species exists mainly on the phenol ring having the methylthio substituent as illustrated in Scheme 3. This is consistent with our previous conclusion that the phenoxyl radical is stabilized to a greater extent by the electron-sharing conjugative effect by the alkylthio group [6a,b]. Overall, it became clear that the electronic structure as well as the stability and the reactivity of phenoxyl radical complexes are greatly influenced by the coordination geometry of the metal center.

#### 4. Supplementary material

A summary of the fundamental crystal data and experimental parameters for structure determinations is given in Table 1. The experimental details including data collection, data reduction, and structure solution and refinement as well as the atomic coordinates and  $B_{iso}/B_{eq}$ , anisotropic displacement parameters, and intramolecular bond distances and angles have been deposited in the Supporting Information (S2).

### Acknowledgements

The present study was financially supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (Molecular Biometallics: 10129218 and 11116219; Electrochemistry of Ordered Interface: 10131242 and 11118244; Creation of Delocalized Conjugated Electronic System: 10146232) from the Ministry of Education, Science, Sports, and Culture of Japan and by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (no. 02410). We also thank Professor Yasushi Kai and his co-workers, particularly, Ms Eiko Mochizuki of Osaka University, for their assistance in the X-ray measurements.

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