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An Artificial Cu^{II} Complex with Intriguing Oxygen Radical-Quenching Profile. X-Ray Structure, Cytochrome c Assay, and ESR Study

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Abstract—A novel artificial peptide named HPH-Pep, comprising a pyridine and two histidine units, was synthesized. The HPH-Pep–Cu^{II} complex had unique pentacoordinated structure as shown by X-ray crystallography and exhibited superoxide-scavenging activity as indicated by ESR spectroscopy. The superoxide-quenching profile of HPH-Pep–Cu^{II} was studied in detail by cytochrome c assay and ESR spin trapping and it was found that (1) HPH-Pep–Cu^{II} did not scavenge hydrogen peroxide or hydroxyl radical and hence the scavenging activity was specific to superoxide, and (2) HPH-Pep–Cu^{II} did not generate hydrogen peroxide upon scavenging superoxide. Copyright © 1996 Elsevier Science Ltd

Introduction

Since reactive oxygen species (ROS) play critical roles in various biological processes, such as defense of human biochemical system or signal transduction pathways associated with immune response and growth control,[1,2] development of oxygen activators or scavengers of oxygen radical is of increasing importance.[3,4] Recently, we have reported that novel ligand, constructed from pyridine and histidine units, namely HPH [Fig. 1(A)], showed potent activity to activate molecular oxygen, as demonstrated by ESR spin trapping.[5] Herein we report our finding that replacement of the secondary amino linkage of HPH by a peptide bond, as envisaged in HPH-Pep [Fig. 1(B)], switches the oxygen-activating capability to the reverse activity to scavenge oxygen radical. In addition, enzyme assay and ESR studies indicated that the Cu^{II} complex of the new ligand HPH-Pep has an intriguing radical-scavenging profile, that is, (1) HPH-Pep–Cu^{II} scavenged superoxide specifically without quenching other ROS, such as hydrogen peroxide and hydroxyl radical, and (2) HPH-Pep–Cu^{II} did not generate hydrogen peroxide upon scavenging superoxide.

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Key words: copper complex, superoxide, hydrogen peroxide, hydroxyl radical and scavenger.

Results

Design, synthesis, and X-ray structure of the HPH-Pep–Cu^{II} complex

Our previous oxygen-activating ligand HPH has been designed by a total structural revision of the iron binding site of antitumor antibiotic bleomycin.[5] The bleomycin metal core contains a pyrimidine ring possessing *erythro*-β-hydroxy-L-histidine and β-amino-L-alanine appendages.[6] The pyrimidine and the histidine residues form a coordination plane through a rigid peptide bonding, while the β-aminoalanine constitutes an axial donor by virtue of a flexible secondary amino linkage [Fig. 2(A)]. HPH ligand was designed by symmetrical arrangement of histidine units [Fig. 2(B)]. It was considered that the flexibility of the HPH ligand, seemingly significant for the oxygen activation via iron complexation, could be constrained by replacing the secondary amine portion by more rigid peptide bonding and such structural modification might alter the physicochemical property of the molecule. A peptide analogue of HPH, namely HPH-Pep [Fig. 1(B)], was thus designed. HPH-Pep was synthesized by peptide condensation of 2,6-pyridinedicarboxylic acid and histidine methyl ester[7] (DPPA[8]/DMF) in 74% yield.

The HPH-Pep–Cu^{II} complex was crystallized and X-ray crystallographic analysis was carried out. HPH-Pep coordinates with Cu^{II} as a pentacoordinate chelator (Fig. 3). The HPH-Pep–Cu^{II} complex reveals a unique

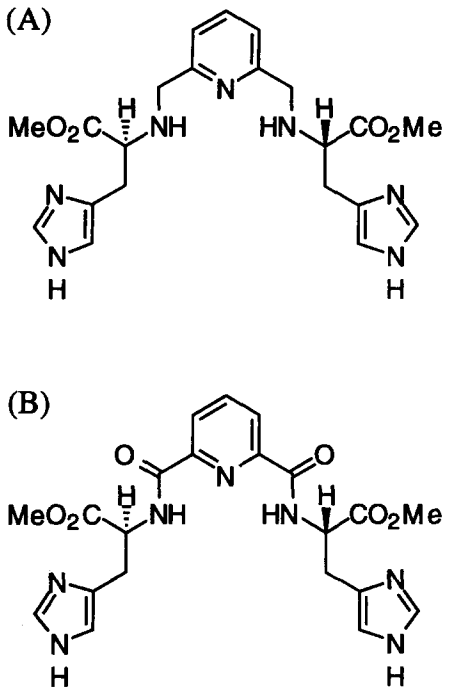


Figure 1. (A) Structure of oxygen-activating ligand HPH. (B) Structure of superoxide-scavenging ligand HPH-Pep.

distorted pentacoordinated configuration in which the pyridine nitrogen, the two deprotonated peptide nitrogens, and the two imidazoles bind to Cu^{II}. The ESR parameters for the HPH-Pep–Cu^{II} complex ($g_{\perp}=2.049$, $g_{||}=2.220$, $A_{||}=156.2$ G) were in accordance with this geometry. This coordination structure of the Cu^{II} complex is totally different from the metal complexes of bleomycin and its analogues that were classified as square pyramidal.[2] Therefore, we considered that the HPH-Pep might exert functions distinct from that of bleomycin.

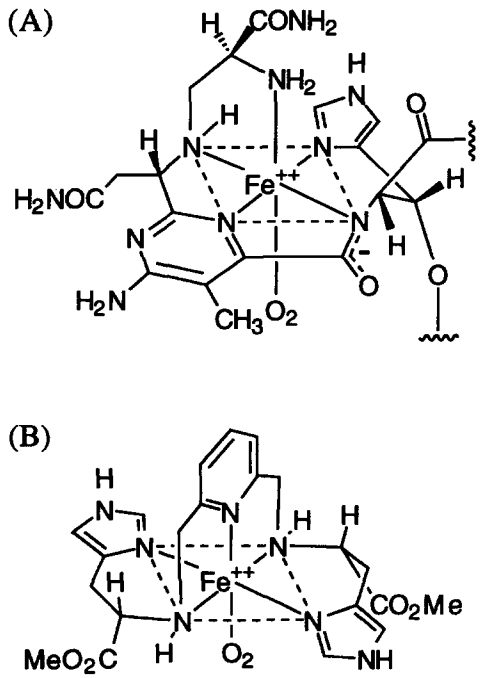


Figure 2. (A) Proposed structure of bleomycin–Fe^{II}–O_2 complex. (B) Design of HPH by arranging two histidine around a pyridine-iron axis in a symmetrical manner.

Superoxide-quenching activity of the HPH-Pep–Cu^{II} complex

We first examined the oxygen-activating capability of the HPH-Pep–Fe^{II} complexes by the ESR spin trapping experiments using *N-tert*-butyl-α-phenylnitrone (BPN).[9] However, any signals for the spin adduct with oxygen radicals could not be detected by ESR. Thus, it was evident that the HPH-Pep–Fe^{II} complex does not generate oxygen radicals.

In contrast, we found that the HPH-Pep–Cu^{II} complex has radical-scavenging activity. Figure 4(A) shows ESR spectrum of potassium superoxide (140 mM of KO_2 in potassium phosphate buffer, 50 mM, pH 7.8; $g_{\perp}=2.006$, $g_{||}=2.080$) observed under liquid nitrogen temperature. This signal of KO_2 was remarkably weakened upon addition of the HPH-Pep–Cu^{II} complex as shown in Figure 4B (recorded at liquid nitrogen temperature). These strongly suggested that the HPH-Pep–Cu^{II} complex does possess superoxide-

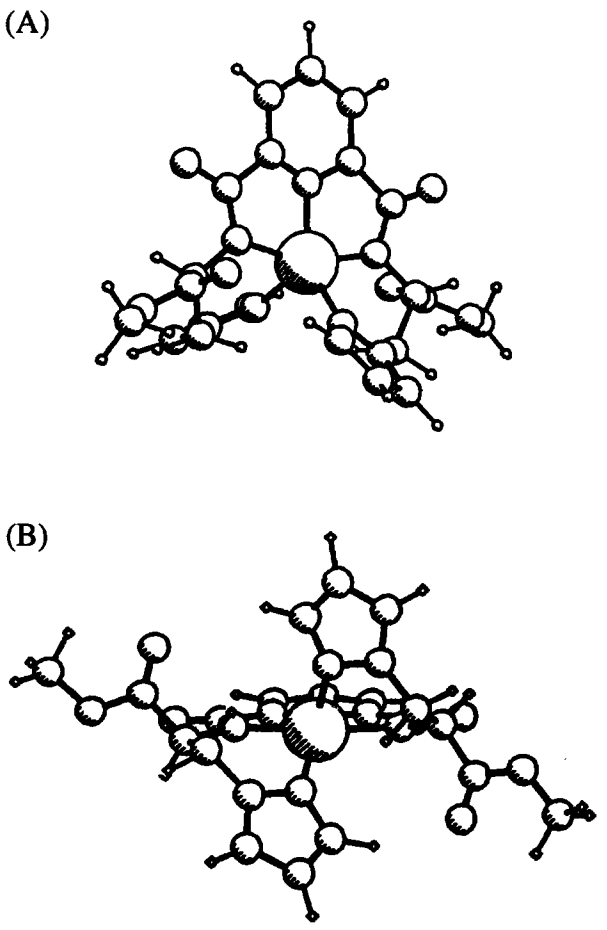


Figure 3. X-ray structure of the HPH-Pep–Cu^{II} complex. (A) A view from the pyridine–Cu^{II} side. (B) A view from the imidazole–Cu^{II}–imidazole side.

scavenging capability instead of oxygen-activating activity. Interestingly, the HPH–Cu^{II} complex had virtually no activity to quench superoxide.

This activity was further studied in detail and confirmed by the well-established xanthine oxidase–cytochrome c assay. This assay is to monitor reduction of cytochrome c by superoxide generated by xanthine oxidase.[10] Time-dependent increase of the increment of 550 nm absorbance of reduced cytochrome c diminished as the concentration of the HPH-Pep–Cu^{II} increased [Fig. 5(A)]. On the other hand, HPH-Pep–Cu^{II} did not influence the production of uric acid formed by the oxidation of xanthine as shown by monitoring absorbance at 292 nm, the λ_{max} for uric acid[11] [Fig. 5(B)]. This ruled out a possibility that the HPH-Pep–Cu^{II} complex might inhibit xanthine oxidase. Accordingly, the observed suppression of absorbance at 550 nm was confirmed to be due to the quench of superoxide produced by xanthine oxidase. The IC_{50} was calculated to be 5.6 μM.

The HPH-Pep–Cu^{II} complex does not convert superoxide to hydrogen peroxide

It is well known that superoxide dismutase (SOD) disproportionate superoxide to hydrogen peroxide and dioxygen.[12] Therefore, we examined the production of hydrogen peroxide by the combination of the Fenton reaction and the ESR spin trapping experiment using BPN. By the Fenton reaction, hydrogen peroxide is transformed to a hydroxyl radical, which in turn is captured by BPN to give a spin adduct to be measured by ESR (Scheme 1).[13]

Figure 6(A) shows the ESR signal for the spin adduct of BPN and the hydroxyl radical formed from hydrogen peroxide in the presence of Fe^{II}, demon-

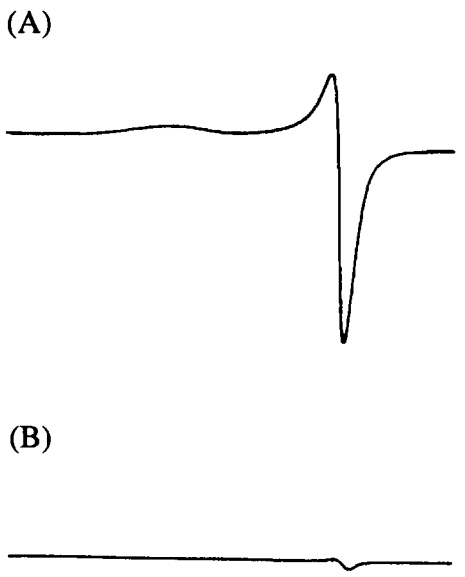


Figure 4. (A) ESR spectrum of KO_2. (B) ESR spectrum of KO_2 reacted with HPH-Pep–Cu^{II}.

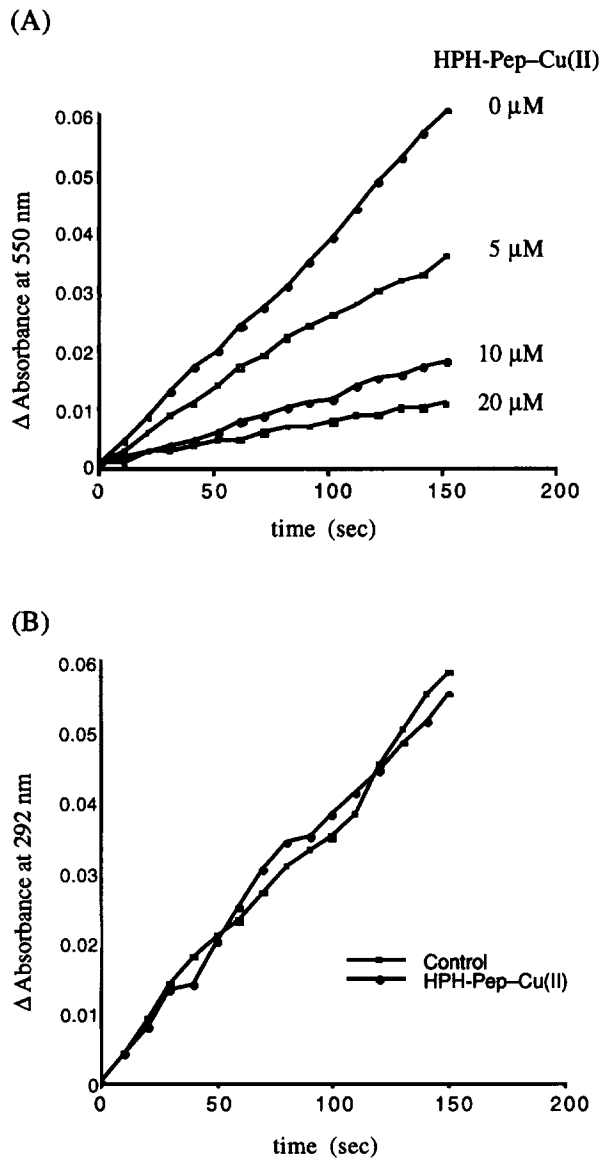
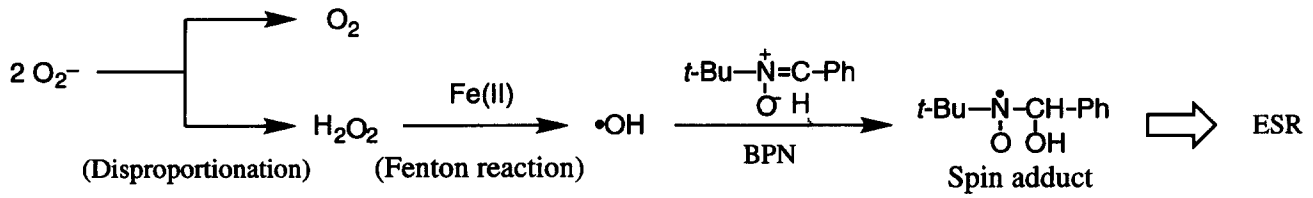


Figure 5. (A) Cytochrome c–xanthine oxidase assay of the superoxide-scavenging activity of HPH-Pep–Cu^{II}. (B) Assay of uric acid produced by catalysis of xanthine oxidase in the presence or absence of HPH-Pep–Cu^{II}.



Scheme 1.

strating the Fenton/BPN technique to be operative. When superoxide was reacted with SOD, the same spin adduct was observed [Fig. 6(B)], in accordance with the formation of the hydrogen peroxide as a disproportionation product. However, replacement of SOD of this system by HPH-Pep–Cu^{II} resulted in a remarkable decrease of the formation of spin adduct, as seen in the signal of Figure 6(C), which is no bigger than that without superoxide [Fig. 6(D)]. This result indicates that the HPH-Pep–Cu^{II} complex did not generate hydrogen peroxide upon scavenging superoxide.

The HPH-Pep–Cu^{II} does not quench either hydrogen peroxide or hydroxyl radicals

We further examined a possibility that the HPH-Pep–Cu^{II} complex might quench ROS other than superoxide, such as hydrogen peroxide and hydroxyl radical. Thus, we compared hydrogen peroxide quenching activity of the HPH-Pep–Cu^{II} with that of catalase[14] by the Fenton/spin trap experiment. When hydrogen peroxide was reacted with catalase and Fe^{II} in the presence of BPN, virtually no ESR signal for the spin adduct was observed [Fig. 7(A)] in contrast to the signal without catalase [Fig. 6(A)]. This shows that hydrogen peroxide was decomposed by catalase before the Fenton reaction occurs. On the other hand, reaction of hydrogen peroxide with HPH-Pep–Cu^{II} and Fe^{II} resulted in the formation of evident ESR signal for the spin adduct of BPN and hydroxyl radical [Fig. 7(B)]. This shows that hydrogen peroxide was not quenched by HPH-Pep–Cu^{II} and hence was converted into hydroxyl radical by Fe^{II}. Other information obtained from Figure 7(B) was that HPH-Pep–Cu^{II} did not quench hydroxyl radical produced by the Fenton reaction. Thus, it was shown that the HPH-Pep–Cu^{II} complex does not quench hydrogen peroxide or hydroxyl radical.

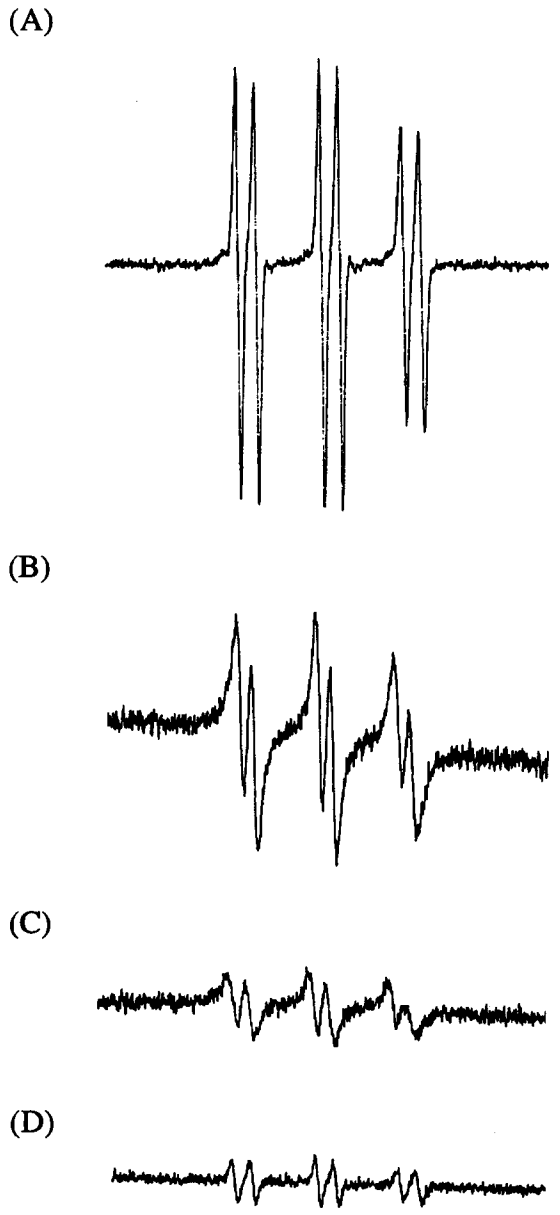


Figure 6. Detection of hydrogen preoxide by ESR spin trapping under the Fenton condition. (A) H_2O_2 and Fe^{II}. (B) KO_2, SOD, and Fe^{II}. (C) KO_2, HPH-Pep–Cu^{II}, and Fe^{II}. (D) HPH-Pep–Cu^{II} and Fe^{II}.

Discussion

It was shown that HPH-Pep is not an oxygen activator but a superoxide scavenger, whereas HPH is not a superoxide scavenger but an oxygen activator. Thus, we are successful to separate the two activities of the closely related ligands in regard to reactive oxygen species. We studied the ROS-removing profile of

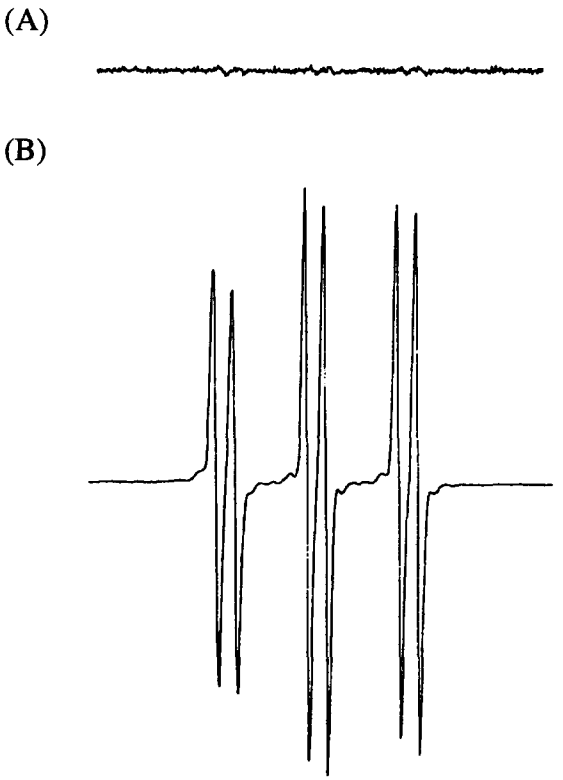


Figure 7. ESR study on the speficity of the ROS-scavenging activity of HPH-Pep–Cu^{II}. (A) H_2O_2, Fe^{II}, and catalase. (B) H_2O_2, Fe^{II}, and HPH-Pep–Cu^{II}.

HPH-Pep–Cu^{II}. The activity of HPH-Pep–Cu^{II} to quench superoxide has been unambiguously demonstrated by directly observing the ESR signal for superoxide itself (Fig. 3). This was further confirmed by the well-established cytochrome c–xanthine oxidase assay. HPH-Pep–Cu^{II} suppressed the 550 nm absorbance of the reduced cytochrome c with IC_{50} of 5.6 μM and the possibility of inhibition of xanthine oxidase in this assay was ruled out (Fig. 4). Therefore, it is no doubt that the HPH-Pep–Cu^{II} complex possesses an activity to quench superoxide.

SOD specifically scavenge superoxide by disproportionation mechanism to generate dioxygen and hydrogen peroxide.[12] It has been well established that SOD converts the first O_2^- molecule into O_2 [eq (1)] then it further transforms the second O_2^- into H_2O_2 to regenerate the initial Cu^{II} state of SOD [eq (2)].[12]

$$Cu^{II} + O_2^- \rightarrow Cu^{I} + O_2 \quad (1)$$

$$Cu^{I} + O_2^- + 2H+ \rightarrow Cu^{II} + H_2O_2 \quad (2)$$

HPH-Pep–Cu^{II} was found to be distinct from SOD in the production of hydrogen peroxide, that is, HPH-Pep–Cu^{II} does not convert superoxide into hydrogen peroxide whereas SOD produces hydrogen peroxide (Fig. 6). This could be reasonably explained by assuming that HPH-Pep–Cu^{II} is dysfunctional in the conversion of the second superoxide into hydrogen peroxide [i.e. eq (2)] and the reaction is constituted solely from the stoichiometric process of eq (1). The superoxide quenching activity was not observed in the absence of Cu^{II} (data not shown), and hence the formation of the Cu^{II} complex with a unique distorted coordination mode (Fig. 3) seemed to be significant for the activity to quench superoxide attributable to the process involving Cu^{I} species. Therefore, it was considered that the reaction ends up with HPH-Pep–Cu^{I} and dioxygen as shown in eq (1). ROS-removing activity of HPH-Pep–Cu^{II} was shown to be specific to superoxide because it did not destroy hydrogen peroxide or hydroxyl radical as demonstrated by ESR spectroscopy (Fig. 7).

Thus, we demonstrated that (1) HPH-Pep–Cu^{II} is capable of quenching superoxide, (2) HPH-Pep–Cu^{II} does not quench hydrogen peroxide or hydroxyl radical, and (3) HPH-Pep–Cu^{II} does not produce hydrogen peroxide upon quenching superoxide.

Of our current interest is the presumed involvement of ROS in the signaling pathway leading to the activation of NF-κB, a transcription factor associated with the expression of various genes essential for the immune responses.[15] Several antioxidants and related molecules, including *N*-acetyl-L-cysteine, a pyrrolidine derivative of dithiocarbamate, and vitamin E derivatives, are known to inhibit the induction of NF-κB.[15–17] However, the ROS-quenching profile of these inhibitors has not been well-characterized and hence they seem to be nonspecific scavengers of ROS. Recently, we found that the Cu^{II} ion blocks activation of NF-κB through inhibiting the signal-induced phosphorylation of IκBα, the inhibitor protein associated with NF-κB.[18] Since the Cu^{II} ion possesses superoxide-scavenging activity,[19–21] it is possible that the inhibitory activity of the Cu^{II} ion against NF-κB activation is related to superoxide. It is a significant characteristic of HPH-Pep–Cu^{II} that it does not produce or quench hydrogen peroxide because hydrogen peroxide is an inducer of NF-κB.[15] This is a great advantage for HPH-Pep–Cu^{II} to examine whether superoxide is involved in the signaling pathway leading to NF-κB. Further, structural modification of HPH-Pep aiming at cell permeability for the application to the in vivo system is currently in progress.

Experimental

Synthesis of HPH-Pep

Diphenyl phosphorazidate (DPPA;[8] 2.1 mL, 9.745 mmol) and triethylamine (Et_3N; 4.6 mL, 33 mmol) were successively added to a solution of 2,6-pyridinedicarboxylic acid (830.3 mg, 4.078 mmol) and L-histidine methyl ester dihydrochloride[7] (1.973 g, 8.148 mmol) in *N*,*N*-dimethylformamide (DMF; 100 mL) at 0 °C under argon. The solution was stirred for 2 h at 0 °C, then 3 days at room temperature and the solvent was removed in vacuo. The residue was dissolved in chloroform and washed with aq $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by a silica gel column chromatography pretreated with Et_3N:Et_2O (1:9) eluted with CH_2Cl_2:MeOH (25:1→9:1) to give HPH-Pep as a colorless powder (1.418 g, 74%), $[\alpha]_D^{18.9}$ −61.45° (*c* 0.5, MeOH); ^1H NMR (DMSO-d_6): δ 3.15 (2H, dd, J=8.0, 14.5 Hz), 3.20 (2H, dd, J=5.0, 14.5 Hz), 3.67 (6H, s), 4.70 (1H, t, J=8.0 Hz), 6.91 (2H, s), 7.56 (2H, s), 8.20 (3H, s), 9.48 (2H, d, J=7.0); FABMS: *m/z* 470 (MH^+). Structure of HPH-Pep was established by the X-ray crystal analysis as described below.

X-ray crystallography

The HPH-Pep-Cu^{II} complex was crystallized from 60–80% aq ethanol. Crystal data: $C_{21}H_{21}N_7O_6Cu \cdot 2H_2O$, M_r=567.02, tetragonal, space group $P4_32_12$, $a=b$= 16.854 (5) Å, c = 19.824 (9) Å, V=5631 (6) Å^3, Z=8, D_{calc}=1.333 g cm^{-3}, λ(Cu-K_α)=1.5418 Å, μ(Cu-K_α)=16.26 mm^{-1}, F(000)=3376. A single crystal with dimensions 0.3 × 0.3 × 0.5 mm was used for X-ray diffraction data collection on a Rigaku AFC-5 diffractometer employing graphite-monochromated Cu-K_α radiation. A total of 2608 independent reflections with 2θ=130° were collected in an ω-2θ scan mode and were corrected for the Lorentz and polarization factors and the absorption effect. Of these, 2213 reflections with $I > 2\sigma(I)$ were used for the structure determination and refinement. The structure was solved by the heavy atom method and refined by the full-matrix least-squares method with the use of anisotropic temperature factors. H atoms were not included in the refinement. The present discrepancy indexes R and Rw are 0.096 and 0.103, respectively. The atomic coordinates, anisotropic thermal parameters, bond lengths

and angles have been deposited at the Cambridge Crystallographic Data Centre.

Cytochrome c–xanthine oxidase assay

The superoxide-quenching activity of HPH-Pep–Cu^{II} was assayed according to the reported procedure of the cytochrome c–xanthine oxidase system,[10] except for omitting EDTA. Uric acid formation was monitored by observing the change in absorbance at 292 nm according to the reported procedure.[11]

Observation of H_2O_2 production by the combination of the Fenton reaction and ESR spin trapping

To a mixture of a solution of *N-tert*-butyl-α-phenylnitrone (BPN) in ethanol (400 μL) and potassium phosphate buffer (50 mM, pH 7.8; 600 μL) were successively added a solution of HPH-Pep–Cu^{II} or Cu–Zn superoxide dismutase (SOD; 20 μL), $Fe(NH_4)_2SO_4$, and KO_2. The whole thing was mixed well and ESR spectra were measured at room temperature.

Examination of H_2O_2-quenching capability of HPH-Pep–Cu^{II} or catalase by ESR spin trapping experiment

To a mixture of a solution of BPN in ethanol (400 μL) and potassium phosphate buffer (50 mM, pH 7.0; 600 μL) were added a solution of HPH-Pep–Cu^{II} or catalase (20 μL) and 30% hydrogen peroxide (14 μL). The whole thing was mixed well and ESR spectra were measured at room temperature.

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