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SYNTHESIS, OXYGEN ACTIVATION, AND DNA-CLEAVING PROPERTY OF A HISTIDINE-PYRIDINE-HISTIDINE LIGAND‡

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Abstract – A novel metal-chelating system comprising a 4-dimethylamino-pyridine and two histidine appendages was synthesized. The two histidines were introduced by different manners; one through an amide linkage and other via a secondary amino linkage. ESR spectrum suggested a distorted pentacoordinate configuration of the copper complex of the ligand. The iron complex of the ligand had oxygen-activating property as shown by ESR spin trapping and DNA-cleaving activity as evaluated by experiments using pUC19 DNA.

INTRODUCTION

DNA-cleaving compounds of natural or artificial origin include a range of metal complexes. Many are used as drugs or biological tools owing to their sequence specificity in DNA cleavage.[1] A naturally occurring cancer chemotherapeutic bleomycin comprises a metal-binding pyrimidine moiety and a DNA-recognizing bithiazole.[2] The iron complex of bleomycin generates active oxygen species, inducing double-strand breaks in DNA.[3] We have previously reported artificial chelating ligands based on the structure of bleomycin, namely, the amine-type ligand HPH[4] and amide-type ligand HPH-Pep[5] (Figure 1). It was intriguing that the HPH-Fe(II) complex activates molecular oxygen while the HPH-Pep-Fe(II) does not. We were interested in hybridizing the structural characteristic of HPH and HPH-Pep to examine metal-chelating, oxygen-activating, and DNA-cleaving function. Herein we report such hybridized ligand (**1**) possessing one histidine introduced through an amide bond and other histidine connected by a

‡This paper is dedicated to Dr. Satoshi Omura on the occasion of his 70th birthday.

secondary amino linkage (Figure 1).

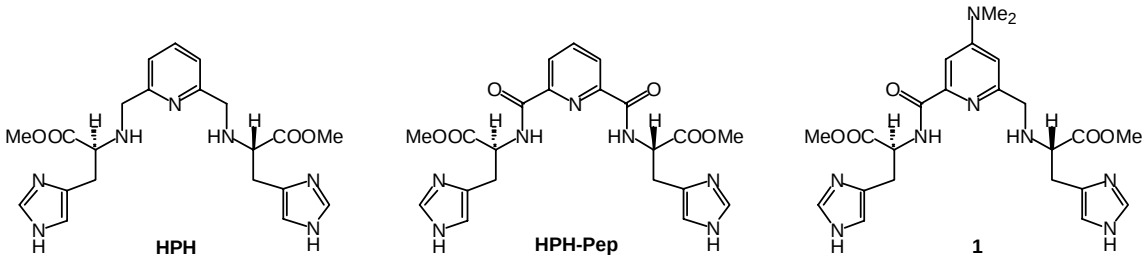
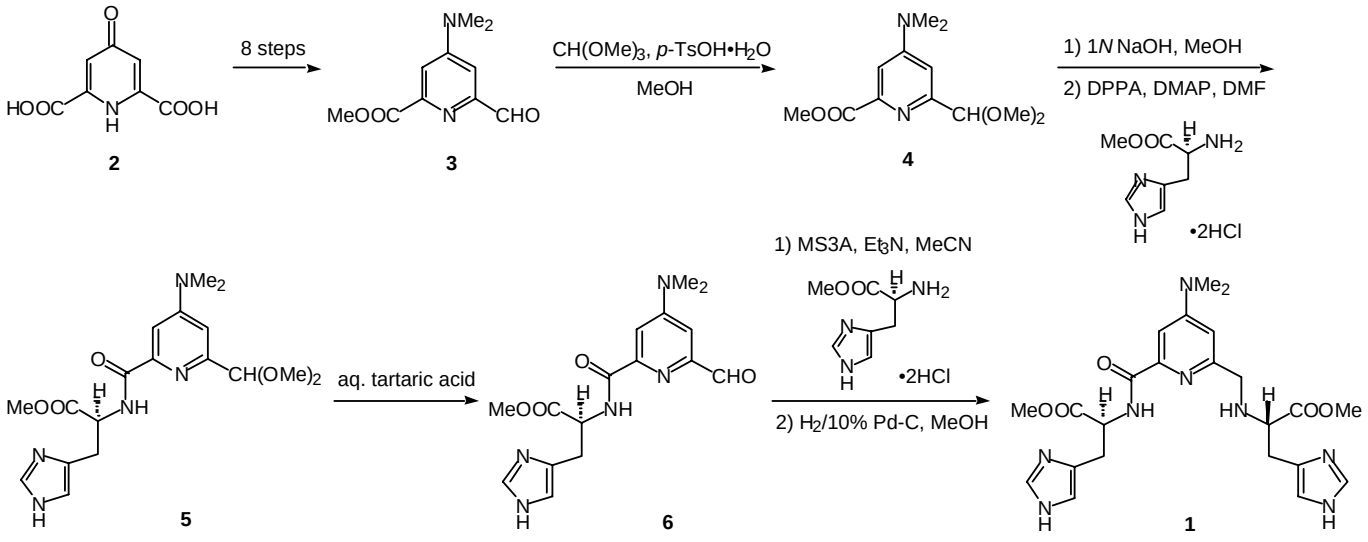


Figure 1. Structure of artificial ligands.

RESULTS AND DISCUSSION

Ligand (**1**) was synthesized as shown in Scheme 1. Formyl pyridine derivative (**3**), prepared in eight steps from chelidamic acid (**2**) according to the procedures previously described[6] was treated with trimethyl orthoformate in the presence of *p*-toluenesulfonic acid monohydrate in methanol to afford the dimethyl acetal (**4**) in quantitative yield. Methyl ester (**4**) was then hydrolyzed and coupled with L-histidine methyl ester dihydrochloride (DPPA,[7] DMAP in DMF) to give amide (**5**) in 46% isolated yield. The subsequent deacetalization was not straightforward as acetal (**5**) and aldehyde (**6**) were inseparable on tlc. Deprotection of acetal group was eventually accomplished by heating at 50°C for two days in the presence of saturated aqueous tartaric acid and extractive workup afforded crude aldehyde (**6**). A mixture of the crude **6** and L-histidine methyl ester dihydrochloride was stirred in acetonitrile in the presence of molecular sieves 3A and triethylamine. The resulting imine was directly hydrogenated with 10% Pd-C to afford the ligand (**1**) in 54% isolated yield.[8]



Scheme 1. Synthesis of ligand (**1**).

The ESR spectrum of the copper complex of **1** suggested typical pentacoordinate complex in which the pyridine nitrogen, the secondary amine nitrogen, the deprotonated peptide nitrogen and the two imidazoles bind Cu(II). The configuration seemed distorted pentacoordinated because of relatively small $A_{//}$ value (13.5 mT) which is indicative of the stability (Figure 2).[9]

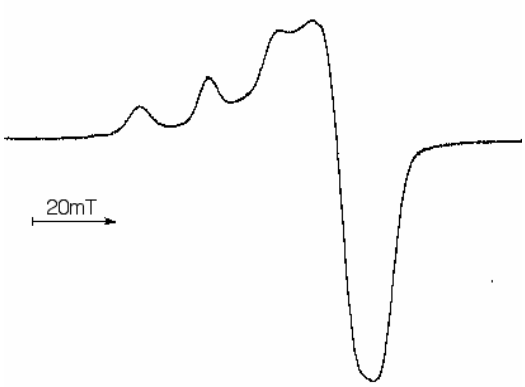


Figure 2. ESR spectrum of the ligand (**1**)-Cu(II) complex at 77K.

The oxygen-activating property of the (**1**)-Fe(II) complex was examined by ESR spin trapping using *N-tert*-butyl-α-phenylnitrone (PBN)[10] at room temperature. The sample was carefully prepared under argon atmosphere and was kept strictly anaerobic until the oxygen bubbling and the ESR measurement. The parameters a^N=1.56mT and a^H=0.36mT found at g=2.0070 of the observed spectrum are essentially identical to these found for the ·CH(OH)CH_3 adduct of PBN.[11] This adduct is presumably formed by the reaction of hydroxyl radical with ethanol in the medium, as shown for ethanol in reaction (A).

$$\cdot OH + CH_3CH_2OH \rightarrow \cdot CH(OH)CH_3 + H_2O \qquad (A)$$

In turn, the carbon centered radicals generated from ethanol and hydroxyl radical, as trapped by 5,5-dimethyl-1-pyrrolinee *N*-oxide (DMPO), showed a six-line pattern with a^N=2.25mT, a^H=1.58mT identical to that reported by Johnson.[12] In aqueous solution, no additional species such as a carbon-centered radical due to the attack of hydroxyl radical was observed. However, the aqueous

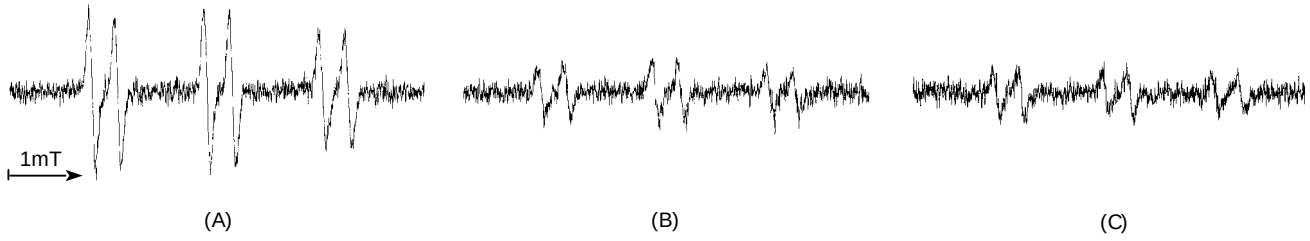


Figure 3. ESR spin trapping of the ligand (**1**)-Fe(II)-O_2 complex system. (A) 1.0 mM **1** and $FeBF_4$•$6H_2O$. (B) 0.2 mM **1** and $FeBF_4$•$6H_2O$. (C) 1.0 mM $FeBF_4$•$6H_2O$.

solution of the (**1**)-Fe(II)-O_2 system that does not contain ethanol showed no signal detectable either with PBN or with DMPO. It was suggested that the chelate complex was stabilized with ethanol in the medium and that hydroxyl radical generated with (**1**)-Fe(II)-O_2 system was trapped with ethanol stabilizing the iron complex. In addition, these signal intensity were concentration-dependent (Figure 3).

The DNA-cleaving ability of the (**1**)-Fe(II) complex was studied by the relaxation of the supercoiled pUC19 DNA to the circular DNA and the linear DNA (Figure 4). Control experiments using $FeSO_4$ and H_2O_2 omitting the ligand (**1**) (lane 1-5) showed DNA cleavage efficiency proportional to the concentration of $FeSO_4$. The cleavage efficiency was clearly enhanced in the case of (**1**)-$FeSO_4$-H_2O_2 systems (lane 6-10).

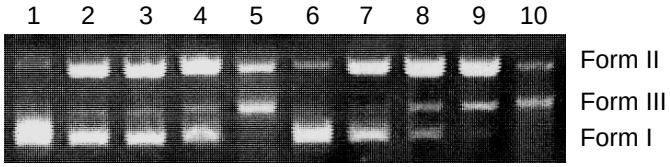


Figure 4. Cleavage of supercoiled pUC19 DNA by the Fe(II) complex in a buffer containing 20 mM Tris borate buffer in the presence of H_2O_2 at 37 ° C. Lane 1-5 : sc pUC19 + 1, 10, 50, 100 and 500 μM $Fe^{II}SO_4$ + 3.3 mM H_2O_2 ; Lane 6-10 : sc pUC19 + 1, 10, 50, 100 and 500 μM HPH-M and $Fe^{II}SO_4$ + 3.3 mM H_2O_2.

Thus, we demonstrated that the new artificial ligand (**1**) having amino- and amido- linked histidines was able to activate molecular oxygen via Fe(II) complex formation as efficiently as the HPH-Fe(II) complex and that the (**1**)-Fe(II)-H_2O_2 may be useful as a warhead of a DNA-cleaving agent. The effort is continuing to assemble a novel DNA-cleaving system based on **1** and specific DNA-recognizing molecules.

EXPERIMENTAL

Melting point were determined on a Yanagimoto Melting Point Apparatus and were uncorrected. IR spectra were recorded on a JASCO IR A-100 Spectrometer. Mass spectra were run on a JEOL JMS-DX303HF Mass Spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-AL300. ESR spectra were recorded with a JEOL TE200 Spectrometer.

2-(Dimethoxymethyl)-4-(dimethylamino)-6-(methoxycarbonyl)pyridine (4)

To a solution of **3** (300mg, 1.44mmol) in MeOH (25mL) was added trimethyl orthoformate (0.8mL, 7.28mmol) and *p*-toluenesulfonic acid monohydrate (547mg, 2.88mmol). The mixture was refluxed for

48 h under argon atmosphere, then concentrated in vacuo. A solution of H_2O-saturated aqueous $NaHCO_3$ (1:1, 25mL) was added and extracted with AcOEt for 3 times. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on silica gel (eluted with n-hexane:AcOEt = 1:2) to afford colorless solid **4** (366mg, 100%); mp 80-88 °C; ^{1}H NMR ($CDCl_3$) δ 3.08 (s, 6H), 3.44 (s, 6H), 3.97 (s, 3H), 5.34 (s, 1H), 6.91 (d, *J*=2.8Hz, 1H), 7.37 (d, *J*=2.6Hz); ^{13}C NMR ($CDCl_3$) δ 39.2, 52.7, 54.3, 105.0, 105.5, 108.3, 147.7, 155.4, 157.9, 166.7; IR (Nujol) 2927, 1709, 1605, 1460, 1377, 1279, 1191, 1104, 976, 789cm^{-1}; MS (EI) *m/z* 254 (M^++H); HRMS (FAB). Calcd for $C_{12}H_{19}N_2O_4$: 255.1345. Found: 255.1388.; Anal. Calcd for $C_{12}H_{18}N_2O_4$: C, 56.68; H, 7.13; N, 11.02. Found C, 56.63; H, 7.24; N, 11.02.

6-(Dimethoxymethyl)-4-(dimethylamino)-2-[[[(*S*)-2-(1*H*-imidazol-4-yl)-1-(methoxycarbonyl)ethyl]-amino]carbonyl]pyridine (5)

To a solution of **4** (762mg, 3.00mmol) in MeOH (12mL) was added dropwise 1*N* aqueous NaOH-MeOH (1:1, 12mL) at 0°C. The mixture was stirred for 10 min at 0°C then for 15 min at rt. Water (15mL) was added and then MeOH was evaporated in vacuo. The solution was neutralized with 10% aqueous citric acid, and the mixture was extracted with $CHCl_3$ for 8 times. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford colorless solid. The solid was dissolved in DMF and the solution was added L-histidine methyl ester dihydrochloride (870mg, 3.60mmol), diphenylphosphoryl azide (900mg, 3.30mmol) and 4-dimethylaminopyridine (450mg, 3.60mmol) at 0°C. The mixture was stirred at from 0°C to rt for 48 h under argon atmosphere, then concentrated in vacuo. The residue was dissolved in $CHCl_3$, and the solution was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on silica gel (eluted with CH_2Cl_2:MeOH = 10:1) to afford yellow oil **5** (537mg, 46%: 2steps from **4**); $[\alpha]^{20}_D$-13.5°(c=1.0, MeOH); ^{1}H NMR ($CDCl_3$) δ 3.05 (s, 6H), 3.24 (d, *J*=5.7Hz, 2H), 3.42 (d, *J*=5.9Hz, 6H), 3.73 (s, 3H), 4.98 (dt, *J*=8.1, 5.7Hz, 1H), 5.23 (s, 1H), 6.83 (s, 1H), 6.84 (d, *J*=2.8Hz, 1H), 7.36 (d, *J*=2.8Hz, 1H), 7.52 (d, *J*=0.9Hz, 1H), 8.81 (d, *J*=8.1Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 29.6, 39.3, 52.3, 52.5, 53.9, 54.2, 104.6, 105.1, 105.4, 120.3, 129.6, 135.2, 149.0, 155.8, 156.4, 165.3, 171.9; IR (Nujol) 2924, 2854, 2360, 1748, 1670, 1607, 1459, 1377, 1061cm^{-1}; MS (FAB) *m/z* 392 $(M+H)^+$; HRMS (FAB). Calcd for $C_{18}H_{26}N_5O_5$: 392.1934. Found: 392.1993.

Methyl (*S*)-2-[(4-Dimethylamino-6-formylpyridine-2-carbonyl)amino]-3-(1*H*-imidazol-4-yl)propionate (6)

A solution of **5** (274mg, 0.70mmol) in saturated aqueous tartaric acid (15mL) was stirred at 50°C, then alkalified to pH 8 with aqueous $NaHCO_3$ and extracted with $CHCl_3$ for 5 times. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give crude **6**, which was used without further purification (R_f 0.30 [MERCK silica gel 60 F_{254} developed with CH_2Cl_2:MeOH =10:1])) (124mg, 52%); ^{1}H NMR

($CDCl_3$) δ 3.09 (s, 6H), 3.27 (d, *J*=5.5Hz, 2H), 3.74 (s, 3H), 5.02 (dt, *J*=7.5, 5.5Hz, 1H), 6.86 (s, 1H), 7.19 (d, *J*=2.6Hz, 1H), 7.54 (d, *J*=2.6Hz, 1H), 7.55(s, 1H), 9.10 (d, 7.5Hz, 1H), 9.95 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 29.7, 39.5, 52.5. 54.0, 105.4, 108.4, 120.2, 129.7, 135.2, 150.1, 151.9, 155.8, 164.5, 171.9, 194.0; MS (FAB) *m/z* 346 $(M+H)^+$.

Methyl (*S*)-3-(1*H*-imidazol-4-yl)-2-[[[6-[[[(*S*)-2-(1*H*-imidazol-4-yl)-1-(methoxycarbonyl)ethyl]-amino]carbonyl]-4-(dimethylamino)pyridine-2-yl]methyl]amino]propionate (1)

To a solution of **6** (86mg, 0.25mmol) and L-histidine methyl ester dihydrochloride (77mg, 0.32mmol) in MeCN (30mL) was added molecular sieves 3A (2.5g) and Et_3N (0.91mL, 0.62mmol). The mixture was stirred at rt for 22 h under argon atmosphere, filtrated on celite, and concentrated in vacuo to give a crude product. The residue was dissolved in MeOH. The solution was added palladium-activated carbon (Pd 10%) (163mg) and stirred at rt for 5 h under hydrogen atmosphere. The mixture was filtrated on celite, and concentrated in vacuo. To the residue was

added saturated aqueous $NaHCO_3$, then the mixture was extracted with $CHCl_3$ for 3 times. The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on NH silica gel (eluted with CH_2Cl_2:MeOH = 20:1) to afford yellow oil **1**(67mg, 54%); $[\alpha]^{20}_D$-4.7°(c=1.0, MeOH); ^{1}H NMR ($CDCl_3$) δ 2.76-3.10 (m, 2H), 2.99 (s, 6H), 3.19 (dd, *J*=14.9, 5.1Hz, 1H), 3.29 (dd, *J*=14.9, 5.1Hz, 1H), 3.64-3.86 (m, 3H), 3.70 (s, 3H), 3.75 (s, 3H), 4.95 (dt, *J*=7.9, 5.1Hz, 1H), 6.40 (d, *J*=2.4Hz, 1H), 6.71 (s, 1H), 6.85 (s, 1H), 7.29 (d, *J*=2.4Hz, 1H), 7.36 (s, 1H), 7.41 (s, 1H), 8.90 (d, *J*=7.9Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 29.6, 30.2, 39.3, 52.0, 52.5, 53.1, 60.6, 61.0, 104.1, 106.8, 116.4, 117.7, 131.6, 133.0, 135.0, 135.1, 149.2, 155.6, 157.3, 165.2, 172.5, 174.5; IR (Film) 3123, 2952, 1737, 1666, 1608, 1523, 1437, 1392, 1201, 1021, 833cm^{-1}; MS (FAB) *m/z* 499 $(M+H)^+$; HRMS (FAB). Calcd for $C_{23}H_{31}N_8O_5$: 499.2417. Found: 499.2473.

ESR spectra

Solution of the Cu(II) complexes were prepared by mixing ligand and copper(II) perchlorate hexahydrate in methanol. Concentration were 1×10^{-3} at a molar ratio of Cu:ligand = 1:1. The pH was adjusted to 9.0 with 0.1*N* aqueous LiOH. ESR spectra were recorded with an X-band ESR spectrometer, JES-TE200, JEOL, Japan, at liquid nitrogen temperature (77K). Instrumental conditions for the ESR measurements were as fellows: Modulation frequency 100 kHz, modulation amplitude 0.63mT and microwave power 1.45 mW. The g-values of the complexes were calibrated using Mn(II) in MgO ($\Delta H_{3\text{-}4}$ = 8.69 mT). The α^2-value for Cu(II) complexes was calculated from ESR parameters using the equation of Kivelson and Neiman (α^2=*A*./*P*+(g-2.0023)+3/7($g_\perp$-2.0023)+0.04, *P*=360cm^{-1}

ESR spin trapping

To a mixture of a solution of *N*-*tert*-butyl-α-phenylnitrone in ethanol (250μL) and potassium phosphate buffer (50mM, pH 7.0, 500μL) were added a solution of ligand and iron(II) tetrafluoroborate hexahydrate

in ethanol (500μL) under fully deaerated condition. The whole thing was mixed well in aerobic condition and ESR spectra were measured at room temperature. Instrumental conditions for the ESR measurements were as fellows: Modulation frequency 100kHz, modulation amplitude 0.32×10^{-1}mT and microwave power 1.00mW.

DNA cleavage assay

A solution of purified pUC19 DNA (2μL of a stock solution containing ca. 260μg mL^{-1}) was incubated at 37°C for 60 min with ligand and $FeSO_4 \bullet H_2O$ in methanol solution (4μL of each concentration of stock solution), Tris borate buffer, pH 8.1 (4μL of a 20mM stock solution), H_2O_2 (2μL of 20mM stock solution) (complemented to give a total volume of 12μL). The mixture was quenched with EDTA and loading buffer (TaKaRa) and the DNA resolved by electrophoresis (Mupid-21)(100V, 1 h) on a 1.0% agarose gel (containing ethidium bromide) in Tris/Boronic acid/EDTA buffer. The bands detected by UV were analyzed and process using densitometer (AE-6900M, ATTO).

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