Antioxidant Effects of Resveratrol and its Analogues against the Free-Radical-Induced Peroxidation of Linoleic Acid in Micelles

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Abstract: The antioxidant effect of resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) and its analogues, that is, 4-hydroxy-*trans*-stilbene (4-HS), 3,5-dihydroxy-*trans*-stilbene (3,5-DHS), 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 3,4,5-trihydroxy-*trans*-stilbene (3,4,5-THS) and 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS), against the peroxidation of linoleic acid has been studied in sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB) micelles. The peroxidation was initiated thermally by a water-soluble azo initiator 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), and the reaction kinetics were studied by monitoring the formation of linoleic acid hydroperoxides. The synergistic antioxidant effect of these compounds with α-tocopherol (vitamin E) was also studied by following the decay kinetics of α-tocopherol and the reaction intermediate, the α-tocopheroxyl radical. Kinetic analysis of the antioxidant process demonstrates that these compounds are effective antioxidants in micelles used either alone or in combination with α-tocopherol. The antioxidative action involves trapping the propagating lipid peroxyl radical and reducing the α-tocopheroxyl radical to regenerate α-tocopherol. It was found that the antioxidant activity of resveratrol analogues depends significantly on the position of the hydroxyl groups, the oxidation potential of the molecule and the reaction medium. Molecules with *ortho*-dihydroxyl and/or *para*-hydroxyl functionalities possess high activity.

Keywords: antioxidation · kinetics · lipids · peroxidation · resveratrol

Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring phytoalexin present in grapes and other plants. It has been suggested that its presence in red wine with concentrations ranging between 0.1 and 15 mg L^{-1}[1] is linked to the low incidence of heart diseases in some regions of France—the so-called "French paradox", that is, that despite a high fat intake, mortality from coronary heart disease is lower due to the regular drinking of wine.[2] In addition, resveratrol has been shown to possess cancer chemopreventive activity.[3–4] Therefore, the past few years have witnessed intense research devoted to the biological activity, especially the antioxidative activity, of this compound,[5–9] since free-radical-induced peroxidation of membrane lipids and oxidative damage of DNA are considered to be associated with a wide variety of chronic health problems, such as cancer, atherosclerosis and ageing.[10–12] Resveratrol has been reported to be a good antioxidant against the peroxidation of low-density lipoprotein (LDL)[6] and liposomes,[7] a potent inhibitor of lipoxygenase,[8] and able to protect rat heart from ischaemia reperfusion injury.[9] These facts, coupled with our recent findings of the antioxidant synergism of vitamin E with green-tea polyphenols,[13] coumarins[14] and β-carotene,[15] motivated us to study the antioxidative behaviour of resveratrol and its analogues, putting emphasis on the structure – activity relationship of these compounds. We report herein kinetic and mechanistic studies on the antioxidation reaction of resveratrol and related *trans*-stilbene analogues, that is 4-hydroxy-*trans*-stilbene (4-HS), 3,5-dihydroxy-*trans*-stilbene (3,5-DHS), 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 3,4,5-trihydroxy-*trans*-stilbene (3,4,5-THS) and 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS) on the peroxidation of linoleic acid. The peroxidation was initiated thermally at physiological temperature by a water-soluble azo initiator 2,2′-azobis(methylpropionamidine) dihydrochloride (AAPH) and conducted in sodium dodecyl sulfate (SDS) and cetyl trimethylammonium (CTAB) micelles to mimic the microenvironment of biomembranes. The interaction of these compounds with α-tocopherol (TOH, vitamin E) was also investigated.

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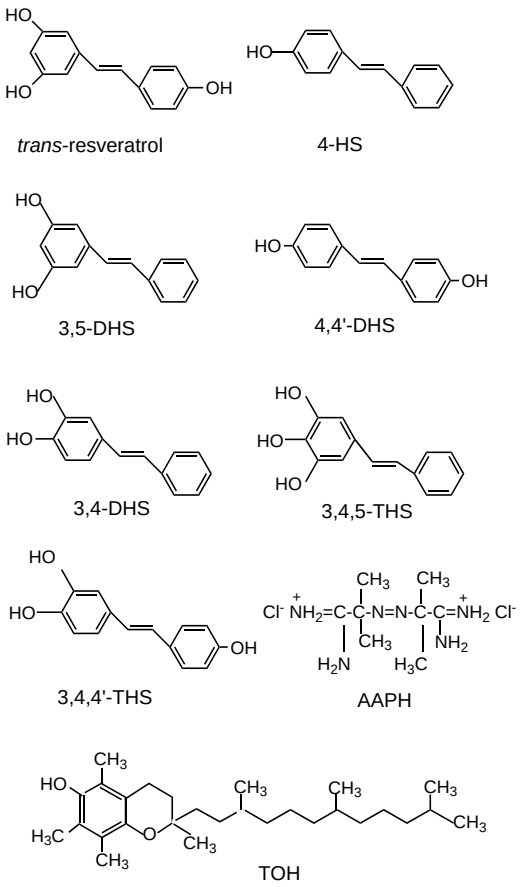

Results and Discussion

Inhibition of linoleic acid peroxidation by resveratrol and its analogues in micelles: Peroxidation of linoleic acid or its esters gives different hydroperoxides depending on the reaction conditions.[16] Hydroperoxide substitution at the C-9 or C-13 positions produces either *trans*,*trans* or *cis*,*trans* conjugated dienes, which are the major products in the absence of antioxidants or in the presence of only small amount of antioxidants, for example, millimolar concentrations of α-tocopherol.[16a,b] It was found recently that these conjugated dienes were formed from the rapid β-scission of the primarily formed bis-allylic 11-peroxyl radical,[16c,d] and that the kinetically controlled product, the nonconjugated 11-substituted hydroperoxide, might become the major product in the presence of high concentrations of antioxidant, for example, molar concentrations of α-tocopherol.[16d] The present experiment used very small amounts of antioxidants (micromolar α-tocopherol and/or resveratrol analogues), hence the production of the nonconjugated 11-hydroperoxide was negligible, and the conjugated hydroperoxides were the predominant products, which showed characteristic ultraviolet absorption at 235 nm[17] that was used to monitor the formation of the total hydroperoxides formed during the peroxidation after separation of the reaction mixture by high-performance liquid chromatography (HPLC). A set of representative kinetic curves of the total hydroperoxides formation during the peroxidation of linoleic acid in SDS micelles is shown in Figure 1. It can be seen from the figure that, upon AAPH initiation, the concentration of the hydroperoxides increased quickly and linearly with time in the absence of antioxidants; this demonstrated the fast

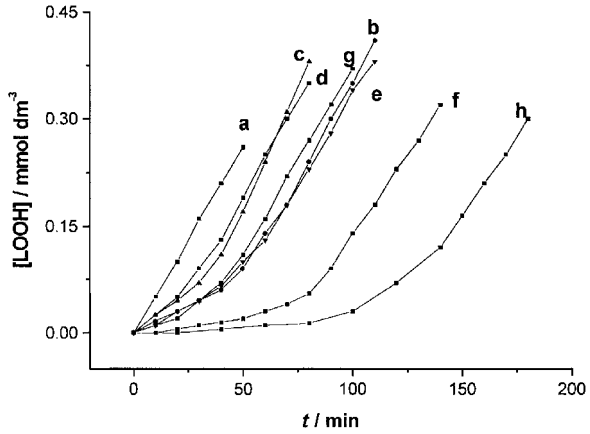

Figure 1. Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in SDS (0.1 mol L^{-1}) micelles at pH 7.5 and 37 °C, initiated with AAPH. $[LH]_0 = 15.2$ mmol L^{-1}, $[AAPH]_0 = 6.3$ mmol L^{-1}, $[ArOH]_0 = 11.2$ μmol L^{-1}. Uninhibited peroxidation (a) or inhibited with b) resveratrol, c) 4-HS, d) 3,5-DHS, e) 4,4′-DHS, f) 3,4-DHS, g) 3,4,5-THS, h) 3,4,4′-THS.

peroxidation of the substrate. The slope of this line corresponds to the rate of propagation, R_p. The peroxides' formation was remarkably inhibited by the addition of resveratrol and its analogues during the so-called "inhibition period" (t_{inh}) or induction period. After the inhibition period, the rate of hydroperoxide formation increased to close to the original rate of propagation; this corresponded to the exhaustion of the antioxidant. During the inhibition period, the concentration of the hydroperoxides also increased approximately linearly with time, and the slope of this line was designated R_{inh}, which also reflects the antioxidative potential of the antioxidant. Similar results were obtained in CTAB micelles (Figure 2), but the kinetic parameters and the

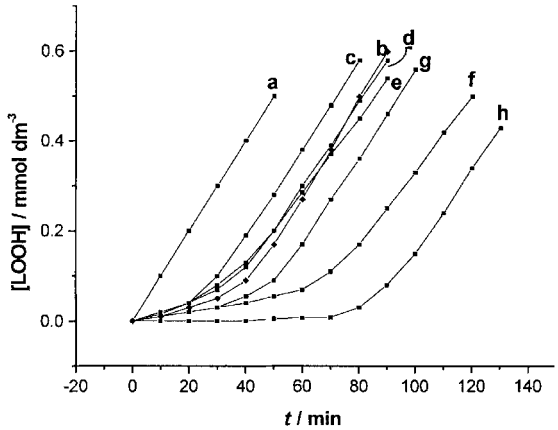

Figure 2. Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in CTAB (0.015 mol L^{-1}) micelles at pH 7.5 and 37 °C, initiated with AAPH. $[LH]_0 = 15.2$ mmol L^{-1}, $[AAPH]_0 = 6.3$ mmol L^{-1}, $[ArOH]_0 = 11.2$ μmol L^{-1}. Uninhibited peroxidation (a) or inhibited with b) resveratrol, c) 4-HS, d) 3,5-DHS, e) 4,4′-DHS, f) 3,4-DHS, g) 3,4,5-THS, h) 3,4,4′-THS.

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relative effectiveness of the antioxidants in the two micelles were appreciably different. The details will be discussed in following sections.

The antioxidant effect of resveratrol and its analogues in the presence of α-tocopherol: α-Tocopherol (TOH), the most abundant and active form of vitamin E, is well known and the principal lipid-soluble chain-breaking antioxidant in plasma and erythrocytes.[18] Its synergistic antioxidative effect with other antioxidants, such as L-ascorbic acid (vitamin C)[19] and green-tea polyphenols,[13] has been well documented. Therefore, it is interesting to see if TOH can also interact synergistically with resveratrol and its analogues. In both SDS and CTAB micelles TOH showed typical antioxidant behaviour against linoleic acid peroxidation (line b in Figures 3 and 4), as reported previously.[13–15] Addition of resveratrol, 3,4-DHS, 3,4,4′-THS or 3,4,5,-THS together with TOH remarkably prolonged the inhibition period of the latter and showed a synergistic antioxidation effect, that is, the inhibition time when the two antioxidants were used in combination was significantly longer than the sum of the inhibition times when they were used individually as illustrated in Figures 3 and 4. 4-HS and 3,5-DHS could also prolong the inhibition time of TOH when they were used together with the latter in both SDS and CTAB micelles, but the effect was only additive, that is, the inhibition time when the two antioxidants were used in combination was the sum of the inhibition times when they were used individually (Figures not shown). The results are summarized in Table 2, later.

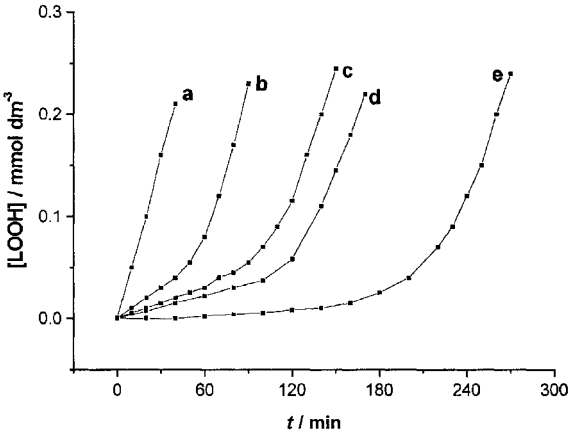

Figure 3. Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in SDS (0.1 mol L^{-1}) micelle at pH 7.5 and 37 °C, initiated with AAPH. $[LH]_0 = 15.2$ mmol L^{-1}, $[AAPH]_0 = 6.3$ mmol L^{-1}, $[TOH]_0 = 5$ μmol L^{-1}, $[ArOH]_0 = 11.2$ μmol L^{-1}. Uninhibited peroxidation (a) or inhibited with b) TOH, c) TOH + resveratrol, d) TOH + 3,4,5-THS, e) TOH + 3,4,4′-THS.

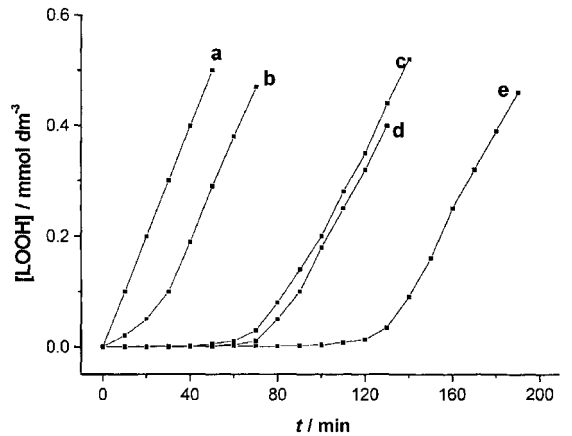

Figure 4. Formation of total hydroperoxides (LOOH) during the peroxidation of linoleic acid (LH) in CTAB (0.015 mol L^{-1}) micelle at pH 7.5 and 37 °C, initiated with AAPH. $[LH]_0 = 15.2$ mmol L^{-1}, $[AAPH]_0 = 6.3$ mmol L^{-1}, $[TOH]_0 = 5$ μmol L^{-1}, $[ArOH]_0 = 11.2$ μmol L^{-1}. Uninhibited peroxidation (a) or inhibited with b) TOH, c) TOH + resveratrol, d) TOH + 3,4,5-THS, e) TOH + 3,4,4′-THS.

Consumption of α-tocopherol: In order to rationalize the mechanism of the antioxidant synergism of α-tocopherol and the resveratrol analogues, the decay of α-tocopherol was studied by HPLC separation of the reaction mixture, followed by electrochemical determination of α-tocopherol. Representative results are illustrated in Figure 5. It was found that the decay of α-tocopherol was approximately linear in the absence of 3,4,4′-THS in the two micelles (lines a and b in Figure 5), in accordance with the kinetic demand for antioxidation reactions [Eq. (4), vide infra]. When 3,4,4′-THS was added, however, the decay of α-tocopherol became much slower before most of 3,4,4′-THS was exhausted (lines c and d). 3,4-DHS and 3,4,5-THS in both micelles, and resveratrol in the CTAB micelle showed a similar effect upon the decay of α-tocopherol, while 4-HS and 3,5-DHS showed no effect (Figures not shown). These results suggest that resveratrol, 3,4-DHS, 3,4,4′-THS and 3,4,5-THS may be able to reduce the α-tocopheroxyl radical to regenerate α-tocopherol and, hence, maintain the concentration of α-tocopherol in the reaction system. Similar α-tocopherol regeneration reactions by vitamin C[19] and green-tea polyphenols have been reported previously.[13]

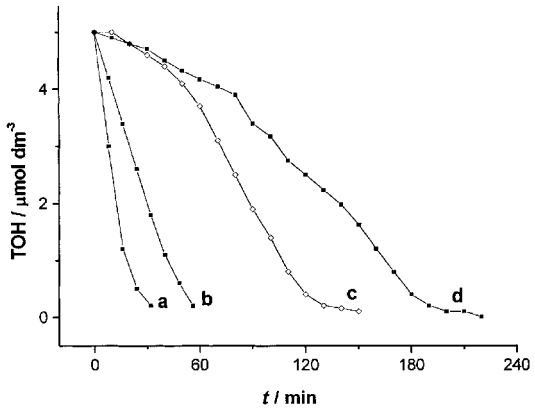

Figure 5. Consumption of α-tocopherol during the inhibition of linoleic acid peroxidation in micelles at pH 7.5 and 37 °C, initiated with AAPH and inhibited by TOH and/or 3,4,4′-THS (ArOH). $[LH]_0 = 15.2$ mmol L^{-1}, $[AAPH]_0 = 6.3$ mmol L^{-1}, $[ArOH]_0 = 11.2$ μmol L^{-1}, $[TOH]_0 = 5$ μmol L^{-1}. a) Decay of TOH in the absence of 3,4,4′-THS in CTAB (0.015 mol L^{-1}) micelle, b) decay of TOH in the absence of 3,4.4′-THS in SDS (0.1 mol L^{-1}) micelle, c) decay of TOH in the presence of 3,4,4′-THS in CTAB micelle, d) decay of TOH in the presence of 3,4,4′-THS in SDS micelle.

Direct determination of the rate of the α-tocopherol regeneration reaction: The α-tocopheroxyl radical (TO$^{\bullet}$) is more persistent in micelles than in homogeneous solutions. The rate constants of the bimolecular self-reaction of TO$^{\bullet}$ were reported to be $3 \times 10^3\,\text{M}^{-1}\,\text{s}^{-1}$ in benzene/di-*tert*-butyl peroxide[20] and $15\,\text{M}^{-1}\,\text{s}^{-1}$ in CTAB micelles,[15a] respectively. They correspond to half-lives of 11 seconds and 38 minutes in the homogeneous solution and the micelle, respectively, taking the initial concentration of TO$^{\bullet}$ as 30 μmol. Therefore, the reaction kinetics of TO$^{\bullet}$ could be easily determined in micelles by using stopped-flow electron paramagnetic resonance (EPR) spectroscopy[21] at ambient temperature. Figure 6 shows the EPR spectrum of TO$^{\bullet}$ recorded in CTAB micelles. Addition of resveratrol through a fast stopped-flow device[21] remarkably increased the decay of TO$^{\bullet}$, which was found to be pseudo-first order in the presence of a large excess of resveratrol (line b in Figure 7). Plotting this first-order rate constant versus the concentration of resveratrol gave a straight line from which the bimolecular rate constant between TO$^{\bullet}$ and resveratrol [Eq. (9), vide infra] could be obtained. 3,4-DHS reacted with TO$^{\bullet}$ much faster than resveratrol (line c in Figure 7). The rate constants for the α-tocopherol regeneration reaction of resveratrol and 3,4-DHS were determined to be 0.23×10^2 and $3.0 \times 10^2\,\text{M}^{-1}\,\text{s}^{-1}$ respectively in CTAB micelles. These EPR experiments confirm unambiguously that the antioxidant synergism of α-tocopherol with the resveratrol analogues is due to the α-tocopherol regeneration reaction by the latter.

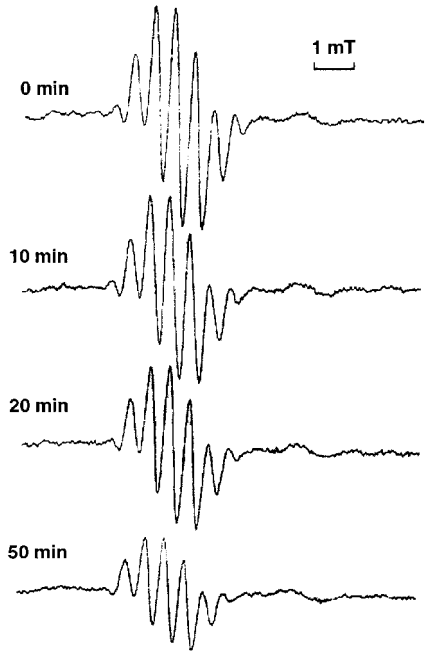

Figure 6. EPR Spectra of the α-tocopheroxyl radical (TO$^{\bullet}$) recorded in CTAB ($15\,\text{mmol}\,\text{L}^{-1}$) micelles at pH 7.4 and room temperature in air. The TO$^{\bullet}$ was generated by oxidizing TOH ($1\,\text{mmol}\,\text{L}^{-1}$) with PbO_2. The initial concentration of TO$^{\bullet}$ was $28\,\mu\text{mol}\,\text{L}^{-1}$.

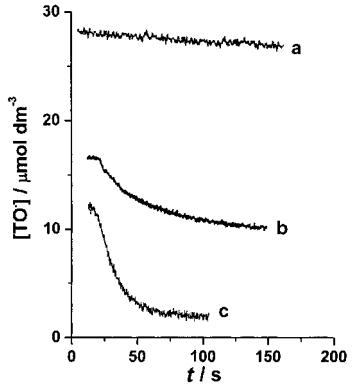

Figure 7. The decay of α-tocopheroxyl radicals in CTAB ($15\,\text{mmol}\,\text{L}^{-1}$) micelles at pH 7.4 and room temperature in air. a) intrinsic decay, b) in the presence of resveratrol ($0.78\,\text{mmol}\,\text{L}^{-1}$), c) in the presence of 3,4-DHS ($0.13\,\text{mmol}\,\text{L}^{-1}$).

Electrochemistry of resveratrol and its analogues: The electrochemistry of resveratrol and its analogues was studied by cyclic voltammetry in both SDS and CTAB micelles. It was found that resveratrol, 4-HS and 3,5-DHS showed irreversible cyclic voltammograms with higher oxidation potentials, while 3,4-DHS, 4,4′-DHS, 3,4,5-THS and 3,4,4′-THS showed reversible cyclic voltammograms with lower oxidation potentials. The oxidation potentials are listed in Table 1.

Kinetics and mechanism: It has been proved that the reaction kinetics of lipid peroxidation in micelles and biomembranes follow the same rate law as that in homogenous solutions.[22] The kinetics of linoleic acid (LH) peroxidation initiated by azo-compounds and its inhibition by chain-breaking antioxidants (AH) have been discussed in detail in our previous papers.[13–14] The rate of propagation (R_p) and the rate of peroxide formation in the inhibition period (R_{inh}) are given by Equations (1) and (2), respectively.

$$\text{d[LOOH]/dt} = R_p = [k_p/(2k_t)^{1/2}]R_i^{1/2}[\text{LH}] \quad (1)$$

$$R_{inh} = k_p R_i [\text{LH}]/(nk_{inh}[\text{AH}]) \quad (2)$$

here k_p, k_t and k_{inh} are rate constants for the chain propagation, chain termination and chain inhibition by antioxidants, respectively, and R_i is the apparent rate of chain initiation, which can be obtained by measuring the inhibition period or decay of the antioxidant (AH), [Eqs. (3) and (4), respectively].[13]

$$R_i = n[\text{AH}]_0/t_{inh} \quad (3)$$

$$R_i = -n\,\text{d[AH]/dt} \quad (4)$$

Here n is the stoichiometric factor that designates the number of peroxyl radicals trapped by each antioxidant molecule. Since the n value of α-tocopherol is generally assumed to be 2,[22] the R_i value can be determined from the inhibition period or the decay rate of α-tocopherol.

The kinetic chain length (kcl) defines the number of chain propagations initiated by each initiating radical and is given by Equations (5) and (6) for uninhibited and inhibited peroxidation respectively. The kinetic parameters deduced from Figures 1 and 2 are listed in Tables 1 and 2, respectively.

$$kcl_p = R_p/R_i \quad (5)$$

$$kcl_{inh} = R_{inh}/R_i \quad (6)$$

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Table 1. Inhibition of AAPH-initiated peroxidation of linoleic acid by resveratrol and its analogues in micelles.[a,b]

Micelle	ArOH	R_p [10^{-8} mol dm^{-3} s^{-1}]	R_{inh} [10^{-8} mol dm^{-3} s^{-1}]	t_{inh} [10^3 s]	k_{inh} [10^4 dm^3 mol^{-1} s^{-1}]	n	kcl_p	kcl_{inh}	E_{pa} [V vs. SCE]
SDS	none	8.3					26.8		
	resveratrol	9.0	2.8	3.1	1.3	0.8	29.1	9.0	0.62
	4-HS	9.2	4.0	2.1	1.2	0.6	29.7	12.9	0.64
	3,5-DHS	7.8	3.8	1.5	1.7	0.4	25.1	12.2	0.85
	4,4′-DHS	8.0	2.6	3.2	1.4	0.9	25.8	8.4	0.40
	3,4-DHS	8.2	0.8	4.9	2.9	1.4	26.4	2.6	0.34
	3,4,5-THS	8.7	1.5	2.4	3.1	0.7	28.1	4.8	0.24
	3,4,4′-THS	6.8	≈ 0	7.2	[c]	2.0	21.9	[c]	0.32
CTAB	none	16.8					20.4		
	resveratrol	16.0	2.7	2.7	0.7	2.0	19.3	3.2	0.67
	4-HS	19.8	3.2	1.7	1.0	1.2	23.9	3.8	0.66
	3,5-DHS	15.6	4.2	2.4	0.5	1.8	18.8	5.1	0.79
	4,4′-DHS	14.8	2.8	2.6	0.8	1.9	17.8	3.4	0.43
	3,4-DHS	14.0	1.6	4.1	0.9	3.0	16.9	1.9	0.36
	3,4,5-THS	16.5	1.6	2.9	1.3	2.1	19.9	1.9	0.23
	3,4,4′-THS	15.5	≈ 0	5.1	[c]	3.8	18.7	[c]	0.34

[a] The reaction conditions and the initial concentration of the substrates are the same as described in the legends of Figures 1 and 2 for reactions conducted in SDS and CTAB micelles respectively. Data are the average of three determinations that were reproducible with a deviation of less than ±10%. [b] Taking R_i as 3.1 and 8.3 nmol L^{-1} s^{-1} in SDS and CTAB micelles, respectively, see text. [c] Could not be calculated because R_{inh} is approximately zero.

It can be seen from Figures 1 and 2 and from Table 1 that resveratrol and its analogues (ArOHs) behave well as chain-breaking antioxidants against AAPH-induced linoleic acid peroxidation in both SDS and CTAB micelles. All of them produced a clear inhibition period in which the rate of propagation and the kinetic chain length are remarkably reduced; this demonstrates that they are able to trap the propagating linoleic acid peroxyl radicals [LOO$^\bullet$, Eq. (7)].

$$LOO^\bullet + ArOH \xrightarrow{k_{inh}} LOOH + ArO^\bullet \quad (7)$$

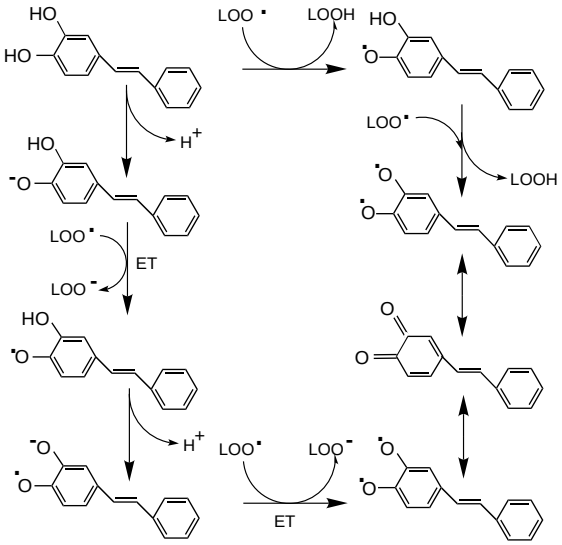
The antioxidant potential of these ArOHs can be assessed by comparing their inhibition rate constant from Equation (7), k_{inh}, the inhibition period, t_{inh}, or the kinetic chain length during the inhibition time, kcl_{inh}. The k_{inh} of ArOHs is about $0.5-3.1\times10^4$ M^{-1} s^{-1}, comparable to that of α-tocopherol (3.6×10^4 and 2.0×10^4 M^{-1} s^{-1} in SDS and CTAB micelles, respectively, see Table 2) and to those of green-tea polyphenols ($0.3-3.7\times10^4$ M^{-1} s^{-1} in micelles).[13b] It can also be seen that the antioxidative activities of 3,4-DHS, 3,4,5-THS and 3,4,4′-THS, that is, the molecules bearing *ortho*-dihydroxyl functionality, are appreciably higher than those of resveratrol and molecules bearing no such functionality. This can be understood because the *ortho*-hydroxyl phenoxyl radical, the oxidation intermediate for these three more active species, is more stable due to the intramolecular hydrogen bonding interaction, as evidenced recently from both experiments[23] and theoretical calculations.[24] The theoretical calculation showed that the hydrogen bond in the *ortho*-OH phenoxyl radical is approximately 4 kcal mol^{-1} stronger than that in the parent catechol, and that the bond dissociation energy (BDE) of catechol is 9.1 kcal mol^{-1} lower than that of phenol and 8.8 kcal mol^{-1} lower than that of resorcinol.[24] In addition, it should be easier to further oxidize the *ortho*-OH phenoxyl

Table 2. Inhibition of AAPH-initiated peroxidation of linoleic acid by resveratrol and its analogues together with α-tocopherol in micelles.[a,b]

Micelle	ArOH	R_p [10^{-8} mol dm^{-3}s^{-1}]	R_{inh} [10^{-8} mol dm^{-3}s^{-1}]	t_{inh} [10^3 s]	k_{inh} [10^4 dm^3 mol^{-1}s^{-1}]	n'[c]	kcl_p	kcl_{inh}	SE [%]
SDS	TOH	8.1	1.1	3.0	3.6	2.0	26.1	3.6	
	esveratrol + TOH	7.0	0.9	6.3	2.0	1.2	22.6	2.9	~ 0
	4-HS + TOH	7.9	0.8	5.3	2.3	1.0	25.5	2.6	~ 0
	3,5-DHS + TOH	8.3	1.0	4.8	2.1	0.9	26.8	3.2	~ 0
	4,4′-DHS + TOH	7.4	0.5	6.6	3.1	1.3	23.9	1.6	~ 0
	3,4-DHS + TOH	6.9	~ 0	12.6	[d]	2.4	22.2	[d]	59.5
	3.4.5-THS + TOH	7.7	0.6	6.6	2.9	1.3	24.8	1.9	22.2
	3,4,4′-THS + TOH	7.0	~ 0	13.8	[d]	2.6	22.6	[d]	35.3
CTAB	TOH	16.7	2.0	1.2	2.0	2.0	20.1	2.4	
	esveratrol + TOH	12.3	0.1	4.4	10.9	2.2	14.8	0.1	12.8
	4-HS + TOH	17.8	0.3	2.5	6.4	1.3	21.4	0.4	~ 0
	3,5-DHS + TOH	13.6	0.3	3.0	6.3	1.5	16.4	0.4	~ 0
	4,4′-DHS + TOH	14.3	0.2	4.5	7.5	2.3	17.2	0.2	18.4
	3,4-DHS + TOH	11.7	~ 0	7.8	[d]	4.0	14.1	[d]	47.2
	3,4,5-THS + TOH	13.0	~ 0	4.9	[d]	2.5	15.7	[d]	19.5
	3,4,4′-THS + TOH	12.8	~ 0	8.1	[d]	4.2	15.4	[d]	28.6

[a] The reaction conditions and the initial concentration of the substrates are the same as described in the legends of Figures 3 and 4 for reactions conducted in SDS and CTAB micelles, respectively. Data are the average of three determinations that were reproducible with a deviation of less than ±10%. [b] Taking R_i as 3.1 and 8.3 nmol dm^{-3} s^{-1} in SDS and CTAB micelles, respectively, see text. [c] $n' = R_i t_{inh}/([ArOH]_0 + [TOH]_0)$. [d] Could not be calculated because R_{inh} is approximately zero.

radical and/or *ortho*-semiquinone radical anion to form the final *ortho*-quinone[23] (Scheme 1). The fact that the stoichiometric factor, n, of 3,4-DHS and 3,4,4′-THS is larger than 1 (Table 1) suggests that the second peroxyl radical is involved



Scheme 1. Antioxidative reaction of 3,4-DHS by hydrogen abstraction and electron transfer.

in the antioxidation reaction that leads to the formation of the corresponding *ortho*-quinones, as shown in the scheme. The 4′-OH group also enhanced the activity, since the 4′-OH group can stabilize the semiquinone radical-anion intermediate by resonance through the *trans* double bond. It has recently been proved that the 4′-OH is more active than the *meta*-dihydroxyl groups in resveratrol.[4, 24, 25] Therefore, the antioxidative activity of 3,4,4′-THS is extremely high. It completely inhibits peroxidation in both SDS and CTAB micelles and produces a longer inhibition period than α-tocopherol (7.2×10^3 and 5.1×10^3 s for 11.2 μmol L^{-1} of 3,4,4′-THS in SDS and CTAB micelles respectively, in comparison with the inhibition period of 3.0×10^3 and 1.2×10^3 s for 5.0 μmol L^{-1} of α-tocopherol in SDS and CTAB, respectively).

It is worth noting that the antioxidant activity of resveratrol analogues is correlated with the electrochemical behaviour of the molecule. Molecules with lower oxidation potentials and reversible cyclic voltammograms, that is, 3,4-DHS, 4,4′-DHS, 3,4,4′-THS and 3,4,5-THS, exhibit higher activity, while molecules with higher oxidation potentials and irreversible cyclic voltammograms, that is, 4-HS, 3,5-DHS and resveratrol, are less active. This correlation between the activity and the oxidation potential of the molecules suggests that electron-transfer antioxidation might take place simultaneously with a direct hydrogen-abstraction reaction, as exemplified in Scheme 1. It is well known that phenoxides undergo electron transfer oxidation more easily to produce relatively stable phenoxide radical anions in alkaline media. Resveratrol, with a pK_{a1} of 6.4,[25] partially dissociates under our experimental conditions (pH 7.4); this makes the electron-transfer reaction feasible. Cooperation between hydrogen-abstraction and electron-transfer processes in antioxidation reactions by phenolic antioxidants has recently been discussed theoretically.[24]

It can be seen from Figures 3 and 4 and Table 2 that addition of the resveratrol analogues (ArOHs) together with α-tocopherol (TOH) significantly increases the inhibition period of the latter. The t_{inh} of TOH and 3,4-DHS when they were used together in SDS micelles was approximately 60% longer than the sum of the t_{inh}s when the two antioxidants were used individually, as expressed by the synergistic efficiency SE% [Eq. (8)].[26]

$$\mathrm{SE}\,\% = \frac{t_{inh}(\mathrm{TOH} + \mathrm{ArOH}) - [t_{inh}(\mathrm{TOH}) + t_{inh}(\mathrm{ArOH})]}{t_{inh}(\mathrm{TOH}) + t_{inh}(\mathrm{ArOH})} \times 100 \qquad (8)$$

As shown in Figures 5 and 7, the antioxidant synergism of TOH and ArOH can be rationalized by the reduction of the α-tocopheroxyl radical by the resveratrol analogues [Eq. (9)], which regenerates α-tocopherol. Since the rate of this TOH-regeneration reaction ($k_{reg} \approx 10^2\,\mathrm{M^{-1}\,s^{-1}}$) is approximately two orders of magnitude slower than that of the antioxidation reaction [Eq. (7), $k_{inh} \approx 10^4\,\mathrm{M^{-1}\,s^{-1}}$], the antioxidant synergism can only be observed when the rate of the TOH-regeneration reaction is high enough to compete with the antioxidation reaction.

$$\mathrm{TO^{\bullet}} + \mathrm{ArOH} \xrightarrow{k_{reg}} \mathrm{TOH} + \mathrm{ArO} \qquad (9)$$

It can also be seen from Tables 1 and 2 that the reaction medium exerts significant influence on the rate of initiation and the antioxidant activity of the resveratrol analogues. The R_i values calculated from the inhibition period [Eq. (3)] are 3.1 and 8.3 nmol L^{-1} s^{-1} in SDS and CTAB micelles, respectively, which are in good agreement with the values of 3.0 and 8.2 nmol L^{-1} s^{-1}, respectively, obtained from the decay of TOH [Eq. (4)]. Taking the concentration of AAPH as 6.3 mmol L^{-1} the R_i value in CTAB micelles corresponds to 1.3×10^{-6}[AAPH] s^{-1}; this is in good agreement with the previously reported value in liposomal dispersions.[27] However, the R_i value of AAPH in SDS micelles is appreciably smaller than that in CTAB micelles. This can be understood because AAPH is positively charged, hence it is prone to being adsorbed onto the surface of SDS micelles; this in turn reduces the effective initiation, due to the cage effect. On the other hand, the inhibition rate constant, k_{inh}, of α-tocopherol and resveratrol analogues is higher in SDS than in CTAB micelles. This is due to the fact that lipid peroxyl radicals are polar (dipole moment of ca. 2.6 Debye) and electrophilic.[22a] Thus, they should move to the surface of micelles and be subject to intermicellar diffusion[28] more quickly in SDS than in CTAB micelles, so as to react with the antioxidant whose reactive phenoxyl functional group resides on the surface of the micelle.[18]

In conclusion, this work demonstrates that resveratrol and its analogues, that is, 4-HS, 3,5-DHS, 4,4′-DHS, 3,4-DHS, 3,4,5-THS and 3,4,4′-THS, are effective antioxidants against linoleic acid peroxidation in SDS and CTAB micelles. The antioxidative action involves trapping the propagating peroxyl radicals (LOO•) on the surface of the micelle and regenerating α-tocopherol (TOH) by reducing the α-toco-

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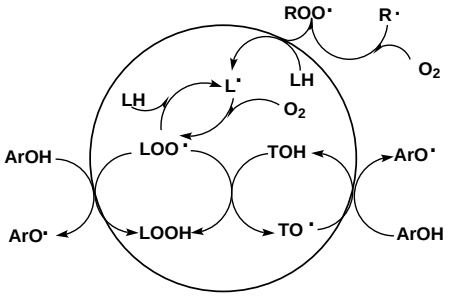
0947-6539/02/0818-4196 $ 20.00+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 18

pheroxyl radical (TO•) as depicted in Scheme 2. The observation that *trans*-stilbene compounds bearing *ortho*-dihydroxyl and/or *para*-hydroxyl functionalities possess remarkably higher antioxidant activity than the ones bearing no such functionalities gives us useful information for antioxidant drug design.



Scheme 2. Antioxidative and TOH-regeneration reactions of resveratrol analogues (ArOH) in micelles

Experimental Section

Materials: Resveratrol and its analogues, that is, 4-HS, 3,5-DHS, 4,4′-DHS, 3,4 -DHS, 3,4,5-THS and 3,4,4′-THS, were prepared according to the available procedures,[29–30] and their structures and purity confirmed by MS, ^{1}H NMR spectroscopy and HPLC. Linoleic acid (Sigma, Chromatographic pure), *dl*-α-tocopherol (Merk, Biochemical reagent, $>99.9\,\%$) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH; Aldrich) were kept under nitrogen in a refrigerator and used as received.

Determination of linoleic acid hydroperoxides: Aliquots of the reaction mixture were taken out of an open vessel at appropriate time intervals and subjected to high performance liquid chromatography (HPLC) analysis on a Gilson liquid chromatograph with a ZORBAX ODS reversed-phase column (6×250 mm, Du Pont instruments), then eluted with methanol/propan-2-ol (3:1, *v*/*v*). The flow rate was set at 1.0 mL min^{-1}. A Gilson 116 UV detector was used to monitor the total linoleic acid hydroperoxides at 235 nm. Every determination was repeated three times, and the experimental deviations were within $\pm10\,\%$.

Determination of α-tocopherol: The procedure was the same as described above for determination of linoleic acid hydroperoxides, except that a Gilson 142 electrochemical detector set at $+700$ mV vs. SCE was used for monitoring TOH. The column was eluted with methanol/propan-2-ol/formic acid (80:20:1, *v*/*v*/*v*) containing sodium perchlorate (50 mmol L^{-1}) as supporting electrolyte.

Determination of α-tocopheroxyl radical: EPR measurements were carried out on a Bruker ER 200D spectrometer operated in the X-band with 100 kHz modulation, a modulation amplitude of 0.25 mT, time constant of 0.2 s and microwave power of 25 mW. A flat quartz flow cell ($0.4\times5.5\times60$ mm) was used for the stopped-flow determination of the reaction kinetics as described previously.[15b] The α-tocopheroxyl radical was generated by vigorously stirring α-tocopherol (1 mmol L^{-1}) and excess lead oxide with a Vortex mixer for 3 min in CTAB (15 mmol L^{-1}) micelles at pH 7.4 and room temperature.

Determination of oxidation potentials: The oxidation potentials of the ArOHs were determined on a PAR 173 potentiostat with a glassy carbon electrode in phosphate buffered micelles at pH 7.4 and room temperature, as described previously.[32] The potential was recorded relative to a saturated calomel electrode.

Acknowledgement

We thank the National Natural Science Foundation of China (NSFC) for financial support (Grant Nos. 29832040 and 20172025).

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Received: February 14, 2002 [F3874]

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