ANTIOXIDANT AND HEPATOPROTECTOR ACTIVITY OF WATER-SOLUBLE 4-PROPYLPHENOLS CONTAINING HYDROPHILIC GROUPS IN ALKYL CHAINS

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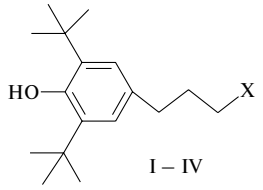
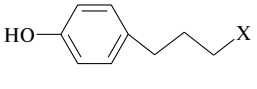
One of the main mechanisms in the pathogenesis of toxic liver damage is destabilization of the membranes of hepatocytes as a result of lipid peroxidation (LPO). In the case of such liver disorders, a pronounced protective action is offered by phenolic antioxidants such as α-tocopherol [1], potassium phenosane, synthetic derivatives of hydroxycynnamic acids, ionol, ubiquinone, and some other natural compounds [2]. According to [3], some derivatives of hydroxycynnamic acids are superior to α-tocopherol with respect to hepatoprotector action. The high hepatoprotector activity of hydroxycynnamic acid derivatives can be due to their hydrophilicity, which accounts for the high rate of transport and biological accessibility of these molecules.

Below we report on a comparative study of the antioxidant activity and hepatoprotector properties of a series of water-soluble 2,6-di-*tert*-butyl-4-propylphenol derivatives (I – IV) and some analogous compounds (V – VIII) not containing *tert*-butyl groups.



I – IV



V – VIII

X = SO_3Na (I, V), SSO_3Na (II, VI), $SC(NH_2)_2^+Cl^-$ (III, VII), $N(C_2H_5)_2 \cdot HCl$ (IV, VIII)

The reference substances were 2,6-di-*tert*-butyl-4-methylphenol (ionol, Acros Organics, USA), β-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate potassium salt (potassium phenosane, Russia), and bis-(gamma-*L*-glutamyl)-*L*-cysteinyl-bis-glycine disodium salt (glutoxime, Russia).

EXPERIMENTAL CHEMICAL PART

Compounds I – VII were synthesized from the corresponding 3-(4′-hydroxyaryl)-1-halogenopropanes as described previously [4, 5] and purified by double recrystallization from ethanol. The main compound content in the samples studied was 97 – 99%. The ^1H NMR spectrum of compound VIII was measured on a 500-MHz Bruker spectrometer (Germany) using $Si(CH_3)_4$ as the external standard. The IR spectrum was recorded using a Vektor 22 Fourier-transform spectrometer (Germany) using samples pelletized with KBr (150 : 1). The melting points were determined using a PTP device (Russia).

[3-(4′-Hydroxyphenyl)propyl]diethylamine (VIII). To a mixture of 4.15 ml (40 mmole) of diethylamine, 2.9 g (20 mmole) of K_2CO_3, and 20 ml dioxane at 50°C was gradually (during 0.5 h) added with stirring a solution of 2.15 g (10 mmole) of 3-(4′-hydroxyphenyl)-1-bromopropane in 20 ml of dioxane. The reaction mixture was kept for 5 h at 50°C, cooled, and extracted with benzene. The benzene extract was washed with a saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4, and saturated with dry hydrogen chloride. The precipitate was separated by filtration, washed with benzene, dried, and recrystallized from water to obtain 0.96 g (40%) of hydrochloride VIII; m.p., 150 – 152°C; ^1H NMR spectrum in D_2O (δ, ppm): 1.060 – 1.090 (t, 6H, $CH_2\underline{CH_3}$), 1.808 (m, 2H, $ArCH_2\underline{CH_2}$), 2.470 – 2.499 (t, 2H, $Ar\underline{CH_2}$), 2.903 – 2.936 (t, 2H, $ArCH_2CH_2\underline{CH_2}N$), 2.986 – 3.029 (q, 4H, $N\underline{CH_2}CH_3$), 6.705 – 6.721 (d, 2H, H_{arom}), 7.012 – 7.028 (d, 2H, H_{arom}); IR spectrum (ν, cm^{-1}): 2958 (CH), 2666 (NH^+); $C_{13}H_{22}ClNO$.

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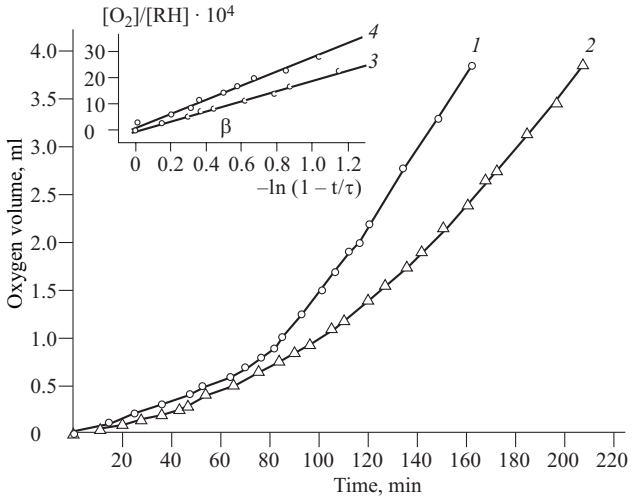


Fig. 1. The kinetics of oxygen absorption in the course of AIBN-initiated methyl oleate oxidation in chlorobenzene at 60°C in the presence of (*1*) ionol (0.3 μM) and (*2*) isothiuronium chloride III (0.5 μM); plots *3* and *4* in the inset represent anamorphoses of curves *1* and *2*, respectively.

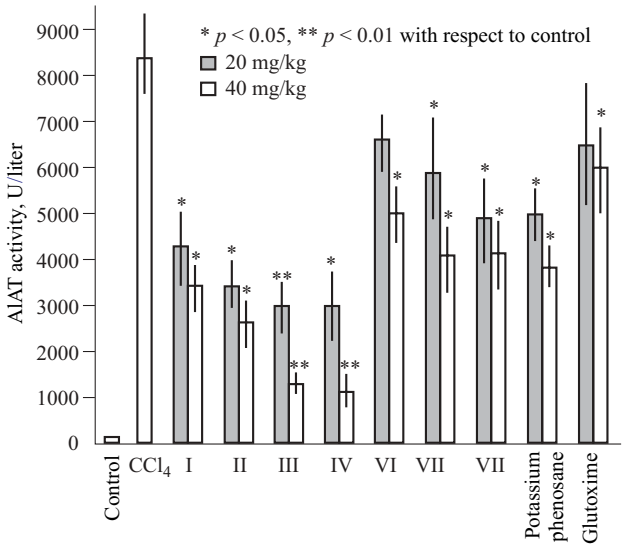


Fig. 2. A histogram showing the effect of compounds I – VIII on the ALT activity in the blood serum of mice with tetrachloromethane hepatitis.

Determining antiradical activity. The antiradical activity of compounds I – IV, ionol, and potassium phenosane was characterized by the rate constant (k_7) of their interaction with hydroperoxide radicals of methyl oleate antiradical activity and the inhibition coefficient (f) equal to the number of chains terminated by a single antioxidant molecule. The k_7 and f values were determined using a model reaction of the AIBN-initiated methyl oleate oxidation in chlorobenzene at 60°C. Methyl oleate (Acros Organics, USA) was preliminary purified from peroxides by double distillation in vacuum (~ 170°C/2 – 3 Torr). The initiator AIBN (Acros Organics) was purified by recrystallization from ethanol [6]. The working concentrations of components in a sample (20°C) were as follows: methyl oleate, 1.48 M; AIBN, 12 mM; antioxidant, 0.3 – 0.6 mM.

The volume of absorbed oxygen was determined by a volumetric technique described in [6]. The oxygen pressure in the system was 1 atm and the volume of oxidized sample was 5 ml (20°C). The induction period (τ) was determined graphically as the point of intersection of two tangents to the kinetic curve, the slopes of which amounted to 0.5 and 0.75 of the slope of a straight line corresponding to the noninhibited oxidation reaction [6].

Figure 1 shows the kinetic curves of oxygen absorption and their anamorphoses plotted as $[O_2]/[RH]$ versus $-\ln(1 - t/\tau)$ for isothiuronium chloride III and ionol. Using the slopes of anamorphoses, it was possible to determine the k_2/k_7 ratio which, for the k_2 value taken from [7], yielded $k_7 = (2.6 \pm 0.4) \times 10^4$ liter/(mole · sec). The coefficient f was determined using the formula

$$f_{AO} = \frac{f_{ionol} \cdot [ionol] \cdot \tau_{AO}}{\tau_{ionol} \cdot [AO]},$$

where $f_{ionol} = 2$ [7] (subscript AO refers to antioxidant).

TABLE 1. Characteristics of the Antiradical and General Antioxidant Activity of the Synthesized Compounds

Compound	k_7, 10^{-4} liter/(mole sec)	f	ID_{50} for LPO, μM	
			Cu^{2+}	Fe^{2+}
I	2.3 ± 0.3	1.1 ± 0.1	23	36
II	2.8 ± 0.5	1.6 ± 0.1	15	15
III	2.7 ± 0.3	1.2 ± 0.1	14	13
IV	1.9 ± 0.2	1.3 ± 0.1	13	14
V	…	…	2800	3000
VI	…	…	400	380
VII	…	…	470	530
VIII	…	…	550	470
Potassium phenosane	1.5 ± 0.1	1.3 ± 0.1	18.5	17
Ionol	2.6 ± 0.4	2.0	15	15

EXPERIMENTAL BIOLOGICAL PART

The general antioxidant activity of the synthesized compounds was evaluated by determining their ability to inhibit the formation of malonic dialdehyde (MDA) during the incubation of low-density lipoproteins (LDLPs) with ambivalent metal ions (Cu^{2+}, Fe^{2+}). The LDLP fraction (1.019 – 1.063 g/ml) was isolated from the blood of donors by the method of sequential ultracentrifugation in KBr solution density gradient (1050000g, 20 h; 105000g, 24 h) [8].

The LDLPs were purified from residual KBr by chromatography on a Sephadex G_{25} column. The protein content in LDLPs was determined by the Lowry method. The isolated LDLPs were oxidized at 37°C in the presence of $CuSO_4$ (5 μM) or $FeSO_4$ (25 μM) [9]. The antioxidant concentrations were 1, 10, or 100 μM. Accumulation of the lipid peroxidation (LPO) product (MDA) capable of interacting with thiobarbituric acid was monitored by fluorescence spectroscopy (Hitachi Model P_{3000} spectrofluorimeter) [10]. The antioxidant properties of the synthesized compounds were evaluated by their effective concentrations producing a 50% inhibition (ID_{50}) of the MDA accumulation during a 30-min incubation of LDLPs in the presence of metal ions.

The experimental toxic hepatitis in (C57Bl/6XCBA)F_1 mice was induced by intraperitoneal injections of a 10% tetrachloromethane solution in olive oil (0.2 ml per animal weighing 25 g). The synthesized compounds were dissolved in physiological solution and introduced intraperitoneally 2 h before tetrachloromethane. The phenolic compounds were used in the concentrations of 5×10^{-5} and 1×10^{-4} M/kg, which corresponds to 20 and 40 mg/kg of glutoxime. The degree of liver damage was evaluated by the alanineaminotransferase (ALT) activity in the blood serum [11] determined 24 h after tetrachloromethane introduction. The ALT activity was estimated by an UV technique using a Biocon reagent kit [12] and expressed in units of enzyme activity per liter blood serum (U/liter). The experimental data were statistically processed in terms of the classical Student's criterion.

RESULTS AND DISCUSSION

Experimental data on the antioxidant activity of the synthesized compounds are presented in Table 1. As can be seen, all the water-soluble 2,6-di-*tert*-butyl-4-alkylphenols (I – IV) exhibit similar k_7 values, close to the corresponding reaction rate constant for ionol. This result is quite natural, since the ionogenic fragments separated from the aromatic nucleus by a hydrocarbon chain cannot significantly influence the reactivity of the phenolic OH group or the stability of the phenoxy radical. At the same time, compounds I – IV and potassium phenosane are inferior to ionol with respect to the inhibition coefficient f.

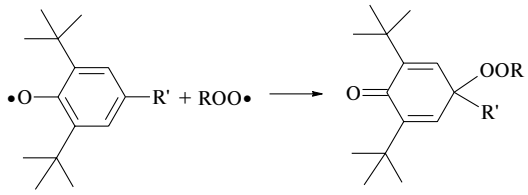
As is known [13], the antioxidant activity of phenolic compounds is based on the reaction of PhOH with a hydroperoxide radical:

$$PhOH + ROO^{\cdot} \rightarrow PhO^{\cdot} + ROOH.$$

Under the conditions of initiated oxidation, the main pathway of decomposition of the phenoxy radicals $PhO^{\cdot}$ is the formation of quinoid peroxides QP via the reaction

$$PhO^{\cdot} + ROO^{\cdot} \rightarrow QP$$

Because of certain features of the spin density distribution and spatial hindrances with respect to *ortho* position, the phenoxy radicals of 2,6-di-*tert*-butyl-4-alkylphenols attach $ROO^{\cdot}$ so as to form predominantly the *para* isomers of QP:



Apparently, the large volume and considerable polarity of the ionogenic fragment R′ = $CH_2CH_2CH_2X$ hinder the attack of the hydroperoxide radical of methyl oleate at the *para* position of phenol ring. This leads to a decrease in the reaction rate constant k_7 and, hence, in the inhibition coefficient f.

The values of k_7 for 2,6-di-*tert*-butyl-4-alkylphenols are usually close to the rate constants for analogous compounds not substituted at the *ortho* position [13]. However, compounds V – VIII virtually do not inhibit the AIBN-initiated oxidation of methyl oleate. This is probably explained by the low stability of the phenoxy radicals formed by these compounds.

Derivatives of the spatially-hindered phenols I – IV exhibit a pronounced inhibition of the LPO of LDLPs, significantly exceeding in this respect the *ortho*-unsubstituted analogs V – VIII. Characterized by a lower antiradical activity than ionol, compounds II – IV are nevertheless comparable with ionol in the ability to inhibit the LPO of LDLPs. The difference between the antiradical and antioxidant activity of these phenols can be related to the antioxidant properties of ionogenic fragments – thiosulfonate, isothiuronium, and alkylammonium. These groups probably account for the antioxidant properties of the *ortho*-unsubstituted phenols VI – VIII. Note that sulfonate V virtually does not affect the oxidation of LDLPs.

With respect to the ability of reducing ALT activity on the toxic hepatitis model, the compounds studied can be arranged in the following order: alkylammonium IV ≈ isothiuronium III > thiosulfonate II > sulfonate I > potassium phenosane ≈ alkylammonium VIII > isothiuronium VII > thiosulfonate VI > glutoxime (Fig. 2).

Thus, the results of our investigation indicate that water-soluble 2,6-di-(*tert*-butyl)-4-(*n*-propyl)phenol derivatives, containing thiosulfonate, isothiuronium, and alkylammonium groups (compounds II – IV) as hydrophilic fragments, can effectively inhibit oxidation of LDLPs *in vitro* and produce a hepatoprotector effect *in vivo*. Therefore, these substances are worthy of further preclinical investigation as hepatoprotector drugs.

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