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New Promising Antioxidants Based on 2,6-Dimethylphenol

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Abstract—Three new sulfur-containing derivatives of 2,6-dimethylphenol were synthesized. Their antioxidative activity, mutagenicity, and genotoxicity were examined by bacterial tests and by calculating the dominant lethal mutations in murine embryonic cells. It was shown that all the compounds synthesized have a marked antioxidative effect and have no genotoxic and mutagenic properties. One of the antioxidants, 4-(3-dodecylthiopropyl)-2,6-dimethylphenol, increases the survival of cells of both the wild-type *Escherichia coli* strain and bacterial strains defective in the genes of repair enzymes and has a more distinct antioxidative effect than the classic antioxidants α-tocopherol and trolox, increasing the survival of cells devoid of repair enzymes.

Key words: oxidative stress, phenol antioxidants, sulfur-containing antioxidants, 4-thiaalkyl-2,6-dimethylphenols

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INTRODUCTION

Aerobic respiration involves the stage-by-stage four-electron reduction of molecular oxygen to water.[2] The intermediates of molecular oxygen, $O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$, are ROS by themselves, and they also generate other ROS when interacting with cell components. One of the main reasons for the development of OS in the organism is the accumulation of ROS, which arise from the disturbance of the balance between the processes of generation of free radicals and the mechanisms of their utilization [1–4]. ROS oxidize biological membrane lipids [5] and destroy proteins and enzymes [6, 7], cellular DNA and RNA [8], and other components of living cells.

A gradual accumulation of mutations in the genome of somatic cells [9, 10] leads to the development of age-related pathologies, which is the main cause of premature aging, mutagenesis, carcinogenesis, and a great number of pathologies of old age [11, 12]. An effective mechanism of protection of cells against ROS is the functioning of the complex of specialized systems of enzymic and nonenzymic antioxidants [2, 13–16]. Enzymes are important first of all for the intracellular defense against ROS. However, radical oxidative processes can also occur in the water and lipid phases where the major role in protection against ROS is played by low-molecular-weight antioxidants. In addition, in OS, antioxidant enzymes are often less effective than low-molecular antioxidants. The reasons for this are the fast inactivation of the constitutive pool of enzymes in cells (especially after they enter the blood flow) and a great deal of time required for the induction of their synthesis. Therefore, freely migrating endogenous and exogenous low-molecular-weight antioxidants in high concentrations play an important role in the protection against OS [17]. At present, a great number of natural and synthetic compounds possessing antioxidative and anticarcinogenic properties have been obtained [18, 19]. Phenol antioxidants [18, 20], including spatially restrained phenols [20], are effective scavengers of radicals.

The studies of the last few years have shown the specificity of the protective action of antioxidants to various organs, tissues, and cells of the man, the differences in the mechanisms of their functioning, as well as the synergism or antagonism of their effects [2, 13, 21–23]. Thus, a study of the effect of the known antioxidants: an extract of the bilberry (*Vaccinium myrtillus*) and Adrusen zinco (a complex of vitamin E with zinc, selene, and ω3-polyunsaturated fatty acids) on the level of oxidized proteins, as well as enzymes having antioxidative functions (superoxide dismutase, catalase, and glutathione peroxidase) in Wistar and OXYS rats

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[2] Abbreviations: ROS, reactive oxygen species; OS, oxidative stress.

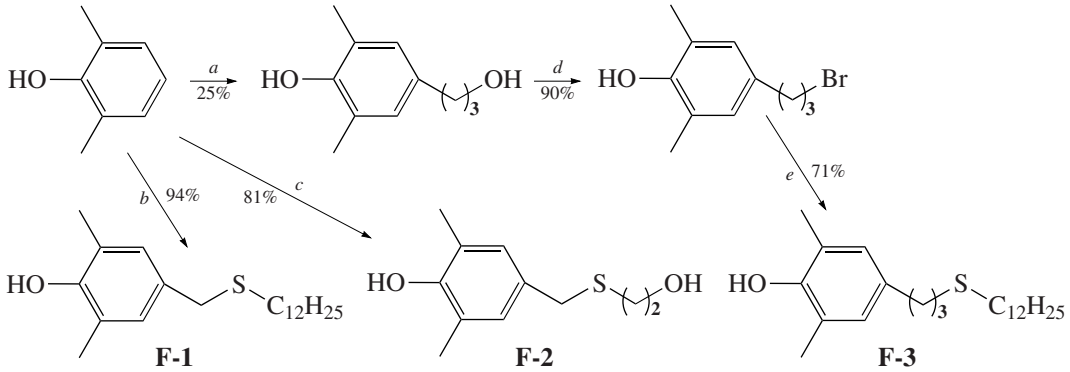
showed that these antioxidants affect differently these indicators in different organs and tissues and differ in their effects on the components of nuclei, mitochondria, and cytosol [24]. The use of the bilberry extract provides a maximal protection of eye tissues against ROS and prevents the formation of cataract, whereas the effect of Adrusen zinco on cataract formation in OXYS rats with genetically determined enhanced sensitivity to ROX is substantially weaker; at the same time, however, it produces a more pronounced protective effect on other organs [24]. This selectivity of action of the antioxidants can be related to the differences in their structures and ability to penetrate into mitochondria, nuclei, and other compartments of living cells. In view of this, the synthesis of novel antioxidants that combine in their molecules several active fragments differing in structure and the mechanism of antioxidant action appears to be a promising task. It is known that sulfur-containing derivatives of alkyl phenols exhibit a high antioxidative activity in vitro and in vivo owing to the synergism of the antiradical activity of the phenol fragment and the antiperoxide effect of sulfur-containing functional groups [25–28].

The primary screening of antioxidants is most often performed using the tests based on the assessment of survival of cells of wild-type *E. coli* strains or oxidative lesions of DNA of genes defective in repair enzymes in the absence or presence of oxidizers and antioxidants [29, 30]. The Ames test estimates the ability of compounds to induce mutations in specially designed strains of *Salmonella typhimurium* (Ames strains) [31]. It is expedient to analyze the lesions of DNA, cell membranes, and whole cells, as well as some organs exposed to ROS in the presence and absence of antioxidants [33, 34] using model animals [32]. The protective properties of antioxidants are also studied using special lines of animals with genetically determined enhanced sensitivity to OS [35–39].

Here we described the synthesis of three novel sulfur-containing compounds based on 2,6-dimethylphenol and showed that the three compounds compare well in their characteristics and are even more promising in some aspects than the previously described antioxidants α-tocopherol and trolox.

RESULTS AND DISCUSSION

Synthesis of novel antioxidants. We examined sulfur-containing phenol compounds F1–F3, obtained on the basis of 2,6-dimethylphenol (scheme). Compounds F1 and F2 were synthesized in one stage by condensing 2,6-dimethylphenol with formalin and the corresponding thiols, and sulfide F3 was obtained through the intermediate synthesis of hydroxy- and bromosubstituted derivatives.



The following reagents and conditions were used: *a*, Allyl alcohol/NaOH; *b*, aqueous H_2CO, *c*, $C_{12}H_{25}SH$/aqueous EtOH; *d*, concentrated HBr; *e*, $C_{12}H_{25}SH$/KOH, EtOH.

A scheme of synthesis of sulfur-containing derivatives based on 2,6-dimethylphenol.

It was shown by an example of the oxidation of model hydrocarbons that compounds F1–F3 are highly reactive towards peroxide radicals (the rate constants for the corresponding reactions are 1.5×10^5 M^{-1} s^{-1}) and inhibit hydrocarbon autoxidation more effectively than phenol antioxidants containing no sulfide groups [40, 41].

Analysis of the Mutagenicity of Compounds by the Ames Test

The mutagenic activity of the compounds synthesized was revealed by the Ames test using the *S. typhimurium* strain TA102. TA102 is a histidine-dependent tester strain developed by Ames [42], which carries an *ocher*-mutation at the histidine operon hisG 428.

TA102 cells contain about 30 copies of plasmid pAQ1 carrying the mutant allele of hisG 428 and seven to eight copies of plasmid pKM101 (*R*-factor), which increases the sensitivity of the strain to mutagens. The genome of strain TA102 also contains mutations common in all Ames strains: *rfa*-mutation, which increases the cell membrane permeability for large molecules, and a deletion in the *uvrB* gene, which causes a destruction of the excision repair system, which further increases the sensitivity of tester strains to some mutagens. The use of strain TA102 makes it possible to reveal the mutagenic activity of hydroperoxides, hydrogen peroxide, quinones, phenylhydrazine, various aldehydes and agents inducing the damage to thymine [31]. The test for mutation induction in the Ames assay is performed by calculating the back mutations from auxotrophicity to prototrophicity at histidine, which arise in response to various mutagenic substances [31]. Strain TA102 is commonly used both to assess the mutagenicity of compounds by analyzing the number of mutants formed by the action of a potential mutagen [31] and to determine the antioxidant activity of antioxidants both from the inhibition of spontaneous mutagenesis in this strain [43] and the decrease in the mutagenicity of oxidants (most often hydrogen peroxide) [44].

It was shown that the addition to TA102 cells of only the compounds dissolved in DMSO at a final concentration of 20 nM leads to the statistically significant formation of a greater number of His^+-revertants than in the case of their spontaneous formation in the control with the cells of this strain not treated with DMSO (Fig. 1). The addition to cells of DMSA alone at the same concentration increases the frequency of formation of revertants, which becomes comparable with, and statistically indistinguishable from that for the control antioxidants α-tocopherol and trolox added in the same amount of DMSO. The effect of compound F1 on the formation of mutants does not differ statistically from the effect of α-tocopherol, and the efficiency of the formation of mutants in the presence of compounds F2 and F3 is only by about 2–3% higher (Fig. 1). Increasing the concentration of the test and reference compounds to 80 nM leads to a slight decrease in the revertant formation efficiency, which becomes statistically indistinguishable from that of control cells treated with DMSO (Fig. 1). Taken together, these data indicate that the three novel compounds (F1–F3), similar to the control antioxidants α-tocopherol and trolox, do not produce any pronounced mutagenic effect and do not decrease the level of spontaneous mutations (Fig. 1).

The main premutational oxidative DNA lesions induced by H_2O_2, the mechanisms of their repair and transformation in the mutation, the induction of the SOS-response by hydrogen peroxide, and the influence of enzymatic and chemical systems of cellular antioxidant defense on the genotoxicity of H_2O_2 have been described in the literature [45–48]. Because some antioxidants effectively reduce the toxic and mutagenic

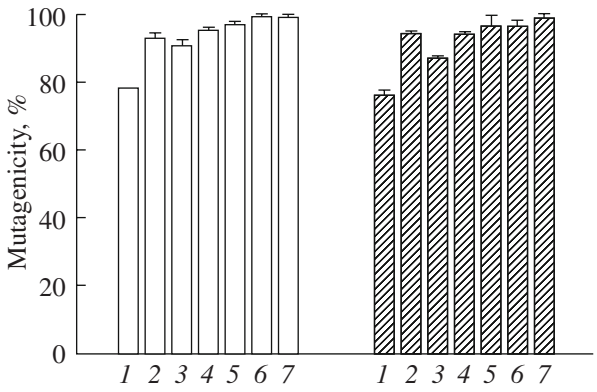


Fig. 1. Analysis of the mutagenic activity of compounds F1–F3 and control antioxidants at a concentration of 20 (open columns) and 80 nM (dashed columns in the absence of hydrogen peroxide; (*1*) control untreated cells; cells treated with (*2*) DMSO, (*3*) trolox solution, (*4*) α-tocopherol, and compounds (*5*) F1, (*6*) F2, and (7) F3 in DMSO. Average values of three separate experiments at each concentration and the standard deviations of these values are given. Maximum values of mutagenicity in the series of these values obtained in experiments are taken to be 100%.

effects of H_2O_2, this oxidant is often used in experiments to estimate the antioxidant properties of various compounds [49–52].

In the Ames test, the optimal concentration of hydrogen peroxide at which it induces mutations but has a relatively low toxicity and does not induce substantial cell death varies within the limits of 3 mM [48]. Solutions of the test compounds in DMSO were added to TA102 cells in different concentrations, and H_2O_2 was added to a final concentration of 3 mM. Cells treated with H_2O_2 were used as a control, and the effect of the solvent was accounted for by treating the cells with hydrogen peroxide in the presence of DMSO (Fig. 2).

It was shown that DMSO does not markedly affect the mutagenic oxidative activity of H_2O_2. Trolox at a concentration of 20 nM had a maximum antioxidative activity (100 ± 1.3%), and the protective properties of α-tocopherol (98 ± 1.2%) and compound F1 (99.4 ± 0.6%) were practically the same. The activity of compounds F2 (84.1 ± 0.4%) and F3 (84.9 ± 1.5%) was somewhat lower. At a concentration of 80 nM, trolox retained the maximum activity (100 ± 0.5%); the efficiency of the protective action of compounds F1 (92.1 ± 0.7%) and α-tocopherol (91.8 ± 0.5%) slightly decreased. However, the antioxidative properties of compounds F2 (96.2 ± 0.6%) and F3 (98.1 ± 0.7%) increased markedly and approached those of trolox. Interestingly, water-soluble trolox was more reactive as antioxidant in this system than water-insoluble α-tocopherol (Fig. 2). Because these compounds are structurally close and their mechanisms of action are similar, this difference in activity may be related to different efficiency of their penetration into cells.

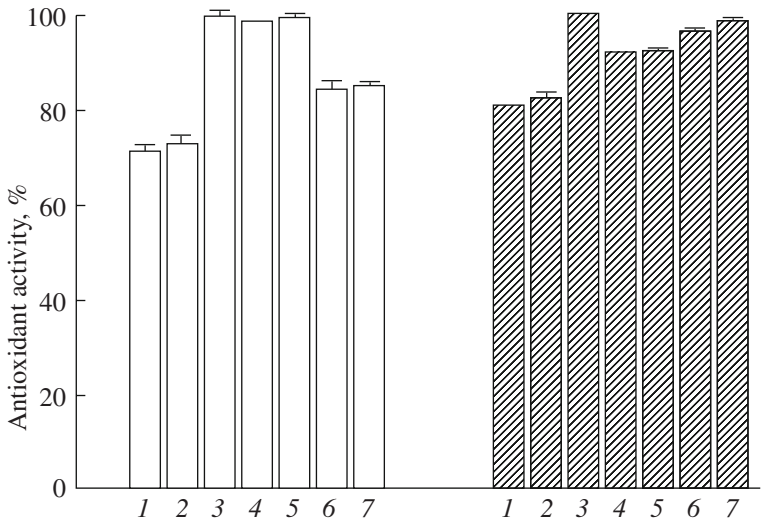


Fig. 2. Analysis of the antioxidative activity of compounds F1–F3 and control antioxidants at a concentration of 20 (open columns) and 80 nM (dashed columns) in the presence of 3 mM H_2O_2; (*1*) control untreated cells; cells treated with (*2*) DMSO, (*3*) trolox solution, (*4*) α-tocopherol, and compounds (*5*) F1, (*6*) F2, and (7) F3 in DMSO. Average values of three separate experiments at each concentration and the standard deviations of these values are given. Maximum values of the antioxidative activity in the series of these values obtained in experiments are taken to be 100%.

Of the three compounds synthesized, only F2 is readilt soluble in water; F1 and F3 are lipophilic like α-tocopherol. The protective effect of compounds F2 and F3 at high concentrations is stronger than that of α-tocopherol. Thus, the Ames test showed that the three novel compounds possess no mutagenic activity, and their antioxidative effect is comparable with that of α-tocopherol and trolox.

Analysis of the antioxidant activity of the compounds using special *E. coli* strains. *E. coli* strains *cc104mutT* and *cc104mutMY*, which are deficient in the system of oxidative DNA damage repair [53, 54] were used. The original *E. coli* strain *cc104* with the entirely intact repair system served as a control.

The strain *cc104mutT* carries a mutation of the gene *mutT*, which encodes 8-oxo-2'-deoxyguanosine-5'-triphosphate pyrophosphohydrolase (8-oxodGTPase or MutT). The protein MutT is not an excision repair enzyme. It has a nucleosidetriphosphate-pyrophosphohydrolase activity and prevents the incorporation of 8-oxodGTP to the DNA from the nucleotide pool during replication, by hydrolyzing it to 8-oxodGMP and pyrophosphate. The inactivation of the *mutT* gene gives rise almost exclusively to AT ⟶ CG transversions; in this case, the frequency of *mutT* mutations in the strain is considerably higher due to a greater amount of 8-oxoGua in the nucleotide pool [55].

The strain *cc104mutMY* carries two mutations: of gene *fpg* encoding DNA-8-oxoguanineglycosylase (MutM protein), which has a DNA-glycosylase, apurine/apyrimidine lyase and 5'-endodeoxyribophosphatase activities [56], and of gene *mutY*, which encodes adenine-DNA-glycosylase (MutY protein), which recognizes G · A- and 8-oxoG · A mismatches and corrects them, using the *N*-glycosylase activity to remove adenine [57]. 8-OxodGua is formed in 8-oxoG · C pairs both during DNA replication and as a result of modification of guanine incorporated into DNA. The generation of the 8-oxoG-oxidative DNA damage is one of the main reasons for the appearance of various mutations, in particular, GC ⟶ TA and AT ⟶ CG transversions. If the base 8-oxoGua would not be removed from DNA by the MutM protein, DNA-polymerase III could "put" adenine in the next replication round opposite 8-oxoGua. The double mutation *mutM-mutY* increases the frequency of spontaneous GC ⟶ TA transversions 20 times compared with the sum of frequencies of transversions due to mutations in genes *mutY* and *mutM* taken separately [58].

We studied the antioxidant activity of the compounds by their ability to increase the survival of bacterial cells treated with hydrogen peroxide. The strains defective in genes *mutT*, *mutM*, and *mutY* exhibited a higher sensitivity to 3 mM H_2O_2 compared with the original strain. A pretreatment of cells of all *E. coli* strains studied with antioxidants increased their survival after the treatment with 3 mM H_2O_2. The addition of 3 mM H_2O_2 together with DMSO in different amounts in control experiments led to different survival of *E. coli* cells of strains *cc104*, *cc104mutT*, and *cc104mutMY* (Table 1). α-Tocopherol only at a high concentration of 80 nM was effective in decreasing the toxicity of H_2O_2 (increased the survival of cells treated with H_2O_2) in the original *E. coli* strain *cc104* as compared with the control (Table 1). At a concentration of 8 and 20 nM, it produced a weak antioxidative effect, and the cell survival was only insignificantly higher than in the control in the presence of H_2O_2 in DMSO.

Table 1. Effect of antioxidants on the cell survival of *E. coli* strains *cc104*, *cc104mutT*, and *cc104mutMY* treated with 3 mM H_2O_2

Strain	*C*, nM	Cell survival, relative units					
		α-Tocopherol	Trolox	F1	F2	F3	DMSO
cc104	8	8.0 ± 0.5	8.7 ± 0.4	4.6 ± 1.2	3.3 ± 1.1	4.3 ± 0.2	3.6 ± 0.7
	20	6.1 ± 2.6	**22.2 ± 2.7**	5.4 ± 1.9	7.9 ± 3.6	**16.2 ± 2.2**	4.9 ± 3.1
	80	**31.6 ± 1.5**	10.4 ± 0.9	–6.8 ± 1.1**	–3.9 ± 1.8	11.0 ± 1.3	10.4 ± 1.1
cc104mutT	8	2.5 ± 1.1	1.3 ± 1.5	0.4 ± 0.7	3.6 ± 0.7	7.4 ± 1.4	4.1 ± 0.6
	20	2.0 ± 3.2	6.3 ± 2.2	**22.8 ± 3.8**	**10.8 ± 2.9**	**25.9 ± 3.8**	3.7 ± 1.6
	80	**26.9 ± 0.8**	**19.3 ± 1.9**	9.1 ± 1.5	3.9 ± 0.8	14.7 ± 1.8	14.2 ± 1.8
cc104mutMY	8	1.6 ± 3.7	5.0 ± 4.3	–0.6 ± 1.4	9.9 ± 1.9	**10.4 ± 3.0**	0.3 ± 1.0
	20	4.4 ± 1.3	6.0 ± 1.2	4.1 ± 1.6	2.0 ± 0.4	3.9 ± 1.6	1.8 ± 0.2
	80	3.4 ± 0.8	**11.6 ± 1.9**	**22.9 ± 2.6**	**10.3 ± 1.5**	2.0 ± 0.1	0.1 ± 0.3

Notes: * Effect of antioxidants was assessed by the difference in cell survival in the presence and absence of the corresponding antioxidant. The average values of cell number and standard deviations of these values ($n = 3$ for each concentration) are given in relative units calculated using the program Image Pro Plus. The statistical processing of the data showed that all differences were statistically significant ($P \le 0.05$). The values of the maximum protecting effects for each compound are shown in boldface type.

** Negative values indicate that the compounds, which exhibit a prooxidant effect.

In the case of the *cc104mutT* strain defective in 8-oxoGTPase, the survival of cells in the presence of 8 and 20 nM α-tocopherol was approximately 1.6–1.9 times lower than in the control; i. e., the compound exhibited a prooxidant effect. Increasing the concentration of α-tocopherol to 80 nM significantly increased the cell survival; with regard for the effect of DMSO, it was only 1.7 times lower compared with the starting strain *cc104*.

The survival of *cc104mutMY* cells in the control experiment (in the presence of H_2O_2 and DMSO) was low. Increasing the concentration of DMSO first led to an increase and then to a decrease in cell growth (Table 1). No prooxidant effect (a decrease in the amount of grown cells compared with the control) was observed. The addition of α-tocopherol (Table 1) increased the cell growth 2.4–5.3 times at a concentration of 8 and 20 nM and 34 times at a concentration of 80 nM; however, in general, the survival of cells was low compared with the values obtained with other antioxidants (Table 1, see below).

In the case of trolox, complicated concentration dependences were observed. On going from 8 to 20 nM concentration (with due regard for the control values at these concentrations), the protective effect in the case of the wild-type strain *cc104* increased approximately 3.4 times, reaching a maximum; then, at a concentration of 80 nM, the cell survival fell to control values (Table 1). A similar complicated concentration dependence for trolox was observed with the *cc104mutT* strain: the cell growth slowed down at a concentration of 8 nM (the prooxidant effect), and the protecting effect at a concentration of 80 nM was lower than in the case of α-tocopherol; however, at a concentration of 20 nM, the cell growth was more intensive than in the case of the fat-soluble analogue. In general, the antioxidative effect of trolox in the case of the *cc104mutT* strain was the lowest compared with the effects of other compounds. At the same time, trolox substantially stimulated the survival of very badly growing cells of the *cc104mutMY* strain: the survival of these cells in the presence of trolox at any concentration increased 3.3–116 times compared with the control (H_2O_2 + DMSO). Thus, despite the structural similarity of the antioxidant groups of fat-soluble α-tocopherol and its water-soluble analogue trolox, the antioxidative effects of these compounds in the case of the wild-type *E. coli* strain and the strains defective in repair enzymes substantially differ.

Of interest are the data obtained for compound F1. This substance at low concentrations (8–20 nM) does not protect the cells of the *cc104* strain and produces the prooxidant effect at a concentration of 80 nM, substantially reducing the rate of cell growth compared with the control. It is noteworthy that F1 has the prooxidant effect at low and high concentrations (8 and 80 nM) but protects well the cells of the *cc104mutT* strain at a concentration of 20 nM, providing the survival 3.6 and 11.4 times higher than with trolox and α-tocopherol.

A somewhat different situation occurs with F1 in experiments with the *cc104mutMY* strain: a prooxidant effect at a concentration of 8 nM and an increase in cell survival 2.3 and 229 times at concentrations of 20 and 80 nM, respectively, compared with the control. The increase in the survival of the *cc104mutMY* strain in the presence of 80 nM F1 is 1.2 and 6.7 times higher compared with the effects of trolox and α-tocopherol, respectively (Table 1). Presumably, in normally functioning cells where repair enzymes effectively remove oxidized bases, this compound at low concentrations can mainly act as a prooxidant, whereas at high concentrations and particularly upon the disturbance of oxida-

Table 2. Lethality of embryos and the level of dominant lethal mutations in embryonic cells of mice

Compound	Stage of spermatogenesis	Number of pregnant females	Potential fertility (number of yellow bodies)	Embryos per one pregnant female			Total death of embryos	Survival, %	Dominant lethals, %	χ^2
				number of implantations	alive	dead				
F3	Mature spermatozoa	42	8.19 ± 0.21	7.45 ± 0.24	7.00 ± 0.29	0.45	14.53	85.47	1.7	0.003
	Late spermatids	34	7.79 ± 0.23	6.79 ± 0.29	6.70 ± 0.30	0.18	12.83	87.13	1.3	0.002
	Early spermatids	38	7.23 ± 0.15	6.42 ± 0.22	6.00 ± 0.22	0.42	17.09	82.91	12.7	0.250
α-Tocopherol	Mature spermatozoa	29	8.13 ± 0.16	6.86 ± 0.27	6.58 ± 0.25	0.28	19.07	80.63	4.3	0.023
	Late spermatids	36	7.58 ± 0.18	6.75 ± 0.21	6.36 ± 0.22	0.39	16.11	83.88	7.5	0.080
	Early spermatids	30	7.40 ± 0.25	6.80 ± 0.23	6.46 ± 0.24	0.33	12.61	87.39	6.1	0.047

tion-reduction processes, it effectively deactivates free radicals.

Compound F2 (Table 2) either had a moderate prooxidant effect or did not exhibit any marked protective effect in the case of two strains (*cc104* and *c104mutT*) at all concentration; only at a concentration of 20 nM it substantially increased the survival of the strain *c104mutT*. At concentrations of 8 and 80 nM, compound F2 was effective in protecting the *cc104mutMY* cells.

We showed that compound F3 significantly increases the survival of cells of all strains examined, but the concentration dependences have a complicated dose-dependent shape for each strain. Interestingly, as distinct from other compounds, including α-tocopherol and trolox, this compound does not produce the prooxidant effect at all concentrations and with all of the *E. coli* strains examined. The rate of growth of *cc104* cells at a concentration of F3 of 20 nM was approximately twofold higher than in the presence of the other compounds studied and only ~1.3 times lower than in the presence of trolox. In the case of the *cc104mutT* strain, compound F3 at a concentration of 20 nM was 12.5 times more effective in protecting cells than α-tocopherol and 4 times more effective than trolox. The efficiency of F3 was 2.5 times higher than that of compound F2 and practically comparable with that of F1. At a concentration of 8 nM, compound F3 was approximately six times more efficient in protecting *cc104mutMY* cells than α-tocopherol and about two times more efficient than trolox.

Thus, of all control and test compounds examined by this assay system, compound F3 had the highest antioxidant activity; it increased the survival of cells of both wild-type strain and the strains defective in the genes of repair enzymes.

Two of the best preparations widely used currently in the therapy of OS, the antioxidants α-tocopherol and trolox, have very complicated concentration–antioxidant effect dependences and can exhibit the prooxidant effect at some concentrations (Table 1). At the same time, the data obtained suggest that compound F3 may be a more promising antioxidant since it has no prooxidant properties in a wide concentration range.

Determination of Dominant Lethal Mutations in Embryonic Murine Cells

The mutagenic effect of compound F3 in the mammalian organism was studied by estimating the level of dominant lethal mutations in embryonic murine cells [59]. A great part of dominant lethals causes the death of embryos during, or shortly after the implantation. An indicator of the level of dominant lethal mutations is the level of postimplantation losses, which is expressed as the ratio of dead embryos to the sum of alive and dead embryos [60]. Compound F3 and α-tocopherol were injected to animals at a concentration of 52 μM, which a fortiori exceeds the therapeutic dose for these compounds. It was shown that the potential fertility of females of all groups used is similar (Table 2). The values of embryonic lethality at all stages of pregnancy of females of the experimental group were comparable with those of the control group. Consequently, F3 does not affect either the pre- or the postimplantation stage of embryogenesis. The frequency of occurrence of dominant lethals in females mated with males treated with F3 did not depend on the spermatogenesis stage (Table 3). In experiments with linear animals, the significance of the increase in the level of postimplantation lethality compared with the control is estimated using the χ^2 criterion for the ratio of the sums of alive and dead embryos. With this approach, the individual sensi-

Table 3. Level of dominant lethal mutations in mice treated with compound F3, α-tocopherol, and cyclophosphane*

Groups of comparison	Spermatogenesis stages	Dominant lethals, %	χ^2
F3/control	Mature spermatozoa	1.7	0.003
	Late spermatids	1.3	0.002
	Early spermatids	12.7	0.250
α-Tocopherol/control	Mature spermatozoa	4.3	0.023
	Late spermatids	7.5	0.080
	Early spermatids	6.1	0.047
F3/α-tocopherol	Mature spermatozoa	6.0	0.055
	Late spermatids	6.0	0.065
	Early spermatids	7.0	0.080
Cyclophosphane/control	Mature spermatozoa	21.79	15.43**
	Late spermatids	37.12	40.54**
	Early spermatids	8.35	4.37***

Notes: * The level of dominant lethal mutations was estimated from the value of postimplantation losses (see the Experimental section). Control animals were given olive oil.
The significance of differences between groups was estimated by the χ^2-criterion.
** The significance of differences between experimental and control groups of animals at $P < 0.01$.
*** The significance of differences between experimental and control groups of animals at $P < 0.05$.

tivity of mice is ignored. The absence of significant differences in the level of dominant lethals in experimental and control groups of animals indicates that F3 produces no mutagenic effect (Table 3).

For comparison, Table 3 presents the frequency of occurrence of dominant lethal mutations at different stages of spermatogenesis in mice treated with the very potent mutagen cyclophosphamide (cyclophosphane) [61]. The injection to animals of cyclophosphane, as opposed to F3 and α-tocopherol, induces intensive mutagenesis at all stages of spermatogenesis. The percent of dominant lethal mutations at the stage of mature spermatozoa after administration of cyclophosphane is 12 and 5 times higher than after the administration of F3 and α-tocopherol, respectively. At the stage of late spermatids, the mutagenicity of cyclophosphane is 28 times higher than that of F3 and 5 times higher than that of α-tocopherol. Thus, these data suggest that compound F3 has no mutagenic effect on the organism of model mice.

Taken together, our data indicate that two novel antioxidants F1 and F3 at optimal concentrations are comparable in properties with α-tocopherol and trolox, and compound F3 may appear a more efficient and promising antioxidant in the treatment of diseases caused by OS.

EXPERIMENTAL

The following preparations were used: trypton, DMSO, potassium dihydrophosphate, sodium hydrophosphate, ammonium chloride, magnesium sulfate, calcium chloride, thiamine (Amerso, United States); agar, (Bacto^{TM}Agar, "BD" (France); H_2O_2, α-tocopherol, trolox (Sigma, United States); cyclophosphane (OAO Biokhimik, Russia), yeast extract, sodium chloride, glucose (Helicon, Russia), 2,6-dimethylphenol (Merck, Germany); 2-mercaptoethanol-1, dodecanethiol-1 (Aldrich, United States); refined olive oil (Greece), bacterial strains of *E. coli*: *cc104*, *cc104mutT* and *cc104mutMY* from the collection of J. Miller (France), *S. typhimurium* TA102 from the collection of B. Ames (United States).

Synthesis of antioxidants. Target compounds F1–F3 were obtained from 2,6-dimethylphenol according to the scheme. 2,6-Dimethylphenol, 2-mercaptoethanol-1, and dodecanethiol-1 were used as initial synthons. ^{1}H NMR spectra (δ, ppm) were recorded on a Bruker DRX500 spectrometer (Bruker, Germany) at an operating frequency of 500.13 MHz in $CDCl_3$ (reference $CHCl_3$). Melting temperatures were determined in a capillary on a PTP instrument (PO Khimlaborpribor, Russia).

Dodecyl-3,5-dimethyl-4-hydroxybenzylsulfide (F1). 2,6-Dimethylphenol (50 g, 0.40 mol), dodecanethiol-1 (81 g, 0.40 mol), KOH (8.87 g, 0.12 mol), and ethanol (90 ml) were heated to boiling in an atmosphere of inert gas, and aqueous formalin (81 ml, 0.80 mol H_2CO) was added dropwise. The mixture was boiled for 2 h, neutralized by hydrochloric acid, and treated with hot water. The organic layer was separated, 250 ml of hot (60°C) water was added, and the solution was cooled under vigorous stirring to room temperature. Precipitated crystals were sucked off, dried, and recrystallized from petroleum ether. Yield of the target product: 129 g (94%), mp 57–58°C. ^{1}H NMR: 0.86 (3 H, t, $(CH_2)_{11}\underline{Me}$); 1.24 (18 H, m, $(C\underline{H_2})_9Me$); 1.54 (2 H, m, $C\underline{H_2}C_{10}H_{21}$); 2.21 (6 H, s, $Ar\underline{Me}$), 2.40 (2 H, t,

$C\underline{H}_2C_{11}H_{23}$); 3.58 (2 H, s, $ArC\underline{H}_2S$); 4.54 (1 H, s, OH); 6.89 (2 H, s, Ar). Found, %: C 75.03, H 10.92, S 9.32. $C_{21}H_{36}OS$. Calculated, %: C 74.94, H 10.78, S 9.53.

2-Hydroxyethyl-(3,5-dimethyl-4-hydroxybenzyl)sulfide (F2). Aqueous formaldehyde (152 ml, 1.64 mol H_2CO) was added dropwise in an atmosphere of inert gas at 80°C to a solution of 2,6-dimethylphenol (100 g, 0.82 mol) and KOH (29.4 g, 0.41 mol) in 2-mercaptoethanol (73.4 g, 0.94 mol) and stirred for 2 h at 80°C. The mixture was cooled, neutralized by hydrochloric acid, and treated with benzene. The extract was washed with hot water, cooled, and precipitated crystals were sucked off, dried, and recrystallized from benzene. Yield of the target product: 141 g (81%); mp 63.5–65°C. ^{1}H NMR: 2.20 (6 H, s, Me), 2.32 (1 H, s, br, $CH_2O\underline{H}$), 2.63 (2 H, t, $SC\underline{H}_2CH_2$), 3.59 (2 H, s, $ArC\underline{H}_2S$); 3.66 (2 H, m, $C\underline{H}_2OH$), 4.95 (1 H, s, $ArO\underline{H}$), 6.89 (2 H, s, Ar). Found, %: C 62.34, H 7.51, S 15.25. $C_{11}H_{16}O_2S$. Calculated, %: C 62.23, H 7.60, S 15.10.

3-(3,5-Dimethyl-4-hydroxyphenyl)propanol-1. 2,6-Dimethylphenol (7.48 g, 61.2 mmol), allyl alcohol (16.8 ml, 0.25 mol), and finely ground NaOH (2.52 g, 61.2 mmol) were placed in an ampoule of heat-stable glass. The ampoule was sealed and kept in a thermostat equipped with a shaker for 5 h at 160°C. After cooling, the mixture was diluted with water, neutralized with HCl, and treated with benzene. The extract was washed with water and dried over Na_2SO_4, and the solvent was distilled off. The residue was distilled in a vacuum to yield 2.76 g (25%) of the target alkanol, mp 69–70°C (from a mixture of toluene and hexane); ^{1}H NMR: 1.50 (1 H, s, br, $CH_2O\underline{H}$), 1.83 (2 H, m, $ArCH_2C\underline{H}_2$); 2.20 (6 H, s, Me), 2.56 (2 H, t, $ArC\underline{H}_2$), 3.65 (2 H, t, $C\underline{H}_2OH$), 4.47 (1 H, s, br, $ArO\underline{H}$), 6.79 (2 H, s, Ar). Found, %: C 73.15, H 8.88. $C_{11}H_{16}O_2$. Calculated, %: C 73.30, H 8.95.

1-Bromo-3-(3,5-dimethyl-4-hydroxyphenyl)propane. A mixture of 3-(3,5-dimethyl-4-hydroxyphenyl)propanol-1 (20 g, 0.11 mol) and hydrobromic acid (97 ml, 0.67 mol HBr) was boiled in an atmosphere of inert gas for 6 h using a Dean–Stark attachment, cooled, and treated with toluene. The extract was washed with water, dried over Na_2SO_4, and the solvent was distilled off. The residue was distilled in a vacuum to yield 24.3 g (90%) of the target bromo derivative, mp 34–35°C (from hexane); ^{1}H NMR: 2.19 (2 H, m, $ArCH_2C\underline{H}_2$), 2.30 (6 H, s, Me), 2.71 (2 H, t, $ArC\underline{H}_2$), 3.46 (2 H, t, CH_2Br), 4.68 (1 H, s, OH), 6.87 (2 H, s, Ar). Found, %: C 54.25, H 6.13, Br 32.75. $C_{11}H_{15}BrO$. Calculated, %: C 54.34, H 6.22, Br 32.86.

Dodecyl-3-(3,5-dimethyl-4-hydroxyphenyl)propylsulfide (F3). 1-Bromo-3-(3,5-dimethyl-4-hydroxyphenyl)propane (2.43 g, 10 mmol) in ethanol (10 ml) was added dropwise in an atmosphere of inert gas to a solution of dodecanethiol-1 (2.02 g, 10 mmol) and KOH (0.69 g, 10.5 mmol) in ethanol (15 ml), and the solution was heated and stirred for 4 h at 30°C. The reaction mass was cooled, neutralized by hydrochloric acid, and treated with toluene. The extract was washed with water, dried over Na_2SO_4, and the solvent was distilled off. After the crystallization of the residue from methanol 2.59 g (71%) of the target product was obtained; mp 50–51.5°C. ^{1}H NMR: 0.89 (3 H, t, $(CH_2)_{11}\underline{Me}$); 1.26 (16 H, m, $(C\underline{H}_2)_8Me$); 1.36 (2 H, m, $C\underline{H}_2C_9H_{19}$); 1.54 (2H, m, $C\underline{H}_2C_{10}H_{21}$); 1.80 (2 H, m, $ArCH_2C\underline{H}_2$), 2.19 (6 H, s, ArMe), 2.40 (4 H, m, $SC\underline{H}_2$), 2.54 (2 H, t, $ArC\underline{H}_2$), 4.30 (1 H, s, OH), 6.71 (2 H, s, Ar). Found, %: C 75.61, H 10.92, S 8.97. $C_{23}H_{40}OS$. Calculated, %: C 75.76, H 11.06, S 8.79.

Determination of the mutagenicity of compounds. In the Ames test, the histidine-dependent strain of *S. typhimurium* TA102 was used, which carries a mutation at the histidine operon [31]. The mutagenic activity of samples was assayed by the standard method without metabolic activation [31]. A night culture of strain OA102 was obtained by a 16-h incubation at 37°C in LB medium with ampicillin. Then cells were seeded on plates with minimal glucose agar, antibiotics, and histidine in such a way as to obtain isolated colonies. A separate bacterial colony was added to LB medium (5 ml) containing ampicillin (50 μg/ml) and tetracycline (2 μg/ml) and incubated on a shaker (130 rpm) for 15 h at 37°C [55].

The Ames test was carried out using the method of double-layer agar as described in [31]. A night culture of *S. typhimurium* strain TA102 (100 μl), one of the three compounds at a final concentration of 20–80 nM (in 5 μl of DMSO), and H_2O_2 to a final concentration of 3 mM were added at 42°C to 2 ml of melted 0.6% upper agar. The mixture was stirred and seeded onto plates with minimal agarized medium A containing 0.2% glucose [55] and 3% agar, distributing the reaction mixture uniformly on the surface of the solidified agar. The plates were incubated for 48 h at 37°C, and the number of His^+-revertants was estimated (see below). Cells incubated with H_2O_2 in the absence of antioxidants were used as positive controls, and bacterial cells grown in the absence of peroxide and antioxidants served as a negative control. The number of His^+-revertants on the plate was counted after a 48-h incubation at 37°C using the program Image Pro Plus V. 4.1 (MEDIA CYBERNETICS). In determinations of the mutagenicity, a maximum amount of revertants, and in determinations of the antioxidant activity, a minimal amount of revertants (the average of three independent experiments) was taken to be 100%. The mutagenicity and the antioxidant activity for the rest of compounds were calculated relative to these values. The statistical processing of the data was carried out using the Student *t*-test (Statistica 5.0). Differences at $P \le 0.05$ were considered to be significant.

Antioxidant activity of compounds was determined using special *E. coli* strains as described [29]. Bacterial cells were grown on a shaker (130 rpm) for 15 h at 37°C in LB medium containing no antibiotics in experiments with strain *cc104* or containing kanamycin

(12.5 μg/ml) in experiments with strain *cc104mutT*, or kanamycin (12.5 mg/ml) and tetracycline (50 μg/ml) in experiments with strain *cc104mutMY* as described in [55]. An inoculate of the night culture was diluted 100 times, and cells were grown in LB medium until the middle of the exponential growth phase. A solution of the antioxidant tested in DMSO (0.5, 1.25, and 5 μl) was added to aliquots of cell cultures (0.9 ml) corresponding to the middle of the exponential growth phase ($A_{405} \sim 0.26$) to a final concentration of 8, 20, or 80 nM, and the corresponding volume of DMSO or medium was added to control samples. The mixture was incubated for 20 min on a shaker at 37°C (130 rpm). Then 25 mM H_2O_2 (100 μl) was added to all samples to a final concentration of 3 mM, and cells were incubated for an additional 20 min under the same conditions. The cell growth was arrested by a tenfold dilution of the incubation mixture with 0.15 M NaCl to a concentration of 2×10^3 colony-forming units/ml, and the cell culture (100 μl) was seeded on dishes with 2% LB agar, three dishes for each oxidant concentration examined. The dishes were incubated for 24 h at 37°C, and the number of grown colonies was calculated using the program Image Pro Plus V. 4.1. Cells that were not exposed to hydrogen peroxide and antioxidants and cells treated with DMSO were used as controls. The survival was estimated by calculating the ratio of the mean number of colonies in experiment to the mean number of colonies in the control having regard to the average data of three separate experiments. The protecting effect of antioxidants was assessed from the difference in cell survival in the presence and absence of the corresponding antioxidant. The statistical processing of the data was carried out using the Student *t*-test (Statistica 5.0). Differences at $P \leq 0.05$ were considered significant.

Determination of dominant lethal mutations in embryonic cells of mice. Experiments were performed with males and females of the genetically homogeneous line CBA/Lac. Each experimental and control group included 20 males weighing 20–25 g. All animals were kept on a 12-h light regime with free access to food and water. A compound being examined was dissolved in olive oil at a concentration of 52 μM and administered per os singly to the animal using a gastric tube, 0.2 ml per animal. For comparison of the genotoxicity of the compound with that of the known compounds, α-tocopherol in the same amount was administered to animals. Cyclophosphane, which has a strong mutagenic effect [61] at a dose of 1 mg per animal, dissolved in the same amount of olive oil, served as a positive control. Control animals were given pure olive oil. Immediately after the treatment, five Virgin females were placed to the cage with a male from the control and experimental groups. Then females were added every week to analyze the dominant lethality according to the stage of spermatogenesis of treated males.

The study was performed for 3 weeks at postmeiosis stages of spermatogenesis. Females were chosen by the presence of a vaginal plug, which indicates the occurrence of mating; the day the plug was detected was considered to be the first day of pregnancy. Females set away from males were autopsied on day 17–18 of pregnancy since the greater portion of dominant lethals causes the death of embryos during, or shortly after the implantation. The number of dead embryos (dark homogenous rounded bodies 2.5-3 mm in diameter) and the number of alive and dead embryos in both uterus horns were calculated. The lethality of embryos in females that became pregnant in the first week after the administration of the chemical compound reflects mutational events that occur in mature spermatozoa, the second week corresponds to late spermatids, and the third week corresponds to early and middle spermatids. The number of pregnant females and the percent of fertility were determined from the number of yellow bodies in ovaries as described in [59]. The frequency of occurrence of dominant lethals was calculated by postimplantation losses by the formula: $F_L\% = [1 -$ number of alive embryos per female in the experimental group/number of alive embryos per female in the control group$] \times 100$ [60]. The data were processed by two-factor dispersion analysis.

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