Free Rad. Res. Comms., Vol. 19, No. 6, pp. 409-423
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INHIBITION OF THE IRON-CATALYSED FORMATION OF HYDROXYL RADICALS BY NITROSOURACIL DERIVATIVES: PROTECTION OF MITOCHONDRIAL MEMBRANES AGAINST LIPID PEROXIDATION

ALAIN RABION[a], JEAN-BAPTISTE VERLHAC[a]*, LAURENT FRAISSE[b], BÉATRICE ROCHE[b] and JEAN-LOUIS SERIS[b]

[a]Laboratoire de Chimie Organique et Organométallique, URA 35 CNRS; Université Bordeaux I, 351 crs de la Libération, 33405 TALENCE CEDEX, France and [b]Groupement de Recherches de Lacq, BP34, 64170 Artix, France

(Received July 22nd 1993; in revised form September 8th, 1993)

A new series of metal ligands containing the 1,3-dimethyl-6-amino-5-nitrosouracil moiety has been synthesized and they have been studied as potential inhibitors of iron-dependent lipid peroxidation. For this purpose, these new derivatives have been tested in the Fenton induced deoxyribose degradation assay, which allows a quantitative measurement of their inhibitory effect towards hydroxyl radical generation. When iron(II) is complexed by these ligands, a strong inhibition of deoxyribose degradation is observed, especially in the case of tris-[2-(1,3-dimethyl-5-nitrosouracil-6-yl)aminoethyl] amine (**5**). This inhibitory effect is clearly related to a specific complexation of iron(II) and is not due to the direct scavenging of hydroxyl radical by the ligand. Inhibition of the iron mediated Fenton reaction presumably results from inactivation of the reactivity of the metal center towards hydrogen peroxide. These derivatives, as well as long alkyl chain substituted nitrosouracils were evaluated in the protection of biological membranes against lipid peroxidation (induced by iron(II)/ dihydroxyfumaric acid and determined with the 2-thiobarbituric acid test). Ligand **5** inhibited lipid peroxidation at a rate similar to Desferal (desferrioxamine B) and slightly higher than bathophenanthroline sulphonate (BPS), which are respectively good iron(III) and iron(II) chelators. When covalently bound with a long alkyl chain, the increase of lipophilic character of the ligand allows its location near the mitochondrial membrane, where lipid peroxidation occurs. Lower concentrations (IC_{50} = 4 μM) are then necessary to inhibit lipid peroxidation. This IC_{50} concentration should be compared to those obtained for Trolox (IC_{50} = 3 μM) or the 21-aminosteroid U74500A (IC_{50} = 1 μM) described previously.

KEY WORDS: Hydroxyl radicals, Fenton reaction, deoxyribose degradation, iron(II) chelators, lipid peroxidation, nitrosouracil derivatives.

ABBREVIATIONS: DETAPAC, diethylenetriaminepentaacetic acid; BPS, bathophenanthrolinesulphonate; DEO, deoxyribose; TBARS, thiobarbituric acid reactive subtance; XO, xanthine oxidase.

INTRODUCTION

The one-electron reduction of molecular oxygen to superoxide ($O_2^{\cdot -}$) has been demonstrated to occur during many biochemical reactions. It is generally accepted that this species is not sufficiently reactive in aqueous solution to account for the

*Author to whom correspondence should be addressed.

type of damage observed in $O_2^{\cdot-}$-generating systems. Many of these damaging effects have been attributed to the formation of the highly reactive hydroxyl radical ·OH, which may be generated during the so-called Haber-Weiss cycle (equations (2) and (3)) including Fenton reaction (3) and using principally iron as catalyst:

$$2\,O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

$$Fe^{3+}\text{-chelate} + O_2^{\cdot-} \rightleftarrows Fe^{2+}\text{-chelate} + O_2 \quad (2)$$

$$Fe^{2+}\text{-chelate} + H_2O_2 \rightarrow {}^{-}OH + \cdot OH + Fe^{3+}\text{-chelate} \quad (3)$$

$$\cdot OH + \text{target molecule} \rightarrow \text{damage} \quad (4)$$

This iron dependent formation of reactive oxygen species is clearly involved in lipid peroxidation process.[1,2,3] Nevertheless the role of ·OH in the initiation process has been discussed by some investigators.[4-8] Involvement of high valent iron/oxygen species (like ferryl or perferryl cations), in which iron plays also a determinant role, cannot be excluded.[6,9]

Catalase, by H_2O_2 consumption, and hydroxyl radical scavengers such as mannitol, show inhibitory activity in these models.[3] In addition, both iron(II) and iron(III) chelators can inhibit hydroxyl radical formation. For instance DETAPAC, BPS, and Desferal have been studied as potential inhibitors of the iron-catalysed Haber-Weiss cycle.[2,5] In contrast to radical scavengers, metal chelators are not consumed during the inhibition process and this may be of interest for the development of drugs in the pharmaceutical field.

It has been suggested that it is the oxygen radicals (or highly reactive iron species) generated near the membrane-bound iron that are responsible for iron-initiated lipid peroxidation.[10] We have recently found that long alkyl chain substituted uric acids and 5,6-diaminouracils were able to protect mitochondrial membranes against lipid peroxidation initiated by the oxygen/iron(II)/dihydroxyfumaric acid system, with an optimal chain length of C_8 to C_{12}.[11] This protection was due to the scavenging activity of these derivatives against lipid radicals, enhanced by their specific localisation into the lipid bilayer. We also noticed that lipophilic nitrosouracils, inactive as radical scavengers, were able to decrease markedly this peroxidation process, probably via iron chelation mechanisms. In this paper, we report the study of the inhibition of the Fenton reaction by various ligands containing the 6-amino-5-nitrosouracil moiety.

MATERIALS AND METHODS

Materials

All chemicals purchased were of the highest quality available (Aldrich or Sigma Chemical Company) and were used without further purification. Reagent grade solvents were purified by distillation. Iron(II) was used as its ammonium sulphate. Compounds **1**–**5** were synthesized according to the procedure described by Pfleiderer[12] and Klemm,[13] compounds **6** and **7** according to Papesh[14] and Goldner[15] (Figure 1). The structure and the purity of the different compounds were established by standard spectroscopic methods and by element analyses. The purity of the tested compounds was above 99% and was checked by HPLC (reverse phase C18, CH_3CN/H_2O)/acetic acid, 90/9/1; 250–350 nm).

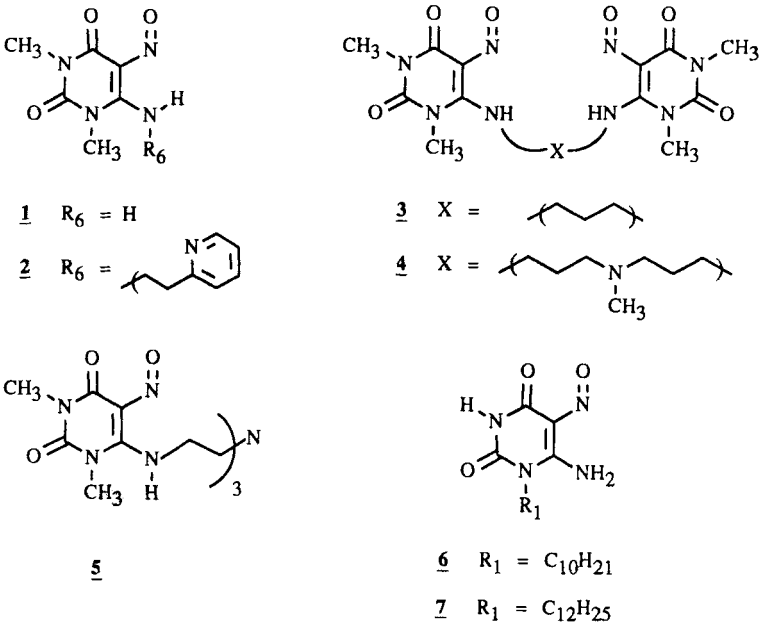

FIGURE 1 General formula of the different ligands tested.

All experiments were carried out with Chelex-resin treated distilled water. The various complexes were prepared as follows: the corresponding ligand is dissolved in a KH_2PO_4-NaOH buffer (20 mM, pH 7.4) at a final concentration of approximately 1 mM and the solution is deoxygenated. Then a degassed aqueous solution of iron(II) is added with a slight excess of ligand. The complexation process is monitored by the specific visible absorption of the complex at 610 nm.

Methods

(i) Deoxyribose degradation assay for hydroxyl radical[16,17]

Three hydroxyl radicals generating systems were employed: iron(II)/O_2, iron(II)/H_2O_2 and iron(III)/$O_2^{\cdot-}$-generating system (xanthine/xanthine oxidase). The reaction was performed in 20 mM KH_2PO_4-NaOH buffer (pH = 7.4) at 30°C. The concentrations of the different reagents and the experimental procedures were indicated in the corresponding figures and tables. Xanthine oxidase activity was evaluated by the O_2-uptake using an Clark-type electrode, and final concentrations of xanthine (1 mM) and xanthine oxidase (0.004 U/ml) were chosen in order to have a sustained $O_2^{\cdot-}$ production and no substrate restriction over 40 min of reaction. The ligands tested did not inhibit the enzyme xanthine oxydase at these concentrations. Deoxyribose degradation was determined by the 2-thiobarbituric acid test as previously described.[18] A special experimental procedure was developed in Fenton assays with H_2O_2: in the acidic conditions arising from the trichloracetic (TCA) treatment of the reaction mixture, iron(II)-nitrosouracil complexes were not stable and free iron (II) was released, allowing the degradation of deoxyribose to continue. Thus it was necessary to scavenge the ·OH produced

and to consume H_2O_2 in order to stop the degradation process before incubation of the mixture with thiobarbituric acid. For this purpose the reaction was stopped by addition of catalase (1000 U) and mannitol (100 mM) before addition of TCA. Under these conditions a maximum of 85% inhibition could be reached. Thiobarbituric Acid Reactive Substances (TBARS) amount was measured by fluorescence (excitation 532 nm, emission 553 nm) on a Shimadzu spectrofluorimeter RF 540. Results are the means of at least three separate experiments and do not differ by more than 5%.

(ii) Catalase-like activity

Oxygen concentration was determined with a Clark-type electrode (Hansatech Ltd).

H_2O_2 concentration was measured using the horseradish-peroxidase-coupled oxidation.[19] Aliquots (5–10 μl) from the O_2-cell were added to the assay system which contained 4-aminoantipyrine (250 μM), sodium 3,5-dichloro-2-hydroxybenzosulphonate (1 mM) and horseradish peroxidase (22 U/ml) in KH_2PO_4-NaOH buffer (0.91 ml, 100 mM, pH 8, 30°C). UV-visible spectrophotometric studies were performed with a Beckman DU 64 spectrophotometer, coupled to a PC-286 computer.

(iii) Lipid peroxidation

Evaluation of the antioxidant properties of the various compounds was performed by formation of thiobarbituric acid reactive substances (TBARS) using bovine heart mitochondria as a support for lipid peroxidation, and iron(II)/dihydroxyfumaric acid (DHF) as initiator.[11] Isolation of bovine heart mitochondria was performed according to a previously described procedure excepted that EDTA was omitted in the buffer used for washings and conservation.[20] Initiation of lipid peroxidation was performed as follows: mitochondria (0.6 mg/ml) were incubated at 37°C in KCl 140 mM, Tris-HCl 30 mM, pH 7.2 containing 1 mM dihydroxyfumarate and different concentrations of tested compounds in DMSO (**6** and **7**) or DMSO alone for control experiments (final DMSO concentration 0.2% v/v). The reaction started with the addition of 30 μM ferrous ammonium sulphate or preformed iron complexes. The formation of TBARS was measured according to reference 11. The amount of TBARS formation was determined on the basis of calibration curves obtained with malondialdehyde bis-diethylacetal diluted in HCl (0.1 N).

RESULTS

Inhibition Effect on Deoxyribose Degradation Initiated by Various Fenton Systems

Hydroxyl radicals (·OH) can be generated by the direct addition of iron (II) salts to an air saturated aqueous solution. When present in the reaction mixture, DEO reacts rapidly with hydroxyl radicals produced by the Fenton reaction, to produce degradation fragments and particularly malondialdehyde (TBARS) that reacts on heating with 2-thiobarbituric acid (TBA) at low pH to form a pink chromogen.[21–23] Under the described reaction conditions and in absence of inhibitors, the rate of degradation was very fast and completion of the DEO degradation occurred in a maximum of 3 minutes (Figure 2A). The degradation rate was measured in the presence of various nitrosouracil derivatives, which are potent iron (II) chelators.

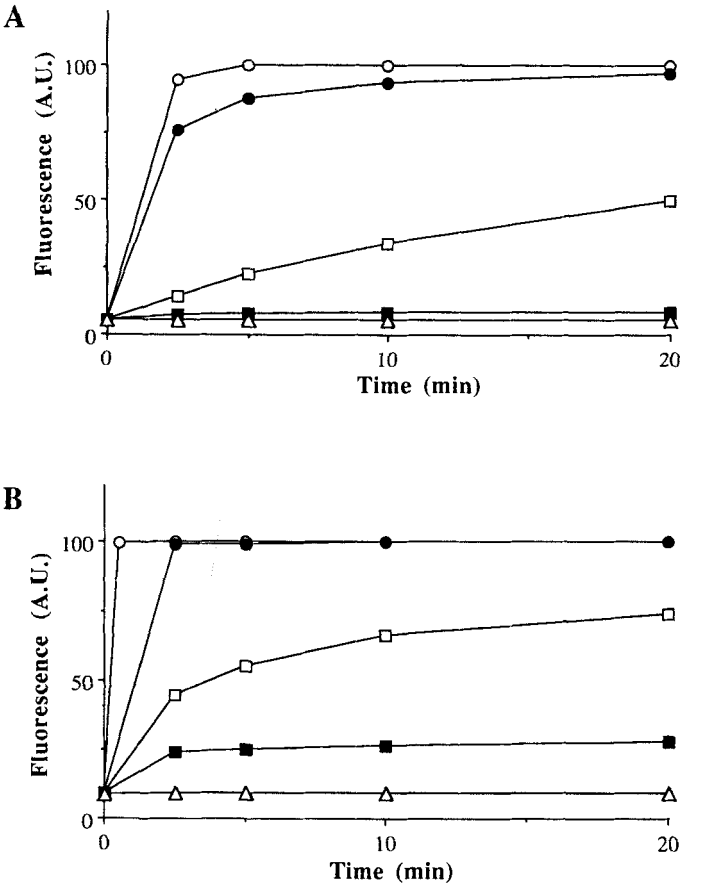

FIGURE 2 Time course of deoxyribose degradation initiated by iron(II) salts and iron(II)-nitrosouracil complexes in the absence (A) or presence (B) of H_2O_2 (100 μM). All samples contained KH_2PO_4-NaOH buffer (20 mM, pH 7.4), deoxyribose (2.5 mM) and (A) air-saturated or (B) hydrogen peroxide (100 μM). Reaction is started by the addition of either iron(II) ammonium sulphate (40 μM, final concentration) or iron(II) complexes (40 μM). - -(control); -●-(ligand 4); -□-(ligand 3); -■-(ligand 5); -△-(blank). The amount of TBARS produced was quantified by fluorescence spectroscopy (ex 532 nm em 553 nm) in arbitrary units.

A five fold excess of the ligand strongly decreased the deoxyribose degradation process. When the ligand was premixed with the iron (II) salt under nitrogen before being added to the reaction mixture, the formation of TBARS was inhibited more efficiently (Table I). The compound 5 inhibited the formation of TBARS in the presence of 80 μM iron(II) in a dose dependent manner (Figure 3). Inhibition was maximal when the stoichiometry (1/1) was overstept. The effect of mannitol, a very efficient hydroxyl radical scavenger with only a weak affinity for iron,[23] is quite different; the inhibition of DEO degradation increased slowly with higher mannitol concentration, because mannitol has to compete with deoxyribose for hydroxyl radicals generated by deoxyribose bound iron ions ("site-specific" Fenton

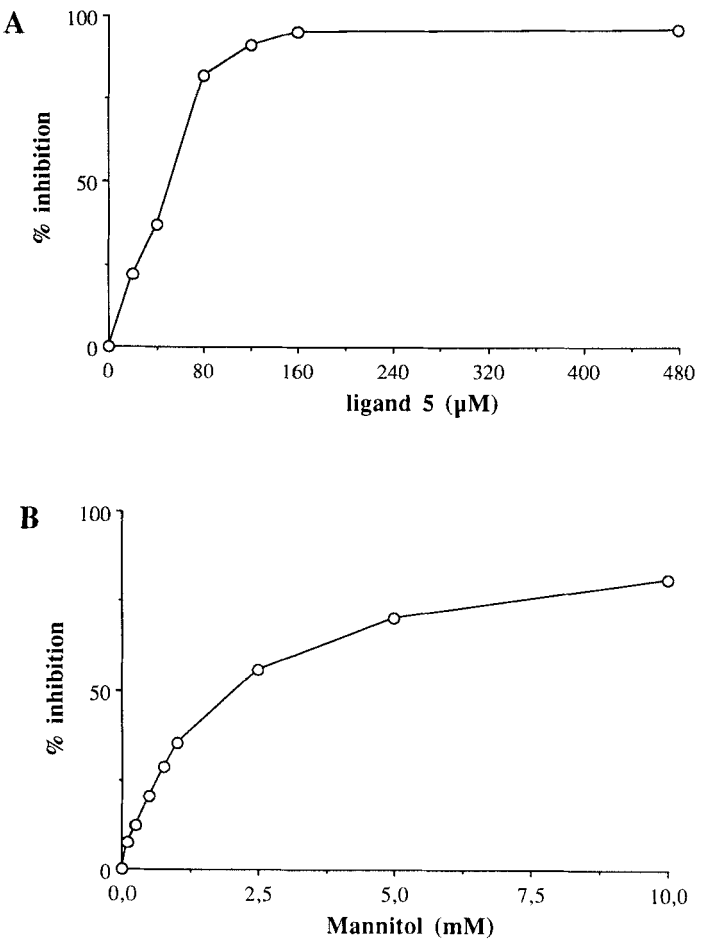

FIGURE 3 Inhibitory effects of the ligand **5** (A) and mannitol (B) as a function of concentration. Incubations were carried out for 20 min at 30°C. A: solutions of ligand and ferrous salt were deoxygenated, mixed, kept under nitrogen, and added to the solution containing KH_2PO_4-NaOH buffer (20 mM, pH 7.4) and deoxyribose (2.5 mM) to start the reaction (final iron concentration 80 μM). B: Reaction is initiated by addition of ferrous salt (final iron concentration 40 μM) to the reaction mixture containing KH_2PO_4-NaOH buffer (20 mM pH 7.4), DEO (2.5 μM), and mannitol.

chemistry).[24] The concentration necessary to reduce by 50% the deoxyribose degradation is 50 fold lower for the iron(II) chelator **5** than for mannitol and is related to the iron concentration. Rate constants for reaction of mannitol and DEO with hydroxyl radicals are similar.[23] This behaviour is not affected by the modification of the iron(II) concentration (40 μM or 80 μM) (not shown).

When hydrogen peroxide is added to the reaction mixture, the Fenton reaction occurred spontaneously (Figure 2B). It was not limited by oxygen reduction and superoxide dismutation. Furthermore, it has been noticed that, at these concentrations, the chelation process of iron(II) by nitrosouracil derivatives was a rather slow one, precluding the inhibition of the rapid DEO degradation. As in the first experi-

TABLE I
Inhibitory effect of nitrosouracil derivatives on the hydroxyl radical dependent deoxyribose degradation initiated by iron(II)/O_2 or iron(II)/H_2O_2 systems after 20 min incubation

	% Inhibition[a]		
	Fe/O_2 system		Fe/H_2O_2 system
	premixed[b]	not premixed[c]	premixed[b]
Compound **1**	90	13	70
2	18	6	38
3	52	22	29
4	2	1	0
5	97	70	79
mannitol(10 mM)	-	81	75
Tris(10 mM)	-	80	-
BPS(200 μM)	-	63	83
catalase(5000 U)	-	65	78

All samples contained KH_2PO_4-NaOH buffer (20 mM, pH 7.4) and deoxyribose (2.5 mM). Reaction is initiated by addition of either the ferrous complexes or ferrous salt (final iron concentration 40 μM). Incubations were carried out for 20 min at 30°C. [a]% Inhibition was calculated as (F.U. compound − F.U. blank)/(F.U. control − F.U. blank) (F.U.: Fluorescence units); concentration of the ligand was calculated according to the stoichiometry of their iron (II) complex determined by the Job method. [b]The complexes were preformed before being introduced into the reaction mixture. [c]Reaction was initiated by the introduction of iron(II) ammonium sulfate (40 μM).

ment, preformation of the complexes allowed a better inhibition rate. This inhibition enhancement is probably due to a better stability of the ligand-iron(II) complex towards H_2O_2 rather than iron(II) salts alone. In the next experiments, the iron complexes were always preformed and kept under nitrogen before use. Under these conditions the nitrosouracils reduced the DEO degradation as in the former experiment (Table I) with lower activities. As in the first experiment, the nitrosouracil derivative **5**, containing three pyrimidine rings, is a very efficient ligand and proved to be the most active in our tests.

For a comparison, we indicated in Table I the inhibition activity of catalase (H_2O_2 dismutation), Tris and mannitol (well-known hydroxyl radical scavengers) and bathophenanthroline sulphonate (an iron(II) chelator).[18,21-23]

These compounds were also assayed in another Fenton system in which hydroxyl radicals were produced by xanthine plus xanthine oxidase ($O_2^{\cdot-}$ and H_2O_2 generating system) and iron(III) at physiological concentrations.[17,22] In these experiments, the conversion of iron(III) to iron(II) is limiting because the production of superoxide radical is rather slow and a large part dismutes spontaneously, thus the concentration of iron(II) remains very small compared to the concentration of the free ligand (relatively to the total iron concentration). The formation of an iron(II) complex is favoured by this large excess of ligand. Indeed, the addition of **5** decreased significantly the TBARS formation (Figure 4). The production of hydroxyl radicals is inhibited drastically after a short period of incubation during which the free iron(II) concentration is lowered by selective complexation by the ligand. When the complexation process was complete, the degradation stopped. In this test, we also checked that, in the absence of iron, the oxygen uptake resulting from the oxidation of xanthine catalysed by the enzyme XO was not modified by the presence of the ligands. Concommitant production of uric acid was also monitored with the aid of its UV absorption at 290 nm.

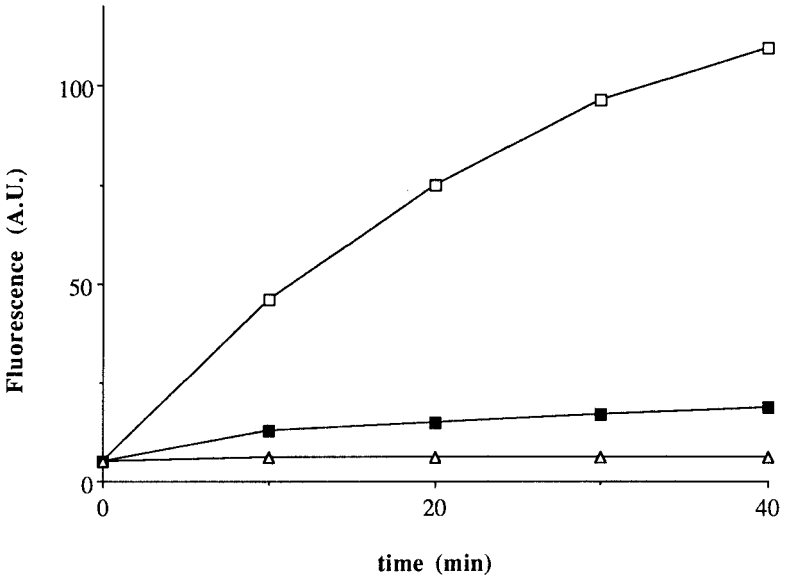

FIGURE 4 Effect of ligand **5** on the degradation of deoxyribose by the iron (III)/xanthine-xanthine oxidase system. The solutions were prepared in a KH_2PO_4-NaOH buffer (20 mM, pH = 7.4, 30°C) containing iron(III) ammonium sulphate (5 μM), deoxyribose (5 mM): -□-(control); -■-(ligand **5** 100 μM); -△-(blank). Reaction is started by addition of xanthine oxidase (0.004 U/ml) and stopped by acidification with TCA (1 ml). The amount of TBARS produced was quantified by fluorescence spectroscopy (ex 532 nm em 553 nm) in arbitrary units.

Stability of Iron(II)-Nitrosouracil Complexes towards H_2O_2 and O_2

The ability of these nitrosouracil derivatives to inhibit iron-dependent deoxyribose degradation appeared to be related to their affinity to iron(II). Nitrosouracil derivatives showed marked spectral changes in the visible range in the presence of iron(II) (characteristic absorption maximum at 610 nm).[25,26] A specificity for iron(II) was suggested by the fact that none of these compounds (except **5** to a small extent) displayed UV or visible spectral changes in the presence of iron(III), suggesting that they have very little affinity for oxidized iron (not shown).

A study of the stability of these iron(II) complexes in the presence of hydrogen peroxide was also performed. The stability was monitored by the absorbance of different solutions at 610 nm. Figure 5 displays examples of H_2O_2-dependent modifications in absorbance with four different complexes after the addition of an excess of H_2O_2 compared to the iron(II) concentration. While **4**-Fe(II) displayed a fast disappearance of the 610 nm peak after addition of H_2O_2, **1**-Fe(II) and especially **5**-Fe(II) absorbance changes were much smaller under the same conditions. Regarding **3**-Fe(II), addition of H_2O_2 resulted in the slow disappearance of the 610 nm peak to a stationary level (when H_2O_2 is in excess compared to iron(II) concentration).

The ligand **5** apparently formed a thermodynamically more stable complex with iron(II) than did other nitrosouracil derivatives. The **5**-ferrous complex was relatively stable over time in the presence of a large excess of EDTA (results not

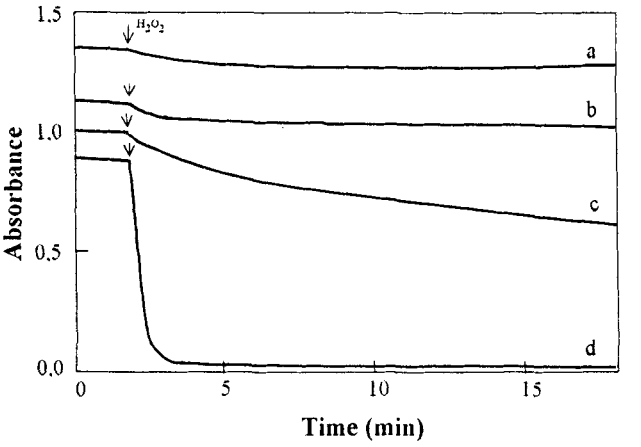

FIGURE 5 Time course degradation of iron(II)-nitrosouracil complexes during their reaction with H_2O_2 (200 μM) monitored by the absorbance changes at 610 nm. Ligand/iron(II) ratio: (a) **5**-Fe^{2+} (320/200 μM); (b) **1**-Fe^{2+} (660/200 μM); (c) **3**-Fe^{2+} (300/200 μM). (d) **4**-Fe^{2+} (300/200 μM). All the solutions were prepared in a KH_2PO_4-NaOH buffer (20 mM, pH 7.4) at 30°C.

shown). Addition of H_2O_2 induced a fast disappearance of **4**-Fe(II) with concomitant liberation of the ligand and apparition of a precipitate (iron(III) hydroxide). The ferrous-complex could be formed again by another addition of ferrous salt to the solution.

We have checked that most of the ligands described were able to inhibit the Fe^{2+} oxidation to Fe^{3+} in the presence of oxygen. Indeed, addition of ligand **5** to a solution of iron II in a closed cell significantly reduced the oxygen consumption (results not shown).

Catalase-like Activities

In order to prove that the inhibition of DEO degradation was not due to catalase-like activity (H_2O_2 dismutation to O_2 and H_2O), several experiments were performed in a closed cell equipped with a Clark-type electrode. Oxygen concentration was measured when Fe(II)-nitrosouracil derivatives were incubated with H_2O_2 in a deoxygenated medium. As shown in Figure 6, ferrous-nitrosouracil derivatives did not produce O_2 in the presence of H_2O_2. In the case of H_2O_2 - reactive iron complexes, no oxygen generation was observed, but concomitant H_2O_2 consumption could be monitored by titration with horseradish peroxidase (Table II). The reactivity of some complexes towards H_2O_2 is in good agreement with their inhibitory effect in the DEO degradation assay. The shape of the curves displaying the time dependent disappearance of either H_2O_2 or the iron complexes (monitored at 610 nm) was very similar, suggesting a 1/1 stoichiometry (not shown). The very low H_2O_2 consumption observed with **5** and **1** corresponded to high inhibition rates of DEO degradation. H_2O_2-reactive complexes (**3**-Fe(II) or **4**-Fe(II)) proved to be poorly active derivatives in the DEO degradation assay.

TABLE II
Inhibitory effect of nitrosouracil derivatives on the decomposition of H_2O_2 initiated by ferrous salts (175 μM)

Compounds	Percent $[H_2O_2]_{cons.}$ [a]
No ligand	100
1 (520 μM)	16
2 (520 μM)	80
3 (300 μM)	56
4 (200 μM)	96
5 (185 μM)	11

Initial H_2O_2 concentration: 116 μM.[a]% H_2O_2 is the relative amount of H_2O_2 consumed without O_2 evolution after correction from the quantity that disappeared by spontaneous dismutation. All the experiments were done in deoxygenated solutions, the iron(II) complexes were prepared before the assay and kept under a nitrogen atmosphere. H_2O_2 concentration was evaluated, after 15 min incubation, by the horseradish peroxidase mediated colorimetric test described in the Material and Method section. All these results were confirmed by the measure of the remaining H_2O_2 by catalase at the end of the reaction.

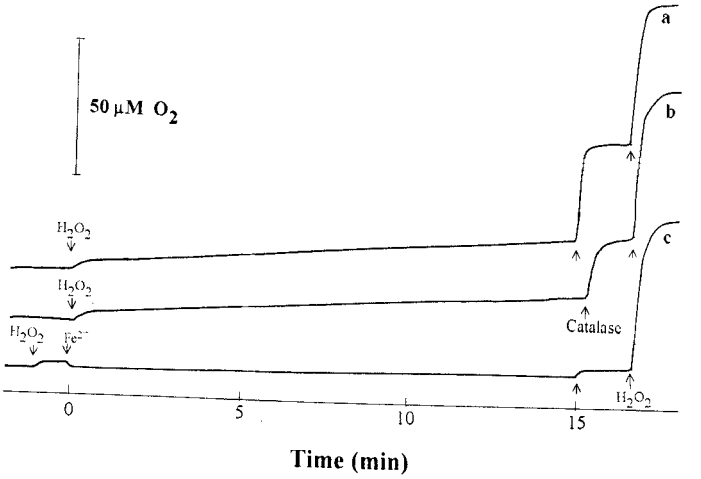

FIGURE 6 Oxygen electrode (Clark-type) measurements showing iron(II) complexes mediated decomposition (or not) of H_2O_2 without Oxygen evolution. The oxygen formation from H_2O_2 (116 μM) was followed after incubation in a deoxygenated KH_2PO_4-NaOH buffer (20 mM, pH 7.4 at 30°C in the presence of either (a) **5**-Fe^{2+} (ligand/iron ratio, 185/175 μM) or (b) **3**-Fe^{2+} (290/175 μM) or (c) iron(II) ammonium sulphate (200 μM). Catalase was added at the time indicated in order to detect the remaining H_2O_2. The subsequent addition of H_2O_2 (116 μM) demonstrate that decomposition of H_2O_2 without O_2 evolution occured in the case of (b) and (c). The curve (a) clearly shows that **5**-Fe^{2+} does not react with H_2O_2.

Lipid Peroxidation Assays

Nitrosouracil derivatives which inhibit Fenton reactions by iron(II) chelation could be potentially regarded as inhibitors of iron dependent peroxidation processes. In agreement with previously described results, addition of 30 μM iron(II) salts to phospholipid membranes of bovine heart mitochondria in the presence of dihydroxyfumaric acid, initiated a lipid peroxidation which could be monitored by measurement of

TABLE III
Inhibitory effect of nitrosouracil derivatives on lipid peroxidation of bovine heart mitochondria

	% Inhibition (ligand concentration in μM) or IC_{50}[a]	
	not premixed	premixed
Compound **1**	12 (200 μM)	80 (100 μM)
2	18 (200 μM)	16 (100 μM)
3	16 (200 μM)	30 (60 μM)
4	10 (200 μM)	6 (60 μM)
5	70 (200 μM)	92 (45 μM)
6	3 μM	
7	4 μM	
Trolox C	3.5μM	
Desferal	93 (200 μM)	
BPS	19 (200 μM)	

Reaction was started by addition of iron(II) ammonium sulphate (30 μM) or preformed iron complex (final iron concentration 30 μM). The results were obtained after incubation for 40 min at 30°C and were compared with appropriate blanks. [a]Each compound was tested in duplicate at several concentrations. Average values for various concentrations were plotted to determine an IC_{50} value (concentration of compound giving 50% inhibition of the peroxidation relatively to a control experiment).

TBARS.[11] Dihydroxyfumaric acid was chosen instead of ascorbic acid as iron reducer since ascorbic acid is known to have antioxydant properties (scavenging of water soluble peroxyradicals, $O_2^{\cdot-}$ and $HO_2\cdot$, inhibition of lipid peroxidation)[27] at the concentration required here (1 mM). Ascorbic acid effectively reduced TBARS formation by 78% at 100 μM in the test. Under our conditions, incubation of bovine heart mitochondria increased linearly during 40 min and produced 0.45 (+/−0.05) nmol TBARS/min/mg protein, whereas without induction no TBARS formation could be detected. DMSO alone (0.2%) had no inhibitory effect on the lipid peroxidation. The active compounds inhibited TBARS formation during 40 min in a dose-dependent manner from 0 to 100%. For less active compounds, the inhibition percentage determined for the highest concentration studied was given instead of an IC_{50}. The effect of various nitrosouracil derivatives on this process was examined (Table III). Ferrous complexes were preformed before being added to the reaction mixture, and lipid peroxidation was evaluated after 40 minutes. For all compounds tested, lipid peroxidation was significantly lowered compared to the effect of free iron(II). If the ligands and iron(II) were not premixed, the inhibitory effect is lowered, probably because the peroxidation process began faster than the complexation one. Without premixing, the very small lipophilic character of the ligand also avoided its localisation where peroxidation occurred and limited the complexation of membrane bound iron. A small excess of the ligand **5** premixed with iron(II) strongly inhibited lipid peroxidation at a rate similar to Desferal and slighly greater than bathophenanthroline sulphonate, a very strong iron(II) chelator which may become prooxidant when mixed with iron(II) in a 1/1 stoichiometry.[2] The ability of nitrosouracil derivatives to inhibit lipid peroxidation was clearly related to their affinity for iron(II).

Some long chain derivatives (**6** and **7**) were synthetized in order to increase the lipophilicity of nitrosouracil derivatives. They strongly inhibited mitochondrial membrane lipid peroxidation with IC_{50} lower than 4 μM. They were not studied for their inhibition activity in the DEO degradation assays since they were not

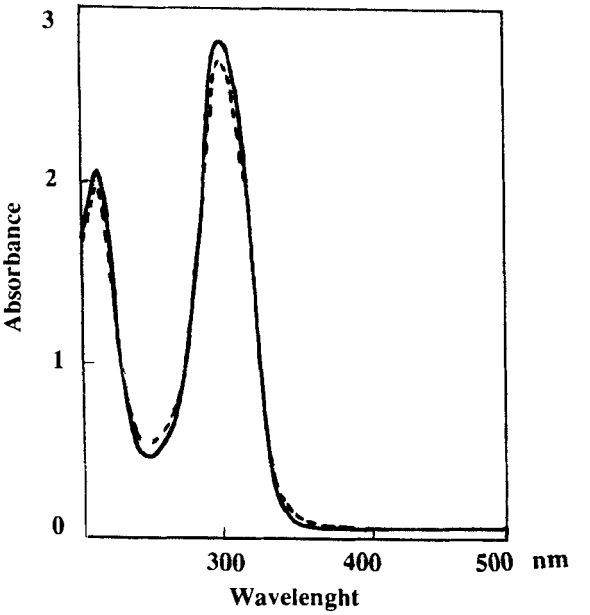

FIGURE 7 UV region spectra of ligand **4** before (solid line) and after Fe^{2+} mediated oxidation with H_2O_2 (dash line). The solutions were prepared in a KH_2PO_4-NaOH buffer (20 mM, pH = 7.4, 30°C). The final spectrum was recorded after addition of iron(II) ammonium sulphate (100 μM), then hydrogen peroxide (100 μM) to a solution of ligand **4** (150 μM) (iron(III) hydroxide precipitate was decanted at the end of the reaction).

soluble in aqueous buffer and the use of organic solvents (methanol or ethanol) was impossible because these solvents are very efficient hydroxyl radical scavengers. These results should be compared with those obtained for Trolox C, a commercial vitamin E analog with antiradical properties (IC_{50} = 3 μM) or for the 21-amino-steroid U74500A (IC_{50} = 1 μM).[10]

DISCUSSION

The deoxyribose assay is a very sensitive test for the detection of hydroxyl radicals in the iron-dependent Fenton reaction. Some nitrosouracil derivatives strongly inhibited this degradation process. This activity is dose-dependent and is clearly due to the inactivation of iron(II) by a complexation process. As it has been suggested by various authors,[28] we chose to perform this test without EDTA because our ligands proved to be very efficient iron (II) chelators. The most active compounds such as **5** and **1** formed stable complexes in the presence of H_2O_2. These compounds did not act as antiradical substances to a large extent because they are not significantly altered when ·OH was produced during H_2O_2 mediated oxidation of the complexes (the complexing ability of **4** was restored upon addition of iron(II) and the UV absorption of the ligand was not modified (Figure 7)), there is not a complete evidence for this statement but if they were destroyed by ·OH generation it would be difficult to explain their inhibitory effect at low concentrations.

Furthermore these derivatives did not present the same behaviour as mannitol towards hydroxyl radicals because they inhibit the DEO degradation at concentration far below that required for a ·OH scavenger with weak iron chelation ability. These complexes did not present a catalase-like activity since oxygen was not produced when hydrogen peroxide was added to a mixture of iron(II) and nitrosouracil derivatives. Furthermore, for the most active compound **5**, addition of catalase immediately elicited an oxygen response corresponding to half of the H_2O_2 added, indicating that catalase activity was close to zero for this compound.

The activities of the ligands are related to the stability of their ferrous complexes under oxidizing conditions, which could explain all the results observed here. Inactivation of iron prevents reaction with O_2 and H_2O_2, avoids generation of reactive oxygen species and limits the resulting degradation processes revealed by TBARS detection. Differences in activities between the complexes may probably be explained by the various structures of these ligands. Compound **5** forms a stable 1/1 complex with iron(II) with no coordination positions accessible to an oxidant or another ligand (H_2O_2 for example) and it is known that at least one free coordination site on iron is necessary for Fenton catalysis.[29] Compound **1** (a bidentate ligand) when mixed with iron(II) in a 3/1 stoichiometry, occupies also the six coordination positions of the metal and forms a very resistant complex towards oxidation. At the opposite, because they have several coordination sites available to the solvent (**4**) or a labile heteroatom (nitrogen of the pyridine ring in **2** or central nitrogen in **4**), complexes **4**-Fe^{2+} and **2**-Fe^{2+} are very reactive towards H_2O_2 or O_2. Because it forms several sort of complexes with iron(II) in solution, compound **3** shows a moderate reactivity – the stoichiometry is not well defined (the spectroscopic determination by the method of JOB[30] gives an apparent stoichiometry between one and two molecules of ligand *per* iron center) (not shown).

The iron-dependent formation of hydroxyl radical from H_2O_2 in the presence of a reducing agent such as superoxide radical is an important step of oxidative stress in cells and tissues.[31] Under these physiological conditions, compound **5** seems to be well-suitable to inhibit the Fenton reaction by iron complexation. These properties could be applied in biological media for the inhibition of degradations induced by oxidative stress.

Some authors have suggested that only membrane bound iron is responsible for lipid peroxidation.[10,11] The present results seem to confirm this hypothesis. For example, **5** or other hydrophilic iron(II) chelators (e.g. **1**) must be at least in a molar excess of the total iron added in order to inhibit significantly the lipid peroxidation process. When a nitrosouracil moiety is covalently linked with a lipophilic long alkyl chain, the inhibitory effect is enhanced by a factor of ten to fifty. It is known that decyl and dodecyl chain have the ideal length to insert an exogeneous compound into the bilayer membranes and the phospholipid environment.[11] Long chain substituted nitrosouracil derivatives effectively inhibited lipid peroxidation at concentrations far below the concentration of added-iron present in the assay. As it has been demonstrated by Braughler *et al.*,[10] lipophilic iron chelators are able to trap the iron which is near the lipid membranes. This iron is responsible of the damage observed at the polyunsaturated fatty acids ("site specific" Fenton chemistry) as ·OH radicals react near their production site. So these lipophilic ligands can stop the lipid peroxidation process at concentration lower than the total concentration of iron.

The new ligand **5** presents some advantages; it forms a complex with iron(II) in the same stability range than EDTA. It did not become prooxidant when mixed with

iron (II) in a 1/1 ratio. These properties make **5** a good challenger of bathophenantroline sulphonate or some 21-amino-steroids as potential iron(II) chelator for a therapeutic use.[2,10] The ligands presented here are also able to complex copper(II). Nevertheless these complexes are instable in the presence of Bovine Serum Albumine and are not presumed to play a major role under physiological conditions. Since some copper complexes, like Cu(II)-BPS, were shown to have a prooxidant activity, the relative specificity of our ligands toward iron(II), by preventing possible copper dependent side effects, may present some interest.[1,2]

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Accepted by Professor B. Halliwell