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Electrochemical and esr spin trap studies of a new iron tetra- catecholamide complex.

N. Cheraïti, [1] M. E. Brik, [2] * B. Keita, [3] L. Nadjo, [3] A. Gaudemer [1]

[1] Laboratoire de Chimie de Coordination Bioorganique et Bioinorganique, ICMO URA, CNRS 1384 Bât 420, Université de Paris-Sud, 91405 Orsay, France. [2] Laboratoire de Chimie Structurale Organique, ICMO URA, CNRS 1384 Bât 410, Université de Paris-Sud, 91405 Orsay, France. [3] Laboratoire d'Electrochimie, Bât 420, Université de Paris-Sud, 91405 Orsay, France

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Summary: A new siderophore, *N*5,*N*6-thiodipropanoyl-bis[*N*1,*N*10-bis(2,3-dihydroxy benzoyl-spermidine)]-Fe (III) complex or H_2LFe has been synthesised. The reaction of the reduced form of this complex with dioxygen has been investigated through electrochemical study and revealed the formation of a new species assumed to be H_2O_2. This species has been confirmed by esr spectroscopy using the diamagnetic compound 5-deutero-2,2,5-trimethylpyrrolidine-1-hydroxyl as spin trap. The resulting persistent radical is 5-deutero-2,2,5-trimethylpyrrolidine-1-yloxy (a_N=16.58 G, a_D= 3.49 G).

Under conditions of ferric stress, many organisms and microbes synthesise potent iron-chelation compounds that are secreted from cell to sequester the available iron for uptake back into the organism where it is provided for essential metabolic process. Enterobactin [1] isolated from cultivated enterobacteria in poor iron medium of *Escherichia coli* and *Salmonella typhimurium* [2] or *Aerobacter aerogenes* [3] is a siderophore belonging to the class of catecholate compounds. This molecule has been extensively studied and many models have been prepared to mimic its metabolic process in bactéria. [4-7] On the other hand, it has been demonstrated that ferrous Fe (II) and ferric Fe (III) iron can act catalytically to generate hydroxyl radicals that are the most potent oxidizing agents known and the source radical in tissues has been assumed to be the Fenton reaction involving a ferrous ion and hydrogen peroxide. [8-10] The later seems to be the by-product of aerobic metabolism and has been shown to be implicated in cell death, aging and a variety of pathology. These problems are mainly due to the attack of °OH upon DNA, producing a multiplicity of different products. [11]

* Correspondence and e-mail: **habbrick@icmo.u-psud.fr**

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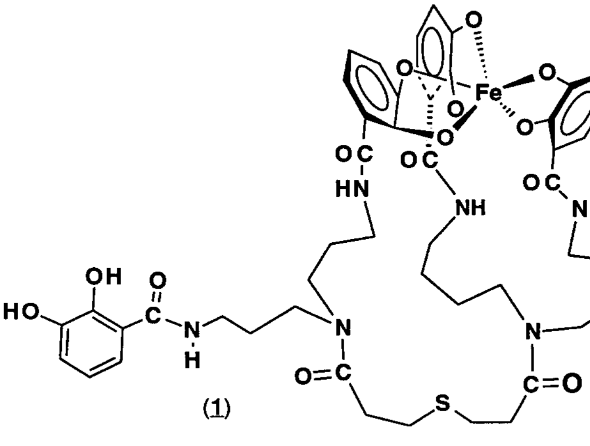
In this paper we report an interesting observation happened during our investigation on the electrochemical study of the new tetra-catecholate ligand *N*5,*N*6-thiodipropanoyl-bis[*N*1,*N*10-bis (2,3-dihydroxybenzoyl-spermidine)] (1) (**Schéma 1**). [12] In fact, we have observed that the interaction of the reduced form of the complex with dioxygen generates a new species in the medium. It is characterised as H_2O_2, and esr spin trapping technique seems to be the most direct method to measure the hydroxyl radical (°OH).



(1)

Scheme 1: Molecular structure of siderophore H_2LFe (1)

Cyclic voltammograms were run in DMSO containing 0.1 NBu_4PF_6 as the supporting electrolyte. The solution of (1) was thoroughly degassed with argon and its voltammogram recorded (Figure 1a). It exhibits a chemically quasi reversible process with E^0 =-411 mV *vs.* SCE and the anodic to cathodic peak potential difference ΔE_p = 485 mV. After electrochemical transformation of all Fe (III) into Fe (II), a new value E^0 = -220 mV *vs.* SCE is obtained (Figure 1b). The solution was then bubbled with dioxygen. Before recording the voltammogram the solution was thoroughly degassed in other to eliminate any traces of unreacted oxygen. The new cyclic voltammogram recorded is different from the initial one, and shows a new redox couple of Fe(III)/Fe(II) E^0 = -587 mV *vs.* SCE, the anodic to cathodic potential ΔE_p =135 mV at scan rate 200mV/s (Figure 2). At the same time a new irreversible oxidation wave with Epa= 501 mV is observed and corresponds to a formation of a free H_2O_2.

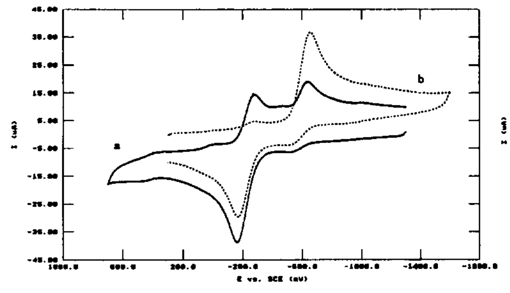


Figure 1: (**a**) Cyclic voltammograms of the initial form 7.75 10^{-4} M of $H_2Fe(III)L$ (1) in DMSO and 0.1 nBu_4NPF_6 under argon. Working electrode was freshly polished-glassy carbon electrode. reference electrode was SCE, scan rate 200mV/s. (**b**) voltammogram obtained after nearly exhaustive reduction of Fe(III) to Fe(II) species.

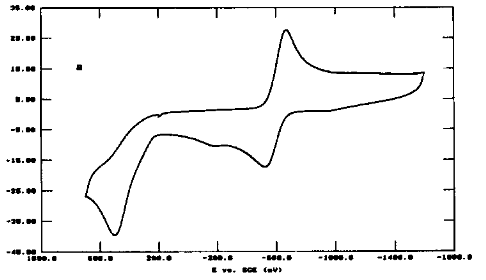
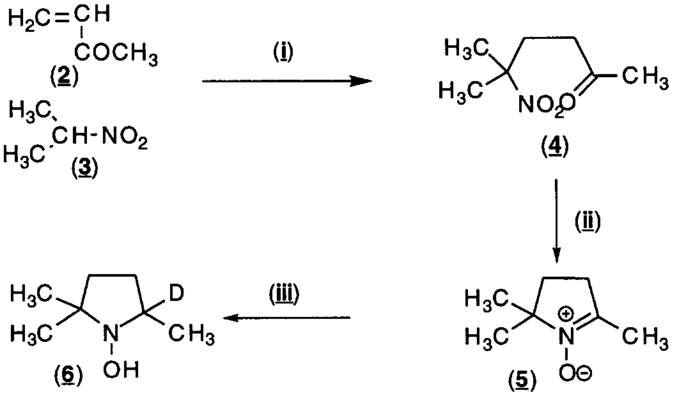


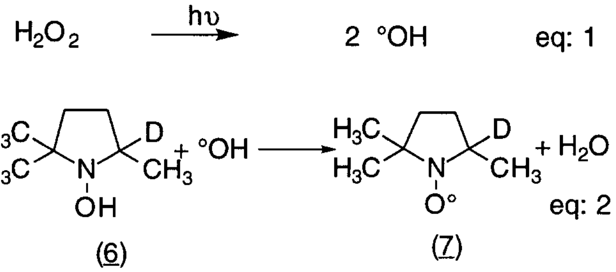
Figure 2 Cyclic voltammogram obtained after bubbling dioxygen and degassing thoroughly with argon. The electrodes are the same as in previous figure 1.

In order to verify the hypothesis of the presence of H_2O_2, we have undertaken epr spectroscopy study using a new spin trap (6) in replacement of the classic DMPO. The spin trap 5-deutero-2,2,5-trimethylpyrrolidine-1-hydroxyl (6) was prepared according to **scheme 2**. [13] The choice of deuterium rather than hydrogen as reported elsewhere, [14] is dictated by the fact that the deuterium-carbon bond is stronger than the hydrogen-carbon bond; consequently, radical (7) (**scheme 3**) is more persistent.



Scheme: 2 i, CH_3OH, CH_3ONa; ii, Zn, NH_4Cl iii, $LiAlD_4$

1 ml of the new electrochemical solution which was assumed to contain H_2O_2 was added to a solution of spin trap (**6**) in DMSO. The mixture was then introduced in a tube of quartz and epr spectra was recorded under UV irradiation (**scheme 3**). If H_2O_2 is present in the solution, the hydroxyl radical (°OH) formed in the medium (eq 1, **scheme 3**) abstracts hydrogen from the hydroxylamine 6 (eq 2, scheme 3). This one-electron oxidation of hydroxylamine leads to the formation of a persistent nitroxide (7) with a nitroxide nitrogen coupling (**a**$_N$= 16.58 G) split by one deuterium (**a**$_D$= 3.49 G) giving the 9 lines spectra (**Figure 3**).



Scheme 3

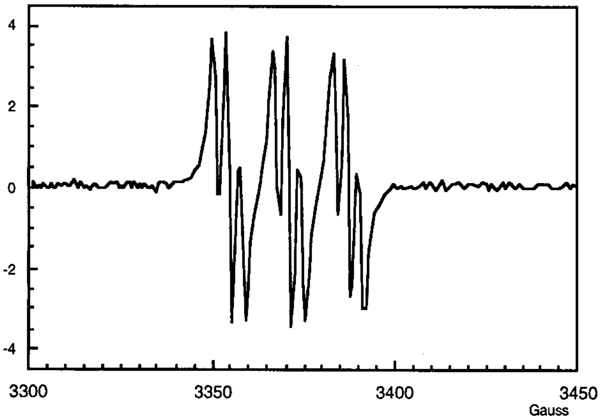


Figure 3: Epr spectra of 1-*D*, 2,5,5-Trimethyl, pyrroline, *N*-oxide (7) obtained after irradiation of the oxygenated reduced form of (1) in the presence of 5-*D*, 2,2,5-Trimethyl, pyrroline, *N*-hydroxy (6) a_N = 16.58G, a_D =3.49 G.

In relation to this, we have recently reported the synthesis of new tris-catecholamide siderophores.[5, 15] The electrochemical studies of their reduced forms bubbled with dioxygen, have been investigated at the same conditions as compound 1, but no formation of H_2O_2 has been detected. It seems that the presence of the fourth catechol which do not take part in the coordination of iron is perhaps responsible for the formation of H_2O_2. The mechanism by which this species was generated is under investigation.

Materials and Methods: Electrochemical experiments were done in DMSO under anaerobic conditions. The solutions were degassed thoroughly for at least 30 mn with pure argon and kept at a positive pressure of the gas during the experiments. n-Bu_4NPF_6(Aldrich) was dried at 80 °C under vacuum for 3 days. The working electrode was a 3-mm diameter vitreous carbon disk (UC, Tukai Japan) for cyclic voltammetry or a large surface area glassy carbon plate (V25, le carbon Loraine) for controlled potential electrolysis and coulometry. The reference electrode (SCE) was kept in a compartment containing an appropriate supporting electrolyte and was separated from the working electrode compartment by a fine porosity glass frit. The counter electrode was a platinum wire of large surface area. All electrode potentials were measured vs. (SCE). The electrochemical set-up was an EG $ 273 A driven by a PC with the 270 software. The esr spectrometer was a Bruker ER200E instrument equipped with a TAM_{100} cavity, a frequence meter and a Gauss meter. Resonance spectra were measured with the use of a flat cell. Photolytic generation of hydroxyl radicals from H_2O_2 was performed with a mercury vapour lamp with a high pressure (OSRAM 200 W). The spectrometer was operated at 100 KHz field modulation, 0.8 modulation amplitude and microwave power levels up to 200mW. Fine structure was obtained at 1G and 10 mw. Spectra were recorded with a gain setting of 10^6, a time constant of 2s and scanned at a rate of 1G/mn.

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13 2,5,5-trimethylpyrroline (4): Freshly distilled crotonaldehyde (2) 20 g (255 mmoles) in anhydrous methanol (100 ml) was added dropwise to a solution of 25 g 2-nitropropane (285 mmoles) (3) in anhydrous methanol (50 ml) containing sodium methoxide (1g of sodium), the temperature being kept at 60°C. After stirred for 3 hours, the solution was left over night. Then, acetic acid (3 ml) was added, the methanol evaporated and the residue poured into water. The product was extracted with ether. The extract was washed with sodium hydrogen carbonate solution, dried on (Na_2SO_4), evaporated and the residue distilled, yielding 30 g (66%). δ_H ($CDCl_3$): 1.36 (s, 6H, $2CH_3$); 1.95 (s, t, 5H, CH_2, CH_3); 2.26 (t, 2H, CH_2); MS: *m/z* = 159 (M, 100).

2,5,5-Trimethyl, pyrroline, *N*-oxide (5). A mixture of 5-methyl-5-nitrohexane-2-one (4) 25 g (157 mmoles g), ammonium chloride 10 g (188 mmoles) and water (250 ml) was heated to 65°C with stirring. 21 g of zinc dust (0.321 mmoles) was added in portions over 30 min keeping the temperature at 65°C. After stirring for 2 h at the same temperature, the mixture was allowed to cool, filtered, and acidified with acetic acid. The layer was then basified with sodium hydroxide (50%) and continuously extracted with chloroform for many times. The chloroform extracts were evaporated and the product distilled to give N-oxide 10.39 (52%). δ_H ($CDCl_3$): 1.24 (s, 6H, $2CH_3$); 1.86 (s and t, 5H, CH_2, CH_3); 2.45 (t, 2H, CH_2). δ_C ($CDCl_3$): 19.60 (s, 1C, $\underline{C}H_3$); 25.88 (s, 1C, $\underline{C}H_2$); 27.62 (s, 1C, $\underline{C}H_2$); 34 (s, 2C, $2\underline{C}H_3$), 58.67 (s, 1C, C-quater); 66.50 (s, 1C, C-tert), MS: *m/z* 127 (M^+, 33); 112 ($(M-15)^+$, 20).

5-*D*, 2,2,5-Trimethyl, pyrroline, *N*-oxide (6). To a solution of 2,2,5-trimethylpyrroline-*N*-oxide (5) 2 g (117 mmoles) in 20 ml of ether at 0°C was added a solution O.5 equivalent of $LiAlD_4$ 0.556 g (15.4 mmoles). The solution was stirred for 30 mn and then hydrolysed at 0 °C. After separation, the aqueous phase was extracted with ether and dried on $MgSO_4$. The ether was evaporated and the product conserved in freezer (1.9 g), yield 90 %.

δ_H ($CDCl_3$): 1.04 (s, 6H, $2CH_3$); 1.21 (s, 3H, CH_3); 1.28 (t, 2H, CH_2); 1.86 (t, 2H, CH_2); 6.27 (s, 1H, OH). δ_C ($CDCl_3$): 12.86 (s, 1C, $\underline{C}H_3$); 24.92 (s, 2C, $\underline{C}H_3$); 28.95 (s, 1C, $\underline{C}H_2$); 31.75 (s, 1C, $\underline{C}H_2$); 72.89 (s, 1C, C-quater), MS: *m/z* 130 (M, 100); 115 ((M-15), 20).

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