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EXPERIMENTAL ARTICLES

Protection of *Saccharomyces cerevisiae* against Oxidative and Radiation-Caused Damage by Alkylhydroxybenzenes

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Abstract—The effects of C_7-alkylhydroxybenzene (C_7-AHB) and *p*-hydroxyethylphenol (tyrosol), chemical analogs of microbial anabiosis autoregulators, on the viability of yeast cells under oxidative stress were investigated. The stress was caused by reactive oxygen species (ROS) produced under γ irradiation of cell suspensions using doses of 10–150 krad at an intensity of 194 rad/s or by singlet oxygen generated in cells photosensitized with chlorin e_6 (10 μg/l). C_7-AHB was found to exert a protective effect. The addition of 0.05–0.16 vol % of C_7-AHB to cell suspensions 30 min before irradiation protected yeast cells from γ radiation (50 krad). The protective effect of C_7-AHB manifested itself both in the preservation of cell viability during irradiation and in the recovery of their capacity to proliferate after irradiation. In our studies on photodynamic cell inactivation, the fact that the phenolic antioxidant C_7-AHB protects cells from intracellular singlet oxygen was revealed for the first time. The analysis of difference absorption spectra of oxidized derivatives of C_7-AHB demonstrated that the protective mechanism of C_7-AHB involves the scavenging of ROS resulting from oxidative stress. The fact that tyrosol failed to perform a photoprotective function suggests that the antioxidant properties of microbial C_7-AHB are not related to its chaperon functions. The results obtained make an important addition to the spectrum of known antioxidant and antistress effects of phenolic compounds.

Key words: stress, yeast, protection, alkylhydroxybenzenes, antioxidant activity, radioprotective activity.

Generation of free radicals, including reactive oxygen species (ROS), is an inevitable phenomenon associated with the aerobic lifestyle. A prerequisite for the functioning and development of cells in an oxygen-containing environment is the existence of protective systems involving specialized enzymes and low-molecular-weight antioxidants. Factors that elicit intracellular ROS accumulation and the disruption of the cell's protective systems cause oxidative stress accompanied by the impairment of subcellular structures. This results in a number of physiological and pathophysiological phenomena, including such processes as aging, the development of malignant tumors, etc. [1].

Recently, much attention has been given to the involvement of low-molecular-weight metabolites, particularly the extracellular autoregulators, in the protection of cells from oxidative stress. In addition to enzymatic protective systems, ROS quenching involves lipids, amino acids, nucleotides, and other compounds. Phenolic compounds are efficient nonenzymatic protectors preventing oxidative stress; these compounds are widespread in microbes and plants [2]. In particular, microbial alkylhydroxybenzenes (AHB) have been shown to possess pronounced antioxidant activity. They perform the function of anabiosis autoinducers, being referred to as factors d_1 [3, 4]. AHB accumulate in developing microbial cultures to the threshold level and cause their transition to the stationary phase. A further increase in the AHB concentration results in the generation of resting forms that are resistant to deleterious factors (including γ irradiation and oxidative stress) [5]. The mechanism of the effect of AHB as anabiosis autoinducers at the molecular level is based on their capacity to increase membrane microviscosity and to induce cell dehydration [6] and on their chemical chaperon properties [7]. AHB, by forming labile complexes with enzyme molecules, change their conformation. This results in changes in their catalytic activity and a significant increase in the stability of protein biopolymers [7, 8]. The involvement of AHB in the nonenzymatic system of antiradical protection of cell structures is of particular importance for resting cells with inhibited metabolic activities, including activities of antioxidant enzymes [9].

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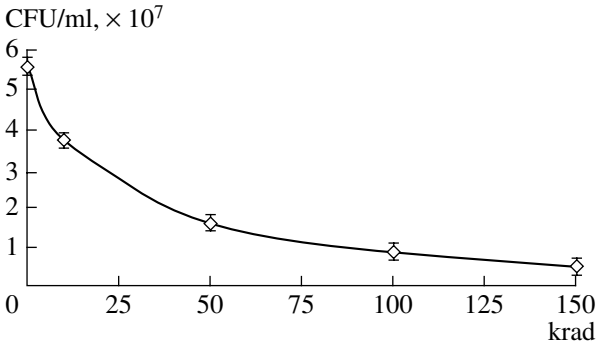

Fig. 1. Influence of the γ-radiation dose on the viability of the yeast *S. cerevisiae.*

Thus far, virtually no studies have been conducted on the protective effect of AHB in vegetative cells under stress. One of the protective effects of AHB involves their operation as ROS quenchers: phenolic compounds have been shown to serve as traps for reactive nitrogen and oxygen species such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide [10–12]. Nevertheless, the properties of phenolic compounds that enable them to protect biological systems from singlet oxygen–mediated reactions have not yet been sufficiently well understood.

Therefore, it seemed expedient to investigate the influence of individual AHB on the viability of yeast cells during oxidative stress caused by ROS formed under γ irradiation or generated in photosensitized cells.

MATERIALS AND METHODS

Strain *Saccharomyces cerevisiae* VKM Y-380 was used in our studies. The yeast was grown in liquid medium (3.5°B wort) in 250-ml flasks containing 50 ml of medium on a shaker (200 rpm) at 28–30°C. An exponential-phase culture served as the inoculum, which was added to yield an initial optical density of 0.2 (Specord, λ = 660 nm, l = 10 mm). Yeast viability was determined from the colony-forming unit (CFU) number, by preparing serial dilutions of cell suspensions and plating them onto wort agar. Inoculation was performed 30 min and 2 h after γ irradiation or monochromic laser illumination.

The studies were conducted using chemical analogs of d_1, bacterial autoregulatory factors. These analogs were C_7-alkylhydroxybenzene (C_7-AHB) in water-soluble form and tyrosol (*p*-hydroxyethylphenol) at concentrations of 0.05–0.16 vol %, which were synthesized at the Lomonosov Moscow Institute of Fine Chemical Technology with a purity degree of 99.9%. The radiation-elicited oxidation reaction was modeled under stationary radiolysis conditions in a γ-^{60}Co setup (GURKh 100000, Frumkin Institute of Electrochemistry, Russian Academy of Sciences) at an irradiation intensity of 194 rad/s.

To investigate the antioxidant properties of AHB with reference to singlet oxygen ($^1O_2$), the photosensitizer (PS) chlorin e_6 was used [12]. PS was added to yeast suspensions at a concentration of 10 μg/l, and the cells were illuminated with a monochromatic laser (662 nm, 20 mW/cm^2). The absorption spectra of AHB solutions were recorded with a Hitachi-550 spectrophotometer (Japan).

Three independent series of experiments were conducted; three repeats of each experiment were done. The results presented are average values. The statistical treatment of the data was carried out using Student's *t*-test. The significance level P was assumed to be below 0.05.

RESULTS

Biological systems are significantly different in respect to their sensitivity to radiation. It was necessary, therefore, to select the γ-radiation dose that corresponded to a cell survival rate of less than 50%. For this purpose, we treated an exponential-phase culture of the yeast *S. cerevisiae* (OD = 4.5) with various doses of ionizing radiation within the range 10–150 krad. The number of viable cells was estimated 30 min after the treatment from the CFU number. An increase in the radiation dose resulted in a decrease in the number of viable cells (Fig. 1). The growth of colonies formed by an irradiated yeast culture was retarded by 1–2 days, depending on the radiation dose used by us. Irradiation also resulted in the formation of numerous microcolonies (over 50% of the total number of colonies), whereas the control culture was dominated by macrocolonies. Our subsequent studies were conducted using a dose of ionizing radiation that was equivalent to 50 krad; the percentage of viable cells in the irradiated culture was 35% of the initial cell number.

Before investigating the protective effects of C_7-AHB during oxidative stress, we elucidated their influence on the viability of yeast cells (Fig. 2). These studies were conducted with samples taken from an exponential-phase culture of *S. cerevisiae*; the sample volume was 3 ml. The experimental systems were supplemented with 0.1 ml of AHB solution and incubated at 28°C without forced aeration for 30 min and for 2 h. The effect of exogenous C_7-AHB (0.1–1.6 g/l) was estimated from the resulting change in CFU number after plating the tested culture onto solid media. The CFU number in samples that were not treated with AHB was assumed to be 100%.

From the data obtained (Fig. 2), it follows that treating yeast with C_7-AHB (0.1–1.0 g/l) for 30 min caused a 12–57% increase in CFU number, which is in quantitative agreement with data on the growth-stimulating effect of phenolic antioxidants [13]. Increasing the C_7-AHB concentration to 1.6 g/l decreased the number of

viable cells by 18%. After 2 h, AHB-untreated yeast samples demonstrated a 20% increase in CFU number, in contrast to samples inoculated 30 min after the beginning of the experiment. After incubating cell suspensions with C_7-AHB (0.5–1.4 g/l) for 2 h, the CFU number decreased by 5–35% (depending on the tested C_7-AHB concentration). Thus, the effect of C_7-AHB on a *S. cerevisiae* culture was dependent on its concentration in the yeast suspension and on the preincubation time.

Therefore, we used C_7-AHB concentrations of 0.1–1.6 g/l in our studies on the radioprotective function of AHB in yeast cells; the pretreatment time was 30 min. The samples preincubated with C_7-AHB for 30 min and the control (AHB-untreated) samples were exposed to a radiation dose of 50 krad. The numbers of viable yeast cells were determined 30 min and 2 h after irradiation (table).

The calculation algorithm included two steps: (i) determining the percentage of viable cells after irradiation for each tested AHB concentration, assuming the number of viable cells in a radiation-untreated culture to be 100% (table, columns 3 and 5); (ii) determining the percentage of viable irradiated cells after AHB treatment, assuming the number of viable cells in an AHB-untreated culture to be 100% (table, columns 4 and 6). From the table, it is evident that C_7-AHB concentrations of 0.1–1.0 g/l fail to protect the cells from irradiation and even potentiate its lethal effect (by 11–24%). The data obtained may be due to the fact that these AHB concentrations stimulate yeast growth (Fig. 2). This is consistent with the data that stress resistance is inversely correlated with the growth rate of microbial cells. A marked protective effect (17–34%) occurred at AHB concentrations exceeding 1.0 g/l (table, column 4).

Another important criterion of the protective effect of AHB is based on their influence on cell reproduction.

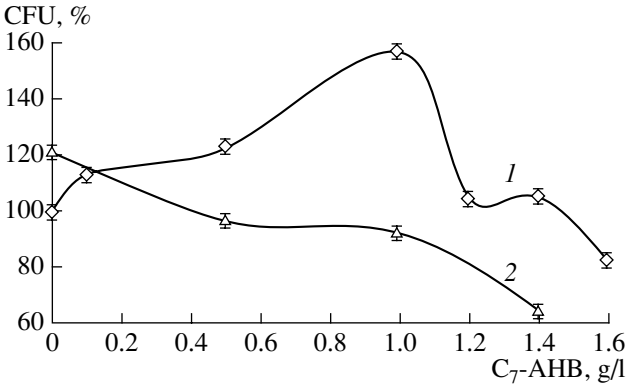

Fig. 2. Effect of the C_7-AHB concentration on the number of viable cells of *S. cerevisiae*. Preincubation time: (*1*) 30 min; (*2*) 2 h. The number of viable cells in AHB-untreated samples was taken as 100%.

It was estimated from the CFU number 2 h after irradiation, because it was viable cells (plus separated buds) that formed colonies. The number of viable cells was considerably higher than that in yeast cultures sampled 30 min after irradiation (table, columns 3 and 5) if the cell suspension contained 0.1–1.0 g/l C_7-AHB. The protective effect of C_7-AHB occurred over the whole tested concentration range. This effect was more significant in samples with 1.2–1.6 g/l AHB; a similar trend was found if the CFU number was counted 30 min after irradiation.

In contrast to the above systems, supplemented with C_7-AHB 30 min before irradiation, the subsequent studies used yeast suspensions that were treated with C_7-AHB immediately after irradiation (the radiation dose was 50 krad). In these studies, no increase in the number of viable cells occurred after incubating the irradiated cells with C_7-AHB for 2 h. Thus, C_7-AHB failed to produce a reactivating effect (Fig. 3).

Number of viable cells in an AHB-pretreated *S. cerevisiae* culture 30 min and 2 h after γ irradiation

C_7-AHB concentration, g/l	Before irradiation	30 min after irradiation		2 h after irradiation	
	CFU/ml, $\times 10^7$ (%)	CFU/ml, $\times 10^7$ (%)	Viable cells, %	CFU/ml, $\times 10^7$ (%)	Viable cells, %
Column no.	2	3	4	5	6
0	5.70 (100)	1.65 (28.9)	100	1.86 (32.63)	100
0.1	6.44 (100)	1.58 (24.5)	84.5	2.33 (36.18)	109.7
0.5	7.05 (100)	1.83 (25.9)	89.3	2.55 (36.17)	109.7
1.0	8.95 (100)	1.98 (22.1)	76.2	2.70 (30.16)	91.4
1.2	5.93 (100)	2.03 (34.2)	118.0	2.26 (38.11)	115.5
1.4	6.05 (100)	2.06 (34.0)	117.2	2.13 (35.20)	106.7
1.6	4.67 (100)	1.82 (38.9)	134.1	1.93 (41.30)	125.2

Note: The algorithm for calculating the values of columns 4 and 6 is described in the text. The values of column 2 were taken to be 100% when calculating the values of columns 3 and 5. The first value of columns 3 and 5 corresponds to the 100% level in columns 4 and 6, respectively.

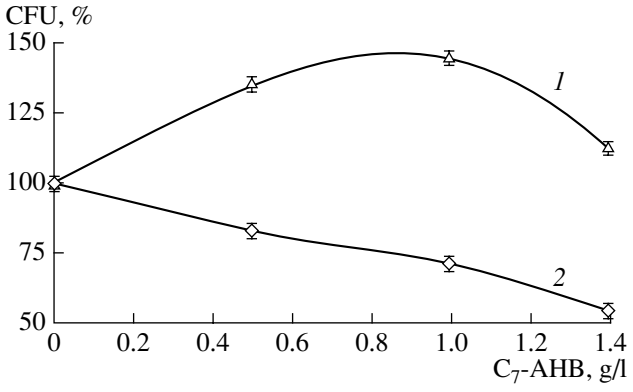

Fig. 3. Number of viable cells of *S. cerevisiae* 2 h after irradiation (50 krad): (*1*) C_7-AHB was added to the yeast suspension prior to irradiation; (*2*) C_7-AHB was added following irradiation. The number of viable cells in AHB-untreated samples was taken as 100%.

Interestingly, AHB-treated yeast suspensions gradually became pink and, subsequently, brown in color. Irradiating pure AHB solutions produced the same effect, indicative of oxidation of AHB that was associated with radiolysis of their solutions and resulted in the formation of colored oxidized products. The antioxidant properties and the biological activities of products of AHB oxidation will be considered in a separate article. Thus, our studies demonstrated a protective effect produced by C_7-AHB, which protect yeast cells from the damage caused by γ irradiation.

In a further series of studies concerning the antioxidant properties of AHB, singlet oxygen was used as a destructive agent. Singlet oxygen was generated in yeast cells supplemented with the photosensitizer chlorin e_6 under photoirradiation. In Fig. 4, the dose-dependent curves of the viability of photosensitized yeast that was illuminated with monochromatic laser light (λ = 662 nm) are plotted. The cells in the control system were treated with the PS chlorin e_6 only, and the cells in the experimental system were simultaneously treated with PS and 1.0 g/l AHB. From the plot it follows that preincubating photosensitized cells with C_7-AHB for 30 min efficiently protected them from $^1O_2$-mediated photodynamic inactivation. A five times lower radiation dose was required in an unprotected cell suspension, compared to a C_7-AHB-treated cell suspension, to yield the same percentage of dead cells. In this system, the protective effect of C_7-AHB, like its radioprotective effect described above, was concentration-dependent. From the data of Fig. 5, it follows that an AHB concentration of 0.1% was optimum for the manifestation of the photoprotective properties of AHB. The photosensitivity of a yeast culture (expressed in $1/LD_{37}$ units; one $1/LD_{37}$ unit corresponds to 37% dead cells) was minimum at this AHB concentration.

Our research on the radio- and photoprotective properties of chemical analogs of microbial autoregulators of anabiosis also addressed the question of whether tyrosol, which was earlier described by us as an autoregulator of resting yeast forms [4], exerts a protective influence on yeast cells. We failed to detect an antioxidant effect of this compound (data not shown).

In an analogy to γ-irradiated systems, PS- and AHB-containing yeast suspensions and aqueous solutions gradually changed their color under illumination (λ = 662 nm). Being pale green initially (due to the presence of chlorin e_6), the systems became orange-green. No color developed if a colorless solution that contained only C_7-AHB was illuminated with monochromatic light. Thus, the formation of colored products only occurred under the photodynamic effect of C_7-AHB. Spectral data confirmed the fact that C_7-AHB had no absorption band in the visible range before or after illumination (Fig. 6, curve *1*). Because the orange-red color of irradiated solutions of PS + C_7-AHB obviously resulted from superposition of the absorption of the colored C_7-AHB derivative and chlorin e_6 (the main

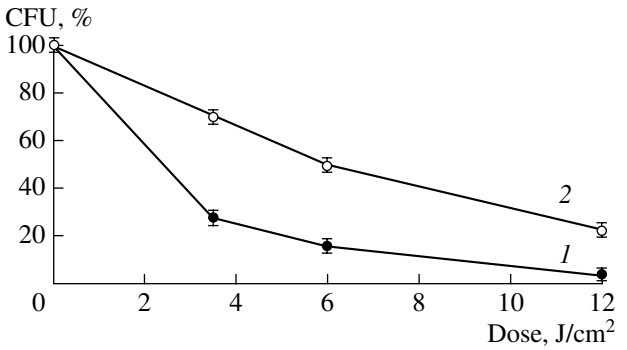

Fig. 4. Dose-dependence curves of the viability of *S. cerevisiae* pretreated, 30 min before illumination (λ = 662 nm), (*1*) with the photosensitizer (PS) chlorin e_6 or (*2*) with PS and 1.0 g/l C_7-AHB.

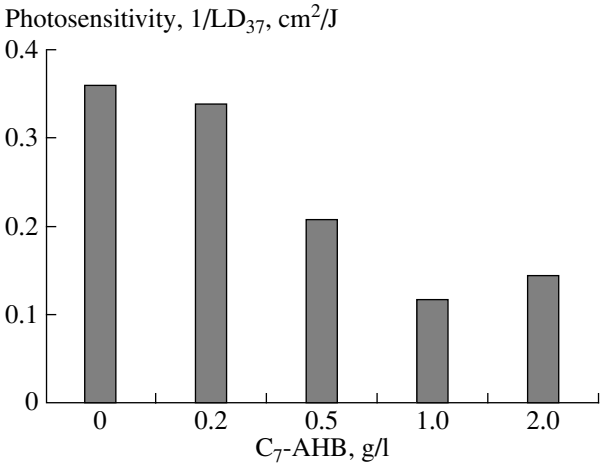

Fig. 5. Concentration dependence of the photoprotective effect of C_7-AHB.

absorption maxima are at about 400 and 650 nm), we recorded the difference absorption spectrum of irradiated PS + C_7-AHB vs. PS (Fig. 6, curve *2*). This allowed us to separate the absorption spectrum of the colored compound resulting from the photodynamic reaction and the absorption spectrum of chlorin e_6, which was also present in the solution of PS. From the plot it is evident that the photodynamically generated colored products of C_7-AHB have a broad absorption band in the blue-green range (430–500 nm). Figure 6 (curve *3*) also shows the absorption spectrum of a highly oxidized C_7-AHB derivative, which is of brown color and forms under the effect of γ irradiation. Hence, photo- and radiooxidized AHB derivatives are spectrally different but have a common feature, the existence of a broad absorption band in the visible range.

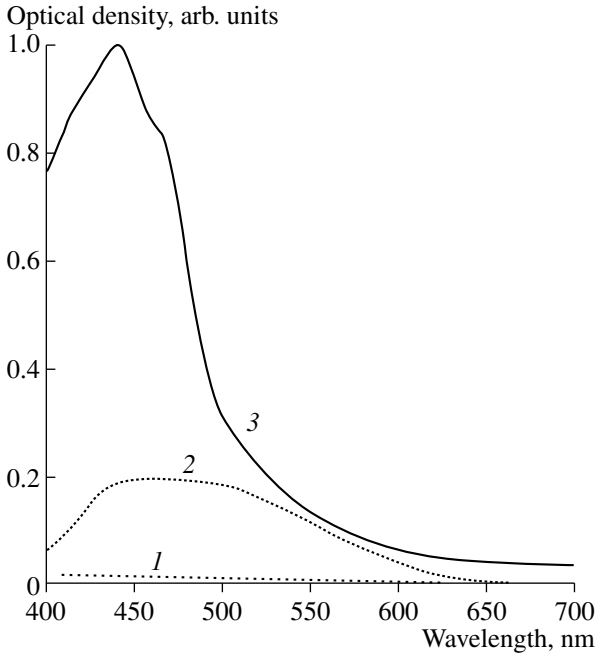

Fig. 6. Absorption spectra of (*1*) C_7-AHB (1.0 g/l; not irradiated or irradiated at λ = 662 nm), (*2*) photooxidized C_7-AHB derivatives in the presence of the PS chlorin e_6, and (*3*) radiochemically oxidized products.

DISCUSSION

The data obtained in our studies of the role of C_7-AHB in the protection of cells of *S. cerevisiae* from radiation are difficult to interpret due to the effects of a large number of factors, including radiation per se, the dose-dependent growth-stimulating or inhibitory effect of AHB, and oxidation of AHB molecules during their interaction with ROS that result from radiolysis or photooxidation. Importantly, yeast cultures were under the combined influence of (i) changes in mass exchange rate during the sampling procedure; (ii) temperature changes; and (iii) radiation/photoirradiation. However, we apparently have sufficient grounds to draw the conclusion that AHB concentrations of 0.5–1.6 g/l exert a protective effect on yeast under γ irradiation or photooxidation.

Determination of the dependence of viable yeast cell number on γ-irradiation dose revealed that, apart from a decrease in CFU number, radiation disrupts cell division, which manifests itself in the formation of an increased number of microcolonies in irradiated cells, in contrast to the control system. Accordingly, the radiation effect results in, apart from the impairment of the genetic and metabolic machinery in some of the cells, disruptions that affect the subsequent yeast generations, changing their division rate and the percentage of dividing cells in the population. Therefore, in our study of the protective properties of AHB, we monitored both their immediate protective effect (30 min after irradiation) and the influence of AHB on the yeast's capacity for reproduction (2 h after irradiation). We found that these effects manifested themselves within distinct ranges of protector (AHB) concentrations. In terms of viability retention, i.e., the retention of the growth capacity immediately after irradiation, the optimum AHB concentrations were 1.2–1.6 g/l; however, the optimum AHB concentrations for the restoration of proliferative activity, i.e., for colony formation after an interval exceeding the generation time, were 0.1–1.6 g/l.

The data obtained in this work suggest that AHB efficiently intercept various kinds of ROS generated in radiochemical processes, including singlet oxygen ($^1O_2$). We demonstrated for the first time that (i) the phenolic antioxidant C_7-AHB protects cells from photodynamic damage and (ii) a colored derivative of C_7-AHB is produced under the effect of $^1O_2$. The fact that this product has no absorption band within the emission range (662 nm) of the laser used in this work militates against the suggestion that the photoprotective effect is due to simple shielding of the cells involved. Hence, the mechanism of the protective effect of AHB is based on the interception of singlet oxygen, which is generated by the PS during its irradiation. This results in the formation of a colored oxidized product of C_7-AHB. Phenolic compounds are known to undergo oxidative polymerization [14, 15] involving peroxidases and hydrogen peroxide. In view of the high reactivity of singlet oxygen, it seems likely that the photodynamic effect results in nonenzymatic oxidative polymerization.

The following subsection briefly considers the mechanisms that enable C_7-AHB to protect yeast from irradiation- and oxidation-caused damage.

Such compounds, by virtue of weak physical and chemical interactions, can form labile complexes with macromolecules and biomembranes, stabilizing their structure and facilitating the dissipation of the excessive energy that causes damage [6–9, 16]. This function of AHB allows us to regard them as chaperons [17]. In addition, AHB trap reactive oxygen species (ROS), i.e.,

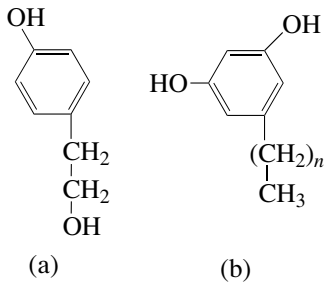

Fig. 7. Structural formulas of (a) tyrosol and (b) C_7-AHB.

function as antioxidants [18]. We demonstrated in our earlier works that AHB of the alkylresorcinol type, which are exemplified by C_7-AHB and C_{12}-AHB, perform both functions: they stabilize the structure of biopolymers and trap ROS. The protective effect of C_7-AHB on yeast exposed to singlet oxygen or γ radiation (that we revealed in this work) is due to the above properties of AHB. The fact that tyrosol exerts no protective effect seems to indicate that it does not efficiently neutralize singlet oxygen, i.e., cannot be considered a potent antioxidant. Thus, the antioxidant properties of hydroxybenzene derivatives are not necessarily related to their chaperon function. Some of them, e.g., tyrosol, may only possess chaperon (stabilizing) properties, whereas other compounds (exemplified by C_7-AHB and C_{12}-AHB) may be characterized by both antioxidant and stabilizing properties. This is conditional on the structure of the compounds involved. Figure 7 shows the structural formulas of tyrosol (2-(4-hydroxyphenyl)-ethanol) and alkylresorcinols. C_7-AHB is an alkylresorcinol. In this context, an important difference between tyrosol and C_7-AHB concerns the position and structure of substituents in the benzene ring. The residue in the tyrosol molecule is an ethanyl radical, which is rather hydrophilic. As for C_7-AHB, it contains an alkyl residue, which is more hydrophobic. Importantly, phenolic compounds readily participate in oxidation reactions [14, 15, 19]. The formation of free radicals [14, 19] that are stabilized by hydrophobic bulky substituents [19] performs a major role in these processes. Therefore, the efficiency of the antioxidant effect of AHB is conditional on the presence of a hydrophobic alkyl radical and on its structure, in addition to other relevant factors. More detailed research on the transformations of structurally diverse AHB during oxidation induced by radiation, light, and chemical agents will be a subject of our publications in the future.

The results obtained in this work make a significant addition to the spectrum of known antioxidant properties of phenolic compounds and mechanisms of their protective effects on living cells exposed to the influence of radiation or oxidants.

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