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THE ROLE OF NO GROUP IN THE ANTIOXIDANT PROPERTIES OF PHENOLS

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The antioxidant activity of a series of phenolic compounds, propofol included, and of their para-nitroso derivatives has been studied in solution and in a natural contest. In the first case we tested the scavenging effect of the molecules on the DPPH radical, while in the second one we considered their inhibitory action on microsomal lipid peroxidation. Data show that the antioxidant properties of substituted phenols depend on the balance between the counteracting steric and electronic effects of their alkyl substituents. From this point of view, the optimum balance is expressed by propofol which acts as a very good scavenger of free radicals and as a potent inhibitor of lipid peroxidation. On the contrary para-nitroso derivative of propofol is scarcely effective in both situations: this is a general behaviour of all the nitroso phenols, and it can be referred to an overcoming electron-withdrawing effect of the NO group.

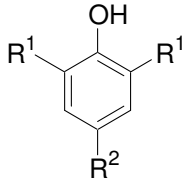
INTRODUCTION

In a recent study (Stevanato, Momo, Marian, Rigobello, Bindoli, Bragadin, Vincenti & Scutari, 2002) on 2,6-diisopropylphenol (propofol or DPP), we demonstrated that 2,6-diisopropyl-4-nitrosophenol (DPPNO), a product of reaction between propofol and S-nitrosoglutathione (GSNO), alters mitochondrial respiration to an extent greater than GSNO and propofol alone. Further DPPNO acts on the phospholipid organization of biological membranes as a stronger perturbating agent than DPP, accumulating, as suggested by ESR spectra, into the interfacial region of the bilayer (Momo, Fabris, Bindoli, Scutari & Stevanato, 2002). This behaviour has been generalized and confirmed by comparing 2,6-dialkylphenols to the corresponding 2,6-dialkyl-4-nitrosophenols (Momo, Fabris, Wisniewska, Bindoli, Scutari & Stevanato, 2003).

The present work is a natural prosecution of this study. In fact, among other effects, general anaesthetics might behave either as antioxidants or prooxidants (Bindoli, Rigobello & Scutari, 2001) therefore influencing the oxidation conditions of biological membranes; in particular propofol acts as a potent antioxidant (Bao, Williamson, Tew, Plumb, Lambert, Jones & Menon, 1998) and, for this reason, the various substituted phenols, and their 4-nitrosoderivatives (Tab. 1) were tested and compared for their reactivity towards the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and for their inhibitory action on lipid peroxidation.

The experimental results show that the antioxi-

Tab. 1. List of the phenolic compounds.

	a: R^1=H, R^2=H	phenol (POH)
	b: R^1=Me, R^2=H	2,6-dimethylphenol (DMP)
	c: R^1=*i*-Pr, R^2=H	2,6-diisopropylphenol (DPP)
	d: R^1=*t*-Bu, R^2=H	2,6-diterbutylphenol (DTP)
	e: R^1=Me, R^2=NO	2,6-dimethyl-4-nitrosophenol (DMPNO)
	f: R^1=*i*-Pr, R^2=NO	2,6-diisopropyl-4-nitrosophenol (DPPNO)
	g: R^1=*t*-Bu, R^2=NO	2,6-diterbutyl-4-nitrosophenol (DTPNO)
	h: R^1=*t*-Bu, R^2=Me	2,6-diterbutyl-4-methylphenol (BHT)

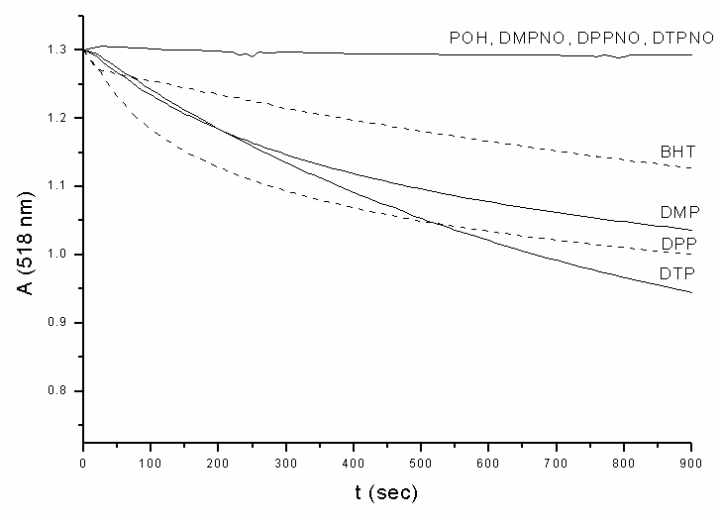


Fig. 1. Time courses of the absorbance values recorded at 518 nm of an ethanol solution containing DPPH 100 μM and phenolic compounds 25 μM, 25°C.

dant properties of these molecules depend on their hydrophobic characteristics and on the steric and electronic effects of their substituents. However, the introduction of the nitroso group in the 4-position almost completely removes the antioxidant properties of the compounds. Taking into account the potential formation of nitric oxide and its derivatives in biological systems, the nitrosation of aromatic ring of antioxidant molecules and the consequent loss of antioxidant capacity, can be considered an *in vivo* occurring condition.

MATERIALS AND METHODS

Chemicals

All chemicals, of the highest available quality, were obtained from Sigma Chemical Co. (St. Louis, USA), while the solutions were prepared with quality milliQ water.

Synthesis of nitrosophenols

The synthesis of 2,6-dialkyl-4-nitrosophenols was carried out following the same procedure as for 2,6-ditertbutyl-4-nitrosophenol, as described in Momo *et al.* (2003).

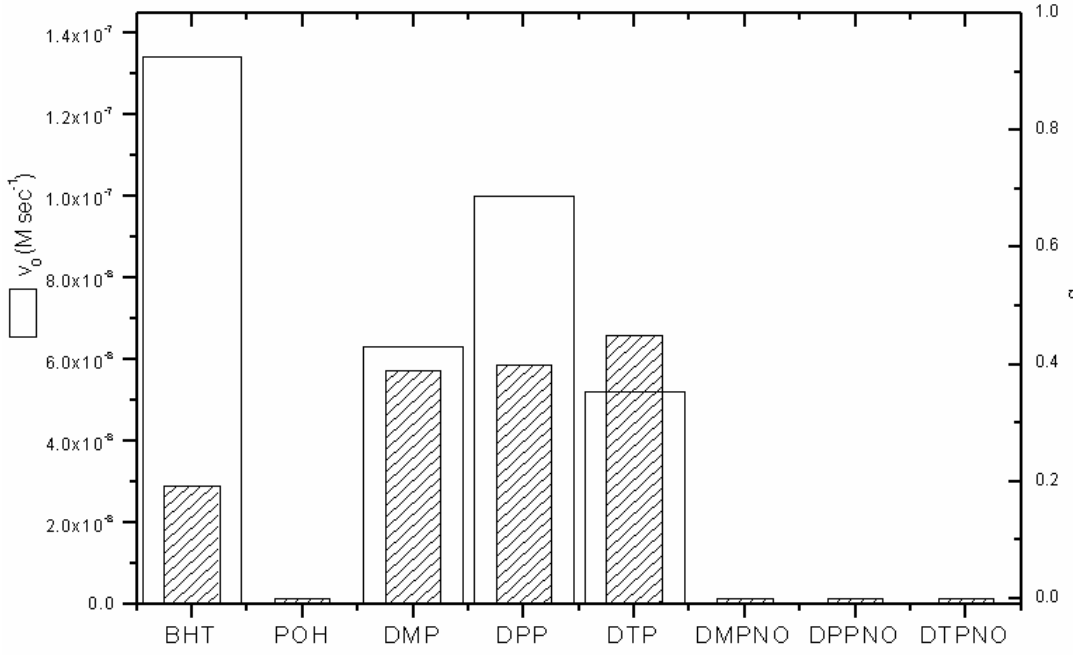


Fig. 2. DPPH scavenging activity of the phenol derivatives measured spectrophotometrically as initial rate of the reaction (Vo) and at the steady state by ESR spectroscopy (RSA_{15}).

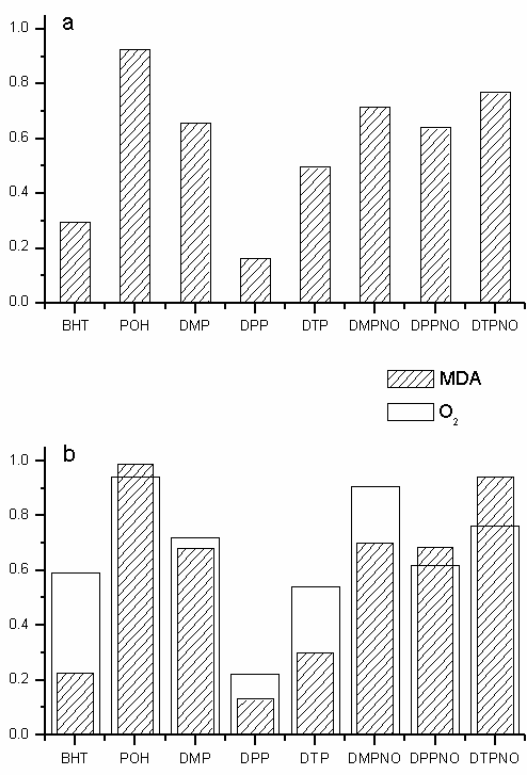


Fig. 3. Effect of the phenol derivatives on lipid peroxidation elicited by a) Fe^{2+}/ascorbate, b) cytochrome P450/cumene hydroperoxide in rat liver microsomes. Rat liver microsomes (1 mg ml^{-1}) were incubated at 25 °C in 0.125 M KCl, 20 mM Hepes/Tris (pH 7.4) and in the presence of the phenols derivatives (20 μM). Y axis is the ratio between MDA formation with and without phenols; analogously for the oxygen uptake.

Antioxidant activity, radical scavenging effect

The radical scavenging activity was estimated by the DPPH (2,2'-diphenyl-1-picrylhydrazyl) method, which is based on the transfer of one hydrogen atom from phenolic compounds to DPPH (Blois, 1958).

The disappearance of DPPH can be followed spectrophotometrically at 518 nm and the fall of absorbance in the first 20 s was used to determine the initial rate of the scavenging reaction (V_o) at 25°C.

Spectrophotometric measurements were recorded on a UV-VIS Beckman DU 640 instrument, equipped with a thermostated quartz cell.

Alternatively, we can use the concentrations at the equilibrium or near the equilibrium, and define the radical scavenging action (RSA) of phenols through the relative concentration of consumed DPPH at a given time:

$$RSA_{15} = 1 - [DPPH]_{15}/[DPPH]_o,$$

where $[DPPH]_{15}$ and $[DPPH]_o$ are the concentrations of DPPH in the presence and in the absence of a phenolic compound after 15 min. The concentrations were determined by EPR spectroscopy, monitoring the central hyperfine line of the DPPH EPR spectrum.

EPR measurements were performed on a Bruker ER200, 9 GHz spectrometer at 20 mW microwave power.

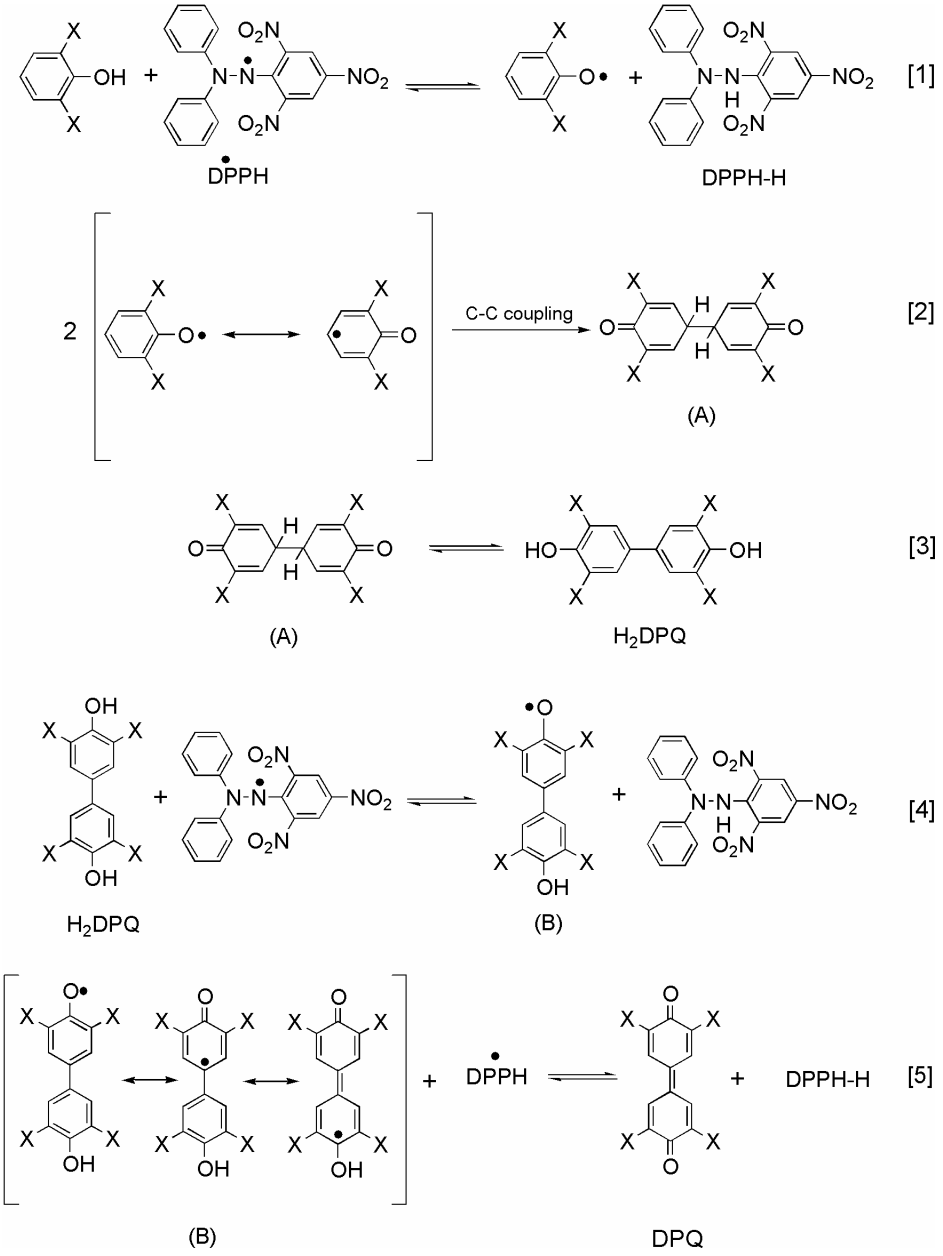
Antioxidant activity, microsomal lipid peroxidation

Liver microsomes were prepared according to Ernster and Nordenbrand (1967) and proteins were measured with the biuret test. Microsomal lipid peroxidation was measured as malondialdehyde (MDA) formation using the 2-thiobarbituric acid assay (Buege & Aust, 1978) or as oxygen uptake estimated polarographically using a Clark-type oxygen electrode connected to a computerized system.

RESULTS

Antioxidant activity

In literature the antioxidant activity, expressed as the ability of scavenging of DPPH, is determined as initial rate of the reaction (V_o) (Ancerewicz, Migliavacca, Carrupt, Testa, Brée, Zini, Tillement, Labidalle, Guyot, Chauvet-Monges, Crevat & Le Ridant, 1998, Basly, Marre-Fournier, Le Bail, Habrioux & Chulia, 2000), as diminution of the concentration of DPPH after a fixed time (RSA) (Fauconneau, Waffo-Teguo, Huguet, Barrier, Decendit & Merillon, 1997; Tait, Ganzerli & Di Bella, 1996; Tang, Kerry, Sheehan & Buckley, 2002) or as the effective concentration (CE) of antioxidant required to decrease DPPH by 50% or by 20% after a fixed time (Ancerewicz *et al*., 1998; Uto, Ishibashi, Nagasawa & Hori, 2002; Arefiev, Domnina, Komarova & Yu, 2000). Even though they are widely used, both RSA and CE are not totally unambiguous parameters. In fact, due to a number of secondary reactions, which in biological system can be really unpredictable, the DPPH concentration at the end of the reaction could represent hardly the equilibrium concentration of the first step scavenging reaction and the true antioxidant properties of the molecule under test. Moreover, as it is clearly shown in Fig. 1, the plots of the kinetics of DPPH, in the presence of dopants, cross, so that RSA and CE may produce different values of the antioxidant activity as a function of the reading-time. Anyway, in the following, we



Scheme 1.

will present the experimental results in terms of RSA after 15 min. (RSA_{15}) as a useful source of information, together with V_o, on the mechanisms and equilibria of the reactions.

On the basis of V_o values (Fig. 2) the compounds can be divided in two well separated groups; phenol and the nitrosophenols which do not show any antioxidant activity, and the 2,6-dialkylphenols which have an antioxidant potentiality comparable with that of BHT, adopted as reference value.

Data show that the antioxidant activity depends strongly on the nature of substituents and that, looking at the null value of phenol, the phenolic OH group is not sufficient by itself to give antioxidant properties to a phenolic compound.

The electron-donating effects of alkyl groups is necessary to stabilize the phenoxyl radicals and to confer to phenols their antioxidant characteristics; on this basis, the tertbutyl group should be expected to be more significant than the isopropyl and methyl groups in the order; as a matter of fact, excessively bulky groups around the hydroxyl group conflict with the radical scavenging capacity because their steric hindrance reduces the accessibility of the OH group. In propofol the electronic and steric effects seem to excellently balance. Anyway the nitroso group, in the 4 position of the

Tab. 2. Values of the reagents concentration at t = 0 ($[]_o$) and at t = 15 min ($[]_{eq}$); $[DPPH]_{consumed} = [DPPH]_o - [DPPH]_{eq}$.

$[XPOH]_0$ (μM)	$[DPPH]_0$ (μM)	[DPPH]consumed (μM)	[DPQ]eq (μM)	[DPPH]consumed/ [DPQ]eq
DMP 25	100	36	1.4	25.7
DPP 25	100	42	7.0	6.0
DTP 25	100	54	10.0	5.4
DTP 12.5	100	28	7.4	3.8

phenolic ring, has electron-withdrawing properties so strong to eliminate the antioxidant capacity.

A similar trend was obtained from the measures of microsomal lipid peroxidation; Figures 3.a and 3.b report the effects of the phenolic compounds on lipid peroxidation in rat liver microsomes elicited by Fe^{2+}/ascorbate and by cytochrome P450/cumene hydroperoxide respectively. Propofol appears as the most efficient inhibitor of lipid peroxidation, DTP has an intermediate behaviour, while DMP is scarcely active as well as the nitroso phenols.

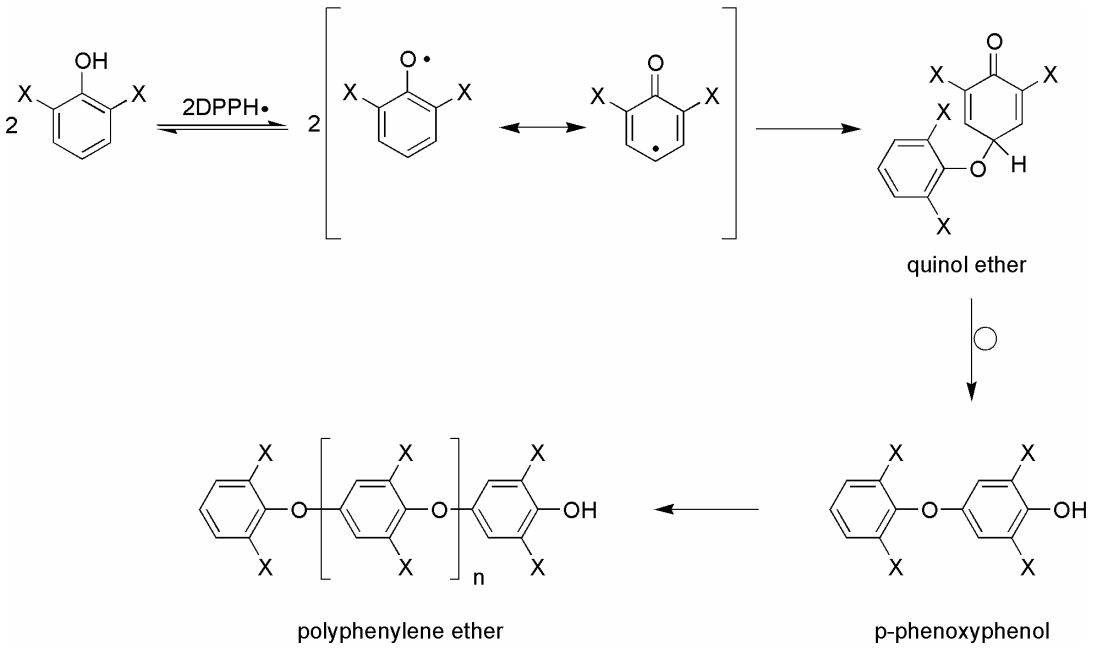
On the bases of these results propofol appears to act both as a very good scavenger of free radicals and also as a potent inhibitor of lipid peroxidation, the nitrosoderivatives are poorly efficient in eliciting a consistent antioxidant effect and DMP and DTP exhibits an intermediate behavior which could be related to different positions inside the membranes.

Mechanisms of reactions

The UV-VIS spectra of decomposition of DPPH in the presence of the phenols show the formation of a new band at 420 nm which can be attributed (Mihailovic & Cekovic, 1971a, Kohara, Fujiyama, Iwai, Nishiyama & Tsuruya, 2000) to a diphenoquinone derivative (DPQ), according with Scheme 1.

The first step of the reaction provides that the phenoxyl radical is generated by abstracting a hydrogen atom from the phenolic compound to DPPH [1]. Then the phenoxyl radical with the unpaired electron at para-position through the C-C coupling forms an unstable dimer (A) [2] which is rearranged tautomerically to H_2DPQ [3].

H_2DPQ reacts with two molecules of DPPH to give first an intermediate (B) and then DPQ. If all the phenolic compounds follow this mechanism of reaction, the ratio between the DPPH consumed and DPQ formed should be four:



Scheme 2.

$$2XPOH + 4DPPH \rightleftharpoons DPQ + 4DPPH\text{-}H$$

As it appears from Tab.2 this is nearly true only for substituents of high steric hindrance. In fact the poor screening of the OH group favours the occurring of other radical reactions, like for example that reported in Scheme 2 (Mihailovic & Cekovic, 1971b).

CONCLUSIONS

The antioxidant properties of some 2,6 dialkylphenols and of their 4-nitroso derivatives has been studied in solution and in a biological system. The different sensitivity in interacting with the stable free radical DPPH, or in inhibiting microsomal lipid peroxidation, demonstrates that substituent characteristics are important and involve polar, electronic and steric factors: the overall sum of these counterbalancing effects makes propofol to act both as a very good scavenger of free radicals and also as a potent inhibitor of lipid peroxidation.
All the nitrosoderivatives demonstrated themselves poorly efficient in eliciting a consistent antioxidant effect, so that a major conclusion resulting from this work is that the introduction of the nitroso group in the 4-position of a phenolic ring almost completely removes the antioxidant properties of the compound. This observation may be of general interest because, in biological systems, it can be easily expected that phenolic antioxidants could interact with nitric oxide losing their scavenging activity.

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