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Reactivity of an Indolinonic Aminoxyl with Superoxide Anion and Hydroxyl Radicals

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The increasing knowledge on the participation of free radicals in many diverse clinical and pathological conditions, has consequently expanded the search for new and versatile antioxidants aimed at combating oxidative stress. Our interest in this field concerns aromatic indolinonic aminoxyls (nitroxides) which efficiently react with alkoxyl, peroxyl, aminyl, arylthiyl and alkyl radicals to give non-paramagnetic species. This prompted us to test their antioxidant activity on different biological systems exposed to free radical-induced oxidative stress and the results obtained so far have been very promising. However little is known about their behaviour towards superoxide and hydroxyl radicals.

Here, we report on the reactivity of an indolinonic aminoxyl, with the two above mentioned radicals using hypoxanthine/xanthine oxidase and potassium superoxide for generating the former and the Fenton reagent for the latter. Besides performing the deoxyribose assay for studying the reaction of the aminoxyl with hydroxyl radical and monitoring spectral changes of the aminoxyl in the presence of superoxide radical, macroscale reactions were performed in both cases and the products of the reactions isolated and identified. The EPR technique was used in this study to help elucidate the data obtained. The results show that this compound efficiently reacts with both hydroxyl and superoxide radicals and furthermore, it is capable of maintaining iron ions in its oxidized form. The results thus contribute to increasing the knowledge on the reactivity of indolinonic aminoxyls towards free radical species and as a consequence, these compounds and/or other aminoxyl derivatives, may be considered as complementary, and sometimes alternative sources for combating oxidative damage.

Keywords: Indolinonic aminoxyl, nitroxide, hydroxyl and superoxide radicals, antioxidants

Abbreviations: BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; TCA, trichlorocacetic acid; TBARS, thiobarbituric acid reactive products; *TLC*, thin layer chromatography

INTRODUCTION

It has now become widely accepted that an increase in the production of free radical species especially oxygen-centered ones (superoxide, hydroxyl, peroxyl, alkoxyl) and/or a decrease in the antioxidant defence systems is responsible for the oxidation of critical cellular biomolecules such as lipids, proteins and nucleic acids leading to the widely accepted phenomenon called "oxidative stress". In this context, the search for new, effective

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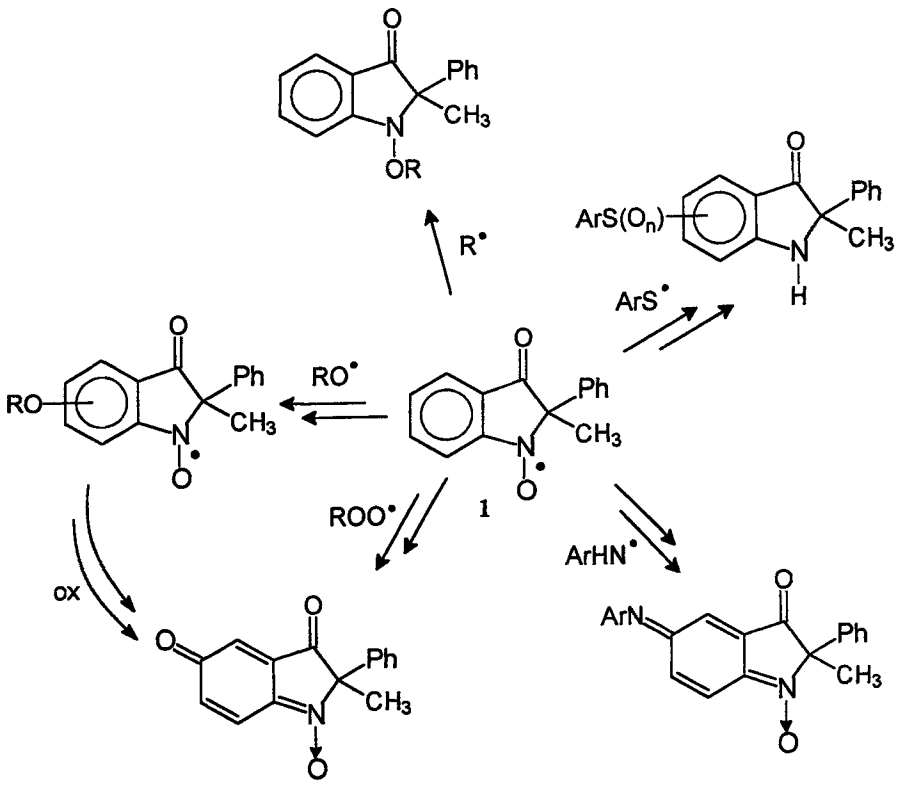
and appropriate antioxidants aimed at detoxifying these oxidizing species and providing defence against free radical-induced stress in diverse clinical and pathological conditions, has greatly expanded.[1]

One such class of novel antioxidants which has received considerable interest in recent years are aminoxyls, more commonly known as nitroxide radicals. The aminoxyls which have been used as antioxidants are remarkably stable and persistent free radicals due mainly to the absence of dimerization and disproportionation.[2] Originally, these compounds were, and still are, exploited as biophysical probes and labels for membrane and protein studies.[2] However, in the past decade there has been an increasing number of literature reports devoted to the versatile antioxidant activity of aminoxyls in different biological systems using a variety of experimental models,[3–5] even if the inhibitory effect of lipid peroxidation by aminoxyls was recognized almost three decades ago.[6]

Our interest in this class of antioxidants concerns with aromatic indolinonic aminoxyls synthesized by us. These aminoxyls efficiently react in chemical systems with alkyl radicals by coupling reaction at the aminoxyl function at a diffusion controlled rate,[7] and with alkoxyl[8] and peroxyl radicals[9] by substitution reaction to give non-paramagnetic species in all three cases. In addition, they also scavenge aminyl[10] and arylthiyl[11] radicals by the same substitution reactions as above (Scheme 1).

This scavenging behaviour toward various types of radical species prompted us to test them as antioxidants in diverse biological systems exposed to free radical-induced oxidative stress.[12–17] The studies undertaken so far with



SCHEME 1 Reactivity of 1,2-dihydro-2-methyl-2-phenyl-3H-indol-3-one-1-oxyl (1) toward alkyl, alkoxyl, peroxyl, aminyl and thiyl radicals.

these compounds and the results obtained have all been promising. However, little is known about their reactivity towards superoxide and hydroxyl radical which are probably the two most important oxygen radical species in biological systems. The former, via reaction with nitric oxide leads to peroxynitrite, a potent oxidizing agent with a chemistry of its own,[18] and to the hydroxyl radical via the metal-catalyzed Haber–Weiss reaction. The latter instead, is the most reactive radical species known to date, whose damage to biomolecules is indiscriminate.[1,19]

Here we test the reactivity of an indolinonic aminoxyl, namely 1,2-dihydro-2-methyl-2-phenyl-3H-indol-3-one-1-oxyl (**1**) (Scheme 1) with the two above mentioned radicals generated by different systems. The hypoxanthine/xanthine oxidase system and potassium superoxide were used for generation of superoxide while the Fenton reagent was used for production of the hydroxyl radical. The deoxyribose assay was also performed for studying the reaction of the nitroxide with $^{\bullet}OH$. The EPR technique was used in this study to help elucidate the data obtained.

MATERIALS AND METHODS

All reagents were of pure analytical grade. Hypoxanthine, xanthine oxidase (E.C. 1.1.3.22), thiobarbituric acid, ferrous sulphate and ferrous ammonium sulphate were purchased from Sigma Chem. Co. 2-Deoxy-D-ribose and potassium superoxide were Aldrich products, lead (IV) oxide, trichloroacetic acid, and hydrogen peroxide were Acros Organics products, while all other compounds and solvents were purchased from Carlo Erba (Milan, Italy). 1,2-Dihydro-2-methyl-2-phenyl-3H-indol-3-one-1-oxyl (**1**), was prepared according to the literature method.[20]

UV–VIS measurements were carried out on a UVIKON 941 Plus spectrophotometer, ^1H and ^{13}C NMR spectra were recorded at room temperature in $CDCl_3$ solution on a Varian Gemini 200 spectrometer (δ in ppm referred to Me_4Si). Mass spectra were recorded on a Carlo Erba QMD 1000 spectrometer in EI^+ mode, EPR measurements were recorded on a Varian E4 spectrometer interfaced with a computer and with a ruby placed in the EPR cavity as a reference, IR spectra were recorded on a Perkin Elmer 298 Infrared spectrometer.

Reactions with Hydroxyl Radical

Deoxyribsose Assay

Deoxyribose 2.3 mM was incubated for 1 h at 37°C with 0.01 mM $[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$ in 10 mM phosphate/saline buffer (PBS) pH 7.4 in the absence or presence of different concentrations of nitroxide dissolved in CH_3CN (< 1%). At the end of the incubation period, 10 µl of 10 mM BHT were added to each sample followed by 1 ml of 1% (w/v) TBA in 50 mM NaOH and 1 ml 2.8% (w/v) TCA and incubated at 90°C for 20 min; when cool the absorbance was read at 532 nm against appropriate blanks.

Macroscale Reaction

To a mixture of 0.4 mmol of aminoxyl (**1**) dissolved in 20 ml CH_3CN and 4 mmol H_2O_2, was added dropwise 4 mmol $FeSO_4 \cdot 7H_2O$ dissolved in 10 ml distilled water everything thoroughly degassed under nitrogen using teflon needles. The reaction mixture darkened upon addition of the ferrous salt. By monitoring the disappearance of the starting aminoxyl using TLC (ethyl acetate/cyclohexane 4 : 6), the reaction was complete after 5 min from the end of the addition. The mixture was extracted with CH_2Cl_2 (2 × 50 ml) washed with distilled water (3 × 50 ml), dried over anhydrous Na_2SO_4 and concentrated to dryness. The mixture was taken up with CH_2Cl_2 and chromatographed on silica gel preparative plates eluting with ethyl acetate/cyclohexane 2.5 : 7.5. From top to bottom compounds **4**, **2** and **3** (Scheme 3) were obtained with the following percentage yields: 39%, 16% and 14% respectively. Seven percent of the starting nitroxide was recovered.

Quinoneimine N-oxides **2** and **3** were identified by comparing their spectroscopic data with those of authentic samples.[8,21] Alkylated hydroxylamine **4** was crystallized from ligroine 60–80°C (pale yellow crystals), m.p. 76°C. The product was identified by its NMR, Mass and IR spectra. ^{1}H-NMR: 1.78 (3H, s, $-CH_3$), 4.37 ÷ 4.54 (2H, $-O-CH_2-CN$, AB, $J = 16.0$ Hz), 7.23 (1H, dt, arom, $J = 7.1$ and 1.1 Hz), 7.32 ÷ 7.57 (6H, m, arom), 7.73 (2H, dt, arom, $J = 7.5$ and 1.1 Hz); ^{13}C-NMR: 19.9, 61.5, 77.3, 116.2, 116.4, 120.9, 125, 127.2, 128.9, 129.3, 137.7, 139.32, 160.5, 198.8; IR: 2300, 1720, 1610, 1300, 1190, 1140, 1050, 960, 920, 760, 740, 700; MS: 278 (M^+, 19), 263 ($M^+ - 15$, 16), 238 (18), 222 (100), 194 (66), 152 (88), 104 (49), 77 (61).

EPR Experiments

A mixture of the aminoxyl (80 µl of a 1.6 mM stock solution), hydrogen peroxide and CH_3CN to a final volume of 2 ml was introduced in one leg of an inverted flat quartz U-cell; in the other leg was added the ferrous salt dissolved in water and CH_3CN to a final volume of 2 ml. The ratios of solvents and reagents were the same as the macroscale reaction. Both solutions were thoroughly degassed under nitrogen using teflon needles, mixed and the EPR spectrum recorded immediately. After this measurement, a tiny amount of PbO_2 was added to the mixture, and the spectrum recorded again using the same recording conditions.

To observe the possible interaction of Fe^{2+} with aminoxyl, EPR experiments using the same molar ratios of aminoxyl and ferrous salt as those used in the deoxyribose assay, i.e. [aminoxyl] = 30 µM vs [Fe^{2+}] = 10, 12.5, 16.6, 25, 50, 100 µM were performed. Routinely, in a test-tube 10 µl of a 3.3 mM solution of aminoxyl in CH_3CN was added to PBS and to this was added the desired amount of a 1.25 mM aqueous ferrous solution to give the final concentration of Fe^{2+} required in a 1 ml final volume. The mixture was immediately vortexed, transferred to the EPR cavity and its spectrum recorded. After this measurement, a tiny amount of PbO_2 was added to the mixture, and the spectrum was recorded again using the same recording conditions. All the EPR experiments described above were performed three times and the results were reproducible in every case.

Reaction with Superoxide Radical

UV Experiments

To a mixture of 100 µM aminoxyl in ethanol (< 1%) and 74 mM hypoxanthine in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.8, final volume 3 ml, 48 mU xanthine oxidase were added after the first scan and the UV spectra were recorded every minute for 10 min. The reaction mixture was then transferred to the EPR cavity and its spectrum was recorded. After this measurement, a tiny amount of PbO_2 was added to the mixture, and the spectrum was recorded again using the same recording conditions.

Macroscale Reaction

To 0.2 mmol of aminoxyl in 12 ml ethanol and 4 ml of distilled water, 0.4 mmol of KO_2 were added under magnetic stirring. On following the reaction by TLC (ethyl acetate/cyclohexane 2 : 8), immediate formation of the reduced form of the nitroxide, i.e. the hydroxylamine (by comparison with an authentic sample)[20] was observed. The reaction was worked up after 30 min, by neutralizing with 1 ml, 1% HCl. The reaction mixture was then concentrated to a small volume and extracted with CH_2Cl_2 (2 × 50 ml), washed with distilled water (2 × 50 ml), dried over anhydrous Na_2SO_4 and concentrated. The reaction mixture was chromatographed on silica gel preparative plates eluting with ethyl acetate/cyclohexane 1.5 : 8.5. Aminoxyl **1** (36%) and the corresponding hydroxylamine **5** (58%) (Scheme 4) were obtained from top to bottom after extraction with ethyl acetate. The hydroxylamine **5** was identified by comparing its spectroscopic data with those of an authentic sample,[20] and by the fact that it easily transforms to the starting aminoxyl upon oxidation with PbO_2.

Appropriate blanks were performed throughout all the experiments.

RESULTS

The deoxyribose assay was used for studying the reaction with •OH according to a literature method.[22] The hydroxyl radical generated by direct addition of a ferrous salt to the reaction mixture containing phosphate buffer attacks the deoxyribose degrading it into fragments, that give a pink chromogen upon heating with thiobarbituric acid at low pH. If an •OH scavenger is added, it competes with deoxyribose for •OH and inhibits chromogen formation.

Figure 1 shows the dose-response curve of the inhibition of •OH-mediated deoxyribose degradation by the aminoxyl. Almost complete inhibition of TBARS formation is observed at 12 μM concentration and above. Two possibilities could exist to explain this apparent protection by the aminoxyl against •OH damage: (a) the aminoxyl oxidizes Fe^{2+} to Fe^{3+}, thus preventing its participation in the Fenton reaction by maintaining the iron salt in its oxidized form; (b) the aminoxyl intercepts the •OH radical by chemically reacting with it. In this case the aminoxyl would be chemically modified. The consequences in both cases (a) and (b) would be a reduction in deoxyribose degradation.

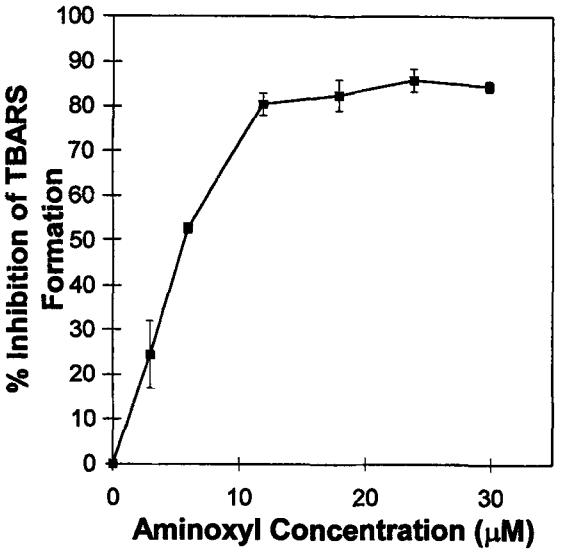
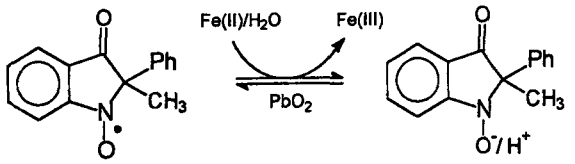


FIGURE 1 Dose-response curve showing the effect of indolinonic aminoxyl **1** on the release of TBARS from 2-deoxy-D-ribose during the deoxyribose assay. Conditions with final reaction concentrations: 2.3 mM deoxyribose was incubated for 1 h at 37°C with 0.01 mM $[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$ in 10 mM PBS pH 7.4 in the absence or presence of different concentrations of aminoxyl dissolved in CH_3CN (< 1%), followed by TBARS-assay.

To verify the first case, the aminoxyl was reacted in the EPR cavity with the same molar ratios aminoxyl : Fe^{2+} as those used in the deoxyribose assay in PBS. When $[Fe^{2+}] < [\text{aminoxyl}]$ there was only a very slight decrease in the EPR signal; on increasing the $[Fe^{2+}]$ the signal of the aminoxyl decreased to almost complete disappearance but it was restored to the starting level (i.e. before iron addition) upon oxidation with PbO_2 (data not shown). The diminution of the signal is due to the formation of the reduced form of the aminoxyl (> NO^-) that could be protonated, being in an aqueous medium, to form the hydroxylamine (> NOH). These two forms, being non-paramagnetic species, could both be responsible for the decrease in the EPR signal and both are easily oxidized back to the aminoxyl by PbO_2 (Scheme 2) explaining the restoration of the EPR signal to the starting level. Lead (IV) oxide is a strong oxidizing agent used to oxidize hydroxylamines to their corresponding aminoxyls.[20] These results suggest that an equilibrium redox reaction exists between the aminoxyl and the ferrous salt, and that to some extent the nitroxide is able to maintain iron in its oxidized form.

To determine whether •OH is intercepted by the aminoxyl, a macroscale reaction was performed

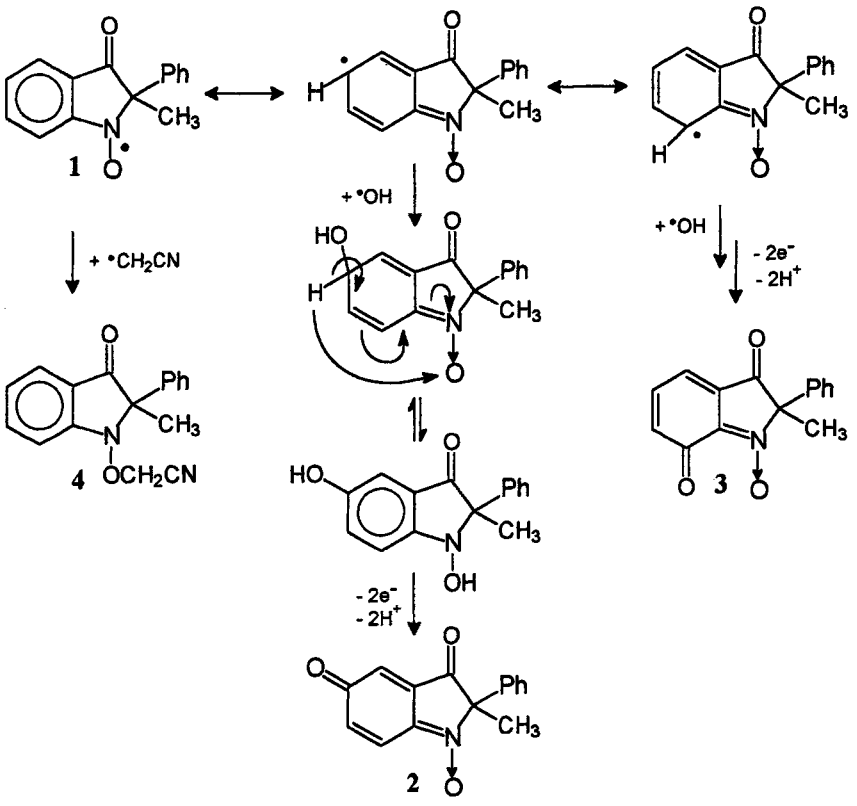


SCHEME 2 Reduction of the indolinonic aminoxyl **1** by Fe(II) to give the reduced form, and oxidation back to the aminoxyl with lead (IV) oxide.

using the Fenton reagent. After reaction work-up the products **2–4** shown in Scheme 3 were obtained. In the same scheme is reported the reaction mechanism whereby the aminoxyl scavenges $^{\bullet}OH$ by entrapment on the conjugated benzene ring followed by oxidation, either by electron and proton transfer or by hydrogen abstraction, to give the quinoneimine N-oxides **2** and **3**. Alkylated hydroxylamine **4** arises from scavenging of $^{\bullet}CH_2CN$ at the N–O$^{\bullet}$ function which is formed by hydrogen abstraction of $^{\bullet}OH$ on the solvent in which the reaction was performed.

The same reaction as above using the same ratios of solvents and reagents was performed in the EPR cavity where an immediate decrease in the EPR signal of the aminoxyl was observed which was not restored upon PbO_2 oxidation (data not shown). This result confirms that there is transformation of the aminoxyl to non-paramagnetic species in the presence of $^{\bullet}OH$, in these experimental conditions.

To assess whether the aminoxyl could react with superoxide radical, its absorbance in the presence of the superoxide radical generating system, hypoxanthine/xanthine oxidase was followed spectrophotometrically. As can be observed in Figure 2, spectral changes occurred in the presence of $O_2^{-\bullet}$. In particular, the peak at 285 nm decreases with time, while the peak at 240 nm shifts to lower wavelengths (234 nm) and increases gradually. The resulting spectrum after 6 min from the start of the reaction was identical to the UV–VIS spectrum of the corresponding hydroxylamine **5**.[20] In fact, when this reaction mixture was transferred to the EPR cavity, a very



SCHEME 3 Reaction scheme of indolinonic aminoxyl **1** with hydroxyl radical generated by the Fenton reagent ($Fe(II)/H_2O_2$) leading to quinoneimine-N-oxides **2** and **3** and with acetonitrile radical to give alkylated hydroxylamine **4**. See Materials and Methods for details of the reaction.

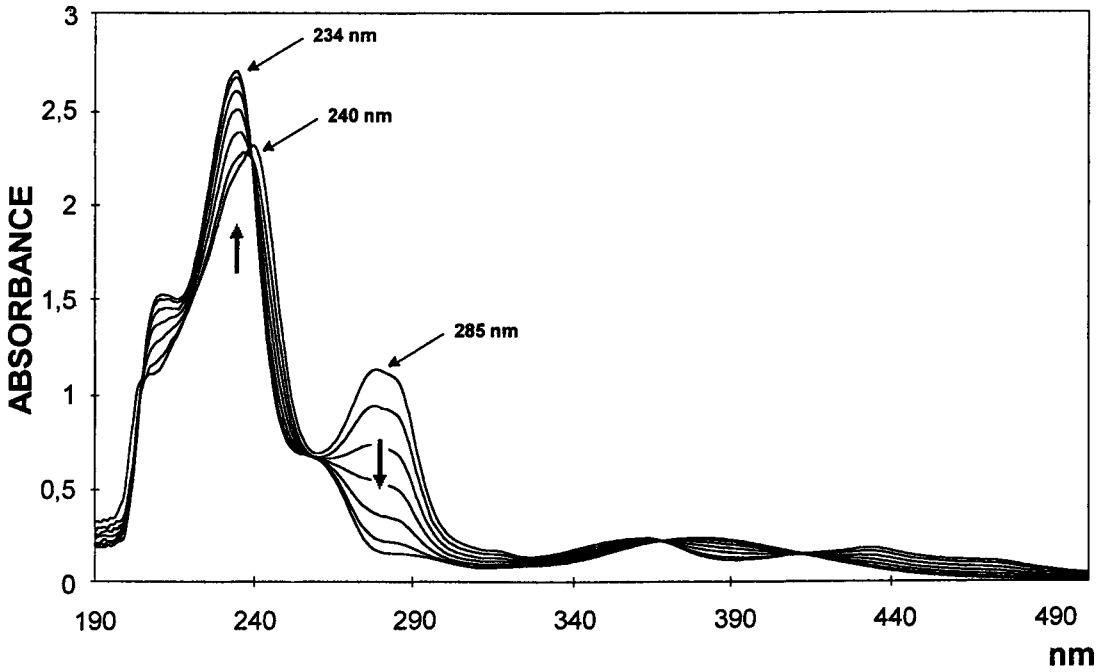


FIGURE 2 Spectral changes of indolinonic aminoxyl **1** in the presence of superoxide radical generated by hypoxanthine/xanthine oxidase. Conditions with final reaction concentrations: to a mixture of 100 μM aminoxyl in ethanol (< 1%) and 74 mM hypoxanthine in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.8, were added 48 mU xanthine oxidase after the first scan and spectra were recorded thereafter every minute for 10 min. After 6 min no more spectral changes were observed.

faint signal of the aminoxyl was recorded, which was completely restored to the starting level i.e. before reaction with superoxide, upon oxidation with PbO_2 (Figure 3). This result suggests that there is electron transfer between $O_2^{-\bullet}$ and aminoxyl to give the reduced form while $O_2^{-\bullet}$ is converted to oxygen (Scheme 4). The reduction potentials of the aminoxyl (−0.26 V vs SCE) and $O_2^{-\bullet}$ (−0.89 vs SCE) are in agreement with this hypothesis.[23]

A macroscale reaction was performed to give support to this hypothesis, using potassium superoxide as generator of superoxide anion radical. The main reaction product was in fact the corresponding hydroxylamine **5** (Scheme 4).

CONCLUSION

The results reported here, show that this indolinonic aminoxyl efficiently reacts with hydroxyl radical by intercepting it on the conjugated benzene ring (Scheme 3). The quinoneimine N-oxides **2** and **3** obtained are the typical reaction products of oxygen-centered radicals with indolinonic aminoxyls.[8,9] Interestingly, the $^{\bullet}OH$ radical is capable of hydrogen abstraction on the solvent (CH_3CN) since the resulting radical ($^{\bullet}CH_2CN$) was also trapped by the aminoxyl by coupling at the N–O$^{\bullet}$ function. Alkylated hydroxylamines are the typical reaction products of carbon-centered radicals with aminoxyls.[7,24]

In addition, in an aqueous medium such as that of the deoxyribose assay, the nitroxide is capable of maintaining iron ions in their oxidized form therefore preventing their participation in metal-catalyzed reactions like the Fenton and Haber–Weiss. This is in accordance with previous studies carried out on the commercial piperidine aminoxyls such as TEMPO which prevent the generation of oxidants via Fenton reactions by maintaining the metals in the oxidized state.[25]

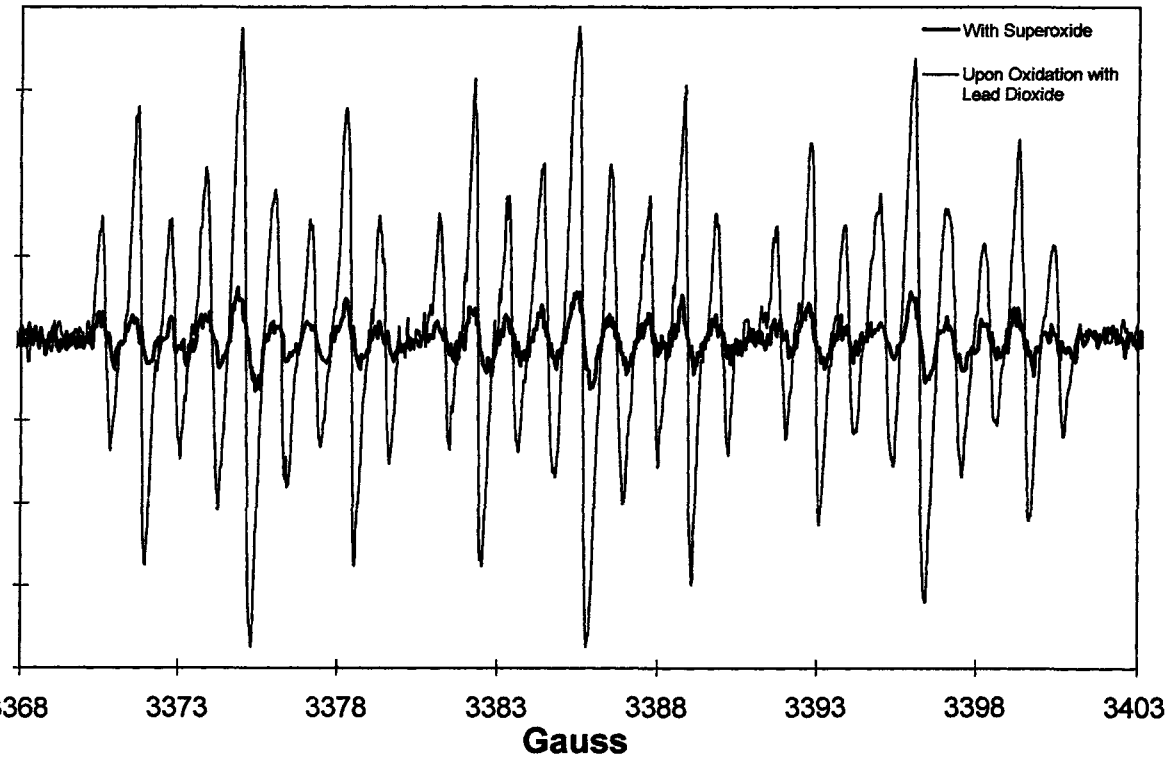
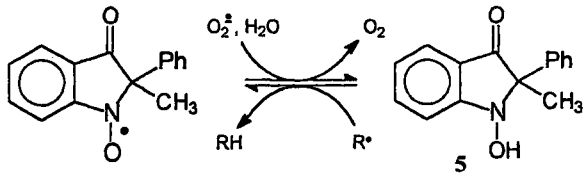


FIGURE 3 EPR spectra of indolinonic aminoxyl. Thick line represents the spectrum recorded after 10 min of the reaction with superoxide radical in the conditions reported in Figure 2. Thin line represents the spectrum obtained after oxidation of the above reaction mixture with lead (IV) oxide.



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SCHEME 4 Reduction of indolinonic aminoxyl **1** by superoxide radical to give the corresponding hydroxylamine **5**, and oxidation back to the aminoxyl via donation of hydrogen to a free radical species denoted R•.

In this way, the indolinonic aminoxyl indirectly contributes to the inhibition of the production of •OH in this system. Therefore both mechanisms, maintenance of iron in its oxidized form and interception of •OH on the conjugated benzene ring could be operating in the prevention of hydroxyl radical-mediated deoxyribose damage observed with the aminoxyl radical. The fact that our aminoxyl reacts with •OH provides a further protective mechanism towards this radical species when compared to piperidine aminoxyls which do not trap oxygen-centered radicals.

This indolinonic aminoxyl also efficiently reacts with superoxide radical which is transformed into oxygen while the aminoxyl is converted into the corresponding hydroxylamine. This is itself an antioxidant by the classical hydrogen donation like vitamin E, thus the aminoxyl is regenerated (Scheme 4).

These results contribute to increasing the knowledge on the reactivity of indolinonic aminoxyls towards free radical species and gives us additional information to explain their antioxidant behaviour in biological systems. These aminoxyls proved efficient in preventing linolenic acid oxidation[12] and copper-mediated low density lipoprotein peroxidation[13] in a dose-dependent fashion. Protein and lipid peroxidation of

microsomes[14] and albumin[15] subjected to radical insult was also reduced in the presence of these aromatic aminoxyls. Recently, these compounds were shown to be also effective in protecting radical-induced DNA damage.[16,17] As a consequence, these results serve further to emphasize the potential utility of aminoxyls as complementary, and sometimes alternative sources for combating oxidative stress because of their interesting and versatile antioxidant properties.[3,26]

Acknowledgments

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