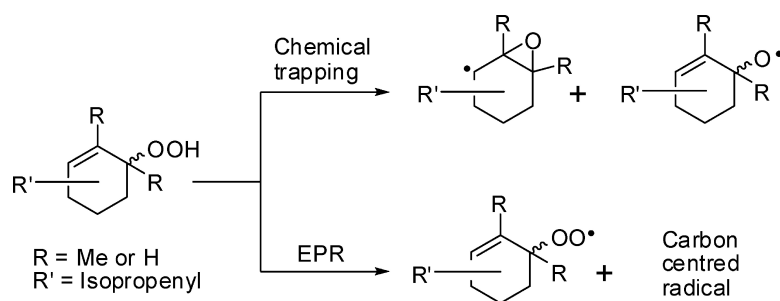


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Carbon- and Oxygen-Centered Radicals Are Equally Important Haptens of Allylic Hydroperoxides in Allergic Contact Dermatitis

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Limonene is one of the most commonly used fragrance compounds in western countries today. When exposed to air, it autoxidises, forming hydroperoxides that are strong contact allergens. To cause allergic contact dermatitis (ACD), the hydroperoxides are considered to bind covalently to proteins in the skin via a radical pathway. Consequently, the nature and reactions of the radicals formed from the hydroperoxides are important. We have examined the radical formation from, and sensitizing potential of, three allylic hydroperoxides. Two of these are found in the oxidation mixture of limonene, while the third is a synthetic structural analogue. The identity of the radicals formed from these hydroperoxides has been studied in radical trapping experiments. Chemical trapping experiments were performed using 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine iron(III) chloride [Fe(III)TPPCl₃] as an initiator and 1,1,3,3-tetramethylisindolin-2-yloxyl as a radical trapper. Electron paramagnetic resonance experiments using photolysis for initiation were performed with and without 5-diethoxy-phosphoryl-5-methyl-1-pyrroline *N*-oxide. Our results demonstrate the ability of the studied hydroperoxides to form peroxy, allyloxy, and oxiranilycarbonyl radicals. These radicals can potentially react with proteins to form immunogenic hapten–protein complexes relevant for ACD. The sensitizing potency of the hydroperoxides was studied in the murine local lymph node assay. All three hydroperoxides were found to be potent sensitizers with some variations, which can be related to the identity and quantity of the radicals formed. The results indicate that both carbon- and oxygen-centered radicals are important intermediates in the formation of hapten–protein complexes and that the sensitizing potency of the hydroperoxides is related to their structures.

Introduction

Contact allergy and its clinical manifestation allergic contact dermatitis (ACD)¹ can be caused by a variety of chemicals after repeated exposure to the skin. It is estimated that 15–20% of the population in the western world are allergic to one or more chemicals in their environment (1). During the last two decades, contact allergy to fragrances has become an increasing health problem (2) due to the increased use in toiletries and common household products. An important category of fragrance compounds is terpenes.

Terpenes can easily be transformed into strong contact allergens via autoxidation. Autoxidation occurs in contact with air and in the presence of an initiator, forming peroxides and hydroperoxides. Although virtually all types of organic materials can undergo air oxidation, terpenes are particularly prone to this reaction. Their isoprenoid skeletons are rich in allylic positions, and the radical abstraction of a hydrogen atom from

one of these positions, forming a stabilized allylic radical in the process, is the key step in the autoxidation reaction of terpenes. These stabilized allylic radicals are the reason for the relative ease of the autoxidation of terpenes. It has been shown that the resulting autoxidation mixtures of the terpenes limonene (3, 4), linalool (5, 6), and geraniol (7) can cause contact allergy. The main sensitizers in all of these oxidation mixtures have been shown to be hydroperoxides (5, 9).

Limonene is one of the most commonly used fragrance terpenes (10). When exposed to air, limonene forms two major hydroperoxides, (4*R*)-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide (**1**) and (5*R*)-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (**2**) (Figure 1). We have previously reported the isolation and identification of these compounds from the autoxidation mixture of limonene (8, 9). Allylic hydroperoxide **2** was also shown to be a strong allergen in guinea pigs (8). In order for a chemical to act as a hapten causing contact allergy, it has to penetrate the skin and react with macromolecules (generally considered to be proteins), thus forming immunogenic hapten–protein complexes. These complexes are recognized by the immune system, and an immunological process starts that ultimately leads to the development of contact allergy (11).

For the majority of organic haptens, the formation of a hapten–protein complex is considered to be an interaction between an electrophilic compound and a nucleophilic amino acid residue in a protein (12). However, in some cases of contact

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¹ Abbreviations: ACD, allergic contact dermatitis; LLNA, local lymph node assay; EC₃, estimated concentration required to produce a stimulation index of 3; HRMS, high-resolution mass spectroscopy; TMIO, 1,1,3,3-tetramethylisindolin-2-yloxyl; DEPMPO, 5-diethoxy-phosphoryl-5-methyl-1-pyrroline *N*-oxide; Fe(III)TPPCl₃, 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine iron(III) chloride.

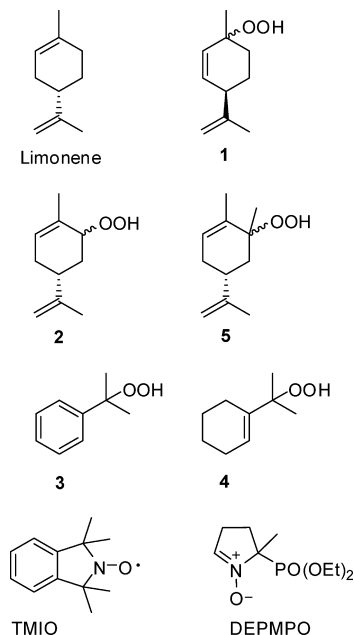


Figure 1. Chemical structures of compounds referred to in this paper. Hydroperoxides **1** and **2** have been identified in the autoxidation mixture of limonene, and **5** is a synthetic analogue of **2**. Cumene hydroperoxide (**3**) and cyclohexyl hydroperoxide (**4**) were used in a previous study (19). TMIO was used in the chemical trapping experiments, and DEPMPO was used in the EPR spin trapping experiments.

allergy, for example, urushiols (13) and hydroperoxides (14, 15), a radical mechanism has been proposed. The proposed mechanism for hydroperoxides starts with the breaking of the oxygen–oxygen bond, thus forming an oxygen-centered alkoxy radical (14). It is believed that heme-containing enzymes (e.g., cytochrome P450) present in skin can achieve this bond cleavage (16). The oxygen-centered radical formed could rearrange into other radical or nonradical species, which can act as haptens.

Only a small number of investigations of how hydroperoxides may modify macromolecules to cause contact allergy by employing a radical reaction mechanism exist (14, 17, 18). These studies, utilizing radical quenchers rather than amino acids or proteins, all reached similar conclusions: Tertiary allylic hydroperoxides prefer to react via an oxiranycarbonyl radical, formed via the 1,3-cyclization reaction of the initially formed allyloxyl radical. However, in a cross-reactivity study in guinea pigs, we have shown that animals induced with cumene hydroperoxide (**3**) cross-reacted with 1-(1-hydroperoxyl-1-methylethyl) cyclohexene (**4**) (Figure 1) (19). These two tertiary hydroperoxides are similar in size but differ in the structure of the six-membered ring attached to the hydroperoxide-bearing carbon. The allyloxyl radical of the cyclohexene hydroperoxide (**4**) can react with a macromolecule, either via an oxiranycarbonyl radical or directly via the primarily formed allyloxyl radical. However, the rearrangement of the cumene alkoxy radical to form the oxiranycarbonyl radical is energetically unfavorable, making antigen formation via this route unlikely (19). A similar case of cross-reactivity was observed in a study of abietic acid and dehydroabietic acid (14). These results indicated that direct addition of the alkoxy radical to the macromolecule plays an important part in the formation of immunogenic hapten–protein complexes of hydroperoxides. Thus, the identity of the formed radicals does not seem to influence the sensitizing and eliciting capacity of the hydroperoxides. However, it is of interest to further investigate the formation of carbon- and oxygen-centered radicals from hydroperoxides in parallel with the sensitizing capacity of the compounds, not only as previously done for

benzylic hydroperoxides vs allylic hydroperoxides (19) but also between structurally similar allylic hydroperoxides like the ones found in the autoxidation mixture of limonene.

The aim of the present study was to investigate the formation of carbon- and oxygen-centered radicals derived from allylic hydroperoxides of limonene, **1** and **2**, as well as from **5**, a structural analogue of **2** (Figure 1). These radicals would be available for the formation of hapten–protein complexes. The different hydroperoxides were subjected to 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine iron(III) chloride [Fe(III)TPPCl₃], which initiates homolytic cleavage of the oxygen–oxygen bond, thus creating the corresponding allyloxyl radicals. Further rearrangement and reactions of these allyloxyl radicals produced new radicals together with nonradical products. Carbon-centered radicals were trapped using 1,1,3,3-tetramethylisindolin-2-yloxy (TMIO) as a radical scavenger, creating stable nonradical products. Isolated nonradical products were identified and characterized using various spectroscopic methods. The hydroperoxides were also subjected to electron paramagnetic resonance (EPR) spin trapping experiments with 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) to detect the presence of carbon- and oxygen-centered radicals. Furthermore, we wanted to investigate the sensitizing potency of the individual hydroperoxides in the murine local lymph node assay (LLNA).

Experimental Procedures

Hazardous Materials. Skin contact with hydroperoxides must be avoided. As these compounds are skin-sensitizing substances, they must be handled with care.

Chemicals. Methylmagnesium iodide solution (3.0 M in diethyl ether), (1*S*)-3,7,7-trimethylbicyclo[4.1.0]hept-2-ene (97%), Fe(II)-TPPCl₃, *N*-benzylphthalimide (99%), (5*R*)-5-isopropenyl-2-methyl-2-cyclohexenone (98%), (5*R*)-5-isopropenyl-2-methyl-2-cyclohexen-1-ol (97%), hydrogen peroxide–urea adduct (30%), hydrogen peroxide (35% in water), and titanium tetrachloride (≥99.0%) were purchased from Sigma-Aldrich (Stockholm, Sweden). Methyl-lithium (1.6 M in diethyl ether), 3-chloroperoxybenzoic acid (mCPBA, 70–75%), and triethyl amine (99%) were purchased from Acros Organics (Geel, Belgium). Methanesulfonyl chloride (98%) was purchased from Lancaster (Lancashire, United Kingdom). Acetone was purchased from Merck (Darmstadt, Germany), and olive oil was purchased from Apoteket AB (Göteborg, Sweden). Spectrophotometric grade acetonitrile (99.8%) and chloroform (99.9%) for the EPR experiments were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Aqueous solutions were prepared with distilled water.

Instrumentation and Mode of Analysis. NMR spectroscopy was performed on a JEOL Eclipse+ 400 instrument at 400 MHz using CDCl₃ as the solvent. Chemical shifts (δ) are reported in ppm relative to CHCl₃ at 7.26 for ¹H and at 77.0 ppm for ¹³C. ¹H and ¹³C NMR spectra were assigned using ¹³C distortionless enhancement by polarization transfer (DEPT), ¹H–¹H correlation spectroscopy (COSY), ¹H–¹³C heteronuclear multiple-quantum coherence (HMQC), and ¹H–¹³C heteronuclear multiple-bond correlation (HMBC).

GC/MS analyses were performed using electron ionization (70 eV) on a Hewlett-Packard model 5973 mass spectrometer connected to a gas chromatograph (Hewlett-Packard model 6890). The GC was equipped with a cool on-column capillary inlet and an HP-5MSi fused silica capillary column (30 m × 0.25 mm, 0.25 μ m, Agilent Technologies, Palo Alto, CA). The temperature program started at 35 °C for 1 min, increased with 10 °C/min, and ended at 280 °C for 10 min. Helium was used as a carrier gas, and the flow rate was 1.2 mL/min.

Preparative HPLC was performed using a Gilson pump model 305, a Gilson UV/vis detector model 119 (Gilson Medical Electron-

ics, Inc., Middleton, WI), and a Zorbax Rx-SiL prepHT column (250 mm $\times$ 21.2 mm i.d., particle size 7 μ m, Agilent). Various concentrations of *tert*-butyl methyl ether in hexane (specified below) were used as the mobile phase, the flow rate was 21.24 mL/min, and the compounds were monitored at 205 nm.

Chiral preparative HPLC was performed using a Gilson 321 pump, a Gilson UV/vis 155 detector set at 220 nm, a PDR-Chiral Inc. peak collector module, a PDR-Chiral Inc. injector module equipped with a 10 mL loop, and a Chiralcel OD-H column (250 mm $\times$ 20 mm i.d., particle size 5 μ m, Chiral Technologies, Exton, PA). Heptane/ethanol 98.5/1.5 was used as a mobile phase, the sample concentration was 50 mg/mL, and the injection volume was 2.25 mL. The flow rate was 15 mL/min, and the separations were run at ambient temperature.

LC/MS analyses were performed on a Hewlett-Packard 1100 HPLC-MS including a vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector, and a single quadrupole mass spectrometer. The HPLC was equipped with a Zorbax SB-C18 column (150 mm $\times$ 3.0 mm i.d., particle size 3.5 μ m, Agilent). The mobile phase consisted of 0.1% formic acid and 5% acetonitrile in water (solvent A) and 0.1% formic acid and 5% water in acetonitrile (solvent B). The flow rate was 0.40 mL/min, and the column temperature was 40 °C. The mass spectrometer was equipped with an atmospheric pressure ionization electrospray (API-ES) interface used in the positive ionization mode with the following spray chamber settings: nebulizer pressure, 40 psig; capillary voltage, 3500 V; drying gas temperature, 350 °C; and drying gas flow rate, 10 L/min. The fragmentor voltage was set to 70–120 V. The mass spectrometer was used in the scan mode detecting molecular ions with *m/z* values ranging from 50 to 1000.

Column chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM) and Fluka aluminum oxide for chromatography (Brockmann activity II, basic, pH 10 $\pm$ 0.5). In some purifications, deactivated silica gel (Merck silica gel 60, 230–400 mesh ASTM) was used. Deactivation was done with 10% triethylamine in hexane.

TLC was performed using silica-plated aluminum sheets (Merck, 60 F₂₅₄ silica gel) and developed with anisaldehyde dip (2.1 mL of acetic acid, 5.1 mL of anisaldehyde, and 7 mL of H₂SO₄ in 186 mL of ethanol) followed by heating.

EPR spectra were recorded on a Bruker ESP 300^E spectrometer equipped with an X-band microwave bridge, 100 KHz modulation, and a variable temperature apparatus (Bruker B-VT 2000). Irradiation of the solutions inside the EPR cavity was performed with a Hamamatsu mercury–xenon lamp (fused silica window, 150 W, λ > 250 nm). Standard spectrometer settings were as follows: field sweep, 100 G; modulation amplitude, 1 G; microwave frequency, 9.8 GHz; and microwave power, 3 mW. Hyperfine splitting assignments were obtained by means of computer simulation using WINEPR SimFonia (version 1.25).

Elemental analysis was performed by H. Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany) and high-resolution mass spectra (HRMS) were obtained from the Department of Chemistry at Lund University (Lund, Sweden).

Synthesis. TMIO. The synthesis was performed as reported in the literature (20).

DEPMPO. The synthesis was performed as reported in the literature (21).

Titanium(IV) Oxide Hydroxide [TiO(OH)₂]. The synthesis was performed as reported in the literature (22).

2,3-Epoxy-3,7,7-trimethylbicyclo[4.1.0]heptane (6). The synthesis was performed as reported in the literature (23). The crude product (a 4:1 mixture of **6** and the starting material) was used directly in the next step without further purification.

(1*R*,4*R*)-4-Isopropenyl-1-methyl-2-cyclohexene-1-ol (7). The synthesis was based on a literature procedure (24). The crude product from the previous step (4.1 g in total, ~21.5 mmol of **6**) and TiO(OH)₂ (0.26 g, 2.7 mmol) in heptane (37 mL) yielded 3.12 g (76%) of crude product that was purified using flash chromatography on silica gel (hexane/ethyl acetate 4:1) to afford 2.1 g (50%)

of the target compound as a colorless oil. ¹H and ¹³C NMR data agreed with published data (25).

(4*R*)-4-Isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide (1). Hydrogen peroxide (aqueous, 35%, 74 mL) and concentrated sulfuric acid (2 drops) were added to a solution of **7** (0.87 g, 5.7 mmol) in pentane (43 mL). The mixture was stirred vigorously at ambient temperature until TLC showed no starting material. The phases were separated, and the organic layer was washed with deionized water (2 $\times$ 250 mL), dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified using flash chromatography on silica gel (hexane/ethyl acetate 85:15) and preparative HPLC to yield two diastereomers of the target compound as separate products, 0.21 (22%) and 0.15 g (16%), respectively. Major diastereomer: ¹H NMR: δ 1.30 (s, 3H, H₇), 1.45–1.57 (m, 1H, H_{A5}), 1.58–1.67 (m, 1H, H_{A6}), 1.73 (s, 3H, H₁₀), 1.89–2.05 (m, 2H, H_{B5} and H_{B6}), 2.76 (m, 1H, H₄), 4.66 (s, 1H, H_{A9}), 4.77 (s, 1H, H_{B9}), 5.68 (dd, 1H, *J* = 10.1, 2.0 Hz, H₃), 5.80 (dd, 1H, *J* = 10.3, 3.3 Hz, H₂), 7.48 (br s, 1H, OOH). ¹³C NMR: δ 21.3 (C₁₀), 24.4 (C₇), 24.9 (C₅), 30.2 (C₆), 42.7 (C₄), 80.9 (C₁), 111.0 (C₉), 130.1 (C₃), 134.6 (C₂), 147.3 (C₈). Minor diastereomer: ¹H NMR: δ 1.33 (s, 3H, H₇), 1.39–1.48 (m, 1H, H_{A6}), 1.66–1.71 (m, 2H, H₅), 1.72 (s, 3H, H₁₀), 2.15 (dt, 1H, *J* = 13.9, 4.8 Hz, H_{B6}), 2.67 (m, 1H, H₄), 4.75 (s, 1H, H_{A9}), 4.77 (s, 1H, H_{B9}), 5.63 (d, 1H, *J* = 10.3 Hz, H₃), 5.82 (dd, 1H, *J* = 10.1, 2.7 Hz, H₂), 7.40 (br s, 1H, OOH). ¹³C NMR: δ 20.9 (C₁₀), 24.5 (C₅), 24.7 (C₇), 31.0 (C₆), 43.6 (C₄), 79.5 (C₁), 111.1 (C₉), 129.4 (C₃), 136.0 (C₂), 147.8 (C₈). Anal. calcd for C₁₀H₁₆O₂: C, 71.39; H, 9.59; O, 19.02. Found (mixture of diastereomers): C, 71.28; H, 9.64; O, 19.15.

(5*R*)-5-Isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (2). (5*R*)-5-Isopropenyl-2-methyl-2-cyclohexen-1-ol (carveol) was purified on silica gel using hexane/ethyl acetate (stepwise gradient 19:1; 9:1, and 4:1) as the eluent. A round-bottomed flask charged with pure carveol (4.3 g, 28 mmol) and dichloromethane (100 mL) was cooled in an ice bath before triethylamine (3.7 g, 37 mmol) and methanesulfonyl chloride (3.9 g, 34 mmol) were added. The solution was stirred at 0 °C until TLC showed no starting material. Evaporation of the solvent resulted in a mix of a white precipitate and a yellow oil. A small amount of hexane was added, the precipitate was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified on deactivated silica gel using pure hexane as the eluent. (5*R*)-3-Chloride-5-isopropenyl-2-methyl-cyclohexene (**8**) was obtained as a colorless oil (4.13 g, 24 mmol, 63%, mixture of diastereomers). This compound was used directly in the next step without further characterization. Compound **8** was dissolved in dimethylformamide (115 mL), hydrogen peroxide–urea adduct (30%, 39.8 g, 423 mmol) was added, and the solution was stirred at room temperature overnight. The reaction mixture was extracted with hexane/ethyl acetate (1:1, 3 $\times$ 400 mL), the extracts were combined, concentrated under reduced pressure to 200 mL, washed with saturated sodium hydrogen carbonate solution (3 $\times$ 500 mL), deionized water (1 $\times$ 400 mL), and brine (1 $\times$ 400 mL), dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified using flash chromatography on silica gel (hexane/ethyl acetate 19:1, 9:1) to afford 1.45 g (48%, mixture of diastereomers 75:25) of the target compound. ¹H NMR: δ 1.73/1.74 (s, 3H, H₁₀), 1.74/1.79 (s, 3H, H₇), 1.40–1.49 (m, 1H, H_{A6}), 1.80–2.39 (m, 3H, H₄, H_{B6}), 2.30–2.37 (m, 1H, H₅), 4.35/4.52 (s, 1H, H₁), 4.74/4.74 (s, 2H, H₉), 5.63 (m, 1H, H₃), 5.74 (d, 1H, *J* = 5.1 Hz, H₃), 7.81/7.47 (s, 1H, H₁₁). ¹³C NMR: δ 19.2/21.3 (C₇), 20.6/21.0 (C₁₀), 30.9/31.2/31.3/32.6 (C₄ and C₆), 35.3/40.6 (C₅), 82.6/84.4 (C₁), 109.1/109.3 (C₉), 127.1/129.8 (C₃), 129.4/132.9 (C₂), 148.8/149.3 (C₈). Anal. calcd for C₁₀H₁₆O₂: C, 71.39; H, 9.59. Found (mixture of diastereomers): C, 71.26; H, 9.50.

(5*R*)-5-Isopropenyl-1,2-dimethyl-2-cyclohexene-1-ol (9). The synthesis was performed as described in the literature (26). The crude product was purified by flash chromatography on silica gel (hexane/ethyl acetate 9:1 and 4:1) to afford 11.5 g (84%, mixture of diastereomers 56:44) of the target compound. Major isomer: ¹H NMR: δ 1.32 (s, 3H, H₁₁), 1.60–1.70 (m, 1H, H_{A6}), 1.72–1.74

(m, 1H, H_A4), 1.73 (br s, 6H, H7 and H10), 1.89–1.97 (m, 1H, H_B6), 2.04–2.16 (m, 1H, H_B4), 2.23–2.36 (m, 1H, H5), 4.73 (s, 2H, H9), 5.38–5.42 (m, 1H, H3). ¹³C NMR: δ 17.0 (C7), 20.8 (C10), 27.0 (C11), 31.4 (C4), 40.3 (C5), 44.7 (C6), 72.6 (C1), 109.2 (C9), 123.0 (C3), 138.5 (C2), 149.1 (C8). MS (API-ES, 70 eV) *m/z* (%): 158 (9), 149 (69), 121 (100), 107 (82), 99 (19), 60 (17). Minor isomer: ¹H NMR: δ 1.30 (s, 3H, H11), 1.47–1.58 (m, 1H, H_A6), 1.73 (br s, 6H, H7 and H10), 1.74–1.76 (m, 1H, H_A4), 1.81–1.89 (m, 1H, H_B6), 2.04–2.16 (m, 1H, H_B4), 2.23–2.36 (m, 1H, H5), 4.70–4.74 (m, 2H, H9), 5.49–5.53 (m, 1H, H3). ¹³C NMR: δ 17.8 (C7), 21.0 (C10), 27.6 (C11), 31.3 (C4), 37.5 (C5), 44.1 (C6), 70.4 (C1), 109.0 (C9), 125.0 (C3), 137.2 (C2), 149.2 (C8). MS (API-ES, 70 eV) *m/z* (%): 158 (11), 149 (70), 121 (100), 107 (79), 99 (22), 60 (22). Anal. calcd for C₁₁H₁₈O: C, 79.46; H, 10.91. Found (mixture of diastereomers): C, 79.30; H, 10.82.

(5*R*)-5-Isopropenyl-1,2-dimethyl-2-cyclohexene-1-hydroperoxide (5). A round-bottomed flask was charged with hydrogen peroxide (aqueous, 35%, 250 mL), concentrated sulfuric acid (6 drops), and compound **9** (11.5 g, 69 mmol) dissolved in pentane. The mixture was stirred vigorously at room temperature, and the reaction was followed by TLC. The organic phase was separated, washed with deionized water (1 × 300 mL), dried over magnesium sulfate, filtered, and concentrated under reduced pressure yielding 11.2 g (88%) of crude product. This was purified using flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 90:10 and 85:15) and chiral preparative HPLC yielding 3.4 g (27%, 95:5 mixture of diastereomers) of the target compound. Major isomer: ¹H NMR: δ 1.33 (t, 1H, *J* = 13.6 Hz, H_A6), 1.35 (s, 3H, H11), 1.73 (br s, 6H, H7 and H10), 1.77–1.87 (m, 1H, H_A4), 2.11–2.21 (m, 1H, H_B4), 2.26–2.33 (m, 1H, H_B6), 2.43–2.53 (m, 1H, H5), 4.71–4.75 (m, 2H, H9), 5.69–5.74 (m, 1H, H3), 7.41 (s, 1H, OOH). ¹³C NMR: δ 18.3 (C7), 21.0 (C10), 23.2 (C11), 31.4 (C4), 37.0 (C5), 38.1 (C6), 82.4 (C1), 108.9 (C9), 129.6 (C3), 133.0 (C2), 149.3 (C8). Anal. calcd for C₁₁H₁₈O₂ (mixture of diastereomers): C, 72.49; H, 9.95; O, 17.56. Found: C, 72.33; H, 10.06; O, 17.43. The mixture did not contain a sufficient amount of the minor isomer to allow characterization.

Chemical Radical Trapping Experiments with TMIO, General Procedure. Fe(III)TPPCl₃ (1 equiv) was added to a solution of hydroperoxide (1 equiv) and TMIO (2 equiv) in acetonitrile/water (1:1). The suspension was stirred at ambient temperature until TLC showed no starting material and was filtered to remove excess Fe(III)TPPCl₃. The filtrate was extracted three times with hexane; the organic phases were pooled, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude products were purified using flash chromatography on silica gel eluting with various mixtures of solvents specified below. When necessary, this was followed by preparative HPLC, using various mixtures of hexane and *tert*-butyl methyl ether as the mobile phase.

Chemical Trapping Experiment with (4*R*)-4-Isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide (1). Starting from 0.32 g (1.9 mmol) of **1**, three products were isolated and characterized. (4*R*)-4-Isopropenyl-1-methyl-2-cyclohexene-1-ol (**7**, 0.08 g, 27%) was isolated in two steps. First, using liquid chromatography on silica gel eluting with solvents with increasing polarity [hexane/diethyl ether (1:1), hexane/ethyl acetate (1:1), and hexane/methanol (9:1)] and then using flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 80:20 and 75:25). ¹H and ¹³C NMR data agreed with published data (25). (4'*R*)-1',2'-Epoxy-4'-isopropenyl-1'-methyl-3'-(1,1,3,3-tetramethylisindolyl-2-oxy)cyclohexane (**10**) was isolated using liquid chromatography on silica gel eluting with solvents with increasing polarity [hexane/diethyl ether (1:1), hexane/ethyl acetate (1:1), and hexane/methanol (9:1)] and then using flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 90:10, 85:15, 80:20, and 50:50). Further purification was done by two isocratic rounds of preparative HPLC using hexane/*tert*-butyl methyl ether (first round, 90:10; second round, 98:2) as the mobile phase. Major isomer (0.14 g, 21%): ¹H NMR: δ 1.36 (s, 6H, H7' and H10), 1.37 (s, 3H, H13), 1.43–1.48 (m, 2H, H5'), 1.56 (s, 3H, H12), 1.57 (s,

3H, H11), 1.78–1.82 (m, 1H, H_A6'), 1.83 (s, 3H, H10'), 1.86–1.95 (m, 1H, H_B6'), 2.16–2.25 (m, 1H, H4'), 3.64 (s, 1H, H2'), 4.10 (dd, 1H, *J* = 9.5, 1.5 Hz, H3'), 4.83 (s, 1H, H_A9'), 4.86 (s, 1H, H_B9), 7.05–7.15 (m, 2H, H4 and H7), 7.20–7.25 (m, 2H, H5 and H6). ¹³C NMR: δ 22.3 (C10'), 25.0 (C7'), 25.2 (C10), 25.3 (C13), 27.5 (C5'), 28.7 (C6'), 29.0 (C11), 31.0 (C12), 41.5 (C4'), 61.5 (C2'), 62.0 (C1'), 67.5 (C1), 68.4 (C3), 82.5 (C3'), 110.8 (C9'), 121.4 (C4 or C7), 121.7 (C4 or C7), 127.2 (C5 or C6), 127.3 (C5 or C6), 145.5 (C8 and C9), 148.0 (C8'). MS (API-ES, 70 eV) *m/z* (%): 343 [M + 2] (28), 342 [M + 1] (100), 174 (5). Anal. calcd for C₂₂H₃₁NO₂: C, 77.38; H, 9.15; N, 4.10; O, 9.37. Found: C, 77.46; H, 9.10; N, 4.02; O, 9.40. Minor isomer (6.4 mg, 1%): ¹H NMR: δ 1.31–1.37 (m, 2H, H5'), 1.33 (s, 6H, H10 and H13), 1.36 (s, 3H, H7'), 1.49 (s, 3H, H11), 1.56 (s, 3H, H12), 1.83 (s, 3H, H10'), 1.86–1.93 (m, 2H, H6'), 2.40 (d, 1H, *J* = 10.8 Hz, H4'), 3.70 (d, 1H, *J* = 2.0 Hz, H2'), 4.44 (m, 1H, H3'), 4.78 (s, 1H, H_A9'), 4.89 (s, 1H, H_B9'), 7.06–7.12 (m, 2H, H4 and H7), 7.20–7.25 (m, 2H, H5 and H6). ¹³C NMR: δ 20.1 (C5'), 22.2 (C10'), 24.3 (C7'), 25.0 (C13), 25.6 (C10), 27.8 (C6'), 29.3 (C11), 31.1 (C12), 40.8 (C4'), 58.9 (C1'), 61.7 (C2'), 67.8 (C1), 68.2 (C3), 78.5 (C3'), 111.8 (C9'), 121.5 (C4 or C7), 121.6 (C4 or C7), 127.3 (C5 or C6), 127.4 (C5 or C6), 144.9 (C8), 145.5 (C9), 145.8 (C8). HRMS (ESI) *m/z* calcd for C₂₂H₃₁NO₂ + H, 342.2433; found, 342.2440.

Chemical Trapping Experiment with (5*R*)-5-Isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide (2). Starting from 0.26 (1.5 mmol) g of **2**, three products were isolated and characterized. (5*R*)-5-Isopropenyl-2-methyl-2-cyclohexene-1-ol (carveol, 7.4 mg, 2.5%) was isolated by two rounds of flash chromatography on silica gel eluting with hexane/ethyl acetate (first round stepwise gradient 8:2, 7:3, and 6:4; second round isocratic 9:1). ¹H and ¹³C NMR data agreed with authentic samples. (5*R*)-5-Isopropenyl-2-methyl-2-cyclohexenone (carvone, 0.10 g, 34%) was isolated by two rounds of flash chromatography on silica gel eluting with hexane/ethyl acetate (first round stepwise gradient 8:2, 7:3, and 6:4; second round isocratic 199:1). ¹H and ¹³C NMR data agreed with authentic samples. (5'*R*)-1',2'-Epoxy-5'-isopropenyl-2'-methyl-3'-(1,1,3,3-tetramethylisindolyl-2-oxy)-cyclohexane (**11**, 7.8 mg, 1.0%) was isolated as a mixture of isomers, in parallel with carvone. Major isomer: ¹H NMR: δ 1.00–1.12 (m, 1H, H_A4'), 1.32 (s, 3H, H10), 1.47 (s, 6H, H11 and H13), 1.48 (s, 3H, H7'), 1.58 (s, 3H, H12), 1.61–1.70 (m, 1H, H_A6'), 1.71 (s, 3H, H10'), 2.00–2.10 (m, 1H, H5'), 2.15–2.22 (m, 1H, H_B6'), 2.43–2.50 (m, 1H, H_B4'), 3.10 (s, 1H, H1'), 4.05 (dd, *J* = 10.6, 5.9 Hz, 1H, H3'), 4.71 (s, 2H, H9'), 7.06–7.12 (m, 2H, H4 and H7), 7.20–7.25 (m, 2H, H5 and H6). ¹³C NMR: δ 19.9 (C7'), 20.8 (C10'), 25.6 (C10), 25.9 (C13), 29.8 (C11), 31.2 (C12), 31.3 (C4'), 34.5 (C5'), 35.0 (C6'), 60.1 (C2'), 62.5 (C1'), 67.3 (C1), 68.6 (C3), 79.1 (C3'), 109.1 (C9'), 121.5 (C4 or C7), 121.6 (C4 or C7), 127.3 (C5 and C6), 145.0 (C8), 145.4 (C9), 148.9 (C8'). MS (EI, 70 eV) *m/z* (%): 341 [M⁺] (1), 326 (3), 191 (10), 176 (100), 160 (7), 145 (8), 129 (3), 107 (3). HRMS (ESI) *m/z* calcd for C₂₂H₃₁NO₂ + H, 342.2433; found, 342.2445. The mixture did not contain sufficient amounts of the minor isomers to allow characterization.

Chemical Trapping Experiment with (5*R*)-5-Isopropenyl-1,2-dimethyl-2-cyclohexene-1-hydroperoxide (5). Starting from 0.31 g (1.7 mmol) of **5**, five products were isolated and characterized. (5*R*)-5-Isopropenyl-1,2-dimethyl-2-cyclohexene-1-ol (**9**, 4.2 mg, 1.5%) was isolated by two rounds of flash chromatography on silica gel eluting with hexane/ethyl acetate (first round stepwise gradient 1:1 and 0:1; second round isocratic 9:1). Further purification was done by preparative HPLC using hexane/*tert*-butyl methyl ether (95:5) as the mobile phase. ¹H and ¹³C NMR data agreed with the above data. (5'*R*)-1',2'-Epoxy-5'-isopropenyl-1',2'-dimethyl-3'-(1,1,3,3-tetramethylisindolyl-2-oxy)cyclohexane (**12**) was isolated using flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 1:1 and 0:1). Further purification was done by two isocratic rounds of preparative HPLC using hexane/*tert*-butyl methyl ether (first round 95:5; second round 99:1) as the mobile phase. Major isomer (0.15 g, 25%): ¹H NMR: δ 1.04–1.16 (m, 1H, H_A4'), 1.32 (s, 3H, H13), 1.37 (s, 3H, H11'), 1.48 (br s, 9H,

H11, H13 and H7'), 1.58 (s, 3H, H10), 1.56–1.61 (m, 1H, H_A6'), 1.71 (s, 3H, H10'), 1.98–2.08 (m, 1H, H5'), 2.04–2.12 (m, 1H, H_B6'), 2.46–2.54 (m, 1H, H_B4'), 4.05 (dd, $J = 10.6, 5.9$ Hz, 1H, H3'), 4.70 (s, 2H, H9'), 7.06–7.12 (m, 2H, H4 and H7), 7.20–7.23 (m, 2H, H5 and H6). ¹³C NMR: δ 16.1 (C7'), 20.1 (C11'), 20.9 (C10'), 25.6 (C13), 26.0 (C11 or C12), 29.7 (C11 or C12), 31.2 (C10), 34.7 (C5'), 35.3 (C4'), 38.0 (C6'), 64.4 (C2'), 64.7 (C1'), 67.3 (C3), 68.6 (C1), 79.8 (C3'), 109.0 (C9'), 121.5 (C4 or C7), 121.6 (C4 or C7), 127.3 (C5 and C6), 145.0 (C9), 145.5 (C8), 149.2 (C8'). MS (API-ES, 120 eV) m/z (%): 357 [M + 2] (22), 356 [M + 1] (83), 193 (14), 192 (100), 174 (12), 160 (21), 159 (11). Anal. calcd for C₂₃H₃₃NO₂: C, 77.70; H, 9.36; N, 3.94. Found: C, 77.47; H, 9.28; N, 3.90. Minor isomer (0.06 g, 9.5%): ¹H NMR: δ 1.33–1.43 (m, 1H, H_A4'), 1.35 (s, 3H, H11'), 1.36 (s, 3H, H11), 1.46 (s, 3H, H10), 1.49 (s, 3H, H12), 1.50–1.60 (m, 1H, H_A6'), 1.58 (s, 3H, H7'), 1.59 (s, 3H, H13), 1.72 (s, 3H, H10'), 2.11 (dd, $J = 14.7, 4.4$ Hz, 1H, H_B6'), 2.19 (d, $J = 13.9$ Hz, 1H, H_B4'), 2.35–2.47 (m, 1H, H5'), 4.16 (dd, $J = 4.9, 3.1$ Hz, 1H, H3'), 4.69 (s, 1H, H_A9'), 4.73 (s, 1H, H_B9'), 7.06–7.12 (m, 2H, H4 and H7), 7.19–7.25 (m, 2H, H5 and H6). ¹³C NMR: δ 19.8 (C7' and C11'), 21.1 (C10'), 25.5 (C11 or C12), 25.6 (C11 or C12), 30.0 (C10), 31.0 (C13), 31.6 (C4'), 34.1 (C5'), 37.5 (C6'), 62.0 (C1' or C2'), 62.1 (C1' or C2'), 67.9 (C1), 68.4 (C3), 79.6 (C3'), 109.2 (C9'), 121.5 (C4 or C7), 121.6 (C4 or C7), 127.2 (C5 and C6), 145.3 (C8), 145.7 (C9), 148.9 (C8'). MS (API-ES, 120 eV) m/z (%): 357 [M + 2] (27), 356 [M + 1] (100), 192 (25), 174 (62), 160 (73), 109 (48). Anal. calcd for C₂₃H₃₃NO₂: C, 77.70; H, 9.36; N, 3.94; O, 9.00. Found: C, 77.84; H, 9.30; N, 3.86; O, 8.83. (5R)-2,3-Epoxy-5-isopropenyl-2,3-dimethyl-cyclohexanol (**13**) was isolated by flash chromatography on silica gel eluting with hexane/ethyl acetate (stepwise gradient 1:1 and 0:1). Further purification was done by two rounds of preparative HPLC using hexane/*tert*-butyl methyl ether (7:3 in both rounds) as the mobile phase. Major isomer (5.1 mg, 1.7%): ¹H NMR: δ 1.31 (ddd, 1H, $J = 13.8, 12.6, 4.8$ Hz, H_A4), 1.37 (s, 3H, H11), 1.44 (s, 3H, H7), 1.53 (dd, 1H, $J = 14.7, 11.0$ Hz, H_A6), 1.70 (s, 3H, H10), 1.72 (ddd, 1H, $J = 13.9, 4.4, 2.2$ Hz, H_B4), 2.08 (ddd, 1H, $J = 14.7, 4.2, 1.8$ Hz, H_B6), 2.14–2.23 (m, 1H, H5), 2.31 (d, 1H, $J = 10.6$ Hz, OH), 3.87 (ddd, 1H, $J = 10.6, 4.7, 2.2$ Hz, H1), 4.69 (s, 1H, H_A9), 4.72 (s, 1H, H_B9). ¹³C NMR: δ 18.6 (C7), 19.9 (C11), 21.1 (C10), 32.8 (C5), 36.8 (C4), 36.9 (C6), 64.5 (C2), 66.5 (C1), 69.0 (C3), 109.4 (C9), 148.3 (C8). MS (EI, 70 eV) m/z (%): 182 [M⁺] (2), 164 (21), 149 (43), 121 (100), 109 (95), 101 (50), 93 (47), 81 (50), 74 (60), 69 (42), 55 (32). HRMS (ESI) m/z calcd for C₁₁H₁₈O₂ + H, 183.1385; found, 183.1380. Minor isomer (4.7 mg, 1.5%): ¹H NMR: δ 1.14–1.26 (m, 1H, H_A4), 1.34 (s, 3H, H11), 1.39 (s, 3H, H7), 1.61 (dd, 1H, $J = 14.4, 11.0$ Hz, H_A6), 1.71 (s, 3H, H10), 1.96–2.16 (m, 3H, H_B4, H5 and H_B6), 3.83–3.93 (m, 1H, H1), 4.69 (s, 1H, H_A9), 4.73 (s, 1H, H_B9). ¹³C NMR: δ 15.9 (C7), 20.1 (C11), 20.9 (C10), 34.7 (C5), 37.1 (C6), 37.4 (C4), 64.5 (C1 and C2), 70.5 (C3), 109.4 (C9), 148.8 (C8). MS (EI, 70 eV) m/z (%): 182 [M⁺] (1), 164 (27), 149 (33), 121 (86), 109 (100), 91 (65), 81 (58), 67 (57), 55 (40). HRMS (ESI) m/z calcd for C₁₁H₁₈O₂ + H, 183.1385; found, 183.1380.

Direct EPR. An oxygen-free acetonitrile solution (30 mL) containing the allylic hydroperoxide (3 mM) was continuously flowed (flow rate, 1–10 mL/h) through a flat quartz cell (0.3 mm optical path length) inside the EPR cavity and directly irradiated at low temperature, typically between 220 and 250 K. The solutions were deaerated prior to use by purging with N₂ gas.

EPR with DEPMPO Spin Trapping. An oxygen-free acetonitrile solution (30 mL) containing the allylic hydroperoxide (3 mM) and DEPMPO (3.6 mM) was continuously flowed (flow rate, 1–10 mL/h) through a flat quartz cell (0.3 mm optical path length) inside the EPR cavity and directly irradiated at a temperature typically between 240 and 278 K. The solutions were deaerated prior to use by purging with N₂ gas. Exactly the same procedure was followed when using chloroform as the solvent.

Sensitization Experiments in Mice. Female CBA/Ca mice, 9 weeks of age, were purchased from Scanbur BK (Sollentuna, Sweden). The mice were housed in individually ventilated cages

Table 1. LLNA Responses for Compounds **1**, **2**, and **5**^a

compd and test concn (% w/v)	^{[3} H] thymidine incorporation (dpm/lymph node)	SI	EC3 value	
			M	% w/v
1			0.019	0.33
0.1	895	1.1		
0.5	3699	4.4		
1.0	6640	7.9		
2.5	15768	18.8		
7.5	18445	22.0		
2			0.049	0.83
0.5	952	2.1		
2.5	3530	7.7		
7.5	5795	12.7		
5			0.071	1.29
0.1	1326	1.4		
0.5	1759	1.8		
1.0	2292	2.4		
2.5	5504	5.7		
7.5	9093	9.4		

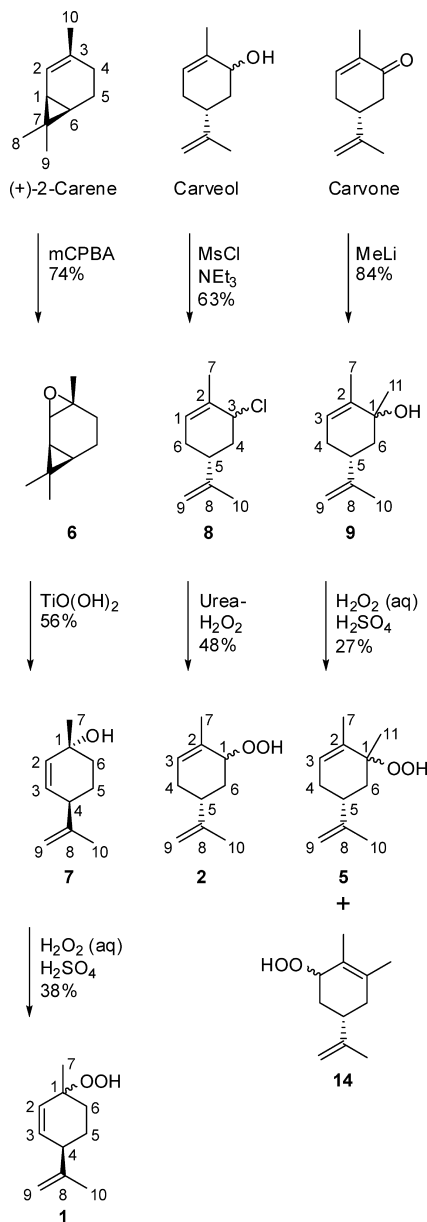
^a The local lymph node experiments were performed as described in the Experimental Procedures. The SIs corresponds to the increase in thymidine incorporation of treated groups relative to vehicle-treated controls. EC3 values (the estimated concentration required to induce an SI of 3) were calculated using linear interpolation.

(BioZone, Margate, United Kingdom) and kept on standard laboratory diet and water ad lib. The local ethics committee in Gothenburg approved the study. For the sensitization experiments, the murine LLNA was used (27). Mice in groups of three or five (Table 1) were treated by topical application on the dorsum of both ears with 25 μ L of the test compound dissolved in acetone:olive oil (AOO) (4:1 v/v) or with a vehicle control. All solutions were prepared freshly for every application. Treatments were performed daily for three consecutive days (days 0, 1, and 2). On day 5, all mice were injected intravenously via the tail vein with 20 μ Ci of [methyl-³H]thymidine (2.0 Ci/mmol, Amersham Biosciences, United Kingdom) in 250 μ L of phosphate-buffered saline (PBS). After 5 h, the mice were sacrificed, the draining lymph nodes were excised and pooled for each group, and single-cell suspensions of lymph node cells in PBS were prepared using cell strainers (Falcon, BD labware, 70 μ m pore size). Cell suspensions were washed twice with PBS, precipitated with 5% TCA and left in the refrigerator overnight. The samples were then centrifuged, resuspended in 1 mL of 5% TCA, and transferred to 10 mL of scintillation cocktail (EcoLume, INC Radiochemicals, United States). Thymidine incorporation was measured by β -scintillation counting on a Beckman LS 6000TA instrument. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), that is, test group/control group ratio. Test materials that at one or more concentrations caused an SI greater than 3 were considered to be positive in the LLNA. EC3 values (the estimated concentration required to induce an SI of 3) were calculated by linear interpolation (28).

Results and Discussion

We have investigated the identity of radicals formed from limonene hydroperoxides. These radicals should potentially be available for the formation of hapten–protein complexes, a prerequisite for the development of ACD. The sensitizing capacities of the hydroperoxides were evaluated in the murine LLNA.

We have previously shown that limonene forms allylic hydroperoxides **1** and **2** during autoxidation (8, 9). Compound **2** has also proved to be a potent allergen in guinea pigs (8). These two hydroperoxides were synthesized together with **5**, a tertiary analogue of **2** (Figure 1). To detect the presence of carbon- and oxygen-centered radicals, the three hydroperoxides were subjected to chemical radical trapping experiments with TMIO and EPR spin trapping experiments with and without

Scheme 1. Synthetic Routes to Allylic Hydroperoxides 1, 2, and 5

DEPMPO. These experiments showed that carbon- as well as oxygen-centered radicals were formed and that the structure of the allylic hydroperoxide has a major impact on the identity and quantity of radicals formed. All of the hydroperoxides were found to be potent allergens in the murine LLNA.

Synthesis. The syntheses of the hydroperoxides **1**, **2**, and **5** are shown in Scheme 1. All of the compounds are based on the limonene structural motif (4-isopropenyl-1-methylcyclohexene) where the positions of the hydroperoxide group and the endocyclic double bond have been altered. In addition, **5** has an extra methyl group in the same position as the hydroperoxide group.

Hydroperoxide **1** was synthesized from (+)-2-carene by epoxidation with mCPBA yielding epoxide **6** (2,3-epoxy-3,7,7-trimethylbicyclo[4.1.0]heptane). This epoxide was subsequently rearranged into alcohol **7** using TiO(OH)_2 as a catalyst, followed by substitution with hydrogen peroxide to generate **1**. Compound **2** was synthesized from carveol via the corresponding chloride **8**, which was successfully turned into the hydroperoxide using urea- H_2O_2 in dimethylformamide. The synthesis of **5** was

achieved by adding methyl lithium to carvone, producing alcohol **9**, which was then converted into hydroperoxide **5** using a two-phase system with pentane and aqueous hydrogen peroxide, employing sulfuric acid as a catalyst (Scheme 1).

Although the synthetic routes produced moderate overall yields of hydroperoxides, they were readily scaled up to provide adequate amounts for further experiments. The conversions of alcohols to their corresponding hydroperoxides were sluggish and contributed significantly to the reduction of the overall yield of hydroperoxides. TLC showed the formation of several byproduct in all hydroperoxide-forming reactions.

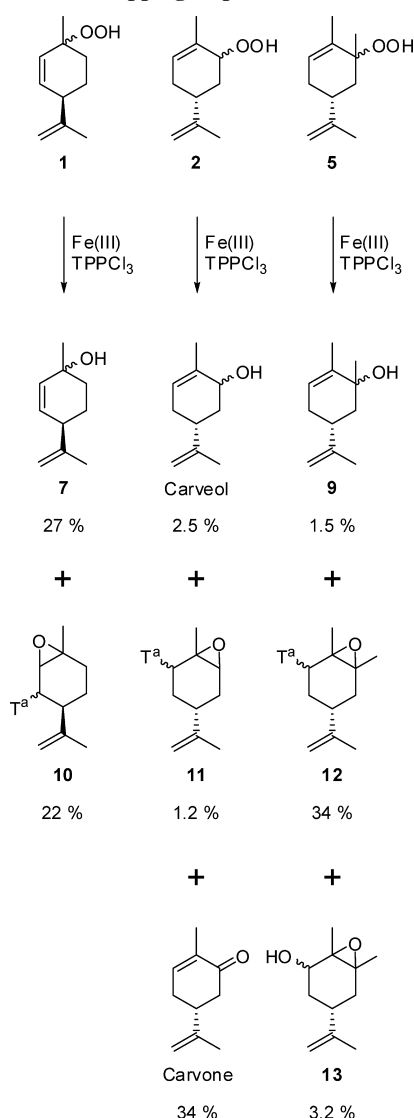
During the formation of **5**, 10% of the regioisomeric hydroperoxide **14** was formed due to rearrangement of the intermediate carbocation. These two regioisomers were separated using chiral preparative HPLC, yielding a 95:5 mixture of diastereomers of the desired product. The mixture did not contain sufficient amounts of the minor isomer to allow full characterization. In the synthesis of **1**, the crude ^1H NMR spectra showed a small amount of the corresponding rearrangement product. The same type of rearrangement as seen in the synthesis of **5** would, in the synthesis of **2**, give enantiomers instead of regioisomers (Scheme 1), thus making them indistinguishable from one another by NMR. The yields were also affected by the low stability of the hydroperoxides toward the workup and purification steps. The formation of **1** was the only hydroperoxide-forming reaction that displayed complete conversion of the starting material.

Chemical Trapping Experiments with TMIO. The chemical trapping experiments were performed to identify and quantify carbon-centered radicals formed from the hydroperoxides (Scheme 2). The experiments were performed with Fe(III)TPP-Cl_3 as an initiator and TMIO as a radical trapper in a 1:1 mixture of acetonitrile and water (17). Fe(III)TPP-Cl_3 is known to cleave the oxygen-oxygen bond homolytically, yielding the allyloxyl radical of respective hydroperoxide (29). TMIO was used as a radical scavenger due to its known specificity toward carbon-centered radicals (30, 31) and its suitability in terms of stability, UV absorbance, and symmetry.

In the radical trapping experiment with **1**, the products isolated and characterized were the corresponding alcohol (**7**, 27%) and the TMIO adduct of an oxiranycarbonyl radical (**10**, 22%). In the radical trapping experiment with **2**, the products isolated and characterized were carvone (34%), carveol (2.5%), and the TMIO adduct (**11**, 1.2%) of the oxiranycarbonyl radical. The fraction containing the TMIO adduct was analyzed by GC/MS. This analysis indicated the formation of four diastereomers. However, only one of the diastereomers was obtained in sufficient amount to allow full characterization. In the radical trapping experiment with **5**, the products isolated and characterized were the TMIO adduct of an oxiranycarbonyl radical (**12**, 34%), the corresponding alcohol (**9**, 1.5%), and an epoxy alcohol (**13**, 3.2%).

The iron(III)-induced homolytic cleavage of the oxygen-oxygen bond in the hydroperoxide group creates an oxygen-centered allyloxyl radical (**15**) (29). From this point, the reaction can follow several different pathways: (i) formation of the corresponding allylic alcohol by H-abstraction (carveol, **7**, and **9**) (32), (ii) 1,2-shift resulting in a 1-hydroxyallyl radical (**16**) (33, 34), or (iii) 1,3-cyclization resulting in an oxiranycarbonyl radical (**17**) (35) (Scheme 3).

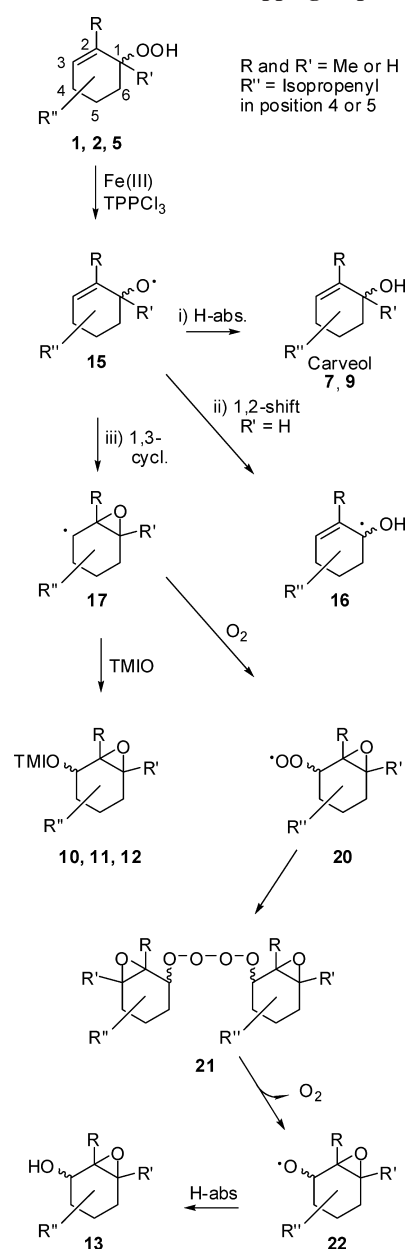
In the chemical trapping experiment with **1**, the possibility of a 1,2-shift is blocked by the methyl group on the carbon bearing the hydroperoxide functionality. Instead, the allyloxyl radical formed from **1** follows pathways i and iii (Scheme 3).

Scheme 2. Products Isolated and Identified in the Chemical Trapping Experiments^a^a T = TMIO.

The corresponding alcohol (**7**, 27%) formed via pathway i and the TMIO adduct (**10**, 22%) formed from the oxiranycarbonyl radical via pathway iii were isolated and identified in roughly equal amounts.

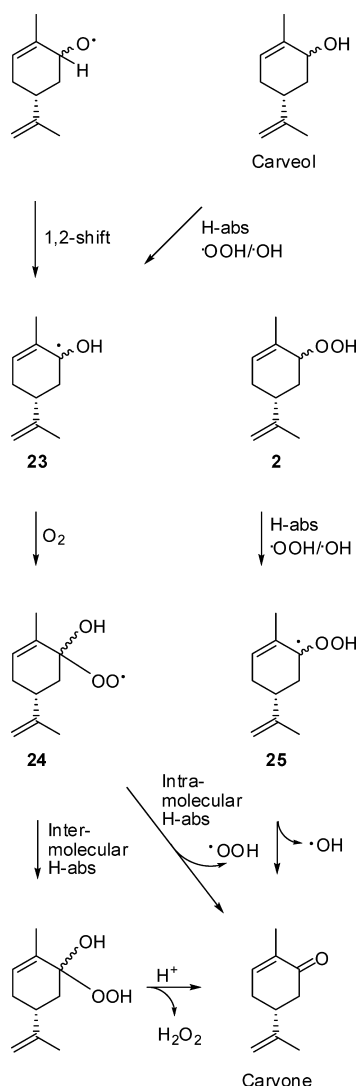
As for **1**, the results from the trapping experiment with **5** indicate that the initially formed allyloxyl radical follows pathways i and iii, since pathway ii is blocked by the methyl group on the carbon bearing the hydroperoxide functionality. Contrary to **1**, only a small amount of the corresponding alcohol (**9**) is formed in this reaction (1.5%). Instead, the 1,3-cyclization and the subsequent trapping by TMIO is the dominating pathway (34%), which indicates that the 1,3-cyclization in this case is faster than the H-abstraction. In the trapping experiment with **5**, a small amount of epoxy alcohol (**13**) was also isolated and identified (3.2%). The origin of this compound can be explained by the reaction of the oxiranycarbonyl radical (**17**) with oxygen, whereafter the formed peroxy radical (**20**) is dimerized giving a tetroxide (**21**). The subsequent expulsion of oxygen from the tetroxide leads to the epoxy alkoxy radical (**22**) that abstracts a proton to yield **13** (Scheme 3).

The allyloxyl radical formed in the experiment with **2** shows evidence of following all three pathways. However, pathway ii

Scheme 3. Mechanism That Can Account for the Products Isolated in the Chemical Trapping Experiments

and the reactions that follow the formation of the 1-hydroxyallyl radical (**23**) seem to dominate the reaction pattern of **2** (Scheme 4). The 1,2-shift of an alkoxy radical has a rate constant of $8 \times 10^6 \text{ s}^{-1}$ (33). However, as the hydroxyallyl radical is stabilized by both the oxygen in the α -position and the allylic double bond, it is likely that the rearrangement in this case is even faster (36, 37). The hydroxyallyl radical (**23**) can capture oxygen (O_2), forming a peroxy radical (**24**) (Scheme 4). This radical can abstract a hydrogen atom in two ways, intermolecularly or intramolecularly. In both cases, the subsequent reaction is an expulsion, forming carveol in the process. If the hydrogen abstraction is intermolecular, hydrogen peroxide is expelled via a general acid catalyzed mechanism. If the hydrogen abstraction is intramolecular, a hydrogen peroxide radical ($\text{HOO}^\bullet$) is expelled. Once the hydrogen peroxide radical ($\text{HOO}^\bullet$) is formed, it can start a separate radical chain reaction by abstracting the allylic hydrogen atom in carveol, generating a new molecule of 1-hydroxyallyl radical (**23**) that will be converted into carveol (Scheme 4). We have previously shown that this reaction pathway is of major importance in the conversion of geraniol

Scheme 4. Mechanism That Can Account for the Formation of Carvone in the Chemical Trapping Experiment with 2



to geranial (7, 38). Allylic hydrogen atom abstraction from **2** would result in a 1-hydroperoxyl allylic radical (**25**) that in turn rapidly and irreversibly would decompose to carvone and a hydroxyl radical (39). The formed hydroxyl and hydroperoxyl radicals can continue to feed these radical chain reactions, adding to the yield of carvone and further diminishing any amount of carveol formed via reaction pathway i. All together, this would account for the high yield of carvone (34%) as well as for the low yield of carveol (2.5%) and TMIO adduct (**11**, 1.2%) formed by pathways i and iii, respectively (Schemes 3 and 4).

In general, the amount of alcohol formed in the chemical radical trapping experiments can be taken as an indication of the amount of allyloxyl radicals available for formation of hapten–protein complexes. Likewise, the amount of TMIO adducts is an indication of the amount of oxiranylcarbiny radicals available for the same reaction. These results indicate that the true hapten in the hapten–protein complex forming reactions is a combination of several active haptens where both carbon- and oxygen-centered radicals play important roles. Depending on the structure of the hydroperoxide, there is a clear preference for different reaction pathways that will swing from a predominantly carbon-reactive pathway for **5**, to a mix of carbon- and oxygen-reactive pathways as for **1**, and further on to a possible combination of radical and classical electrophilic pathways for **2**. However, we believe that for **2** the radical

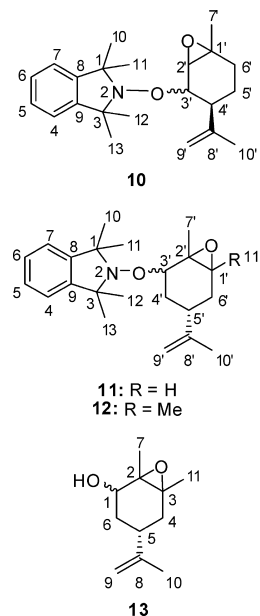


Figure 2. Adducts of oxiranylcarbiny radicals isolated from the chemical trapping experiments.

pathway is the prevailing one, since studies in guinea pigs revealed no cross-reactivity between carvone and hydroperoxides found in the oxidation mixture of limonene (8). Furthermore, if a purely electrophilic reaction pathway was to be assumed for **2**, the EC₃ value for carvone, 0.86 M (13% w/v) (40), classifying carvone as a weak sensitizer, can not alone explain the high sensitizing potential of **2**.

These results indicate that the sensitizing potential of the hydroperoxides might be affected by the identity and quantity of radicals formed. If so, the sensitizing potential of **1** might differ slightly from the sensitizing potential of **2** and **5**, because more radicals, both carbon- and oxygen-centered, are formed from **1**.

Structural Analysis of Carbon Radical Adducts. To determine their structure and identity, the oxiranylcarbiny radical adducts derived from the hydroperoxides investigated in this work were subjected to NMR analysis. The identities and structures were also supported by HRMS or elemental analysis. From the same kind of oxiranylcarbiny radical, three TMIO adducts (**10–12**) and one epoxy alcohol (**13**) (Figure 2) were isolated in the chemical trapping experiments. For each of compounds **10**, **12**, and **13**, two diastereomers were isolated. The structures of these compounds were determined by the combination of 1D and 2D NMR experiments (¹H, ¹³C, DEPT, COSY, HMQC, and HMBC). The combined application of ¹H–¹H and ¹H–¹³C correlation experiments allowed identification of both short, ¹J(C, H), and long-range, ²J(C, H), ³J(C, H), ²J(H, H), and ³J(H, H), couplings between atoms and provided the means for determining the structures of the adducts.

For both the TMIO adducts and the epoxy alcohol, full connectivity of the hydrogens in the terpene ring and the isopropenyl group was displayed in the ¹H–¹H correlation experiment (COSY). The ¹H–¹³C correlation experiments (HMQC and HMBC) confirmed the connectivity of the hydrogens and the carbons in and between the cyclohexane ring and the isopropenyl group. The epoxide ring was characterized by the couplings between the adjacent methyl and methylene hydrogens and the epoxide carbons. The binding of TMIO or OH was indicated by the shifts of the respective carbons as well as the shifts of the hydrogens located on these carbons. The

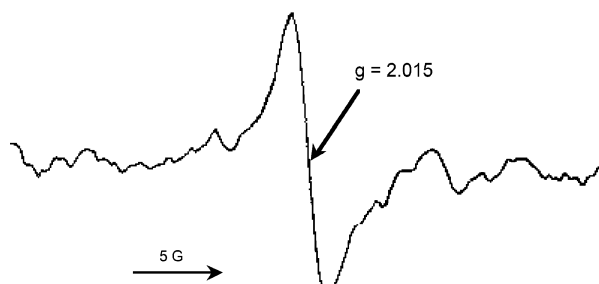


Figure 3. Direct EPR spectrum of the peroxy radical formed from irradiation of a solution of **2** in acetonitrile (3 mM). The solution was continuously flowed at 2 mL/h, and the temperature was 240 K.

assignments of the individual atoms (see the Experimental Procedures) are supportive of the proposed structures (Figure 2).

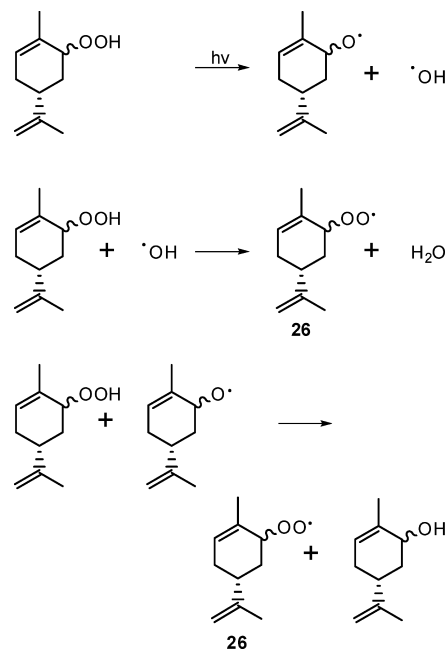
In the HMBC experiments with the TMIO adducts, the hydrogens in positions 3' and 5' for **10**, respectively, 4' and 6 for **11** and **12**, displayed $^2J(\text{C}, \text{H})$ couplings to the 4', respectively, 5' tertiary carbons. The 3' and 6' hydrogens also correlated with the 1' and 2' carbons via $^2J(\text{C}, \text{H})$ and $^3J(\text{C}, \text{H})$ couplings. The 7' (**10**–**12**) and 11' (**12**) methyl hydrogens correlate to the 1' and 2' carbons via $^2J(\text{C}, \text{H})$ and $^3J(\text{C}, \text{H})$ couplings. The 4' (**10**) and 5' (**11** and **12**) hydrogens correlate to the 8' carbon. In the HMQC, the 3' hydrogens displayed a $^1J(\text{C}, \text{H})$ coupling to the carbon in the 3' position.

In the HMBC experiments with the epoxy alcohol (**13**), the hydrogens in the 4 and 6 positions displayed $^2J(\text{C}, \text{H})$ couplings to the 5 carbon. The 1 and 4 hydrogens also correlated with the 2 and 3 carbons via $^2J(\text{C}, \text{H})$ and $^3J(\text{C}, \text{H})$ couplings. The 7 and 11 methyl hydrogens correlate to the 2 and 3 carbons via $^2J(\text{C}, \text{H})$ and $^3J(\text{C}, \text{H})$ couplings. The 5 hydrogen correlates to the 8 carbon via a $^2J(\text{C}, \text{H})$ coupling. In the HMQC, the 1 hydrogen displayed a $^1J(\text{C}, \text{H})$ coupling to the carbon in the 1 position.

EPR Experiments. The reactions following oxygen–oxygen bond cleavage of the hydroperoxides were studied in EPR experiments. With the EPR technique, it is possible to directly detect radical intermediates. In combination with a radical trapper, the spin trapping EPR technique can be used to detect short-lived radicals without the need to form stable products suitable for purification. The reactions were initiated with photolysis and carried out with and without DEPMPO as a radical trapper. The experiments with DEPMPO studied the formation of oxygen-centered radicals since these radicals were not expected to be trapped by TMIO.

Direct EPR. Reactions without DEPMPO were carried out to detect oxygen-centered radicals derived from the hydroperoxides directly, without the need of spin trapping. A broad-line singlet with a peak-to-peak line width of 8 G was detected when a solution of **2** in acetonitrile (3 mM) was irradiated at low temperature (240 K) in the EPR cavity (Figure 3). The g factor (gyromagnetic ratio) value of the singlet was higher ($g = 2.015$) than that of typical organic radicals. Comparison with literature g values indicated that an alkylperoxy radical $\text{R}-\text{OO}^\bullet$ (**26**) was formed (41, 42). Because EPR spectra of peroxy radicals have no hyperfine structure, except that due to the ^{17}O nuclear interaction, the g value provides the only index to distinguish them from other radicals such as carbon- and nitrogen-centered free radicals. The line width of alkylperoxy radicals is broad for a free radical in solution due to a large spin–rotation interaction. The experiments were carried out at low temperature as alkylperoxy radicals have a short lifetime and a high reactivity at room temperature. The mechanism that could

Scheme 5. Mechanism That Can Account for the Formation of the Alkylperoxy Radical Derived from Photolysis of **2 and Observed in the Direct EPR Experiments**



account for the formation of the alkylperoxy radical (**26**) is shown in Scheme 5.

Direct irradiation of solutions of **1** and **5** in acetonitrile also gave a signal corresponding to an alkylperoxy radical ($g = 2.015$). However, in the case of these two tertiary allylic hydroperoxides, it was necessary to drastically increase the flow (10 mL/h) to get a detectable signal.

DEPMPO Spin Trapping. The spin trapping technique was used to detect the alkylperoxy radicals formed as well as other species too reactive to be detected directly by EPR. Combined with EPR spectroscopy, the spin trapping technique has been extensively used for the detection and study of free radicals generated in biological milieu. Indeed, it is a versatile technique for detecting transient radical species by addition of a spin trap that will yield a more persistent paramagnetic species detectable by EPR.

A variety of cyclic nitrones, especially pyrroline *N*-oxides, exhibit interesting properties as spin traps (43). Among them, DEPMPO (Figure 1) has gained wider acceptance as a spin trap because of the very characteristic EPR spectra that are observed upon reaction with free radicals (44). The phosphorus coupling affords valuable information on the trapped radical and, this way, great reliability to the EPR spectrum assignments. Moreover, a big advantage of DEPMPO is the higher persistency of oxygen-centered radical adducts and especially of the superoxide and the alkylperoxy radicals (45).

The first experiments with **1**, **2**, and **5** were carried out in acetonitrile/water solutions. However, it became obvious that the best results and resolution were obtained in organic milieus. Depending on the solvent, acetonitrile or chloroform, somewhat different results were obtained. When **2** was photolyzed in the presence of DEPMPO in deaerated acetonitrile, the EPR spectrum shown in Figure 4A was obtained. This spectrum is the superimposition of two different signals, which can be attributed to the spin adducts arising from the addition of the formed $\text{R}-\text{OO}^\bullet$ (**26**) on both faces of the nitron (Scheme 6). The major signal (●) corresponds to the *trans*-diastereomer, resulting from the addition of $\text{R}-\text{OO}^\bullet$ (**26**) to the less hindered

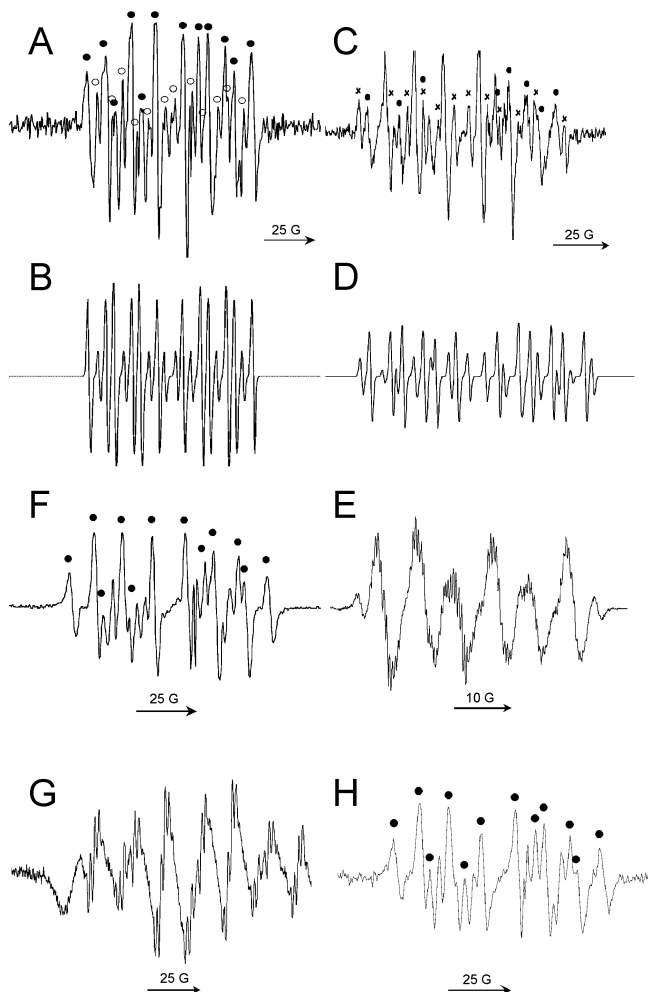
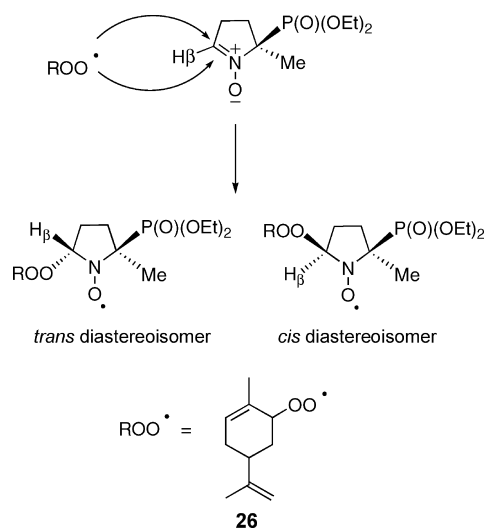


Figure 4. (A) EPR spectrum of DEPMPPO spin adducts formed from photolysis of **2** in acetonitrile (flow, 5 mL/h; $T = 278$ K). A mixture of two isomers is observed as follows: *trans*-isomer (●) with coupling constants $a_p = 46.93$ G, $a_N = 12.79$ G, $a_H = 8.78$ G, and $g = 2.0064$; and *cis*-isomer (○) with coupling constants $a_p = 38.63$ G, $a_N = 12.75$ G, $a_H = 7.34$ G, and $g = 2.0064$. (B) Spectrum simulated by WINEPR SimFonia (version 1.25). (C) EPR spectrum of DEPMPPO spin adducts formed from photolysis of **2** in chloroform (flow, 5 mL/h; $T = 283$ K). The major *trans*-DEPMPPO-OOR isomer was observed (●) with coupling constants $a_p = 48.05$ G, $a_N = 12.41$ G, $a_H = 8.06$ G, and $g = 2.0063$, together with a carbon radical DEPMPPO spin adduct (×) with coupling constants $a_p = 40.60$ G, $a_N = 13.40$ G, $a_H = 20.25$ G, and $g = 2.0065$. (D) Spectrum simulated by WINEPR SimFonia (version 1.25). (E) Half EPR spectrum of *trans*-DEPMPPO-OOR spin adduct formed from photolysis of **2** in chloroform (flow, 5 mL/h; $T = 283$ K) recorded at low modulation amplitude (0.5 G). The lines were split by weak couplings with the pyrrolidine ring hydrogens ($a_{H\gamma} = 0.45$ and 1.01 G). (F) EPR spectrum of DEPMPPO spin adducts formed from photolysis of **5** in acetonitrile (flow, 5 mL/h; $T = 278$ K). Under the same reaction conditions, an identical spectra is obtained for **1**. Only the major *trans*-DEPMPPO-OOR isomer was observed (●) with coupling constants $a_p = 47.50$ G, $a_N = 12.70$ G, $a_H = 8.70$ G, and $g = 2.0066$ in the case of **5** and $a_p = 47.75$ G, $a_N = 12.70$ G, $a_H = 8.70$ G, and $g = 2.0066$ in the case of **1**. (G) Half EPR spectrum of the *trans*-DEPMPPO-OOR spin adduct recorded at low modulation amplitude (0.5 G). The lines were split by weak couplings with the pyrrolidine ring hydrogens with $a_{H\gamma} = 0.46$ and 0.90 G for **5** and $a_{H\gamma} = 0.45$ and 1.02 G for **1**. (H) EPR spectrum DEPMPPO spin adducts formed from photolysis of **1** in chloroform (flow, 5 mL/h; $T = 283$ K). Under the same reaction conditions, an identical spectra is obtained for **5**. The major *trans*-DEPMPPO-OOR isomer was observed (●) with coupling constants $a_p = 47.75$ G, $a_N = 12.70$ G, $a_H = 8.70$ G, and $g = 2.006$ in the case of **1** and $a_p = 47.50$ G, $a_N = 12.70$ G, $a_H = 8.70$ G, and $g = 2.0066$ in the case of **5**.

face of the molecule, and the minor signal (○) corresponds to the *cis*-diastereomer. The EPR signal of the *trans*-diastereomer

Scheme 6. Radical Addition of Peroxyl Radicals on DEPMPPO



had the expected 12 lines ($a_p = 46.93$ G, $a_N = 12.79$ G, and $a_H = 8.78$ G) and showed an alternating line width. This pattern has already been described for the spin adducts of *tert*-BuOO• or superoxide with DEPMPPO and analogues (46, 47). The signal of the *cis*-diastereomer was also composed of 12 lines ($a_p = 38.63$ G, $a_N = 12.75$ G, and $a_H = 7.34$ G). When conducting the same experiment in chloroform, the *trans*-DEPMPPO-OOR spin adduct was observed. Most interestingly, a signal corresponding to the trapping of a carbon-centered radical was also observed (Figure 4C). The experimental signal was in good agreement with simulations using the calculated parameters $a_p = 40.60$ G, $a_N = 13.40$ G, and $a_H = 20.25$ G, $a_H > a_N$ being characteristic of a trapped carbon-centered radical (48). Most probably the signal of the minor *cis*-DEPMPPO-OOR diastereomer was hidden by the signal of the carbon radical DEPMPPO spin adduct. When the spectra were recorded at low modulation amplitude, the *trans*-DEPMPPO-OOR spin adduct clearly exhibited the alternating line width mentioned above, and the lines were further split by weak couplings with the pyrrolidine ring hydrogens ($a_{H\gamma} = 0.45$ and 1.01 G) (Figure 4E). It has been suggested in the literature that the alternating line width is generated by a slowed down rotation around the O—O peroxy bond (Figure 5), as the same phenomenon is not observed when trapping alkoxyl R—O• radicals (44, 47). This rotation induces conformational changes resulting in diverse forms of the DEPMPPO-OOR spin adduct for which the hyperfine coupling constants have different values. The fact that the spectra changed drastically when the experiments were carried out at lower temperatures with a widening of the signals supports this hypothesis.

For the tertiary hydroperoxides **1** and **5**, the behavior was slightly different in the sense that only and exclusively the *trans*-DEPMPPO-OOR spin adduct was detected. The same results were obtained in both acetonitrile (Figure 4F,G) and chloroform (Figure 4H), and the characteristics of the adducts were exactly

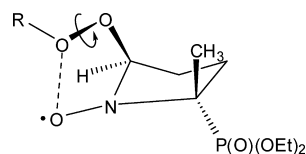


Figure 5. DEPMPPO-OOR *trans*-diastereomer: Conformational exchanges are suggested to be the result of the hindered rotation around the O—O bond.

the same as described for **2**. No traces of a carbon-centered radical adduct were detected.

Sensitizing Capacity of the Hydroperoxides. The murine LLNA (27) was used to evaluate the sensitizing potency of hydroperoxides **1**, **2**, and **5** (Table 1). The EC₃ value of hydroperoxide **1** was 0.019 M (0.33% w/v), of hydroperoxide **2** was 0.049 M (0.83% w/v), and of hydroperoxide **5** was 0.071 M (1.29% w/v). This is in accordance with the values of several other hydroperoxides that have been tested previously (6, 7, 18, 49), and all three hydroperoxides can be considered potent contact allergens. Although small, the variation in EC₃ values may be indicative of a notable difference in the sensitizing potential. If so, this difference might be caused by the different identity and quantity of radicals formed from the hydroperoxides, as displayed in the chemical trapping experiments and the EPR studies.

Conclusion

We conclude that peroxy, allyloxy, and oxiranycarbonyl radicals are readily formed from hydroperoxides **1**, **2**, and **5**. Our results support the hypothesis that these radicals are the true haptens in the proposed radical reaction mechanism for the formation of immunogenic hapten–protein complexes of hydroperoxides. Furthermore, the structures of the hydroperoxide clearly influence the identity as well as the quantity of the formed radicals. This is displayed in the product distribution of the chemical trapping experiments as well as in the EPR experiments. All three hydroperoxides gave a positive response in the LLNA and can be considered potent sensitizers. However, there was a small difference between the sensitizing capacities of the hydroperoxides. This difference could originate from the difference in radical formation, thus indicating that different radicals have different sensitizing potential. In summary, our results show that the structure of the allylic hydroperoxide will dictate the amount of formed radicals and indicate that carbon and oxygen radicals are equally important as haptens in ACD of allylic hydroperoxides.

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Supporting Information Available: ¹H and ¹³C NMR data for 2,3-epoxy-3,7,7-trimethylbicyclo[4.1.0]heptane (**6**). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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