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Dedicated to Professor Valer Farcasan on the occasion of his 95th anniversary

ESTIMATION OF RADICAL SCAVENGING CAPACITY OF SOME DIARYL- AND PHENOTHIAZINYL-NITRONES

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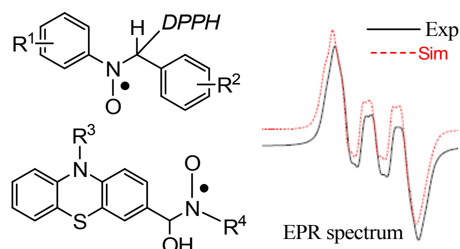
A series of phenyl and phenothiazinyl nitrones were tested as spin traps for hydrazyl and hydroxyl radicals. UV-Vis spectrophotometric DPPH free radical assay indicated excellent free radical scavenging activity of diphenyl nitrones with fast reaction kinetics and high steady state conversions. EPR spectroscopic assay afforded the detection of paramagnetic nitroxide adducts formed by spin trapping of hydroxyl radical. DNA binding interaction was not confirmed by electrophoresis experiments.



INTRODUCTION

Radicals are reactive species containing at least one unpaired electron in the outer electronic shell responsible for promoting chain reactions which might be very useful at the synthetic chemistry level, but unpredictable and most dangerous at biochemical level. Free radical reactive oxygen species (ROS) and nitrogen species (RNS) can be generated *in vivo* by various endogenous systems[1] and oxidative damage to various cellular components may occur in the absence of antioxidants capable of keeping the balance.[2] Singlet oxygen and free radicals such as hydroxyl radical, superoxide anion radical, nitric oxide radical, and peroxynitrite radical may induce damage to relevant biological macromolecules (DNA, proteins, carbohydrates and lipids).[3] The most common health diseases associated to free radicals damage and oxidative stress are: ageing, cancer, myocardial infarction, rheumatoid arthritis, chronic inflammation, stroke, as well as cardiovascular and inflammatory disease.[4]

The quantification of radical-scavenging capacity (RSC) of natural extracts and synthetic compounds can be addressed by several methods such as: the colorimetric DPPH radical oxidative assay,[5,6] the oxygen radical absorbance capacity (ORAC),[7] the Electron Paramagnetic Resonance (EPR) spin trapping method which was successfully applied in the detection of transient free radicals forming stable paramagnetic adducts.[8]

Nitrones can be used as spin traps designated to detect, identify, quantify and locate the species responsible for oxidative stress in biological chemistry.[9] For instance, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) is a widely used spin trap for carbon- and oxygen-centered radicals generated in chemical and biochemical systems.[10] Other nitrones have been evaluated as therapeutics in the

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context of free radical biochemical and cellular processes considered important in pathologic conditions and age-related diseases and were proved chain-breaking antioxidants due to the stability of nitroxide adducts formed by the capture of reactive paramagnetic species.[11-13]

Phenothiazine derivatives are well known for important applications as neuroleptic drugs and antioxidants for lubricants, both taking benefit of the redox-active character of the heterocyclic unit readily involved in reversible one electron exchange processes.[14,15]

In this work we describe the spin trap properties of a series of diphenyl and phenothiazinyl nitrones recently reported by our group,[16] by screening their hydrazyl and hydroxyl radical scavenging capacity. The standard DPPH radical oxidative assay and alternatively the EPR spectroscopic assay were employed for the detection of the generated nitroxide spin adducts. We also reported here a DNA binding test of (hetero)aryl nitrones under investigation, as potential tools for chemical biology.

RESULTS AND DISCUSSION

The structures of diphenyl and phenothiazinyl nitrones used in this study are depicted in Fig. 1. Substituents with typical electron donor (-OH, $-OCH_3$), electron withdrawing ($-NO_2$, $-CO-CH_3$) effect, as well as halogens (-Cl, -Br) were located in different positions of the aromatic rings adjacent to the nitrone structural motif in order to modulate their molecular electronic properties.

DPPH radical oxidative assay

The radical scavenging capacity (RSC) of nitrones **1-29** was estimated by the standard colorimetric DPPH radical oxidative assay. The protocol applied took care of the sensitivity of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical to light and of the spectrophotometric sensitivity range. According to Beer's law, the optimal detectable range of a spectrophotometric determination corresponds to a DPPH concentration between 25 and 70 μM affording absorbance values in the range 0.221-0.698.[5] Thus, methanolic solutions of radical and spin traping nitrone respectively were kept in the dark at room temperature before and after the mixing in molar ratio DPPH : nitrone 1:2. The bleaching of the deep purple color of DPPH radical (λ_{max} = 520 nm) upon formation of a spin adduct was quantitatively detected by monitoring the decrease of the absorbance maxima over a period of 2 hours (Fig. 2).

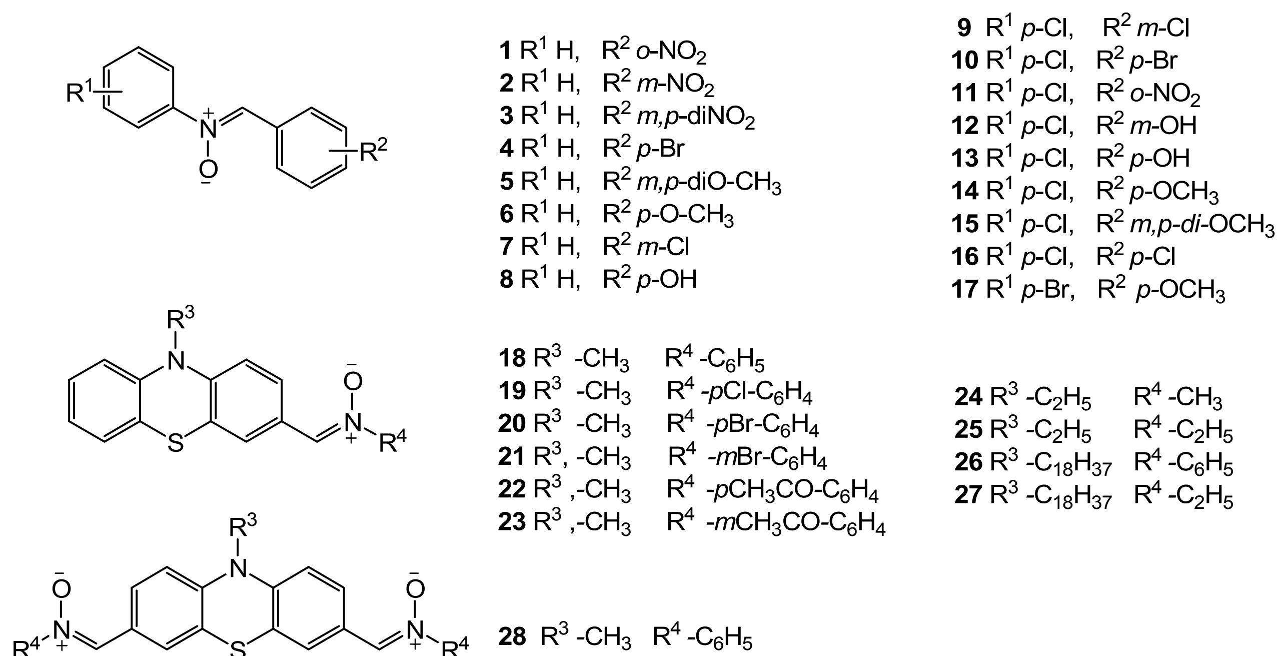


Fig. 1 – Substituted diphenyl-nitrones and phenothiazinyl-nitrones under investigation.

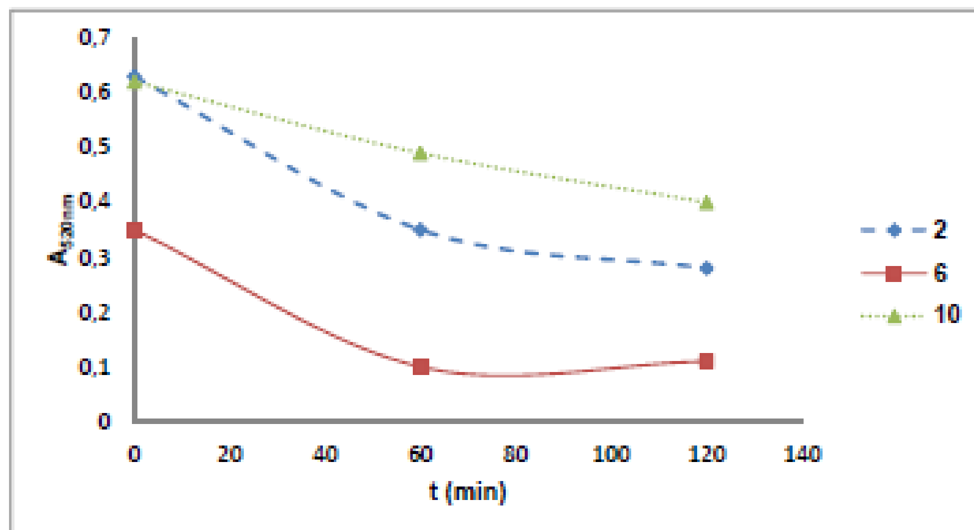
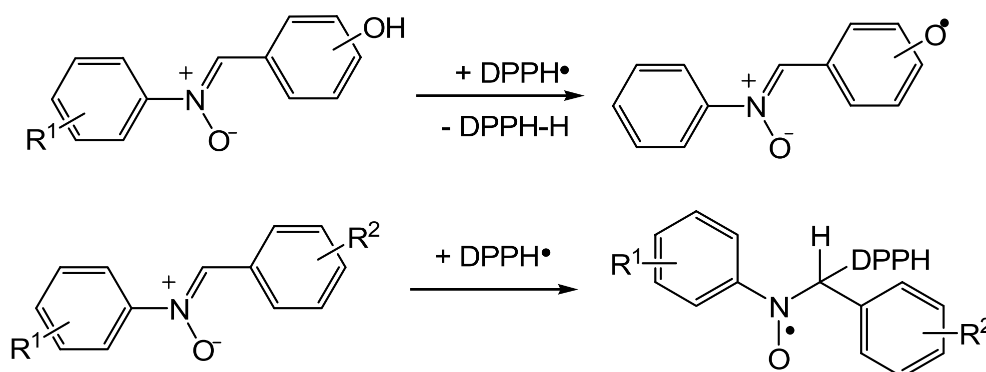


Fig. 2 – Scavenging of DPPH free radical by nitrones.



Scheme 1

DPPH radical scavenging may occur by two different reaction pathways: i) by hydrogen capture from nitrones containing phenolic units (**8, 12, 13),** or ii) by nitroxide adduct formation under electrophilic addition to the carbon center of nitrones (Scheme 1).

The radical scavenging capacity (RSC) of diphenyl and phenothiazinyl nitrones was calculated as a percentage of DPPH bleaching using equation (1)

$$RSC = 100 \times [1-(A_s/A_{ref})]\ \% \qquad \text{eq (1)}$$

where, A_s represents the absorbance of the solution containing DPPH in the presence of nitrone and A_{ref} is the absorbance of blank solution containing DPPH, at 520 nm.

RSC was determined at three time intervals: immediately after mixing (t_0), after 60 (t_{60}) and 120 (t_{120}) minutes respectively; the results are summarized in Table 1.

The reaction of DPPH radical with diphenyl nitrones **1-18** was characterized by a fast kinetic with a time required to reach the steady state saturation of approximately 1 hour. The phenolic nitrones **8,12,13** as well as the $-OCH_3$, -Br, -Cl and $-NO_2$ substituted nitrones (**6, 4, 7, 11, 14, 15**) appeared to be effective spin traps with RSC higher than 90% after 1 hour, even though the spin trap mechanism might be different. Slower reaction kinetics and lower conversions of the DPPH radical were observed in the case of dinitro- and dihalogeno-nitrones (*e.g.* **3, 10** Table 1).

The RSC of phenothiazinyl nitrones **18-28** occured within the range of 0 to 22%, values which classifies them as almost inactive. The DPPH radical assay might not be completely appropriate for assigning the RSC of these heteroaryl-nitrones due to the sterical hindrance induced by the folded phenothiazine unit which reduces the accessibility of the large size DPPH radical to the reaction center and as a consequence these results remain questionable.

Table 1

Radical scavenging capacity of diphenyl and phenothiazinyl-nitrones

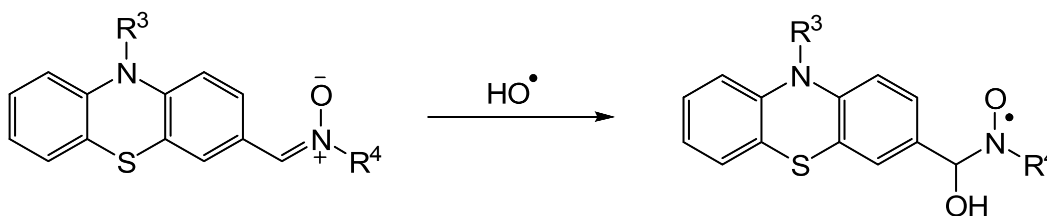
Nitrone	RSC %		
	t_0	t_{60}	t_{120}
1	14	81	88
2	10	51	55
3	4	31	38
4	20	90	91
5	11	86	88
6	59	91	90
7	44	92	92
8	23	93	93
9	2	71	79
10	11	28	35
11	47	93	93
12	45	93	93
13	49	92	93
14	52	92	92
15	48	90	91
16	22	69	73
17	25	33	49
18	0	0	8
19	4	9	11
20	3	10	14
21	5	8	6
22	3	14	19
23	6	7	8
24	0	0	0
25	3	5	22
26	5	5	0
27	3	4	3.5
28	1	8	18

EPR spectroscopic assay

The radical scavenging capacity of selected diphenyl- and phenothiazinyl-nitrones was tested in the presence of oxygen centered hydroxyl radicals generated by Fenton reagent (Fe^{2+}/H_2O_2) by EPR spectroscopic detection of the longer-lived paramagnetic adduct formed according to Scheme 2.

Nitroxide paramagnetic adducts gave rise to a strong EPR signals characterized by *g*-factor value 2.003 and hyperfine splittings consistent mainly to the coupling of the unpaired electron with the nitrogen nucleus and the α-proton. In Fig. 3 are depicted the recorded and simulated EPR spectra of the nitroxide adducts generated by diphenyl-nitrone **12** and phenothiazinyl-nitrone **19**, respectively. In some cases, the high resolution spectra emphasized additional hyperfine splitting due to couplings with other protons in proximity and a second nitrogen atom.

The recorded EPR spectral parameters for all nitroxide adducts were in agreement with the simulated spectra and the data are summarized in Table 2. Unfortunately, this method was not suitable for quantification.



Scheme 2

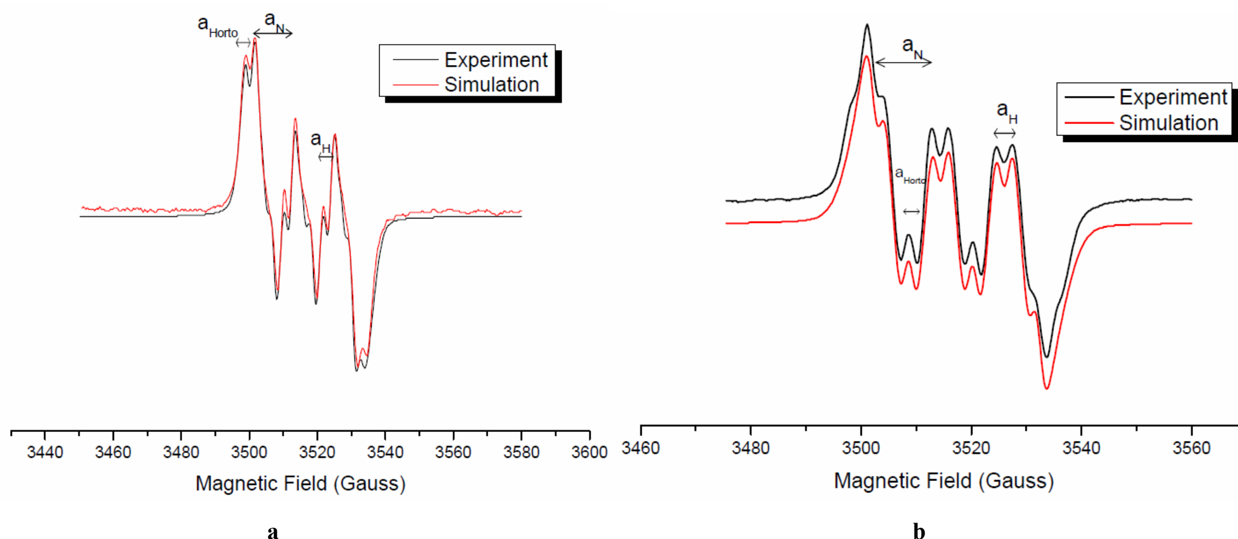


Fig. 3 – Experimental and simulated EPR spectra of nitroxide adducts generated in the presence of Fenton reagent solution by: a) diphenyl-nitrone **12** and b) phenothiazinyl-nitrone **19**.

Table 2

EPR spectral parameters of paramagnetic nitroxide adducts formed by hydroxyl radical scavenging

Nitrone	$R^1(R^3)$	R^2 (R^4)	g	a_N	a_H	a_H	a_{N1}
5	H	*m,p*-diMeO	2.003	11.506	4.518	1.170	-
8	H	*p*-OH	2.003	11.715	4.617	2.186	-
11	*p*-Cl	*o*-NO_2	2.003	11.579	4.396	2.659	2.710
12	*p*-Cl	*m*-OH	2.003	11.504	4.819	3.020	-
13	*p*-Cl	*p*-OH	2.003	11.554	4.770	3.024	-
19	Me	Ph	2.003	11.760	4.417	1.925	-
22	Me	*m*-Br-Ph	2.003	11.452	4.495	2.869	-
25	Me	Et	2.003	11.709	4.438	2.907	-
26	Et	Et	2.003	11.606	4.653	2.962	-

DNA binding interaction

The interaction of diphenyl nitrones **6, 13** and phenothiazinyl nitrones **26, 27, 28** with plasmid DNA was studied by means of electrophoresis on agarose gel at pH=8. As it can be seen in Fig. 4, no modifications of the plasmid DNA were detected, thus leading to the conclusion that the investigated nitrones are not capable of interactions with plasmid DNA.

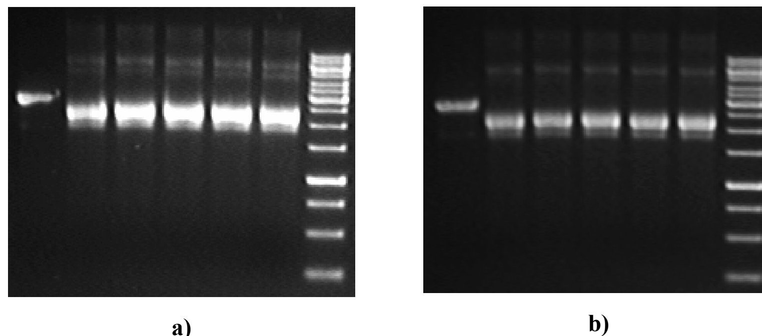
EXPERIMENTAL

Nitrones **1-28** were prepared according to previously reported method.[16] All reagents and solvents were purchased and used without purification.

UV-Vis measurements were performed with Perkin Elmer Lambda 35 UV-Vis Spectrometer in methanol/water 2:1 v/v solvent system using quartz cuvettes. EPR spectra for nitrones for nitrones were recorded in continuous wave (CW), at room temperature, with a Bruker Elexsys 580-EPR spectrometer, operating at the X-band (9.5 GHz) and using the following parameters: modulation frequency 100 kHz, microwave power 3.768 mW, modulation amplitude 2G.

DPPH assay

2 mL (94 µM) from a freshly prepared DPPH solution in methanol was mixed with 1 mL (10^{-4} M) methanolic solution of the corresponding nitrone. The absorbance was measured at 520 nm immediately after mixing (t_0), after 60 (t_{60}) and after 120 (t_{120}) minutes against a blank solution, containing only DPPH solution in methanol. The samples were kept in the dark.



a) b)

Fig. 4 – Interaction of chemical complexes with plasmid DNA: a) – compound **6**; b) – compound **26**; Lane 1 – liniar plasmid DNA (digested with restriction enzyme *Eco*RI); 2 – plasmid DNA; 3 to 6 – plasmid DNA with 4, 6, 8 and 10µl of tested compound; 7 – GeneRuler 1 kb DNA ladder (ThermoScientific).

EPR Spectroscopic assay

50 µL (3.32 mM) solution of Iron(II) sulphate, 10 µL (6.6 mM) solution of H_2O_2 and 100 mL (100 mM) nitrone solution were mixed at room temperature. The EPR spectra were recorded immediately after mixing in quartz capillaries (0.9 mm) filled with 50 µL of sample. WinSim package was used for the simulation of EPR spectra.

DNA binding

Tested chemical complexes were mixed with 800 ng plasmid DNA (pTZ57R) at different ratios. The plasmid DNA was purified from an overnight culture of *Escherichia coli* DH5α cells using the DNA-spin™ Plasmid DNA Purification Kit (iNtRON). The mixtures were loaded into an agarose gel 1% in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) without ethidium bromide. After migration, gels were stained for 30 min in water containing ethidium bromide (1.5 µg/mL), according to standard procedures.[17]

CONCLUSIONS

Based on the DPPH assay, diphenyl-nitrones may be considered excellent spin traps for hydrazyl free radical, as most of them leaded to fast kinetic processes with a time required to reach the steady state saturation with high conversions of approximately 1 hour. Phenothiazinyl nitrones showed very low reactivity in DPPH spin trapping experiments.

EPR spectroscopic study demonstrated that both diphenyl- and phenothiazinyl-nitrones are efficient spin trapping agents for hydroxyl free radicals generated by Fenton reagent.

According to the electrophoresis experiments, the investigated nitrones are not capable of interactions with circular plasmid DNA.

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