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New Phenolic Antioxidants of PYA and PYE Class Increase the Resistance *S. cerevisiae* Strain SP4, its SOD- and Catalase-Deficient Mutants to Lipophilic Oxidants

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ABSTRACT. Was demonstrate the protection ability against reactive oxygen species afforded to *S. cerevisiae* (wild-type strain SP4 and its mutants deficient in major antioxidant enzymes – catalase T and A, CuZnSOD) by PYA and PYE, new groups of phenolic antioxidants (quaternary ammonium salts of dihydrocinnamic acid amino esters with different alkyl chains; synthesized in this laboratory). The survival of strains exposed to the lipophilic oxidation inducers *tert*-butyl hydroperoxide (TBHP) and 1,1′-azobis(4-cyclohexane carbonitrile) (ACCN) with or without antioxidant pretreatment was determined by plating. *S. cerevisiae* mutant deficient in SOD was found to be hypersensitive to TBHP and ACCN while the sensitivity of the strain deficient in catalase T and A was about the same as in the wild-type strain. A 1-h preincubation of cells of both the wild-type and the mutant strains with the phenolic antioxidants prior to exposure to TBHP or ACCN substantially increased the cell survival. The magnitude of protection depended on the strain and the length of the alkyl chain of the antioxidant; the best average protection against TBHP was provided by PYE and PYA compounds with 12- and 16-membered alkyl chains whereas PYE-8 and PYA-12 derivatives afforded the best average protection against ACCN.

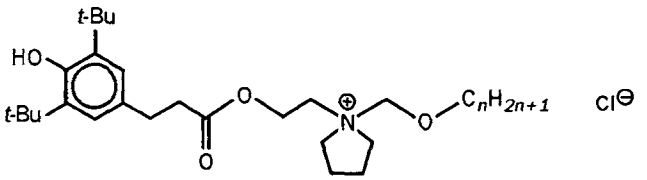
Reactive oxygen species (ROS), such as the superoxide radical $O_2^{-}\bullet$, the hydroxyl radical HO•, H_2O_2 or organic peroxides, are generated endogenously in all aerobic cells by incomplete reduction of molecular oxygen to water in the electron transport chain (Pinkus *et al.* 1996). Their action on cell constituents leads to oxidative stress that can cause lipid and protein peroxidation, changes in the structure of nucleic acids, and can contribute to many severe diseases such as atherosclerosis (Yagi 1994), cancer (Halliwell and Gutteridge 1999) or AIDS (Pace and Leaf 1995). To survive in an oxidative environment, cells have developed antioxidant defense mechanisms to prevent and/or repair the oxidative damage (Gille and Sigler 1995). These mechanisms include nonenzymatic antioxidants, such as glutathione and enzyme defenses, *e.g.*, superoxide dismutases (SOD), catalases, peroxidases, *etc.* (Pinkus *et al.* 1996). Under certain conditions, all these natural defense systems may become inadequate, especially in damaged or old cells. Synthetic antioxidants are therefore often used in medicine and/or environmental protection to prevent serious cell damage or death. They comprise a variety of compounds bearing different reactive centers (phenols, thiols, *etc.*) and with widely differing hydrophobicity. Phenolics are efficient antioxidants since they display at least three important activities: they quench free radicals that oxidize lipids (Neužil *et al.* 1997), chelate transient metals that often cause metal-catalyzed oxidation of cell constituents (Luo *et al.* 1996) and quench the dangerous oxidant singlet oxygen (Pinkus *et al.* 1996).

We synthesized a new class of antioxidants, quaternary ammonium salts with a hindered phenol substituent as an antioxidant function. These compounds can incorporate into the lipid matrix of biological membranes and exert their protective free-radical scavenging action there. The protective effect of these compounds was tested on *S. cerevisiae*, which is ideal for investigating oxidative stress responses because it is genetically well defined and its ROS defense systems are well characterized (Evans *et al.* 1998). The cells were exposed to the strong lipophilic oxidants *tert*-butyl hydroperoxide (TBHP) and 1,1′-azobis(4-cyclohexane carbonitrile) (ACCN) and their survival was determined in the absence and presence of the above antioxidants. The role of major antioxidant enzymes in prevention of damage by the oxidants was examined by using yeast mutants deficient in CuZnSOD or catalases T and A.

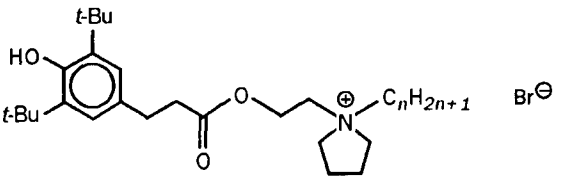
MATERIALS AND METHODS

Chemicals. PYA and PYE antioxidants (Fig. 1) were synthesized in our laboratory by quaternarization of (4-hydroxy-3,5-di-*tert*-butyl)dihydrocinnamic acid 2-pyrrolidine ethyl ester with *n*-alkyl bromides (PYA-*n*) or *n*-alkyl-chloromethyl ethers (PYE-*n*). The structure and purity of the compounds were confirmed

by NMR spectra. TBHP, ACCN, AAPH (1,1'-azobis(3-amidinopropane) dihydrochloride), $FeSO_4 \cdot 7H_2O$ and components of the GO medium were from *Sigma*.



PYE-*n* (*n* = 8, 12, 16)



PYA-*n* (*n* = 8, 12, 16)

Fig. 1. The chemical structure of phenolic antioxidants PYA and PYE.

Yeast strains and growth conditions. The experiments were performed with the wild-type *S. cerevisiae* strain SP4 (*a, leu, arg*) and its isogenic derivatives A50 (*a, leu, arg, catt, cata*) and DSCD1-1C (*a, leu, lys, met, sod*). All strains were obtained from Prof. T. Biliński (*Rzeszów Pedagogical University*, Poland). The cells were grown in GO medium (2 % yeast nitrogen base w/o amino acids, 2 % glucose, essential amino acids) at 28 °C under shaking for 24 h to reach he exponential phase (2×10^7 cells per mL).

Exposure to oxidants and antioxidant treatment. Aliquots of 4.5 mL of the exponential-phase culture were supplied with different PYE or PYA antioxidants (0, 1 and 10 μmol/L) for 1 h. After this time the oxidants (0.1 or 1 mmol/L TBHP, or 10 or 100 μmol/L ACCN) were added and the cell suspension was incubated for another 1 h. All treatments were carried out at 28 °C with shaking. Samples from each suspension were diluted with fresh GO medium and plated in duplicate on GO-containing agar plates containing essential amino acids.

RESULTS AND DISCUSSION

The strains were relatively highly resistant to hydrophilic oxidation inducers such as the azo compound AAPH or Fe^{2+} (Table I). In general, yeast cells are able to adapt to a range of oxidants and free radical-generating agents but they often exhibit sensitivity to lipophilic oxidants that attack unsaturated lipids and may damage the cell membranes. Accordingly, we found that the lipid-soluble oxidation inducers TBHP and ACCN were highly toxic to all yeast strains under investigation. The fact that the most sensitive was the mutant strain DSCD-1 deficient in cytosolic CuZnSOD seems to indicate the role of this enzyme in preventing yeast membrane damage by oxidants.

The PYA and PYE antioxidants seemed to be only slightly toxic to the *S. cerevisiae* strains under study (Table II). The only exception was again the DSCD-1 mutant strain, which was sensitive to PYE compounds, whereas the A50 strain deficient in catalase T and A exhibited about the same low sensitivity as the wild-type strain SP4 (Table II). Pretreatment of cells of all strains with the PYA or PYE antioxidants (Fig. 1) substantially increased their resistance to both TBHP and ACCN (Figs 2 and 3). The magnitude of protection depended on the oxidant in question and on the yeast strain. PYA-12 and PYE-12 with 12-membered alkyl chains afforded the best overall protection of all three strains against TBHP (Fig. 2 *middle, left and right*). PYE-16 provided the best protection of strains SP4 and A50 at 10 μmol/L used but gave only a slight protection of the CuZnSOD-deficient strain DSCD1-1C (Fig. 2 *right, bottom*). At 10 μmol/L, PYA-8 gave nearly full protection to the wild-type strain SP4 and the CuZnSOD-deficient strain DSCD1-1C (Fig. 2 *left, top*) but was less effective with the catalase-deficient strain A50. PYE-8 was also the least effective with strain A50 and its ability to protect strain DSCD1-1C against TBHP decreased with increasing concentration of the oxidant (Fig. 2 *right, top*). This shows that not all of the antioxidants can compensate in full the lack of antioxidant enzymes.

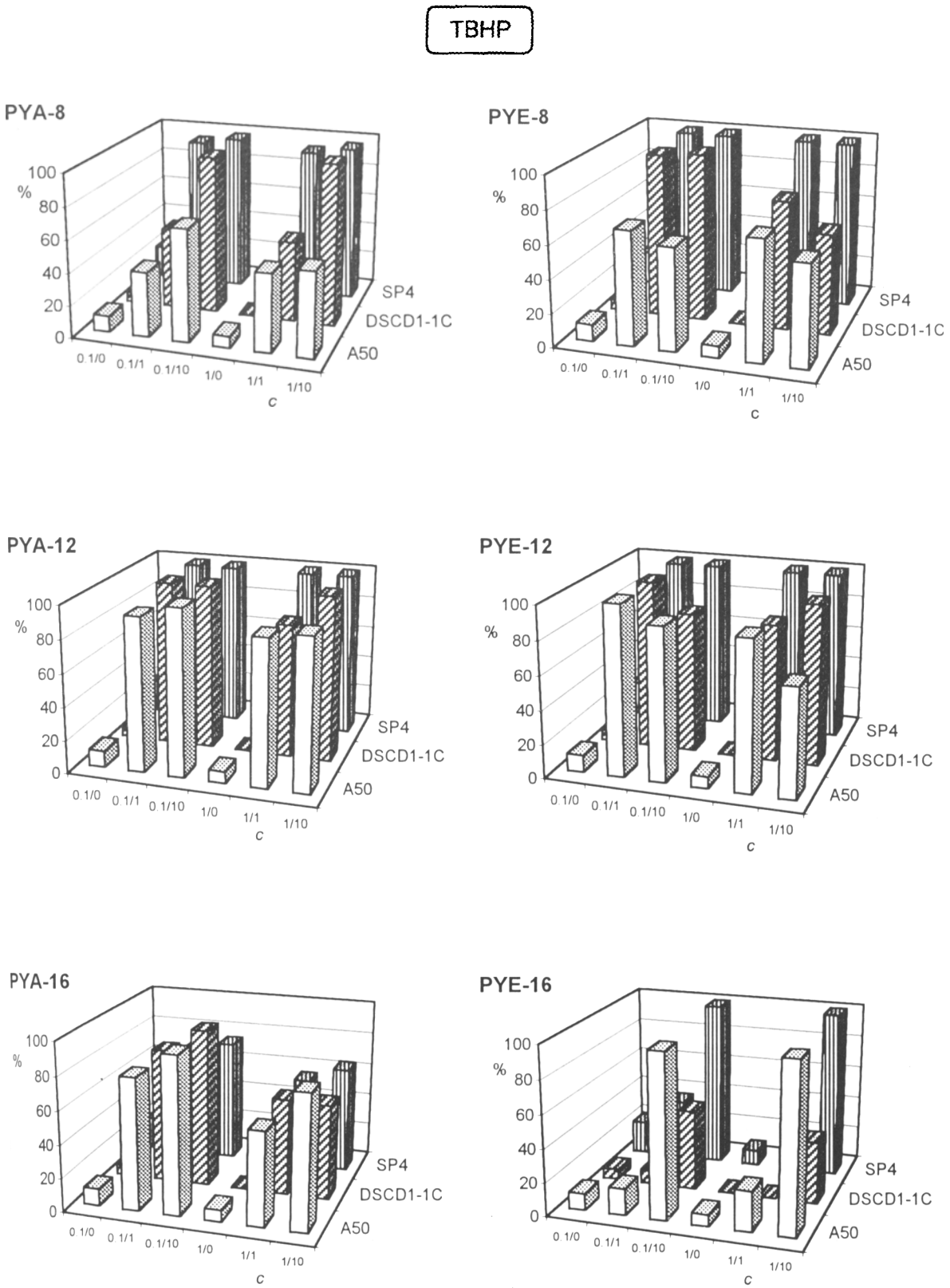


Fig. 2. Effect of pretreatment with **PYA-*n*** and **PYE-*n*** antioxidants on the survival (%) of *S. cerevisiae* strains **A50** (*open columns*), **DSCD1-1C** (*hatching*) and **SP4** (*striping*), and exposed to 0.1 and 1 mmol/L TBHP. **0.1/0** – 0.1 mmol/L TBHP without antioxidant; **0.1/1** – 0.1 mmol/L TBHP after preincubation with 1 μmol/L PYA-*n*/PYE-*n*; **0.1/10** – 0.1 mmol/L TBHP after preincubation with 10 μmol/L PYA-*n*/PYE-*n*; **1/0** – 1 mmol/L TBHP without antioxidant; **1/1** – 1 mmol/L TBHP after preincubation with 1 μmol/L PYA-*n*/PYE-*n*; **1/10** – 1 mmol/L TBHP after preincubation with 10 μmol/L PYA-*n*/PYE-*n*.

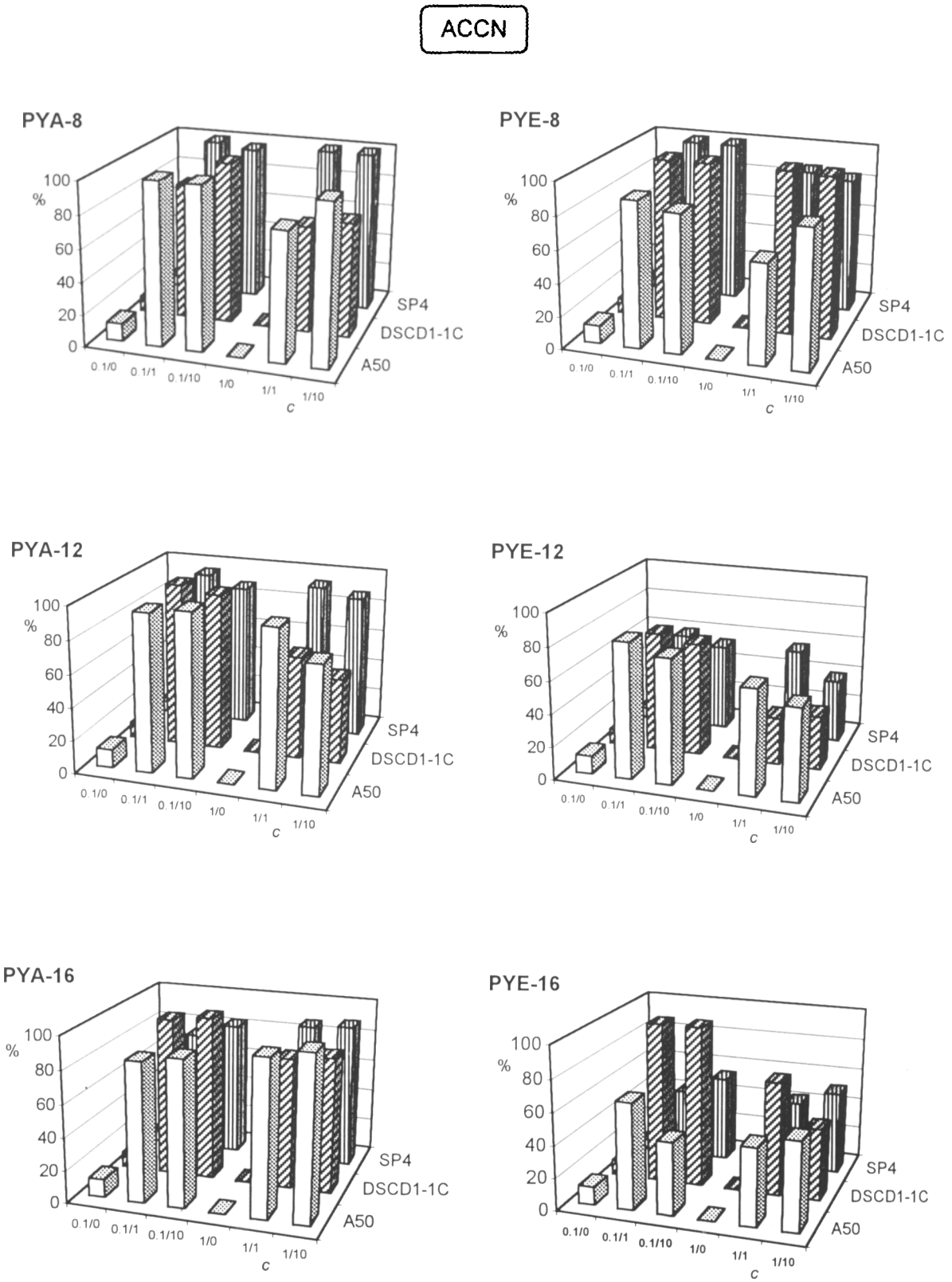


Fig. 3. Effect of pretreatment with PYA-*n* and PYE-*n* antioxidants on the survival (%) of *S. cerevisiae* strains A50 (*open columns*), DSCD1-1C (*hatching*) and SP4 (*striping*), and exposed to 0.1 and 1 mmol/L ACCN. 0.1/0 – 0.1 mmol/L ACCN without antioxidant; 0.1/1 – 0.1 mmol/L ACCN after preincubation with 1 μmol/L PYA-*n*/PYE-*n*; 0.1/10 – 0.1 mmol/L ACCN after preincubation with 10 μmol/L PYA-*n*/PYE-*n*; 1/0 – 1 mmol/L ACCN without antioxidant; 1/1 – 1 mmol/L ACCN after preincubation with 1 μmol/L PYA-*n*/PYE-*n*; 1/10 – 1 mmol/L ACCN after preincubation with 10 μmol/L PYA-*n*/PYE-*n*.

As concerns the protective action against ACCN, antioxidants PYA-8 and PYE-8 with eight carbon atoms in the alkyl chain were highly effective. PYA-12 and PYA-16 were better protectants against ACCN than their PYE counterparts (Fig. 3 *middle and bottom left vs. middle and bottom right*). The structure of the antioxidant molecule thus plays a significant role in the protective effect against ROS. This is obviously connected with the readiness of the compound to form micelles and incorporate into the cell membrane. One can hypothesize that the site of action of the best antioxidants is identical with the site of action of oxidation inducers.

Table I. Toxic effect of concentration of the lipophilic oxidation inducers TBHP, ACCN, the hydrophilic AAPH, and Fe^{2+} on cell survival (%)*S. cerevisiae* strains SP4, A50 and DSCD1-1C[a]

Oxidation inducer	Concentration mmol/L	SP4	A50	DSCD1-1C
TBHP	0.01	100	78	59
	0.1	20	10	6
	1	9	7	0
ACCN	0.01	11	9	6.5
	0.1	0	0	0
	1	0	0	0
AAPH	0.01	100	100	100
	0.1	100	100	100
	1	100	100	97
Fe^{2+}	0.01	100	100	80
	0.1	100	100	76
	1	100	100	67

[a]Determined by plating; means of two measurements.

Phenolic antioxidants had the highest protective effect in the mutant strain DSCD-1 lacking cytosolic superoxide dismutase, followed by the A50 mutant strains lacking catalase. Very low concentrations (1–10 μmol/L) of all antioxidants were effective against considerably higher concentrations of the oxidants. Conventional phenolic antioxidants such as di-*tert*-butylhydroxytoluene (BHT), which are used in food industry, are active in concentrations of about 10 mmol/L (Kleszczyńska *et al.* 1998). The fact that our PYA and PYE antioxidants are active in much lower concentrations may result from their ready incorporation into biological membranes (Kleszczyńska *et al.* 1998; Witek *et al.* 1999) and the quenching of ROS immediately at the sites of their generation.

Table II. Effect of concentration of the lipophilic PYA and PYE antioxidants on the survival of *S. cerevisiae* strains SP4, A50 and DSCD1-1C[a]

Antioxidant	Concentration μmol/L	SP4	A50	DSCD1-1C
PYA-8	1	100	100	2
	10	76	76	1
PYA-12	1	100	100	87
	10	79	100	81
PYA-16	1	64	100	100
	10	55	78	62
PYE-8	1	74	75	2
	10	67	65	1.5
PYE-12	1	78	100	6
	10	76	99	2
PYE-16	1	79	89	9
	10	71	77	4

[a]See footnote to Table I.

Superoxide dismutase catalyzes the conversion of the superoxide radical ($O_2^{\bullet-}$) to H_2O_2, which is then decomposed by catalase. Our phenolic antioxidants thus seem to protect the cells from these two ROS and, to a certain extent, make up for the lack of these two enzymes in mutant strains. Although the exact mechanism of their protective action is not yet known, they may be of potential use in preventing cell damage by lipophilic oxidants.

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