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Amphiphilic Amine-*N*-oxides with Aliphatic Alkyl Chain Act as Efficient Superoxide Dismutase Mimics, Antioxidants and Lipid Peroxidation Blockers in Yeast

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ABSTRACT. Amphiphilic 3-(alkanoylamino)propyldimethylamine-*N*-oxides with different length of the alkyl chain, *i.e.* different hydrophilic–lipophilic balance, act in micromolar concentrations as SOD mimics by lifting the inhibition of aerobic growth caused by SOD deletions in *Saccharomyces cerevisiae*. They also enhance the survival of *sod* mutants of *S. cerevisiae* exposed to the hydrophilic superoxide-generating pro-oxidant paraquat and the amphiphilic hydroperoxide-producing *tert*-butylhydroperoxide (TBHP), and largely prevent TBHP-induced peroxidation of isolated yeast plasma membrane lipids. Unlike the SOD-mimicking effect, the magnitude of these effects depends on the alkyl chain length of the amine-*N*-oxides, which incorporate into *S. cerevisiae* membranes, causing fluidity changes in both the hydrophilic surface part of the membrane and the membrane lipid matrix. Unlike wild-type strains, the membranes of *sod* mutants were found to contain polyunsaturated fatty acids; the sensitivity of the mutants to lipophilic pro-oxidants was found to increase with increasing content of these acids. *sod* mutants are useful in assessing pro- and antioxidant properties of different compounds.

Abbreviations

HLB	hydrophilic–lipophilic balance
LPO	lipid peroxidation
NBD-PE	*N*-(7-nitrobenz-2-oxa-1,3diazol-yl)-1,2-dihexadecanoyl-*sn-glycero*-3-phosphoethanolamine, triethylammonium salt
PDA	3-(alkanoylamino)propyldimethylamine-*N*-oxide
PUFA	oligounsaturated fatty acids
PCR	polymerase chain reaction
ROS	reactive oxygen species
SOD	superoxide dismutase (EC 1.15.1.1)
SUV	small unilamellar vesicles
TBHP	*tert*-butylhydroperoxide
TBRS	2-thiobarbituric acid-reactive substances
TMA-DPH	*N,N,N*-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium 4-toluenesulfonate

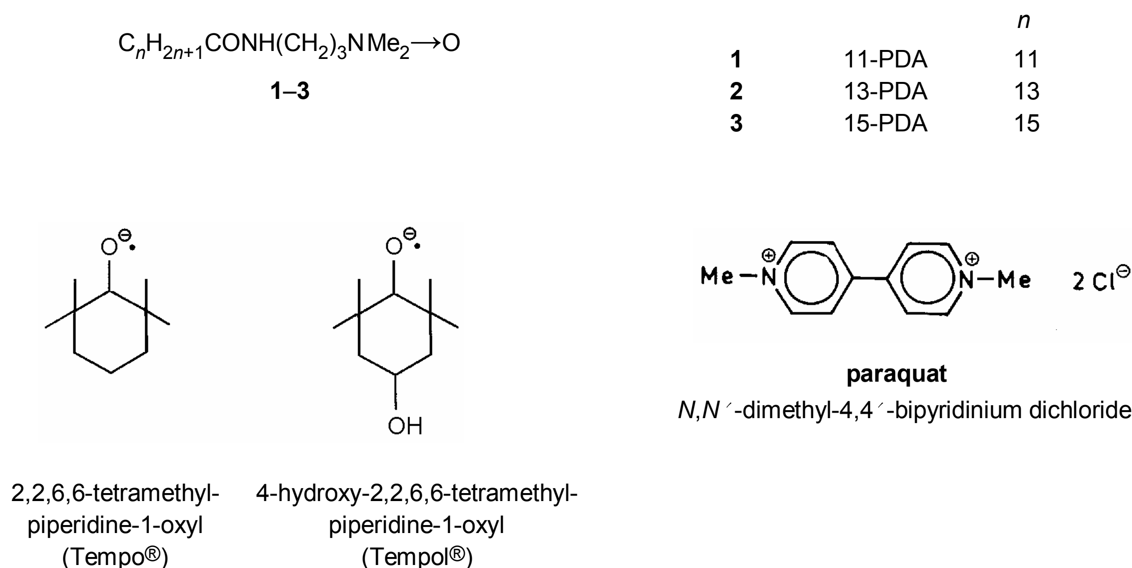
One of the major processes caused by ROS such as hydrogen peroxide, superoxide or hydroxyl radicals and damaging cells by affecting membranes containing long-chain unsaturated acyl substituents is LPO. An effective antioxidant preventing LPO should: (*i*) be present and active in both lipophilic and hydrophilic compartments, (*ii*) be nontoxic, and (*iii*) react with a wide range of reactive species.

Some years ago, considerable antioxidant properties have been revealed in nitroxides – stable, nontoxic radicals, which interact with other radicals by several mechanisms, *e.g.*, trapping of carbon and oxygen radicals (Blough *et al.* 1988), terminating radical chain reactions (Nilsson *et al.* 1989) or catalytic dismutation of the superoxide anion (O_2^{-}•) (Offer *et al.* 1998). Thanks to this last function, nitroxides can function as metal-free SOD mimics.

Unlike exogenously added SOD, nitroxides are not immunogenic and penetrate readily into the cell (Boccu *et al.* 1982). Apart from nitroxides, several metal chelates that catalyze the dismutation of O_2^{-}• have previously been reported as SOD mimics (Weiss *et al.* 1996; Benov and Fridovich 1996; Batinic *et al.* 1997) but unlike nitroxides they are susceptible to dissociation and might exert pro-oxidative activity (Nagele and Lengfelder 1996).

The best known and described nitroxides are cyclic synthetic Tempo® (2,2,6,6-tetramethylpiperidine-1-oxyl) and Tempol® (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; Samuni and Barenholz 2003) which are used as spin probes in electron paramagnetic resonance spectroscopy and as contrasting agents for magnetic resonance imaging (Kocherginsky and Swartz 1995). Both exert their protective effect mainly in the aqueous phase by scavenging ROS formed near the lipid bilayer (Samuni *et al.* 1997; Samuni and Barenholz 2003).

Based on the idea that other compounds containing the N–O group and differing in the HLB parameter might also exhibit antioxidant properties, we synthesized three 3-(alkanoylamino)propyldimethylamine-*N*-oxides (**1–3**) with different length of the alkyl chain and examined their efficacy in inhibiting the oxidative damage of yeast lipids and alleviating ROS-induced growth suppression.



We focused on the damage of lipids from *S. cerevisiae* membranes by two pro-oxidants – paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride) that produces superoxide anion, and TBHP, a generator of hydroperoxides, and on the protective action of amine-*N*-oxides against the damage. Another part of our study concerned the survival of intact cells of *S. cerevisiae sod* mutants treated by paraquat and TBHP, and the protective effect of the amine-*N*-oxides on these cells.

MATERIALS AND METHODS

Strains and growth conditions. Mutant strains of *S. cerevisiae* with deletions in *SOD1* (JS-C50), *SOD2* (JS-C55) and both *SOD1*, *SOD2* (JS-C128) genes were constructed on the genetic background of parental strain US50-18C (JS-C66) (*MATα PDR1-3 ura3 his1*) (Table I).

Table I. Strains used

Strain	Origin	Genotype
JS-C66	US50-18C (Balzi *et al.* 1987)	*MATα PDR1-3 ura3 his1*
JS-C50	*this work*	*MATα PDR1-3 ura3 his1 Δsod1*
JS-C55	*ditto*	*MATα PDR1-3 ura3 his1 Δsod2*
JS-C128	*ditto*	*MATα PDR1-3 ura3 his1 Δsod1 Δsod2*

Deletions of respective *SOD1* and *SOD2* genes were performed by the short flanking homology method (Wach *et al.* 1998) with replacement marker *loxP–kanMX–loxP* (Güldener *et al.* 1996). Marker gene *kanMX* contains at its ends short *loxP* sequences homologous to each other. The action of the Cre recombinase on these sequences allows the excision of the marker gene from the chromosome, permitting multiple

deletions of genes in the same strain. Proper integration of disruption cassettes and subsequent *kanMX* rescue was verified by analytical PCR.

Strains were grown in YEPD medium (1 % yeast extract, 2 % bactopeptone, 2 % glucose) at 30 °C for 20 h in 750 mL flasks containing 500 mL medium, the flask volume-to-medium ratio of 1.5 : 1 ensuring microaerophilic conditions (Manfredini *et al.* 2005). All strains (parental and *sod* mutants) grew similarly under these conditions; after 20 h they were in the end-exponential phase of growth.

Chemicals. 3-(Alkanoylamino)propyldimethylamine-*N*-oxides **1–3** were synthesized by acylation of *N*,*N*-dimethyl-propanediamine-1,3 with long-chain acyl chlorides followed by oxidation with hydrogen peroxide. The purity of the compounds was verified using ^{1}H-NMR spectra (*Bruker* Avance DRX 300) in $CDCl_3$ solution with tetramethylsilane as internal standard. The fluorescent dyes TMA-DPH and NBD-PE were purchased from *Molecular Probes* (USA). Lecithin was obtained from *Lipid Products* (UK). Pro-oxidants included paraquat (*Fluka*, Poland) and *tert*-butylhydroperoxide (*Sigma-Aldrich*, Poland); 2-thiobarbituric acid and trichloroacetic acid were obtained from *Fluka*, Poland.

Analysis of membrane fatty acid composition. The GC analysis was performed according to Prescha *et al.* (2001). The fatty acids quantified in *S. cerevisiae* membranes included C_{10}, C_{12}, C_{14}, C_{15}, C_{16}, C_{18}, C_{20} and C_{22} saturated fatty acids, $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, and $C_{22:1}$ monounsaturated, and $C_{18:2}$ and $C_{18:3}$ di- and tri-unsaturated fatty acids.

Isolation of plasma membrane lipids. The method of isolation of plasma membranes was based on that used by Dufour *et al.* (1988) for *Schizosaccharomyces pombe*, with modifications by Krasowska *et al.* (2002). Lipids were isolated by the method of Allen and Good (1971) with modifications (*see* Krasowska *et al.* 2002).

Lipid peroxidation was measured in plasma membrane lipids. The pro-oxidants paraquat and TBHP (both at a final concentration of 100 μmol/L) were allowed to induce the oxidation of a lipid containing the antioxidants under study. The end products of lipid peroxidation, *i.e.* malonaldehyde and other TBRS, were quantified by a method adapted from Aust (1994). In brief: 1-mL samples containing 1.5 μg of lipids and 3 and 30 μmol/L antioxidants were incubated for 15 min at 30 °C. The pro-oxidants were added to a concentration of 100 μmol/L and the samples were incubated for further 15 min at the same temperature. Then 2 mL of reagent A (15 % trichloroacetic and 0.37 % 2-thiobarbituric acids in 0.25 mol/L HCl) was added and the mixture was thoroughly blended. Test tubes were heated at 100 °C for 15 min, cooled under running tap water and centrifuged (2000 ***g***, 10 min). Absorbance A_{535} was measured on a *Hach* Odyssey spectrophotometer against a reference blank containing the 2-thiobarbituric acid reagent and distilled water.

Antioxidant activity tests. With the aim of using yeast *sod* mutants for rapid screening of the effect of antioxidants, the experiments with the survival of strains exposed to 10 μmol/L oxidants paraquat and TBHP and supplied with 3 and 30 μmol/L antioxidants were performed in 96-well microplates in 100 μL YPG medium (surface-to-volume ratio 0.28). After a 20-h incubation at 30 °C, absorbance A_{630} was measured on *Dynatech* MR 5000 microplate reader.

*Estimation of amine-*N*-oxide localization in the lipid phase* was done by determining the effect of the amine-*N*-oxides **1–3** on the fluorescence of two probes, TMA-DPH and NBD-PE, incorporated in SUV produced from PML and 0.6 mmol/L lecithin. The SUV were prepared according to Lentz (1988) with modification (*see* Jemioł-Rzemińska *et al.* 1996).

The effects of **1–3** on the fluorescent probes variously immersed in the SUV were assayed by measuring the fluorescence quenching on a *Kontron* SFM 25 spectrofluorimeter at the following excitation and emission wavelengths: for TMA-DPH λ_{ex} = 355 nm, λ_{em} = 450 nm, for NBD-PE λ_{ex} = 460 nm, λ_{em} = 534 nm. The fluorescence intensity measurements yielded polarization coefficients, C_p, which were calculated according to the formula (Jemioł-Rzemińska *et al.* 1996):

$$C_p = \frac{I_{||} - GI_{\perp}}{I_{||} + GI_{\perp}}$$

where $I_{||}$ is the intensity of fluorescence emitted in the direction parallel to the polarization plane of excitation light, $I_{\perp}$ is the intensity of fluorescence emitted in the direction perpendicular to the polarization plane and G is a wavelength-dependent diffraction constant. Interaction of an amine-*N*-oxide with the lipid phase in the vicinity of the probe resulting in lipid fluidization or rigidization should bring about a change in the C_p value. Changes in NBD-PE fluorescence reflect changes occurring in the hydrophilic part of the lipid bilayer, *i.e.* at the level of the phosphate residues of phospholipids, whereas TMA-DPH fluorescence reports on interactions inside the bilayer – below the C-4 atom in the phospholipid alkyl chains (Krasowska *et al.* 2001). In all experiments, a 0.5-mg sample of liposomes was added to 2.7 mL 140 mmol/L NaCl in 10 mmol/L

Tris-HCl (pH 7.4). Aqueous solutions of **1–3** were added at 5-min intervals to a final concentration of 4.3–43 µmol/L.

RESULTS

Alleviation of growth defects in sod *mutants by amine*-N-*oxides.* At 3 µmol/L, compounds **1–3** markedly increased the survival of *sod* mutants and this protective effect was largely independent of the length of the alkyl chain. However, at 30 µmol/L the survival, which was substantially higher in 11-PDA-treated *sod* mutants than in the parent strain JS-C66, tended to decrease with increasing alkyl chain length (Table IIA,B). This decrease in survival, which could be due to the higher toxicity of 13-PDA and 15-PDA at this concentration, occurred only in *sod1* and *sod2* mutants but, surprisingly, not in the *sod1 sod2* mutant where the survival increased with increasing aliphatic chain length. At concentrations >30 µmol/L the amine-*N*-oxides were toxic for all strains, especially for *sod* mutants (*data not shown*); Tempo® had no protective effect.

Table II. Survival (%) of *S. cerevisiae* strains in the presence of antioxidants (Tempo®, 11-PDA, 13-PDA, 15-PDA; **A**, **B**), pro-oxidants (paraquat, TBHP; **C**), and after treatment by *1*) paraquat (**D**) or TBHP (**E**), and *2*) with 30 µmol/L of antioxidants[a]

Strain	Tempo®	11-PDA	13-PDA	15-PDA	Control
A	3 µmol/L of antioxidants				
JS-C66	91 ± 9.1	82 ± 4.5	61 ± 19	66 ± 6.8	100 ± 11.3
JS-C50	83 ± 2.3	177 ± 26	171 ± 9.1	160 ± 12.5	100 ± 31
JS-C55	91 ± 3.4	159 ± 25	137 ± 28	104 ± 18	100 ± 34
JS-C128	79 ± 4.5	152 ± 16	161 ± 18	153 ± 7.9	100 ± 25
B	30 µmol/L of antioxidants				
JS-C66	89 ± 6.8	60 ± 26	61 ± 26	65 ± 19	100 ± 12.6
JS-C50	72 ± 3.1	160 ± 6.3	89 ± 12.1	72 ± 32	100 ± 30
JS-C55	89 ± 5.2	155 ± 6.3	70 ± 24	88 ± 8.4	100 ± 34
JS-C128	71 ± 3.1	126 ± 24	146 ± 7.3	165 ± 19	100 ± 25
C		10 µmol/L paraquat	10 µmol/L TBHP		
JS-C66		101 ± 8.1	71 ± 13.7		100 ± 6.2
JS-C50		40 ± 11.2	34 ± 9.3		100 ± 7.0
JS-C55		75 ± 11.8	47 ± 3.7		100 ± 6.2
JS-C128		37 ± 13.7	14.9 ± 2.5		100 ± 4.3
	11-PDA	13-PDA	15-PDA	Control[b]	
D	10 µmol/L paraquat				
JS-C66	1.8 ± 3.7	3.3 ± 5.1	4.0 ± 4.8	2.6 ± 5.9	
JS-C50	41 ± 6.8	31 ± 3.3	12.1 ± 5.3	5.1 ± 3.7	
JS-C55	26 ± 2.4	21 ± 5.5	19.0 ± 4.4	7.3 ± 2.7	
JS-C128	10.3 ± 2.9	10.3 ± 4.2	5.5 ± 6.4	–4.8 ± 1.8	
E	10 µmol/L TBHP				
JS-C66	17.7 ± 3.6	240 ± 3.7	31 ± 5.7	2.2 ± 3.7	
JS-C50	11.2 ± 7.1	30 ± 4.0	55 ± 14.6	–4.9 ± 2.2	
JS-C55	1.5 ± 1.2	11.2 ± 3.1	18 ± 10.8	–4.4 ± 2.5	
JS-C128	29 ± 7.4	580 ± 6.4	68 ± 7.0	–7.4 ± 2.5	

[a]Values ± confidence interval; $n = 3$, $\alpha = 0.05$. [b]Tempo®.

Effect of amine-N-*oxides on pro-oxidant-exposed* sod *mutants.* Exposure to the superoxide generator paraquat and hydroperoxide-producing TBHP strongly suppressed the survival of *sod* mutants. At 10 µmol/L,

TBHP exhibited a somewhat stronger toxicity than paraquat, both pro-oxidants being more toxic for *sod1* and *sod1sod2* mutants than for the *sod2* counterpart (Table IIC). At higher concentrations, however, paraquat was much more toxic than TBHP for all *sod* mutants (*data not shown*).

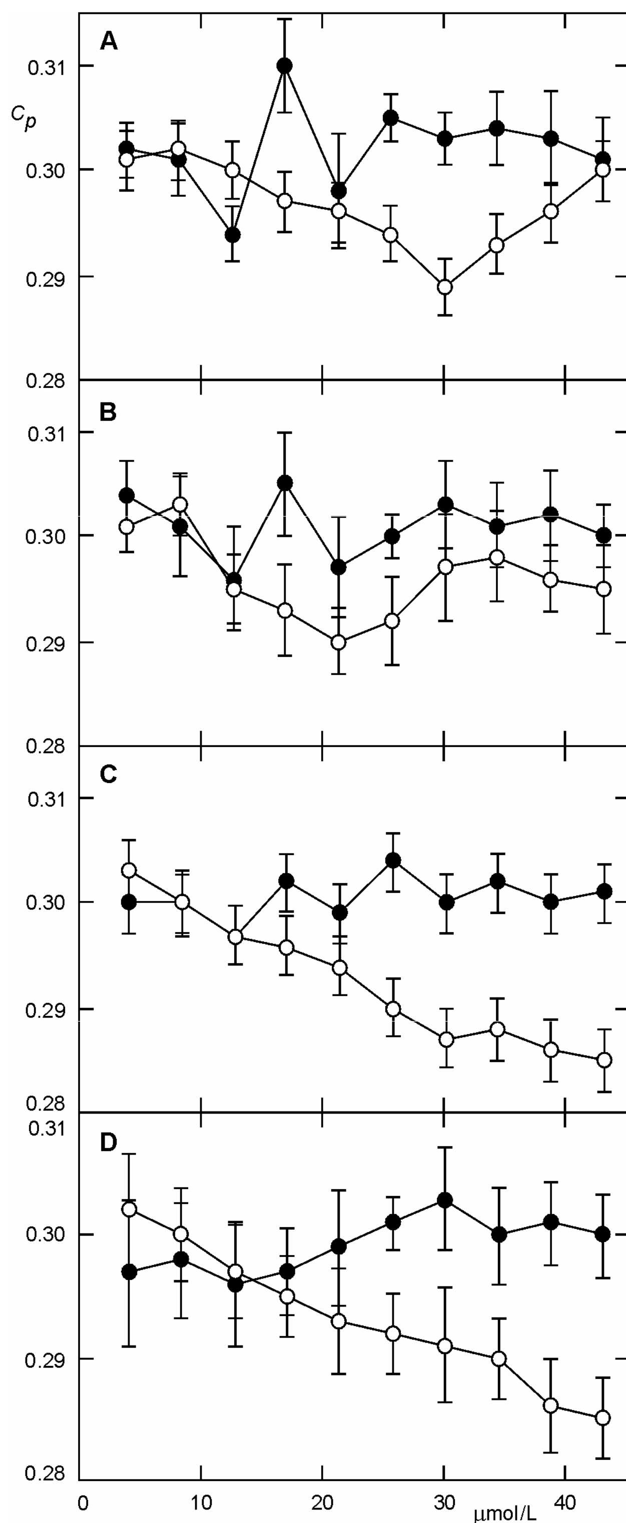


Fig. 1. Polarization coefficient C_p of TMA-DPH fluorescence in SUV reconstituted from the membranes as affected by 11-PDA (*closed symbols*) and 15-PDA (*open symbols*) (both in µmol/L); values ± confidence interval; $n = 3$, $\alpha = 0.05$; **A** – JS-C66, **B** – JS-C50, **C** – JS-C55, **D** – JS-128.

Like with the *sod* mutants exposed only to oxygen during growth, our amine-*N*-oxides **1–3** (with aliphatic alkyl chains) were substantially more effective antioxidants than the cyclic Tempo®, which in fact in some cases (*sod1 sod2* mutant exposed to paraquat, all *sod* mutants exposed to TBHP) slightly decreased the survival (Table IID,E).

In all *sod* mutants, the protection provided by 30 µmol/L amine-*N*-oxides against the toxic action of 10 µmol/L hydrophilic paraquat was higher than in the parent JS-C66 strain and decreased with increasing alkyl chain length. The best protection was observed with the *sod1* mutant followed by *sod2* strain, the survival of the *sod1 sod2* mutant being increased by a mere 5–10 % (Table IID).

In both the parent strain JS-C66 and its *sod* mutants, amine-*N*-oxides efficiently alleviated the growth suppression caused by the lipophilic TBHP. In contrast to the paraquat-induced damage, the protective effect was the stronger the longer was the alkyl chain and was highest in the *sod1 sod2* mutant (nearly 70 % with 15-PDA), followed by the *sod1* strain. The enhancement of survival caused by the amine-*N*-oxides in the *sod2* strain (≈20 % with 15-PDA) was smaller than that observed in the parent strain (Table IIE).

*Effect of amine-*N*-oxides on membrane fluidity at different depths.* The differences in the antioxidant action of different amine-*N*-oxides on individual *S. cerevisiae sod* mutants can reflect their possible influence on the cell membrane lipid matrix, which could be reflected in a change in membrane fluidity. Fig. 1 shows the dependence of membrane fluidity in SUV formed from membrane lipids on the concentration of 11-PDA and 15-PDA.

In all strains, increasing concentrations of both 11-PDA and 15-PDA caused a slight increase in C_p of the membrane surface-localized NBD-PE probe. The slope of this concentration dependence tended to decrease in the sequence *sod1* > *sod2* > *sod1 sod2* ≈ parent strain. However, the changes fell mostly within the confidence intervals and the effect of the amine-*N*-oxides on membrane fluidity in the region monitored by the NBD-PE probe was thus marginal.

In all strains, the C_p of the TMA-DPH probe situated within the membrane lipid matrix exhibited only slight changes with increasing 11-PDA concentration, whereas 15-PDA caused either a C_p drop followed by an increase at higher concentrations (in the parent strain and the *sod1* mutant) or a marked C_p decrease throughout the concentration range (*sod2* and *sod1 sod2* mutants). Tempo® showed only negligible effects on the fluorescence (*data not shown*).

*Peroxidation of plasma membrane lipids by paraquat and TBHP and protective effect of amine-*N*-oxides.* Plasma membrane lipids from *sod* mutants were oxidized by high concentration (100 µmol/L) of paraquat and TBHP and, as a control, subjected to autooxidation at 30 °C. The level of lipid peroxidation caused by paraquat was about equal or only slightly higher than autooxidation while TBHP induced a large increase of TBRS levels in all three mutants, which rose in the order *sod1* < *sod2* < *sod1 sod2* (Table III).

Table III. Peroxidation of plasma membrane lipids (TBRS, mmol/kg) of *S. cerevisiae* strains after autooxidation (AO) and application of 100 µmol/L paraquat and TBHP[a]

Strain	AO	Paraquat	TBHP
JS-C66	160 ± 48	210 ± 75	96 ± 55
JS-C50	300 ± 55	520 ± 75	1440 ± 75
JS-C55	490 ± 89	580 ± 34	2380 ± 96
JS-C128	710 ± 82	840 ± 82	2820 ± 34

[a]Values ± confidence interval; $\alpha = 0.05$.

For the *sod1 sod2* mutant, the protective effect of amine-*N*-oxides against TBHP-induced peroxidation of membrane lipids increased with increasing antioxidant concentration and with increasing alkyl chain length (Table IV).

Table IV. Inhibition of plasma membrane lipids peroxidation[a] (%) at strain JS-C128 by 3 and 30 µmol/L amine-*N*-oxides[b]

µmol/L	Tempo®	11-PDA	13-PDA	15-PDA
3	11.8 ± 1.97	15.1 ± 4.93	23.0 ± 3.61	40.1 ± 5.09
30	26.3 ± 1.97	44.0 ± 2.14	51.9 ± 3.12	57.1 ± 5.09

[a]Caused by 100 µmol/L TPBH. [b]Values ± confidence interval; $n = 3$, $\alpha = 0.05$.

Contents of unsaturated fatty acids in the membranes. The GC analysis of the fatty acid composition of membranes of individual strains showed that, somewhat surprisingly for *S. cerevisiae*, the membranes of *sod* mutants contain, in addition to saturated and monounsaturated fatty acids, also PUFA (Table V).

DISCUSSION

In the presence of oxygen, *sod* mutants of *S. cerevisiae* display serious growth defects, which can be further aggravated by additional exposure to various pro-oxidants. Hence, they offer the possibility to test antioxidants under the conditions of graded oxidative stress and we used them as a convenient system for evaluating the antioxidative properties of conventional antioxidants (vitamin A, vitamin E, quercetin, β-carotene) (Krasowska *et al.* 2003).

Nitroxides with a five- or six-membered ring in the molecule, previously studied by Samuni and coworkers (Samuni *et al.* 1997; Samuni and Barenholz 2003), were found to protect lipid bilayers against γ-irradiation-induced LPO. They act catalytically and are self-replenished, without any pro-oxidative effects, and their mode of action was reported to involve scavenging radicals in the aqueous phase at or near the bilayer surface, no correlation being found between their lipophilicity and the protective effect. Also, no difference was found between the antioxidant efficiency of nitroxides differing solely in the depth of their location in the bilayer. In yeast, the nitroxide Tempol® was found to be a less effective antioxidant than, *e.g.*, ascorbate and glutathione (Lewinska *et al.* 2004).

The amine-*N*-oxides **1–3** with various HLB caused by different lengths of the hydrophobic alkyl chain were found to act as efficient SOD mimics, lifting the aerobic growth suppression caused by SOD deletions in *S. cerevisiae*. Due to the presence of the polar $^{+}NMe_2{\rightarrow}O^{-}$ grouping in their molecules, which, on interaction with membranes, is located in the hydrophilic surface part of the lipid bilayer, and the alkyl chains which are incorporated into the membrane lipid matrix, their antioxidant action may encompass both the hydrophilic environment at the membrane surface and the lipophilic environment inside the membrane. At 3 μmol/L concentration, the protective action of all three amine-*N*-oxides **1–3** was about the same and significantly exceeded the protection provided by the cyclic Tempo® nitroxide while at 30 μmol/L it was smaller, reflecting apparently an adverse effect of **1–3** on the cells. This toxic effect seems to increase with increasing alkyl chain length.

Table V. The contents of saturated, monounsaturated, oligounsaturated and other fatty acids (% of total) in the membranes of individual strains[a]

Strain	Saturated	Monounsaturated	Oligounsaturated	Other[b]
JS-C66 (control)	37.4	61.4	0.5	0.7
JS-C50 (*sod1*)	36.1	53.7	3.2	7.0
JS-C55 (*sod2*)	50.6	44.8	2.8	1.8
JS-C128 (*sod1 sod2*)	41.0	46.4	5.9	6.7

[a]The relative error of the determination was ≈0.15 %.
[b]The exact structure of these acids could not be determined.

Their potential toxicity notwithstanding, 30 μmol/L amine-*N*-oxides considerably enhanced the survival of *sod* mutants exposed to superoxide- and hydroperoxide-generating pro-oxidants. In this, their protective effect against the action of the pro-oxidants was highest if the HLB of both the pro-oxidant and the amine-*N*-oxide was the same. Longer-chain amine-*N*-oxides were more effective against hydroperoxides generated in the lipid phase by TBHP whereas shorter-chain amine-*N*-oxides eliminated better the paraquat-produced hydrophilic superoxide anion (which has a low ability to penetrate into the lipid bilayer; Frimer *et al.* 1996). However, superoxide may undergo reactions in the lipid bilayer if the active site of the substrate lies at or near the lipid–water interface (Frimer *et al.* 1996) and it may give rise to lipid-oxidizing hydroxyl radicals in the Haber–Weiss reaction (Halliwell and Gutteridge 1989).

Plasma membrane lipids isolated from the *sod1 sod2* mutant were hardly sensitive to the oxidizing action of paraquat and the level of peroxidation induced by this agent approximately corresponded to the level of autooxidation. This is in keeping with the known poor penetration of superoxide anion into isolated bulk lipids. On the other hand, the peroxidating activity of the lipophilic TBHP and its product hydroperoxides, which are efficient lipid oxidants, was much higher and increased in the sequence parent strain < *sod1* < *sod2* < *sod1 sod2*. The protective effect against the peroxidation of plasma membrane lipids from the *sod1 sod2* mutant caused by 100 μmol/L TBHP was perceptible even with 3 μmol/L amine-*N*-oxides **1–3**, reaching 40 % with **3**. At 30 μmol/L the inhibition of peroxidation attained with the least effective **1** was nearly twice that provided by Tempo® while **3** displayed a >50 % inhibition of peroxidation.

In contrast to cyclic nitroxides (Samuni *et al.* 1997; Samuni and Barenholz 2003), the protective effects of our amine-*N*-oxides **1–3** on pro-oxidant-exposed whole cells of *sod* mutants and the suppression of peroxidation of isolated plasma membrane lipids are obviously closely associated with their HLB, *i.e.* with the length of the alkyl chain. Accordingly, compounds **1–3** affect the fluidity of membrane lipids. A similar relationship between lipophilicity and antioxidative properties has been described in another group of antioxidants – bifunctional surfactants, *viz.* pyrrolidinium and piperidinium bromides with 7–15 carbon atoms in their aliphatic chain (Kleszczyńska *et al.* 2002). However, both the bifunctional surfactants and the amine-*N*-oxides have considerable antioxidant and membrane-protective effects in micromolar concentrations that have yet no discernible effects on membrane fluidity.

The efficiency of the antioxidative protection was also found to depend on the kind of the *sod* mutation; the protection of the *sod1* mutant exposed to the hydrophilic paraquat is the largest while the *sod1 sod2* mutant is less protected. Conversely, the overall protection of this double mutant by all amine-*N*-oxides against TBHP-generated hydroperoxides is the highest.

The high lipid peroxidation rates of membrane lipids from the *sod* mutants and the strong stress-alleviating action of lipophilic amine-*N*-oxides on cells exposed to TBHP are hard to explain if, as traditionally believed, *S. cerevisiae* is in all circumstances unable to synthesize PUFA and does not require protection

by lipophilic antioxidants (*see*, *e.g.*, Żyracka *et al.* 2005). Our analysis of fatty acid composition of membranes revealed a novel feature, *viz.* the presence of PUFA in the membrane of *sod* mutants. Consistent with this finding, the lipid-phase-situated oxidative processes were found to be the most severe in the *sod1 sod2* double mutant, which shows the highest content of PUFA, and the protection afforded by the more lipophilic **2** and **3** was correspondingly the highest.

According to some authors, the plasma membrane in aerobically grown *S. cerevisiae* contains only saturated and monounsaturated fatty acids (*e.g.*, Paltauf *et al.* 2002), while others found a certain proportion of PUFA in cell lipids (1.1–4.6 % depending on conditions; Singh *et al.* 1990). Likewise, the membrane of anaerobically grown brewer's yeast *S. cerevisiae* has recently been reported to contain 2.5 % of PUFA (Blagović *et al.* 2005). Growth under microaerophilic conditions may have the same effect; in contrast to Lewinska *et al.* (2004) we found 1.3 % of linoleic acid in the membrane of wild-type *S. cerevisiae* strain SP-4 cultured under identical conditions (Krasowska *et al.* 2002). Our present data suggest that deletions in *sod* genes can have a similar effect as anaerobic culturing, and/or potentiate the effect of cultivation under microaerophilic conditions, increasing the proportion of polyunsaturated fatty acids in the plasma membrane.

Manfredini *et al.* (2005) showed that, compared with wild-type strain, cells of *sod* mutants of *S. cerevisiae* grown under microaerophilic conditions have enhanced level of GSH and a higher total and free iron content whether exposed to oxidative stress or not. They also exhibit high levels of malonaldehyde, a product of LPO that obviously reflects a sizable level of oxidation-prone lipids in the cells. All these features were suggested to be part of an adaptive response to enhanced basal oxidative damage. Thus in SOD-deficient cells, oxidative stress, which is assumed to involve also the generation and action of HO•, could give rise to PUFA in the membrane by an as yet unknown mechanism.

Due to their high sensitivity to oxidative assault readily discernible as growth suppression, and simple performance of the tests of antioxidant action (Żyracka *et al.* 2005), *sod* mutants of *S. cerevisiae* are a highly suitable tool for first-line testing of the efficiency of a wide variety of antioxidants.

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