LIMONENE HYDROPEROXIDES IN ALLERGIC CONTACT DERMATITIS

Radical Formation, Sensitizing Capacity and Immunogenic Complex Formation

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UNIVERSITY OF GOTHENBURG

DOCTORAL THESIS

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Limonene Hydroperoxides in Allergic Contact Dermatitis

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Rock n Roll ain't noise pollution

Abstract

Contact allergy to fragrance compounds is an increasing problem in the western countries today. *R*-Limonene is one of the most common fragrance compounds; it is used in hygiene products and cosmetics as well as in industrial products such as hand cleansers and degreasers. *R*-Limonene is prone to autoxidation and it has been shown that 2-3% of consecutive dermatitis patients are allergic to oxidized limonene or the hydroperoxide fraction of the oxidation mixture.

This thesis examines limonene hydroperoxides, what radicals they can form, their sensitizing capacities, and a possible mechanism for immunogenic complex formation. Six structurally similar hydroperoxides were studied. Two of these are naturally occurring in oxidized limonene (limonene-1-hydroperoxide and limonene-2-hydroperoxide), while the others are synthetic structural analogues used for SAR-studies.

The formation of radicals was studied in radical trapping experiments using iron porphyrin as a model for enzyme-initiated radical formation. All hydroperoxides formed large amounts of radicals and the trapping experiments showed that the identity and quantity of radicals formed depend on the structure of the hydroperoxide. In combination with the sensitizing capacities, the results also indicate that the alkoxyl radicals are the most important in the immunogenic complex formation.

The sensitizing capacities were studied in the local lymph node assay (LLNA) and all hydroperoxides were found to be potent sensitizers. In a modified LLNA, comprising non-pooled lymph nodes and statistical evaluation, limonene-1-hydroperoxide was significantly more sensitizing compared to two other hydroperoxides. The clinical relevancy of this result was demonstrated in a limited study where more allergic reactions to limonene-1-hydroperoxide compared to limonene-2-hydroperoxide were recorded in individuals with known contact allergy to oxidized limonene.

The immunogenic complex formation of limonene-2-hydroperoxide was studied in a model using amino acids. Limonene-2-hydroperoxide forms carvone that reacts with thiyl radicals from cysteine according to the thiol-ene reaction. The identification of a carvone-

cysteine adduct indicates a possible radical mechanism for the immunogenic complex formation of olefinic hydroperoxides.

The combined results indicate that the immunogenic complex formation of hydroperoxides may include two phases. The formation of large amounts of radicals in the skin weakens the antioxidant defense; this facilitates the addition of a compound derived from the hydroperoxide to a protein via a radical mechanism, resulting in a specific immunogenic complex. This form of action explains why all hydroperoxides are strong sensitizers with very small differences in their sensitizing capacities.

In summary, the results presented in this thesis demonstrate that the radical formation of the hydroperoxides depends on their structure and influence the sensitizing capacity of the hydroperoxide. In addition, the formation of protein radicals and addition of a compound originating from the hydroperoxide via the thiol-ene reaction is proposed as a possible mechanism of immunogenic complex formation of olefinic hydroperoxides.

Keywords: allergic contact dermatitis, contact allergy, immunogenic complex, limonene hydroperoxides, local lymph node assay, patch testing, radicals, sensitizing capacity, skin, structure activity relationship.

Contents

List of Publications

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I – IV. The papers are appended at the end of the thesis. Reprints are made with permission from the publishers.

I. Carbon and Oxygen Centered Radicals are Equally Important Haptens of Allylic Hydroperoxides in Allergic Contact Dermatitis. Staffan Johansson, Elena Giménez-Arnau, Morten Grøtli, Ann-Therese Karlberg, and Anna Börje. *Chemical Research in Toxicology*, **2008**, 21, (8), 1536–1547.

II. Limonene Hydroperoxide Analogues Differ in Allergenic Activity. Johanna Bråred-Christensson, Staffan Johansson, Lina Hagvall, Charlotte Jonsson, Anna Börje, Ann-Therese Karlberg. *Contact Dermatitis*, **2008,** 59, 344-352.

III. Identification of a Radical Mechanism for Formation of Specific Immunogenic Complexes - A Key Step in Allergic Contact Dermatitis to Olefinic Hydroperoxides. Staffan Johansson, Theres Redeby, Timothy M. Altamore, Ulrika Nilsson, Anna Börje. *Submitted for publication.*

IV. Radicals are the Active Haptens of Alkylic Limonene Hydroperoxide Analogues in Allergic Contact Dermatitis. Staffan Johansson, Katarina Emilsson, Morten Grøtli, Anna Börje. *Manuscript in preparation.*

Contribution Report

Paper I Contributed to the formulation of the research problem, performed all synthesis and radical trapping experiments, contributed significantly to the interpretation of the results and writing of the manuscript.

Paper II Contributed to the formulation of the research problem, performed the synthesis of the investigated compounds, contributed to the interpretation of the results and writing of the manuscript.

Paper III Performed the large scale trapping experiments, isolation and characterization of adducts, major contribution to the interpretation of the results and writing of the manuscript.

Paper IV Major contribution to the formulation of the research problem, performed or supervised all experimental work, major contribution to the interpretation of the results and writing of the manuscript.

Abbreviations

ACD	Allergic Contact Dermatitis
APC	Antigen Presenting Cells
BDE	Bond Dissociation Enthalpy
DEPMPO	5-diethoxy-phosphoryl-5-methyl-1-pyrroline *N*-oxide
EPR	Electron Paramagnetic Resonance
DCM	Dichloromethane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
Fe(III)TPPCl	5,10,15,20-tetraphenyl-21*H*,23*H*-porphine iron(III) chloride
GSH	Glutathione
HPLC	High Pressure Liquid Chromatography
LC/MS	Liquid Chromatography Mass Spectroscopy
LLNA	Local Lymph Node Assay
NAc-Cys-OMe	2*R*-Acetamido-3-mercaptopropanoic acid methyl ester
NMR	Nuclear Magnetic Resonance
PTSA	*p*-Toluenesulfonic acid
RT	Room temperature
S_N2	Substitution, Nucleophilic, 2^{nd} order
TEA	Triethylamine
TES	Triethylsilyl
TEMPO	2,2,6,6-Tetramethylpiperidine-1-oxyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TMIO	1,1,3,3-tetramethylisoindolin-2-yloxyl
TMS	Trimethylsilyl

Introduction

Redness, drying, swelling, itching, and blistering are some of the clinical manifestations that characterizes eczema, an inflammation in the skin that can have different causes [1]. One of these is the hypersensitivity to chemicals in our everyday environment. More commonly known as contact allergy, it affects 20% of the population in the western countries today [2]. Nickel and fragrance compounds are among the most common contact allergens. Contact allergy is a chronic disease, meaning that once a person is sensitized the only way to avoid the eczema is to avoid exposure to the allergen [3]. The level of exposure is affected by occupation, personal habits, the general use in society, and legislation; for example the use of scented or unscented cosmetic and hygiene products and an increased use of fragrances in everyday products. Legislation can limit the use of known allergens or demand clearer labeling to allow customers a conscious choice. One example is the European Union Cosmetics Directive [4] that requires labeling of cosmetic products and detergents for 24 individually named fragrances, if present above set concentration limits.

Fragrances are used, not only for their pleasant scent, but also to hide foul smell, in numerous everyday and industrial products such as soaps, shampoos, lotions, perfumes, degreasers, cutting fluids etc. The use of fragrances has increased during the last decades and this is accompanied by an increase in contact allergy to fragrances [5].

One of the most common fragrance compounds is *R*-limonene, which is not a contact allergen itself but forms allergenic compounds when exposed to air. Reactions to the oxidation mixture are seen in 2-3% of consecutive dermatitis patients in Europe [6-9]. Several oxidation products have been identified, among these the hydroperoxides have been shown to be strong allergens [10-12]. In order for the hydroperoxides to trigger the outbreak of eczema they have to bind to a protein in the skin [3]. This is believed to happen through a radical mechanism [13-17]. This thesis examines limonene hydroperoxides, what radicals they can form, their sensitizing capacities, and a possible mechanism for the immunogenic complex formation. The results presented expand the

knowledge of the mechanisms of contact allergy and allergic contact dermatitis to limonene hydroperoxides.

1.1 Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is the clinical manifestation of contact allergy [3]. It is caused by repeated exposure of chemicals to the skin and results in eczema. The immunologic response of ACD is cell-mediated, a mechanism that requires 24 – 72 hours from exposure to fully developed eczema in a sensitized person. This makes ACD a delayed type hypersensitivity reaction.

ACD involves two phases: the sensitization phase, when an individual becomes sensitized, and the elicitation phase, during which eczema develops. Both phases start with exposure to a hapten (Figure 1). Haptens are chemical compounds or metal ions with physicochemical properties that allow them to penetrate stratum corneum into epidermis and react with proteins. This generates immunogenic hapten-protein complexes, a prerequisite of ACD since the haptens are too small to elicit an immune response themselves [18]. The complexes are processed by antigen-presenting cells (APC) before they are presented as a hapten-modified peptide in association with major histocompatability complexes (MHC) on the cell surface [19]. If the immunogenic complex is formed outside the APC, it is internalized, processed via the exogenous pathway and presented as an antigen on MHC class II molecules to $CD4^+$ T-cells. Lipophilic haptens can enter the cell before forming the immunogenic complex. These internal complexes are processed via the endogenous pathway resulting in presentation on MHC class I molecules to $CD8^+$ T-cells. However, there is a large degree of overlap between the two routes from hapten to antigen, for instance can external immunogenic complexes be processed by the endogenous pathway [19], and the precise contribution of $CD4^+$ and $CD8^+$ cells in human ACD is unknown [20].

In the sensitization phase, a special type of APC situated in epidermis, Langerhans cells, will migrate to the lymph nodes where they present the hapten-modified peptides as antigens to naïve T-cells. Recognition of the antigen by the naïve T-cells causes them to mature to effector and memory T-cells that circulate the blood and lymphatic system. The

memory T-cells cells constitute an immunological memory as they will recognize the antigen on repeated exposure [3].

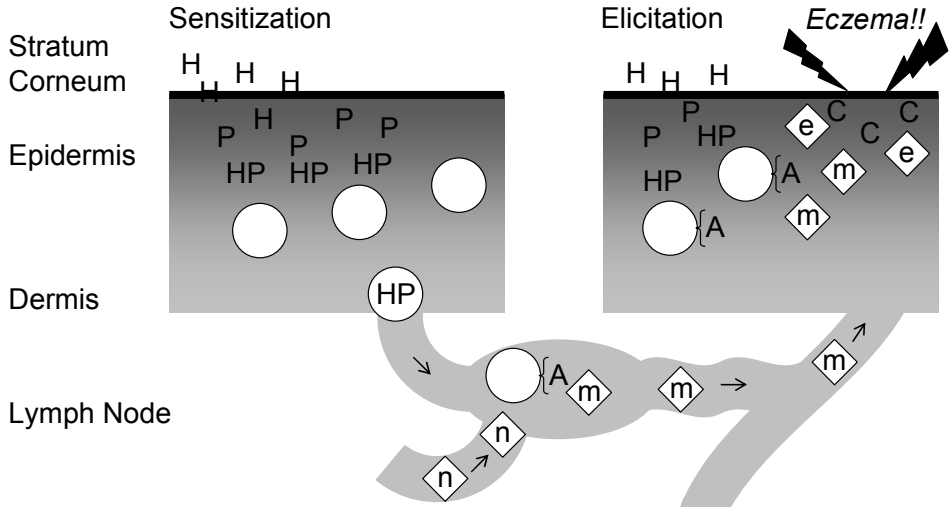


Figure 1. Schematic representation of the immunogenic mechanism of ACD. (A) Antigen, (C) inflammatory cytokines and chemokines, (e) effector T-cells, (H) hapten, (HP) immunogenic hapten-protein complex, (m) memory T-cells, (n) naïve T-cells, (P) protein, (○) antigen presenting cells (APC). In the sensitization phase, a special type of ACD called Langerhans cells will migrate from epidermis to the lymph node.

In the elicitation phase, re-exposure to the hapten results in formation of the same immunogenic hapten-protein complex that will be internalized, processed and presented by APC in the skin. Memory T-cells that recognize the antigen will now be activated by APC, resulting in formation of effector T-cells and the release of pro-inflammatory cytokines and chemokines. These substances cause and enhance the immunological response leading to the development of eczema at the site of exposure. Memory T-cells for a specific antigen circulate the body of a sensitized person in higher concentrations compared to the naïve T-cells specific for the same antigen in a non-sensitized person. Thus, a much lower concentration of the hapten is needed in the elicitation phase compared to the sensitization phase. If two different haptens form antigens that are so similar that the T-cells can not differentiate one from the other, the haptens are said to cross-react. This is evident when animals sensitized to one hapten react to another hapten [13, 21].

1.1.1 Formation of Immunogenic Complexes

The formation of immunogenic hapten-protein complexes, a prerequisite for the immunological mechanisms of ACD, is achieved by the formation of a covalent or coordination bond between the hapten and a protein in the skin [3]. The bond formation can proceed via different reactions and depends on the nature of the hapten.

Coordination reactions

Metals such as nickel, chromium, and cobalt form positively charged ions that readily accept electrons from the nucleophilic side chains of amino acids [22]. The ions are said to coordinate to the amino acids and the bonds are called coordination bonds.

Electrophilic – Nucleophilic reactions

The majority of organic compounds that cause ACD are electrophiles [23]. They form covalent bonds with nucleophilic amino acid side chains in reactions common in organic chemistry, for example S_N2, Michael addition or nucleophilic addition to carbonyls.

Radical Reactions

It has been proposed that for instance urushiols [24, 25] and hydroperoxides [13-17] (Figure 2), form immunogenic complexes via radical reactions. For hydroperoxides oxidation of proteins has been discussed as a possible mechanism for formation of unspecific immunogenic complexes. This would cause cross-reactivity between structurally different hydroperoxides. However, investigations show no cross-reactivity between such hydroperoxides, instead it is concluded that hydroperoxides form specific immunogenic complexes via hapten-protein binding [21]. For hydroperoxides the initial step of the immunogenic complex formation would be the homolytic cleavage of the oxygen-oxygen bond in the hydroperoxide group [26, 27]. This results in an alkoxyl radical that either reacts directly with a protein or rearranges into another radical that reacts with a protein. Rearrangement to other radicals is demonstrated by the formation of carbon-centered radicals from linalyl hydroperoxide [14, 17]. The alkoxyl radicals can also rearrange into haptens that react as electrophiles. One example of this is the formation of allergenic epoxides from 15-hydroperoxyabietic acid [13]. However, to the

best of our knowledge, no specific radical mechanisms have been proposed for the immunogenic complex formation of hydroperoxides.

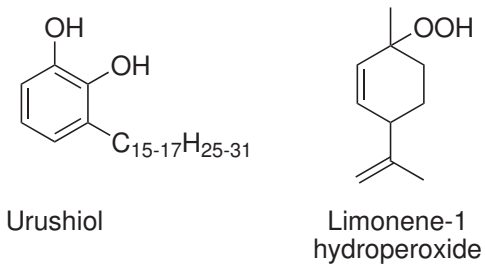


Figure 2. General structure of urushiol and an example of a hydroperoxide.

1.2 Radicals

Electrons reside in orbitals surrounding the atom nucleus. When atoms combine into molecules, the atomic orbitals combine into molecular orbitals. Each atomic or molecular orbital can contain two electrons and when they do so the specie is, in general, stable. Atoms, ions or molecules with orbitals that contain only one electron are called radicals and the lone electron is said to be unpaired. Radicals are highly reactive and react in such ways as to fill their half empty orbitals with electrons (Figure 3).

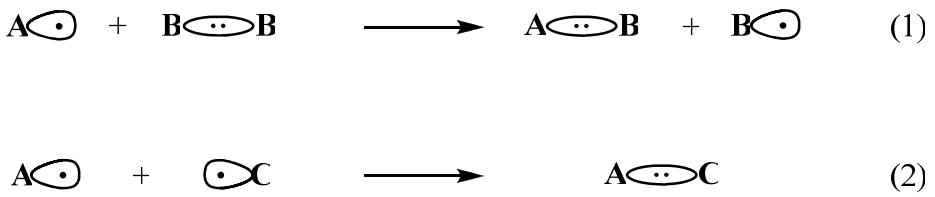


Figure 3. Schematic representation of how radicals react to fill their half-empty orbitals. (1) Radical (A·) will either abstract another radical (B·) from a non-radical specie (BB), creating a new radical (B·) and a new non-radical (AB) in the process, or (2) react with another radical (C·) to form a non-radical (AC).

1.2.1 Studying Radicals - Electron Paramagnetic Resonance

Observation of radicals is difficult as their high reactivity results in short life-times, but can be done using Electron Paramagnetic Resonance (EPR) Spectroscopy [28]. The basis of this technique is the interaction of the unpaired electron with micro-wave radiation in a

magnetic field. The electron spins align themselves parallel or anti-parallel to the magnetic field. This gives rise to two energy levels and by absorbing micro-wave radiation the state of the electrons can change from the lower to the higher level (Figure 4 left). The energy absorption is monitored and converted into a spectrum. The gap between the energy levels is dependent on the strength of the local magnetic field around the electrons. This is the sum of an external magnetic field and the magnetic fields originating from the spins of nearby nuclei in the molecule. The nuclear spins align themselves parallel or anti-parallel to the external magnetic field, thereby increasing or decreasing the local magnetic field. The radicals in a sample will be evenly distributed between nuclei increasing or decreasing the local magnetic field. Thus, the electrons of different radicals will have different local magnetic fields around them and will need different amounts of energy to change energy level. This is observed as a splitting of the EPR-signal, resulting in more than one peak in the spectrum (Figure 4 right). The size and number of the splitting is dependent on the identity of the nearby nuclei, as different nuclei have different magnetic moments and spins. Thus, information about the nuclei in the molecule close to the radical can be extracted from the EPR-spectrum and aid in the identification of the radical.

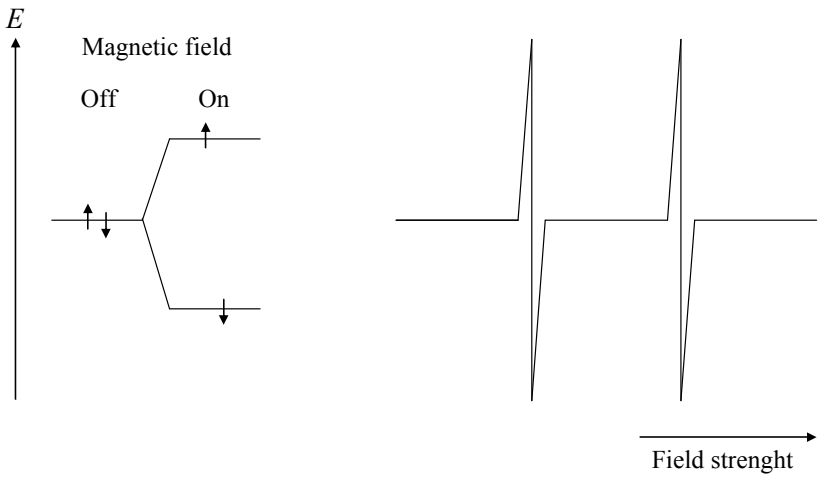


Figure 4. Schematic representations of the energy states of an electron in a magnetic field (left) and the splitting of the EPR-signal (right).

The majority of molecules are not EPR-active since they have no unpaired electrons. This constraint of the EPR-technique is also its advantage since it gives a high degree of

specificity and the possibility to observe radicals in complex matrices, e.g. biological samples. The generally short lifetime of radicals is countered by the use of spin-traps, compounds that react with the radicals and in doing so creates new radicals with longer lifetimes. Interaction with the nuclei of the spin-trap can give extra information about the identity and structure of the radical. Nitrones are among the most commonly used spin-traps (Figure 5) and a multitude of these and other spin-traps has been synthesized to be used in specific experiments [29].

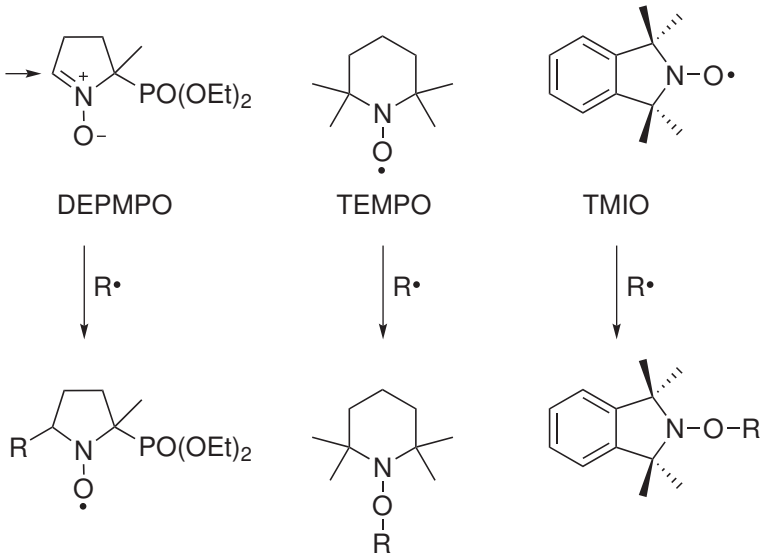


Figure 5. DEPMPO is a nitrone used in EPR spin-trapping; the arrow marks the position where radicals add. The new radical (bottom row, left) is delocalized over the oxygen and the adjacent nitrogen. TEMPO and TMIO are examples of stable radicals used as radical trappers (vide infra). (R•) Radical.

1.2.2 Studying Radicals - Radical Trapping

Indirect observation of radicals can be done by radical trapping. That is the formation of a stable compound by reaction of the radicals with a radical trapper (Figure 5). In theory, any molecule that reacts with a radical can be seen as a radical trapper. If the trapper doesn't contain unpaired electrons before the reaction with the radical, the new molecule will also be a radical (as in EPR spin-trapping). In reality the best radical trappers are radicals themselves. Molecules that contain an un-paired electron but for steric and electronic reasons have a low reactivity are called stable radicals (Figure 5). They are frequently used as radical trappers and the product is a non-radical. However, radical

trapping can be done without stable radicals as trappers, if so the formation of a stable product usually requires more than one reaction step. The stable products, and thus the former radical, can be purified and analyzed by conventional chromatographic and spectroscopic methods.

1.2.3 Radicals in biology

Radicals and radical reactions are of major importance in biology [30]. For most organisms they are necessary both for function and survival as well as constantly damaging the molecular components of the organism. Examples of the necessity are that many enzymes have a free radical located at their active site and that redox reactions are involved in both intra- and intercellular signaling. Examples of the damage caused by radicals are the oxidation of lipids, proteins and DNA, for instance the oxidation of unsaturated fatty acids [31]. This may lead to uncontrolled leakage through cell membranes, damage membrane proteins, and inactivate ion channels [30].

Numerous radicals are constantly produced and present in our cells. The level of radicals is balanced by antioxidants. If this balance is disturbed in such a way that the level of radicals increases, the cell is said to be under oxidative stress. The response from the cell depends on the severity of the oxidative stress. Mild stress can cause proliferation and adaptation whereas intense stress results in damage and cell death. The production of radicals is affected not only by disease or injury but also by xenobiotics. Radicals and oxidative stress are associated with many different diseases. However, very few cases are known where radicals are the primary cause of the disease, more commonly oxidative stress is a consequence of the disease [30].

In the context of ACD radicals are thought to be involved in the formation of immunogenic complexes from e.g. hydroperoxides [13-17] and urushiols [24, 25]. Hydroperoxides can be formed by autoxidation of terpenes (vide infra); a group of natural products that includes many fragrance compounds, commonly used in cosmetics and everyday products.

1.3 Terpenes

Terpenes form a diverse family of organic molecules made up of two or more isoprene units (Figure 6). Condensation of the isoprene units form the carbon skeletons of mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}) and tetraterpenes (C_{40}) [32]. These can be further modified to include closed rings or oxygen atoms. Terpenes are produced in a wide variety of plants and their pleasant smells make them ideal as scents. They are thus frequently used as fragrance compounds in perfumes, toiletries and household products. Well-known examples are geraniol, which is the scent of roses and the primary ingredient in rose oil, and linalool, which is responsible for the scent of lavender (Figure 6). The scent of citrus fruits most commonly originates from *R*-limonene, the major constituent of citrus peel oil. The oil is produced by pressing the citrus peel followed by distillation. *R*-Limonene is used not only in perfumes but also in cosmetic products, detergents, paints, degreasers, rinsing agents, and disinfectants in concentrations ranging from <0.1% to 100% [33]. Due to the presence of allylic positions, terpenes are prone to autoxidation (vide infra).

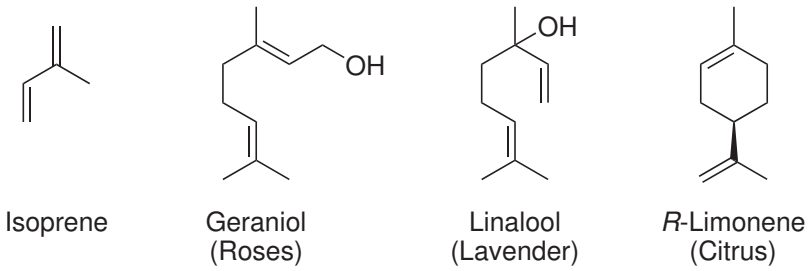


Figure 6. Structures of isoprene and three monoterpenes commonly used as fragrance compounds.

1.4 Autoxidation of Terpenes

Autoxidation is a radical chain reaction between an organic compound and molecular oxygen resulting in various oxidation products. It requires the parent compound to be in contact with molecular oxygen and an initiator such as metal ions, heat or ultraviolet light. Autoxidation of terpenes follows the mechanism of olefin oxidation [34, 35] generating hydroperoxides as the primary oxidation products (Figure 7). These can be

further oxidized to secondary oxidation products such as alcohols, aldehydes, ketones, epoxides etc.

The first step of the autoxidation sequence is the abstraction of a hydrogen atom from the parent compound by the initiator resulting in the formation of a radical. The ease and location of the hydrogen atom abstraction is strongly influenced by the stability of the formed radical. Thus, hydrogens in allylic positions and α-positions of heteroatoms are prone to abstractions due to the stabilizing effect of the allylic double bond and the heteroatom [36].

The second step is the reaction with molecular oxygen resulting in a peroxyl radical that abstracts another hydrogen atom, thus propagating the reaction, to form hydroperoxides as the primary oxidation product.

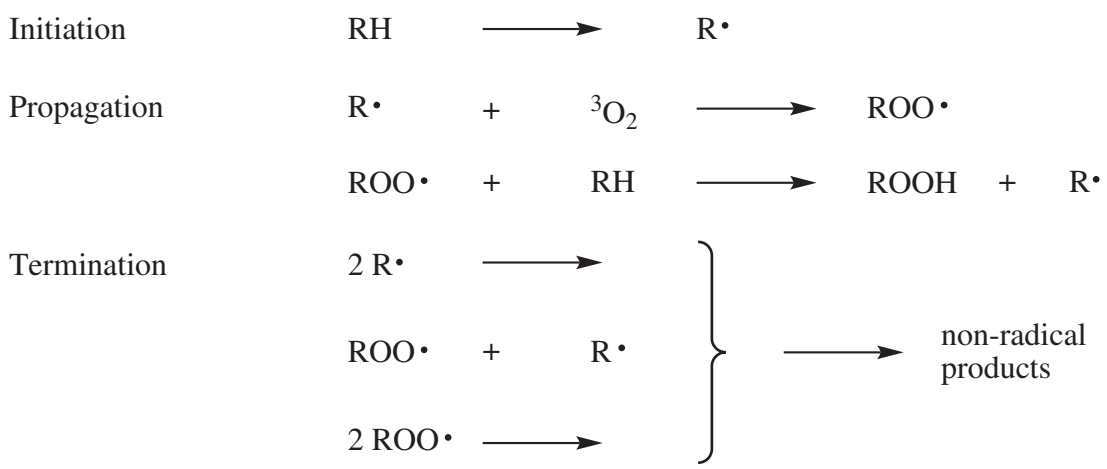


Figure 7. General mechanism for the formation of hydroperoxides and non-radical products via autoxidation.

1.5 Hydroperoxides

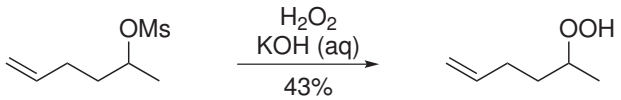
Hydroperoxides have the general formula ROOH were R is an organic structure. Hydroperoxides are mostly used as oxidants in organic chemistry, in recent years becoming increasingly important in the synthesis of enantiomerically pure compounds as chiral hydroperoxides can be used as induce asymmetry in the product [37]. The stability of hydroperoxides is largely dependent on the size of the R-group and the level of substitution of the hydroperoxide bearing carbon. Less than five carbon atoms per hydroperoxide make the hydroperoxide potentially explosive, whereas a large R-group and a high level of substitution generally mean a more stable hydroperoxide.

1.5.1 Synthesis of Hydroperoxides

Several methods for synthesis of hydroperoxides are available; all of these utilize reagents where the oxygen-oxygen bond is already present to construct the carbon-oxygen bond [37, 38].

Synthesis from hydrogen peroxide or the hydrogen peroxide anion

Both hydrogen peroxide and the hydrogen peroxide anion are strong nucleophiles which can be used in substitution reactions together with e.g. alcohols, carboxylates, halides, and sulfonates to give hydroperoxides (Scheme 1). Primary and secondary hydroperoxides can be made from halides or sulfonates under S_N2-conditions [12, 39]; yields are sometimes low due to the base sensitivity of these hydroperoxides. Higher yields of primary, secondary, and tertiary hydroperoxides can be achieved by reaction of bromides or iodides with hydrogen peroxide in the presence of silver trifluoroacetate or silver tetrafluoroborate.



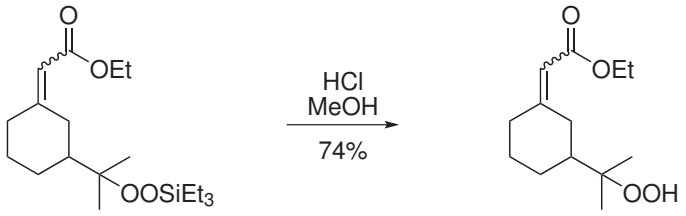
Scheme 1. Example of synthesis of hydroperoxide from sulfonate and hydrogen peroxide [39].

Synthesis from superoxide anion

The superoxide anion can also be used in S_N2-reactions together with halides or sulfonates followed by reduction and protonation to yield hydroperoxides [40, 41]. DMF is a good solvent for this reaction that sometimes suffers from low yields due to competing formation of alcohol and dialkyl peroxide.

Synthesis from peroxide precursors

Peracetals, peraminals, perketals, peroxyesters, and silyl peroxides can be converted to hydroperoxides (Scheme 2). Reaction conditions are harsh for peracetals whereas they are mild for perketals, peroxyesters, and silyl peroxides [37, 42]. Acidic hydrolysis of peroxyesters in the presence of bis(tributyltin)oxide is a good way of making primary hydroperoxides, as these conditions avoid the base-catalyzed decomposition of hydroperoxides and results in good yields. Racemic hydroperoxides can be resolved by conversion to peracetals or perketals followed by separation and re-conversion.



Scheme 2. Example of synthesis of a hydroperoxide from silyl peroxide [42].

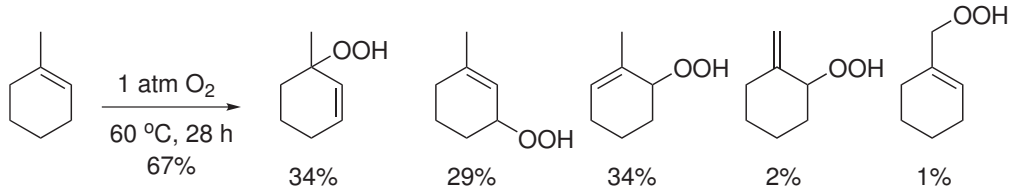
Synthesis by ozonolysis

Reaction of alkenes with ozone gives carbonyl oxides [37]. Reaction of this intermediate with alcohol or water yields α-alkoxyhydroperoxides and 1-hydroxyhydroperoxides, respectively [43, 44].

Synthesis by autoxidation

Autoxidation is the spontaneous radical reaction of hydrocarbons with molecular oxygen (vide supra). The reaction is promoted by high concentration of the substrate and stabilization of the initially formed radical [38]. Hydroperoxides are formed by reaction of this radical with molecular oxygen, generating a peroxyl radical that abstracts a proton

to furnish the hydroperoxide. Enols, phenols, hydrazones, imines, alkenes, dienes and polyenes can all serve as substrates (Scheme 3).



Scheme 3. Example of synthesis of allylic hydroperoxides by autoxidation [45]. Individual yields are reported as proportion of total peroxide yield (67%), with a conversion of 30%.

Synthesis by photooxidation

In photooxidation an alkene is transformed into an allylic hydroperoxide in an ene-type reaction [46, 47]. The active reagent is singlet oxygen which is generated from triplet oxygen by triplet sensitizers such as Rose Bengal, methylene blue or porphyrins. The products and product distribution obtained in a photooxidation can differ from the products of autoxidation of the same substrate.

Synthetic methods used in this thesis

The hydroperoxides investigated in this thesis were synthesized in substitution reactions or from silyl peroxides. Substitution started from an alcohol, a sulfonate or a hydrazine using hydrogen peroxide or the hydrogen peroxide anion as nucleophiles (Papers I-IV). Silyl peroxides were generated from alkenes and converted to hydroperoxides by acidic hydrolysis (Paper IV).

1.5.2 Hydroperoxides in Reactions with Iron(III) Porphyrins

Hydroperoxides are believed to form immunogenic complexes in the skin via a radical mechanism [13-17]. This makes it interesting to study the radical formation of hydroperoxides in reactions mimicking their metabolism. Hydroperoxides are metabolized by cytochrome P450 enzymes [26, 27] and iron(III) porphyrin complexes are frequently used as biomimetic models for these enzymes [48 and references therein].

Cytochrome P450 is a large family of metabolic enzymes present mainly in the liver but also in other tissues, e.g. the skin [49]. The P450s are responsible for a large number of metabolic transformations; one of these is the cleavage of the oxygen-oxygen bond in

hydroperoxides. The active site of the P450s contains a heme unit [26] and as biomimetic models iron(III) porphyrin complexes have been extensively studied. Investigations presented in the literature regarding reactions of iron(III) porphyrins with hydroperoxides conclude that the oxygen-oxygen bond can be cleaved either homolytically or heterolytically [48 and references therein]. Which of the reaction pathways that dominate is dependent on the reaction conditions, the structure of the hydroperoxide and the electronic properties of the iron(III) porphyrin complex. Hydroperoxides with electron-donating alkyl groups, for example *t*-butyl hydroperoxide, and electron-rich porphyrin complexes, such as Fe(III)TPPCl (Figure 8), will promote the homolytic cleavage of the oxygen-oxygen bond [48].

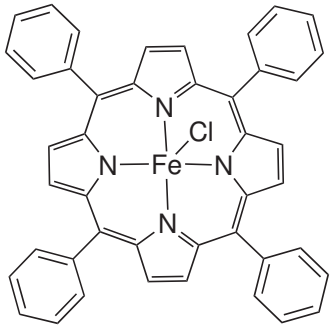


Figure 8. The iron(III) porphyrin complex used in this work. Studies by Nam *et. al.* shows that this complex cleaves the oxygen-oxygen bond of *t*-butyl hydroperoxide homolytically [48].

1.5.3 Hydroperoxides in Allergic Contact Dermatitis

In the context of ACD, hydroperoxides have received attention since the middle of the 20th century when eczema among painters was attributed to hydroperoxides in turpentine [50] Hydroperoxides from Δ^3-carene were identified as the "eczematogenic factor" but no structure was reported [51-54]. Hydroperoxides are formed in the autoxidation of terpenes and it has been shown that the oxidation mixtures of colophony, limonene, linalool, and geraniol are sensitizing [55-58]. Individual oxidation products have been tested and hydroperoxides have been demonstrated to be strong sensitizers [12, 47, 58]. The oxidation of terpenes has a clinical relevance as positive reactions to both oxidation mixtures of terpenes and hydroperoxide fractions from these mixtures are observed in dermatitis patients [6, 59, 60].

1.6 Diagnosis of Contact Allergy in Patients

Diagnosis of contact allergy in patients is performed by so called patch testing. This means that the most well known contact allergens are applied to the skin under controlled forms [61]. The compound or mixture of interest is dissolved in white petrolatum and applied on the upper back of the patient in a small aluminum cup held in place by adhesive tape. The concentration is chosen to provoke an allergic reaction in a sensitized patient, while simultaneously causing minimal risk of sensitizing a non-sensitized patient. The test material is left under occlusion for 48 h and the reaction is evaluated twice, on day 2-4 and on day 5-7. Reactions are classified based on their morphological characteristics according to the scale in Table 1. Patients are tested for contact allergy using a base-line series containing the most common compounds or mixtures of compounds that cause ACD. Additional compounds commonly used, in for instance different professions, may be added if considered appropriate.

Table 1. Morphological characteristics of patch test reactions.

Classification	Reaction	Morphological characteristics
−	Negative	
irr	Irritant	Irritant reaction of different types
?	Doubtful	Faint erythema only
+	Weak or moderate positive	Erythema, infiltration, possibly papules
++	Strong positive reaction	Erythema, infiltration, papules, vesicles
+++	Very strong positive	Intense erythema, infiltration, coalescing vesicles,

1.7 Local Lymph Node Assay

The local lymph node assay (LLNA) is a method for estimation of the sensitizing capacity of a compound [62, 63], accepted and recommended by both the U.S. Food and Drug Administration (FDA) and the Organisation for Economic Co-operation and Development (OECD). It is an animal test where mice in different groups are subjected to different concentrations of the compound under investigation (Figure 9). The basis of the LLNA is that if the immune system of the mice responds to the compound, the cells of

the local lymph nodes proliferate and in this process they incorporate thymidine. Through the administration of radioactive labeled thymidine it is possible to measure the proliferation in the lymph nodes. A strong sensitizer induces more proliferation compared to a weak sensitizer. For each concentration a stimulation index (SI) is calculated, this is the proliferation in the test group divided with the proliferation in the control group. The final outcome of a LLNA experiment is an EC3-value. This is defined as the concentration (% w/v) of compound where the proliferation of cells in the lymph nodes is three times as high as in the control group. Ranging from 0% to 100% it is a measurement of the sensitizing capacity of the compound, lower EC3-value means a stronger sensitizer. Sensitizing compounds are roughly divided in four classes; 0 – 0.1% extreme, 0.1 – 1% strong, 1 – 10% moderate and 10 – 100% weak or non-sensitizing [64].

Figure 9. The local lymph node assay (LLNA). The compound to be tested is dissolved in a vehicle, usually acetone/olive oil (4/1 v/v) and applied on the back of the ears of mice on day 0, 1, and 2. The mice are divided into 3 – 5 groups with 3 – 5 mice in each. The different groups receive different concentrations of the compound, from no compound (control group) up to pure compound (no vehicle) depending on the expected sensitizing capacity of the compound. On day 5 [methyl-^{3}H]-thymidine is injected in the tail vein, after five hours the mice are sacrificed and the draining auricular lymph nodes from each ear are excised. The lymph nodes from all mice receiving the same concentration of compound are pooled and single-cell suspensions are prepared. The incorporation of the radioactive thymidine is measured and the stimulation indexes and EC3 value are calculated [62, 63].

Aims of the Thesis

The overall aim of the work presented in this thesis was to provide knowledge about the mechanism of immunogenic complex formation of limonene hydroperoxides in allergic contact dermatitis. The purpose was to investigate the relation between structure, radical formation, and sensitizing capacity of limonene hydroperoxides and structural analogues.

Specific aims were to study:

- The radical formation of limonene hydroperoxides and structural analogues (Papers I and IV).
- The sensitizing capacity of limonene hydroperoxides and structural analogues (Papers I and IV).
- If there is a significant difference in sensitizing capacities of three allylic hydroperoxides in mice and if any found difference in sensitizing capacities is clinically relevant for the two major hydroperoxides occurring in the oxidation mixture of limonene (Paper II).
- The formation of adducts between limonene-2-hydroperoxide and cysteine as a model for immunogenic complex formation (Paper III).

Results and Discussion

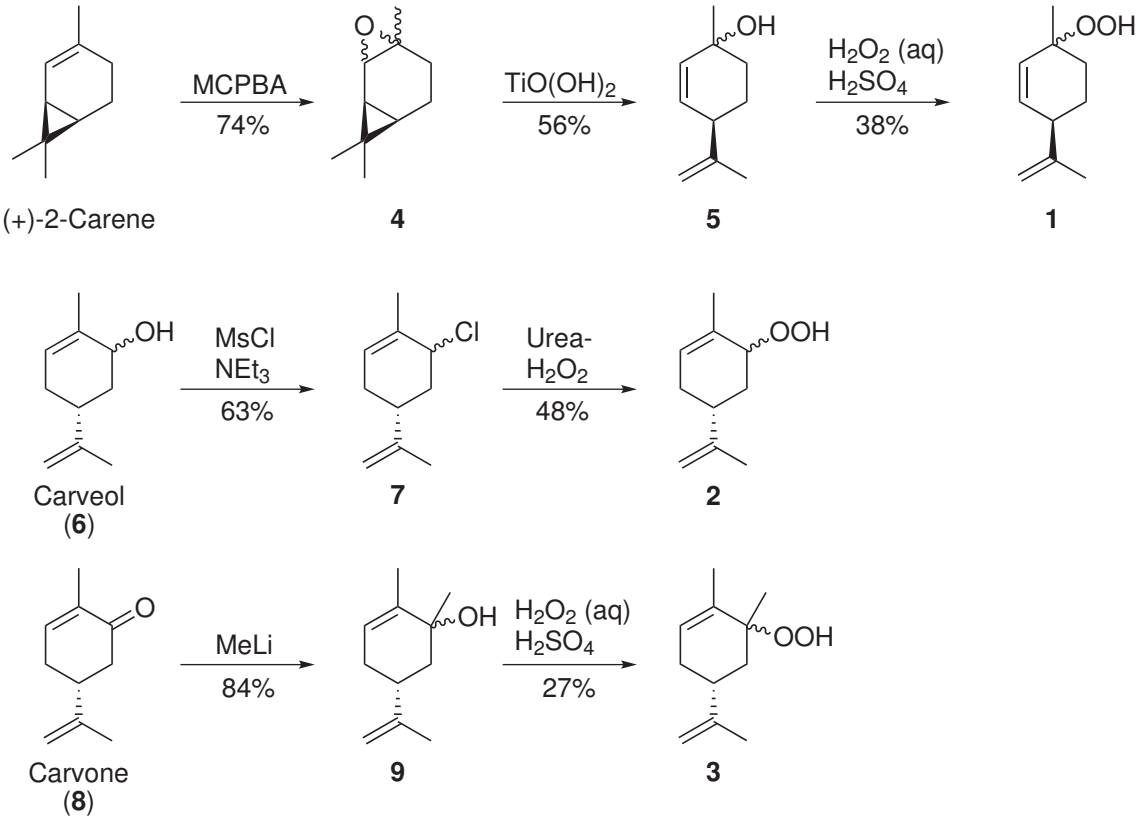
3.1 Radical Formation and Sensitizing Capacity of Allylic Limonene Hydroperoxides (Paper I)

The increased use of scented products has caused an increase in ACD to fragrance compounds [5]. Among the most commonly used fragrance compounds is *R*-limonene, which is not a sensitizer itself, but forms allergenic hydroperoxides **1** (limonene-1-hydroperoxide, Scheme 4) and **2** (limonene-2-hydroperoxide) on air-exposure. The formation of immunogenic complexes between the hapten and a protein in the skin is a prerequisite of ACD; for hydroperoxides a radical mechanism is postulated for this reaction [13-17].

The aim of this paper was to study the radical formation and sensitizing capacity of limonene hydroperoxides. Three hydroperoxides were included in the study. Two of them (**1** and **2**) are naturally occurring in the autoxidation mixture of limonene; the third (**3**) is a synthetic analogue, included in order to further study the difference between secondary and tertiary hydroperoxides. Formed radicals are potentially the chemical entities that form covalent bonds to proteins in the skin and thereby immunogenic complexes of hydroperoxides.

Hydroperoxide **1** was synthesized from (+)-2-carene by epoxidation and subsequent rearrangement of the epoxide (**4**) into the corresponding alcohol (**5**, Scheme 4). Acid catalyzed treatment with hydrogen peroxide furnished the hydroperoxide. Hydroperoxide **2** was synthesized from carveol (**6**) via the corresponding chloride (**7**) in two substitution reactions, utilizing methanesulfonyl chloride and urea-hydrogen peroxide adduct as reagents. Hydroperoxide **3** was synthesized from carvone (**8**) by addition of a methyl group to furnish the corresponding alcohol (**9**) which was converted into the hydroperoxide by acid catalyzed treatment with hydrogen peroxide. To the best of our knowledge synthetic procedures for hydroperoxides **1** and **3** have not been published before whereas a similar synthesis for hydroperoxide **2** is known [12]. The reaction

pathways produced moderate overall yields but were readily scaled up to produce sufficient amounts of hydroperoxides for further investigations.



Scheme 4. Synthesis of Allylic Hydroperoxides **1**, **2** and **3**.

The sensitizing capacity was tested in the LLNA. All of the hydroperoxides were found to be potent sensitizers with the following EC3-values: **1** 0.019 M (0.33%), **2** 0.049 M (0.83%) and **3** 0.071 M (1.29%). These sensitizing potencies correspond to previously tested hydroperoxides [14, 15, 47, 58].

The radical formation was studied in radical trapping experiments (Figure 10) and with EPR spectroscopy (vide infra). The trapping experiments were performed in a 1:1 mixture of acetonitrile and water, using 1.1 equivalent of Fe(III)TPPCl as radical initiator and 2 equivalents of TMIO as radical trapper. The oxygen-oxygen bond of the hydroperoxide group was cleaved homolytically with Fe(III)TPPCl. Reactions and radical rearrangement resulted in non-radical products and carbon centered radicals, of which the latter were trapped by TMIO.

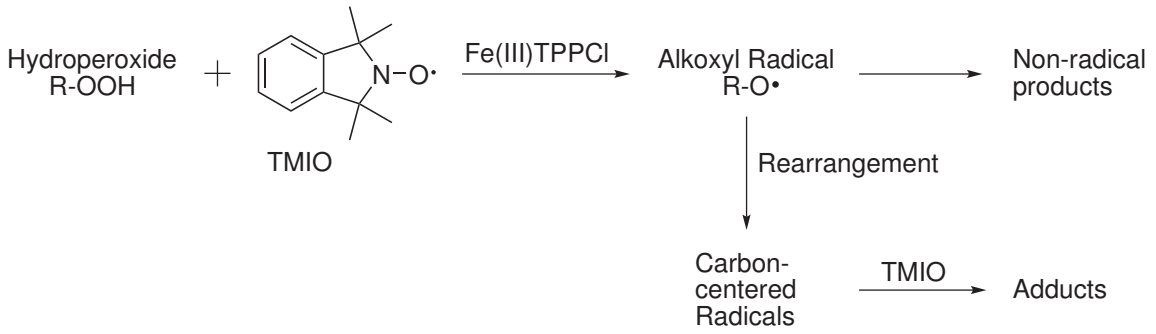
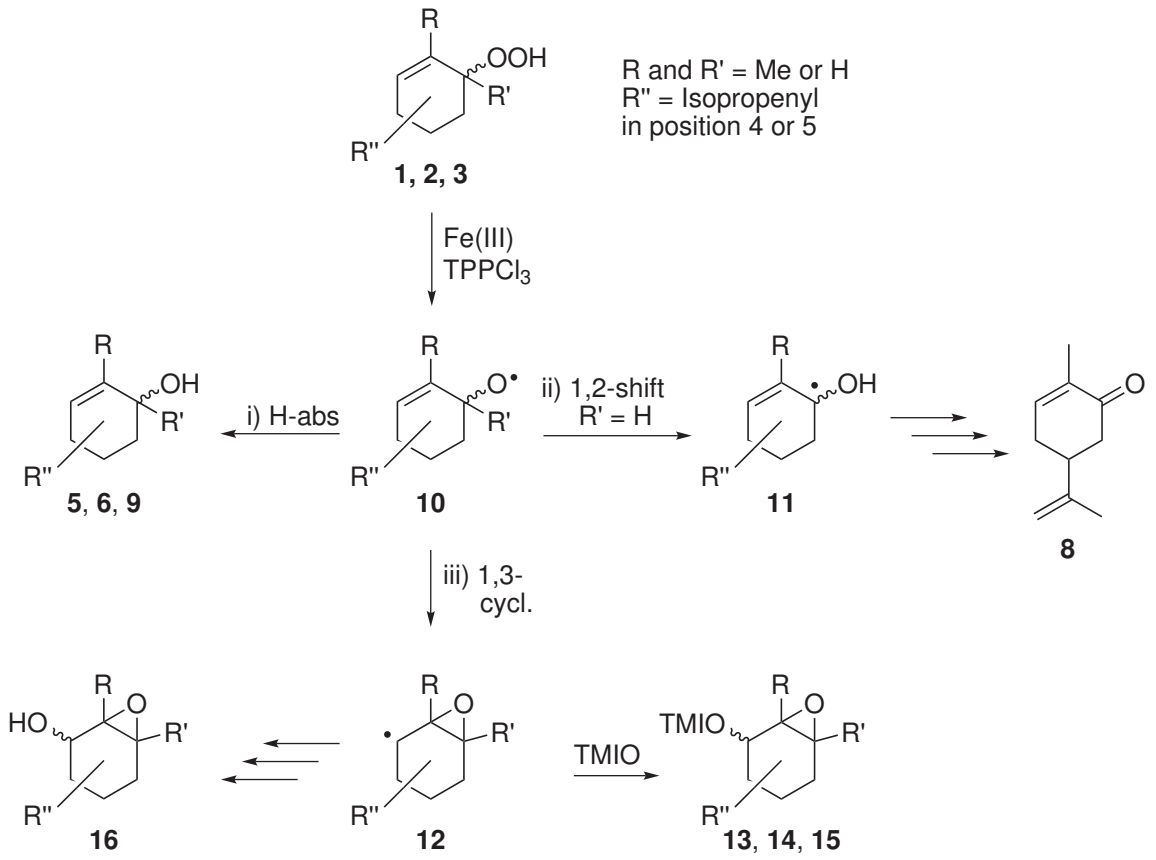


Figure 10. Schematic representation of the TMIO experiments performed in Papers I and IV.

The initial cleavage of the oxygen-oxygen bond creates oxygen centered alkoxyl radicals (**10**, Scheme 5). This radical can react or rearrange according to several different pathways.



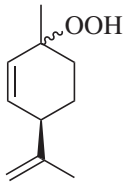
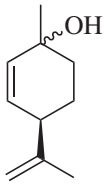
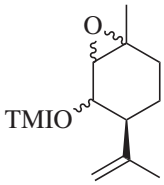
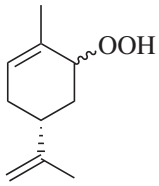
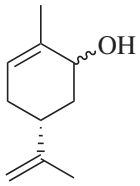
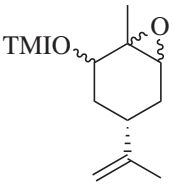
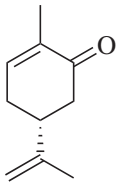
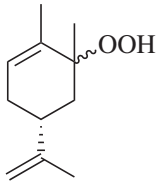
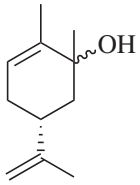
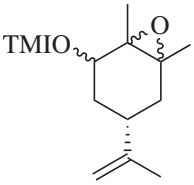
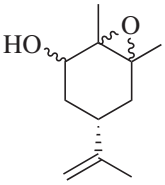
Scheme 5. Mechanistic proposal for the formation of products identified in the trapping experiments with hydroperoxides **1**, **2** and **3**.

Products from three major pathways have been identified: pathway i) hydrogen abstraction resulting in the corresponding alcohol (**5**, **6**, **9**); pathway ii) 1,2-shift resulting in a 1-hydroxyallyl radical (**11**); and pathway iii) 1,3-cyclization resulting in a oxiranylcarbinyl radical (**12**). In pathways ii) and iii) the formed radicals react further to form non-radical products that have been isolated and identified. The outcome of the trapping experiments is governed by the balance between the different pathways, which is in turn governed by the structure of the parent hydroperoxides.

Hydroperoxide **1** reacted according to pathways i) and iii), resulting in the corresponding alcohol (**5**) and the TMIO-adduct (**13**) of the oxiranylcarbinyl radical being formed in approximately equal amounts (Table 2). No products formed by pathway ii) were detected. This is in accordance with **1** being a tertiary hydroperoxide and the 1,2-shift requiring a hydrogen atom in position 2.

The products isolated and identified in the radical trapping experiments with hydroperoxide **2** corresponds to all three pathways. Since **2** is a secondary hydroperoxide alkoxyl radical **10** can react according to pathway ii). The rapid 1,2-shift of this pathway and the following reactions results in carvone (**8**) being the major product in the trapping experiment with hydroperoxide **2**. Small amounts of alcohol **6** (carveol) and the 1,3-cyclization product **14** were also isolated and identified. The ratio of the products was approximately 30:2:1, favoring carvone over carveol and the TMIO-adduct. No epoxidized products were isolated in the trapping experiment with hydroperoxide **2**. This indicates that Fe(III)TPPCl cleaves the oxygen-oxygen bond of the secondary hydroperoxide **2** homolytically [48].

Table 2. Product distribution in the radical trapping experiments with TMIO and allylic hydroperoxides **1**, **2** and **3**; %-values correspond to purified yields.

Hydroperoxides	Products		
 **1**	 **5** 27%	 **13** 22%	
 **2**	 **6** 2.5%	 **14** 1.2%	 **8** 34%
 **3**	 **9** 1.5%	 **15** 34%	 **16** 3.2%

In the trapping experiment with hydroperoxide **3** three different products formed by pathways i) and iii) were isolated and identified: the corresponding alcohol (**9**) and two products originating from the oxiranylcarbinyl radical, the TMIO-adduct (**15**) and the epoxy alcohol (**16**). The products were formed in a 1:25 ratio, favoring the oxiranylcarbinyl derived products. Similar to hydroperoxide **1** the 1,2-shift of pathway ii) is blocked since **3** is a tertiary hydroperoxide.

The formation of immunogenic hapten-protein complexes of hydroperoxides is proposed to follow a radical mechanism [13-17]. The alcohols (**5**, **6**, **9**) identified in the radical trapping experiments is a measure of the amount of alkoxyl radicals (**10**) available for this reaction. Likewise, the amount of TMIO-adducts (**13**, **14**, **15**) and the epoxy alcohol

(**16**) is a measure of the amount of oxiranylcarbinyl radicals available for the same reaction.

Low amounts of alcohol **6** (carveol) and TMIO-adduct **14** were isolated from the trapping experiment with hydroperoxide **2**. This indicates low amounts of alkoxyl radicals available for formation of an immunogenic complex. The high amount of carvone can not account for the sensitizing capacity of hydroperoxide **2** since carvone is a weak sensitizer [65]. Formation of carvone is proposed to proceed via the 1-hydroxyallyl radical **11** but no adducts with this radical was isolated.

High amounts of products derived from the oxiranylcarbinyl radical were isolated in the trapping experiment with hydroperoxide **3**. Even so, there is no substantial difference in the sensitizing capacities of hydroperoxides **2** and **3**. This might indicate the importance of the oxygen centered alkoxyl radical, since roughly equal amounts of alcohol were detected in the respective trapping experiments.

Hydroperoxide **1** displays the highest amount of alcohol as well as the highest total amount of products in the trapping experiments. Since the experiments with hydroperoxides **2** and **3** indicate that the oxygen centered alkoxyl radicals may be more important compared to the carbon-centered radicals, this result indicates that hydroperoxide **1** may be a more potent sensitizer compared to hydroperoxides **2** and **3**.

The reactions following cleavage of the oxygen-oxygen bond in the hydroperoxides were studied in EPR experiments (Section 1.2.1). Experiments were carried out in acetonitrile or chloroform at temperatures ranging from 220 to 283 K and the samples were continuously flowed through a flat quartz cell where they were irradiated with a mercury-xenon lamp to initiate the radical reactions. The first experiments were carried out without a spin-trap present and peroxyl radicals were detected from all three hydroperoxides. This radical can be formed by hydrogen abstraction from the hydroperoxide group by alkoxyl or hydroxyl radicals formed by the cleavage of the oxygen-oxygen bond of another hydroperoxide. In experiments with the tertiary hydroperoxides **1** and **3** in the presence of the spin-trap DEPMPO (Figure 5, Section 1.2.1) the same peroxyl radicals were detected. When performing the same experiment with hydroperoxide **2**, two different radicals were detected: the peroxyl radical and a

carbon-centered radical. As the carbon-centered radical was only detected from hydroperoxide **2** it may be the 1-hydroxyallyl radical (**11**) that forms via a 1,2-shift from the initially formed alkoxyl radical.

In summary, all three hydroperoxides formed large amounts of radicals and were found to be potent sensitizers according to the LLNA. The identities and amounts of the individual radicals were clearly affected by the structure of the hydroperoxides. The product distribution in the radical trapping experiments indicates that the alkoxyl radicals may be more important compared to the carbon-centered radicals in the immunogenic complex formation.

3.2 Limonene Hydroperoxide Analogues Differ in Allergenic Activity (Paper II)

The fragrance compound *R*-limonene readily autoxidizes on air-exposure. The oxidation mixture causes positive patch test reactions in 2-3% of consecutive dermatitis patients [6-9] and hydroperoxides formed in the autoxidation of limonene have been shown to be strong sensitizers [9, 12 and Paper I]. Hydroperoxides are believed to form immunogenic complexes via a radical mechanism [13-17] and Paper I revealed the formation of high amounts of radicals from limonene hydroperoxides.

The aim of this paper was to further investigate the sensitizing capacities of the limonene hydroperoxides from Paper I together with pure and oxidized limonene. The sensitizing capacities of pure and oxidized limonene as well as the individual oxidation products were determined in the LLNA. In addition, limonene hydroperoxides **1**, **2** and **3** (Scheme 4) were tested in a modified LLNA including non-pooled lymph nodes and statistical analysis to investigate if there was a significant difference in the sensitizing capacities of the hydroperoxides. Clinical studies were performed using both oxidized limonene and the pure limonene hydroperoxides **1** and **2** to investigate the clinical relevance of the results from the modified LLNA.

The sensitizing capacity of limonene is markedly increased by air-exposure and the subsequent oxidation (Figure 11). Pure limonene has an EC3-value of 2.2 M (30%) whereas limonene oxidized for 10 weeks has an EC3-value of 0.22 M (3.0%). Testing of the individual oxidation products reveals that the hydroperoxides have the highest sensitizing capacities [65, 66 and Paper I]. Thus, the high sensitizing capacity of the oxidation mixture is mainly attributed to the hydroperoxides.

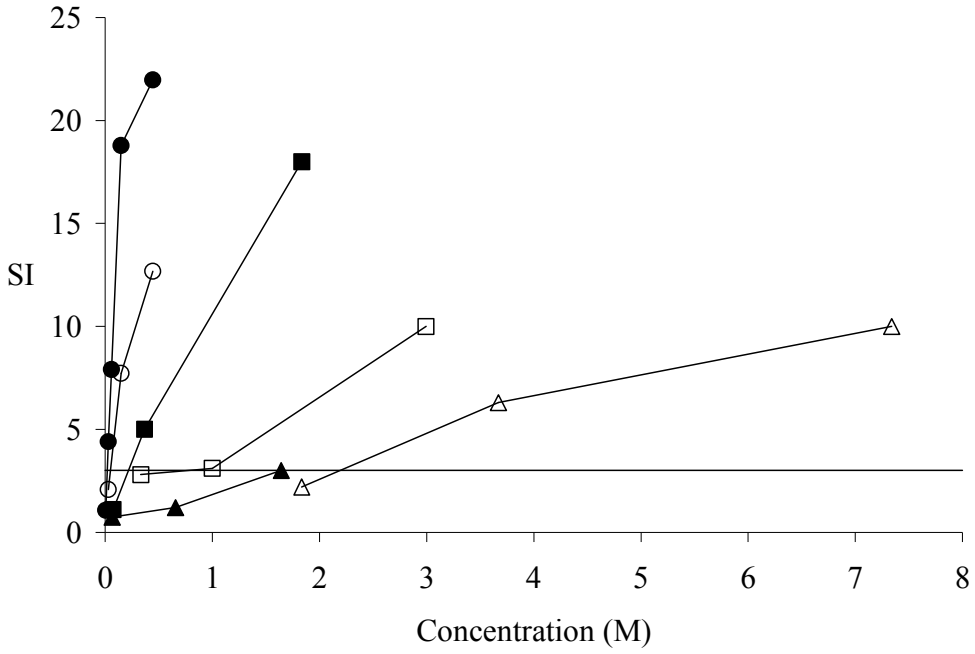


Figure 11. LLNA-results for pure and oxidized limonene and individual autoxidation products. (●) Hydroperoxide **1**, (○) hydroperoxide **2**, (■) *R*-limonene oxidized for 10 weeks, (□) *R*-carvone, (▲) limonene epoxide, (Δ) *R*-limonene. Stimulation index (SI) is the quotient of the proliferation between the test groups and the control group. The horizontal line marks a stimulation index of 3, the cut-off limit for a compound to be considered a sensitizer. The EC3-values are the concentrations were the curves intersect the horizontal line.

Hydroperoxides **1**, **2** and **3** were tested in a modified LLNA. In the ordinary LLNA the lymph nodes from all mice receiving the same concentration of compound are pooled before single-cell suspensions are prepared and the proliferation is measured. In the modified LLNA single-cell suspensions were prepared of the lymph nodes from each individual mouse which made it possible to perform a statistical analysis. The result of this analysis show that hydroperoxide **1** have a significantly higher sensitizing capacity compared to hydroperoxides **2** and **3** ($P = 0.0008$, Figure 12), that there is no statistical difference between hydroperoxides **2** and **3**, and that all hydroperoxides differ significantly from the controls ($P = 0.0008$). This modification of the LLNA offers a new possibility to compare compounds with similar sensitizing capacities and has, to the best of our knowledge, not been published before.

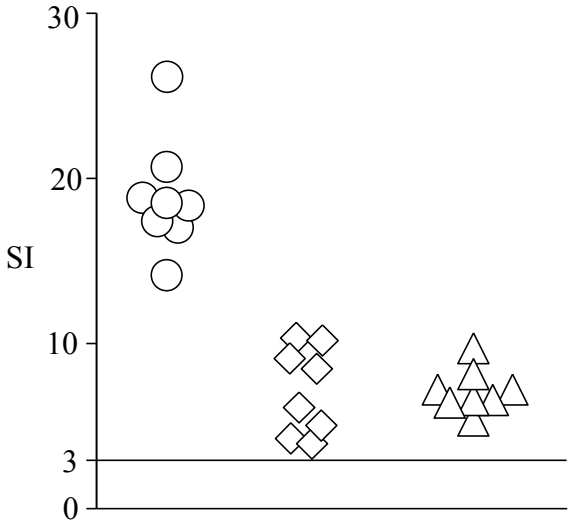


Figure 12. Results from the modified LLNA. (○) Hydroperoxide **1**, (◊) hydroperoxide **2**, (Δ) hydroperoxide **3**, (SI) stimulation index. The horizontal line marks a stimulation index of 3, the cut-off limit for a compound to be considered a sensitizer.

In the clinical study, seven patients that had previously reacted positive to oxidized limonene were retested with pure limonene, oxidized limonene and different concentrations of limonene hydroperoxides **1** and **2**. All patients displayed positive reactions to hydroperoxide **1**, whereas only three patients reacted to hydroperoxide **2**. Reactions to lower concentrations of hydroperoxide **1** compared to hydroperoxide **2** were seen in all patients that reacted to both hydroperoxides (Table 3). This shows the clinical relevance of the results from the modified LLNA.

Table 3. Results from clinical testing with pure and oxidized limonene and hydroperoxides **1** and **2**.

	Number of patients tested	Number of positive reactions
Limonene	7	0
Oxidized limonene	7	6
Hydroperoxide **1**	7	7
Hydroperoxide **2**	7	3

The impact of the hydroperoxides on the sensitizing capacity of the oxidized limonene is demonstrated by the LLNA experiments with pure and oxidized limonene and the individual oxidation products. Hydroperoxides **1** and **2** have the highest sensitizing capacities of the compounds tested in the LLNA and the oxidized limonene has a higher sensitizing capacity compared to carvone and limonene oxide (Figure 11). These results are in accordance with previous testing of oxidation mixtures and oxidation products of linalool and geraniol [47, 57, 58].

In Paper I the radical formation of hydroperoxides **1**, **2** and **3** was studied. It was found that both hydroperoxide **1** and **3** formed high amounts of carbon-centered radicals, and that hydroperoxide **1** formed almost 20 times more alkoxyl radicals compared to hydroperoxide **3**. The significantly higher sensitizing capacity of hydroperoxide **1** and the higher number of positive reactions to hydroperoxide **1** in the clinical tests further indicates the importance of the alkoxyl radicals in the formation of immunogenic complexes of limonene hydroperoxides.

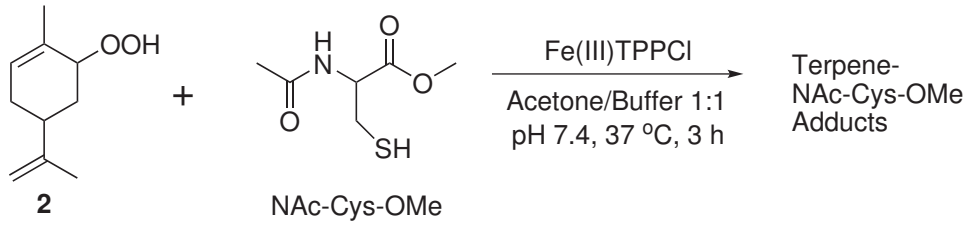
In summary, the sensitizing capacity of limonene is markedly affected by oxidation and the sensitizing capacity of the oxidation mixture is mainly attributed to the hydroperoxides. Hydroperoxide **1** was shown to be a significantly stronger sensitizer compared to hydroperoxides **2** and **3** in the modified LLNA. This proved clinically relevant as more positive reactions to hydroperoxide **1** were found in dermatitis patients. In combination with the results from Paper I this indicates the importance of the alkoxyl radicals in the immunogenic complex formation of limonene hydroperoxides. The finding that structurally related hydroperoxides differ significantly in sensitizing potential refutes the proposal of formation of unspecific antigens and supports formation of specific antigens from hydroperoxides [21].

3.3 Identification of a Radical Mechanism for Formation of Immunogenic Complexes (Paper III)

It has been discussed that hydroperoxides would form immunogenic complexes by an unspecific oxidation of proteins. This suggestion has been refuted by the lack of cross-reactivity between structurally different hydroperoxides [21], a finding that strongly indicates the formation of specific immunogenic complexes, which is proposed to follow a radical mechanism [13-17]. Hydroperoxide **2**, a strong sensitizer in the LLNA, forms large amounts of radicals but only weakly allergenic products in the radical trapping experiment presented in Paper I.

The aim of this paper was to further investigate the formation of immunogenic hapten-protein complexes of olefinic hydroperoxides via a radical mechanism. This was done by studying the formation of adducts between **2** and protected amino acids or glutathione (GSH). Radical reactions were initiated by Fe(III)TPPCl and formed adducts were identified by LC/MS and NMR.

Reactions between hydroperoxide **2** and the amino acids or GSH were initiated by a catalytic amount of Fe(III)TPPCl in a 1:1 mixture of acetone and phosphate buffer at 37 °C. An initial screening was performed using glutathione and protected alanine, leucine, cysteine, histidine, lysine, tryptophan and tyrosine. LC/MS analysis of the reaction mixtures revealed large amounts of adducts in the experiments with cysteine and GSH, whereas no or only small amounts of adducts were detected with the other amino acids. Therefore, the cysteine experiment was scaled up to isolate sufficient material for NMR-analysis (Scheme 6).



Scheme 6. Reaction of hydroperoxide **2** with Fe(III)TPPCl in the presence of NAc-Cys-OMe.

The scaled up reaction mixture was fractionated by preparative HPLC. MS-analysis identified possible adducts in four fractions that were subjected to NMR-analysis resulting in two fully characterized adducts (Figure 13). The first adduct (**17**) corresponds to the Michael addition product of NAc-Cys-OMe to carvone. This compound was also synthesized as a reference compound in a Michael addition reaction. The second adduct (**18**) is the result of addition of the thiol group of NAc-Cys-OMe over the double bond in the isopropenyl group of carvone. The formation of this adduct can not be explained by an ionic reaction.

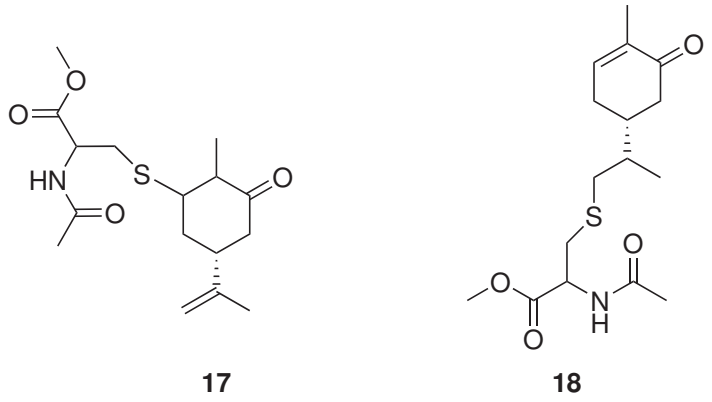
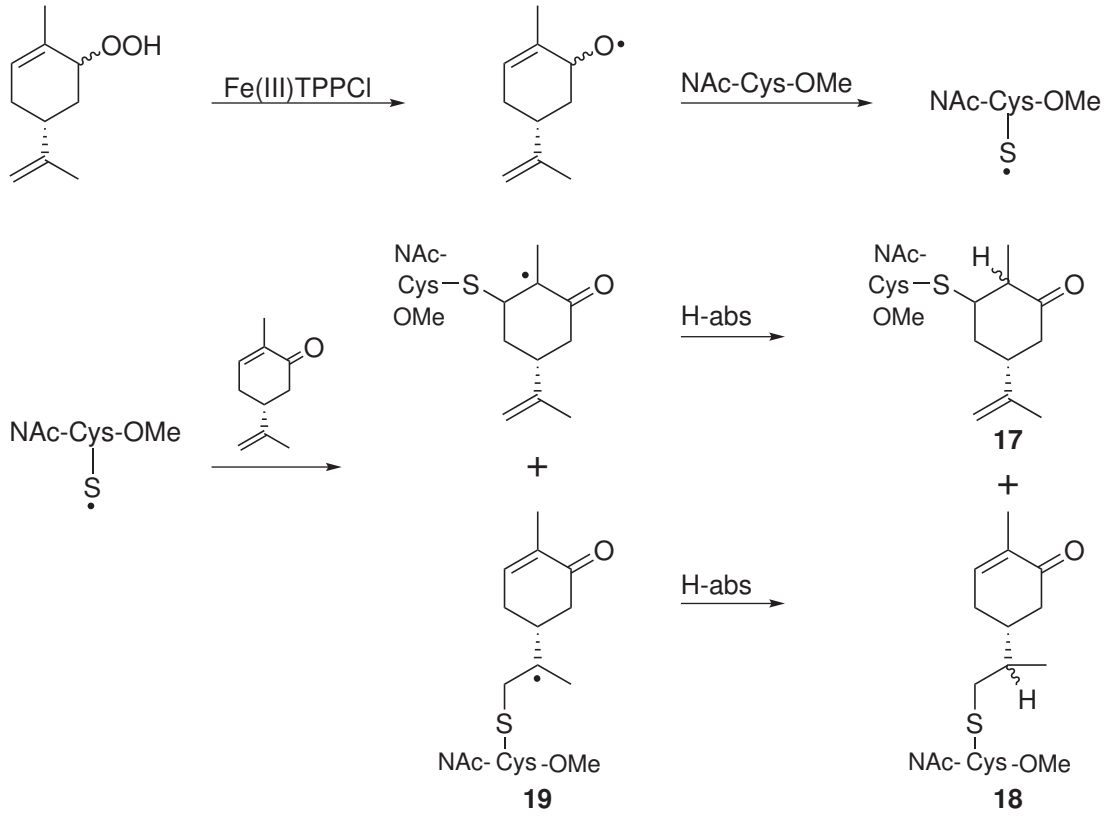


Figure 13. Structures of identified m/z 328 adducts isolated from the reaction of hydroperoxide **2** with Fe(III)TPPCl in the presence of NAc-Cys-OMe.

The studies presented in Paper I show that a lot of radicals, as well as carvone and carveol, are formed in the reaction of hydroperoxide **2** with Fe(III)TPPCl. If any of these radicals abstract a hydrogen atom from the thiol group of NAc-Cys-OMe a thiyl radical is formed. Thiyl radicals are known to add to olefinic double bonds in an anti-Markovnikov fashion via the thiol-ene reaction [67]. This is followed by hydrogen abstraction to generate the non-radical product. The results presented in Paper I together with the thiol-ene reaction accounts for the formation of adduct **17** and **18** (Scheme 7).



Scheme 7. Mechanistic proposal for the formation of the identified adducts (**17** and **18**) in the reaction of hydroperoxide **2** and Fe(III)TPPCl in the presence of NAc-Cys-OMe.

Several other products were detected in the reaction mixture and HPLC-fractions from the reaction of **2** with Fe(III)TPPCl in the presence of NAc-Cys-OMe. The major product was the dimer of NAc-Cys-OMe. The formation of a diastereomer of adduct **2** is explained by a planar symmetry around the carbon radical (**19**) formed by the addition of the thiyl radical. The carbon radical is formed at the quaternary carbon in the isopropenyl group and the subsequent hydrogen abstraction can take place on both sides of the plane, resulting in diastereomers. The addition of NAc-Cys-OMe to the endocyclic double bond of carveol is consistent with the formation of carveol from **2** and the thiol-ene mechanism. The addition of one molecule of NAc-Cys-OMe to each carbon-carbon double bond of carvone is in agreement with the detection of adducts **17** and **18**.

Hydroperoxide **2** was reacted with Fe(III)TPPCl in the presence of GSH under the same conditions as in Scheme 6. In this reaction, two diastereomers corresponding to the

addition of GSH to the isopropenyl double bond of carvone was detected. No GSH-adducts corresponding to the Michael addition adduct were detected.

Although it has been suggested that a radical mechanism is involved in the formation of specific antigens of hydroperoxides [13-17], no specific mechanism has been proposed. The results in this paper demonstrate that the formation of an amino acid centered radical and the addition of compounds derived from the hydroperoxide are possible. Thus, the thiol-ene reaction and the addition of compounds derived from the hydroperoxide offer a potential mechanism for the formation of specific immunogenic complexes of olefinic hydroperoxides.

All hydroperoxides tested are strong sensitizers with small differences in their sensitizing capacities [14, 15, 47, 58 and Paper I]. A possible explanation for this is an initial weakening of the antioxidant defenses in the skin by the formation of large amounts of radicals [68, 69] that will ease the formation of a specific immunogenic complex by radical addition.

The formation of immunogenic complexes corresponding to adduct **18** also offers an explanation for the lack of cross-reactivity between carvone and hydroperoxide **2** [12], as the hapten-peptide formed by the addition of carvone to a cysteine residue via the isopropenyl double bond would be different to the hapten-peptide formed via addition to the endocyclic double bond.

In summary, the formation of specific immunogenic complexes of olefinic hydroperoxides by a two-step process is proposed. First the depletion of antioxidants and formation of protein thiyl radicals by large amounts of radicals formed from the hydroperoxide, thereafter addition of the thiyl radical to a compound originating from the hydroperoxide via the thiol-ene reaction. This two-step process also explains the small difference in sensitizing capacities between different hydroperoxides.

3.4 Radical Formation and Sensitizing Capacity of Alkylic Limonene Hydroperoxides (Paper IV)

The results presented in Papers I and II demonstrate a link between the structure, the radical formation and the sensitizing capacities of the allylic hydroperoxides. In Paper II a significant difference in sensitizing capacities between hydroperoxide **1** and hydroperoxides **2** and **3** was demonstrated. In Paper I three major pathways for radical formation was observed, one of these was the 1,3-cyclization including the endocyclic double bond. This led to the formation of carbon-centered radicals that subsequently reacted with the radial trapper TMIO. Both hydroperoxide **1** and **3** formed large amounts of carbon-centered oxiranylcarbinyl radicals, but only hydroperoxide **1** formed large amounts of alcohol, the result of an intermolecular reaction of the alkoxyl radical. Together these results indicate the importance of the alkoxyl radicals in the formation of immunogenic complexes of the hydroperoxides.

The aim of this paper was to study the radical formation and sensitizing capacity of three alkylic analogues to the previously studied allylic hydroperoxides (Paper I). In these analogues the endocyclic double bond is removed (**20**, **21**, **22**, Figure 14). This will prevent formation of the oxiranylcarbinyl radicals and may increase the preference to intermolecular reactions of the alkoxyl radicals. This could possibly increase the sensitizing capacity of the hydroperoxides as the immunogenic complex formation requires an intermolecular reaction. The radical formation was studied in radical trapping experiments utilizing Fe(III)TPPCl as radical initiator and TMIO as radical trapper. The sensitizing capacities of the hydroperoxides were investigated in the LLNA.

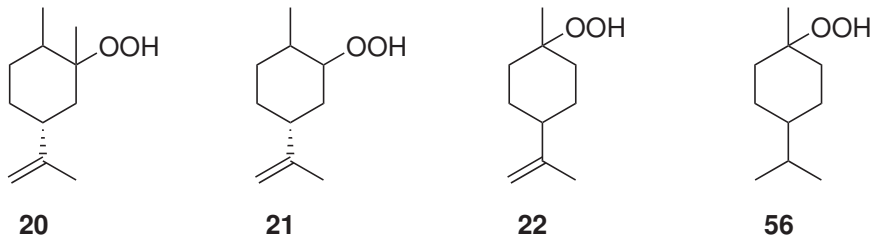


Figure 14. The alkylic hydroperoxides referred to in this chapter.

The first attempts to synthesize hydroperoxide **20** were made using the same acid-catalyzed substitution methodology that proved successful in the synthesis of hydroperoxides **1** and **3**. However, starting from alcohol **23**, the major product (**24**) originates from elimination of water instead of the anticipated nucleophilic substitution (Figure 15); addition to the isoprene unit (**25**, **26**), typically in a Markovnikov manner, was also observed [70]. Different combinations of peroxide reagents (H_2O_2, urea- H_2O_2 adduct, or Na_2O_2), acids (H_2SO_4, PTSA, acetic acid, or TFA), equivalents, temperatures, reaction times and solvents (pentane, DCM, DMF, or THF) were used (Appendix I). Products resulting from elimination of the hydroxyl group or addition to the isopropenyl group were also obtained when trying to substitute the hydroxyl group into a chloride, bromide, mesylate, or tosylate leaving group (**27**). The carbocation formation was projected to proceed smoothly from the tertiary alcohol (**23**) under acidic conditions in polar solvents. Conversely, according to our experience, these reactions were sluggish. Four fifths of the reactions did not consume the starting material, in some reactions only one of the two diastereomers of the starting material reacted and in six attempts no reaction at all was observed (Appendix I). These observations could possibly be explained by the structure of the starting material. In the conformations where both the isopropenyl and the hydroxyl group have axial positions, a hydrogen bond can form between the hydroxyl group and the double bond. This will stabilize the conformation and if the stabilization is large enough this might be the most common conformation. The formation of elimination products, once the intermediate carbocation is formed, is readily explained by the low energy of the fully substituted double bond, which stabilizes these products.

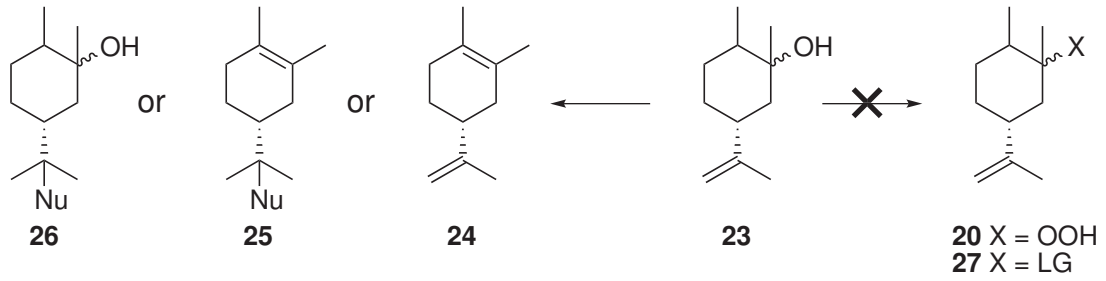
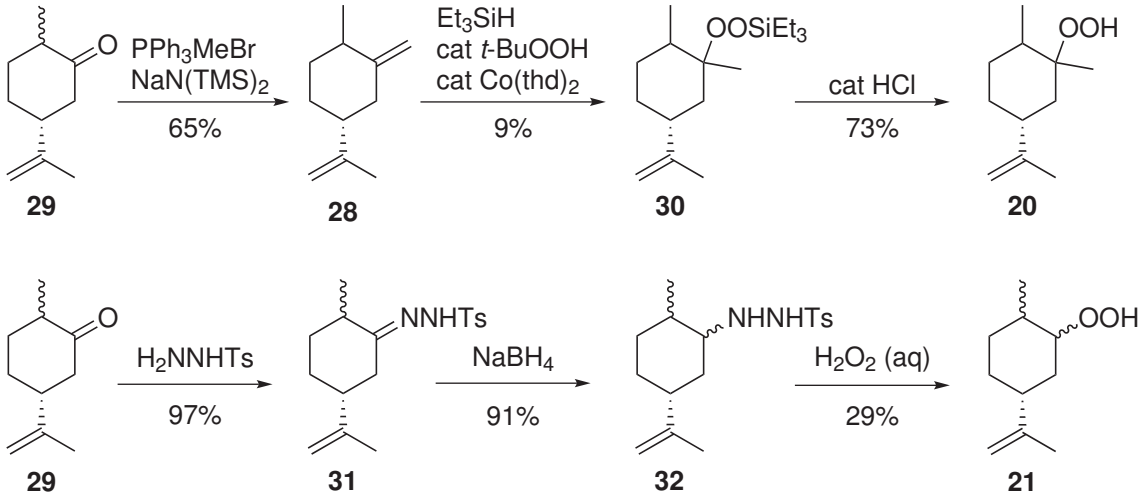


Figure 15. Products identified in the first attempts to synthesize hydroperoxide **20** via substitution of the corresponding alcohol (**23**). Several combinations of peroxide reagents, acids, equivalents, temperatures, reaction times and solvents were used (Appendix I). (Nu) Nucleophile, (LG) leaving group.

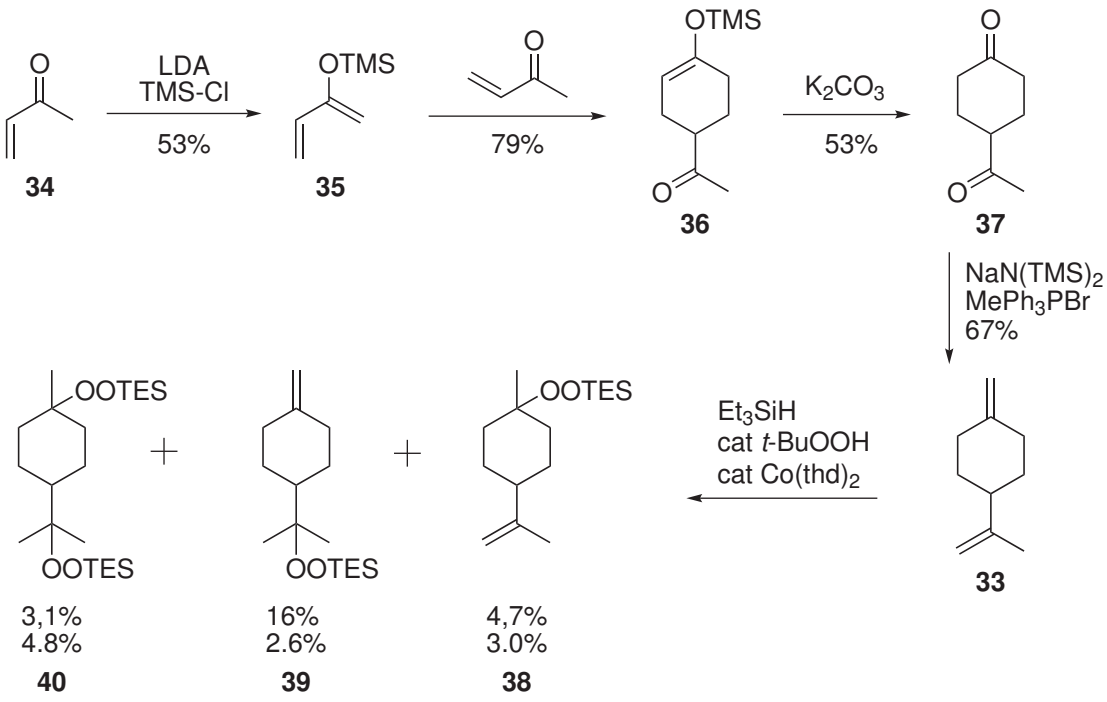
Instead the synthesis of hydroperoxide **20** was performed using cobalt catalyzed peroxidation of the corresponding alkene **28** (Scheme 8). The alkene was synthesized from dihydrocarvone (**29**) in a Wittig reaction and the peroxidation product (**30**) was deprotected in acidic methanol to yield hydroperoxide **20**. Hydroperoxide **21** was synthesized from dihydrocarvone (**29**) via the corresponding hydrazone (**31**) that was reduced to hydrazine **32** and treated with aqueous hydrogen peroxide yielding hydroperoxide **21**.



Scheme 8. Syntesis of hydroperoxides **20** and **21**.

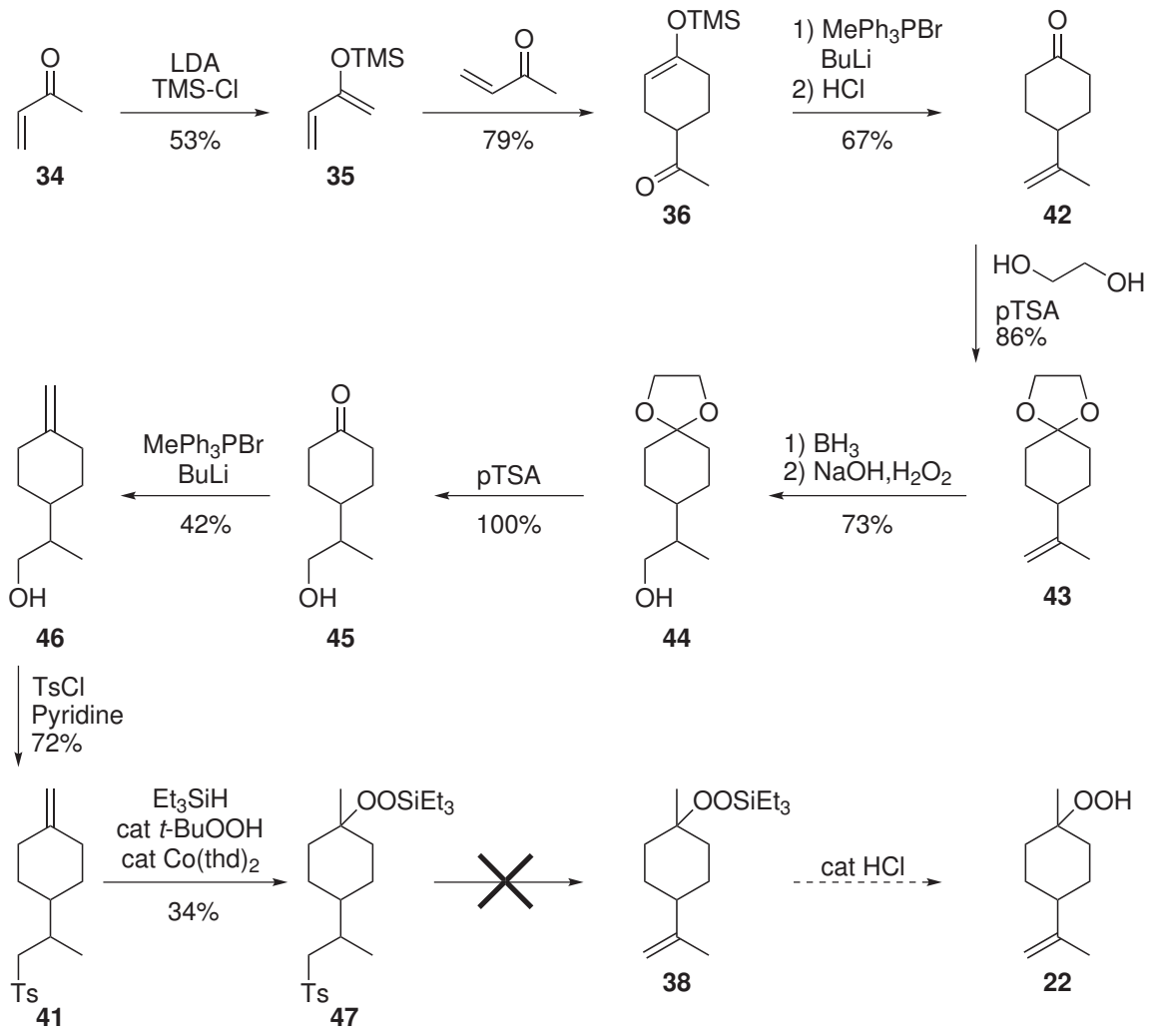
Two attempts to synthesize hydroperoxide **22** were made. The first one aimed at using the same strategy that proved successful for hydroperoxide **20** (Scheme 9). The synthesis of the corresponding dialkene (**33**) started with the Diels-Alder reaction of methyl vinyl ketone (**34**) with its corresponding TMS-enolate (**35**). The Diels-Alder product (**36**) was deprotected yielding **37** and a Wittig transformation of the carbonyl groups furnished **33**. The peroxidation was successful in the sense that the correct product (**38**) was isolated, in two reactions the isolated yields were 4.7% and 3.0%, respectively. In substrates with multiple double bonds, the cobalt catalyst has a higher affinity for electron-rich double bonds [42]. The double bonds of alkene **33** have similar electronic properties, thus the similar yields of silyl peroxides (**38**) and (**39**). Long reaction times will reduce the yield of silyl peroxide **38** by double peroxidation (**40**). The isolated yields of product **38**, in combination with the synthetic work required for the starting material **33**, were

considered insufficient for an effective synthesis of the amounts needed for further experiments and the strategy was abandoned.



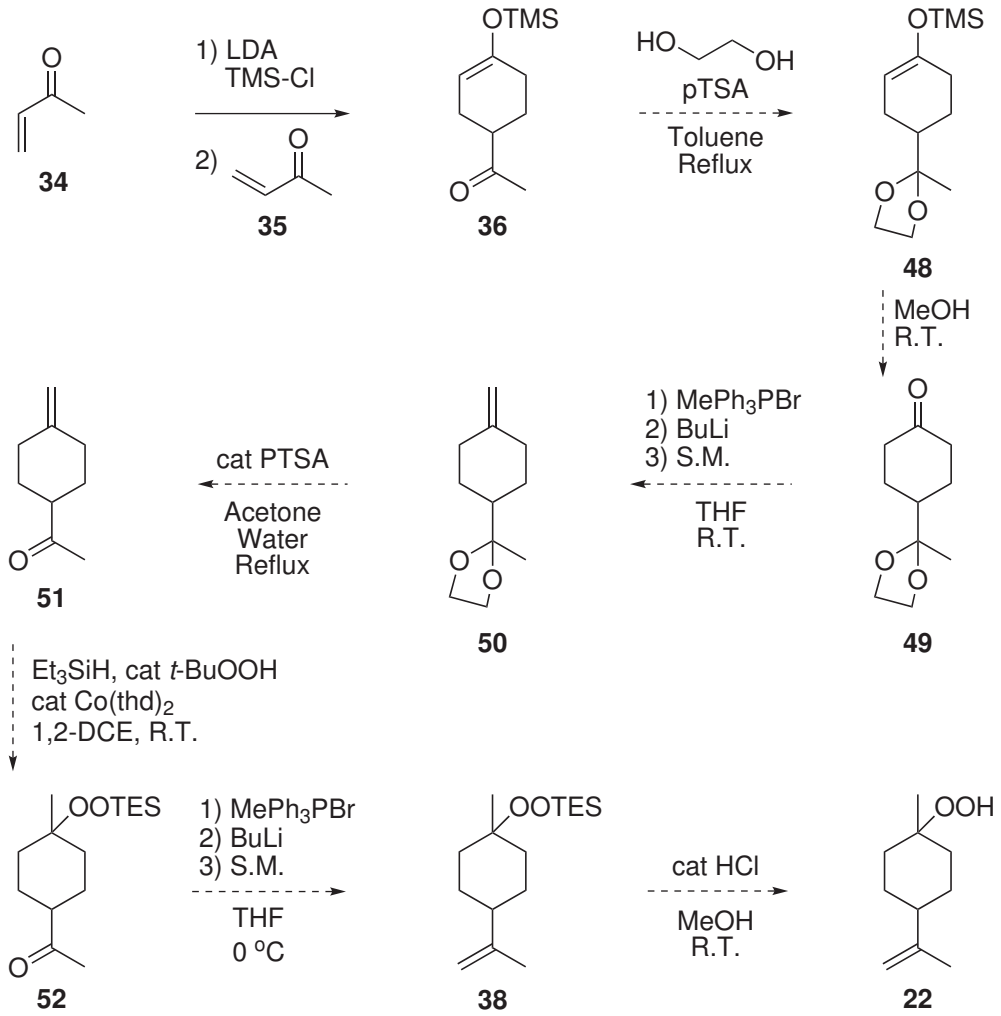
Scheme 9. First attempt to synthesize hydroperoxide **22** using the cobalt catalyzed peroxidation method on diene **33**.

The second attempt to synthesize hydroperoxide **22** aimed at using the cobalt catalyzed peroxidation on a monoalkene (**41**, Scheme 10). Starting with the same Diels-Alder reaction of methyl vinyl ketone (**34**) and its corresponding TMS-enolate (**35**) as in the first attempt, Wittig transformation of **36** followed by TMS-deprotection resulted in **42**. In order to avoid reduction the ketone was protected as an acetal (**43**) before hydroboration was used to convert the isopropenyl group to a propanoyl group (**44**). The acetal was removed (**45**), the ketone transformed to an alkene (**46**), and the hydroxyl group converted to a tosylate (**41**). The peroxidation step was successful but the projected elimination of the tosyl group (**47**) failed despite several attempts. Most successful was the use of *t*-BuOK in *t*-BuOH/DMSO at 80 °C which gave the desired alkene (**38**) as a minor product. Attempts using refluxing collidine, refluxing TEA, *t*-BuOK in THF at RT, and collidine using microwave assisted heating at 100 – 170 °C for 5 – 40 min resulted in degradation of the starting material (**47**) without any detectable formation of the desired product (**38**). As the starting material (**47**) was not compatible with the reaction conditions needed for the elimination a new strategy was drawn up.



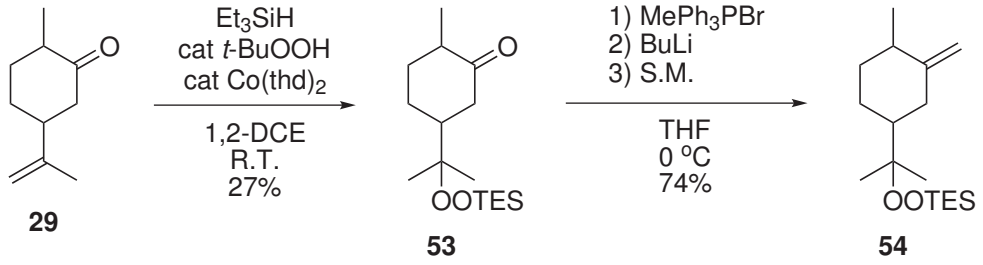
Scheme 10. Second attempt to synthesize hydroperoxide **22** using the cobalt catalyzed peroxidation method on a monoalkene (**41**).

The third strategy for synthesis of hydroperoxide **22** is outlined in Scheme 11. To avoid the fatal elimination reaction, the carbonyl group of the Diels-Alder product (**36**) is protected as an acetal (**48**). Deprotection of the TMS-enolate (**48**) is followed by a Wittig-transformation of the carbonyl (**49**) to an alkene (**50**) and restoration of the acetyl group (**51**). The introduction of the peroxyl functionality (**52**) is followed by conversion of the acetyl group to an isopropenyl group (**38**) and deprotection of the silylperoxide to furnish the hydroperoxide (**22**).



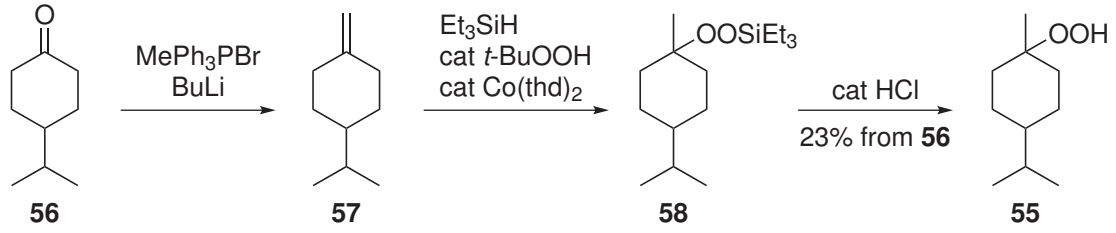
Scheme 11. Third synthetic strategy for the synthesis of hydroperoxide **22**. Dashed arrows implies projected reactions.

In order to evaluate some of the synthetic transformations of the third strategy to synthesize hydroperoxide **22** a short test synthesis was made (Scheme 12). Commercially available dihydrocarvone (**29**) was peroxidized using the same conditions as in the planned synthesis yielding silyl peroxide **53**. This was used in a Wittig reaction, transforming the carbonyl group into alkene **54** (see Appendix II for experimental procedures). The success of these reactions proves the compatibility of the different functional groups with the planned reaction conditions.



Scheme 12. Reactions used to evaluate the compatibility of functional groups with the projected reaction conditions in the third strategy for the synthesis of hydroperoxide **22**.

As the attempts to synthesize hydroperoxide **22** were unsuccessful, no reactions including the isopropenyl unit had been observed in Paper I and in view of the mechanistic proposal in Paper III, it was decided to synthesize the fully saturated hydroperoxide **55**. Thus, ketone **56** was transformed into alkene **57** in a Wittig reaction, (Scheme 13) followed by cobalt catalyzed peroxidation yielding silyl peroxide **58,** that was deprotected in acidic methanol furnishing hydroperoxide **55**.

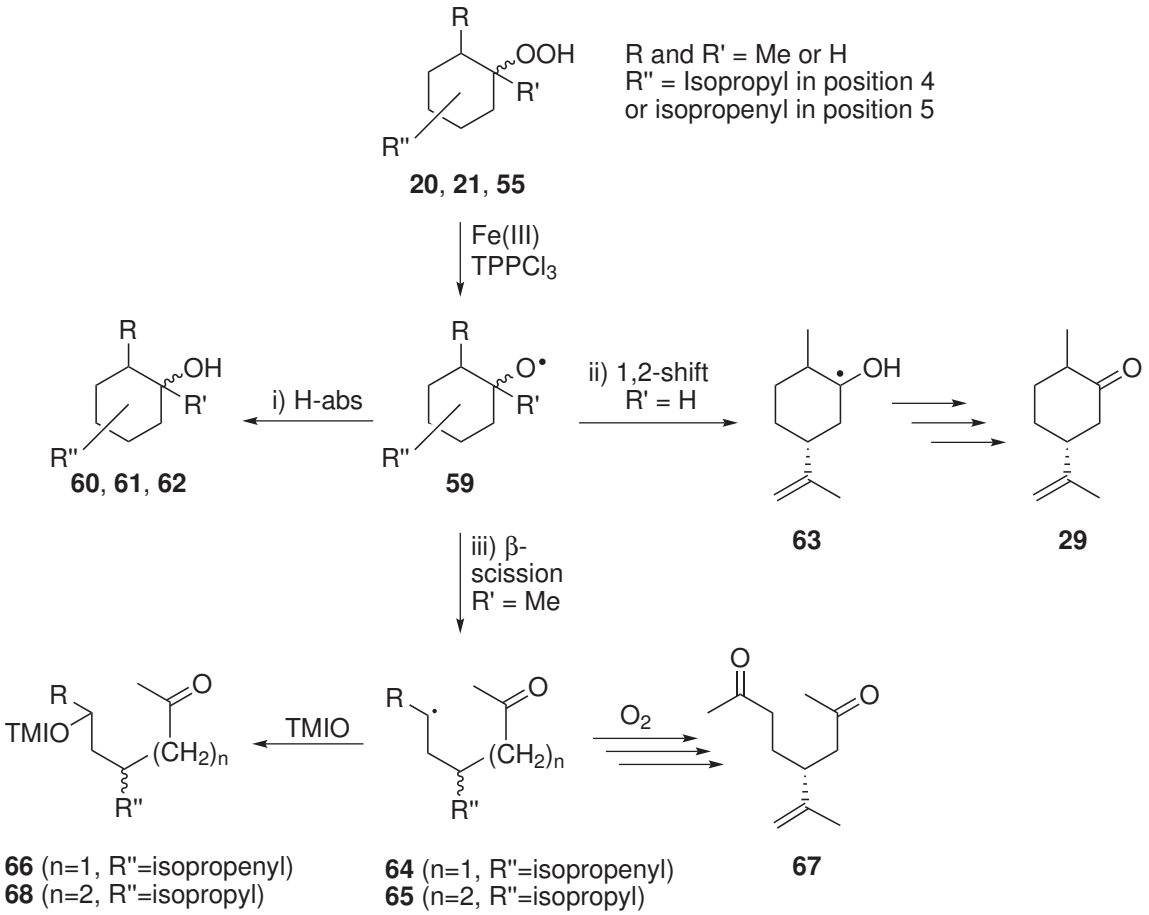


Scheme 13. Synthesis of alkylic hydroperoxide **55**.

The sensitizing capacity of hydroperoxide **20** and **21** was tested in the LLNA and the EC3-values obtained were 0.065 M (1.10%) and 0.037 M (0.68%), respectively. This makes both hydroperoxides potent sensitizers and is equivalent to previously investigated hydroperoxides [14, 15, 47, 58]. Testing of hydroperoxide **55** in the LLNA is planned for early summer of 2009.

The radical trapping experiments were performed in a 1:1 mixture of acetonitrile and water, using 1.1 equivalent of Fe(III)TPPCl as radical initiator and 2 equivalents of TMIO as radical trapper (Figure 10, Section 3.1). The reactions were stirred at room temp until TLC showed no hydroperoxide. The products were isolated using flash chromatography as well as preparative HPLC and identified by NMR and MS.

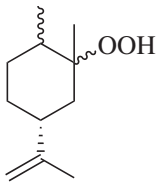
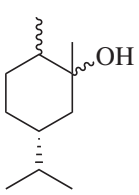
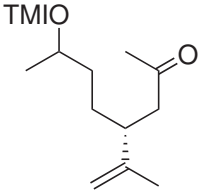
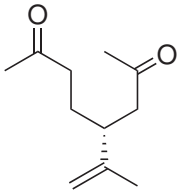
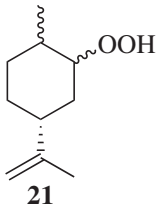
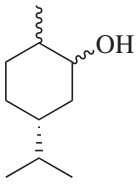
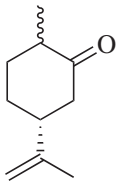
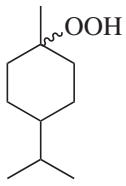
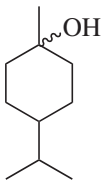
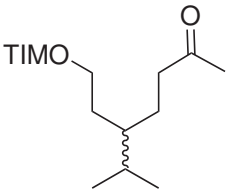
In the radical trapping experiments the oxygen-oxygen bond of the hydroperoxide functional group is cleaved homolytically by Fe(III)TPPCl. The alkoxyl radical (**59**) thus formed can react according to three different pathways (Scheme 14): i) hydrogen abstraction to form the corresponding alcohol (**60**, **61**, **62**); ii) 1,2-shift to form a 1-hydroxyl radical (**63**) that subsequently forms dihydrocarvone (**29**); or iii) β-scission that opens the cyclohexane ring and forms a carbon-centered radical (**64**, **65**) that can be trapped by TMIO or dioxygen. The structure of the hydroperoxide and the reaction rates of the different pathways determine the product distribution of the trapping experiments.



Scheme 14. Mechanistic proposal for the formation of products identified in the trapping experiments with hydroperoxides **20**, **21** and **55**.

Hydroperoxide **20** forms large amounts TMIO-adduct **66** and ketone **67**, which both are derived from the carbon-centered radical that is the result of β-scission, as well as small amounts of alcohol (**60**, Table 4**)**. From hydroperoxide **21** the major product is dihydrocarvone (**29**) together with small amounts of alcohol (**61**). The major products from hydroperoxide **55** are the corresponding alcohol (**62**) and TMIO-adduct **68**. The corresponding ketone is not formed because the 1,2-shift requires a hydrogen atom on the hydroperoxide bearing carbon.

Table 4. Product distribution in the radical trapping experiments with TMIO and alkylic hydroperoxides **20**, **21**and **55**; %-values correspond to purified yields.

Hydroperoxides	Products		
 **20**	 **60** 3.8%	 **66** 47%	 **67** 14%
 **21**	 **61** 3.2%	 **29** 41%	
 **55**	 **62** 24%	 **68** 15%	

Similar to the hydroperoxides in Paper I, there seems to be a correlation between the radical formation and the sensitizing capacities of hydroperoxides **20** and **21**. In the trapping experiment with hydroperoxide **21** a small amount of alcohol corresponding to an intermolecular reaction of the alkoxyl radical was isolated. The sensitizing capacity of

the hydroperoxide can not be explained by the formation of dihydrocarvone as this compound is not a sensitizer according to guinea pig studies [71]. This indicates the importance of the alkoxyl radical in the formation of the immunogenic complexes. In analogy with hydroperoxide **2** formation of dihydrocarvone is proposed to proceed via the 1-hydroxyl radical **63** but no adducts with this radical was isolated.

The same amount of alcohol was isolated in the trapping experiment with hydroperoxide **20** as with hydroperoxide **21**, together with large amounts of products derived from the carbon-centered radical. The formation of high amounts of carbon-centered radicals without any substantial increase in the sensitizing capacity indicates that these radicals may be less potent compared to the alkoxyl radicals in the formation of immunogenic complexes. Relatively large amounts of both alcohol and TMIO-adduct were isolated in the trapping experiment with hydroperoxide **55**. If the sensitizing capacity correlates to the radical formation in the same way as for the allylic hydroperoxides in Paper I, **55** will be a stronger sensitizer compared to hydroperoxides **20** and **21**.

In comparison with the allylic hydroperoxides in Paper I, roughly the same amounts of radicals and products were isolated in the radical trapping experiments with the alkylic analogues investigated in this paper (Tables 2 and 4). However, the mechanistic pathway for formation of the carbon-centered radicals is different. The allylic alkoxyl radicals rearranged into carbon-centered radicals via a 1,3-cyclization including the double bond whereas the alkylic alkoxyl radicals reacts according to a β-scission mechanism that opens the cyclohexane ring. The preference for the different reactions is in accordance with the general ease of the reactions and the stability of the formed radicals. It is interesting that the formation of primary carbon-centered radicals via ring-opening from hydroperoxide **55** is identified, while the formation of a methyl radical and the corresponding ketone is not observed.

In summary, all hydroperoxides were potent sensitizers in the LLNA and the same correlation between radical formation and sensitizing capacity was observed as for the allylic hydroperoxides in Paper I, albeit the formation of carbon-centered radicals follows a different mechanism. The results indicate that alkoxyl radicals may be more important

compared to carbon-centered radicals in the immunogenic complex formation, even if the latter are formed in larger amounts.

3.5 Stability of Hydroperoxides towards Fe(III)TPPCl

Most metabolizing enzymes in the body belong to the P450 cytochrome family that has a heme group in their catalytic sites [26]. As a model for the heme group, iron porphyrins have been extensively studied [48 and references therein]. The stability of hydroperoxides **1**, **2** and **3** (Figure 16) towards catalytic amounts of the iron porphyrin Fe(III)TPPCl was investigated in NMR experiments (see Appendix II for experimental details). The intensity of the signals from the olefinic protons was used as a measure of the hydroperoxide concentration.

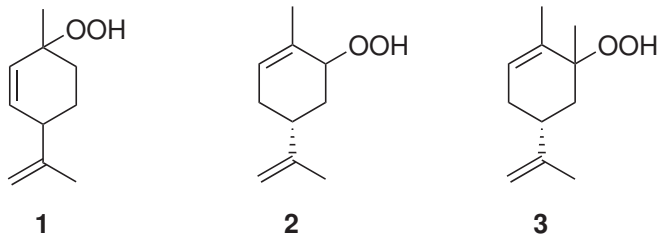


Figure 16. Hydroperoxides investigated for stability towards Fe(III)TPPCl.

The results of the investigations are that the half-life for hydroperoxide **1** was 3.9 min, whereas it was 66 min for hydroperoxide **2**, and 13.5 min for hydroperoxide **3** (Figure 17).

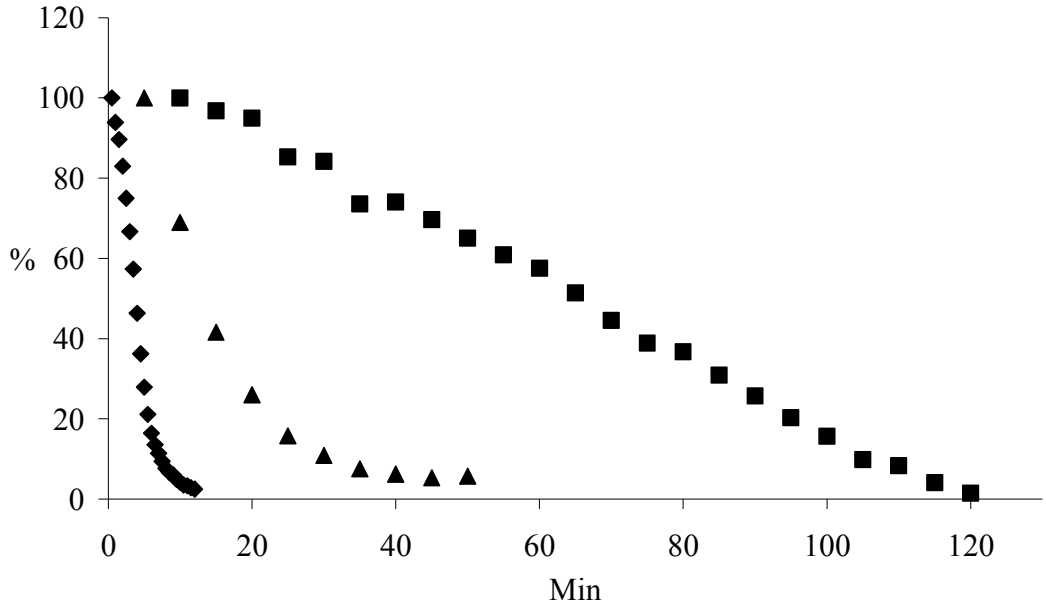


Figure 17. Stability of hydroperoxides **1** (♦), **2** (■) and **3** (▲) towards Fe(III)TPPCl.

These results indicate that the concentration of radicals formed in the skin from the same amount of hydroperoxide may differ between the investigated hydroperoxides. This is important as formation of large amounts of radicals in the skin will deplete the antioxidants [68, 69] and are likely to result in the formation of more immunogenic complexes. The number of immunogenic complexes are likely to be important both to pass the threshold for the immunogenic response and for the strength of the response [72].

General Discussion

This thesis investigates the mechanisms of immunogenic complex formation of limonene hydroperoxides. The studies have focused on the connection between structure, radical formation and sensitizing capacities of the hydroperoxides. Studies of the reaction between hydroperoxide **2** (limonene-2-hydroperoxide) and cysteine identified a possible mechanism for the formation of immunogenic complexes from olefinic hydroperoxides and proteins.

The radical formation has been studied in trapping experiments employing TMIO as a radical trapper for carbon-centered radicals. The isolation and identification of TMIO-adducts and other non-radical products indicated what radicals were potentially available for immunogenic complex formation from each hydroperoxide (Papers I and IV). In these experiments, Fe(III)TPPCl was used as a model for the metabolizing enzymes, cleaving the oxygen-oxygen bond of the hydroperoxides homolytically, thereby initiating the radical reactions. All investigated hydroperoxides formed large amounts of radicals; however, several reaction pathways are available after formation of the initial alkoxyl radical. The structure of the hydroperoxide and thus the alkoxyl radical governs what pathways will dominate the following reactions and thereby also the identity and quantity of the formed radicals.

All investigated hydroperoxides formed the corresponding alcohols by hydrogen abstraction. However, the yields of alcohol differed between the hydroperoxides. This is a result of the structure of the hydroperoxides influencing the balance between the different pathways, and thus the product distribution in the radical trapping experiments (Tables 2 and 4).

For the secondary hydroperoxides **2** and **21** the 1,2-shift is the dominating pathway, resulting in the formation of large amounts of the corresponding 1-hydroxyl radicals. Further reactions ultimately form carvone (**8**) and dihydrocarvone (**29**), respectively, as the major products in the trapping experiments.

For the tertiary hydroperoxides the 1,2-shift is not possible due to the absence of a hydrogen on the hydroperoxide bearing carbon. The allylic hydroperoxides **1** and **3** primarily react according to the 1,3-cyclization pathway, resulting in high amounts of oxiranylcarbinyl radicals. The preference for this reaction compared to hydrogen abstraction is influenced by the stability of the formed epoxide; this is displayed by the larger amount of cyclization products formed from hydroperoxide **3** compared to the amount formed from hydroperoxide **1**.

The omission of the endocyclic double bond prevents the alkylic hydroperoxides **20** and **55** to react according to the 1,3-cyclization pathway. Instead the preferred reaction is a β-scission resulting in opening of the cyclohexane ring and the simultaneous formation of a carbon-centered radical. The ease of this is mainly depending on the stability of the formed carbon radical. Thus, considerably more secondary radicals were formed from **20** compared to primary radicals formed from **55**, and consequently higher yields of products corresponding to the secondary radicals were isolated.

The sensitizing capacities were investigated in the LLNA and all hydroperoxides tested were found to be potent sensitizers (Papers I and IV). This is in accordance with previously studied hydroperoxides [14, 15, 47, 58]. In a modified LLNA comprising non-pooled lymph nodes and statistical calculations hydroperoxide **1** was demonstrated to be a significantly stronger sensitizer compared to hydroperoxides **2** and **3**. Investigations of statistical differences in the sensitizing capacities of compounds with similar EC3-values using this modification of the LLNA have, to the best of our knowledge, not been published before. When developing new methods to replace current animal based assays of allergenic activity this possibility to differentiate the sensitizing capacities of structurally similar compounds with comparable EC3-values is important.

The results from the modified LLNA proved clinically relevant as seven out of seven patients who previously showed positive reactions to oxidized limonene also reacted to hydroperoxide **1**, while only three of them reacted to hydroperoxide **2** (Paper II). In previous testing with oxidized limonene and linalool and their respective hydroperoxide fractions 40 – 60% of the patients reacted to both the oxidation mixture and its hydroperoxide fraction [6, 9, 59]. This is likely caused by some patients being allergic to

secondary oxidation products (aldehydes, epoxides, ketones, etc.) in the mixtures. However, the clinical study in Paper II is limited and needs to be repeated with a larger number of patients to verify the results.

The difference in EC3-values from the ordinary LLNA is too small to differentiate between the sensitizing capacities of the hydroperoxides. Thus one must be careful when drawing conclusions about the correlation between radical formation and sensitizing capacity. However, in the modified LLNA hydroperoxide **1** was found to be a significantly stronger sensitizer compared to hydroperoxides **2** and **3**. Hydroperoxide **1** also formed considerably higher amounts of alcohol in the trapping experiments compared to hydroperoxides **2** and **3**. This indicates a higher preference of the alkoxyl radical from **1** to participate in an intermolecular reaction which is likely to be important for the immunogenic complex formation and sensitizing capacity.

Paper III investigates the formation of adducts between hydroperoxide **2** and amino acids as a model system for immunogenic complex formation. When hydroperoxide **2** was reacted with Fe(III)TPPCl carvone was formed, and when the reaction was performed in the presence of cysteine two adducts between carvone and cysteine were isolated and identified. This revealed a possible radical mechanism for immunogenic complex formation of olefinic hydroperoxides: formation of thiyl radicals from cysteine, followed by addition to a compound originating from the hydroperoxide, and hydrogen abstraction to furnish the non-radical product (Paper III). This mechanism explains how the specificity of the immunogenic complex of an olefinic hydroperoxide is obtained as an exogenous molecular structure originating from the hydroperoxide is covalently bound to the protein.

The mechanistic proposal in Paper III also explains the importance of the alkoxyl radicals, displayed by the combined results of the trapping experiments (Paper I), the modified LLNA and the clinical studies (Paper II). The formation of thiyl radicals is most likely achieved via hydrogen abstraction. This can be done by both the oxygen centered and the carbon-centered radicals mentioned above. However, the bond dissociation energy (BDE) of an O-H bond is larger compared to that of a C-H bond [73] making hydrogen abstraction by an oxygen-centered radical more energetically favorable. Thus,

it is possible that a larger amount of thiyl radicals are formed from oxygen-centered radicals, compared to the amount of thiyl radicals formed from the same amount of carbon-centered radicals. In the skin this would correspond to more thiyl radicals being formed from the same amount of hydroperoxide, thereby creating more possibilities for formation of immunogenic complexes. A higher number of immunogenic complexes are likely to cause a stronger immunogenic response as a larger number of memory T-cells will be activated [72].

The formation of protein radicals as part of the immunogenic complex formation of hydroperoxides points to another important aspect of radical reactions in the skin; the formation of large amounts of radicals will deplete the antioxidant reserve [68, 69] and thereby ease the formation of covalent hapten-protein bonds via radical mechanisms. In this aspect the identity of the radicals are likely to be less important compared to the quantity. This is a possible explanation for the observation that all tested hydroperoxides are potent sensitizers with only small differences in EC3-values [14, 15, 47, 58 and Paper I].

The quantity of radicals depends on the amount of hydroperoxide, but also the stability of the hydroperoxides towards the metabolizing enzymes. In the stability experiments (Section 3.5) hydroperoxide **1** was the least stable of the investigated hydroperoxides towards the P450-mimicing Fe(III)TPPCl. This means that more radicals are formed from the same amount of hydroperoxide within a given time period. In the skin this will contribute to the weakening of the antioxidant defenses, thus increasing the possibility of immunogenic complex formation by radical reactions and the sensitizing capacity of the hydroperoxide.

Most likely there are several other mechanisms available for the formation of immunogenic complexes of hydroperoxides. One indication of this is the reported cross-reactivity between cumene hydroperoxide and a cyclohexenyl analogue together with quantum chemical calculations [21]. These results support the direct attachment of the alkoxyl radicals to a protein as rupture of the aromatic system of cumene hydroperoxide would be energetically unfavorable. Another possible mechanism for the immunogenic complex formation of hydroperoxides is the rearrangement of the initially formed

radicals into non-radical haptens capable of forming hapten-protein bonds in nucleophilic-electrophilic reactions. One example of this is the formation of allergenic epoxides from 15-hydroperoxyabietic acid HPA [13]. However, not all electrophilic compounds that can be formed by radical rearrangement are strong sensitizers that can explain the sensitizing capacity of the initial hydroperoxide. One example of this is hydroperoxide **2** that forms carvone (Paper I) that have a lower sensitizing capacity in the LLNA compared to hydroperoxide **2** (Paper II). Another example is linalyl hydroperoxide, that forms epoxides that are non-sensitizers according to the LLNA [14].

In summary, the work presented in this thesis demonstrates a correlation between structure, radical formation and sensitizing capacity of limonene hydroperoxides. All investigated hydroperoxides were potent sensitizers in the LLNA and formed large amounts of radicals in the trapping experiments. The identity and quantity of formed radicals are influenced by the structure of the hydroperoxides. Furthermore, the results indicate that alkoxyl radicals may be the most important radicals in the formation of immunogenic complexes of hydroperoxides. A statistically significant difference in sensitizing capacities between hydroperoxide **1** and hydroperoxides **2** and **3** is demonstrated in a modified LLNA and supported by clinical data, results that further emphasize the formation of specific antigens from hydroperoxides. The isolation of an adduct between cysteine and carvone makes us propose that the formation of thiyl radicals and reaction of these with an olefin originating from the hydroperoxide in the thiol-ene reaction is a possible mechanism for the formation of specific immunogenic complexes of olefinic hydroperoxides. Finally, the formation of large amounts of radicals in the skin will weaken the antioxidant defenses, easing the formation of hapten-protein bonds via a radical mechanism. This offers an explanation to all hydroperoxides being potent sensitizers with similar EC3-values, seemingly without regard to individual structure.

Outlook

The results presented provide an insight into the mechanism of immunogenic complex formation of limonene hydroperoxides but also the realization that a lot of interesting and important questions still remain. Future investigations should include a clinical study with a larger number of patients to verify the clinical results from Paper II. Further studies of the sensitizing capacities of hydroperoxides **20**, **21,** and **22** as well as **1**, **22** and **55** using the modified LLNA should be conducted in order to increase the understanding of the correlation between structure and sensitizing capacity. Trapping experiments with amino acids, peptides and possibly proteins are also very interesting for further elucidation of the mechanism of immunogenic complex formation. Especially hydroperoxide **1**, which is a significantly stronger sensitizer, and the analogues **22** and **55** are interesting in this aspect.

The results point to the importance of considering oxidative stress as a part of the immunogenic complex formation mechanism. When part of a mixture hydroperoxides can deplete antioxidant defenses and thus facilitate the formation of immunogenic complexes not only of hydroperoxides but also of other haptens able to react via radical mechanisms.

The use of antioxidants as a preventive treatment of ACD caused by limonene-2-hydroperoxide have been investigated in guinea pigs with positive results [74]. This thesis further indicates the importance of radical reactions in ACD and presents valuable knowledge that can be used in investigations of the relationship between hydroperoxides and antioxidant levels as well as the development of better treatment methods.

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Appendix I

Reagents and reaction conditions used in the attempts to synthesize hydroperoxide **20** by substitution of alcohol **23**.

The tables are adopted from *Emilsson* [70] with permission from the author.

Table 1. Reactions with H_2O_2 (aq), using different acids, equivalents of acid and peroxide, temperatures, solvents and reaction times in order to synthesize **20** from **23**.

Acid	Eq H^+	Eq peroxide	Solvent	Temp.	Time	All s.m. consumed	Elim[a].	Other prod.[a]
H_2SO_4	Drop	145	Pentane	RT	1d	No	ND	NR
H_2SO_4	Drop	100	Pentane	RT	1h	Yes	Yes	AI
H_2SO_4	Drop	100	DCM	RT	1h	Yes	Yes	NI
H_2SO_4	Drop	100	DMF	RT	5d	Yes	ND	AI
pTSA	1	100	DMF	RT	5d	No	ND	NI
H_2SO_4	Drop	100	DCM	0 °C	15 min	No	ND	NI
H_2SO_4	Drop	100	DCM	0 °C	1 min	No	ND	NI
H_2SO_4	Drop	100	DCM	8 °C	7 min	No	Yes	NI
H_2SO_4	Drop	100	DCM	RT	1 min	No	Yes	NI
H_2SO_4	Drop	100	DCM	0 °C → RT	days	No	ND	NI

[a] *NR = no reaction; NI = formed but not identified, AI = addition to isoprene unit, ND = not detected*

Table 2. Reactions with TFA, using different peroxide reagents, equivalents of acid and peroxide, temperatures, solvents and reaction times in order to synthesize **20** from **23**.

Eq H^+	Peroxide	Eq peroxide	Solvent	Temp.	Time	All s.m. consumed	Elim. [a]	Other prod.[a]
2	UHP	20	DMF	RT	1d	No	ND	NR
0	None	None	DMF[b]	RT	1d	No	ND	NI
2	Na_2O_2	5	DCM	RT→40 →RT	o/n	No	ND	NI
2	Na_2O_2	5	DCM	RT	1h	No	Yes	NI
10	Na_2O_2	5	DCM	RT	1h	No	Yes	AI
2	Na_2O_2	5	THF[c]	RT	1d	No	ND	NR
10	Na_2O_2	5	THF[c]	RT	1d	No	ND	NI
2	Na_2O_2	5	THF[c]	RT	1d	No	ND	NR

[a] *NR = no reaction; NI = formed but not identified, AI = addition to isoprene unit, ND = Not detected.*
[b] *DCM added in equal amounts as DMF.* [c] *THF dried prior to use.*

Table 3. Reactions with pTSA and Na_2O_2 in DCM, using different equivalents of acid and peroxide, temperatures and reaction times in order to synthesize **20** from **23**.

Eq pTSA	Eq Na_2O_2	Temp.	Time	All s.m. consumed	Elim. [a]	Other prod.[a]
3	5	RT	5h	No	Yes	NI
3	5	RT	2h	No	Yes	NI
3	5	RT	2h	No	Yes	AI
3	50	RT	days	No	Yes	NI
3	5	Reflux	2h	Yes	ND	AI
3	5	RT	days	No	ND	NI
0 → 17 %	5	RT	days	No	ND	NI
3	0	RT	3h	No	Yes	NI
3	0	Reflux	3h	Yes	ND	NI, DS

[a] *NI = not identified, ND = formed but not detected, AI = addition to isoprene unit, DS = degradation of starting material*

Table 4. Miscellaneous reactions using different acids, peroxide reagents, equivalents of acid and peroxide, solvents and reaction times at room temperature in order to synthesize **20** from **23**.

Acid	Eq acid	Peroxide	Eq peroxide	Solvent	Time	All s.m. consumed	Elim. [a]	Other prod.[a]
Acetic acid	3	Na_2O_2	5	DCM	5h	No	Yes	NI
H_2SO_4	3	Na_2O_2	5	DCM	30 min	Yes	Yes	ND
Amberlyst	100 wt%	H_2O_2 aq	100	THF	7d	No	ND	NR
Amberlyst	100 wt%	Na_2O_2	5	DCM	3h	OI	ND	NI

[a] *NR = no reaction; NI = formed but not identified, ND = not detected, OI = only one isomer consumed*

Table 5. Reactions with MsCl or TsCl, using different bases, equivalents of substrate and base, temperatures, solvents and reaction times in order to substitute **23** with a chloride, mesylate or tosylate group.

Additive	Eq additive	Reagent	Eq substrate	Solvent	Temp.	Time	All s.m. consumed	Elim. [a]	Other prod.[a]
NEt_3	1.3	MsCl	1.2	DCM	0 °C	2h	No	Yes	ND
Pyr	Solvent	MsCl	1.2	Pyr	0 °C	2h	No	Yes	NI
H_2O_2	16	MsCl	1.2	Pyr	0 °C	5d	No	Yes	ND
NEt_3	1.3	TsCl	1.2	DCM	0 °C	2d	No	ND	NR
NEt_3	1.3	MsCl	1.2	DCM	0 °C	2.5h	Yes	Yes	ND
None	None	HCl konc	Excess	DCM	RT	o/n	Yes	ND	DS
None	None	HCl konc	Excess	DCM	RT	1h	Yes	ND	DS

[a] *NR = no reaction; NI = formed but not identified, ND = not detected, DS = degradation of starting material*

Table 6. Reactions with halogen reagents, using different equivalents, temperatures, solvents and reaction times in order to substitute **23** with a bromide or chloride group.

Reagent	Eq reagent	Solvent	Temp.	Time	All s.m. consumed[a]	Elim. [a]	Other prod.[a]
LiBr	1.5	2 eq HBr	-10 °C	1h	No	Yes	NI, DS
BBr_3	1.2	DCM	0 °C	2h	No	Yes	AI, NI
PBr_3	2.2	DCM	0 °C → RT	1d	No	Yes	AI, NI
PCl_3	1.2	Pentane	0 °C → RT	3h	No	Yes	NI
PCl_3	1	DCM	0 °C	3h	OI	Yes	NI
$POCl_3$	1	DCM	0 °C	5h	No	No	NI
$POCl_3$	2.5	DCM[b]	8 °C	3d	No	Yes	NI
$POCl_3$	2.5	DCM	RT	2d	OI	Yes	NI
$SOCl_2$	1.1	$CHCl_3$	0 °C → RT	30h	OI	Yes	NI
$POCl_3$	3	DCM	0 °C → RT	1d	OI	Yes	NI

[a] NI = formed but not identified, ND = not detected, AI = addition to isoprene unit, OI = one isomer consumed, DS = degradation of starting material, [b]one drop of pyridine added

Appendix II

Experimental procedures discussed in section 3.4 and 3.5

Instrumentation. NMR spectroscopy was performed on a JEOL Eclipse+ 400 instrument at 400 MHz using deuterated chloroform ($CDCl_3$) as solvent. Chemical shifts (δ) are reported in ppm relative to $CHCl_3$ at 7.26 for ^1H, and at 77.0 for ^{13}C. Coupling constants are reported in Hz.

Column chromatography was performed using Merck silica gel 60 (230-400 mesh ASTM) and TLC was performed using silica plated aluminium sheets (Merck, 60 F_{254} silica gel) that were developed with anisaldehyde dip (2.1 mL of acetic acid, 5.1 mL of anisaldehyde and 7 mL of H_2SO_4 in 186 mL of ethanol) followed by heating.

Synthesis. 35 - 37 were synthesized according to literature [75].

4-Isopropenyl-1-methylenecyclohexane (**33**). Sodium bis(trimethylsilyl)amide (2 M in THF, 9.00 mL, 18.0 mmol) was added dropwise to a stirred suspension of methyltriphenylphosphonium bromide (6.42 g, 18.0 mmol) in dry THF (30 mL) under N_2 at room temperature. After 15 min, 4-acteyl-cyclohexanone (1.10 g, 7.82 mmol) dissolved in THF (6.5 mL) was added dropwise and the reaction mixture was heated to reflux. After 1 h, the reaction mixture was allowed to reach room temperature and n-pentane (35 mL) and water (35 mL) were added. The aqueous phase was extracted with n-pentane (35 mL), and the combined organic phases were washed with water (3 × 65 mL). Due to heavy precipitation both aqueous and organic phases were filtered, the aqueous phase was extracted with pentane (100 mL), the organic phases were pooled, washed with water (125 mL) and brine (125 mL), dried over MgSO4, and concentrated under reduced pressure at 0 °C. The crude product was purified by flash chromatography on silica gel (100% n-pentane) affording 0.72 g (67%) of the target compound: ^1H NMR δ 1.21 – 1.34 (m, 2H), 1.71 (s, 3H), 1.79 – 1.88 (m, 2H), 1.98 – 2.12 (m, 3H), 2.29 – 2.38 (m, 2H), 4.59 – 4.62 (m, 2H), 4.67 (s, 2H); ^{13}C NMR δ 21.0, 33.1, 35.0, 45.1, 107.0, 108.6, 149.4, 150.2.

Silylperoxides 38 – 40. Triethylsilane (0.35 mL, 2.19 mmol) and *t*-butylhydroperoxide (5.5 M in decane, 0.11 μmol, 20 μL) were added to a stirred solution of **33** (0.31 g, 2.26 mmol) in 1,2-dichloroethane (90 mL) under oxygen atmosphere at 0 °C. $Co(thd)_2$ (0.03 g, 0.08 mmol) was added, the mixture was stirred for 45 min at 0 °C and concentrated. The crude product was purified by flash chromatography (hexane/ethyl acetate 99:1) yielding starting material **33** (71 mg, 24%), silylperoxide **38** (19 mg, 3.0%), silylperoxide **39** (16 mg, 2.6%), and silylperoxide **40** (30 mg, 4.8%).

Characteristic data: **38** TLC (hexane 100%) R_f 0.31; ^{1}H NMR δ 1.70 (s, 3H, (CH_3)C(CH)(CH_2)), 4.63 – 4.69 (m, 2H, CH_2); **39** TLC (hexane 100%) R_f 0.25; ^{1}H NMR δ 0.61 – 0.70 (m, 6H, Si(CH_2CH_3)$_3$), 0.94 – 1.00 (m, 9H, Si(CH_2CH_3)$_3$), 1.12 (s, 6H, C(CH_3)$_2$Si), 4.57 – 4.59 (m, 2H, CH_2); **40** TLC (hexane 100%) R_f 0.10; ^{1}H NMR δ 0.61 – 0.71 (m, 6H, Si(CH_2CH_3)$_3$), 0.93 – 1.01 (m, 9H, Si(CH_2CH_3)$_3$), 1.13 (s, 3H, (CH_3)C(CH_2)$_2$Si)), 1.55 (s, 6H, C(CH_3)$_2$Si).

4-isopropenylcyclohexanone (**42**) was synthesized according to literature [76]. Methyltriphenylphosphonium bromide (14.5 g, 40.1 mmol), butyllithium (2.5 M, 17.0 mL, 42.5 mmol), **36** (7.16 g, 33.7 mmol). The reaction mixture was concentrated, dissolved in hexane (50 mL) and water (50 mL), the aqueous phase extracted with hexane (3 × 50 mL), the combined organic phases washed with water (1 × 200 mL) and brine (1 × 200 mL), dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 19:1 and 9:1) yielding 3.11 g (67%) of the target compound. Characterization data corresponds to literature values [76].

1-(1',3'-dioxolane)-4-isopropenylcyclohexane (43). *p*-Tolunesulfonic acid (14.6 mg, 0.08 mmol), 4-isopropenylcyclohexanone (1.69 g, 12.3 mmol), 1,2-ethanediol (0.74 mL, 13.3 mmol), and toluene (10 mL) was added to a roundbottomed flask fitted with a Dean-Stark water separator. The mixture was heated to reflux for 3.5 hours, allowed to cool to room temperature, washed with aqueous sodium hydroxide (10%, 1 × 50 mL), water (1 × 50 mL) and brine (1 × 50 mL). The aqueous phases was extracted with toluene (2 × 200 mL), the organic phases were combined, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel

eluting with hexane/ethyl acetate (stepwise gradient 19:1 and 9:1) yielding 3.04 g (74%) of the target compound as a yellow oil: ^{1}H NMR δ 1.51 – 1.58 (m, 4H), 1.69 – 1.82 (m, 7H), 1.88 – 1.98 (m, 1H), 3.94 (s, 4H), 4.66 – 4.72 (m, 2H); ^{13}C NMR δ 21.0, 29.1, 34.9, 44.3, 64.3, 64.4, 108.81, 108.83, 149.8.

1-(1',3'-dioxolane)-4-(propan-1-ol-2-yl)-cyclohexane (44). To a stirred solution of compound **51** (100 mg, 0.55 mmol) in dry THF (5 mL) at 0 °C under N_2 was added 1 M BH_3 in THF (1 M, 0.28 mmol). The mixture was stirred at 0 °C for 2 h before aqueous NaOH (3 M, 1.11 mmol) was slowly added at 0 °C followed by H_2O_2 (30% in water, 1.42 mL, 16.5 mmol). The mixture was stirred for 3 h in RT, water (10 mL) was added and the mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic phases was washed with brine (75 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel eluting with hexane/ethyl acetate (1:1) yielding 0.08 g (73%) of the target compound: ^{1}H NMR δ 0.89 (d, J = 6.96 Hz, 3H), 1.35 – 1.42 (m, 2H), 1.45 – 1.60 (m, 4H), 1.60 – 1.68 (m, 2H), 1.72 – 1.79 (m, 2H), 3.44 – 3.62 (m, 2H), 3.92 (s, 4H); ^{13}C NMR δ 13.6, 25.8, 28.0, 34.8, 35.0, 38.1, 40.1, 64.25, 64.30, 66.4, 109.1.

4-(propan-1-ol-2-yl)-cyclohexanone (45). Compound **44** (234 mg, 1.17 mmol) was added to a solution of *p*-toluensulfonic acid (121 mg, 0.64 mmol) in acetone (8 mL) and water (5 mL) and heated to reflux until TLC showed no starting material. Water (13 mL) and DCM (25 mL) was added to the reaction mixture, the aqueous phase was extracted with DCM (4 × 25 mL), the combined organic phases was washed with saturated aqueous sodium carbonate (2 × 125 mL), the combined sodium carbonate phases was extracted with DCM (4 × 125 mL), all organic phases were combined and dried over $MgSO_4$ and concentrated under reduced pressure yielding 0.18 g (100%) of the target compound that was used without further purification: ^{1}H NMR δ 0.92 (d, J = 6.96 Hz, 3H), 1.40 – 1.72 (m, 4H), 1.82 – 1.93 (m, 1H), 1.95 – 2.05 (m, 2H), 2.33 – 2.44 (m, 3H), 3.52 – 3.65 (m, 2H); ^{13}C NMR δ 13.5, 28.4, 30.7, 37.6, 39.6, 41.1, 41.3, 66.2, 212.3.

1-methylene-4-(propan-1-ol-2-yl)-cyclohexane (46). Butyllithium (2.5 M in hexane, 3.73 mL, 9.34 mmol) was added dropwise to a stirred suspension of methyltriphenylphosphonium bromide (3.34 g, 9.35 mmol) in dry THF (34 mL) under N_2

at room temperature. After 45 min, compound **45** (1.22 g, 7.78 mmol) dissolved in THF (10 mL) was added dropwise and the reaction mixture was heated to 60 °C for 4 h. The reaction mixture was allowed to reach room temperature before hexane (50 mL) and water (50 mL) were added. The aqueous phase was extracted with hexane (100 mL), the combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure at 0 °C yielding a yellow oil mixed with white crystals. The oil was transferred to a new flask and the crystals were washed with several portions of hexane that was combined with the oil and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 9:1, 4:1) yielding 0.50 g (42%) of the target compound: ^{1}H NMR δ 0.87 (d, J = 6.77 Hz, 3H), 1.00 – 1.22 (m, 2H), 1.45 – 1.58 (m, 2H), 1.69 – 1.77 (m, 2H), 1.94 – 2.07 (m, 2H), 2.25 – 2.34 (m, 2H), 3.42 – 3.63 (m, 2H), 4.56 – 4.58 (m, 2H); ^{13}C NMR δ 13.5, 29.9, 32.2, 34.9, 35.1, 38.9, 40.4, 66.4, 106.7, 149.9.

1-methylene-4-(propan-1-tosyloxyl-2-yl)-cyclohexane (41). Compound **46** (499 mg, 3.23 mmol) was added to a solution of p-toluenesulfonyl chloride (1.26 g, 6.61 mmol) in pyridine (8 mL) and stirred at RT under N_2 until TLC show no starting material. The reaction mixture was poured onto crushed ice and water (5 mL) and extracted with toluene (3 × 100 mL). The aqueous phase was extracted with toluene (3 × 100 mL), the organic phases were combined, washed with aqueous HCl (1M, 2 × 300 mL), dried over $MgSO_4$ and concentrated under reduced pressure yielding 0.76 g (72%) of crude product that was used without further purification: ^{1}H NMR δ 0.83 (d, J = 6.83 Hz, 3H), 0.91 – 1.11 (m, 2H), 1.39 – 1.50 (m, 1H), 1.55 – 1.64 (m, 2H), 1.64 – 1.73 (m, 1H), 1.85 – 2.01 (m, 2H), 2.20 – 2.29 (m, 2H), 2.44 (s, 3H), 3.82 – 3.96 (m, 2H), 4.55 – 4.57 (m, 2H), 7.31 – 7.36 (m, 2H), 7.75 – 7.80 (m, 2H); ^{13}C NMR δ 13.4, 21.7, 29.7, 31.7, 34.6, 34.7, 37.3, 38.5, 73.6, 107.0, 128.0, 129.9, 133.1, 144.8, 149.2.

1-methyl-4-(propan-1-tosyloxyl-2-yl)-cyclohexane-1-triethylsilyl peroxide (47). Triethylsilane (0.97 mL, 6.07 mmol) and *t*-butylhydroperoxide (5.5 M in decane, 0.10 μmol, 0.02 μL) were added to a stirred solution of **41** (0.65 g, 2.00 mmol) in 1,2-dichloroethane (26 mL) under oxygen atmosphere. $Co(thd)_2$ (0.03 g, 0.06 mmol) was added to the clear solution at room temperature, whereupon it instantly turned dark green. The mixture was stirred for 3 h, filtered through silica and concentrated. The crude

product was purified by flash chromatography on silica gel eluting with hexane/ethyl acetate (19:1) yielding 0.32 g (34%) of the target compound: ^{1}H NMR δ 0.58 – 0.70 (m, 6H), 0.83 (d, *J* = 7.14 Hz, 3H), 0.92 – 1.00 (m, 9H), 1.10 – 1.72 (m, 12H), 1.87 – 1.95 (m, 1H), 2.44 (s, 3H), 3.80 – 4.00 (m, 2H), 7.31 – 7.36 (m, 2H), 7.75 – 7.80 (m, 2H); ^{13}C NMR δ 4.0, 6.9, 13.2, 21.7, 23.2, 25.5, 25.7, 34.4, 34.6, 37.6, 37.9, 73.8, 79.8, 128.0, 129.9, 133.2, 144.7.

2-methyl-5-(propan-2-triethylsilyl peroxyl-2-yl)-cyclohexanone (53). Triethylsilane (0.85 mL, 5.32 mmol) and *t*-butylhydroperoxide (5.5 M in decane, 0.10 μmol, 0.02 μL) were added to a stirred solution of **29** (0.31 g, 2.01 mmol) in 1,2-dichloroethane (12 mL) under oxygen atmosphere. $Co(thd)_2$ (0.03 g, 0.06 mmol) was added to the clear solution at room temperature, whereupon it instantly turned dark green. The mixture was stirred for 2 h, filtered through silica and concentrated. The crude product was purified by flash chromatography on silica gel eluting with hexane/ethyl acetate (4:1) yielding 0.16 g (27%) of the target compound: ^{1}H NMR δ 0.30 – 2.60 (m, 32H); ^{13}C NMR δ 3.9, 6.8, 14.4, 21.8, 22.1, 26.6, 34.8, 43.3, 45.0, 46.7, 83.4, 213.2.

1-methylene-2-methyl-5-(propan-2-triethylsilyl peroxyl-2-yl)-cyclohexane (54). Butyllithium (2.5 M in hexane, 0.26 mL, 0.65 mmol) was added dropwise to a stirred suspension of methyltriphenylphosphonium bromide (0.24 g, 0.67 mmol) in dry THF (3 mL) under N_2 at 0 °C. After 20 min, compound **53** (0.16 g, 0.53 mmol) dissolved in THF (2 mL) was added dropwise and the reaction mixture was stirred at 0 °C for 2 h. Hexane (10 mL) and water (10 mL) were added to the reaction mixture, the aqueous phase was extracted with hexane (2 × 10 mL), the combined organic phases were washed with water (20 mL) and brine (20 L), dried over Na_2SO_4 and concentrated under reduced pressure yielding beige crystals. The crystals were filtered and washed with several portions of hexane that was combined and concentrated under reduced pressure yielding 0.12 g (74%) of crude product: ^{1}H NMR δ 0.61 – 0.69 (m, 6H), 0.77 – 0.93 (m, 1H), 0.93 – 1.00 (m, 9H), 1.01 – 1.04 (m, 3H), 1.12 – 1.16 (m, 6H), 1.16 – 1.32 (m, 1H), 1.62 – 1.75 (m, 2H), 1.75 – 1.87 (m, 3H), 1.87 – 1.98 (m, 1H), 4.55 – 4.58 (m, 1H), 4.65 – 4.68 (m, 1H); ^{13}C NMR δ 4.0, 6.9, 18.3, 21.7, 22.1, 27.5, 36.5, 37.4, 37.7, 47.0, 84.1, 104.6, 153.9.

General procedure for studying the stability of hydroperoxides 1, 2 and 3 towards Fe(III)TPPCl. Fe(III)TPPCl (1.60 mg, 2.28 μmol) dissolved in base washed $CDCl_3$ (75 μL) was added to a solution of hydroperoxide (77 μmol) in base washed $CDCl_3$ (0.7 mL) in a NMR tube at t = 0. The solution was thoroughly mixed and the degradation of the hydroperoxide was followed by ^1H NMR, where the signal corresponding to the endocyclic olefinic proton was used as a measure of hydroperoxide concentration.