Mechanistic insights on skin sensitization to linalool hydroperoxides: EPR evidence on radical intermediates formation in reconstructed human epidermis and ^{13}C -NMR reactivity studies with thiol residues

Salen Kuresepi,[†] Bertrand Vilen^{‡,§} Jean-Pierre Lepoittevin,[†] Elena Giménez-Arnau^{*,†}

[†] Dermatochemistry Laboratory, University of Strasbourg, CNRS, UMR 7177, F-67000 Strasbourg, France

[‡] POMAM Laboratory, University of Strasbourg, CNRS, UMR 7177, F-67000 Strasbourg, France

[§] French EPR Federation of Research, REseau NAional de Rpe interDisciplinaire, RENARD, Fédération IR-RPE CNRS 3443, France

Corresponding Author

* Elena Giménez-Arnau
e-mail: egimenez@unistra.fr
Tel: +33 3 68 85 15 25

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ABSTRACT

Linalool is one of the most commonly used fragrance terpenes in consumer products. While pure linalool is considered as non-allergenic because it has a very low skin sensitization potential, its autoxidation on air leads to allylic hydroperoxides that have been shown to be major skin sensitizers. These hydroperoxides have the potential to form antigens *via* radical mechanisms. In order to obtain in-depth insights of such reactivity, we first investigated the formation of free radicals derived from linalool hydroperoxides *in situ* in a model of human reconstructed epidermis by electron paramagnetic resonance combined with spin trapping. The formation of carbon and oxygen centered radical species derived from the hydroperoxides was especially evidenced in an epidermis model, mimicking human skin and thus closer to what may happen *in vivo*. To further investigate these results, we synthesized linalool hydroperoxides containing a ^{13}C -substitution at positions precursor of carbon radicals to elucidate if one of these positions could react with cysteine, its thiol chemical function being one of the most labile groups prone to react through radical mechanisms. Reactions were followed by mono- and bi-dimensional ^{13}C -NMR. We validated that carbon radicals derived from allylic hydrogen abstraction by the initially formed alkoxyl radical and/or from its β -scission, can alter directly the lateral chain of cysteine forming adducts *via* radical processes. Such results provide an original vision on the mechanisms likely involved in the reaction with thiol groups that might be present in the skin environment. Consequently, the present findings are a step ahead towards the understanding of protein binding processes to allergenic allylic hydroperoxides of linalool through the involvement of free radical species and thus of their sensitizing potential.

INTRODUCTION

Linalool is one of the most commonly used fragrance terpenes in consumer products because of its flowery-fresh odor.¹ It is also found in many essential oils as lavender, ylang-ylang and rosemary oils.² Linalool is one of the 26 compounds (EU Cosmetic Regulation 1223/2009) that is mandatory to label in the packaging of cosmetic products when the concentration exceeds 10 ppm in leave-on products and 100 ppm in rinse-off products.³ Pure linalool is considered as non-allergenic because of its very low skin sensitization potential.⁴ Though, it easily autoxidizes once exposed to air. Autoxidation is a free radical chain reaction in which hydrogen atom abstraction with addition of oxygen forms hydroperoxides as primary oxidation products. Primary autoxidation products of linalool Lina-7-OOH and Lina-6-OOH (Chart 1) have been shown to be major skin sensitizers in the oxidation mixtures when tested in the murine local lymph node assay.⁴ Besides, high prevalence of allergic contact dermatitis (ACD) to a mixture of these hydroperoxides (herein referred as Lina-OOHs) has been reported in several dermatological multicenter studies when patch testing consecutive dermatitis patients with autoxidized linalool at the suggested optimal patch test concentration (6% in petrolatum, containing Lina-OOHs 1% w/w).⁵⁻⁹ Also, a repeated open application test study indicated that oxidized linalool can elicit ACD when applied repeatedly in lower concentrations in previously sensitized individuals, as for daily use of cosmetic products. As low a concentration as 0.3% w/w of oxidized linalool (0.056% w/w of Lina-OOHs) gave reactions in participants within the test period of three weeks.¹⁰

ACD results from an adverse reaction of the immune system to low molecular weight chemicals. It is a T-cell mediated delayed-type hypersensitivity reaction based on a sensitization and a further elicitation phase.¹¹ The OECD has approved the mechanism behind the sensitization phase as an adverse outcome pathway (AOP) built on four “key events”.¹² Following entry of the chemical into the skin, the opening key event is chemical. Skin sensitizers are unable to stimulate directly an immune

response. Immunogenicity is attained by chemical reaction with skin proteins, forming stable antigenic conjugates that will be recognized and processed for presentation to the immune system.¹³ The common process for this reaction is the formation of a covalent bond between the chemical and skin proteins *via* a two electrons mechanism. Yet, many allergens do not fit this pattern and there is a strong belief that radical mechanisms are involved in the antigen formation. This is especially the case for Lina-OOHs that have the potential to form antigens *via* radical mechanisms arising from the easy cleavage of the O-O bond when considering its weak dissociation energy (175 kJ.mol^{-1}). This process affords unstable alkoxyl ($\text{RO}^\bullet$) radicals, which can lead via intramolecular cyclization, fragmentation or hydrogen abstraction to the formation of more stable carbon centered radicals ($\text{RC}^\bullet$). Consequently, we believe that Lina-OOHs intrinsic chemical reactivity constitutes the needed trigger that provides information to the immune system, alerting defense mechanisms of the organism. A further key event of the skin sensitization AOP is the activation of dendritic cells (DCs), antigen-presenting cells that take up and process the antigenic complex, mature and migrate to the local lymph nodes ensuring the presentation of altered peptides to naïve T-lymphocytes. We have recently shown that the THP-1 cell line, employed in the h-CLAT *in vitro* test as a surrogate for DCs,¹⁴ is activated by Lina-OOHs with up regulation of cell surface markers (CD54, CD86) and activation of the Nrf2 pathway.¹⁵ We also demonstrated that $\text{RO}^\bullet$, peroxy ($\text{ROO}^\bullet$) and $\text{RC}^\bullet$ radicals were formed from iron activated Lina-OOHs by using electron paramagnetic resonance (EPR) and spin trapping (ST),¹⁶ a powerful approach toward the characterization of transient radicals in chemical and biological systems.¹⁷ Further, we studied the reactivity toward amino acids prone to radical reactions by liquid chromatography combined to mass spectrometry, and we proved that $\text{RC}^\bullet$ radicals issued from Lina-OOHs altered directly the lateral chain of cysteine and the thiol group of glutathione (GSH) forming adducts *via* radical processes after iron induced radical initiation.^{16,18} We suggested thus the involvement of $\text{RC}^\bullet$ intermediates in the biological answers related to Lina-OOHs sensitization process. However, EPR-ST studies were at that time carried out in solution (aqueous buffer or semi-organic), yet with experimental conditions being far

away from real life sensitization, and reactivity studies did not allow to conclude on the specific carbon centered radical $RC^\bullet$ reacting with thiol groups. To overcome these drawbacks, we report herein more evidences and mechanistic insights on Lina-OOHs mechanisms of action by assessing (i) the formation of carbon radicals in a reconstructed human epidermis (RHE) 3D model, much closer to what may happen *in vivo*, by EPR-ST and (ii) the molecular structure of adducts resulting from reactivity with thiol groups using ^{13}C -substituted Lina-OOHs at positions precursor of radical intermediates and ^{13}C -NMR.

MATERIALS AND METHODS

Caution: Linalool hydroperoxides must be handled with caution, as they are skin sensitizers.

EPR Spin Trapping Studies

Studies in Solution. A mixture of Lina-7-OOH ((*5E*)-7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol) and Lina-6-OOH (6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol) (Lina-OOHs, ratio 2:3) was synthesized from linalool as previously described in the literature.¹⁶ 5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was synthesized as reported in the literature.¹⁹ HEPES ($\geq 99.5\%$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and acetonitrile (CH_3CN , 99.8%) were acquired from Sigma-Aldrich (Saint-Quentin Fallavier, France). Aqueous solutions were prepared with deionized water. To prepare a HEPES buffer solution *i.e.* 10 mM at pH 6.8, 1.19 g HEPES were dissolved in 400 mL deionized water, 4 g sodium chloride and 0.1 g potassium chloride were added. To reach pH 6.8, sodium hydroxide pellets were added. If the pH went too high, it was lowered back by adding hydrochloric acid until the pH remained stable to 6.8. Finally, deionized water was added to a final volume of 500 mL. Stock solutions were prepared for Lina-OOHs (10 mM in HEPES/ CH_3CN 9/1), DEPMPO (100 mM in HEPES) and $FeSO_4 \cdot 7H_2O$ (10 mM in deionized water). 12.5 μL of DEPMPO solution were mixed with $FeSO_4 \cdot 7H_2O$ (*i.e.* 0.5 μL for a final concentration in the reaction mixture of 0.1 mM), supplemented by 5 μL Lina-OOHs solution and final volume completed to 50 μL . This way,

final concentrations in the reaction mixture were 25 mM DEPMPO and 1 mM Lina-OOHs. The reaction mixture was subjected to stirring and further transferred into a glass capillary (Hirschmann, 25 μ L) sealed on both ends prior EPR investigation. EPR spectra were recorded on a X-band spectrometer (EMXplus, Bruker Biospin GmbH, Germany) equipped with a high sensitivity resonator (HSW, Bruker Biospin GmbH, Germany). The g calibration standard was Bruker strong pitch of known g factor 2.0028. The spectrometer was operated at ca. 9.8 GHz and principal spectrometer settings were 100 kHz modulation frequency, microwave power of ca. 4.5 mW, sweep width 150 G, modulation amplitude 1 G, sweep time 120 s, conversion time ca. 130 ms and time constant ca. 40 s. A couple of spectra were accumulated to ensure a decent signal-to-noise S/N ratio. Spectra were recorded at room temperature ($295\text{K}\pm 1\text{K}$) as soon as possible after mixing the reagents.

Studies in Reconstructed Human Epidermis. EpiSkinTM (Lyon, France) is a RHE from normal human keratinocytes cultured for 13 days on a collagen matrix at the air-liquid interface. The 0.38 cm² format was chosen. Immediately after arrival in the laboratory, the RHE were removed from the agarose-nutrient solution in the shipping multiwell plate under a sterile airflow. They were placed in a plate in which each well was previously filled with 2 mL EpiSkinTM maintenance or growth medium at room temperature. Samples were placed in the incubator at 37 °C, 5% CO₂ and saturated humidity, at least 24 h before incubation. EpiSkinTM (Lyon, France) furnished the assay medium used for incubations. RHE were topically treated first with DEPMPO (20 μ L, 250 mM in HEPES) and post-incubated (37 °C, 5% CO₂) during 15 min. After the incubation time, the RHE were placed in an EPR tissue cell equipped with a silica window (Wilma, #ER162TC-Q) and Lina-OOHs (20 μ L, 10 mM in acetone) were applied to the epidermis taking care to ensure that the solution was only applied to it. EPR spectra were then recorded on a X-band spectrometer (ESP300E, Bruker Biospin GmbH, Germany) equipped with a rectangular Te₁₀₂ resonator (Bruker Biospin GmbH, Germany). The g calibration standard was performed with Bruker strong pitch. The spectrometer was operated at ca. 9.8 GHz and spectrometer settings were 100 kHz modulation frequency, microwave power of ca. 5.1 mW,

sweep width 160 G, sweep time 328 s, modulation amplitude 1 G and time constant of ca. 164 s. Spectra were recorded at room temperature ($295^{\circ}\text{K} \pm 1^{\circ}\text{K}$).

EPR Simulations. All experimental EPR spectra were analyzed by means of computer simulation using labmade scripts based on Easyspin toolbox under Matlab (MathWorks) environment.²⁰

Synthesis of (^{13}C)-Lina-OOHs

Lina-OOHs (mixture Lina-7-OOH/Lina-6-OOH) were synthesized containing a ^{13}C -substitution at carbon atom positions 5 or 4 (Chart 1). Here the synthesis of 5-(^{13}C)-Lina-OOHs is described as it afforded the most important reactivity information. The synthesis of 4-(^{13}C)-Lina-OOHs and results obtained, corroborating that information, are detailed in the Supporting Information.

Chemicals and Reagents. Anhydrous solvents were obtained by distillation under an inert atmosphere of argon and kept on molecular sieves (4 Å). Ethanol was distilled on sodium hydroxide. Tetrahydrofuran, diethyl ether and dichloromethane were dried through activated alumina columns. All reactions needing anhydrous conditions were carried out under argon atmosphere in flame-dried glassware. 1-(^{13}C)-Acetic acid was purchased from Euriso-top (Saint-Aubin, France). All other reagents and solvents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) or Fisher Scientific (Illkirch-Graffenstaden, France) and were used as received. Reactions were followed by thin layer chromatography (TLC) using silica gel plates (Merck 60 F₂₅₄; thickness 0.25 mm). After elution, the plates were revealed under short-wave UV radiation (254 nm) or immersed in a phosphomolybdic acid solution (5 g phosphomolybdic acid and 2 g cerium sulfate tetrahydrate in a mixture of 12 mL sulfuric acid and 188 mL distilled water) or a potassium permanganate solution (1.5 g potassium permanganate, 1.25 mL sodium hydroxide 10% and 10 g potassium carbonate in 200 mL water) followed by heating. Column chromatography purifications were performed using silica 60 (Merck; Geduran, 40-63 μm). ^1H and ^{13}C -NMR spectra were recorded on Bruker Avance 300 (300 MHz for ^1H and 75 MHz for ^{13}C) and Bruker Avance 500 (500 MHz for ^1H and 125 MHz for ^{13}C) spectrometers.

Chemical shifts (δ) are reported in ppm and coupling constants (J) are reported in Hz. Chemical shifts are indirectly referenced to TMS via the solvent signal CHCl_3 (CDCl_3 δ ^1H = 7.26 ppm; δ ^{13}C = 77.2 ppm). Multiplicity is denoted as s (singlet), sl (singlet large), d (doublet), dd (doublet of doublets), t (triplet), q (quadruplet) and m (multiplet).

2-Bromo-1-(^{13}C)-ethyl acetate ((^{13}C)-1). In a flame-dried double-necked flask under argon, to a solution of 1-(^{13}C)-acetic acid (2.0 g, 33.3 mmol, 1 equiv) and phosphorus tribromide (1.60 mL, 16.66 mmol, 0.5 equiv) was slowly added bromine (6.0 mL, 116.56 mmol, 3.5 equiv). The mixture was stirred during 30 min at room temperature, then heated at 60 °C for 6 h. After that time the mixture was cooled to room temperature before anhydrous ethanol (3.6 mL, 63.28 mmol, 1.9 equiv) was added. After one night at room temperature, the reaction mixture was hydrolyzed with ethanol (10 mL) and distilled water (30 mL). The mixture was extracted with diethyl ether (3 $\times$ 30 mL). Collected organic phases were neutralized using a saturated solution of sodium bicarbonate (2 $\times$ 30 mL) and washed with a saturated solution of sodium thiosulfate (30 mL, then 20 mL). Organic phases were dried over magnesium sulfate, filtered and concentrated under reduced pressure. 2-Bromo-1-(^{13}C)-ethyl acetate ((^{13}C)-1 (3.67 g) was obtained as an orange oil and was used without further purification. ^1H NMR (CDCl_3) δ 1.28 (t, 3H, $^3J_{\text{HH}}=7.5$ Hz, OCH_2CH_3), 3.81 (d, 2H, $^2J_{\text{HC}}=5.0$ Hz, CH_2), 4.22 (qd, 2H, $^3J_{\text{HH}}=7.0$ Hz, $^3J_{\text{HC}}=3.0$ Hz, OCH_2CH_3). ^{13}C NMR (CDCl_3) δ 14.1 (CH_3), 26.3 (d, $^1J_{\text{CC}}=64.6$ Hz, CH_2Br), 62.4 (OCH_2CH_3), 167.4 ($^{13}\text{C}=\text{O}$).

2-(Diethoxyphosphoryl)-1-(^{13}C)-ethyl acetate ((^{13}C)-2). In a flame-dried round bottom flask under argon triethyl phosphite (4.7 mL, 26.21 mmol, 1.2 equiv) was added to ((^{13}C)-1 (3.67 g, 21.84 mmol, 1equiv). The mixture was heated at 140 °C for 3 h and then cooled to room temperature. The product was purified by silica gel chromatography (EtOAc/pentane 8/2). 2-(Diethoxyphosphoryl)-1-(^{13}C)-ethyl acetate ((^{13}C)-2 was obtained as a yellow oil (4.95 g, 90 %). ^1H NMR (CDCl_3) δ 1.26 (t, 3H, $^3J_{\text{HH}}=7.0$ Hz, OCH_2CH_3), 1.32 (t, 6H, $^3J_{\text{HH}}=7.5$ Hz, 2 $\times$ OCH_2CH_3), 2.94 (dd, 2H, $^2J_{\text{HP}}=22.0$ Hz, $^2J_{\text{HC}}=7.5$ Hz, CH_2), 4.06-4.20 (m, 6H, 3 $\times$ OCH_2CH_3). ^{13}C NMR (CDCl_3) δ 14.1 (OCH_2CH_3), 16.4 (d,

$^3J_{\text{CP}}=6.5$ Hz, $2 \times \text{OCH}_2\text{CH}_3$), 34.5 (dd, $^1J_{\text{CC}}=59.0$ Hz, $^1J_{\text{CP}}=134.0$ Hz, CH_2), 61.6 (OCH_2CH_3), 62.7 (d, $^2J_{\text{CP}}=6.5$ Hz, $2 \times \text{OCH}_2\text{CH}_3$), 166.0 (d, $^2J_{\text{CP}}=6.1$ Hz, $^{13}\text{C}=\text{O}$).

Ethyl-1-(^{13}C)-3-methyl but-2-enoate ((^{13}C)-3). Sodium hydride (60 % in mineral oil, 0.87 g, 22.10 mmol, 1 equiv) was introduced in a flamed dried flask under argon. 2-(Diethoxyphosphoryl)-1-(^{13}C)-ethyl acetate ((^{13}C)-2 (4.95 g, 22.10 mmol, 1 equiv) dissolved in anhydrous tetrahydrofuran (50 mL) was added at 0 °C. The mixture was stirred for 15 min. Anhydrous acetone (1.57 mL, 20.1 mmol, 0.95 equiv) was added to the reaction and the mixture was stirred during 30 min at 0 °C then allowed to warm to room temperature overnight. The reaction was quenched using a saturated solution of ammonium chloride (25 mL). The aqueous phase was extracted with diethyl ether (3×80 mL), organic fractions collected, dried over magnesium sulfate, filtered then concentrated under reduced pressure. The product was purified by silica gel column chromatography (pentane/diethyl ether 97/3), to obtain ((^{13}C)-3 as a yellow oil (1.46 g, 51 %). ^1H NMR (CDCl_3) δ 1.25 (t, 3H, $^3J_{\text{HH}}=7.1$ Hz, OCH_2CH_3), 1.88 (s, 3H, CH_3 *cis*), 2.14 (s, 3H, CH_3 *trans*), 4.15 (dd, 2H, $^3J_{\text{HH}}=7.1$ Hz, $^3J_{\text{HC}}=3.1$ Hz, CH_2), 5.67 (m, 1H, $=\text{CH}$). ^{13}C NMR (CDCl_3) δ 13.9 (OCH_2CH_3), 19.9 (CH_3 *cis*), 27.7 (CH_3 *trans*), 59.2 (OCH_2CH_3), 116.0 (d, $^1J_{\text{CC}}=75.4$ Hz, $=\text{CH}$), 156.5 ($(\text{CH}_3)_2\text{C}=\text{C}$), 166.7 ($^{13}\text{C}=\text{O}$).

1-(^{13}C)-3-Methylbut-2-en-1-ol ((^{13}C)-4). A diisobutylaluminum hydride solution in dichloromethane (27.50 mL, 27.50 mmol, 2.4 equiv) was introduced into a flame-dried round bottom flask equipped with an addition funnel and cooled at -78 °C. ((^{13}C)-3 (1.46 g, 11.43 mmol, 1 equiv) dissolved in anhydrous dichloromethane (20 mL) was slowly added. The reaction mixture was allowed to warm to room temperature overnight. The reaction was stopped by adding ethyl acetate (10 mL) and water (10 mL). The solution was filtered on celite and the organic phase separated. The aqueous phase was extracted with diethyl ether (2×10 mL). Combined organic phases were dried over magnesium sulfate, filtered and concentrated under reduced pressure. 1-(^{13}C)-3-Methylbut-2-en-1-ol ((^{13}C)-4 (1.65 g) was obtained as a transparent oil and used without further purification. ^1H NMR (CDCl_3) δ 1.69 (s, 3H, CH_3 *cis*), 1.75 (s, 3H, CH_3 *trans*), 4.10 (dd, 2H, $^3J_{\text{HH}}=7.1$ Hz, $^1J_{\text{HC}}=142.1$ Hz, $^{13}\text{CH}_2$), 5.41 (m, 1H, $=\text{CH}$).

=CH). ^{13}C NMR (CDCl_3) δ 17.8 (CH_3 *cis*), 25.7 (CH_3 *trans*), 59.3 ($\text{HO}^{13}\text{CH}_2$), 123.6 (d, $^1J_{\text{CC}}=47.0$ Hz, =CH), 136.3 ($(\text{CH}_3)_2\text{C}=\text{C}$).

1-(^{13}C)-1-Bromo-3-methyl but-2-ene ((^{13}C)-5). To a solution of 1-(^{13}C)-3-methylbut-2-en-1-ol (^{13}C)-4 (1.65 g, 19.17 mmol, 1 equiv) in dichloromethane (7 mL) cooled at 0 °C was added hydrobromic acid (48%, 24.3 mL, 214.2 mmol, 11.2 equiv). The mixture, protected from daylight, was vigorously stirred for 2 h. Magnesium sulfate (6.69 g, 55.72 mmol, 2.9 equiv) and dichloromethane (30 mL) were then added and the solution was allowed to warm to room temperature. The organic phase was separated and the aqueous phase was extracted using dichloromethane (2 $\times$ 20 mL). The organic layers were assembled and dried over magnesium sulfate. The solvent was evaporated under reduced pressure. 1-(^{13}C)-1-Bromo-3-methyl but-2-ene (^{13}C)-5 (1.44 g) was obtained as brown oil and used without further purification. ^1H NMR (CDCl_3) δ 1.73 (s, 3H, CH_3 *cis*), 1.78 (s, 3H, CH_3 *trans*), 4.15 (dd, 2H, $^3J_{\text{HH}}=8.5$ Hz, $^1J_{\text{HC}}=153.0$ Hz, CH_2), 5.48-5.56 (m, 1H, =CH). ^{13}C NMR (CDCl_3) δ 17.5 (CH_3 *cis*), 25.8 (CH_3 *trans*), 29.7 ($^{13}\text{CH}_2$), 121.6 (d, $^1J_{\text{CC}}=47.0$ Hz, =CH), 137.9 ($(\text{CH}_3)_2\text{C}=\text{C}$).

Ethyl-3-(^{13}C)-2-acetyl-5-methylhex-4-enoate ((^{13}C)-6). In a flame-dried round bottom flask, sodium ethoxide (4.34 mL, 11.62 mmol, 1.2 equiv) was added to a solution of ethyl acetoacetate (2.47 mL, 19.37 mmol, 2 equiv) in anhydrous ethanol (20 mL). The mixture was cooled to 0 °C and a solution of (^{13}C)-5 (1.44 g, 9.6 mmol, 1 equiv) in ethanol (10 mL) was added to the mixture. After stirring 1 h at 0 °C, the solution was refluxed for 4 h. The solvent was evaporated under reduced pressure, then water (5 mL) was added and the aqueous phase was extracted with ethyl acetate (3 $\times$ 15 mL). The combined organic phases were dried over magnesium sulfate and evaporated under reduced pressure. The crude product was purified using silica gel chromatography (pentane/diethyl ether 9/1) and ethyl-3-(^{13}C)-2-acetyl-5-methylhex-4-enoate (^{13}C)-6 was obtained as a colorless oil (1.289 g, 67%). ^1H NMR (CDCl_3) δ 1.25 (t, 3H, $^3J_{\text{HH}}=7.1$ Hz, OCH_2CH_3), 1.63 (s, 3H, CH_3 *cis*), 1.67 (s, 3H, CH_3 *trans*), 2.22 (s, 3H, COCH_3), 2.53 (m, 2H, $^{13}\text{CH}_2$), 3.40 (m, 1H, CH), 4.19 (q, 2H, $^3J_{\text{HH}}=7.1$ Hz, OCH_2CH_3), 4.98-5.06 (m, 1H, =CH). ^{13}C NMR (CDCl_3) δ 14.0 (OCH_2CH_3), 17.6 (CH_3 *cis*), 25.8 (CH_3 *trans*), 29.7 ($^{13}\text{CH}_2$), 121.6 (d, $^1J_{\text{CC}}=47.0$ Hz, =CH), 137.9 ($(\text{CH}_3)_2\text{C}=\text{C}$).

trans), 27.1 ($^{13}\text{CH}_2$), 59.8 (d, $^1J_{\text{CC}}=34.2$ Hz, CH), 60.4 (COCH_3), 61.4 (OCH_2CH_3), 120.1 (d, $^1J_{\text{CC}}=44.1$ Hz, $=\text{CH}$), 134.9 ($((\text{CH}_3)_2\text{C}=\text{C})$), 169.6 ($((\text{O})\text{C}=\text{O})$), 203.0 ($\text{C}=\text{O}$).

4-(^{13}C)-6-Methylhept-5-en-2-one (^{13}C)-7. An aqueous solution of sodium hydroxide (10%, 18 mL) was added to a solution of (^{13}C)-6 (1.29 g, 6.50 mmol, 1 equiv) in ethanol (20 mL) at 0 °C. The mixture was refluxed during 5 h before allowing warming to room temperature. Then it was acidified using a solution of hydrochloric acid 10% down to pH 4-5. The aqueous phase was extracted with diethyl ether (3 × 50 mL). The combined organic phases were dried over magnesium sulfate and evaporated under reduced pressure. 4-(^{13}C)-6-Methylhept-5-en-2-one (^{13}C)-7 (0.26 g) was obtained as colorless oil and was used without further purification. ^1H NMR (CDCl_3) δ 1.61 (s, 3H, CH_3 *cis*), 1.67 (s, 3H, CH_3 *trans*), 2.13 (s, 3H, COCH_3), 2.36 (m, 2H, CH_2CO), 2.53 (m, 2H, $^{13}\text{CH}_2\text{CH}$), 5.01-5.08 (m, 1H, $=\text{CH}$). ^{13}C NMR (CDCl_3) δ 17.6 (CH_3 *cis*), 22.5 ($^{13}\text{CH}_2\text{CH}$), 25.7 (CH_3 *trans*), 29.9 (COCH_3), 44.3 (d, $^1J_{\text{CC}}=34.5$ Hz, CH_2CO), 122.6 (d, $^1J_{\text{CC}}=44.2$ Hz, $=\text{CH}$), 132.7 ($((\text{CH}_3)_2\text{C}=\text{C})$), 208.9 ($\text{C}=\text{O}$).

5-(^{13}C)-3,7-Dimethylocta-1,6-dien-3-ol; 5-(^{13}C)-linalool (^{13}C)-8. In a flame-dried round bottom flask, vinyl magnesium bromide (4.12 mL, 4.12 mmol, 2 equiv) was added to a solution of 4-(^{13}C)-6-methylhept-5-en-2-one (^{13}C)-7 (0.26 g, 2.06 mmol, 1 equiv) in anhydrous tetrahydrofuran (10 mL) previously cooled to 0 °C. After stirring 1 h at 0 °C, the reaction was stopped using a saturated solution of ammonium chloride (10 mL). The aqueous phase was extracted with diethyl ether (3 × 30 mL). The combined organic phases were dried over magnesium sulfate and evaporated under reduced pressure. The crude product was purified by silica gel column chromatography (pentane/diethyl ether 8/2) and 5-(^{13}C)-linalool (^{13}C)-8 was obtained as a yellow oil (0.198 g, 63 %). ^1H NMR (CDCl_3) δ 1.27 (s, 3H, CH_3), 1.52-1.57 (m, 2H, $=\text{CHCH}_2\text{CH}_2$), 1.60 (s, 3H, CH_3 *cis*), 1.67 (s, 3H, CH_3 *trans*), 1.92-2.09 (m, 2H, $=\text{CH}^{13}\text{CH}_2\text{CH}_2$), 5.05 (dd, 1H, $^3J_{\text{HH}}=1.1$ Hz, $^2J_{\text{HH}}=10.8$ Hz, $\text{CH}=\text{CH}_2$ *trans*), 5.11 (m, 1H, $=\text{CH}$), 5.20 (dd, 1H, $^3J_{\text{HH}}=1.1$ Hz, $^2J_{\text{HH}}=17.3$ Hz, $\text{CH}=\text{CH}_2$ *cis*), 5.90 (dd, 1H, $^3J_{\text{HH}}=10.8$ Hz, $^2J_{\text{HC}}=17.3$ Hz, $\text{CH}^{13}\text{CH}_2$). ^{13}C NMR (CDCl_3) δ 17.7 (CH_3 *cis*), 22.8 ($^{13}\text{CH}_2\text{CH}$), 25.7 (CH_3 *trans*), 27.9 (CH_3), 42.0 (d, $^1J_{\text{CC}}=34.4$ Hz, CH_2COH), 73.5 (COH), 111.7 ($=\text{CH}_2$), 124.3 (d, $^1J_{\text{CC}}=43.6$ Hz, $=\text{CH}$), 131.9

$((\text{CH}_3)_2\text{C}=\text{CH})$, 145.0 ($\text{CH}_2=\text{CH}$).

5-(^{13}C)-(5*E*)-7-Hydroperoxy-3,7-dimethylocta-1,5-dien-3-ol (^{13}C)-9 and **5-(^{13}C)-6-hydroperoxy-3,7-dimethylocta-1,7-dien-3-ol (^{13}C)-10**. A microemulsion was prepared by slowly adding a sodium molybdate solution (0.239 g in 1.27 mL distilled water) to a suspension of sodium dodecyl sulfate (1.975 g) in butanol (2.41 mL) and dichloromethane (11.76 mL). At the end of the addition, the microemulsion became clear after 5 min stirring. 5-(^{13}C)-Linalool (^{13}C)-8 (0.198 g, 1.26 mmol, 1 equiv) was introduced in the microemulsion at room temperature. Hydrogen peroxide (aqueous solution 35%, 71 μL one fraction) was then added. The solution was instantly red colored. After 20 min stirring the solution became yellow. Fourteen other fractions of hydrogen peroxide (aqueous solution 35%, 71 μL each) were added successively every 10 min. The resulting yellow solution was stirred overnight at room temperature. When the reaction was thoroughly translucent, 5-(^{13}C)-linalool (^{13}C)-8 was completely oxidized. The solvent was removed under reduced pressure at 40 $^{\circ}\text{C}$. The semi-solid white product obtained was suspended in dichloromethane (65 mL) and vigorously stirred for 48 h. The suspension was filtered to recover sodium molybdate and sodium dodecyl sulfate. The filtrate, partially evaporated under reduced pressure, was washed with distilled water (3×50 mL) and the aqueous phase was extracted with dichloromethane (50 mL). Combined organic phases were dried over magnesium sulfate, filtered and concentrated under reduced pressure to give a yellow oil. A mixture of hydroperoxides (^{13}C)-9 and (^{13}C)-10 (ratio 2:3) was obtained using silica gel column chromatography (petroleum ether/EtOAc 8/2, then 6/4) as a yellow oil (0.238 g, 1.25 mmol, 99%). ^1H NMR (CDCl_3): Isomer (^{13}C)-9 (mixture of two enantiomers) δ 1.27 (s, 3H, $\text{CH}_3\text{C}(\text{OH})$), 1.31 (s, 6H, $(\text{CH}_3)_2\text{C}(\text{OOH})$), 2.28 (m, 2H, $\text{CH}_2\text{C}(\text{OH})$), 5.05 (dd, 1H, $^3J_{\text{HH}}=1.1$ Hz, $^2J_{\text{HH}}=10.8$ Hz, $\text{CH}_2(\text{CH})\text{C}(\text{OH})$), 5.21 (dd, 1H, $^3J_{\text{HH}}=1.1$ Hz, $^2J_{\text{HH}}=17.4$ Hz, $\text{CH}_2(\text{CH})\text{C}(\text{OH})$), 5.50-5.64 (m, 2H, $^{13}\text{CHCH}$), 5.92 (dd, 1H, $^3J_{\text{HH}}=10.7$ Hz, $^2J_{\text{HH}}=17.3$ Hz, $\text{CHC}(\text{OH})$); Isomer (^{13}C)-10 (mixture of four diastereoisomers) δ 1.29 (s, 6H, $2 \times \text{CH}_3\text{C}(\text{OH})$), 1.51-1.66 (m, 4H, $2 \times ^{13}\text{CH}_2\text{CH}_2$), 1.73 (s, 6H, $2 \times \text{CH}_3(\text{C})\text{CH}(\text{OOH})$), 4.29 (m, 2H, $2 \times \text{CH}(\text{OOH})$), 5.00 (m, 4H, $2 \times \text{CH}_2(\text{C})\text{CH}(\text{OOH})$), 5.07 (dd, 2H,

$^3J_{\text{HH}}=1.1$ Hz, $^2J_{\text{HH}}=10.8$ Hz, $2 \times \text{CH}_2(\text{CH})\text{C}(\text{OH})$), 5.17 (dd, 1H, $^3J_{\text{HH}}=1.1$ Hz, $^2J_{\text{HH}}=17.4$ Hz, $\text{CH}_2(\text{CH})\text{C}(\text{OH})$), 5.20 (dd, 1H, $^3J_{\text{HH}}=1.6$ Hz, $^3J_{\text{HH}}=17.4$ Hz, $\text{CH}_2(\text{CH})\text{C}(\text{OH})$), 5.86 (dd, 1H, $^3J_{\text{HH}}=10.8$ Hz, $^2J_{\text{HH}}=17.4$ Hz, $\text{CHC}(\text{OH})$), 5.88 (dd, 1H, $^3J_{\text{HH}}=10.8$ Hz, $^2J_{\text{HH}}=17.4$ Hz, $\text{CHC}(\text{OH})$). ^{13}C NMR (CDCl_3): Isomer (^{13}C)-**9** (mixture of two enantiomers) δ 24.4 ($2 \times (\text{CH}_3)_2\text{C}(\text{OOH})$), 27.8 ($\text{CH}_3\text{C}(\text{OH})$), 45.1 (d, $^1J_{\text{CC}}=43.5$ Hz, $\text{CH}_2\text{C}(\text{OH})$), 72.8 ($\text{C}(\text{OH})$), 82.1 ($\text{C}(\text{OOH})$), 112.3 ($\text{CH}_2(\text{CH})\text{C}(\text{OH})$), 126.7 ($\text{CH}_2(^{13}\text{CH})\text{CH}$), 138.0 (d, $^1J_{\text{CC}}=70.9$ Hz, $\text{CHC}(\text{OOH})$), 144.8 ($\text{CHC}(\text{OH})$); Isomer (^{13}C)-**10** (mixture of four diastereoisomers) δ 17.1 ($2 \times \text{CH}_3(\text{C})\text{CH}(\text{OOH})$), 25.1 (d, $^1J_{\text{CC}}=3.5$ Hz, $2 \times ^{13}\text{CH}_2(\text{CH})\text{OOH}$), 28.2 and 28.4 ($2 \times \text{CH}_3\text{C}(\text{OH})$), 37.7 and 37.8 (d, $^1J_{\text{CC}}=23.8$ Hz, $2 \times \text{CH}_2\text{C}(\text{OH})$), 73.1 ($2 \times \text{C}(\text{OH})$), 89.2 and 89.7 (d, $^1J_{\text{CC}}=20.5$ Hz, $2 \times \text{CH}(\text{OOH})$), 112.3 ($2 \times \text{CH}_2(\text{CH})\text{C}(\text{OH})$), 114.2 and 114.3 ($2 \times \text{CH}_2(\text{C})\text{CH}(\text{OOH})$), 143.6 ($2 \times \text{C}(\text{CH})\text{OOH}$), 144.7 and 144.8 ($2 \times \text{CHC}(\text{OH})$).

^{13}C -NMR reactivity studies

5-(^{13}C)-Lina-OOHs (^{13}C)-**9** and (^{13}C)-**10** (ratio 2:3) (1 mg, 5.37 μmoles , 1 equiv) dissolved in 300 μL of deuterated acetonitrile were incubated with *N*-acetyl-L-cysteine-methyl ester (1.94 mg, 10.74 μmoles , 2 equiv) dissolved in 300 μL of deionized water, and in the presence of a catalytic amount of iron sulfate heptahydrate (0.5 μmoles , 0.1 equiv). The whole mixture was introduced into an NMR tube to follow the reaction by ^{13}C -NMR. The reactions were followed by mono-dimensional ^{13}C -NMR on a Bruker Avance 500 spectrometer at 125 MHz. Chemical shifts (δ) are reported in ppm in comparison to tetramethylsilane, using the residual signal of acetonitrile ($\delta ^1\text{H} = 21.94$ ppm; $\delta ^{13}\text{C} = 118.26$ ppm) as internal standard. The potential structure of the products formed during the reactions was assigned by heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments. Chemical shifts were compared with those calculated using ACD/CNMR and ACD/HNMR Predictor software. Same methodology was employed in the case of 4-(^{13}C)-Lina-OOHs (Supporting Information).

RESULTS

EPR Spin Trapping Studies. EPR-ST is a perfect tool for the identification of transient radicals in chemical and biological systems. Radical intermediates that are too short-lived or of too low concentration to be directly detected react with a spin-trap to form persistent radicals (spin-adducts) observed by EPR with a signature depending on the trapped transient reactive species.¹⁷ To study Lina-OOHs (mixture Lina-7-OOH/Lina-6-OOH, ratio 2:3), we employed our EPR-ST method that allows investigating *in situ* the formation of free radicals derived from xenobiotics in the EpiSkinTM reconstructed human epidermis (RHE) model.²¹ EpiSkinTM RHE are normal multi-layered keratinocytes cultures, the major cell type in the epidermis playing a key role in skin inflammatory reactions. Initially, the aim was to establish in solution the optimal experimental conditions providing a good S/N ratio while keeping Lina-OOHs concentrations as low as possible, consistent with real life exposure conditions. DEPMPO was the spin-trap chosen based on (i) our previous studies,²¹ (ii) the aptitude to distinguish oxygen and carbon centered radicals,²² and (iii) the high persistency of the superoxide and alkyl-peroxyl radicals spin-adducts.²³ Radical initiation was triggered by the known catalytic Fe(II)-induced reductive cleavage of the hydroperoxide chemical function in a Fenton-like reaction.²⁴ HEPES was preferred to phosphate buffer (PB) to avoid the formation of insoluble Fe(III) through Fe(II) oxidation catalyzed by PB, decreasing the Fe(II) content available.^{21,24} Experimental EPR spectrum in Figure 1 was obtained with DEPMPO 25 mM and Lina-OOHs 1 mM, supplemented by a Fe(II) catalytic amount (0.1 mM) to trigger radical initiation. The spectrum showed the spin-adduct corresponding to hydroxyl radicals (DEPMPO-OH spin-adduct), together with species testimony of carbon centered radicals (DEPMPO-R1 and DEPMPO-R2 spin-adducts) and potential alkoxyl radicals (DEPMPO-OR spin-adduct), all of them with characteristic hyperfine coupling constant (*hfccs*) values (Table 1).^{23,25,26}

Experiments were further carried out in RHE based on a topical application procedure to get closer to real life sensitization. RHE samples were initially loaded with DEPMPO in HEPES (15 min, 37 °C, 5% CO₂) to ensure that the spin-trap is present at the site of radical generation before application of Lina-OOHs.²⁷ When pre-treated RHE were then exposed topically to different concentrations of Lina-OOHs in acetone, DEPMPO spin-adducts were observed from a 10 mM concentration (Figure 2). As for previous studies, concentrations of spin-trap and target compounds needed in the case of EPR-ST studies in RHE were ten-fold higher (250 mM and 10 mM, respectively) than those giving a good S/N ratio signal in solution.²¹ This is probably due to the fact that RHE are complex 3D structures including a *stratum corneum* and therefore skin penetration concerns need to be considered, together with the content in phospholipids and ceramides that may influence RHE barrier properties.²⁸ As for the investigations in solution, the fingerprint of a carbon centered radical DEPMPO spin-adduct (DEPMPO-R, Figure 2) was detected. Moreover, both hydroxyl radical (DEPMPO-OH spin-adduct) and alkoxyl radical (DEPMPO-OR spin-adduct) were also detected in the RHE when put in presence of Lina-OOHs. Noteworthy, spin-adducts for RHE were assigned despite of low S/N as being based on the direct comparison with corresponding investigations in solution (Table 1). This is especially the case for the latter one (DEPMPO-OR) that is barely visible in the RHE. Even though we are here at the detection limit, shallow shoulders are noticeable within the experimental spectrum that are compatible with *hfccs* of DEPMPO-OR spin-adduct. Besides, the incubation of the sole spin-trap in RHE gave no signal, indicating that observed radicals intermediates originate rather from Lina-OOHs than from RHE biomolecules. On another hand, RHE incubation with single Lina-OOHs gave also no signal. Lina-OOHs generate radical intermediates thus pointing out the necessity to use the EPR-ST methodology for these studies. It is important to stress here that no Fe(II) was added in RHE experiments, indicating that Lina-OOHs radical initiation has been induced by the RHE itself.

Synthesis of 5-(¹³C)-Lina-OOHs. In our laboratory, we have developed NMR techniques in association with ¹³C-substituted molecules to probe hapten-protein interaction mechanisms in buffered

or semi-organic solutions and in RHE.²⁹ Herein, to study the reactivity toward thiol groups, Lina-OOHs were synthesized ¹³C-substituted at carbon positions 4 or 5 in order to follow the reactivity toward thiol groups by ¹³C-NMR. Indeed, we have showed previously that Lina-7-OOH decomposes in the presence of ferrous salts affording carbon radicals that could potentially be centered at these positions (Scheme 1).¹⁶

The synthetic route developed for the synthesis of 5-(¹³C)-Lina-OOHs is illustrated in Scheme 2 (synthesis of 4-(¹³C)-Lina-OOHs with a ¹³C-substitution at position 4 is detailed in the Supporting Information). Initially, (¹³C)-**1** was synthesized from 1-(¹³C)-acetic acid in a two-step's reaction. 1-(¹³C)-Acetic acid (source to introduce the ¹³C-substitution) was submitted to a Hell-Vollhardt-Zellinsky reaction allowing bromination of the α-carbon to the carbonyl chemical function in presence of bromine and phosphorous tribromide, followed by esterification using ethanol. A nucleophilic substitution of bromine by triethyl phosphite afforded 2-(diethoxyphosphoryl)-1-(¹³C)- ethylacetate (¹³C)-**2** through a Michaelis-Arbuzov reaction. Based on the literature, the stabilized phosphonate carbanion of (¹³C)-**2** was then obtained by treatment with a strong base and a Horner-Wadsworth-Emmons reaction with acetone afforded (¹³C)-**3**.³⁰ Further reduction to allylic alcohol (¹³C)-**4** was carried out by using diisobutyl aluminum hydride. The alcohol functionality of (¹³C)-**4** was then converted into a good bromine-leaving group leading to (¹³C)-**5**, which reacted with ethyl acetoacetate deprotonated at the α position of the carbonyl group to afford (¹³C)-**6**. In order to obtain the key ketone intermediate (¹³C)-**7**, β-keto ester (¹³C)-**6** was first saponified and the carboxylic acid function obtained decarboxylated in presence of hydrochloric acid. Linalool ¹³C-substituted at position 5 (¹³C)-**8** was then synthesized by treating the ketone with vinyl magnesium bromide. Finally, (¹³C)-**8** was subjected to the ene or “Schenck reaction” allowing the preparation of allylic hydroperoxides from alkenes containing allylic hydrogens via singlet oxygen (¹O₂) reaction as we described previously.¹⁶ ¹O₂ was quantitatively generated by disproportionation of hydrogen peroxide induced catalytically by sodium molybdate in

alkaline aqueous solutions.³¹ A microemulsion consisting of water, dichloromethane (organic phase), sodium dodecyl sulfate (surfactant), and butanol (cosurfactant) was used as reaction medium to avoid epoxidizing competitive reactions.³² In a 99% yield reaction, a mixture of (¹³C)-**9** and (¹³C)-**10** was obtained with a 2:3 ratio measured by NMR. Due to the difficulty of isolating these two position isomers, the mixture (¹³C)-**9**/¹³C)-**10** was subsequently used for thiol reactivity studies followed by ¹³C-NMR. We considered that the use of the mixture was not a limitation as (¹³C)-**9** contains the ¹³C-substitution on a vinyl carbon atom and (¹³C)-**10** on a methylene group, both giving distinct easily recognizable ¹³C-NMR chemical shifts. Moreover, it is actually the mixture that is used in clinical patch testing studies and repeated open application tests with Lina-OOHs.

¹³C-NMR reactivity studies. The reaction of (¹³C)-**9**/¹³C)-**10** with *N*-acetyl-L-cysteine methyl ester (*N*-Ac-Cys-OMe) in CD₃CN/H₂O 1/1 was followed by mono-dimensional ¹³C-NMR at room temperature during one week (Figure 3). A catalytic amount of iron (II) sulfate heptahydrate (0.1 mM) was sufficient to induce radical initiation as for the EPR studies in solution. Control experiments were realized without the presence of iron (II) as initiator.

As shown in Figure 3, ¹³C peaks of 5-(¹³C)-Lina-7-OOH (¹³C)-**9** at 125.7 ppm and of 5-(¹³C)-Lina-6-OOH (¹³C)-**10** at 24.3 ppm disappeared with time and new peaks appeared at 122.1 ppm and 28.8 ppm. Prediction of ¹³C-NMR chemical shifts by computer simulation suggested that ¹³C-peak at 122.1 ppm could correspond to compounds (¹³C)-**11** or (¹³C)-**12**, derived from (¹³C)-**9**, and ¹³C-peak at 28.8 ppm to compound (¹³C)-**13**, derived from (¹³C)-**10** (Figure 4). Compounds (¹³C)-**11** and (¹³C)-**12** have theoretically similar chemical shifts for several parts of the terpene unit. We observed by HSQC bi-dimensional NMR a direct correlation between the ¹³C-peak at 122.1 ppm (position d) and a hydrogen atom of a vinyl group at 5.54 ppm. Also, HMBC bi-dimensional NMR showed long-distance correlations with protons at 1.18 ppm (methyl group, position b in Figure 4), 5.60 ppm (hydrogen atom of a vinyl group, position a) and 5.70 ppm (hydrogen atom of a vinyl group, position e). These data indicated that both vinyl groups of (¹³C)-**9** had been preserved. More interestingly, we could suggest

that a cysteine unit was linked as there was also a long-distance correlation with a proton at 2.16 ppm, this chemical shift being characteristic of a >CH- group having a thiol substituent (position c). However, many of the chemical shifts described above could also match with a structure such as (¹³C)-**12**, that could derive from β-scission of the initially formed alkoxyl radical RO[•]. Consequently, the cohabitation of both compounds cannot be neglected, as we are not able to assign one chemical structure unambiguously upon the ¹³C-NMR data. On another hand, ¹³C-peak at 28.8 ppm (position β in (¹³C)-**13**) was correlated directly to protons at 1.45 ppm, this being characteristic of a methylene -CH₂ carbon. Also, the HMBC bi-dimensional NMR spectrum showed long-distance correlations with protons at 1.19 ppm (methyl group, position δ), 1.35 ppm (methylene -CH₂, position γ) and, more precisely, with a -CH proton at 3.93 ppm. This chemical shift was distinctive of the secondary alcohol methyl allyl function (position α) of compound (¹³C)-**13**, itself resulting from radical degradation of (¹³C)-**10**. When using a large excess of *N*-Ac-Cys-OMe (10 equiv) the results were identical and the reaction was much faster (a few hours). However, the intensity of carbon peaks corresponding to *N*-Ac-Cys-OMe was much more important and prevented a good interpretation of NMR mono- and bi-dimensional spectra. Complementary studies were carried out with 4-(¹³C)-Lina-OOHs, having a tendency to corroborate results above concerning the hypothetical formation of compounds (¹³C)-**11** and (¹³C)-**12** *via* radical degradation processes (see Supporting Information).

DISCUSSION

Chemical and molecular understanding on the mode of action of skin sensitizers is necessary to improve risk assessment procedures and thus help to protect producers and consumers. Linalool (CAS: 78-70-6; EC: 201-134-4) is listed in the European inventory as a deodorant and perfuming agent, playing an indispensable role in fragrances and cosmetics,³³ sharing the spotlight with limonene. These terpenes readily oxidize on exposure to air and the resulting hydroperoxides have shown to have high

frequencies of positive patch tests reactions in several European and international studies.^{7,8} Although a great step forward has been made in understanding their mechanism of action involving the intervention of radical mechanisms, essential informations are still lacking to unravel the link between these allergens and the still unknown chemical “radical structural alerts” at the basis of their sensitizing potential.

Our previous studies in solution on Lina-7-OOH demonstrated the formation of oxygen and carbon centered radicals derived from the hydroperoxide, by using the spin trapping technique combined to EPR spectroscopy and Fe(II)/Fe(III) radical initiation.¹⁶ Here, we have evidenced that these radical intermediates are well and truly formed in a RHE model, histologically similar to human epidermis. EpiSkinTM RHE (Lyon, France) is a very good replicate of human epidermis architecture, and is used for irritation and penetration tests of cosmetic and chemical compounds replacing *in vivo* testing.³⁴ As a linalool oxidation mixture containing both Lina-7-OOH and Lina-6-OOH has been used for patch testing in multicenter studies in European dermatological clinics (6.0% oxidized linalool in petrolatum, 1% w/w of Lina-OOHs content),^{5,7,8} our studies were conducted with a Lina-7-OOH/Lina-6-OOH (2:3 ratio) mixture that we synthesized from linalool following a procedure identical to that described for the obtention of (¹³C)-**9** and (¹³C)-**10** from (¹³C)-**8**. EPR technical adjustments needed firstly to be established to obtain EPR fingerprints with reasonable S/N ratio. Preliminary studies were carried out in solution to get a first accurate picture of radical intermediates issued from Lina-7-OOH/Lina-6-OOH. Further, the optimized method in RHE was based on a topical application procedure to get closer to real life sensitization.³⁵ The fingerprint of a carbon centered radical DEPMPO spin-adduct, formed by addition of transient short-lived carbon radicals to the α -carbon of the nitronyl group of the spin-trap (see Supporting Information), was identified. The experimental data was in good agreement with simulations using the calculated parameters $a_N=14.8$ G, $a_H=21.2$ G and $a_P=48.2$ G, such fingerprint being testimony of trapping carbon radicals.²¹ The HO[•] radical, highly reactive and with a very short life in all kinds of media, was also detected by spin trapping with DEPMPO in treated RHE

with $hfccs$ $a_N=13.8$ G, $a_H=13.7$ G and $a_P=47.1$ G. However, one must be careful when interpreting EPR experiments in aqueous media, and keep in mind that detection of the DEPMPO-OH nitroxide does not necessarily mean that $HO^\bullet$ has been trapped, as non-spin trapping reactions involving nucleophilic addition of water can lead to similar EPR spectrum. For example, the Forrester-Hepburn reaction, consisting in a nucleophilic addition on the spin-trap followed by oxidation of the so formed hydroxylamine (e.g. by dioxygen present in the medium),³⁶ must be always considered in aqueous media, especially in the presence of traces of metal ions which can by complexation activate the nitron function making it more sensitive to the addition of water.³⁷ We performed several control experiments by incubating exclusively the spin-trap (also in the solution experiments with Fe(II)) and any spin-adducts were never observed, suggesting that maybe we could rule out this competition reaction with water in our studies. Finally, when considering the $hfccs$ values obtained in RHE and those obtained in solution (Table 1) variances are noticeable. This could be explained by the very different environment of the paramagnetic probe in mere solution when compared to that of a complex heterogeneous tissue such as RHE. Indeed, the magnitude of the $hfccs$, testimony of the delocalization extent of the nitroxide unpaired electron, is known to be sensitive to its close surrounding especially regarding the local polarity: the spin density on the ^{14}N (i.e. the corresponding $hfcc$) increases with the polarity of the solvent.^{38,39}

It is worth to compare the amount of Lina-OOHs topically applied on the RHE in our study with the amounts of Lina-OOHs used in clinical ACD diagnostic tests. An investigation on the optimal patch test concentration for detection of contact allergy to oxidized linalool suggested the mostly nowadays used concentration of 6% in petrolatum, containing 1% w/w of Lina-OOHs.⁴⁰ A dose per unit area of approximately 2.4 mg/cm^2 was considered when 20 mg oxidized linalool was tested in small Finn Chambers[®] (diameter 8 mm, inner area of 0.5 cm^2), which means $400 \text{ }\mu\text{g/cm}^2$ of Lina-OOHs. Further, to identify threshold concentrations for elicitation of ACD caused by oxidized linalool in allergic individuals with repeated exposures, repeated open application tests were performed on 6 patients

simulating daily exposure over a period of three weeks on their forearms (circular area 3.6 cm diameter).¹⁰ Two types of model product containing oxidized linalool were tested in parallel, solutions in ethanol to mimic a 'fine fragrance', and cream base formulations with 15% glyceryl stearate in water to mimic a scented cosmetic 'cream'. 5/6 participants reacted to the 'cream' containing 3% oxidized linalool, thus 0.56% w/w of Lina-OOHs. The correspondent calculated dose per unit area was of 273 $\mu\text{g}/\text{cm}^2$ for oxidized linalool, thus approximately 50 $\mu\text{g}/\text{cm}^2$ for Lina-OOHs. With preparations containing 0.3% oxidized linalool, i.e. 0.056% w/w of Lina-OOHs, 2/6 participants reacted to the 'cream' and 1/6 to the 'fine fragrance'. The correspondent calculated dose per unit area was of 27 $\mu\text{g}/\text{cm}^2$ for oxidized linalool, thus about 5 $\mu\text{g}/\text{cm}^2$ for Lina-OOHs. In our studies, 20 μL Lina-OOHs (10 mM in acetone) were topically applied to 0.38 cm^2 RHE, meaning a dose per unit area of approximately 100 $\mu\text{g}/\text{cm}^2$. This is four times less than the dose per unit area used in patch test clinical studies. It is necessary to mention here that the optimal patch test concentration established was considerably high to get the maximum number of answers and thus avoid missing false positives. Indeed, concentrations used in patch testing are often much higher than concentrations found in commercial products. However, with repeated exposure to a hapten in everyday products, low concentrations may be sufficient to cause or worsen eczema in allergic individuals. 100 $\mu\text{g}/\text{cm}^2$ is approximately two times more than the dose per unit area used for the more concentrated solution in the repeated open application tests and 20 times more than the dose per unit area used for the less concentrated one. This would mean that in our studies we are approaching the elicitation dose per unit area when exposing patients to a repeated exposure, and that the EPR-ST methodology in RHE has sensitivity in good agreement with what could happen *in vivo*. Though, further experiments will be carried out to improve the sensitivity of the methodology in order to highlight the formation of radicals with lower doses, which have clinically proven elicitation of allergy to Lina-OOHs, and this under repeated exposure.

It is important to note that after penetrating the RHE, Lina-OOHs radical initiation should have been induced by RHE itself as no extra Fe(II) was added in RHE experiments. The reaction of organic hydroperoxides in the skin in the presence of one-electron donor agents (e.g. amino and thiol groups present in amino acids, metal complexes, enzymes) may start the electron transfer processes under radical oxidation conditions and further skin proteins haptentation.⁴¹ In this sense, we have conducted some very preliminary studies of reactivity with the system Lina-OOHs/*N*-Ac-Cys-OMe/DEPMPO in a Fe(II) free solution. Spin-adducts EPR fingerprints pointing to HO[•], carbon centered or alkoxyl based radicals were detected (see Supporting Information) indicating at a first sight that cysteine induces radical generation from the target compounds.

Knowing that carbon centered radicals can be formed in the epidermis, our thereafter objective was to identify which of these radicals are able to react with amino acids and form potential antigenic entities. Reactivity of (¹³C)-Lina-OOHs towards cysteine, probably the most labile residue involved in radical reactions triggered by peroxides, was studied. We had reported previously the reactivity of Lina-7-OOH towards *N*-Ac-Cys-OMe and GSH triggered by Fe(II) and followed by liquid chromatography combined to mass spectrometry.¹⁸ We validated that carbon radicals issued from Lina-7-OOH are able to directly alter thiol chemical groups, forming adducts *via* radical processes, and we suggested chemical structures for some of the adducts observed (Figure 5). Regardless of the experimental conditions used we observed prevailing oxidation of *N*-Ac-Cys-OMe to form a cysteine dimer together with suggested adducts **14** and **15**. We proposed that **14** and **15** could result from reaction of the thiol group with carbon radicals derived from allylic hydrogen abstraction by initially formed Lina-7-O[•] (RO[•]), possible at a single position for Lina-7-OOH, and from a β-scission process, respectively. The same kind of chemistry was proposed with GSH (adduct **16**) together with a nucleophilic addition of GSH on the α,β-unsaturated ketone derived from β-scission of Lina-7-O[•] (adduct **17**). Findings reported herein, where reactivity towards *N*-Ac-Cys-OMe has been followed by

^{13}C -NMR using ^{13}C -substituted Lina-OOHs, are in agreement with these previous results in the case of Lina-7-OOH ((^{13}C)-**9** in the mixture studied). ^{13}C -NMR data, and more precisely direct and long distance ^1H - ^{13}C correlations, were in line with compounds (^{13}C)-**11** and/or (^{13}C)-**12**. The possibility to form adduct (^{13}C)-**11** is a very good evidence that allylic hydrogen abstraction is a major radical pathway to form carbon radicals from Lina-7-OOH. A suggestion for a mechanistic pathway explaining the formation of (^{13}C)-**11** is given in Scheme 3, based on a probable concerted mechanism. Initially, 5- (^{13}C)-Lina-7-OOH (^{13}C)-**9** is able to oxidize Fe(II) to Fe(III) generating $\text{RO}^\bullet$ radicals through a Fenton-like reaction, and the thiol group of *N*-Ac-Cys-OMe can then be oxidized by reduction of Fe(III) to Fe(II). Then, *N*-Ac-Cys-OMe dimer can be formed. Concomitantly, $\text{RO}^\bullet$ radicals may abstract an allylic hydrogen to form carbon centered radicals at position 4 that afterwards attack on the cysteine dimer to form adduct (^{13}C)-**11** and release a thiyl radical. On another hand, hypothetical compound (^{13}C)-**12** confirms that β -scission processes could occur in parallel and must not be neglected, though addition of the thiol moiety as in **15** and **17** was not observed here. No reactivity with the thiol group was observed in the case of Lina-6-OOH ((^{13}C)-**10** in the mixture studied). Only radical decomposition to form an allylic alcohol could be detected. This would probably mean that Lina-7-OOH is much more reactive than Lina-6-OOH towards cysteine and thiol containing peptides. This could be essential information pointing the radical at carbon atom 4 of Lina-7-OOH as a potential “radical structural alert” that could give an explanation to its sensitizing potential. Reactivity studies carried out with 4- (^{13}C)-Lina-OOHs corroborated the preferential reactivity of Lina-7-OOH compared to Lina-6-OOH (see Supporting Information). It is worth noting that 5- (^{13}C)-Lina-OOHs reactivity studies gave better information on radical reactivity at the hypothetically preferential position 4 than 4- (^{13}C)-Lina-OOHs, the ^1H - ^{13}C NMR long distance correlations having a higher resolution in the spectra.

Resuming, EPR-ST confirmed the formation of carbon radicals issued from Lina-OOHs in RHE. ^{13}C -NMR reactivity studies with ^{13}C -substituted compounds suggested carbon atom position 4 as a potential “radical structural alert” reacting with thiol groups. It is worthy to note that ^{13}C -NMR studies

allowed detection only of diamagnetic adducts. However, as EPR studies in solution showed, other radical (paramagnetic species) are coexisting in solution and it is not excluded that they could react with other amino acids prompt to radical reactions. It would be essential to study by EPR-ST which carbon radical is specifically formed in RHE. As we have shown here, nitrones such as DEPMPO are excellent traps for carbon radicals derived from Lina-OOHs in RHE. We have shown that spin-adducts formed are particularly stable, though the S/N ratio is relatively poor. It would be further necessary to use spin-traps more lipophilic than DEPMPO, able for instance to pass through the cell membrane and so allowing to distinguish if radical generation is intra or intercellular in the case of Lina-OOHs. Though, nitrones rarely allow the different carbon radicals formed to be distinguished (carbon-radical at position 4 *versus* position 5), as the coupling patterns observed in the spectrum are always very similar. A very good tool to overcome this drawback is the introduction of a ^{13}C -substitution in the positions of the molecule where a carbon radical can be formed such as for 5-(^{13}C)-Lina-OOHs and 4-(^{13}C)-Lina-OOHs. If the trapped radical is centered on a ^{13}C -substituted carbon, an additional hyperfine coupling will be observed on the EPR spectrum due to its nuclear spin, which is then in the beta position, as we have shown recently when studying the sensitizing cumene hydroperoxide.⁴² This property can thus be used to elucidate reaction mechanism(s).

CONCLUSION

To complement previous studies in solution on the identification of reactive radical intermediates issued from linalool sensitizing hydroperoxydes, we have now taken a step forward demonstrating that these are indeed formed in a reconstructed human epidermis 3D model, much closer to what may happen *in vivo*. For this purpose, we demonstrated that EPR-ST is a very powerful tool. Studying the reactivity of these radicals towards amino acids prone to radical reactions such as cysteine has given new insights on the mechanisms most likely to be involved in the reaction with thiol groups that might be present in the skin environment. The use of ^{13}C -substituted sensitizing compounds at positions

precursor of carbon radicals and further follow up of the reactions by ^{13}C -NMR is thus also an efficient tool to give innovative understanding on the reactivity of allylic hydroperoxides towards proteins and thus their sensitizing potential.

ASSOCIATED CONTENT

Supporting Information.

Experimental data on synthesis and reactivity of 4-(¹³C)-Lina-OOHs; General mechanism for the detection of free radicals by spin trapping; Data on EPR spin trapping experience with cysteine.

AUTHOR INFORMATION

Corresponding Author

* Elena Giménez-Arnau
e-mail: egimenez@unistra.fr
Tel: +33 3 68 85 15 25

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Notes

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Table 1

Table 1. Hyperfine coupling constants ($hfccs$) of spin-adducts identified in solution and in RHE

Spin-adducts	Coupling constants*		
	a_N (G)	a_H (G)	a_P (G)
In solution			
DEPMPO-OH	14.1	13.3	47.6
DEPMPO-R1	15.0	22.5	47.2
DEPMPO-R2	14.4	20.9	47.1
DEPMPO-OR	13.6	8.5	48.2
In RHE			
DEPMPO-OH	13.8	13.7	47.1
DEPMPO-R	14.8	21.2	48.2
DEPMPO-OR	15.2**	7.2**	45.9**

* All g factors were measured and found to be of ca. 2.0055 ± 0.0002 .

** Values given on an indicative basis following the best simulations attempts considering the S/N

FIGURE LEGENDS

Chart 1. Chemical structures of linalool, Lina-7-OOH ((*5E*)-7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol) and Lina-6-OOH (6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol).

Scheme 1. Potential carbon centered radicals resulting from Lina-7-OOH justifying the synthesis of ^{13}C -linalool at positions 5 and 4, further oxidized to the corresponding target (^{13}C)-Lina-OOHs.

Scheme 2. Synthesis of Lina-OOHs ^{13}C -substituted at position 5.

Scheme 3. Possible mechanistic pathway for the formation of (^{13}C)-**11** through the involvement of a carbon radical at position 4. ^{13}C -NMR only allows detection of diamagnetic adducts formed by reaction with the thiol group of cysteine. This does not mean there are not in the solution other radical intermediates, as EPR studies have shown, that could potentially react with other amino acids in the skin.

Figure 1. EPR experimental spectrum (Exp) of Lina-OOHs (1 mM)/DEPMPO (25 mM)/Fe(II) (0.1 mM) in solution, together with computer simulation (Sim) and deconvolution affording spectra of spin-adducts DEPMPO-OH, DEPMPO-R1, DEPMPO-R2 and DEPMPO-OR. Values of the *hfccs* are shown in Table 1. All *g* factors were measured and found to be of ca. 2.0055 ± 0.0002 . Control experiments with single Lina-OOHs or single DEPMPO did not give any signal.

Figure 2. EPR experimental spectrum (Exp) of Lina-OOHs (10 mM)/DEPMPO (250 mM) in RHE, together with computer simulation (Sim) and deconvolution affording spectra of spin-adducts DEPMPO-OH, DEPMPO-R and DEPMPO-OR. Values of the *hfccs* are shown in Table 1. All *g* factors were measured and found to be of ca. 2.0055 ± 0.0002 . Control experiments with single Lina-OOHs or single DEPMPO did not give any signal.

Figure 3. ^{13}C -NMR spectra obtained from the reaction (^{13}C)-**9**/ ^{13}C)-**10** (5.37 μmoles , 1 equiv)/ *N*-Ac-Cys-OMe (10.74 μmoles , 2 equiv)/Fe(II) (0.1 equiv) in $\text{CD}_3\text{CN}/\text{H}_2\text{O}$: (a) (^{13}C)-**9**/ ^{13}C)-**10** control with ^{13}C peaks at 125.7 ppm and 24.30 ppm corresponding to (^{13}C)-**9** and (^{13}C)-**10**, respectively; (b) *N*-Ac-Cys-OMe control; (c) reaction mixture at day 0; (d) reaction mixture at day 3; (e) reaction mixture at day 7.

Figure 4. Hypothetical chemical structures of compounds (^{13}C)-**11**, (^{13}C)-**12** and (^{13}C)-**13** based on HMBC interpretation.

Figure 5. Comparison of the chemical structures of the products suggested in previous liquid chromatography-mass spectrometry studies (**14-17**)¹⁸ with those identified in this ¹³C-NMR study ((¹³C)-**11**, (¹³C)-**12**).

Chart 1

Scheme 1

Scheme 2

Scheme 3

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5