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## **Skin sensitisation to fragrance hydroperoxides: interplay between dendritic cells, keratinocytes and free radicals**

**Short title:** Dendritic cell activation and radicals of terpene fragrance hydroperoxides

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#### **What's already known about this topic?**

- Allylic hydroperoxides of commonly used fragrance terpenes are important contact allergens.
- Hydroperoxides have the potential to form free radicals.

#### **What does this study add?**

- DC activation in the presence of keratinocytes by hydroperoxides of limonene, linalool, and citronellol.
- In case of limonene-1-OOH were donor variations coming from keratinocyte even more pronounced than variations arising from MoDC donors.
- Amounts of limonene-1-OOH and limonene-2-OOH inducing DC activation in human.
- Radical intermediates derived from hydroperoxides of limonene are formed *in situ* in a reconstructed human epidermis model and computational calculations confirm a preferential radical degradation pathway for limonene-1-OOH.

#### **What is the translational message?**

- This study provides new insights on interindividual differences in DC response to sensitising hydroperoxides and on the amounts needed for induction of sensitisation to hydroperoxides from limonene.

## **Summary**

**Background:** Skin sensitisation to hydroperoxides (R-OOHs) of the commonly used fragrance terpenes limonene, linalool and citronellol is frequently reported. R-OOHs are believed to initiate the

process leading to sensitisation and allergic contact dermatitis (ACD) through mechanisms involving radical intermediates. Thus, radical intermediates, keratinocytes and dendritic cells (DC) may act in concert to initiate the process.

**Objectives:** To evaluate individual DC activation profiles in the context of keratinocytes by R-OOHs with regard to frequency, specificity and magnitude of upregulation.

**Methods:** 2D and 3D cocultures with keratinocytes/reconstructed human epidermis (RHE) and DC to evaluate cell surface levels of the costimulatory molecules CD86, CD80 and the adhesion molecule CD54 on cocultured DC. Analysis of radical formation from limonene hydroperoxides in RHE by EPR-ST.

**Results:** R-OOHs induce donor-dependent DC activation. Major differences were found between the limonene-OOHs. Limonene-1-OOH was stronger with respect to both, frequency and magnitude of responses. Using a 3D coculture model, no DC activation was detected after topical application of 0.2% limonene-OOHs (20  $\mu\text{g}/\text{cm}^2$ ), while 1.2% limonene-1-OOH or 2% limonene-2-OOH induced DC activation. Furthermore, we demonstrated differences in the formed carbon and oxygen radicals from the limonene-OOHs using RHE, mimicking what may happen *in vivo*.

**Conclusions:** We report clear individual differences in DC maturation by the most important hydroperoxides. Both, response rates and magnitude of response indicate that very small structural alterations in the hydroperoxides are translated into specific DC responses. In addition, we provide more insights into the amounts of hydroperoxides that are able to activate DC and induce sensitisation.

## Introduction

Allergic contact dermatitis (ACD) is the most common cause of occupational and life-style related skin disease<sup>1</sup>. Fragrance compounds remain the most commonly detected contact allergens<sup>2</sup>, generally used in fine perfumes, cosmetics and household products, but also found in essential oils. Some of them autoxidise on contact with air, forming sensitisers which can lead to an adaptive immune response. This is particularly the case for hydroperoxides (R-OOHs) derived from autoxidation of natural terpenes. The best-known fragrance R-OOHs are those derived from limonene (limonene-OOHs), linalool (linalool-OOHs) and citronellol (citronellol-OOHs)<sup>3,4</sup>. Hydroperoxides are believed to initiate the multistep process leading to skin sensitisation through mechanisms involving radical intermediates. Potential formation of immunogenic complexes via radical intermediates has already been investigated for representative molecules<sup>5,6</sup>. In contrast to the parent molecules, fragrance R-OOHs are categorised as strong sensitisers according to<sup>7</sup>. However, a quantitative link between the amounts of R-OOHs in fragrance-containing products and sensitisation and ACD is unclear. Existence of that link is so far supported by few cases<sup>8-10</sup>. Investigations using patch test studies to assess whether the observed high prevalence of reactions observed for several R-OOHs are specific for their parent molecules found no evidence for cross-reactivity. Even the structurally close related limonene-1-OOH and limonene-2-OOH showed no unspecific reactions or cross-reactivity in patch test studies (<sup>11</sup>and references therein). Thus, the question remains what the cause for sensitisation and ACD is, and which concentrations are able to sensitise and elicit ACD. Therefore, it is important to evaluate whether target cells, like skin keratinocytes and dendritic cells (DC), are highly responsive to R-OOHs.

We demonstrated lately by electron paramagnetic resonance (EPR) spectroscopy combined with the spin trapping (ST) technique that in reconstructed human epidermis (RHE) oxygenated and carbon-centred radicals are formed after exposure to cumene-OOH<sup>12-14</sup>, to ascaridole<sup>14</sup> and linalool-OOHs using concentrations of clinical patch test studies<sup>13</sup>. The formed radicals may initiate the formation of specific antigens that are taken up, processed and cause maturation of DC. Moreover, radical-initiated formation of reactive oxygen species (ROS) and/or H<sub>2</sub>O<sub>2</sub> may reach DC. For instance, H<sub>2</sub>O<sub>2</sub> can signal between different cells due to its relatively high stability, and as a result may enter DC via

diffusion or aquaporins in the plasma membrane<sup>15,16</sup>. This way keratinocytes could influence both the maturation of DC, including upregulation of costimulatory molecules (such as CD40, CD80 and CD86), as well as the adhesion molecule CD54 involved in the formation of the immunological synapse, and migration to draining lymph nodes, where they instruct naïve T cells proliferation and participate in their differentiation into appropriate effector and regulatory T cells. In line with ROS promoting DC activation, H<sub>2</sub>O<sub>2</sub>-treated human monocyte-derived DC (MoDC) were efficient in promoting T cell proliferation and showed upregulation of MHC molecules, CD40 and CD86<sup>17,18</sup>. Accordingly, studying DC activation in the presence of keratinocytes and analysis of formed radicals and intermediates in these cells provide a deeper insight into the subject.

Based on this background, we carried out upstream investigations aiming at understanding the reactions induced by a series of hydroperoxides (Fig. 1a), and focused on the impact of keratinocytes to induce DC maturation. We used MoDC cocultured together with a keratinocyte cell line (HaCaT). In order to define more precisely the contribution coming from primary keratinocyte donors, we also used DC surrogate cells, namely monocytic THP-1 cells, cocultured either with keratinocytes (2D cocultures) or RHE (3D coculture). This way, we could investigate MoDC- and keratinocyte- donor-dependent effects that helps to figure out the role of keratinocytes in the DC activation and sensitization to R-OOHs in different individuals. Consequently, we evaluated the differences observed for limonene-OOHs by performing an in-depth analysis of the radicals issued from limonene-1-OOH and limonene-2-OOH in RHE.

## Materials and Methods

### Generation of human monocyte-derived dendritic cells (MoDC) and culture of THP-1 cells

CD14<sup>+</sup> cells (buffy coats, 18 to 45 years) were differentiated as described<sup>19</sup> (characterisation of MoDC see appendix S2, Fig. S1). THP-1 cells were cultured as described<sup>20</sup>.

### Keratinocyte cell culture

Normal human epidermal keratinocytes (NHEK, healthy donors, 23 to 51 years, passages 2-5) and HaCaT keratinocytes were cultured as we described<sup>20,21</sup> and seeded ( $4.8 \times 10^4$  cells/cm<sup>2</sup>).

### **Establishment and treatment of cocultures**

MoDC/HaCaT: MoDC ( $8 \times 10^4$  cells) were cocultured on top of HaCaT and incubated with the compounds for 24h. THP-1/NHEK: THP-1 ( $8 \times 10^4$  cells) were placed on top of NHEK and exposed (see Tab. S1). Details on synthesized hydroperoxides are given in appendix S1.1. Concentrations for R-OOHs (300  $\mu$ M) and DNCB (20  $\mu$ M) are based on previous experiments<sup>22</sup> and allowed for direct comparison of the results.

### **Analysis of cell surface expression of CD80, CD86 and CD54**

MoDC and THP-1 cells were collected and cell surface expression (percent positive cells, geometric mean fluorescence intensities [MFI]) of CD80, CD86 and CD54 was measured by flow cytometry. Details are given in appendix S3 (Fig. S2).

### **Activation of THP-1 cells cocultured with human epidermis**

Topical application (20-200  $\mu$ g/cm<sup>2</sup> in acetone:olive oil 4:1) of limonene-1-OOH and limonene-2-OOH on RHE (SkinEthic model, Episkin, France) was performed as we described<sup>23</sup>. We used two RHE covering three donors, in detail, one RHE consisted of NHEK from one donor and the other of NHEK from 2 donors.

### **EPR-ST studies with limonene-1-OOH and limonene-2-OOH in solution**

The mixture 50 mM DEPMPO/1 mM hydroperoxide/0.1 mM Fe(II) was analysed as described<sup>13</sup>. EPR spectra were recorded at room temperature (295K  $\pm$ 1K) on an EPR X-band spectrometer (ESP300E, Bruker Biospin GmbH, Germany) equipped with standard TE102 or high sensitivity (HSW) resonators. Principal experimental parameters were: microwave power 5 mW, modulation amplitude 1 G and resulting sweep time of *ca.* 164 s for a single scan of 1024 points.

### **EPR-ST studies with limonene-1-OOH and limonene-2-OOH in RHE**

RHE (EpiSkin model, Episkin, France) were topically pretreated with DEPMPO in dimethylsulfoxide/HEPES 1:1 (250 mM, 20  $\mu$ L) and afterwards topically treated with limonene-1-OOH (100 mM in acetone, 20  $\mu$ L) and limonene-2-OOH (10 mM in acetone, 20  $\mu$ L) as described<sup>13</sup>. Principal experimental parameters were: microwave power 5 mW, modulation amplitude 1 G and resulting sweep time of *ca.* 328 s for a single scan of 1024 points. Up to 10 scans were accumulated to enhance the S/N. Representative results for one RHE consisting of NHEK of one donor are shown.

### **EPR Simulations**



EPR spectra were analysed by means of computer simulation using labmade scripts based on Easyspin toolbox under Matlab (MathWorks) environment<sup>24</sup>.

### **Computational studies**

DFT calculations were performed using Gaussian 09 package (Gaussian 2009, version D.01) at Density Functional level of Theory (DFT) with  $\omega$ B97XD functional<sup>25</sup>. Atoms were described by the 6-31+G\*\* basis set<sup>26</sup>. Water solvent was modeled through a polarised continuum model<sup>27</sup>. Structures were fully optimised and the nature of the encountered stationary point characterised by a frequency calculation. The minima connected by a transition state were checked by an intrinsic reaction coordinate calculation following the imaginary frequency. Gibbs free energies were extracted from the frequency calculation done within the harmonic approximation. For each studied molecule, the energy reference is the initial alkoxy radical.

### **Statistical analysis**

Differences between two groups were evaluated by one-tailed Wilcoxon matched-pairs signed rank test (GraphPad Prism 5).

## **Results**

### **R-OOHs induce donor-dependent DC activation**

Keratinocytes and DC conduct dynamic communication and exchange material with each other<sup>28</sup>. Also different responsiveness of DC and THP-1 in coculture with keratinocytes was reported for contact allergens<sup>29,30</sup>. Here, we investigated the potential of important hydroperoxides to activate DC in the presence of keratinocytes without adding a radical initiator (Fig. 1b, representative histograms: Fig. S2). Here, we also included cumene-OOH, as it is known to generate radicals as well as oxidative stress in 2D cultured human keratinocytes<sup>31</sup>. First, we studied MoDC (Fig. S1) in coculture with HaCaT keratinocytes in order to address interindividual differences arising from MoDC (n=12). Second, for the evaluation of donor-dependent effects arising from keratinocytes, we cocultured NHEK (n=6) together with THP-1 cells that mimic DC activation (CD86, CD54)<sup>32</sup>, and which are also used in a non-animal testing strategy to identify chemical sensitizers<sup>33</sup>. A comprehensive

comparison of MoDC and THP-1 cells evaluating a large set of chemical sensitizers found comparable magnitudes of CD86 upregulation, while CD54 was not used for quantitative comparison<sup>34</sup>.

MoDC from all individuals reacted to lipopolysaccharide (LPS) and all R-OOHs, though to a different extent, upregulated the co-stimulatory molecules CD86 and CD80 on cocultured MoDC. In detail, while all individuals upregulated CD86, 3 donors upregulated CD80 in response to limonene-1-OOH, limonene-2-OOH and citronellol-6/7-OOH (MoDC23-25). Cells from one other donor (MoDC24) upregulated CD80 in response to citronellol-6/7-OOH only and another (MoDC30) to cumene-OOH only. Quantitative analysis of CD54 was not included due to high basal expression on cocultured MoDC (Fig. 1c, upper panels). In sum, compound-dependent upregulation was remarkably different between the two limonene-OOHs. MoDC from all tested individuals reacted in 2D coculture to limonene-1-OOH, while only 50% responded to limonene-2-OOH.

Using the 2D model consisting of NHEK and THP-1 cells, again differences for the two limonene-OOHs were found, both based on CD86 and CD54 upregulation (Fig. 1c, lower panels). Here, the donor-dependent upregulation varied 5-fold (CD86) and 20-fold (CD54), respectively. These results are notable when looking at the results for 2,4-dinitrochlorobenzene (DNCB) with 3-fold (CD86) and 6-fold (CD54) variation between different donors. Thus, these data indicate that keratinocytes significantly contribute to DC activation by R-OOHs.

Taken the results from both 2D cocultures together, both, donor variation from MoDC and keratinocytes contribute to the inter-individual variation in response to R-OOHs. Collectively, every tested individual responded strongly to limonene-1-OOH (16/16, 100%). Thirteen of 14 individuals responded to citronellol-OOHs (93%) followed by cumene-OOH (11/14, 79%). Comparable response rates were found for limonene-2-OOH (11/16, 69%) and linalool-OOHs (8/12, 67%).

Focusing on the individual susceptibility to the hydroperoxides, we found that the majority of individuals (10/16, 63%) responded to all tested hydroperoxides (considering those donors, which were tested for at least three hydroperoxides). For the other individuals (6/16) no particular pattern was obvious (Fig. 1d). Looking at the magnitude of the responses to limonene-1-OOH and limonene-2-OOH significant differences were evident, although no differences were found in case of three individuals (Fig. 1e).

### Different potentials of limonene-1-OOH and limonene-2-OOH in 3D models

First, we determined the amounts that lead to DC activation after topical application of the two compounds on RHE (SkinEthic), thereby giving a clearer image of what may happen *in vivo*. To achieve this, we placed THP-1 cells under the RHE (Fig. 2a, for details see<sup>23</sup>). As recommended, we tested RHE covering three donors<sup>35</sup>. Data presented in Fig. 2b show clear upregulation of CD86 and CD54 on THP-1 when 120  $\mu\text{g}/\text{cm}^2$  (1.2%) limonene-1-OOH or 200  $\mu\text{g}/\text{cm}^2$  limonene-2-OOH was applied, while no or only marginal upregulation was observed for 20  $\mu\text{g}/\text{cm}^2$  (0.2%).

An EPR-ST methodology was developed to study radical intermediates of the limonene-OOHs (Fig. 2c-d). First, studies in solution were performed to define the radical landscape in a simple system (i.e. cell-free conditions, ratio: 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide, DEPMPO/limonene-OOHs). Initiating the reaction with Fe(II), limonene-OOHs gave similar EPR signatures pointing to spin-adducts (Fig. 2e) testimony of carbon (e.g. methyl) and oxygen-centered radicals (e.g. hydroxyl, peroxy, alkoxy), (appendix S4, Fig. S3-S4). Second, investigations were performed in RHE (EpiSkin model), but with higher DEPMPO/Lim-OOHs concentrations as radicals are harder to probe in this matrix. Indeed, the RHE matrix is intrinsically much more complex than the one defined for the study in solution. In turn, it offers manifold targets for radicals to react (e.g. proteins, lipids) and cellular antioxidant defenses that can silent them, all competing with the spin-trap and drastically lowering the EPR signal-to-noise ratio (S/N). Overall, EPR fingerprints found in RHE appeared compatible to those obtained in solution, pointing to similar radicals (Fig. 2e). Notwithstanding poor S/N, RHE EPR analysis was attempted relying on the signature found beforehand in solution through the hyperfine coupling constants (*hfccs* appendix S5, Fig. S5-S6). This was valuable for the prediction of oxygen-centered radicals, barely detected by EPR and consequently hardly predictable based on the sole RHE results. Importantly, RHE studies were conducted without addition of supplementary Fe(II). Therefore, radical initiation is assumed to be driven by endogenous skin constituents (e.g. metals, amino acids).

Computational chemistry (density functional theory, DFT) was then applied to highlight potential preferential paths of radical degradation. DFT investigation demonstrated different pathways for limonene-OOHs, starting with the O-O bond cleavage to form alkoxy radicals (detailed description in appendix S6). For limonene-1-OOH, a predominant carbon radical could be formed (path A), which is

the most likely based on kinetic considerations (Fig. 2f). Path A has the lowest barrier (10.8 kcal.mol<sup>-1</sup>) and consists of the C1-C7 bond cleavage to form an  $\alpha,\beta$ -unsaturated ketone, the reaction being exergonic ( $\Delta G = -4.7$  kcal mol<sup>-1</sup>). A highly reactive methyl radical is released and if remaining in the vicinity of the ketone can further react through H-transfer with a low barrier ( $\Delta G$  7.7 kcal mol<sup>-1</sup>) giving a  $\alpha,\beta$ -unsaturated ketone with a very stable delocalised radical at C4 ( $\Delta G$  -34.4 kcal mol<sup>-1</sup>). Thus, more specific carbon-centred radical generation and/or faster kinetics may occur in the epidermis in the case of limonene-1-OOH. On the other hand, carbon radicals that can be formed from limonene-2-OOH are very similar in terms of Gibbs free energies (Fig. 2f) and one could thus expect a less specific generation in the epidermis. In sum, the DFT analysis demonstrates that many different radicals could be formed in skin through endogenous initiators and that these initial processes are already different between the two limonene-OOHs.

## Discussion

Hydroperoxides (R-OOHs) have been identified in clinical patch test studies as potent skin sensitisers in the autoxidation mixtures of the frequently used fragrances limonene, linalool and citronello<sup>3</sup>. To be sensitisers, R-OOHs must associate with skin proteins through stable covalent bonds and provide signals for dendritic cell (DC) maturation through upregulation of surface receptors/ligands (e.g. CD80, CD86, CD54), a very crucial step in this process<sup>36-39</sup>. There is now a growing realisation that skin keratinocytes and innate immune cells influence the intensity and quality of DC maturation, determining the sensitisation potential and potency. Keratinocytes could be involved by determining the effective dose and contributing maturation signals. Focusing on keratinocytes and the early events of hydroperoxide activation, we reported lately the generation of radicals by linalool hydroperoxides and cumene hydroperoxide *in situ* using reconstructed human epidermis (RHE)<sup>13,40</sup>. Here we evaluated the individual responsiveness and magnitude of DC maturation by important hydroperoxides in 2D and 3D coculture models with keratinocytes.

Individual response variations were observed in both 2D cocultures, indicating MoDC and keratinocyte dependency. Especially for limonene-1-OOH, keratinocytes from different individuals

altered the response even stronger than MoDC, which was not the case for DNCB. This suggests that donor variation coming from keratinocyte is even more important in responses to limonene-1-OOH than variation arising from MoDC donors.

Remarkable differences were found for the limonene hydroperoxides (see Fig. 1a, structures 1,2). Namely, every donor responded to limonene-1-OOH and the magnitude of response is partly comparable to lipopolysaccharide. In contrast, only 11 of 16 individuals responding to limonene-1-OOH reacted to limonene-2-OOH (69%), and a pairwise analysis supported the differences. In line with our *in vitro* approach, human patch test studies led to the same conclusion, ranking limonene-1-OOH as the more potent isomer<sup>41-43</sup>. Thus, we conclude that the frequency and magnitude of the DC response are good indicators for reactions to hydroperoxides.

Looking at citronellol-OOHs and linalool-OOHs as well as cumene-OOH, we also found high responsiveness of individuals. Overall, only 6 individuals (6/16, 38%) did not react to all tested hydroperoxides. However, other response rates are observed under natural exposure conditions. For example, in clinical multicenter patch test studies, about 25% of those who reacted to either patch test preparation with oxidised limonene containing limonene-OOHs and oxidised linalool containing linalool-OOHs in standardised concentrations, also reacted to the other mixture (<sup>44</sup>and references therein). In sum, our insights, showing that different R-OOHs induce distinct DC maturation, both with regard to frequency of response and magnitude of upregulation, supplement investigations demonstrating that hydroperoxides act as specific compounds<sup>45</sup>.

From a chemical point of view, the first activation step for R-OOHs needs low dissociation energy (*circa* 175 kJ mol<sup>-1</sup>) and results in the formation of highly reactive alkoxy radicals via cleavage of the O-O bond. The radicals may undergo radical rearrangements (e.g. intramolecular cyclisation, allylic hydrogen abstraction,  $\beta$ -scission) and form carbon radicals with longer half-life. Characterising the extremely short-lived radicals from limonene-OOHs in the context of a RHE using the EPR-ST technique, we found carbon- as well as oxygen-centered radicals. In contrast to limonene-2-OOH, the predominant radical-induced molecular rearrangement pathway of limonene-1-OOH likely involves the formation of methyl radicals. Noteworthy, the intramolecular cyclisation at C2 forming an epoxide radical was considered for longtime as a key path for limonene-1-OOH radical rearrangement processes in the skin<sup>6,46</sup>, but our DFT studies excluded this possibility. The next steps

would be to clarify whether extracellular and/or intracellular agents or metabolic enzymes mediate the cleavage of the O-O bond.

Further evaluation of limonene-1-OOH and limonene-2-OOH using the RHE model and assessment of DC activation by placing THP-1 cells underneath (3D coculture) allowed us to even closer monitor what may happen *in vivo*. Studying dose-response relationships for the two molecules, we found no DC activation after topical application of 0.2% limonene-OOHs, while 120  $\mu\text{g}/\text{cm}^2$  (equal to 1.2%) limonene-1-OOH or 2% limonene-2-OOH were required for DC activation. Although the human sensitisation thresholds for the hydroperoxides are unknown, these amounts are comparable to the sensitising doses observed in experimental mice models (local lymph node assay<sup>41,42</sup>) and are, as expected, more than 100-fold higher compared to concentrations inducing elicitation observed in a repeated open application study<sup>47</sup>. Thus, for the first time, our results showed distinct DC activation profiles in the presence of keratinocytes, which can degrade hydroperoxides via radical mechanisms. At this moment, we cannot directly conclude that the radicals we detected by EPR-ST and/or their rearrangements generate the danger signals responsible for our findings.

In conclusion, these results provide new insights on how individuals respond to sensitising hydroperoxides. Our observed individual susceptibilities indicate that small structural alterations, resulting in specific radical degradation pathways, can be translated into variable but specific biological DC responses (i.e. limonene-1-OOH *versus* limonene-2-OOH). Knowing that maturation of DC is the crucial step in sensitisation, we provide hints for the amounts needed for induction of this critical step.

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## Figure legends

**Figure 1. Chemical structures of the studied hydroperoxides, setup of the 2D cocultures and DC responses.** Chemical structures of the investigated hydroperoxides (**A**). 2D coculture models of monocyte-derived dendritic cells (MoDCs, 12 single donors) with HaCaT keratinocytes (**B, lower panel**) and THP-1 cells with keratinocytes (6 single NHEK donors, HaCaT as black symbol) (**B, upper panel**). Cells were exposed (300  $\mu$ M each) to lin-6/7-OOHs [mixture of lin-6-OOH (**3**) and lin-7-OOH (**4**), ratio 6:4], citr-6/7-OOHs [mixture of citr-6-OOH (**5**) and citr-7-OOH (**6**), ratio 6:4], cum-OOH (**7**), lim-1-OOH (**1**) or lim-2-OOH (**2**). Lipopolysaccharide (LPS, 1  $\mu$ g/mL) and 2,4-dinitrochlorobenzene (DNCB, 20  $\mu$ M) served as controls. Expression of CD86, CD80 and CD54 was analysed on collected MoDC/THP-1 (24 h) by flow cytometry. Results are shown as compound-induced change (mean fluorescence intensity, MFI), considering an MFI increase of  $>8$  for CD86,  $>2$  for CD80 and  $>300$  for CD54 as a positive response (dotted line). Each color represents one individual donor (MoDC: **C, upper panel**, NHEK: **C, lower panel**). For comparison, THP-1/HaCaT is depicted as dark blue dot (**C, lower panel**). Individual susceptibility to tested hydroperoxides are visualised (**D**) and a quantitative comparison of limonene-1-OOH and limonene-2-OOH is depicted (**E**). \*,  $p \leq 0.05$ ; \*\*\*  $p = 0.001$

**Figure 2. Setup of the 3D coculture, DC responses to lim-OOHs and radicals detected by EPR-ST.** THP-1 cells were placed below a 3D epidermis model (2 RHE covering 3 donors) and compounds were topically applied (**A**). Cell surface expression of CD86 and CD54 was analysed on collected THP-1 cells by flow cytometry (24h) and stated as MFI (**B**). Formation of spin-adducts in the form of nitroxide radicals by scavenging free radicals with DEPMPO (**C**). Sealed capillaries used for in solution experiments and flat cell for RHE experiments (**D**); Left: EPR experimental spectrum (Exp) of limonene-1-OOH (1mM)/DEPMPO (50 mM)/Fe(II) (0.1 mM) in 10 mM HEPES solution

(pH 6.8) together with computer simulation (Sim), and comparison with EPR experimental spectrum of limonene-1-OOH (100 mM)/DEPMPO (250 mM) in RHE; Right: EPR experimental spectrum (Exp) of limonene-2-OOH (1mM)/DEPMPO (50 mM)/Fe(II) (0.1 mM) in solution together with computer simulation (Sim), and comparison with EPR experimental spectrum of limonene-2-OOH (10 mM)/DEPMPO (250 mM) in RHE. Control experiments in RHE with single limonene-OOHs or single DEPMPO did not give any signal (**E**). Radicals that could be formed from limonene-1-OOH and limonene-2-OOH (**F**) according to DFT computational studies. Only the most favored mechanisms are presented. Gibbs energies ( $\Delta G$ ) are given in kcal.mol<sup>-1</sup>.



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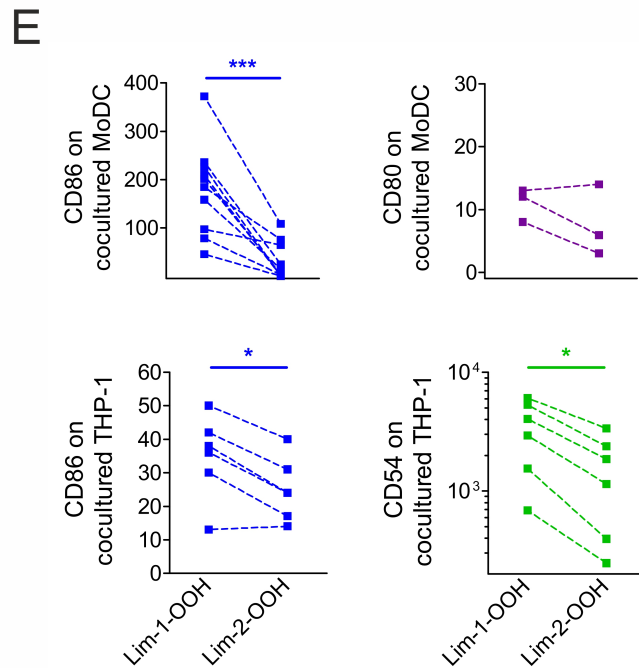
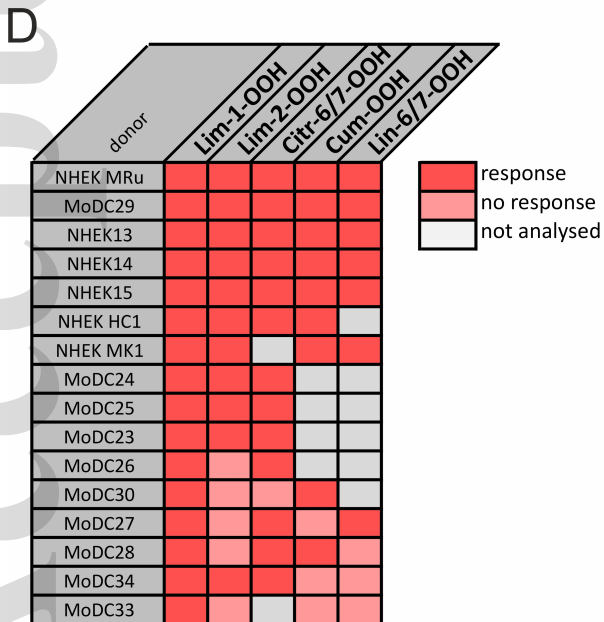
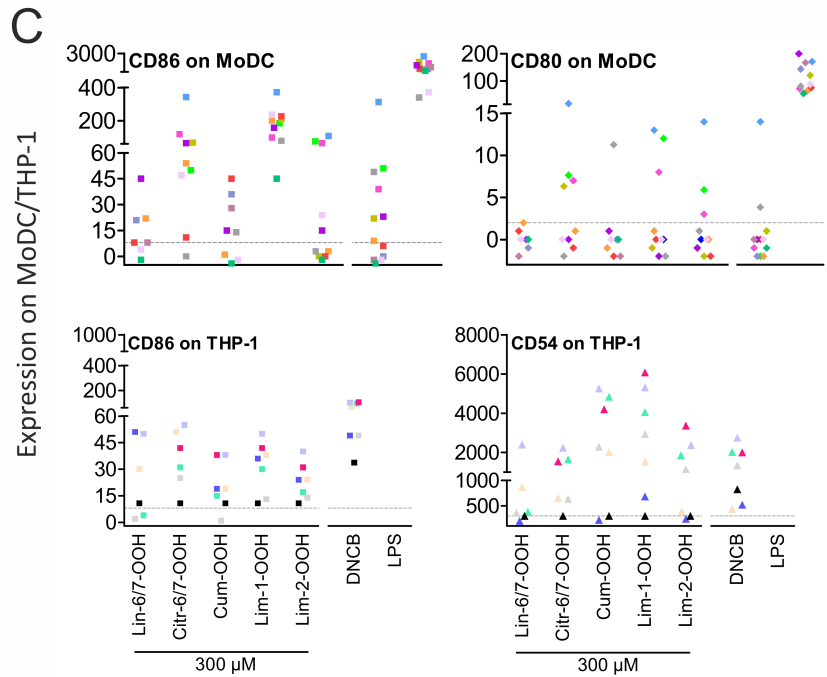
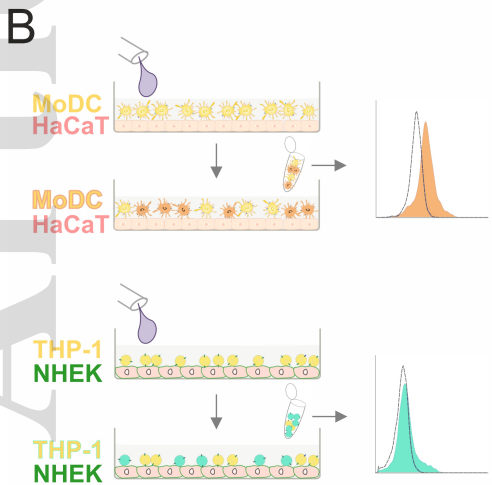
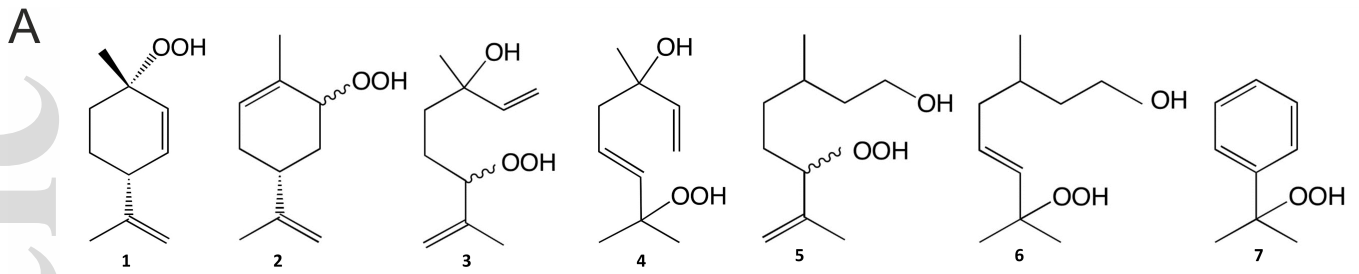
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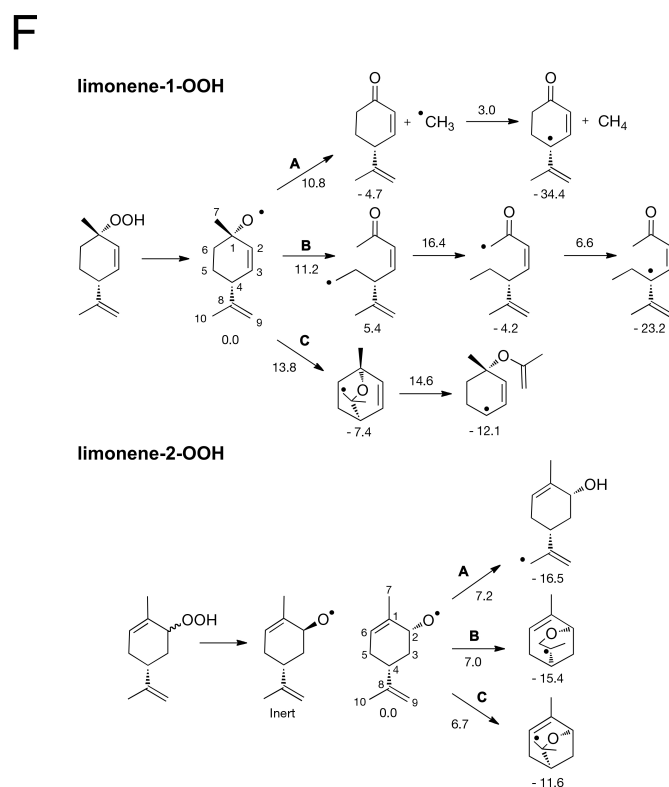
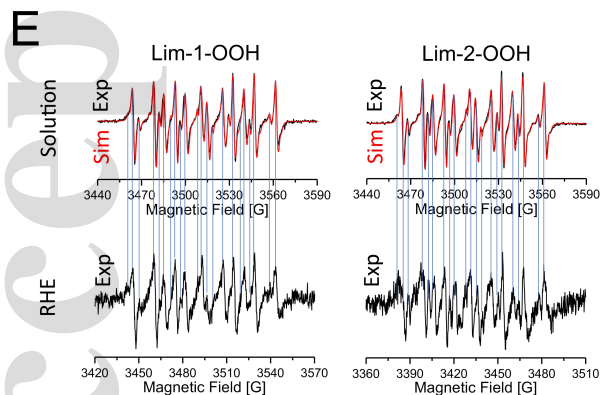
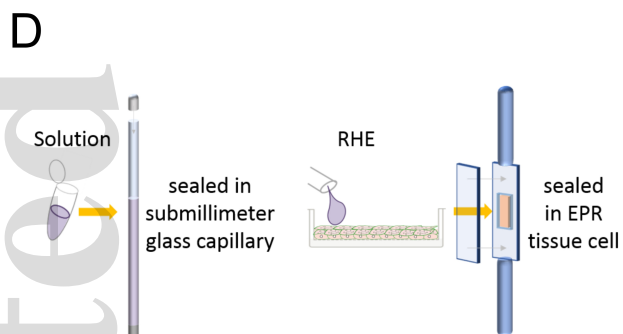
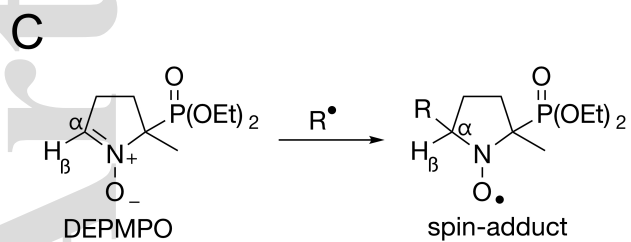
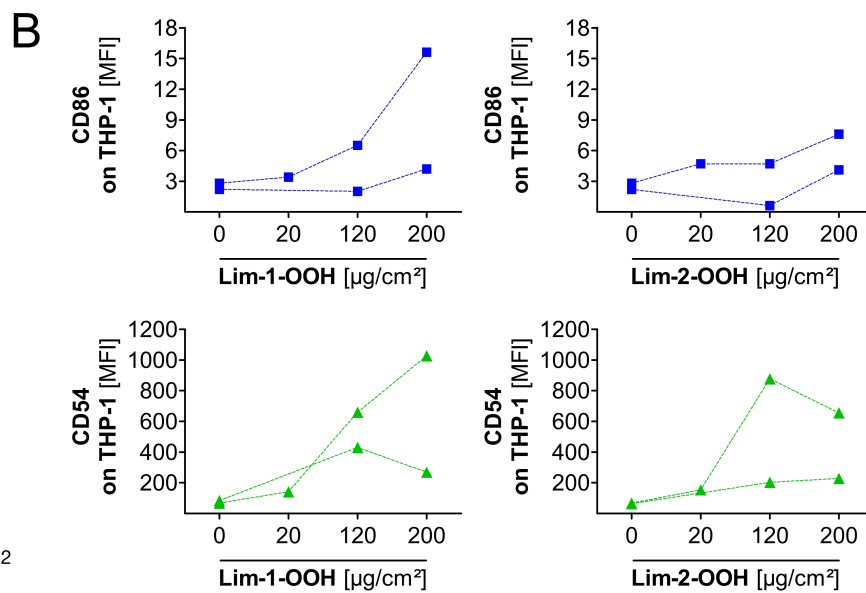
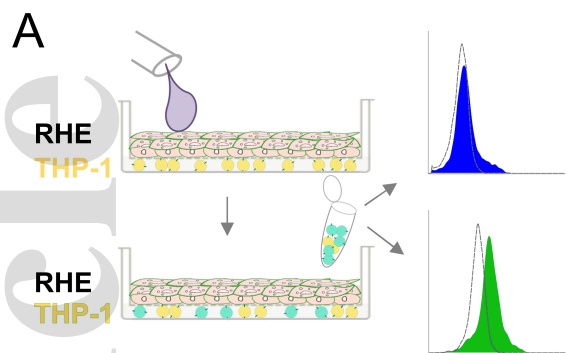
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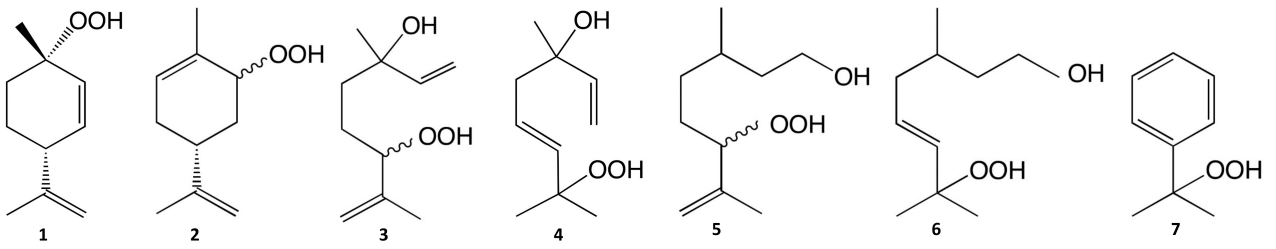


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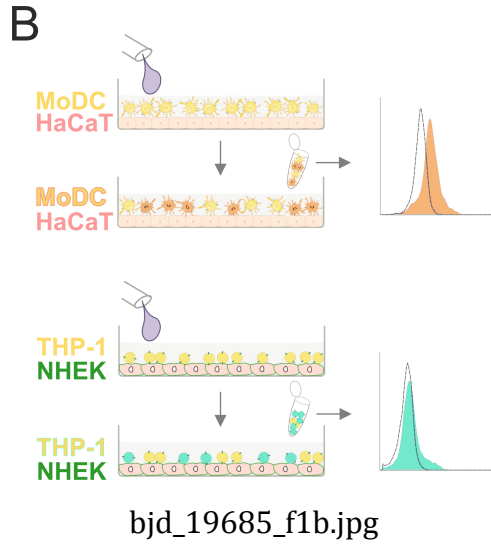
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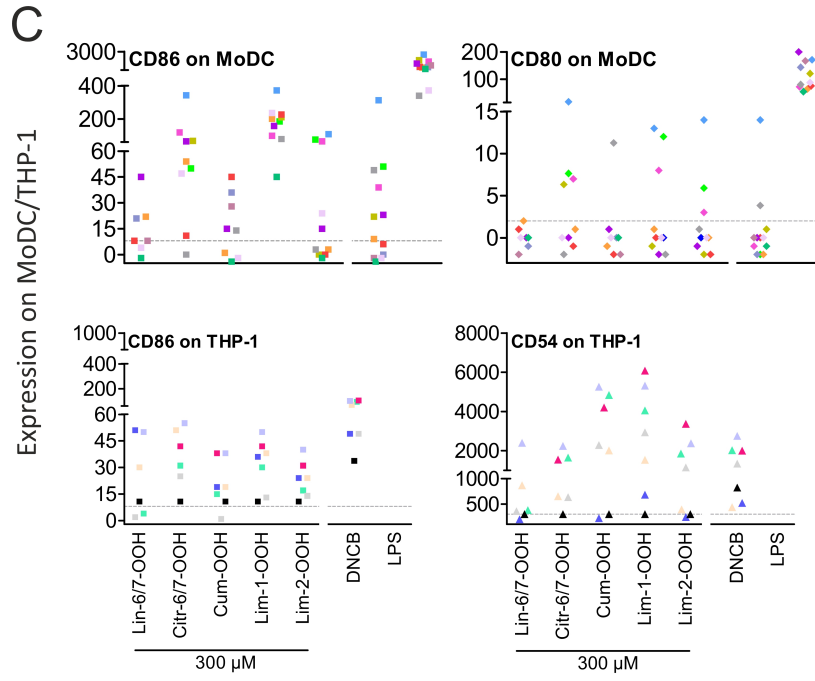
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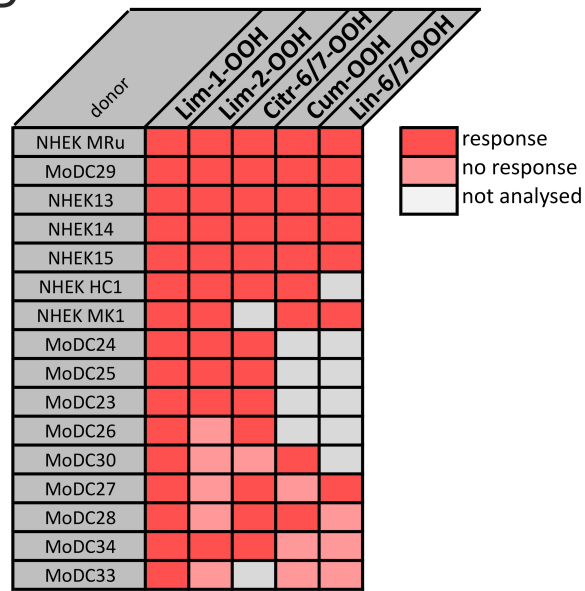






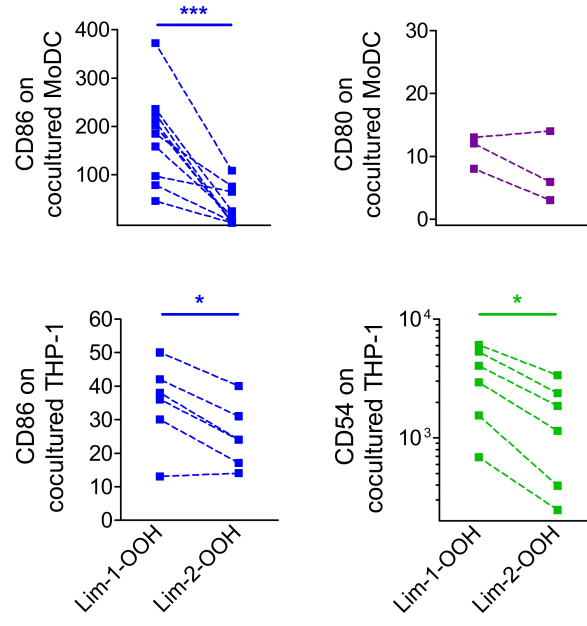
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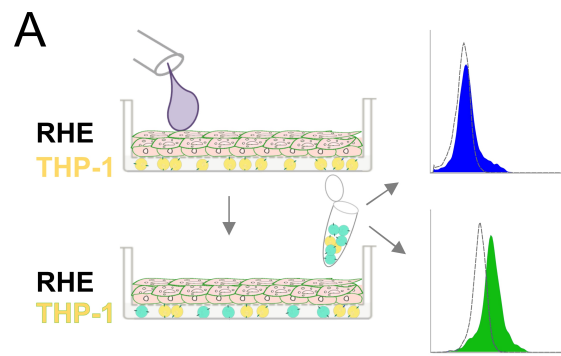


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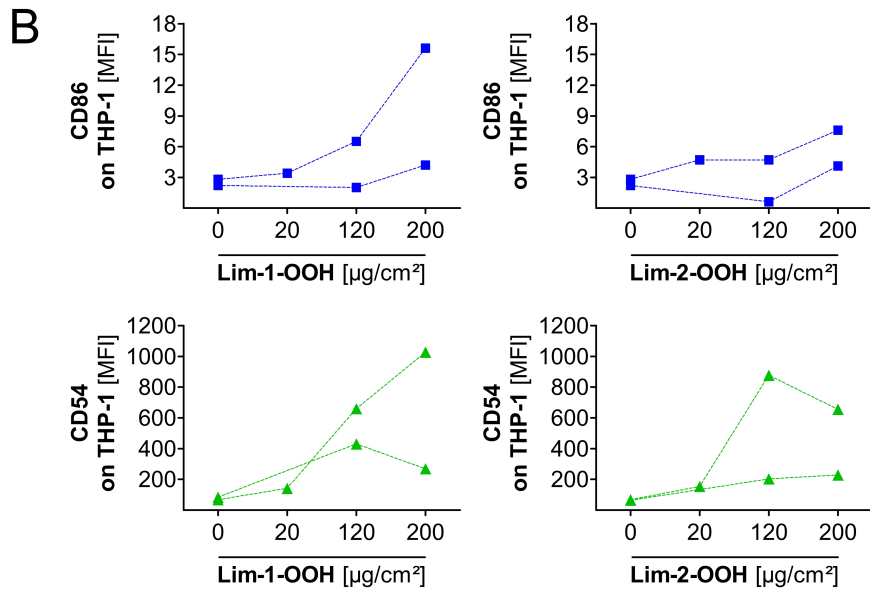
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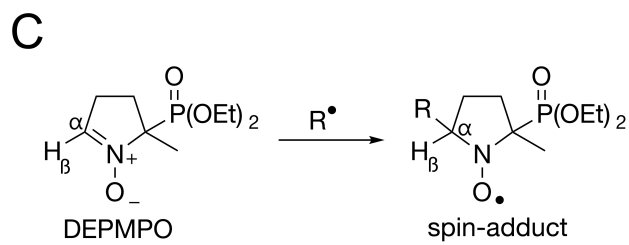
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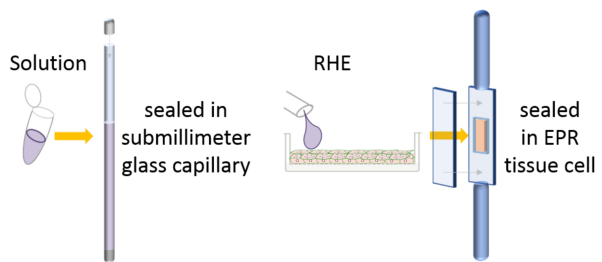


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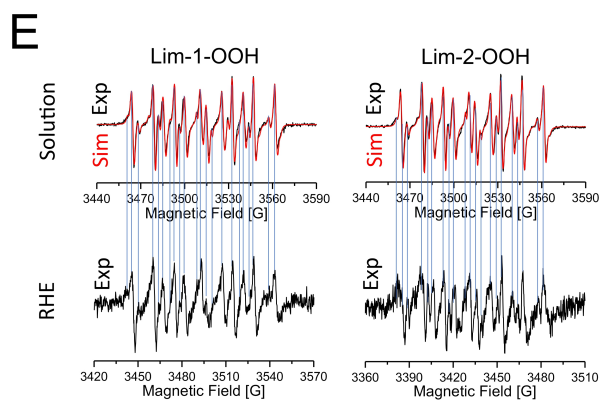
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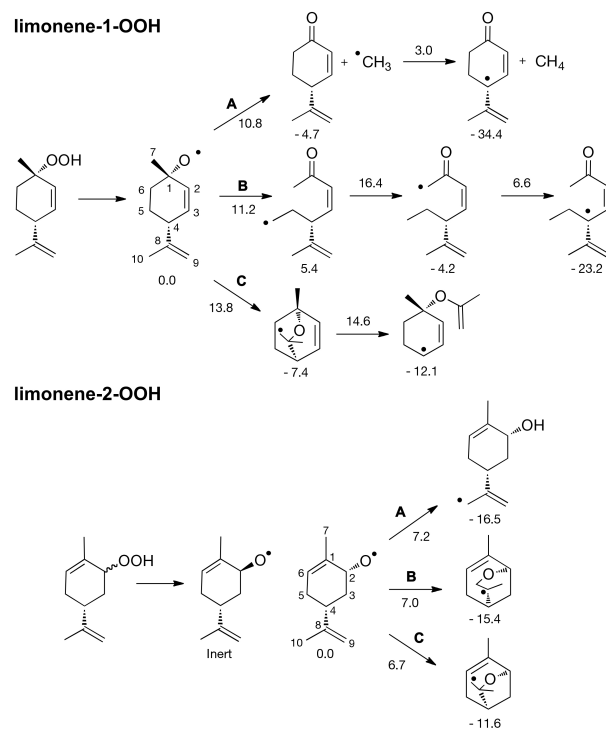
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