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The effect of aromatic amines and phenols in the thiyl-induced reactions of polyunsaturated fatty acids

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HIGHLIGHTS

- LH micelles were used for the parallel study of peroxidation/*cis–trans* isomerization.
- Both 2-mercaptoethanol and diphenylamine alone protect LH from oxidation.
- Aminyl radicals promote thiyl-radical-induced *cis–trans* isomerization of LH in air.

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ABSTRACT

Thiols are well known for their role in cellular redox homeostasis, while aromatic amines and phenols are the best known classes of chain-breaking antioxidants. On the other hand, thiyl radicals are known to catalyse the double bond isomerization in PUFA. We investigated the role and interplay of 2-mercaptoethanol and diphenylamine in the parallel processes of peroxidation and *cis–trans* isomerization of linoleic acid (LA) during gamma radiolysis, both in solution and micelles. Both compounds, used alone were able to protect LA from oxidation; however pro-oxidant activity and enhanced isomerization was observed when they were used together, depending on the experimental settings. Instead, α-tocopherol protected LA from both oxidation and isomerization in the presence of thiols under any tested settings. The mechanistic scenario is discussed highlighting the role of diphenylaminyl radicals in promoting thiyl-radical-induced *cis–trans* isomerization in the presence of oxygen.

1. Introduction

Due to the crucial roles of lipids in structural and signaling activities, the control of lipid reactivity and transformations is an interdisciplinary research field extending over chemistry to biology and medicine (Chatgilialoglu et al., 2014; Halliwell and Gutteridge, 2007). In this context, the protection against their degradation under oxidative and free radical conditions is of special interest. The reactions of polyunsaturated fatty acids (PUFA) with free radicals are known to occur via two main processes: (i) lipid peroxidation (Niki, 2012) and (ii) *cis–trans* isomerization (Lykakis et al., 2015; Ferreri and Chatgilialoglu, 2012). The mechanism and products of each process have been studied extensively and are now fairly well documented and understood. Scheme 1 shows the interplay of the two processes in the case of linoleic acid moiety and the corresponding main products. The initial step of peroxidation is hydrogen abstraction from the bisallylic position, which can be performed by a variety of radicals, followed by the reaction with oxygen. Conjugated diene hydroperoxides having the *trans,cis* double bond geometry are the initial stable products (Yin et al., 2011). In free radical isomerization, the addition–elimination of a thiyl radical is enough to produce mono-*trans* geometrical isomers (Chatgilialoglu and Ferreri, 2005). An overall damaging potential is produced, that must be carefully considered for its consequences in a biological scenario, since peroxidation is a chain reaction (Yin et al., 2011) and isomerization is a catalytic process (Ferreri et al.,

Abbreviations: ArOH, α-tocopherol; LH, Linoleic acid; PUFA, Polyunsaturated Fatty Acids; $Ph_2NO^{\bullet}$, diphenylnitroxyl radicals; LOOH, Lipid hydroperoxide; PB, phosphate buffer; $RS^{\bullet}$, thiyl radicals; Ψ, volume part of the solvent in a solvent mixture

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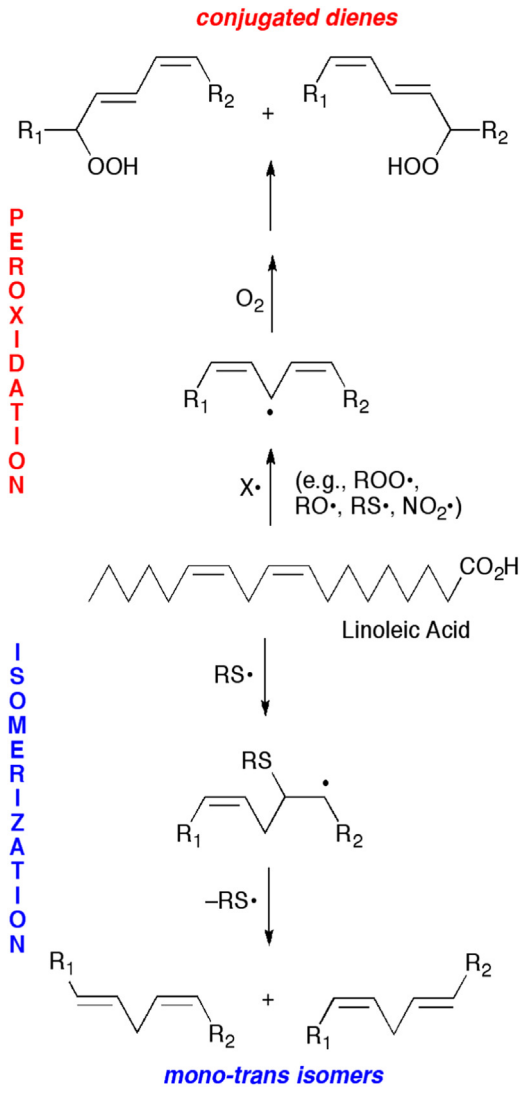


Scheme 1. Radical-based peroxidation and *cis–trans* isomerization processes of linoleic acid.

2001; Ferreri et al., 2004). In the presence of oxygen and thiols, both processes were detected for the first time in the biomimetic model of linoleic acid micelles, thus confirming the double-sworded reactivity of thiols towards unsaturated lipids (Scheme 1) (Mihaljević et al., 2011). It was demonstrated that conjugated diene hydroperoxides and mono-*trans* isomers can be formed to comparable extent.

Phenols are very efficient antioxidants with fairly well-known chemistry and mechanisms (Valgimigli and Pratt, 2012). We were interested in extending the investigation to other common antioxidants, such as aromatic amines. They are also well known protective agents against PUFA peroxidation, whereas their role in protecting against PUFA isomerization has remained unknown until present. Diphenylamine has been largely used as antioxidant in food products in several countries, particularly to preserve the "fresh aspect" of fruits and vegetables during storage and transportation. However, its use has been banned in the EU due to its significant toxicity (Leisso et al., 2013). Albeit relevant, free radical chemistry associated to amine toxicity is still not completely understood (Beland and Kadlubar, 1985; Benigni and Passerini, 2002). Aiming at filling this gap of knowledge we have investigated the combined role of Ph_2NH and $HOCH_2CH_2SH$ in the radical stress induced to linoleic acid (LH) under diverse biomimetic conditions. The results highlight the role of diphenylaminyl radicals as "promoters" of the *cis–trans* isomerization in the biomimetic model systems consisting of the micellar solution of LH in the presence of thiols and molecular oxygen, contributing to a better understanding of complex interactions related to free radical stress and antioxidant control, as well as of the mechanisms of aromatic amines toxicity.

2. Materials and methods

Unless otherwise noted, solvents and chemicals were of the highest grade commercially available (Aldrich, Fluka, Sigma) and were used as received.

Initially γ-irradiation of phosphate buffered air-equilibrated or N_2O-saturated solutions Ψ (EtOH: H_2O)=(1:1) (pH 5) containing 0.5 mM of LH and TWEEN®-20 (a non-ionic surfactant soluble in ethanol/water), in the presence and absence of $HOCH_2CH_2SH$ was studied (Mihaljević et al., 2011). After 100 Gy of irradiation, lipid components were extracted with a solvent mixture Ψ (CH_2Cl_2:MeOH)=(2:1), deaerated and analyzed for quantitative determination of LOOH by the spectrophotometric ferric thiocyanate method, as described earlier (Mihaljević et al., 1996). In parallel the analysis of *trans* fatty acid formation at 100 Gy was carried out by GC, after treatment of the lipid extracts with an ethereal solution of CH_2N_2 in order to transform quantitatively the free fatty acid into the corresponding methyl ester. In the absence of thiol no trace of *trans* fatty acid was detected. By replacing TWEEN®-20 with another non-ionic surfactant Brij 35, similar results were obtained (Mihaljević et al., 2011).

Next, model system containing mixed surfactant micelles and buffer was prepared by slow solubilization of LH in non-ionic surfactant micelles previously formed by mixing TWEEN -20® or Brij 35 and NaH_2PO_4 (PB), pH 6.5. The composition of the investigated systems was typically 0.5 mM LH, 0.28 mM TWEEN-20 and 5 mM PB (pH∼5). Gamma radiolysis of the solutions with typical composition and in the presence of 2.8 mM $HOCH_2CH_2SH$ and/or 83 μM Ph_2NH, which was added prior to irradiation, was performed. It should be pointed out that solubility of Ph_2NH in micellar solutions is much lower than in homogeneous solutions so its concentration had to be reduced to 83 μM.

Radiolysis was performed at room temperature using panoramic ^{60}Co source at dose rate 2.4 and 274.8 Gy min^{-1}. The "transit time" (the time during the movement of the source to and from irradiation position) was 2.2 s, corresponding to the transit irradiation dose of about 0.10 Gy at the dose rate of 2.4 Gy min^{-1} and 10 Gy at the dose rate of 274.8 Gy min^{-1}, which had to be taken into consideration at doses lower than 100 Gy. Micelles were irradiated in equilibrium with air or after saturation with N_2O. After irradiation, lipid components were extracted with a solvent mixture of Ψ (CH_2Cl_2:MeOH)=(2:1), deaerated by nitrogen, and an aliquot of the sample was taken out from the lower layer for the quantitative determination of LOOH. All further analysis was carried out in three independently prepared solutions. The concentration of LOOH was determined by the spectrophotometric ferric thiocyanate method following a published procedure (Mihaljević et al., 1996), using UV/vis spectrophotometer Varian Cary 4000. The rest of the lipid extract was used for GC analysis of geometrical isomers using the known conditions for the separation of *cis* and *trans* isomers (Ferreri et al., 2001; Mihaljević et al., 2011; Chatgilialoglu et al., 2002; Chatgilialoglu et al., 2005). In order to transform linoleic acid and its geometrical isomers into the corresponding methyl esters the reaction solutions were treated with an ethereal solution of diazomethane (Glastrup 1998). A Varian 450 gas chromatograph equipped with a flame ionization detector and a Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropylpolysiloxane capillary column;

105 m × 0.25 mm) was used with the following oven program: temperature started from 180 °C, held for 35 min, followed by increase of 10 °C min^{-1} up to 250 °C and held for 5 min. Methyl esters were identified by comparison with the retention times of authentic samples, which are commercially available.

3. Results and discussion

3.1. Irradiation of linoleic acid in homogeneous solutions

LH is soluble in Ψ (EtOH:H_2O)=(1:1). The γ-radiolysis of ethanol/water led to the transient species shown in Eqs. (1) and (2), where $R^{\bullet}$ represent the produced alcohol radicals (i.e., $^{\bullet}CH_2CH_2OH$, $CH_3{}^{\bullet}CHOH$, $CH_3CH_2O^{\bullet}$) (Asmus et al., 1973). Solvated electrons ($e_{sol}{}^{-}$) in N_2O-saturated solutions are transformed into $HO^{\bullet}$ radical [Eq. (3)]. Hydrogen abstraction from ethanol by $HO^{\bullet}$ radical and $H^{\bullet}$ atoms increases the production of alkyl radicals [Eq. (4)]. Alkyl radicals react with $HOCH_2CH_2SH$ to give the corresponding thiyl radicals [Eq. (5), $k_5=2\times10^7\ M^{-1}\ s^{-1}$] (Buxton et al., 1988; Ross et al., 1998). In O_2-atmosphere alkyl radicals give alkylperoxyl radicals [Eq. (6) ($k_6\approx1\times10^9\ M^{-1}\ s^{-1}$)] (Valgimigli and Pratt, 2012). Under these conditions thiyl radicals generated by Eq. (7) [$k_7\approx10^2\ M^{-1}\ s^{-1}$] (Denisov et al., 2009) are effective in inducing the isomerization process, although much less than under N_2-atmosphere. In the presence of Ph_2NH alkyl radical $R^{\bullet}$ generates diphenylaminyl radical [Eq. (8), $k_8\approx1\times10^3\ M^{-1}\ s^{-1}$, estimated from the value for 4,4′-diacyldiphenilamine with primary alkyl radicals as $1.3\times10^3\ M^{-1}\ s^{-1}$ at 25 °C (Hanthorn et al., 2012b)]. Similarly in the presence of oxygen aminyl radicals are formed by reaction of Ph_2NH with peroxyls [Eq. (9), $k_9=1.5\times10^4\ M^{-1}\ s^{-1}$ at 50 °C (Lucarini et al., 1999) and nitroxyl radicals $Ph_2NO^{\bullet}$ can also be formed Eq. (10)]. Finally, quenching of thiyl radicals by Ph_2NH [Eq. (11)] could be possible. The $ROO^{\bullet}$ and $HOCH_2CH_2S^{\bullet}$ radicals are expected to react with LH generating the bisallylic radicals $L^{\bullet}$ [Eq. (12)] (Porter, 1986), which in the presence of oxygen propagate the lipid peroxidation chain reactions (Scheme 1).

$$CH_3CH_2OH \rightsquigarrow e_{sol}{}^{-}, R^{\bullet} \quad (1)$$

$$H_2O \rightsquigarrow e_{sol}{}^{-}, HO^{\bullet}, H^{\bullet} \quad (2)$$

$$e_{sol}{}^{-} + N_2O + H_2O \rightarrow N_2 + HO^{-} + OH^{\bullet} \quad (3)$$

$$HO^{\bullet}/H^{\bullet} + CH_3CH_2OH \rightarrow H_2O/H_2 + R^{\bullet} \quad (4)$$

$$R^{\bullet} + HOCH_2CH_2SH \rightarrow RH + HOCH_2CH_2S^{\bullet} \quad (5)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (6)$$

$$ROO^{\bullet} + HOCH_2CH_2SH \rightarrow ROOH + HOCH_2CH_2S^{\bullet} \quad (7)$$

$$R^{\bullet} + Ph_2NH \rightarrow RH + Ph_2N^{\bullet} \quad (8)$$

$$ROO^{\bullet} + Ph_2NH \rightarrow ROOH + Ph_2N^{\bullet} \quad (9)$$

$$Ph_2N^{\bullet} + ROO^{\bullet} \rightarrow Ph_2NO^{\bullet} + RO^{\bullet} \quad (10)$$

$$HOCH_2CH_2S^{\bullet} + Ph_2NH \rightarrow HOCH_2CH_2SH + Ph_2N^{\bullet} \quad (11)$$

$$ROO^{\bullet}/HOCH_2CH_2S^{\bullet} + LH \rightarrow ROOH/HOCH_2CH_2SH + L^{\bullet} \quad (12)$$

Based on the rate constants of 1.2×10^{10} and $9.1\times10^9\ M^{-1}\ s^{-1}$ for the reaction of $e_{aq}{}^{-}$ with $HOCH_2CH_2SH$ (2.8 mM) and N_2O (20 mM) (Buxton, et al., 1988; Ross et al.,1998), in the experimental conditions 85% of hydrated electrons are trapped by N_2O to increase the formation of $HO^{\bullet}$ radicals [Eq. (3)]. The $HO^{\bullet}$ radicals and $H^{\bullet}$ atoms formed also can react with thiol to give thiyl radicals [Eq. (13)], the isomerizing species.

$$HO^{\bullet}/H^{\bullet} + HOCH_2CH_2SH \rightarrow H_2O/H_2 + HOCH_2CH_2S^{\bullet} \quad (13)$$

Table 1 shows the effect of $HOCH_2CH_2SH$ and Ph_2NH on peroxidation and isomerization processes of LH. When the thiol was added to the system the decrease of LOOH observed in air-equilibrated solutions indicated a lower degree of lipid peroxidation accompanied with the relevant formation of *trans* isomers of LH, thereby confirming the double-face behavior of thiols, which are able to protect lipids from oxidation but cause radical damage (thiyl radical-mediated isomerization). The amount of irradiation-induced lipid peroxidation at 100 Gy in control solution was at low dose rate 13% higher than at the higher dose rate. In solutions with addition of diphenylamine alone the LOOH concentration was about 60% higher at the 2.4 Gy min^{-1} than at 274.8 Gy min^{-1}. With addition of thiol the LOOH concentration was double at the low dose rate relative to that obtained at the higher dose rate. Furthermore, irradiation of the solutions with both diphenylamine and thiol at the lower dose rate promoted three times higher LOOH concentrations than those determined at the higher dose rate. Generally, lower dose rates were found to be more efficient in LOOH formation in these systems, indicating the validity of the inverse square-root relationship with dose rate expected in homogeneous systems (Metwally and Moore, 1987; AlSheikhly and Simic, 1989; Katušin-Ražem and Ražem, 2000).

Ph_2NH alone inhibited the oxidation confirming the antioxidant role of aromatic amine. However, in the presence of amine there was no evidence for *cis–trans* isomerization, indicating that diphenylaminyl radicals produced by reactions (8) and (9) do not induce LH isomerization. However, under anaerobic, and particularly under aerobic conditions, when both Ph_2NH and $HOCH_2CH_2SH$ were present in the system, *cis–trans* isomerization was more marked than in the presence of the thiol alone. Furthermore, only at the lower irradiation dose rate, in the presence of both amine and thiol, LOOH formation was enhanced indicating a prooxidant role of the $Ph_2NH/HOCH_2CH_2SH$ couple. One possible explanation for the observed loss of protective activity of aromatic amines towards the oxidation/isomerization of LH in the presence of $HOCH_2CH_2SH$ is the occurrence of chain transfer/propagation reactions caused by aminyl radicals [Eq. (-11)]. Based on the calculated gas phase S–H Bond Dissociation Enthalpy (BDE) for thiols (e.g. cysteine BDE=87.3 kcal/mol) (Roux et al., 2010), and diphenylamine (BDE=86.4 kcal/mol) (Hanthorn et al., 2012a), respectively at G3 and CBS-QB3 level, chain transfer [Eq. (-11)] should be only slightly endothermic, hence feasible in the presence of biomimetic concentrations of thiols (the rate constants of alkylaminyl radical with *t*-BuSH and PhSH are 5×10^6 and $1\times10^3\ M^{-1}\ s^{-1}$ 25 °C, respectively (Musa et al., 1996)). In a study by Montevecchi and Navacchia (1998), the rate of H-atom abstraction from $ArNH_2$ by $PhS^{\bullet}$ radical was found to be similar to that of thiyl addition to an arylalkyne group, which, in turn, has been determined as $7.9\times10^5\ M^{-1}\ s^{-1}$ by Ito et al. (1982). Based on the thermochemistry of equilibrium (11) it can, therefore, be estimated that the rate constant for reaction (-11) is $k_{-11}\approx1.7\times10^5\ M^{-1}\ s^{-1}$.

$$HOCH_2CH_2SH + Ph_2N^{\bullet} \rightarrow HOCH_2CH_2S^{\bullet} + Ph_2NH \quad (-11)$$

From Table 1 it can be seen that isomerization is much more effective at the low dose rate. The level of *trans* isomers of LH in N_2O-saturated solutions was much higher than under air-equilibrated conditions, when thiol was present, either alone or with amine. As it was expected, in N_2O-saturated solutions LOOH

Table 1
The amounts of LOOH of air-equilibrated Ψ (EtOH:H_2O)=(1:1) solutions (third column) and *trans* LH isomers of air-equilibrated or N_2O-saturated Ψ (EtOH:H_2O)=(1:1) solutions generated by gamma irradiation with 100 Gy as function of dose rate; 0.5 mM LH, 0.28 mM TWEEN-20®, 5 mM PB, pH 5.

Dose rate/ Gy min^{-1}	Additives	[LOOH]/10^{-6} M	% Isomers LH 9c,12c/9c,12t/ 9t,12c/9t,12t*	
			Air	N_2O
2.4	None (control)	27.5 ± 0.4	100/0/0/0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$	18.8 ± 0.7	90.5/4.7/4.8/0	81.4/4.7/3.7/10.2
	0.5 mM Ph_2NH	23.8 ± 0.9	100/0/0/0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$ and 0.5 mM Ph_2NH	36.1 ± 1.2	69.4/13.7/13.5/3.5	9.9/20.2/19.9/50.0
274.8	None (control)	24.3 ± 3.2	100/0/0/0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$	9.3 ± 0.4	95.2/2.2/2.2/0.4	17.8/23.1/22.1/37
	0.5 mM Ph_2NH	14.9 ± 0.5	100/0/0/0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$ and 0.5 mM Ph_2NH	12.9 ± 1.6	93.2/3.3/3.1/0.4	21.1/23.8/22.8//32.3

* Reported values represent the mean of three independent measurements ($p < 0.05$); errors are less than ± 5%.

concentrations were below detection limit.

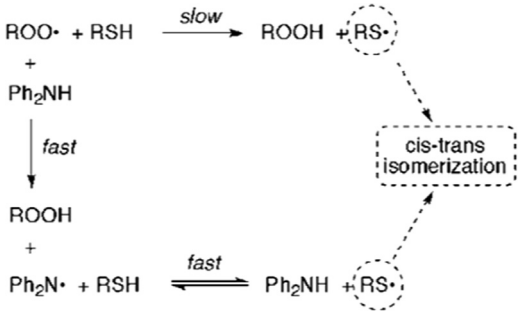
In the absence of literature in this regard we wondered whether the diphenylnitroxyl radicals $Ph_2NO^{\bullet}$ which can be formed from the corresponding $Ph_2N^{\bullet}$ according to Eq. (10), could contribute to the enhanced isomerization of LH. In order to clarify whether and to which extent $Ph_2NO^{\bullet}$ radicals are involved in *cis–trans* isomerization we turned to EPR spectroscopy and product studies. The results allowed excluding that under our experimental settings $Ph_2NO^{\bullet}$ or in general persistent nitroxyl radicals can induce significant *cis–trans* isomerization (see Supporting Information for full details). The mechanism for the *cis–trans* isomerization in the presence of $HOCH_2CH_2SH$ and Ph_2NH is summarized in Scheme 2.

3.2. Irradiation of linoleic acid in micelles

Irradiated N_2O-saturated samples in the presence or absence of thiol/amine did not show any formation of LOOH. Fig. 1 shows dose-profiles of LOOH formation under air-equilibrated conditions. LOOH is produced in the presence (○) or absence of thiol (◆). However, LOOH was negligible when Ph_2NH was added, either alone (▲) or with $HOCH_2CH_2SH$ before irradiation (●), showing a good protective activity from lipid peroxidation in micelles, despite the lower concentration as compared to experiments in homogeneous solution.

The same samples were analysed for geometrical isomers distribution during lipid peroxidation. Fig. 2 (A) displays the development of geometrical isomers as a function of dose under N_2O-saturated conditions in the presence of $HOCH_2CH_2SH$ (open symbols) or in the presence of $HOCH_2CH_2SH$/Ph_2NH (solid symbols). In both cases the results show the disappearance of 9c,12c–C18:2 (●,○) being replaced by the formation of mono-*trans* isomers (▲,△) and 9 t,12 t–C18:2 (■,□).

As expected, isomerization was more marked in the absence of



Scheme 2. Role of aminyl radicals in promoting *cis–trans* isomerization in the presence of O_2.

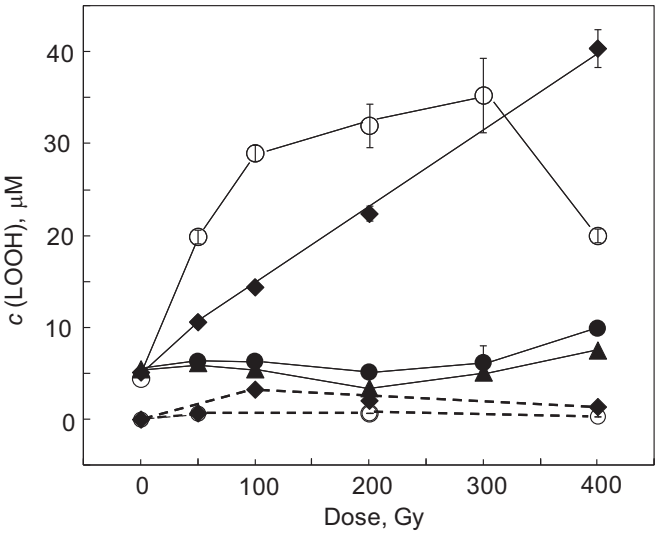


Fig. 1. The LOOH concentrations in micelles (0.5 mM LH, 0.28 mM Tween®-20, 5 mM PB at pH 5) as a function of irradiation dose (dose rate 274.8 Gy min^{-1}) under air-equilibrated (solid lines) or N_2O-saturated conditions (dashed lines): (◆) no other additives, (○) with 2.8 mM $HOCH_2CH_2SH$, (▲) with 83 μM Ph_2NH, (●) with 2.8 mM $HOCH_2CH_2SH$ and 83 μM Ph_2NH.

oxygen, however under air-equilibrated conditions the *cis–trans* isomerization still operates. The disappearance of 9c,12c–C18:2 (●) matches well with the formation of mono-*trans* isomers (▲) and 9t,12t–C18:2 (■), under the conditions where the LOOH formation was detectable (cf. Fig. 1).

Results obtained in this part of work are summarized in Table 2. As compared with the results obtained in homogeneous LH solutions, in LH micelles the Ph_2NH/thiol couple inhibited peroxidation processes with modest effect on isomerization level. Low concentration of Ph_2NH (83 μM) slightly inhibited isomerization when the thiol is present in aerobic and anaerobic conditions. This effect can be explained by quenching of thiyl radicals by Ph_2NH [Eq. (11)].

However, at variance with what was observed in homogeneous solution, Ph_2NH had negligible effect on thiyl radical-mediated isomerization in the absence of oxygen. This result can, in part, be explained by the lower concentration of Ph_2NH as compared to homogenous-phase experiments; however, it could also depend on different interactions of aminyl radicals and thiol due to partition effects in the heterogeneous environment.

In comparison with the results of lipid transformation processes investigated as function of dose rate in homogeneous solutions, LOOH concentrations formed at the same dose and at the

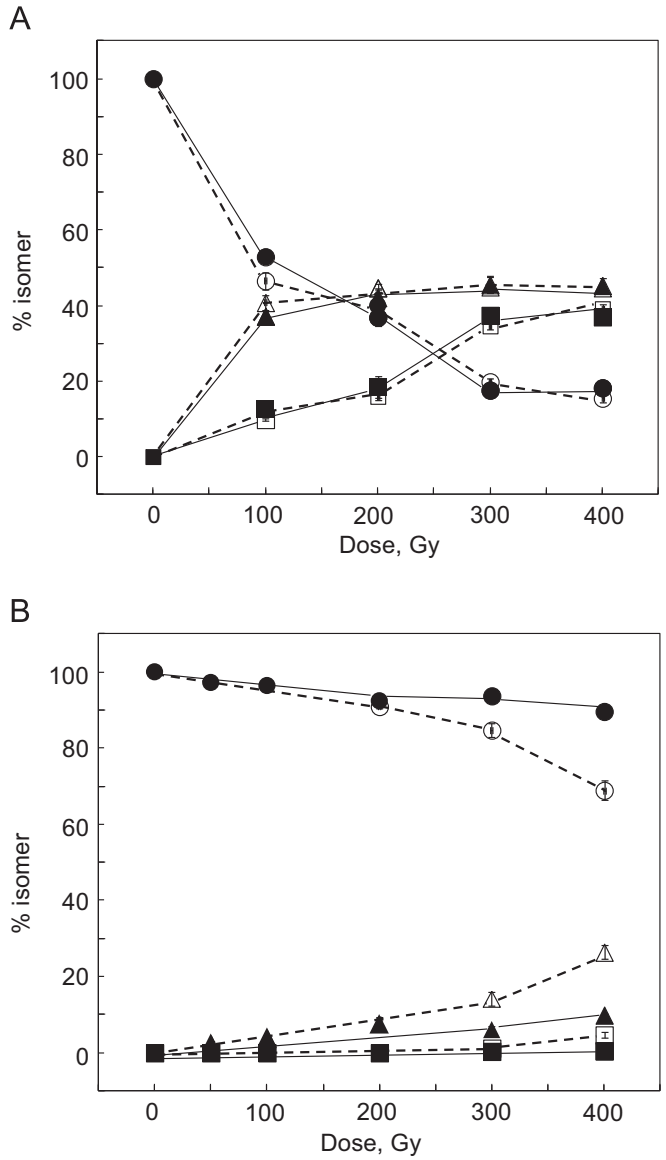


Fig. 2. The geometrical isomers distribution of LH as function of irradiation dose (dose rate 274.8 Gy min^{-1}) in micelles (0.5 mM LH, 0.28 mM Tween®-20, 5 mM PB at pH 5) under N_2O-saturated (A) or air-equilibrated (B) conditions; the disappearance of 9c,12c–C18:2 (●,○), the formation of 9t,12c–C18:2+9 c,12t–C18:2 (▲,△), and the formation of 9t,12t–C18:2 (■,□); experiments were performed either in the presence of 2.8 mM $HOCH_2CH_2SH$, open symbols (○,△,□), or in the presence of 2.8 mM $HOCH_2CH_2SH$ and 83 μM Ph_2NH, solid symbols (●,▲,■).

dose rate of 2.4 Gy min^{-1} in control solution and in solutions with added diphenyl amine were 80% and 70%, respectively, higher than those obtained at 274.8 Gy min^{-1}. However, the presence of both diphenylamine and thiol at the lower dose rate promoted 65% higher LOOH concentrations than those determined at the higher dose rate. With the exception for the case when thiol alone was added to control micellar solution, the lower dose rate was found to be more efficient in LOOH formation. Although no inverse square-root correlation with dose rate was originally tested in those systems, this dose rate dependence of peroxidation found in our model system indicates that an inverse square root relation between the radiation-chemical yield of LOOH and dose rate in homogeneous medium may also hold in our micellar model system.

In order to compare the activity of Ph_2NH in radiation induced peroxidation and thiyl radical-catalyzed *cis–trans* isomerization

Table 2
The amounts of LOOH under aerobic conditions (third column) and *trans* isomers of LH generated by gamma irradiation with 100 Gy of LH micelles in aerobic and anaerobic conditions as function of dose rate; 0.5 mM LH, 0.28 mM Tween-20®, 5 mM PB, pH 5.

Dose rate/ Gy min^{-1}	Additives	[LOOH]/10^{-6} M	% Isomers of LH 9c,12c/9c,12t/9t,12c/ 9t,12t*	
			Air	N_2O
2.4	None (control)	76.7 ± 3.1	100/0/0/ 0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$	7.0 ± 0.2	88.3/5.4/ 5.3/1.0	11.2/20.8/ 16.3/52.3
	0.5 mM Ph_2NH	18.5 ± 0.9	100/0/0/ 0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$ and 83 μM Ph_2NH	17.9 ± 0.9	94.3/3.1/ 2.7/0	17.7/24.2/ 20.1/38.0
274.8	None (control)	14.4 ± 0.4	100/0/0/ 0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$	29.0 ± 0.9	96.4/2.0/ 1.6/0	46.5/21.7/ 19.1/2.7
	0.5 mM Ph_2NH	5.5 ± 0.1	100/0/0/ 0	100/0/0/0
	2.8 mM $HOCH_2CH_2SH$ and 83 μM Ph_2NH	6.3 ± 0.2	96.3/1.9/ 1.9/0	52.9/19.6/ 17.6/9.9

* Reported values represent the mean of three independent measurements ($p < 0.05$); errors are less than ± 5%.

we repeated the experiments in LH micelles using α-tocopherol (ArOH) as one of the most efficient and biologically relevant radical scavengers (Valgimigli and Pratt, 2012).

In the presence of α-tocopherol the alkyl radical R• generates the aryloxyl radical from α-tocopherol [Eq. (14), $k_{14}=6.0\times10^5\ M^{-1}\ s^{-1}$ at 25 °C] (Franchi et al., 1999).

$$R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet} \quad (14)$$

$$ROO^{\bullet} + ArOH \rightarrow ROOH + ArO^{\bullet} \quad (15)$$

$$HOCH_2CH_2S^{\bullet} + ArOH \rightarrow HOCH_2CH_2SH + ArO^{\bullet} \quad (16)$$

In O_2-atmosphere peroxyl radicals also give aryloxyl radicals from α-tocopherol [Eqs. (6) and (15), $k_{15}=3.2\times10^6\ M^{-1}\ s^{-1}$ (Valgimigli and Pratt, 2012)]. Fig. 3 shows the formation of LOOH as function of dose in LH micelles. The antioxidant activity of α-tocopherol is confirmed by a significant decrease of LOOH formation when dose increases in the presence of α-tocopherol. Protection is even higher upon combination with the thiol. While isomerization was induced in the presence of thiol alone ((♦) Fig. 4), under aerobic, and especially under anaerobic conditions, this process was significantly inhibited upon addition of 50 μM α-tocopherol. The good protection of α-tocopherol against lipid peroxidation/isomerization in the presence of thiol can be explained based on the available kinetic information for reaction (16). The reaction of thiyl radicals (e.g. from cysteine) with tocopherol's analog Trolox is known to occur by hydrogen abstraction [Eq. (16)] with $k_{16}=1\times10^8\ M^{-1}\ s^{-1}$, whereas for the reverse reaction only a limiting value ($k_{-16}<10^5\ M^{-1}\ s^{-1}$) is available (Davies et al., 1988; De Koning 2002).

Therefore, the presence of biologically significant concentrations of α-tocopherol (50 μM) inhibited lipid peroxidation with simultaneous inhibition of thiyl-induced *trans* isomerization of LH (Fig.4). The inhibition of isomerization by α-tocopherol was more effective than what was observed with even higher concentration of the aromatic amine (Fig. 2A).

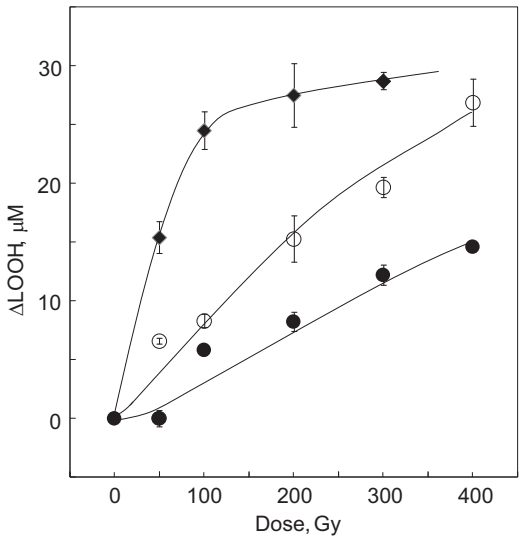


Fig. 3. Effect of irradiation dose on the concentration change of LOOH in LH micelles under aerobic conditions in the presence/absence of α-tocopherol: (♦) with 2.8 mM $HOCH_2CH_2SH$; (○) with 50 μM ArOH; (●) with 50 μM ArOH and 2.8 mM $HOCH_2CH_2SH$. Micelles: 0.5 mM LH, 0.28 mM Tween-20®, 5 mM PB, pH 5; P=274.8 Gy min^{-1}.

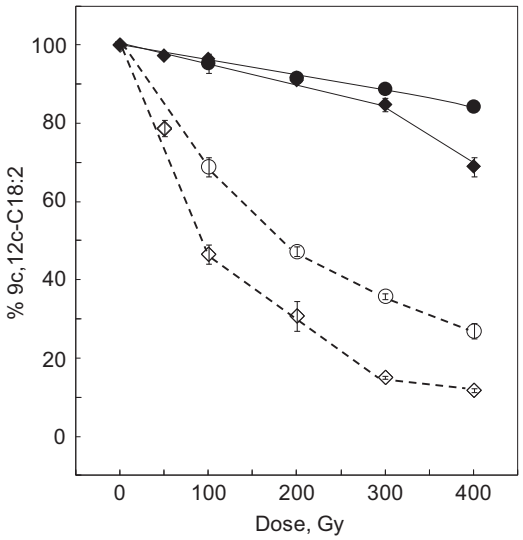


Fig. 4. : Effect of irradiation dose on disappearance of the *cis* isomer in LH micelles under air-equilibrated (solid lines) and N_2O-saturated (dotted lines) conditions: (♦) 2.8 mM $HOCH_2CH_2SH$, (●) 50 μM ArOH and 2.8 mM $HOCH_2CH_2SH$. Micelles: 0.5 mM LH, 0.28 mM Tween-20®, 5 mM PB, pH 5; P=274.8 Gy min^{-1}.

4. Conclusions

Antioxidant diversity is very important for achieving satisfactory protection of a biological environment and so far thiols and secondary amines are mostly evoked for their protective role against peroxidation of polyunsaturated fatty acids (Mihaljević et al., 2011; Brownlie and Ingold, 1966; Lucarini et al., 1999; Hanthorn et al., 2012b). On the other hand, the harmful effects of aromatic amines are known to be associated with adducts formation (Beland and Kadlubar, 1985; Benigni and Passerini, 2002), whereas in this work another potential mechanism of toxicity has been brought to light, involving thiols and unsaturated lipids. In homogenous solution the radical reactivity of aromatic amines with thiols increased *cis–trans* isomerization of PUFA, as summarized in Scheme 2. This observation clearly differentiates aromatic amines from physiological antioxidants like α-tocopherol, despite the ability of both antioxidants to protect against peroxidation. Free radical chemistry described in this work is expectedly relevant in biological environments, where thiols are the most abundant endogenous antioxidants (at mM levels) and diphenylamine might be introduced with the diet as food preservative. The aminyl radical reactivity bringing to overgeneration of thiyl radicals stimulates further investigation of a novel process of toxicity of secondary aromatic amines.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.radphyschem.2015.11.018.

References

AlSheikhly, M., Simic, M.G., 1989. Chain-propagation length of linoleic acid peroxidation in aqueous monomeric and micellar systems. J. Phys. Chem. 93, 3103–3106.
Asmus, K.-D., Möckel, H., Henglein, A., 1973. Pulse radiolytic study of the site of hydroxyl radical attack on aliphatic alcohols in aqueous solution. J. Phys. Chem 77, 1218–1221.
Beland, F.A., Kadlubar, F.F., 1985. Formation and persistence of arylamine DNA adducts in vivo. Environ. Health Perspect. 62, 19–30.
Benigni, R., Passerini, L., 2002. Carcinogenicity of the aromatic amines: from structure-activity relationships to mechanisms of action and risk assessment. Mutat. Res. 511, 191–206.
Brownlie, I.T., Ingold, K.U., 1966. The inhibited autoxidation of styrene, Part V. The kinetics and deuterium isotope effect for inhibition by diphenylamine, phenyl-α-naphtylamine and phenyl-β-naphtylamine. Can. J. Chem. 44, 861–868.
Buxton, G.V., Greenstock, C.L., Helman, P.W., Ross, A.B., 1988. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (.OH/.O^-) in aqueous solution. J. Phys. Chem. Ref. Data 17, 513–886.
Chatgilialoglu, C., Ferreri, C., 2005. Trans lipids: the free radical path. Acc. Chem. Res. 38, 441–448.
Chatgilialoglu, C., Altieri, A., Fischer, H., 2002. The kinetics of thiyl radical-induced reactions of monounsaturated fatty acid esters. J. Am. Chem. Soc. 124, 12816–12823.
Chatgilialoglu, C., Samadi, A., Guerra, M., Fischer, H., 2005. The kinetics of *Z*/*E* isomerization of methyl oleate catalyzed by photogenerated thiyl radicals. ChemPhysChem 6, 286–291.
Chatgilialoglu, C., Ferreri, C., Malchiorre, M., Sansone, A., Torreggiani, A., 2014. Lipid Geometrical Isomerism: From Chemistry to Biology and Diagnostics. Chem. Rev. 114, 255–284.
Davies, M.J., Forni, L.G., Willson, R.L., 1988. Vitamin E analogue Trolox C. E.s.r. and pulse-radiolysis studies of free-radical reactions. Biochem. J. 255, 513–522.
De Koning, A.J., 2002. The antioxidant ethoxyquin and its analogues: a review. Int. J. Food Prop. 5, 451–461.
Denisov, E.T., Chatgilialoglu, C., Shestakov, A.F., Denisova, T.G., 2009. Rate constants and transition-state geometryof reactions of alkyl, alkoxyl, and peroxyl radicals with thiols. Int. J. Chem. Kinet. 41, 284–293.
Ferreri, C., Chatgilialoglu, C., 2012. Lipid isomerization in encyclopedia of radicals in chemistry. In: Chatgilialoglu, C., Studer, A. (Eds.), Biology and Materials. John Wiley & Sons Ltd, Chichester, UK, pp. 1599–1622.
Ferreri, C., Samadi, A., Sassatelli, F., Landi, L., Chatgilialoglu, C., 2004. Regioselective cis-trans isomerization of arachidonic double bonds by thiyl radicals: the influence of phospholipid supramolecular organization. J. Am. Chem. Soc. 126, 1063–1072.
Ferreri, C., Costantino, C., Perrotta, L., Landi, L., Mulazzani, Q.G., Chatgilialoglu, C., 2001. Cis-trans isomerization of polyunsaturated fatty acid residues in phospholipids catalyzed by thiyl radicals. J. Am. Chem. Soc. 123, 4459–4468.
Franchi, P., Lucarini, M., Pedulli, G.F., Valgimigli, L., Lunelli, B., 1999. Reactivity of

substituted phenols toward alkyl radicals. J. Am. Chem. Soc. 121, 507–514.
Glastrup, J., 1998. Diazomethane preparation for gas chromatographic analysis. J. Chromatogr. A 827, 133–136.
Halliwell, B., Gutteridge, J.M.C., 2007. Antioxidant defences in free radicals in biology and medicine, 4th ed. University Press, Oxford, pp. 105–245.
Hanthorn, J.J., Valgimigli, L., Pratt, D.A., 2012a. Incorporation of ring nitrogens into diphenylamine antioxidants: striking a balance between reactivity and stability. J. Am. Chem. Soc. 134, 8306–8309.
Hanthorn, J.J., Amorati, R., Valgimigli, L., Pratt, D.A., 2012b. The reactivity of air-stable pyridine- and pyrimidine-containing diarylamine antioxidants. J. Org. Chem. 77, 6895–6907.
Ito, O., Omori, R., Matsuda, M., 1982. Determination of addition rates of benzenethiyl radicals to alkynes by flash photolysis. Structures of produced vinyl-type radicals. J. Am. Chem. Soc. 104, 3934–3937.
Katušin-Ražem, B., Ražem, D., 2000. Absolute Rate constants of elementary reactions steps in radiation-induced peroxidation of unsaturated fatty acids. J. Phys. Chem. A 104, 1482–1494.
Leisso, R., Buchanan, D., Lee, J., Mattheis, J., Rudell, D., 2013. Cell wall, cell membrane, and volatile metabolism are altered by antioxidant treatment, temperature shifts, and peel necrosis during apple fruit storage. J. Agric. Food Chem. 61, 1373–1387.
Lucarini, M., Pedrielli, P., Pedulli, G.F., Valgimigli, L., Gigmes, D., Tordo, P., 1999. Bond dissociation energies of the N–H bond and rate constants for the reaction with alkyl, alkoxyl, and peroxyl radicals of phenothiazines and related compounds. J. Am. Chem. Soc. 121, 11546–11553.
Lykakis, I.N., Ferreri, C., Chatgilialoglu, C., 2015. Biomimetic chemistry on the protection of cis phospholipid from the thiyl radical isomerization by common antioxidants. ARKIVOC 2015 (iii), 140–153. http://dx.doi.org/10.3998/ark.5550190.p008.984.
Metwally, M.M., Moore, J.S., 1987. Oxygen uptake during the gamma-irradiation of fatty acids. Int. J. Radiat. Biol. 52, 253–267.
Mihaljević, B., Katušin-Ražem, B., Ražem, D., 1996. The reevaluation of the ferric thiocyanate assay for lipid hydroperoxides with special considerations of the mechanistic aspects of the response. Free Radic. Biol. Med. 21, 53–63.
Mihaljević, B., Tartaro, I., Ferreri, C., Chatgilialoglu, C., 2011. Linoleic acid peroxidation vs. isomerization: a biomimetic model of free radical reactivity in the presence of thiols. Org. Biomol. Chem. 9, 3541–3548.
Montevecchi, P.C., Navacchia, M.L., 1998. Sulfanyl radical-mediated cyclization of aminyl radicals. Tetrahedron Lett. 39, 9077–9080.
Musa, O.M., Horner, J.H., Shahin, H., Newcomb, M., 1996. A kinetic scale for dialkylaminyl radical reactions. J. Am. Chem. Soc. 118, 3862–3868.
Niki, E., 2012. Lipid peroxidation in encyclopedia of radicals in chemistry. In: Chatgilialoglu, C., Studer, A. (Eds.), Biology and Materials 2012. John Wiley & Sons Ltd, Chichester, UK, pp. 1577–1597.
Porter, N.A., 1986. Mechanisms for the autoxidation of polyunsaturated lipids. Acc. Chem. Res. 19, 262–268.
Ross, A.B., Mallard, W.G., Helman, W.P., Buxton, G.V., Huie R.E., Neta, P., 1998. NDRL-NIST Solution Kinetic Database – Ver. 3, Notre Dame Radiation Laboratory, Notre Dame, IN and NIST Standard Reference Data, Gaithersburg, MD, 1998.
Roux, M.V., Foces-Foces, C., Notario, R., Ribeiro da Silva, M.A.V., Ribeiro da Silva, M. D.M.C., Santos, A.F.L.O.M., Juaristi, E., 2010. Experimental and computational thermochemical study of sulfur-containing amino acids: L-cysteine, L-cystine, and L-cysteine-derived radicals. S–S, S–H, and C–S bond dissociation enthalpies. J. Phys. Chem. B. 114, 10530–10540.
Valgimigli, L., Pratt, D.A., 2012. Antioxidants in Biology and Medicine in Encyclopedia of Radicals in Chemistry, Biology and Materials. In: Chatgilialoglu, C. Studer, A. (Eds.) , John Wiley & Sons Ltd, Chichester, UK, pp. 1623–1677.
Yin, H., Xu, L., Porter, N.A., 2011. Free radical lipid peroxidation: mechanisms and analysis. Chem. Rev. 111, 5944–5972.