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# A New Copper(II) Complex as an Efficient Catalyst of Luminol Chemiluminescence

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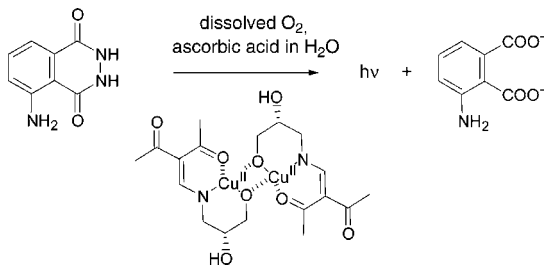
## ABSTRACT




**A new copper complex (2) has exhibited highly efficient catalytic activity of luminol chemiluminescence in water in the presence of ascorbic acid and dissolved $O_2$ under conditions that conventional catalysts such as $Cu(OAc)_2$, hemin or cyclen–Cu(II) did not show significant activity.**

Chemiluminescence (CL) has attracted considerable attention as a versatile and highly sensitive detection tool within diverse fields such as biology, biotechnology, bioimaging, and analytical technology.[1] Luminol is oxidized by strong oxidants in the presence of a catalyst such as peroxidase, metal ions, or metal complexes to produce chemiluminescence, leading to its use in a variety of analytical methods.[2] Recently, the amplified detection of DNA has been achieved by using catalyst-conjugated probes, in which $H_2O_2$ generated by doxorubicin-electrocatalyzed reduction of $O_2$ is detected by luminol CL with horseradish peroxidase,[3] or by the use of the hemin−(Fe(III) complex with protoporphyrin IX)[4]−DNA complex, which exhibits peroxidase-like activity.[5] It is expected from these and other examples of catalyst-conjugated probes[6] that small molecular weight catalysts for CL have potential benefits for ultrasensitive sensoring of biomolecules because of their easy synthesis and handling, high stability, and compatibility in physiological conditions. Here, we report on a new dicopper complex as an artificial peroxidase model to produce efficient luminol chemiluminescence with the use of dissolved $O_2$.

Luminol CL is initiated by oxidation of luminol to luminol radicals by strong oxidants including horseradish peroxidase, metals such as cobalt, copper, and iron, and organic complexes of these metals. Despite a huge number of studies, the mechanism of the reactions leading to enhancement or inhibition of luminol CL is still not fully understood,[7] hampering the development of a new molecular catalyst of CL. At the initial stage in designing a new molecular catayst, we became interested in dicopper complexes because of their

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oxidase-, oxygenase-, and catalase-like activity,[8] and a new $\beta$-ketoenamine ligand (**1**) and a new dicopper complex (**2**) were designed.[9] It has been reported that $H_2O_2$ is formed concurrently with quinone formation catalyzed by a dicopper complex.[10] In this study, it has been shown that a dicopper complex catalyzes both the reduction of dissolved $O_2$ and the following oxidation of luminol with $H_2O_2$ to yield efficient CL (Figure 1).

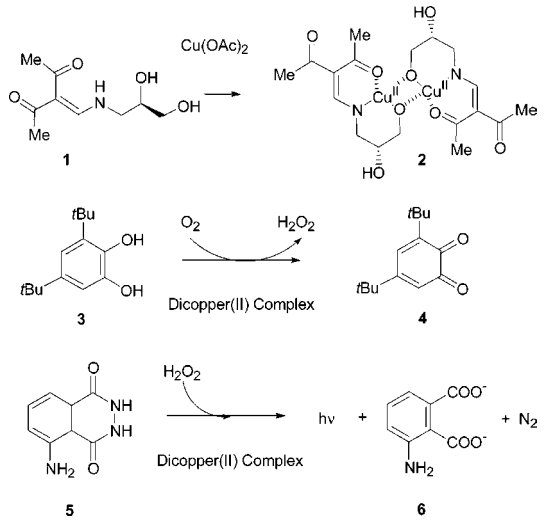


**Figure 1.** Design of a new catalyst for luminol CL using $O_2$ and a dicopper complex.

The $\beta$-ketoenamine ligand (**1**) was synthesized with (*R*)-3-amino-1,2-propanediol and 3-ethoxymethyleneacetylacetone. The ligand (**1**) and $Cu(OAc)_2$ were complexed in methanol, in the presence of triethylamine, to produce pale blue crystals. Its ESI-MS supported the formation of the dicopper complex, and its UV/vis spectrum was similar to the reported one with a d−d band at $\lambda = 620$ nm and a ligand-to-metal charge-transfer absorption at $\lambda \approx 350$ nm.[9a] As the complex was not characterized by X-ray crystallography, determination of the dicopper complex (**2**) needs further investigation. According to the structure of a similar dicopper complex,[11] we propose a structure **2** as described in Figure 1.

The oxidase-like activity of the dicopper complex **2** was evaluated by using 3,5-di-*tert*-butylcatechol (**3**) and ascorbic acid (Figure 2). The complex **2** showed significantly higher activity in the catechol oxidation from **3** to the quinone **4** compared to $Cu(OAc)_2$. Oxidation of ascorbic acid was faster than catechol oxidation. Catechol oxidation by the complex **2** was completely inhibited in the presence of ascorbic acid. Considering that the concentration of ascorbic acid is less than the dissolved $O_2$ concentration under standard conditions (2.5 mM)[12] and that the reaction did not reach completion, $O_2$ consumption is not the reason for the inhibition. As the oxidation mechanism of ascorbic acid by a dicopper complex is similar to that of chatechol oxidation,[13] inhibition of catechol oxidation may be due to competitive binding of ascorbic acid at the oxidation site of the complex **2**.

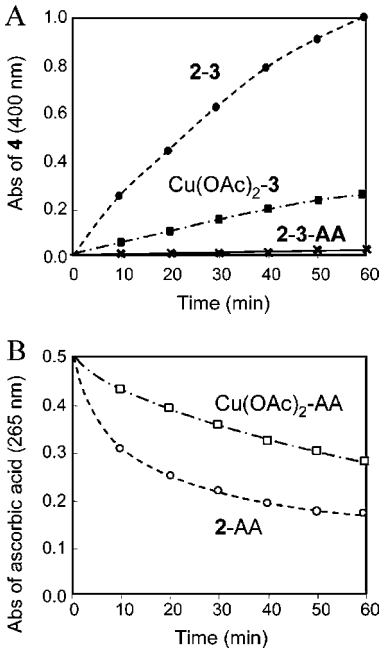


**Figure 2.** Oxidase-like activity of the copper complex **2** and $Cu(OAc)_2$. A: Oxidation of **3** (1 mM) was performed using the copper catalyst (0.02 mM) in methanol and the formation of **4** was followed at 400 nm. 1 mM of ascorbic acid (AA) was used for inhibition of the oxidation of **3**. B: Oxidation of ascorbic acid (AA, 0.1 mM) was performed using the copper catalyst (4 $\mu$M), followed by reduction of absorbance at 265 nm.

As the oxidase-like activity of **2** has been proven, we next tested **2** as a catalyst for oxidation of luminol. As it has been reported that dicopper(II) complexes possess catalase-like activity,[14] the effect of **2** on luminol CL was evaluated in the presence of $H_2O_2$ and compared with the already-known catalysts including $Cu(OAc)_2$, $K_3[Fe(CN)_6]$, hemin, and Cu(II)−cyclen complex (Figure 3A).

It should be noted that **2** exhibited catalytic activity as high as the hemin system, which is a potent catalyst for

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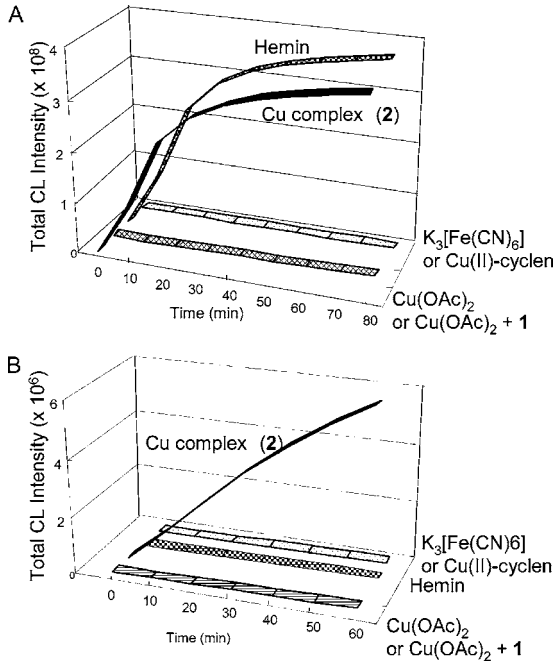


**Figure 3.** Comparison of the catalytic activity of the Cu(II) complex (**2**) and other metal catalysts (A) in the presence of $H_2O_2$ and (B) in the presence of ascorbic acid. Reaction was performed using luminol sodium (0.1 mM) and catalyst (4 $\mu$M) in the presence of $H_2O_2$ (0.5 mM) (A) or in the presence of ascorbic acid (0.1 mM) (B) in 50 mM $H_3BO_3$−NaOH buffer at pH 9.1.

luminol CL in the presence of $H_2O_2$. Significant CL was not induced by either $Cu(OAc)_2$ or a simple mixture of Cu(II) and the ligand **1**, suggesting that the high activity of **2** may be attributable to the dicopper complex. From the expectation of $H_2O_2$ production during the oxidation of **3**, we next tested the catalytic activity of **2** for CL in the presence of **3** without the addition of any $H_2O_2$, but no significant CL was observed. In contrast, it was found that addition of ascorbic acid into the solution of **2** induced efficient CL (Figure 3B). Hemin, $Cu(OAc)_2$, $Cu(OAc)_2$ + **1**, Cu(II)−cyclen, or $K_3[Fe(CN)_6]$ did not produce significant CL. Other reducing agents such as cysteamine, uric acid, 2-hydroxyacetophenone, or glutathione did not produce significant CL. To our knowledge, this is the first example of effective catalysis by a dicopper complex for luminol CL with the use of dissolved $O_2$.

Possible mechanisms of catalase and oxidase-like activity of dicopper catalysts have been proposed, some of which are summarized in Scheme 1. To reveal the oxidants in this CL reaction, the effects of inhibitors were used as a specific probe (Table 1). From the fact that $NaN_3$ did not inhibit CL,

**Scheme 1.** Summary of Proposed Mechanism for Catalase and Oxidase-like Activity of Dicopper Complexes (a Part of Catalase-like Activity Is Not Shown for Clarity)[20]

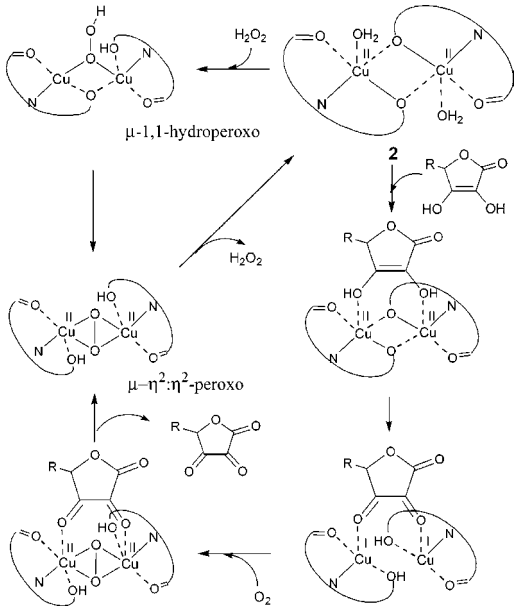


**Table 1.** Inhibition of Luminol CL by Different Inhibitors[a]

| | inhibitors (%) | | |
|---|---|---|---|
| oxidants | $NaN_3$ | SOD | catalase |
| **2**, $H_2O_2$ | no inhibition | 10 | 0 |
| **2**, ascorbic acid | no inhibition | no inhibition | 0 |

[a] 1 mM $NaN_3$, 10 units SOD in 1 mL, and 160 units catalase from bovine liver in 100 $\mu$L were added to the reaction vessel as described in the Figure 3 caption.

singlet oxygen is excluded as an oxidant.[15] Superoxide dismutase (SOD)[16] effectively inhibited CL in the reaction with **2**−$H_2O_2$ to about 10%. Almost complete inhibition of CL in both the **2**−$H_2O_2$ and **2**−ascorbic acid systems was observed with catalase. These results suggest that the superoxide anion radical ($O_2^{\bullet-}$) is the major oxidant in the reaction of **2**−$H_2O_2$, similar to the reports for CL by the hemin−$H_2O_2$ system,[17] in which an oxoiron(IV) porphyrin radical cation (oxene) acts as the oxidant of luminol and $H_2O_2$ to form luminol radicals and $O_2^{\bullet-}$, repectively. The addition of $H_2O_2$ to **2** in methanol induced a red shift in the d−d band from $\lambda$ = 620 to 650 nm, suggesting the formation of the $\mu$-1,1-hydroperoxo-Cu(II) speices $(LCu(II))_2$-OOH) (Scheme 1).[8] Such hydroperoxo species may be candidate oxidants for the formation of $O_2^{\bullet-}$. Complete inhibition of CL by catalase has indicated that other $H_2O_2$-related species besides $O_2^{\bullet-}$ are included as the major oxidants in the **2**−ascorbic acid system and also in a portion of the **2**−$H_2O_2$ system. The catalytic activity of **2** in the ascorbic acid system was shown to be proportional to the concentration of **2** at higher than 2 $\mu$M, indicating that active catalytic species are

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retained, at least, at higher concentrations than 2 $\mu$M. Further elucidation is needed to identify the active catalyst as well as the active oxidants in this new CL system.

We compared the oxidation rates of ascorbic acid and the rates of CL in the presence of **2** (Figure 4A). Oxidation of ascorbic acid was completed within 5 min. When 0.5 mM of ascorbic acid was used, the reaction stopped at 0.22 mM, which is in good agreement with expectations based on consumption of all of the dissolved $O_2$ (0.25 mM under standard conditions) to form $H_2O_2$ with a 1:1:1 stoichiometry of ascorbic acid, $O_2$, and $H_2O_2$. [18] Compared to the rapid oxidation of ascorbic acid, increases in CL intensity were delayed (Figure 3B), suggesting that the formed $H_2O_2$ was used for luminol oxidation gradually. It should be mentioned here that CL efficiency depends on the buffer system and is in the order of $Na_2HPO_4-NaH_2PO_4$ > $H_3BO_3-NaOH$ in the case of CL by the hemin$-H_2O_2$ system. In contrast, efficiency of CL by the **2**$-H_2O_2$ system is almost the same in either $H_3BO_3-NaOH$ or $Na_2HPO_4-NaH_2PO_4$. It turned out that the buffer system was a critical determinant for efficient CL by the **2**−ascorbic acid system, and that CL by the **2**−ascorbic acid system was obtained only in $H_3BO_3-NaOH$ buffer.

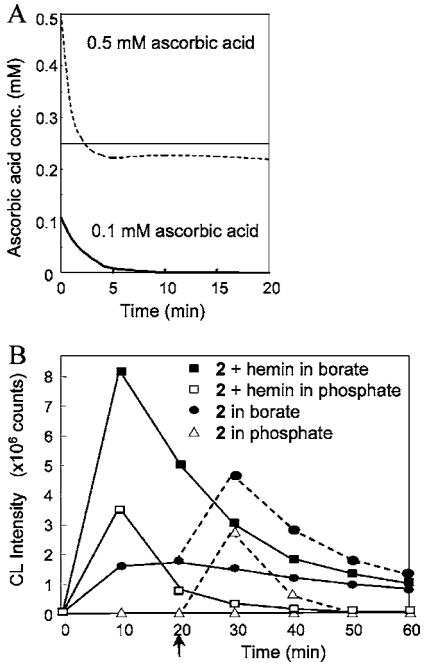


**Figure 4.** Oxidation of ascorbic acid by **2** (A) and effects of hemin on CL in the **2**-ascorbic acid system (B). A: Acorbic acid concentrations were plotted against time. Oxidation was performed using 0.5 mM or 0.1 mM ascorbic acid, 20 $\mu$M of **2** in 50 mM $H_3BO_3-NaOH$ buffer at pH 9.1. B: The luminol CL reaction was performed using 4 $\mu$M of **2**, 0.1 mM ascorbic acid in the absence or presence of 4 $\mu$M of hemin in a borate buffer (50 mM $H_3BO_3-NaOH$) or in a phosphate buffer (50 mM $Na_2HPO_4-NaH_2PO_4$). Hemin (4 $\mu$M) was added to reaction mixtures of **2** in both the borate buffer and the phosphate buffer after 20 min.

In order to investigate differences in the reactivity of $H_2O_2$ species in both buffer systems, CL by the **2**−ascorbic acid system was measured in the presence or absence of hemin (Figure 4B). Hemin enhanced higher CL in the borate buffer than in the phosphate buffer (closed and open rectangles in Figure 4B). When hemin was added to the **2**−ascorbic acid system after 20 min, CL was induced in both buffer systems (Figure 4B, dotted lines), showing that $H_2O_2$ species had been present at this point. As it has been reported that dehydroascorbic acid is oxidized by $H_2O_2$ to form hydroperoxide species and then decomposed in aqueous solution,[19] the produced $H_2O_2$ is presumably trapped as such organic hydroperoxide species, which are used as oxidants for CL in the hemin system. From the fact that no CL was induced by the **2**−ascorbic acid system in the phosphate buffer, it is assumed that the complex **2** cannot employ such hydroperoxide species as oxidants for CL in the phosphate buffer.

Interestingly, the dicopper complex (**2**) can utilize $H_2O_2$ species as oxidants for luminol CL in the borate buffer. It has been reported that $H_2O_2$ in a borate buffer is in equilibrium with monoperoxoborate ($HOOB(OH)_3^-$) and diperoxoborate ($(HOO)_2B(OH)_2^-$).[21] Accordingly, it may be assumed that $H_2O_2$ species are trapped as peroxoborates in the borate buffer in addtion to hydroperoxide spieces of dehydroascorbic acid and then gradually used for luminol oxidation. Delayed oxidation by use of peroxoborates may also account for long-lasting CL in the borate buffer compared to that in the phosphate buffer (closed rectangles and circles in Figure 4B).

In this study, we designed a new dicopper complex (**2**) as a catalyst with oxidase and catalase-like activity and have shown that the complex (**2**) acts as an efficient catalyst for luminol CL in the presence of $H_2O_2$, with a similar efficiency as hemin. More importantly, based on its catalase-like activity, the dicopper complex (**2**) demonstrated the first example of efficient catalysis for luminol CL using dissolved $O_2$ in the presence of asorbic acid as a reducing agent. As the dicopper complex (**2**) can be obtained easily in quantity and is chemically stable, it will find a variety of applications for sensitive sensors, catalyst-conjugated probes, and so on.

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**Supporting Information Available:** Experimental procedure, UV spectra, and $^1H$ NMR and ESI-MS spectra of compound **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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