3-*O*-Alkylascorbic Acids as Free Radical Quenchers. II. Inhibitory Effects on Some Lipid Peroxidation Models

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We previously found that 3-*O*-dodecylcarbamethylascorbic acid (3-RASA, 3, HX-0112) exhibited a potent inhibitory effect on biochemical lipid peroxidation and that 3-RASA (3) alleviated myocardial lesions induced by ischemia-reperfusion treatment in rats. In this study we examined the mode of action of 3-RASA (3) on the inhibition of lipid peroxidation. There was no reducing activity by 3-RASA (3) (*i.e.*, no oxide was produced) against ferric ions and superoxide anion radicals. The low reducing activity of 3-RASA (3) against a radical as compared to that of α -tocopherol was obtained by using a stable radical. However, 3-RASA (3) had a potent inhibitory effect, almost equal to that of α -tocopherol, in the model of lipid peroxidation dependent on enzymatic superoxide generation. 3-RASA (3) very strongly inhibited the chain-reaction of the peroxidation induced by Fe^{2+} -linoleic acid hydroxyperoxide. On the basis of these findings, it appears that the anti-lipid-peroxidative effects of 3-RASA (3) are due to the inhibition of the radical chain-reaction, as a chain-breaking antioxidant.

Keywords 3-*O*-alkylascorbic acid; 3-*O*-dodecylcarbamethylascorbic acid; 3-*O*-decylascorbic acid; HX-0112; HX-0113; lipid peroxidation; antioxidant; linoleic acid hydroxyperoxide; site specific Fenton reaction; chain-breaking antioxidant

It has recently become clear that active oxygen species (AOS; such as superoxide anion radical, hydroxyl radical, $\cdot\text{OOH}$ *etc.*) and free radicals derived from the biochemical utilization of O_2 and the prooxidant stimulation of O_2 metabolism participate in the development of exacerbation of various kinds of diseases: ischemia-reperfusion disturbances in the brain and heart, rheumatism, inflammation, gastric ulcers, and cancer.¹⁾

Although the exact mechanism of lipid peroxidation and the roles of AOS in this biochemical phenomenon are not entirely clear, there have been numerous reports that disturbances of membranes which result in cell damage are derived from AOS-induced lipid peroxidation.²⁾ The average level of lipid peroxidant in plasma is higher in some disease cases than in normal cases³⁾; the lipid peroxidation has been suggested to result from excess superoxide anion radical (superoxide) production.⁴⁾

In fact, unsaturated fatty acids, the constituents of cell membranes, are substantially susceptible to oxidation. Additionally, there is considerable evidence that inhibitors of biochemical lipid peroxidation, such as α -tocopherol (Toc.),⁵⁾ idevenone,⁶⁾ and cyanidanol⁷⁾ have demonstrably valuable pharmacological effects. A thiazolidine derivative, CS-045,⁸⁾ an ascorbic acid derivative, CV-3611,⁹⁾ and ebselene, a selenium compound,¹⁰⁾ which were developed as new antioxidants, have been studied for the treatment of angiopathy, postischemia-reperfusion disturbance and inflammation, respectively.

We synthesized a novel series of 3-*O*-alkylascorbic acids (3-RASA) in order to develop new antioxidants. We found that 3-*O*-decylascorbic acid (3-RASA (2), HX-0113) and 3-*O*-dodecylcarbamethylascorbic acid (3-RASA (3), HX-0112) were strong inhibitors of lipid peroxidation in rat liver microsomes.¹¹⁾ HX-0112 and HX-0113, which are stable lipophilic ascorbic acid derivatives, alleviated myocardial lesions induced by ischemia-reperfusion treatment in rats.¹²⁾ This paper deals with 3-RASA's mode of antioxidative action, using chemical models of lipid peroxidation. We discuss, especially, whether 3-RASA (3) scavenges AOS to participate in biochemical lipid peroxidation or inhibits the

radical chain-reaction in linoleic acid peroxidation.

Results

The chemical structures of the various 3-RASA (1–4) investigated are presented in Fig. 1.

Blois proposed a method for assessing antioxidant activity in which such activity was determined by using a stable radical, α, α -diphenyl- β -picryl hydrazyl (DPPH).¹³⁾ The reducing activity of 3-RASA, ascorbic acid and Toc. was

Fig. 1. Chemical Structure of 3-RASA

3-RASA; (1) $\text{R} = \text{CH}_3\text{CH}_2-$, (2) $\text{R} = \text{CH}_3(\text{CH}_2)_9-$, (3) $\text{R} = \text{CH}_3(\text{CH}_2)_{11}\text{COCH}_2-$, (4) $\text{R} = \text{CH}_3(\text{CH}_2)_{17}-$.

Fig. 2. Reducing Activity against DPPH hydrazone with 3-RASA and Toc.

1, 3-RASA (1); 2, 3-RASA (2); 3, 3-RASA (3); 4, Toc.; 5, ascorbic acid. DPPH 0.1 mM and equimolar amount of compound exist in EtOH. The decrease of absorption of DPPH is continuously monitored by the absorption change at 517 nm.

determined by using DPPH, which is generally available in laboratories in which electron spin resonance experiments are conducted. The stable free radical was reduced by the test compounds, 3-RASA (1–3), ascorbic acid, and Toc. (Fig. 2). The reducing activities of 3-RASA (1–3) against this radical was much lower than that of ascorbic acid and Toc.

The redox potential is a valuable index for the electron donating potency required for a biologically beneficial radical scavenger. 3-RASA required much higher potentials than ascorbic acid in electrode oxidation, but the potentials of 3-RASA were estimated to be lower than those of superoxide/hydrogen peroxide¹⁴ and those of $\text{Fe}^{3+}/\text{Fe}^{2+}$ (Table I). Our electrochemical results suggest that 3-RASA (3) must be oxidized by superoxide and Fe^{3+} ; however, a strong reducing agent has sometimes acted on the prooxidant and stimulated lipid peroxidation. For instance, a transition metal and ascorbic acid at a low concentration synergistically stimulate nonenzymic induced lipid peroxidation.¹⁵ The reducing activity of ascorbic acid, Toc., and 3-RASA against Fe^{3+} was examined. Ascorbic acid and Toc. reduced against Fe^{3+} immediately, but 3-RASA could not reduce against Fe^{3+} (Table I). However, 3-RASA (1, 2), but not 3-RASA (4) which bears an octadecyl group, demonstrated reducing activity against Fe^{3+} after the

TABLE I. Redox Potential of 3-RASA and Reducing Activity against Fe^{3+} : *o*-Phenanthroline

Compound	Redox potential ^{a)} (mV)	Reducing Fe^{3+} (%) ^{b)}	
		FeCl_3 alone	FeCl_3 + <i>o</i> -phenanthroline
3-RASA (1)	305	0	39
3-RASA (2)	300	0	32
3-RASA (3)	380	0	12
3-RASA (4)	—	0	3
Toc.	—	83	83
Ascorbic acid	210	87	87

a) The redox potentials are determined by differential pulse voltammetry; an Ag/AgCl reference electrode and a glassy carbon working electrode are used, voltammetric analysis is conducted in 0.01 M phosphate buffer at pH 7.4. b) The compound exists at the molar ratio of 2.1 parts of Fe^{3+} .

Fig. 3. Effect of 3-RASA and Toc. on XO-Dependent Peroxidation of Detergent-Dispersed Linoleate

1, control; 2, (–)-EDTA- Fe^{3+} ; 3, SOD (10 g/ml); 4, α -Toc (10 μM); 5, 3-RASA (2) (50 μM). The micelle solution of linoleate is made by sodium linoleate (5.7 mM) with XO (6.6 munits/ml), Lubrol (1%), CH_3CHO (35 mM), and EDTA- Fe^{3+} (0.11 mM EDTA, 0.1 mM FeCl_3) in 30 mM NaCl, pH 7.5. Diene conjugation during lipid peroxidation is continuously monitored at 234 nm.

addition of *o*-phenanthroline (Table I).

The effects of 3-RASA (1–4) and Toc. on the lipid peroxidation of linoleic acid (LA), which is dependent on enzymatic superoxide generation, were also studied. The addition of ethylenediaminetetraacetic acid (EDTA)- Fe^{3+} to the xanthine oxidase (XO)-system resulted in the lipid peroxidation of LA, although no activity was observed in the absence of iron (Fig. 3). The effects of 3-RASA (1–4), Toc., superoxide dismutase (SOD), and catalase on the lipid peroxidation of the system are shown in Table II. Our results agreed with Tien's data¹⁶ which showed that the addition of iron as an EDTA-chelate was necessary for XO-dependent peroxidation (XO-peroxidation); and further, indicated that SOD had an almost complete inhibiting effect and that catalase had a partially inhibiting effect. It appeared that 3-RASA (1–4) and Toc., at the molar ratio of 1 : 100 parts of LA, almost completely inhibited XO-peroxidation. Mannitol, at the molar ratio of about 2 : 1 parts of LA, resulted in partial inhibition of activity; 3-RASA (2) and Toc., which are more lipophilic compounds than 3-RASA (1), were capable of inhibiting activity at the molar ratio of $1/5 \times 10^3$ to LA. It is clear, as with the inhibitory effect of SOD, that XO-peroxidation is dependent on the generation of superoxide.

We assessed the effects of 3-RASA (2, 3) on the XO superoxide generation system by continuous monitoring of the reduction against Fe^{3+} -cytochrome c. 3-RASA (2, 3) did not affect the generating speed and level of superoxide; the ultraviolet (UV) spectra of 3-RASA (2, 3) were not changed under these conditions (data not shown).

The effects of 3-RASA (2, 3) and Toc. on the site-specific induction of lipid peroxidation by iron in charged micelles were studied according to the method of Fukuzawa.¹⁷ At pH 3.5 both Fe^{2+} and H_2O_2 were necessary to induce lipid peroxidation in the positively charged tetradecyltrimethylammonium bromide (TTAB) micelles, in spite of the linoleic acid hydroperoxide (LOOH) which was present in LA. However, at pH 7, peroxidation of LA was induced by the addition of Fe^{2+} alone (Fig. 4). The Fe^{2+} -induced

TABLE II. Inhibitory Effect of Various Scavengers and Antioxidants on XO-Dependent Peroxidation of Dispersed Linoleate

Addition		ΔA_{234} (nm/min)	Inhibition (%)
None		0.029 ± 0.002	—
3-RASA (1)	50 μM	< 0.002	> 93
	10 μM	0.022 ± 0.003	24
3-RASA (2)	50 μM	< 0.002	> 93
	10 μM	0.011 ± 0.002	62
	1 μM	0.023 ± 0.026	20
3-RASA (3)	50 μM	< 0.002	> 93
	10 μM	0.013 ± 0.002	55
3-RASA (4)	50 μM	< 0.002	> 93
	10 μM	0.017 ± 0.003	41
Toc.	50 μM	< 0.002	> 93
	10 μM	< 0.002	> 93
	1 μM	0.011 ± 0.003	62
SOD	10 $\mu\text{g/ml}$	< 0.002	> 93
Mannitol	10 mM	0.024 ± 0.004	18
Catalase	10 $\mu\text{g/ml}$	0.017 ± 0.005	41

The micelle solution of linoleate is made by sodium linoleate (5.7 mM) with XO (6.6 munits/ml), Lubrol (1%), CH_3CHO (35 mM), and EDTA- Fe^{3+} (0.11 mM EDTA, 0.1 mM FeCl_3) in 30 mM NaCl, pH 7.5. The rate of diene conjugation is lined up to approximately 4 min, after which a decrease in rate is observed. The rate is determined from the initial velocity of the reaction (mean $\pm$ S.D., $n = 5$).

Fig. 4. Lipid Peroxidation of LA in TTAB Micelles

1, H_2O_2 ; 2, Fe^{2+} ; 3, Fe^{3+} ; 4, $\text{TPP} + \text{Fe}^{2+}$; 5, $\text{H}_2\text{O}_2 + \text{Fe}^{2+}$; 2a, $\text{Fe}^{2+} + 3\text{-RASA}$ (2), $10\ \mu\text{M}$; 2b, $\text{Fe}^{2+} + 3\text{-RASA}$ (2), $50\ \mu\text{M}$. The reaction mixture contains LA ($5\ \text{mM}$) with H_2O_2 ($100\ \mu\text{M}$), FeCl_3 ($20\ \mu\text{M}$), FeSO_4 ($20\ \mu\text{M}$), and TPP ($80\ \mu\text{M}$), in $50\ \text{mM}$ TTAB micelle. Diene conjugation during lipid peroxidation is continuously monitored at $234\ \text{nm}$.

TABLE III. Inhibitory Effect of Ascorbic Acid Derivatives and Toc. on Lipid Peroxidation by Fe^{2+} in TTAB Micelles

Compound		Rate ΔA_{234} (nm/min)	Inhibition (%)
Control		0.263 ± 0.020	—
3-RASA (2)	$50\ \mu\text{M}$	0.025 ± 0.005	90
	$10\ \mu\text{M}$	0.040 ± 0.015	84
3-RASA (3)	$10\ \mu\text{M}$	0.027 ± 0.005	90
Toc.	$50\ \mu\text{M}$	0.013 ± 0.001	95
	$10\ \mu\text{M}$	0.025 ± 0.005	90
EDTA	$10\ \mu\text{M}$	0.039 ± 0.024	84
TPP	$80\ \mu\text{M}$	>0.002	100

The reaction mixture contains LA ($5\ \text{mM}$) with FeSO_4 ($20\ \mu\text{M}$) in $50\ \text{mM}$ TTAB micelle at pH 7. The rate of diene conjugation is lined up to approximately 4 min, after which a decrease in rate is observed. The rate is determined from the initial velocity of the reaction (mean $\pm$ S.D., $n=5$).

peroxidation at pH 7 was not observed using Fe^{3+} and triphenylphosphine (TPP) pretreatment to reduce against LOOH. The peroxidation was partially inhibited by EDTA at 1/2 molar ratio to Fe^{2+} , and it was completely inhibited by TPP at a molar ratio of almost 1:1 to LOOH (Table III). The peroxidation was strongly inhibited by 3-RASA (2, 3) and Toc. at a molar ratio to LA of 1/500 and a molar ratio to LOOH of 1/7.

Discussion

We synthesized various novel antioxidants and selected 3-RASA (2, HX-0113) and 3-RASA (3, HX-0112) for further clinical testing. We determined their inhibitory effects of lipid peroxidation in rat liver microsomes and their stability against autooxidation in aqueous solution.¹¹⁾ In this paper, we attempt to clarify 3-RASA's mode of action as an antioxidant. This attempt has been a significant addition to understanding the pharmacological effects of the inhibition of lipid peroxidation.

However, the reactive form(s) of AOS involved in superoxide-dependent lipid peroxidation has not been unequivocally established. Certainly there is much evidence of superoxide generation during many biological reaction.¹⁸⁾ Although superoxide itself is relatively unreactive, the hydroxyl radical is a very highly reactive AOS toward

many compounds, including polyunsaturated fatty acids.¹⁹⁾ However, the hydroxyl radical is too short-lived in living organisms and thus it cannot reach the membrane from a distant place in extracellular fluid.²⁰⁾

Samuni *et al.* suggested a site-specific Fenton mechanism in which the binding of a transition metal ion to the biological target is a prerequisite for the production of hydroxyl radical-mediated cell damage.²¹⁾ The hydroxyl radical is probably derived from the superoxide as follows²²⁾:

Recently, Fukuzawa *et al.* demonstrated lipid peroxidation induced by a site-specific Fenton mechanism in charged micelles.²³⁾

On this fairly reasonable hypothesis, we experimented with 3-RASA to find out the reactivity toward superoxide, the hydroxyl radical, and Fe^{3+} . No reactivity of 3-RASA was observed against superoxide and Fe^{3+} , in spite of 3-RASA's having a redox-potential suitable for oxidation with these entities. Only after the addition of *o*-phenanthroline was 3-RASA (1) capable of reducing activity against Fe^{3+} . The reducing ability of 3-RASA toward the Fe^{3+} : *o*-phenanthroline complex was inhibited by the steric hindrance of a long alkyl group. The shielding of the hydroxyl group of 3-RASA from Fe^{3+} is attributable to 3-RASA's long alkyl chain. The oxidation of 3-RASA with the hydroxyl radical was reported by Cabelli *et al.*²⁴⁾ However, it is too difficult to make an exact comparison of the reactivity of an antioxidant with the hydroxyl radical because of the high reactivity of hydroxyl radical. We chose the stable radical (DPPH),¹³⁾ which Blois proposed as an assay for the antioxidant. 3-RASA obviously had less reducing activity against DPPH than did Toc., a typical inhibitor²⁵⁾ of lipid peroxidation. The low reducing activity and the high redox potential of 3-RASA were in conflict with its potent inhibitory effect on lipid peroxidation. In other words, based on our data, 3-RASA will be characterized as a stable antioxidant.

While many organic compounds which have reducing activity, except for transition metal complexes,²⁶⁾ are hardly oxidized or only slowly oxidized in aqueous solution by superoxide,^{19a)} 3-RASA was not oxidized in the enzymatic generation of superoxide, although it demonstrated complete inhibition toward lipid peroxidation dependent on superoxide generation. The XO-dependent peroxidation which became necessary with the addition of EDTA- Fe^{3+} might occur via the hydroxyl radical¹⁶⁾ (see. Eqs 1—3).

The 3-RASA, which bear a long alkyl chain, and Toc. showed potent inhibitory effects, but mannitol and 3-RASA (1), which bear an ethyl group, are lower in activity than the lipophilic hydroxyl-radical scavengers on XO-dependent peroxidation. SOD was also an effective protectant for the oxidation, but catalase did not lead to complete inhibition. The fact that SOD exists in the enzymatic superoxide generation area (outside the micelles) and that it is efficiently able to dismutate superoxide,²⁷⁾ and the fact that catalase is sensitive to superoxide,²⁸⁾ account for this phenomenon [see the following section for further details]. On the basis of

our results, it is suggested that lipid peroxidation occurring from the site-specific Fenton reaction cannot be effectively inhibited by a hydrophilic antioxidant. In fact, the antioxidant efficiency of 3-RASA was influenced by its hydrophobic action on lipid peroxidation in rat liver microsomes.¹¹⁾ 3-RASA, bearing a long alkyl chain, would be located in the lipid layer and might react as an antioxidant. Our hypothesis is also supported by the fact that the inhibitory effect of 3-RASA (1), which bears an ethyl group, was lower than that of 3-RASA (2), which bears a decyl group, on XO-dependent peroxidation.

It was reported that the antioxidant efficiency of Toc. in liposomes depends on its ability to react mainly with the chain-initiating and chain-propagating lipid radicals.²⁹⁾ On the basis of these findings, since the mode of antilipid peroxidative action of the lipophilic 3-RASA (2, 3) is thought to be similar to that of Toc., the effects of 3-RASA on the lipid peroxidation model which involved site-specific chain-initiating lipid radicals, not initiated by AOS, were studied. The Fe²⁺-LOOH-initiated peroxidation, which is a minor modification of the lipid peroxidation induced in TTAB micelles by site-specific Fenton reaction,²³⁾ was suitable for our purpose. In this peroxidation, because of the results obtained from TPP treatment and the addition of Fe³⁺, induction is clear, as shown in the following equations:

The potent inhibitory effects of 3-RASA (2, 3) and Toc. on Fe²⁺-LOOH-induced peroxidation are obviously due to reaction with the lipid radical (s) derived from LOOH. These lipid radicals, like the hydroxyl radical, show very high reactivity. These finding resolved the previous conflict, between the low reducing activity of 3-RASA (2, 3) and the potent inhibitory effects of these compounds on lipid peroxidation models. That is, the conflict probably involves the difference in chemical reactivity between DPPH as a radical and the lipid radicals. Since lipid peroxidation consists of high reactive chain-reactions, the difference in chemical reducing activity between Toc. and 3-RASA against low potential radicals, DPPH, will be compensated for by the high reactivity of lipid radicals, or may be compensated for by the negligible levels of each. Of course, this consideration is based on the localization of 3-RASA, like Toc.,³⁰⁾ in the lipid layer as a site for the radical chain-reaction. Additionally, 3-RASA would be expressed in scavenging activity for same considerably reactive AOS (such as ¹O₂,³¹⁾ oxygen and iron complex³²⁾), but this scavenging activity against remarkably reactive AOS, which is generated only at a distant place from the membrane, may not be very important as a protectant of the biomembranes. On the basis of these findings, it appears that the potent antioxidative effects of 3-RASA (2, 3) on biochemical lipid peroxidation are not due to scavenging activity for AOS which participates in the peroxidation, XO-dependent lipid peroxidation, but that 3-RASA (2, 3) inhibit the radical chain-reaction in linoleic acid peroxidation as a chain-breaking antioxidant.

Materials and Methods

Materials TTAB, bovine erythrocyte SOD (2900 units/mg protein),

cytochrome c, beef liver catalase (30000 units/mg protein purified by redialysis before use) and XO (purified by redialysis before use) were purchased from the Sigma Chemical Co., St. Louis, LA, TPP, DPPH, Toc., and mannitol were obtained from Wako Pure Chemical Industries, Osaka. Lubrol was from Nakarai Tesque, Kyoto. 3-O-Ethylascorbic acid (3-RASA (1)), 3-RASA (2), 3-RASA (3), and 3-O-octadecylascorbic acid (3-RASA (4)) were synthesized by the method outlined in the previous paper.¹¹⁾ All buffer and reagent solutions were passed through Chelex 100 (Bio-Rad Laboratories) ion-exchange resin to free them of contaminants.

Determination of the Reducing Activity of the Stable Radical DPPH¹³⁾

The test compound, in 40 μ l dimethylformamide (DMF) (1×10^{-2} M), was added to a solution of a stable radical, DPPH, in 4 ml EtOH (1×10^{-4} M). The decrease of absorption during the reduction of DPPH was continuously monitored at 517 nm.

Determination of 3-RASA Redox Potentials Voltammetric analysis was conducted in 0.01 M phosphate buffer, at pH 7.4, which was degassed with argon before use. An Ag/AgCl reference electrode, a glassy carbon working electrode (3 mm i.d.), and a Pt counter electrode (0.05 mm i.d. $\times$ 2 cm) were used (BSA systems). The redox potentials were determined by using a BSA 100A electrochemical analyzer to obtain the peak potential in the voltammograms by using the differential pulse voltammetry mode. The experimental conditions were: initial E (mV) = -100, final E (mV) = 500, V (mV/s) = 4, pulse amplitude (mV) = 50, pulse width (ms) = 60, pulse ratio (ms) = 1000. Test compound solutions were added to the buffer (final conc. 0.05 mM, final conc. of MeOH 5%). Electrochemical reversibility was assessed by analyzing the reversed differential pulse voltammograms (initial E (mV) = 500, final E (mV) = -100).

Measurement of Ferrous Ion Concentration³³⁾ FeCl₃ 5 mM dissolved in 1 N-HCl was prepared for Fe³⁺ stock solution before use. The test sample was dissolved in MeOH. Reaction mixtures contained 0.5 mM FeCl₃ and 1 mM test compound in MeOH, which were mixed for 30 s and allowed to stand at room temperature for 3 min. The absorption of the solution was measured at 362 nm to assess the reduction of Fe³⁺. In separate experiments, reaction mixtures contained 0.05 mM FeCl₃, 0.1 mM test compound, and 0.13 mM *o*-phenanthroline in MeOH:2.5 M acetate buffer at pH 4.2 (9:1). The reaction was initiated by the addition of FeCl₃, and the production of Fe²⁺ for 25 min was monitored by measuring the absorbance at 510 nm.

XO-Dependent Peroxidation in Linoleate Dispersed Solution¹⁵⁾ Linoleate stock solution was made by suspending linoleic acid (100 mg) in N₂-purged Chelexed water and adding 5–10 drops of 6 N-NaOH to yield a clear solution. The pH of this solution was then slowly lowered to 7.5 by the addition of 6 N-HCl. The micelle solution of linoleate was made by incubating sodium linoleate (5.7 mM) with XO (6.6 munits/ml), Lubrol (1%), 35 mM CH₃CHO, and EDTA-Fe³⁺ (0.11 mM EDTA, 0.1 mM FeCl₃) in 30 mM NaCl, pH 7.5, at 20–22 °C, under air. Reactions were initiated by the addition of XO; the test sample solutions (1%; 3-RASA dissolved in DMF) were added before the addition of the enzyme. Diene conjugation during lipid peroxidation was continuously monitored at 234 nm.³³⁾ The rate of diene conjugation was lined up to approximately 4 min, after which a decrease in rate was observed. All rates were determined from the initial velocity of the reaction.

Measurement of Enzymatic Superoxide Generation²⁷⁾ Reaction mixtures contained 0.1 mM EDTA, 200 units/ml catalase, 80 μ M cytochrome c, 35 mM CH₃CHO, and XO (6.6 munits/ml) in Chelexed 50 mM potassium phosphate buffer, pH 7.5, at 20–22 °C. The reduction of cytochrome c during the generation of superoxide was continuously monitored at 550 nm. The test sample was dissolved in DMF, and the DMF solution (1%, v/v) was added to the reaction mixture. Reactions were initiated by the addition of XO.

Iron-Induced Lipid Peroxidation in TTAB Micelles²³⁾ LA micellar solutions were prepared as follows: The interior base of the apparatus was filmed with LA 25 μ M (and test sample) by evaporation of the solvent. Five ml of TTAB 50 mM solution was added to the film, followed by vortexing and sonication in a Sanyo sonic bath. The pH was adjusted by adding 5% acetic acid and 5% NaHCO₃. Any LOOH present in LA was reduced by treatment with TPP in chloroform solution³⁴⁾ before the preparation of micelles. Reactions were initiated by the addition of iron. The peroxidation was monitored as described in the XO-dependent peroxidation section. The LOOH content of LA was determined iodometrically.³⁵⁾

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