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Synthesis and electrochemical study of a new chiral tris-catecholamide analogue of enterobactin.

N. Cheraïti,[1] M. E. Brik,[2*] A. Gaudemer, [1] G. Kunesh [1]

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

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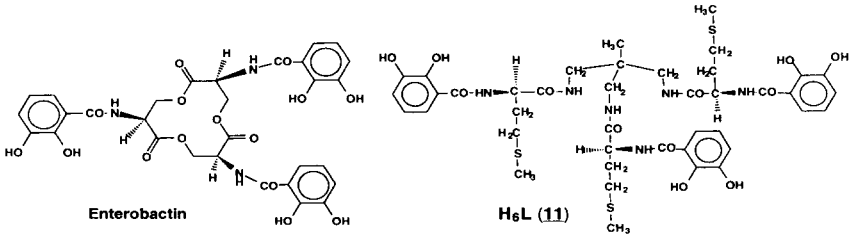
Summary: The comparison of siderophore complex redox potentials with those of physiological reductants may aid in the clarification of the mechanism of iron metabolism. In this paper, a new chiral tris-catecholamide compound *N,N',N''*-tris-(2,3-dihydroxybenzoyl)-1,1,1-tris-(L-methioninemehyl)-ethane or H_6L (**11**) has been synthesised in nine steps, and may mimic the release of iron from enterobactin to the agents which are directly involved in cell metabolism. The choice of methionine as a constituent of the siderophore incorporates divalent sulphur which leads to the increase of the reduction potential of the siderophore, and consequently facilitates the iron release [Fe(III)/Fe(II) redox potential $E_{1/2}$=-0.749 V *vs* (SCE)].

Siderophores[1-2] are specific chelating agents for iron Fe(III). They are characterised by their low molecular weights and their ability to form stable complexes with this ion. These compounds are isolated from cultured bacteria. Their role is to solubilise, bind and assimilate extracellar iron, and to transport it into the cell or to inhibit the iron catalysed production of hydroxyl (OH°) via the Fenton reaction (Fe^{2+}/H_2O_2).[3] There are two big siderophore families in which the hydroxamate and catecholate are the major functional groups involved in iron complexation respectively . Due to this iron chelating specificity, they are extensively exploited in biology and medicine.[4-5] One of the well known siderophores extensively studied and used in clinical applications is the microbial natural enterobactin [6] which binds ferric ion to form a macro-bridged hexacoordinate trianion and takes part in the uptake iron in certain bacterial cells. This molecule was isolated for the first time in 1970 from cultivated enterobacteria *Escherichia coli* and *Salmonella typhimurium* or *Aerobacter aerogenes* [6-7] in an iron poor medium.



Scheme 1: Molecular structure of Enterobactin and siderophore H_6L (**11**).

* **habbrick@icmo.u-psud.fr**

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The enterobactin/Fe(III) complex possesses the highest known formation constant among the natural siderophore compounds (K_f= 10^{52}) [8] and during the last twenty years, it has been the subject of intense research. [9-10]

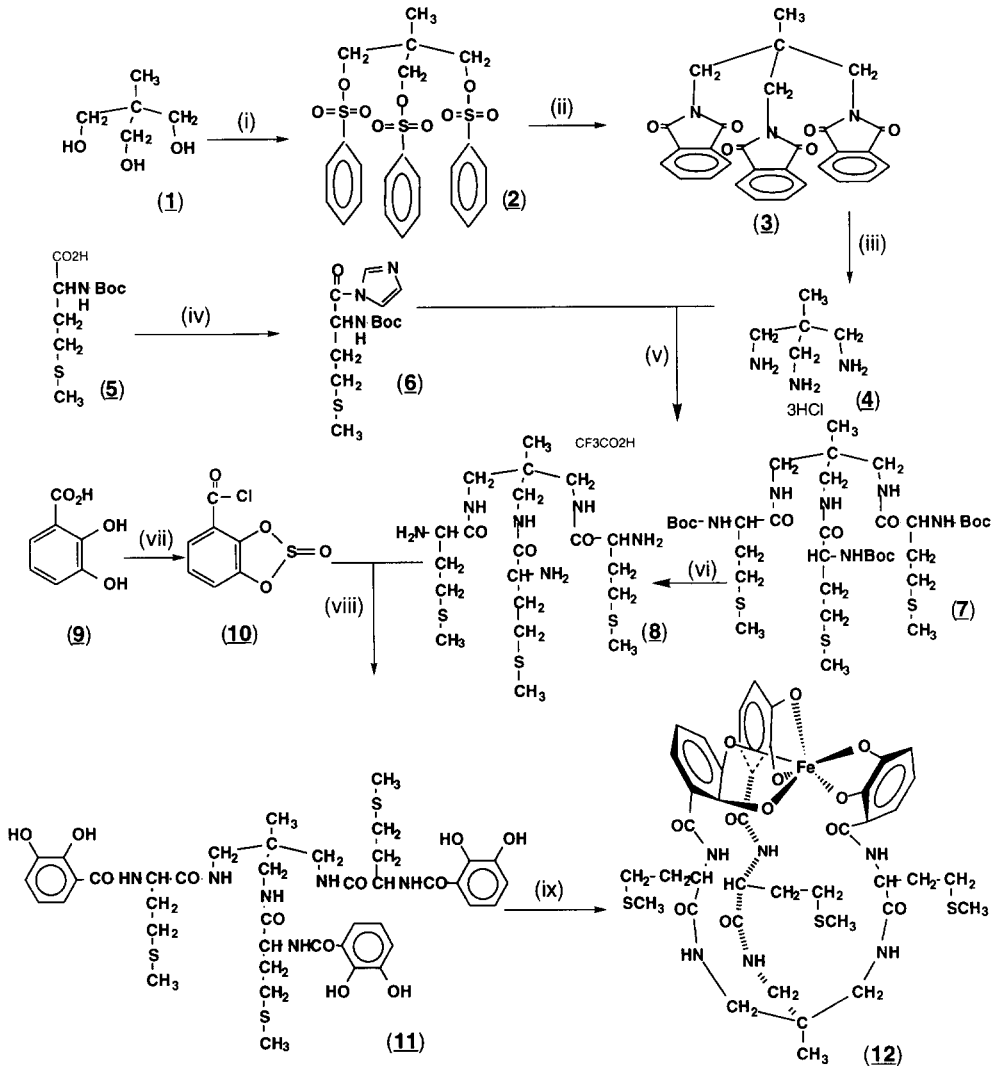
It is now established that the chirality,[11] the shape, [12] as well as the reduction of conformational space of the ligand to assure maximal binding efficacy,[13-14] are important factors involved in the binding process. Under conditions of iron stress, the siderophore is synthesized and the Fe(III)-siderophore complex interacts with a specific receptor in the outer cell membrane receptor protein-FepA which allows it to pass into the periplasmic space. The ferric siderophore is then bound to a periplasmic binding protein-FepB and hydrolysed by an esterase Fes [15] into the monomeric (2,3-dihydroxybenzoyl)serine units, which would be expected to tightly bind the ion . Finally, iron is released into the cytoplasm as Fe(II) after reduction by an NAD(P)H-dependent ferri-reductase.[16] However transfert of iron from enterobactin to the agents which are directly involved in cell metabolism necessitates its destruction by hydrolysis. Many models and mechanisms have been proposed for the release of iron from enterobactin and have been the subject of great controversy.[17-20] In fact, non-hydrolyzable analogues of enterobactin were synthesized and were still able to support growth of enterobactin strains under low iron conditions. To explain this intriguing behaviour, electrochemical studies have indicated that iron removal occurs via a reduction and protonation mechanism, which could occur in the acidic environment of the periplasmic space of *Escherichia coli* . [18, 20-21]

Based on this knowledge, we planned the synthesis of a new tris-(catecholate) siderophore analogue *N,N',N''*-tris-(2,3-dihydroxybenzoyl)-1,1,1-tris-(L-methioninemehyl)-ethane H_6L (**11**). This compound is characterised by its low molecular weight and possesses three catechol functions. This configuration gives the complex a filiform aspect which allows the transport of iron across cell membranes. However, the salient feature of this new ferric ion sequestering agent is the incorporation of a methionine group into the backbone. The sulphur atom of which is expected to enhance its reductive properties and consequently to facilitate the iron release. Therefore, the comparison of siderophore complex redox potentials with those of physiological reductants may aid in the clarification of the mechanism of iron metabolism.

The synthesis of ferric siderophore (**12**) was accomplished in nine steps. The triamine or 1,1,1-tris-(aminomethyl)-ethane (**4**), was prepared by converting 1,1,1-tris-(hydroxymethyl)-ethane (**1**) to 1,1,1-tris-(benzenesulfonyloxymethyl)-ethane (**2**) [22] and then into the 1,1,1-tris-(phtalimidomethyl)-ethane (**3**) by condensation with potassium phtalimide in freshly distilled dry dimethylformamide at 170 °C over 15 h.

The hydrolysis of (**3**) was carried out with aqueous KOH in a high-pressure autoclave (20 h at 180 °C) to give 70 % of 1,1,1-tris-(aminomethyl)-ethane (**4**). The solution was diluted, acidified to pH=3 and adsorbed directly onto a column containing strong-acid cation-exchange resin (H^+ form) [23] to give a white crystalline structure after evaporation of water. The fourth step is the preparation of the activated form (**6**) of Boc-L-methionine (**5**) using carbonyldiimidazole (CDI). [24] No attempt was made to isolate this intermediate which was directly treated with 3 equivalents of triethylamine and one

equivalent of triamine (4) in dichloromethane to give 1,1,1-tris-(Boc-L-methioninemehyl)-ethane (7) in 75 % yield after chromatography. Removal of the Boc groups from (7) using CF_3CO_2H followed by evaporation under reduced pressure gave the 1,1,1-tris-(L-methioninemehyl)-ethane (8) (100 %).



Scheme 2: Reagents and conditions: i, $PhSO_2Cl$, ii, Potassium phtalimide, 170 °C, DMF, iii, autoclave, KOH, 180 °C, iv, CDI, CH_2Cl_2, V, CH_2Cl_2 48 h, 25°C, vi, CF_3CO_2H, vii, $SOCl_2$, 54 °C, 3 h, viii, CH_2Cl_2, Et_3N, $NaHCO_3$, ix, $Fe(acac)_3$, NaOH, MeOH.

The seventh step involves initial treatment of dihydroxybenzoic acid (9) with thionyl chloride, [25] which serves in one step, to activate the acid as its acid chloride as well as to protect the 2,3-

dihydroxy groups of (**9**) in giving 2,3-dioxosulfinyl benzoyl chloride (**10**). Finally, the pure enterobactin analogue *N,N',N''*-tris-(2,3-dihydroxybenzoyl)-1,1,1-tris-(L-methioninemehyl)-ethane H_6L (**11**) was obtained by coupling triamine (**8**) and acid chloride (**10**). The crystalline compound (**11**) was then treated with Fe(acac)3 in methanol to give ferric complex (**12**) as an analogue of ferric enterobactin (**Scheme 2**).

These compounds [26] have been characterised by ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy.

The reduction of ferric siderophore (**12**) was examined by cyclic voltammetry, applying an electrolytic potential across a freshly polished glassy carbon disk and saturated calomel reference electrode (SCE). The complex (**12**) exhibited a quasi-reversible one-electron oxidation-reduction wave with $E_{1/2}$ value of -0.749 V, vs. SCE (**Figure 2a**). From the reversibility of the couple it may be inferred that not only the Fe (III) but the Fe(II) complex is fully formed in the pH range of 9-11

This value is near the range of the physiological reducing agents NAD(P)H but much higher than those reported at pH>10 for the natural siderophore enterobactin [27] and the synthetic siderophore TREMCAM [28] which show one-electron oxidation-reduction wave with $E_{1/2}$ values of -1.230 V and -1.1 V [29] respectively, vs. the saturated calomel electrode (SCE).

Below pH=9, the cyclic voltammogram of ferric siderophore exhibits an irreversible wave at $E_{1/2}$=-0.893 mV *vs.* SCE (**Figure 2b**).

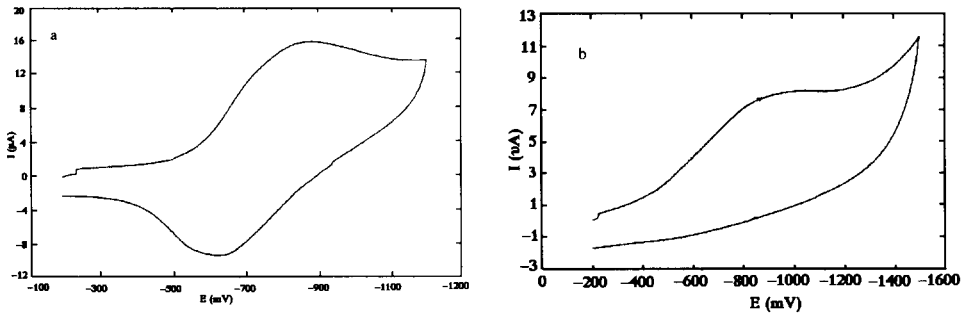


Figure 2: Cyclic voltammogram for 5 10^{-3} Fe (III) complex in H_2O and 0.1 M NaCl working electrode freshly polished glassy carbon electrode, reference electrode was SCE, Scan rate: 200 mV/s. (**a**) at pH=9.5, (**b**) at pH=6.5

The extremely low redox potential of ferric enterobactin suggested the synthesis of tris catecholate ligands containing reducing functionality. This leads to the increase of the siderophore redox potential, approaching the range of the physiological reducing agent. We have prepared another new catecholate siderophore structure incorporating divalent, sulphur which shows a considerable increase in the reduction potential. [30]

Materials and Methods: All reagents were of the finest quality available commercially. All solvents were distilled prior to use. Ethyl ether was distilled and stored over sodium. DMF was distilled and freshly used. The triethylamine was stored over potassium hydroxide. ^{1}H-NMR spectra were run on Bruker AM 250 and AC 200 instruments at 250 and 200 MHz, ^{13}C NMR were run on Bruker AC

200 instruments at 50 MHz. Chemical shifts are reported in parts permillion (δ) down field from internal Me_4Si. The abbreviations used are: s= singlet, d=doublet, t= triplet, q^{qt}= quadruplet. m= multiplet. J= coupling constant (Hz). IC-MS spectra determined on Nermag spectrometer using direct insertion probe with source pressure 10^{-1} torr and ammoniac as reactant gas. Therefore (M^+ +1) and (M^++ 18) values are reported. Mass spectra were recorded at 70 eV on Nermag spectrometer, electrospray were recorded on Finigan spectrometer MAT 95. Electrochemical experiments were done in water and under anaerobic conditions. The solutions were thoroughly degassed for at least 30 mn with pure argon, and kept at a positive pressure of the gas during the experiments. The working electrode was a freshly polished glassy carbon disk. The reference electrode (SCE) was kept in a compartiment containing the appropriate supporting electrolyte, and was separated from the working electrode compartiment by a fine porosity glass frit. The counter electrode is in platinum. The electrochemical set up was an E G $ G 273 A driven by a PC with the 270 software.

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26 1,1,1-Tris-(benzenesulfonyloxymethyl)-ethane (**2**) δ_H ($CDCl_3$) 0.90 (s, 3H), 3.90 (s, 6H), 7.56 (t, 6H), 7.68 (t, 3H), 7.81 (d, 6H); δ_C ($CDCl_3$) 15.94 (s, CH_3), 39.34 (s, C, quat), 69.69 (s, $(CH_2)_2$), 127.72 (s, C-Arom); 129.37 (s, C-Arom); 134.15 (s, C-Arom); 134.72 (s, C-Arom); IC-MS (NH_4^+) *m/z* 558 ($(M+18)^+$, 100).

1,1,1-Tris-(phtalimidomethyl)-ethane (**3**) δ_H ($CDCl_3$) 0.99 (s, 3H), 2.90 (d, 1/3 (2 CH_3)), 3.82 (s, 6H),7.74 (m, 6H), 7.86 (m, 3H); δ_C ($CDCl_3$) 19.23 (s, $\underline{C}H_3$), 29 (s, C-quat), 44.81 (s, $(\underline{C}H_2)_2$), 123.40 (s, C-Arom), 131.85 (s, C-Arom), 134.12 (s, C-Arom), 168.91 (s, C=0); IC-MS (NH_4^+) *m/z* 508 ($(M^+{+}1)^+$, 100); 525 ($(M+18)^+$, 35).

1,1,1-Tris-(aminomethyl)-ethane trichloride (**4**). δ_H (D_2O) 1.09 (s, 3H), 3.04 (s, 6H); δ_C (D_2O) ($CDCl_3$ as reference) 17.20 (s, $\underline{C}H_3$); 35.61 (s, -C-quat); 43.00 (s, -$\underline{C}H_2$); ESI-MS (positive): *m/z* 118 $(M+1)^+$

1,1,1-Tris-(Boc-L-methioninemehyl)-ethane (**7**). δ_H ($CDCl_3$) 0.81 (s, 3H); 1.43 (s, 12H); 1.89 (m, 6H); 2.08 (s, 9H); 2.56 (t, 6H); 2.97 (d, 6H); 4.21 (m, 3H, CH). 5.46 (d, 3H, NH); 7.59 (d, 3H, NH); δ_C ($CDCl_3$) 15.35 (s, S-$\underline{C}H_3$); 19.14 (s, $\underline{C}H_3$); 28.24 (s, -$(\underline{C}H_3)_3$); 30.21 (s, -S-$\underline{C}H_2$); 31.63 (s, S-CH_2-$\underline{C}H_2$); 41.39 (s, C-quat); 42.56 (s, -$\underline{C}H_2$-NH-); 54.25 (s, -$\underline{C}$H-); 80.06 (s, O-C-quater); 155.49 (s, -$\underline{C}$O-O); 172 (s, NH-$\underline{C}$O-CH-); IC-MS (NH_4^+): *m/z* (811 ((M+1)+, 37).

1,1,1-Tris-(L-methioninemehyl)-ethane (**8**) δ_H (D_2O), 0.65 (s, 3H, CH_3); 1.87 (s, 9H); 1.95 (m, 6H); 2.39 (t, 6H); 2.93 (d, 6H); 3.90 (t, 3H); δ_C 14.42 (s, S-$\underline{C}H_3$); 18.44 (s, $\underline{C}H_3$); 28.31 (s, $\underline{C}H_2$-S-); 30.98 (m, -$\underline{C}H_2$-CH_2); 40.92 (s, $\underline{C}H_2$-NH); 43.38 (s, C-quat); 51.76 (m, $\underline{C}$H); 168.84 (s, C=O); IC-MS (NH_4^+): *m/z* 811 (M+1).

2,3-dioxosulfinyl benzoyl chloride (**10**). δ_H ($CDCl_3$) 7.3 (t, H-Arom); 7.5 (d, H-Arom); 7.9 (d, H-Arom); MS-*m/z*: 219 (^{37}Cl) (M^+, 16); 218 (^{35}Cl) (M^+,46); 183 ($(M-Cl)^+$, 100); 135 ($(M-Cl+SO)^+$, 29).

N,N',N''-Tris-(2,3-dihydroxybenzoyl)-1,1,1-Tris-(L-methioninemehyl)-ethane (**11**) δ_H (DMSO-d_6) 0.72 (s, 3H); 2.01 (m, 6H); 2.07 (s, 9H), 2.84 (m, 6H); 2.90 (m, 6H); 4.58 (d, 3H), 6.65-6.71 (t, 3H, H-Arom), 7.37-7.40 (d, 3H, H-Arom), 8.25 (d, 3H, NH); 8.90 (d, 3H, NH), 9.37 (s, 3H, OH), 11.90 (s, 3H, 0H), δ_C ($CDCl_3$) 15.28 (s, S-$\underline{C}H_3$); 19.67 (s, $\underline{C}H_3$); 31.27 (s, $\underline{C}H_2$-S), 32.18 (s, $\underline{C}H_2$-CH_2-S); 43.02 (s, $\underline{C}H_2$-NH), 43.92 (s, C-quat); 54.79 (s, -$\underline{C}H_2$-NH-); 114.32 (s, C-Arom), 117.93 (s, C-Arom), 119.83 (s, C-Arom), 121.42 (s, C-Arom), 149.54 (s, C-Arom), 153.54 (s, C-Arom) 170.36 (s, -$\underline{C}$O); 174 (s, $\underline{C}$O), IC-MS (NH_4^+): *m/z* 919 ($(M+1)^+$, 17).

Na_3FeL (**12**) ESI-MS (negative) 991 *m/z* (M-2Na)

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29 The value reported for the redox potential of TREMCAM is $E_{1/2}$=-1.04 V vs. (NHE) ref 28 and for best comparison with our results, we have measured this value vs. (SCE).

30 Cheraïti, N.; Brik. M. E.; Bricard, L.; Keita, B.; Nadjo, L.; Gaudemer, A. *submitted.*