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SYNTHESIS AND ESR STUDY OF NEW DIHYDROXAMIC ACID SIDEROPHORES S AS SCAVENGERS OF HYDROXYL RADICALS

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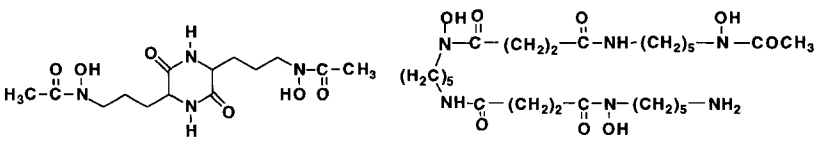
Abstract: Five new dihydroxamic acid ligands (**L**) (**8**, **10a**, **10b**, **10c** and **13**) have been synthesised and characterised as potential chelating agents for iron (Fe^{3+}). The log stability constants of Fe_2L_3 and FeL^+ from Fe^{3+} and L^{2-} have been estimated to be $\log\beta$=61.96 and $\log\beta 1$= 22.8 respectively. The ability of these compounds to scavenge hydroxyl radicals (°OH) responsible for cell damage have been studied by esr spectroscopy.

Due to their excellent chelating properties particularly for iron (Fe^{3+}), some natural siderephores belonging to di- and trihydroxamate families are extensively used for in vitro experiments to remove metal ions from the reaction system. For example, the iron chelator desferrioxamine (DFO) produced by *Streptomyces pilosus* is a powerful clinical drug used to treat some iron-overload patients. [1] It is also used to inhibit the iron-catalysed production of hydroxyl radicals (°OH) by superoxide generating systems [2-5] or in certain conditions implying the use of UV-irradiation [6-7] or via the Fenton reaction (Fe^{2+}/H_2O_2) systems respectively. In addition to these very interesting properties, it has been found recently, that D-aspartic acid β-hydroxamate and hydroxycarbamide two types of hydroxamic acid compounds, present an anti-HIV-1 activity in infected resting peripheral blood lymphocytes. [8]

In comparison with rhodotorulic Acid (RA) a dihydroxamic acid isolated from *Rhodotorula pilimanae* and produced in high yields by yeast, [9] new dihydroxamic acid compounds (**8**), (**10a**), (**10b**), (**10c**) and (**13**) have been prepared according to the **scheme 2**. Synthesis of each ligand involves four subsequent steps.



RA DFO

Scheme 1: Molecular structure of Rhodotorulic Acid RA and Desferrioxamine DFO

The reaction of diethyl malonate (**1**) with bromoalkane in ethanol led to the formation of diethyl 2-alkylmalonate (**2**) which was coupled to ethylenediamine to give 6-alkyl-1,4,8,11-tetraazaundecane (**5**)

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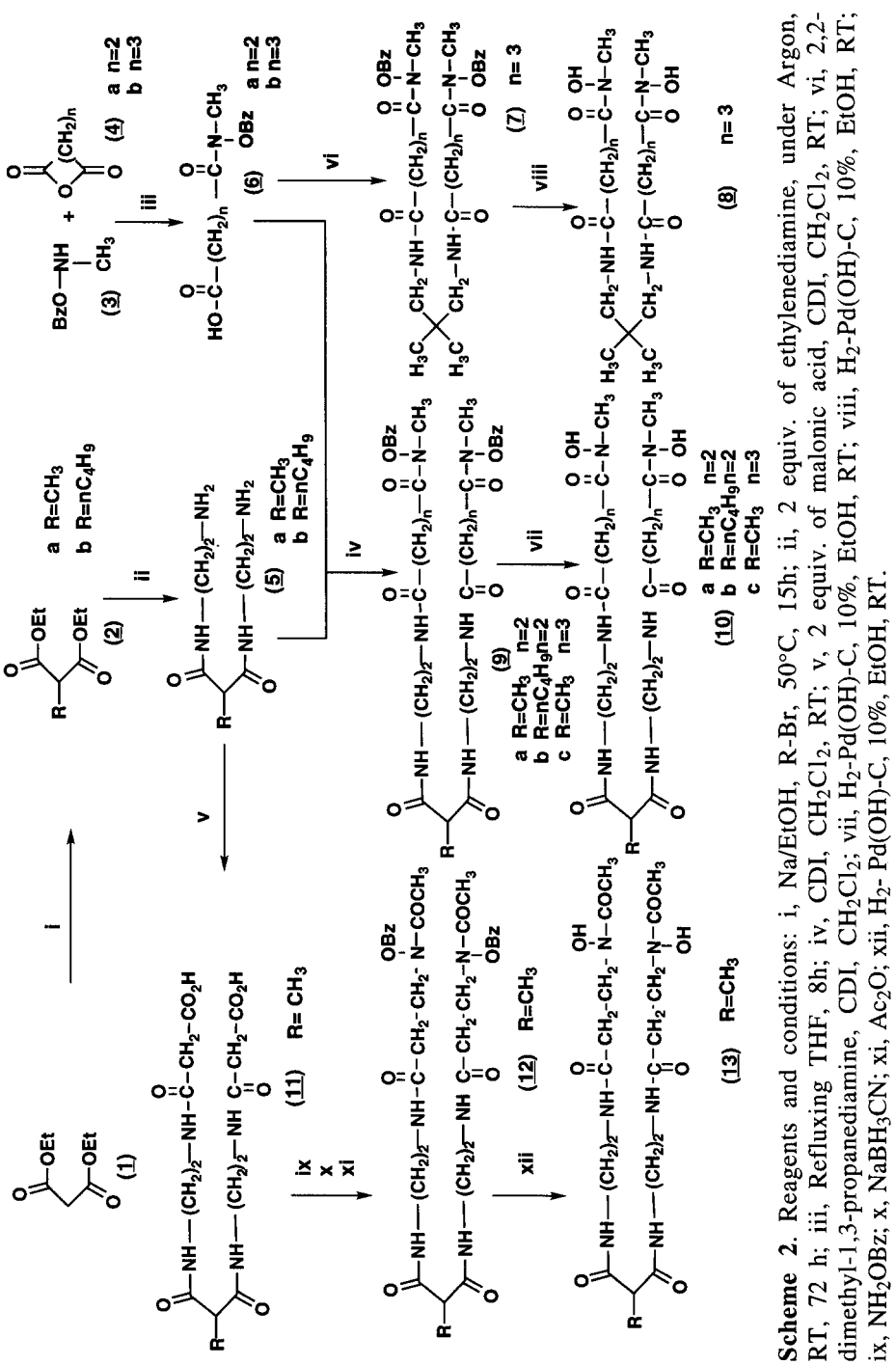


Scheme 2. Reagents and conditions: i, Na/EtOH, R-Br, 50°C, 15h; ii, 2 equiv. of ethylenediamine, under Argon, RT, 72 h; iii, Refluxing THF, 8h; iv, CDI, CH_2Cl_2, RT; v, 2 equiv. of malonic acid, CDI, CH_2Cl_2, RT; vi, 2,2-dimethyl-1,3-propanediamine, CDI, CH_2Cl_2; vii, H_2-Pd(OH)-C, 10%, EtOH, RT; viii, H_2-Pd(OH)-C, 10%, EtOH, RT; ix, NH_2OBz; x, $NaBH_3CN$; xi, Ac_2O; xii, H_2- Pd(OH)-C, 10%, EtOH, RT.

in yields ranging from 50 to 70%.[10] In the other side, compounds (**6a**) and (**6b**) were prepared by treatment of *N*-methyl-*O*-benzyl hydroxylamine (**3**) with succinic anhydride (**4a**) and glutaric anhydride (**4b**) respectively in refluxing THF.[11] The stoechiometric reaction of two equivalents of (**6**) with one equivalent of (**5**) gave by use of *N,N'*-carbonyldiimidazole (CDI)[12] the compound (**9**) which was in turn converted to dihydroxamic acid (**10**) by catalytic hydrogenation. The dihydroxamic acid (**8**) was prepared using the same method. However for the ligand (**13**) the procedure described elsewhere[13] has been used.

The molecular structures of these compounds [14] have been characterised by ^{1}H-NMR, ^{13}C-NMR and mass spectroscopies and their efficacy as radical scavenging antioxidant has been investigated by esr spectroscopy. The stability constants of the ferric complexes of (**L**) have been determined potentiometrically and spectrophotometrically at 25°C according to the method described by Raymond et *al.*[15] The predominant species at neutral pH is the dimmer Fe_2L_3, in which each iron is bound to three hydroxamate groups. At acidic pH this complex dissociates into the monomer, FeL^+, in which each iron is bound to two hydroxamates. The stability constants of β_1, β_2 and β are defined as follows.

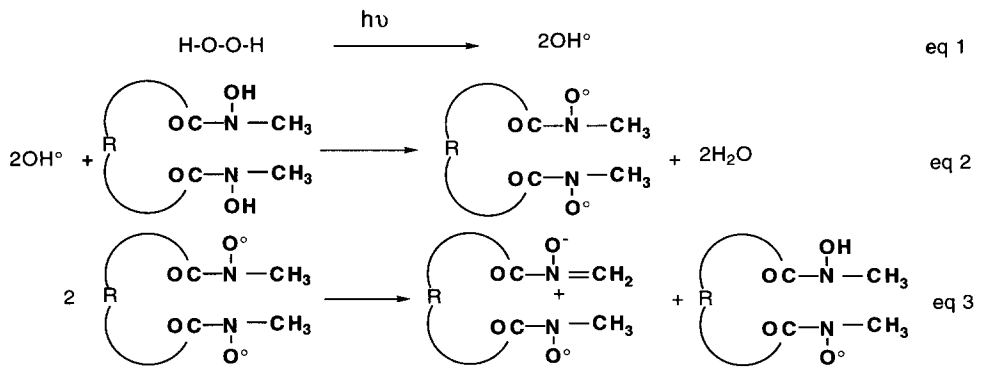
$$Fe^{3+} + L^{2-} \rightleftharpoons FeL^{+} \qquad \beta_1$$
$$2FeL^{+} + L^{2-} \rightleftharpoons Fe_2L_3 \qquad \beta_2$$
$$2Fe^{3+} + 3L^{2-} \rightleftharpoons Fe_2L_3 \qquad \beta$$

Their log values are estimated to be $\log\beta_1 = 22.8$ for FeL^+ and $\log\beta = 61.9$ for Fe_2L_3 in which $\beta = \beta_1^2\beta_2$. These results are comparable with those obtained and reported for rhodotorulic acid RA where $\log\beta_1 = 21.99$ and $\log\beta = 62.2$ for $FeRA^+$ and Fe_2RA_3 respectively. [16] In the other hand, it is established that the power of hydroxamic acid functions to react with the oxyradical derivatives gives these substances a protector role of biological tissues against oxidative effects. When H_2O_2 was exposed to UV irradiation in the presence of one of the five ligands (**8**), (**10a**), (**10b**), (**10c**) and (**13**), the hydroxyl radicals °OH formed in the medium (eq 1, scheme 3) abstract hydrogen from hydroxamic acid functions (eq 2, scheme 3). The one electron oxidation of each hydroxamic acid function of compound (**13**) leads to the formation of stable nitroxide free radical. An acetoxynitroxide nitrogen coupling (a_N=7.90 G) is spliting two protons (a_H= 6.30 G) from neighbouring CH_3 group giving the 9 lines spectra. In the cases of hydroxamic acids (**10a**), (**10b**), (**10c**) and (**8**), the oxidation leads to the formation of persistent nitroxides with a methylnitroxidenitrogen coupling (a_N= 7.60 G) is splitting three protons (a_H= 8.80 G) from neighbouring CH_3 group giving the 12 lines spectra (**Table 1**).

Hydroxamic acids	Nitroxides	Spectral lines	a_N (G)	a_H (G)	Ref
DFO	**N-O°**	9	7.85	6.35	6
RA	**N-O°**	9	7.60	6.60	16
(**13**)	**N-O°**	9	7.90	6.30	*
(**8**)	**N-O°**	12	7.60	8.80	*
(**10a**)	**N-O°**	12	7.72	8.60	*
(**10b**)	**N-O°**	12	7.70	8.81	*
(**10c**)	**N-O°**	12	7.73	8.81	*

Table1: Esr spectral characteristics of nitroxides generated by UV-irradiation of H_2O_2 in the presence of dihydroxamic acids (a_N and a_H are hyperfine splitting constants). * Present work.

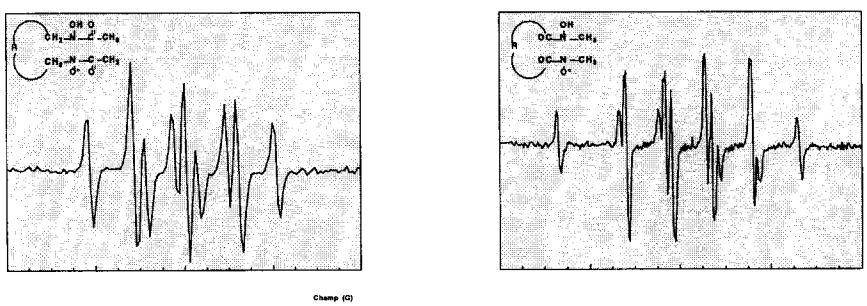
On the basis of the esr spectra recorded, only one function of hydroxamic acid group is oxidised to nitroxide and it is not possible to determine which hydroxamic acid of the molecule is under attack, due to similarities of the environment surrounding the nitroxide group as it has been mentioned for DFO. [17]



Scheme 3

However, dinitroxide generated from dihydroxamic acids could be formed in the medium but its epr spectra relative to the exchange interaction (**J**) was not observed due firstly, to the large distance between the two paramagnetic centres and their relative positions. Indeed, in epr spectroscopy, a possibility to maximise spectral changes observed with the stable nitroxide labels would be to use specific interaction between two nitroxides which are near to each other. When the nitroxide groups are close enough, electron exchange is fast on the esr scale ($J >> a_N$) and the signal of the odd electrons is split by both nitrogens. In contrast, when the nitroxides are far apart and ($J << a_N$), a spectrum indistinguishable from that of the mononitroxide is observed. The effective range for detection of changes in the spectrum for "Frozen" dinitroxide can be as large as 17 Å for dipolar broadening [18-19] but the range for direct electron exchange only extends to about 6 Å.[20]

Secondly, it has been reported that nitroxides without α hydrogens are the most persistent radicals and show no tendency for dimerization, but for those which having α hydrogens (DFO, RA, (**8**), (**10a**), (**10b**), (**10c**) and (**13**)) disproportionate rapidly giving nitrone and hydroxylamine (eq 3, scheme 3). [21]



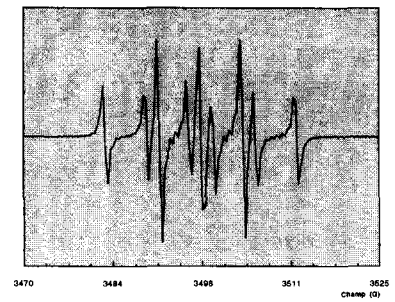


Figure 1: a) Epr experimental spectrum of nitroxide generated by photolysis of 30% H_2O_2 solution of dihydroxamic acid (**13**) (20 mM). Spectrometer Gain 2.5 10^4, Mod. amp. 0.8 G, Power b) computer simulation of the spectrum. The parameters used for this simulation are (a_N=7.90 G, $\mathbf{a_H}$= 6.30 G), line width = 0.6 G.

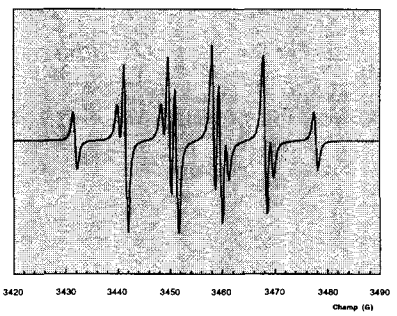


Figure 2: a) Epr experimental spectrum of nitroxide generated by photolysis of 30% H_2O_2 solution of dihydroxamic acid (**8**) (20 mM). Spectrometer Gain 2.5 10^4, Mod. amp. 0.8 G, Power b) computer simulation of the spectrum. The parameters used for this simulation are ($\mathbf{a_N}$=7.60 G, $\mathbf{a_H}$= 8.80 G), line width = 0.6 G.

In this paper, we have reported the synthesis of new dihydroxamic acid siderophores presenting excellent chelating properties of iron and exhibiting a great ability to scavenge radicals responsible for cells damage. More detailed investigations into the nature of the bacterial activity and calculations of complexation constants are in progress.

Materials and methods: All organic reagents were purchased from (Aldrich and Acros) and used without further purification. Desferrioxamine (DFO) and Rhodotorulic Acid (RA) were from Ciba-France. ^1H NMR and ^{13}C NMR spectra were unregistered on a bruker AM 250 spectrometer, the chemical shifts (δ) are relative to the solvent: D_2O and CD_3OD: 4.80 ppm; $CDCl_3$: 7.24 ppm. Mass Spectra (CI) were recorded on Ribermag R-10-10. High resolution mass spectra (FAB) were taken on a Kratos MS-80. Solutions of Hydroxamic acids were made up in distilled water and adjusted to the pH required immediately before use. Experiments were carried out at 25 ± 1°C.

The ESR spectrometer was a Bruker instrument ER200E equipped with a TM_{100} cavity. Frequencemeter and Gauss meter. Resonance spectra were measured with the use of a flat cell. Photolytic generating of hydroxyl radicals from H_2O_2 was performed with a mercury vapor 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 200 mW. Spectra were recorded with a gain setting of 10^6, a time constant of 2s. Computer simulation for determination of the coupling constants was performed on PC computer using Win epr program from NIEHS (USA).

Abbreviations: DFO, Desferrioxamine, RA, Rhodotorulic Acid, esr, electron spin resonance, HA, hydroxylamine.

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14 (**10a**) 1,1 bis((10-N-hydroxy)-2,5,10-triaza-1,6,9-trioxo undecanyl) ethane
^{1}H NMR ($CDCl_3$) : 1.22 (d, 3H, CH_3); 2.33 (t, 4H, CH_2-8); 2.67 (t, 4H, CH_2-7); 3.05 (s, 6H, CH_3N); 3.18 (m, 8H, CH_2-3-4); 3.10 (q, 1H, CH). ^{13}C NMR (D_2O): 14.91 (CH_3); 28.14 et 31.08 (CH_2-7-8); 36.70 (CH_3-N); 39.30 and 39.73 (CH_2-3-4); 48.6 (CH); 174.07 (CO); 174.83 (CO); 176.22 (CO). MS-FAB: M+H$^+$=461
(**10b**) 1,1 bis (10-N-hydroxy)-2,5,10-triaza-1,6,9-trioxo undecanyl) pentane
^{1}H NMR (CD_3OD): 0.76 (t, 3H, CH_3); 1.16 (m, 4H, CH_2CH_2); 1.65 (q, 2H, CH_2CH); 2.29 (t, 4H, CH_2-8); 2.67 (t, 4H, CH_2-7); 2.93 (t, 1H, CH); 3.03 (s, 6H, CH_3N); 3.16 (m, 8H, CH_2-3-4). ^{13}C-NMR (CD_3OD): 14.25 (CH_3); 23.47 (CH_2); 28.62 (CH_2-8); 30.75 and 31.38 (CH_2-7 and $2CH_2$); 36.31 (CH_2N); 39.82 and 40.22 (CH_2-3-4); 55.20 (CH); 172.93 (CO); 174.58 (CO); 175.44 (CO). MS-FAB: M+H$^+$=503
(**10c**) 1,1 bis ((11-N-hydroxy)-2,5,11-triaza-1,6,10-trioxo dodecanyl) ethane
^{1}H NMR (CD_3OD): 1.20 (d, 3H, CH_3); 1.74 (q, 4H, CH_2-8); 2.08 (t, 4H, CH_2-7); 2.34 ((t, 4H, CH_2-9); 3.10 (q, 1H, CH); 3.13 (s, 6H, CH_3N); 3.15 (m, 8H, CH_2-3-4).
^{13}C NMR (CD_3OD): 15.77 (CH_3); 22.11 (CH_2-8); 32.28 (CH_2-7-9); 36.37 (CH_3-N); 39.74 and 40.37 (CH_2-3-4); 49.27 (CH); 173.74 (CO); 175.28 (CO); 175.94 (CO).
MS.FAB: M+Na$^+$= 511, (M+H$^+$) = 489.
(**8**) 1-methyl-1,1 bis ((8-N-hydroxy)-2,8 diaza-3,7-dioxo nonanyl) ethane
^{1}H NMR (CD_3OD): 0.85 (s, 6H, CH_3); 1.95 (q, 4H, CH_2-5); 2.25 (t, 4H, CH_2-6); 2.47 (t, 4H, CH_2-4); 2.98 (d, 4H, CH_2-1); 3.16 (s, 6H, CH_3-9). ^{13}C NMR (CD_3OD): 22.32 (C5) 23.83 (2 CH_3). 32.34 (C9); 36.24 (C4); 37.53 (C6); 36.60 (C-β); 47.31 (C-1); 175.94 and 175.96 (C-7, C-3)
(**13**) 1,1-Bis ((9-N-hydroxy)-2,5,9 triaza-1,6,10-trioxo undecanyl)) ethane
^{1}H NMR (CD_3OD):1.21 (d, 3H, CH_3); 1.96 (s, 6H, $COCH_3$); 2.36 (t, 4H, CH_2-7); 3.10 (m, H_1); 3.17 (m, 8H, CH_2-4, CH_2-5); 3.74 (t, 4H, CH_2-8). ^{13}C NMR (D_2O): 15.03 ($CH_3\beta$; 20.20 (C-11); 34.17 (C-7); 39.43-39.74 (C-3, C4); 45.58 (C-8); 48.68 (C_α); 174.13-174.92 (C-1, C-6); 181.40 (C-10).
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