Fragrance Compound Geraniol Forms Contact Allergens on Air Exposure. Identification and Quantification of Oxidation Products and Effect on Skin Sensitization

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · MAY 2007

Impact Factor: 3.53 · DOI: 10.1021/tx700017v · Source: PubMed

6 AUTHORS, INCLUDING:

Lina Hagvall
University of Gothenburg
20 PUBLICATIONS **322** CITATIONS
SEE PROFILE

Gunnar Nyman
University of Gothenburg
138 PUBLICATIONS **2,709** CITATIONS
SEE PROFILE

Anna Börje
University of Gothenburg
52 PUBLICATIONS **1,360** CITATIONS
SEE PROFILE

Ann-Therese Karlberg
University of Gothenburg
176 PUBLICATIONS **4,146** CITATIONS
SEE PROFILE

Fragrance Compound Geraniol Forms Contact Allergens on Air Exposure. Identification and Quantification of Oxidation Products and Effect on Skin Sensitization

Lina Hagvall,[†] Carina Bäcktorp,[‡] Sophie Svensson,[§] Gunnar Nyman,[‡] Anna Börje,[†] and Ann-Therese Karlberg*[,†]

Department of Chemistry, Dermatochemistry and Skin Allergy, Göteborg University, SE-412 96 Göteborg, Sweden, Computational Chemistry, Department of Chemistry, Göteborg University, SE-412 96 Göteborg, Sweden, and Occupational and Environmental Medicine, Sahlgrenska Academy and University Hospital, SE-405 30 Göteborg, Sweden

Received January 15, 2007

Fragrances are common causes of contact allergy. Geraniol (*trans*-3,7-dimethyl-2,6-octadiene-1-ol) is an important fragrance terpene. It is considered a weak contact allergen and is used for fragrance allergy screening among consecutive dermatitis patients. Analogous to other monoterpenes studied, such as limonene and linalool, geraniol has the potential to autoxidize on air exposure and form highly allergenic compounds. The aim of the present study was to investigate and propose a mechanism for the autoxidation of geraniol at room temperature. To investigate whether allergenic compounds are formed, the sensitizing potency of geraniol itself, air-exposed geraniol, and its oxidation products was determined using the local lymph node assay in mice. The results obtained show that the allylic alcohol geraniol follows an oxidation pattern different from those of linalool and limonene, which autoxidize forming hydroperoxides as the only primary oxidation products. The autoxidation of geraniol follows two paths, originating from allylic hydrogen abstraction near the two double bonds. From geraniol, hydrogen peroxide is primarily formed together with aldehydes geranial and neral from a hydroxyhydroperoxide. In addition, small amounts of a hydroperoxide are formed, analogous to the formation of the major linalool hydroperoxide. The autoxidation of geraniol greatly influenced the sensitizing effect of geraniol. The oxidized samples had moderate sensitizing capacity, quite different from that of pure geraniol. The hydroperoxide formed is believed to be the major contributor to allergenic activity, together with the aldehydes geranial and neral. On the basis of the present study and previous experience, we recommend that the possibility of autoxidation and the subsequent formation of contact allergenic oxidation products are considered in risk assessments performed on fragrance terpenes.

Introduction

Contact allergy and its clinical manifestation, allergic contact dermatitis, are caused by chemicals that can penetrate the skin and form antigens by reactions with macromolecules (proteins) in the skin. Some compounds, prohaptens, are not allergenic themselves but are activated outside the body, for example, by air oxidation or in the skin by metabolism. Our research is focused on identifying potential prohaptens and increasing the understanding of structure−activity relationships involving activation steps. Thus, the basis for the prediction of contact sensitizers on the basis of chemical structure is enlarged. We have, in previous studies, shown that the commonly used fragrance terpenes, limonene (*p*-mentha-1,8-diene) and linalool (3,7-dimethyl-1,6-octadien-3-ol) **1** (Figure 1), themselves are weak sensitizers or non-sensitizers but autoxidize spontaneously on air exposure forming oxidation products with varying sensitizing potencies (*1*−*3*). The primary oxidation products, the hydroperoxides, are strong contact allergens (*2*, *4*). Clinical studies have shown oxidized limonene and linalool to be frequent causes of contact allergy (*5*, *6*).

Fragrances are, next to nickel, the most common causes of contact allergy in the western world (*7*). A fragrance mix (FM I[1]), consisting of seven fragrance chemicals and one natural mixture, is used for the screening of fragrance allergy among consecutive dermatitis patients in dermatology clinics. One of the fragrance chemicals included in FM I is geraniol (*trans*-3,7-dimethyl-2,6-octadiene-1-ol) **2** (Figure 1). It is considered to be a weak allergen and is responsible for only 5% of the positive patch test reactions to the individual compounds of FM I (*8*). Geraniol occurs naturally in large amounts in various plant materials, for example, in rose, citronella, and palmarosa. It is an important fragrance terpene, widely used because of its fresh flowery odor. Geraniol was detected in 76% of investigated deodorants on the European market (*9*) and in 41% of domestic and household products (*10*). Studies also show that geraniol is present in 33% of the cosmetics based on natural ingredients (*11*).

Antigen formation is normally considered to take place between an electrophilic hapten and nucleophilic moieties in amino acid side chains in skin proteins. Geraniol is not electrophilic and should consequently not possess any contact

* To whom correspondence should be addressed. E-mail: karlberg@chem.gu.se.
[†] Dermatochemistry and Skin Allergy, Göteborg University.
[‡] Computational Chemistry, Göteborg University.
[§] Sahlgrenska Academy and University Hospital.

[1] Abbreviations: EC3, estimated concentration required to produce a SI of 3; FIA, flow injection analysis; FM I, fragrance mix I; HPAA, *p*-hydroxyphenylacetic acid; HRP, horse radish peroxidase; LLNA, local lymph node assay; SI, stimulation index.

10.1021/tx700017v CCC: $37.00
Published on Web 04/12/2007

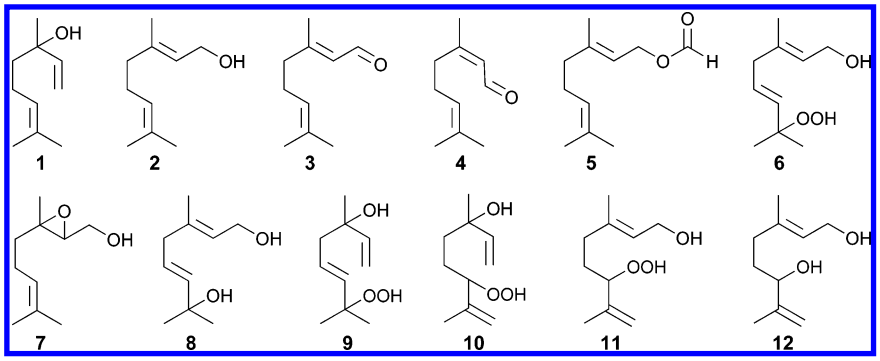

Figure 1. Structures of linalool **1** and its hydroperoxides **9**−**10**, geraniol **2** with identified oxidation products **3**−**8**, and synthesized reference compounds **11** and **12**.

allergenic activity. However, analogously to linalool (*3*), it has the potential to autoxidize on air exposure at normal handling and storage, but to the best of our knowledge, there are no published studies on the autoxidation of geraniol. Thus, it is important to investigate the pattern of geraniol autoxidation and to study the sensitizing capacity of the oxidation products formed. At autoxidation, allylic hydrogen atoms are the most plausible targets, considering hydrogen abstraction in a radical chain process. Geraniol possesses six allylic positions for hydrogen abstraction, whereas the previously studied linalool only has three. Therefore, in the oxidation mixture, the formation of not only the corresponding hydroperoxides seen in the linalool oxidation (*3*) is expected but also other oxidation products originating from allylic hydrogen abstraction at the first trisubstituted double bond bearing the allylic alcohol.

The aim of the present study was to investigate the autoxidation of geraniol at room temperature. In order to investigate whether allergenic compounds are formed on air exposure, the sensitizing potency of geraniol itself, air-exposed geraniol, and its oxidation products was determined. We also wanted to propose a mechanism for the major route of autoxidation.

Experimental Procedures

Caution: *Oxidized geraniol contains contact allergenic substances, and therefore, skin contact must be avoided and the compounds handled with care.*

Chemicals. Geraniol **2**, without antioxidant and 99% after distillation, *tert*-butyl hydroperoxide, 5.0−6.0 M in nonane, 88% cumene hydroperoxide, horse radish peroxidase (HRP), *p*-hydroxyphenylacetic acid (HPAA), and hydrogen peroxide 35% w/v in water were purchased from Sigma Aldrich (Germany). 98% Geranial (*trans*-3,7-dimethyl-2,6-octadienal) **3**, >99% neral (*cis*-3,7-dimethyl-2,6-octadienal) **4**, and geranyl formate (*trans*-formic acid 3,7-dimethyl-octa-2,6-dienyl ester) **5** were obtained from Bedoukian Research, Inc. (USA). Acetone, p. a. and HPLC grade methanol were purchased from Merck (Germany). Olive oil was purchased from Apoteket AB (Sweden).

Instrumentation and Mode of Analysis. Chromatographic separations were performed using Merck silica gel 60 (230−400 mesh ASTM). TLC plates (Merck, 60 F_{254} silica gel) were sprayed with a visualizing agent containing anisaldehyde, sulfuric acid, and acetic acid in ethanol after development.

GC analyses were performed using a Hewlett-Packard (HP) 6890 gas chromatograph equipped with an on-column injector and a flame ionization detector, using a 30 m fused silica column (HP-5; ID 0.25 mm, 0.25 μm film thickness) and nitrogen as the carrier gas. The column temperature was 35 °C at injection, held isothermally for 2 min, raised to 185 °C at a rate of 5 °C/min, and finally held at 185 °C for 5 min. The detector temperature was 250 °C, and 1,2,3,5-tetramethylbenzene was used as the internal standard.

Electron impact (EI) mass spectral data (70 eV) were obtained on an HP mass selective detector 5973 (scanned *m*/*z* 40−500), connected to a GC HP 6890.

HPLC analyses were conducted on a Merck LaChrom 2000 instrument with a diode array detector. An analytical silica column, Purospher STAR (4.6 × 250 mm, 5 μm particles, Merck) was used. The column and sample rack were kept at a constant temperature of 20 °C. Mobile phase: *tert*-butyl methyl ether/*n*-hexane 1:9, isocratic elution for 10 min, a linear gradient for 10 min reaching *tert*-butyl methyl ether/*n*-hexane 6:4, and isocratic elution with *tert*-butyl methyl ether/*n*-hexane 6:4 for an additional 20 min. The flow rate was 1 mL/min. Blank subtractions of the gradient were performed.

Preparative HPLC was performed using a Gilson pump model 305 and a Gilson UV/VIS detector model 119. Photo-oxidation was performed using a Rayonet reactor equipped with 16 RPR 350 nm lamps.

Semiquantitative Merckoquant peroxide colorimetric analytical test strips of the scale 1−100 mg/L were used to estimate hydrogen peroxide content in the mixtures of air-exposed geraniol. The quantification of hydrogen peroxide was carried out on a flow injection analysis (FIA) system consisting of a Gilson Pretech 233XL autosampler and a Jasco PU-980 HPLC pump connected to a Jasco FP-920 fluorescence detector.

^{1}H and ^{13}C NMR spectra were recorded on a JEOL eclipse+ 400 MHz spectrometer using $CDCl_3$ as the solvent. Chemical shifts (δ) are reported in ppm relative to $CHCl_3$ at δ 7.25 and δ 77.0 for ^{1}H and ^{13}C, respectively.

Air Exposure Procedure. A distilled sample of geraniol (98%) was air-exposed in an Erlenmeyer flask, covered with aluminum foil to prevent contamination. It was stirred at room temperature for 1 h, 4 times a day, as previously described (*1*). Samples were taken on a regular basis and stored in the freezer under nitrogen to be analyzed with GC and HPLC-UV to determine the concentrations of geraniol and its major oxidation products.

Isolation of Major Oxidation Products Using Flash Chromatography. Samples of air-exposed geraniol were subjected to flash chromatography on silica gel columns. Starting with about 5 g of oxidized material, repeated purifications were made. Mixtures of ethyl acetate and *n*-hexane were used for elution, starting with ethyl acetate/hexane 1:9, after which the proportion of ethyl acetate was increased. Isolated compounds were characterized with NMR and GC-MS using reference compounds.

Quantification of Geraniol and Oxidation Products Using HPLC. The amounts of geraniol **2**, geranial **3**, neral **4**, and geraniol hydroperoxide **6** were determined in geraniol air-exposed from 0 to 45 weeks using HPLC. Pure reference compounds were used to make external calibration curves from which the concentrations of the substances in the air-exposed geraniol could be determined. Retention times for reference compounds and isolated compounds were determined and compared.

Quantification of Oxidation Products using GC. The amounts of the secondary oxidation products geranylformate **5**, epoxygeraniol **7**, and 3,7-dimethyl-octa-2,5-diene-1,7-diol (**8**), were determined

Table 1. Determination of the Selectivity of HRP for Hydrogen Peroxide over Hydroperoxides

compound	relative response[a] (%)
hydrogen peroxide (standard)	100
linalool hydroperoxides **9**, **10**	0.65
cumene hydroperoxide	0.09
tert-butyl hydroperoxide	0.81

[a] The relative responses of hydrogen peroxide and hydroperoxides were determined. The relative response for hydrogen peroxide was set to 100%, and the relative responses of hydroperoxides were normalized and compared to this.

using GC. Analyses were made on pure reference compounds with added internal standard (1,2,3,5-tetramethylbenzene) to determine the response factors. The same amount of internal standard was added to the dissolved samples of air-exposed geraniol. Using the response factor, the amount of each compound in the samples could be determined.

Quantification of Hydrogen Peroxide. Hydrogen peroxide was quantified in the air-exposed samples by a FIA method with fluorescence detection, previously described by Heinmöller et al. (*12*) and further modified by Svensson et al. (*13*). This derivatization method involves an enzymatic reaction with HRP and a substrate, HPAA. The enzyme catalyzes a redox reaction between hydrogen peroxide and the substrate, which produces a radical precursor that dimerizes to form the highly fluorescent compound detected. Samples of air-exposed geraniol were diluted in milli-Q water and thereafter subjected to the derivatization reaction. A solution of HRP and HPAA was prepared with concentrations of 2.5 U/mL and 1.5 mM, respectively. Fifteen microliters of this solution was added to 150 μL of sample solution. Standards and blanks (water) were prepared in the same way. Hydrogen peroxide in water was used to make an external calibration curve from which the amount of hydrogen peroxide in the samples of air-exposed geraniol could be determined. The background fluorescence of unreacted substrate was subtracted from the fluorescence measurements of samples and standards. The mobile phase consisted of methanol/water 9:11, with analytical grade water. To evaluate the selectivity of the method for hydrogen peroxide over hydroperoxides, four hydroperoxides, *tert*-butyl hydroperoxide, cumene hydroperoxide, and the linalool hydroperoxides **9** and **10** were tested in the derivatization reaction, where **9** and **10** were tested as a mixture. After subsequent analysis of the reaction mixtures in the FIA fluorescence detection method, the relative response was determined for each hydroperoxide and compared to that of hydrogen peroxide. The ratios were very low for all hydroperoxides tested, which indicates that these substances are poor substrates for the HRP enzyme. The experiment thus showed that the enzyme is selective toward hydrogen peroxide (Table 1) because the relative response of the hydroperoxides was <1% of that of hydrogen peroxide.

Isolation and Identification of 7-Hydroperoxy-3,7-dimethyl-octa-2,5-dien-1-ol (6) Using Preparative HPLC. Air-exposed geraniol was fractionated using flash chromatography, after which the most polar fraction was subjected to preparative HPLC. A Zorbax Rx-SIL preparative column (21.2 × 250 mm, 7 μm particles, Agilent Technologies) was used. The eluent consisted of *tert*-butyl methyl ether/hexane/iso-propanol 30:19:1, and the flow rate was 24.2 mL/min. The compounds were monitored at 205 nm. Compound **6** was identified with NMR using a synthesized reference compound (see below).

Synthesis of Reference Compounds. 7-Hydroperoxy-3,7-dimethyl-octa-1,5-dien-3-ol (9) and 6-Hydroperoxy-3,7-dimethyl-octa-1,7-dien-3-ol (10). The photo-oxidation reaction was performed as described in the literature (*3*). Starting from **1** (0.58 g, 3.8 mmol) and after a reaction time of 5 h, the crude product was purified with flash chromatography (20:80 ethyl acetate/hexane) to give a colorless oil consisting of a 5:3 mixture of **9** and the two diastereomers of **10**, in 63% total yield (0.43 g). ^{1}H and ^{13}C NMR data agree with published data (*3*).

7-Hydroperoxy-3,7-dimethyl-octa-2,5-dien-1-ol (6) and 6-Hydroperoxy-3,7-dimethyl-octa-2,7-dien-1-ol (11). The photo-oxidation reaction was performed as described in the literature (*3*). Starting from **2** (0.7 g, 4.54 mmol) and after a reaction time of 2 h, the crude product was purified with flash chromatography (35:65 ethyl acetate/hexane) to give a colorless oil consisting of a 5:3 mixture of **6** and **11**, in 41% total yield (0.35 g). To be able to characterize the hydroperoxides, the compounds were separated using preparative HPLC.

Compound 6. ^{1}H NMR δ 5.7−5.5 (2H, m) 5.4 (1H, t, J = 6.96 Hz), 4.1 (2H, d, J = 6.96 Hz), 2.7 (2H, d, J = 6.22 Hz), 1.6 (3H, s), 1.3 (6H, s); ^{13}C NMR δ 138.2, 135.7, 128.7, 124.3, 82.1, 59.4, 42.3, 24.4 (2 C), 16.5.

Compound 11. ^{1}H NMR δ 5.4 (1H, t, J = 6.96), 5.0 (2H, s), 4.2 (1H, t, J = 6.77), 4.1 (2H, d, J = 6.96), 2.1−1.9 (2H, m), 1.7 (3H, s), 1.6 (3H, s), 1.6−1.5 (2H, m); ^{13}C NMR δ 143.9, 138.9, 123.8, 114.2, 89.0, 59.3, 35.6, 28.8, 17.2, 16.2.

3,7-Dimethyl-octa-2,5-diene-1,7-diol (8) and 3,7-Dimethyl-octa-2,7-diene-1,6-diol (12). Compounds **6** and **11** were prepared as described above. The mixture of hydroperoxides was dissolved in diethyl ether, and 1.1 equiv of triphenyl phosphine was added. After the completion of the reaction, the solvent was evaporated, and the two diols were separated by flash chromatography. ^{1}H and ^{13}C NMR data agree with published data (*14*).

Compound 8. EI-MS (70 eV), *m*/*z* (%) 152 (12), 134 (41), 119 (93), 91 (100), 67 (49), 51 (12).

Compound 12. EI-MS (70 eV), *m*/*z* (%) 137 (18), 110 (19), 84 (43), 67 (100), 55 (45).

[3-Methyl-3-(4-methyl-pent-3-enyl)-oxiranyl]-methanol (Epoxygeraniol) (7). The synthesis was performed as described in the literature (*15*), starting from **3**. ^{1}H and ^{13}C NMR data agree with published data. EI-MS (70 eV), *m*/*z* (%) 139 (6), 121 (8), 109 (74), 95.1 (30), 82.1 (100), 69.1 (97), 55.1 (39).

Sensitization Experiments in Mice. The sensitizing potency of the chemicals was investigated using the local lymph node assay (LLNA) (*16*) as previously described (*3*).

All animal procedures were approved by the local ethics committee. Each compound was tested in three or five different concentrations (Table 2) using mice in groups of four or three, respectively. In the case of hydrogen peroxide, the vehicle acetone/glycerol/water (8:1:1 v/v) was used. Pretests were performed to determine the maximum nonirritating concentration of hydrogen peroxide in the acetone/glycerol/water vehicle. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), that is, the test group/control group ratio. Test materials that at one or more concentrations caused an SI of 3 or higher were considered to be positive in the LLNA. EC3 values (estimated concentration required to produce an SI of 3) were calculated by linear interpolation and used to compare the sensitizing capacities of the different test materials (*17*). The sensitizing potency of the test substances was classified according to the following: EC3 < 0.1%, extreme; EC3 ≥ 0.1 − < 1%, strong; EC3 ≥ 1 − < 10%, moderate; EC3 ≥ 10 − < 100%, weak (*18*).

Computational Method. In this study, all calculations were performed using the GAUSSIAN 03 (Gaussian, Inc., Pittsburgh, PA) program package (*19*). The geometry optimization for all stationary points was performed using the 6-31G(d, p) basis set and the density functional theory method, B3LYP (*20*, *21*). The harmonic vibrational frequencies were calculated at the same level of theory to ensure the nature of the corresponding stationary point. These calculations also generate the thermochemistry data from which ΔH (298 K) was calculated.

Results and Discussion

The allylic alcohol geraniol shows a different oxidation pattern compared to those of linalool and limonene, which autoxidize forming hydroperoxides as the only primary oxidation products (*1*, *3*). The autoxidation of geraniol follows two paths originating from allylic hydrogen abstraction near the two double

Table 2. LLNA[a] Responses for Pure Geraniol, Two Samples of Geraniol Air-Exposed for 10 and 45 Weeks, and Oxidation Products

test material and concn (% w/v)	[^{3}H]thymidine incorporation (DPM/lymph node)	SI	EC3 value (%)	EC3 value (M)	classification
geraniol **2**			22.4	1.45	weak
control	1030				
5%	1008	0.98			
10%	1301	1.3			
15%	1980	1.9			
20%	2241	2.2			
30%	5787	5.6			
air-exposed geraniol (10 weeks)			4.4	0.28	moderate
control	671				
1%	695	1.0			
3%	1275	1.9			
6%	2908	4.3			
10%	6469	9.7			
20%	10370	16			
air-exposed geraniol (45 weeks)			5.8	0.37	moderate
control	724				
0.5%	763	1.1			
1%	790	1.1			
3%	819	1.1			
6%	2295	3.2			
10%	4216	5.8			

[a] The sensitization experiments were performed as described in Experimental Procedures. The SIs correspond to the increase in the thymidine incorporation of treated groups relative to vehicle-treated controls. EC3 values were calculated using linear interpolation. The sensitizing potency of the test substances was classified according to the following: EC3 < 0.1%, extreme; EC3 ≥ 0.1 − < 1%, strong; EC3 ≥ 1 − < 10%, moderate; EC3 ≥ 10 − < 100%, weak.

bonds. From geraniol, mainly hydrogen peroxide is formed together with the aldehydes geranial and neral from a hydroxyhydroperoxide. In addition to these oxidation products, hydroperoxide **6** is also formed, analogous to the major hydroperoxide formed in the autoxidation of linalool (*3*). Sensitization experiments show that geraniol hydroperoxide gives the most important contribution to the allergenic activity of air-exposed geraniol, together with geranial and neral, whereas hydrogen peroxide was found to be non-sensitizing.

Air Exposure of Geraniol. The concentration of geraniol started to decrease immediately on air exposure at room temperature. The rate of autoxidation was similar to that of linalool (*3*), that is, 80% of geraniol remained at 10 weeks of air exposure, whereas 20% remained at 45 weeks (Figure 2). The oxidation mixture polymerized with time, and the samples taken out beyond 45 weeks were no longer suitable for HPLC or GC analysis because of their viscosity.

Discovery and Quantification of Hydrogen Peroxide in Air-Exposed Geraniol. Analysis of the oxidation mixture with semiquantitative peroxide test strips gave a strong positive result that could not be verified by TLC as hydroperoxides. Hydrogen peroxide was expected to give this result, which was confirmed by analysis with a selective derivatization and fluorescence detection method recently described (*13*). During the autoxidation process, the amount of hydrogen peroxide increased until week 7, where a maximum of 0.37% (w/w) was reached (Figure 3). After 10 weeks, the amount of hydrogen peroxide had decreased to 0.31% (w/w). The formation rate of hydrogen peroxide is similar to the combined formation rate of the aldehydes **3** and **4** until week 5, after which time the concentration of hydrogen peroxide reaches a plateau. The slopes for

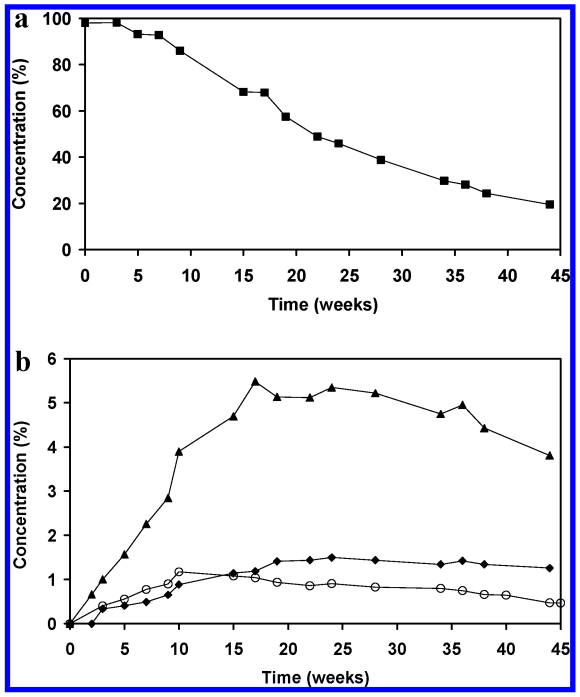

Figure 2. Degradation of geraniol. Concentrations of (a) geraniol **3** (■) and (b) oxidation products geranial **4** (▲), neral **5** (♦), and hydroperoxide **6** (○) in air-exposed geraniol over time. Quantification was performed with HPLC-UV at 205 and 230 nm for the detection of geraniol, **6**, and the aldehydes.

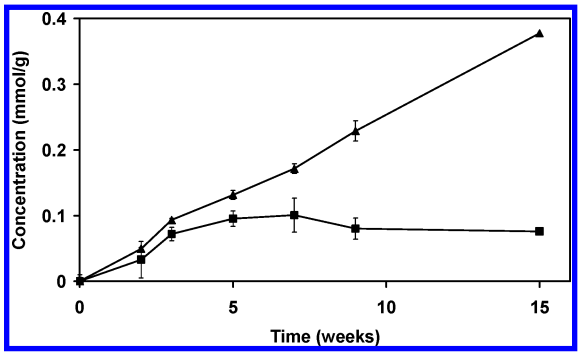

Figure 3. Formation of hydrogen peroxide (■) and aldehydes **3** and **4** (▲) in air-exposed geraniol. The formation rates during the first five weeks are calculated to be the same, within the 95% confidence interval.

hydrogen peroxide and the aldehydes were compared for parallelism between 2 and 5 weeks by means of a two-sample *t*-test, within the 95% confidence interval. There was no evidence for differences in the formation rates during this time. This supports the proposed mechanism where aldehyde and hydrogen peroxide are formed from a hydroxyhydroperoxide. After 5 weeks, the reaction of hydrogen peroxide with other components of the mixture becomes faster than the formation of hydrogen peroxide itself. The amount of aldehydes continues to increase for some time, which indicates that more hydrogen peroxide is formed but that it is readily consumed because of its reactivity. In our investigation of the selectivity of the fluorescence detection method for hydrogen peroxide compared to that for organic hydroperoxides, we showed that the mass recovery of hydroperoxides is <1% of that of hydrogen peroxide (Table 1). Thus, the concentration of hydroperoxide in air-exposed geraniol was too low to affect the quantification of hydrogen peroxide with this method.

Other methods used to detect hydrogen peroxide are, for example, titrations with iodine (*22*) or chemoluminescence detection by reaction with luminol (*23*). These methods are

sensitive to both hydrogen peroxide and organic peroxides because the reactive species, iodide ion and luminol, do not discriminate between hydrogen peroxide and hydroperoxides. Iodide ion cleaves the oxygen−oxygen bond in peroxides, hydroperoxides, and hydrogen peroxide. Luminol reacts with hydroxyl radicals, which can originate from peroxides, hydroperoxides, and hydrogen peroxide alike. Thus, there are many difficulties in detecting and quantifying hydrogen peroxides in the presence of hydro peroxides, and to date, no completely selective method has been presented. The method for measuring hydrogen peroxide used in this article is the most selective one available, but because hydroperoxides can accumulate to concentrations 100-fold that of hydrogen peroxide, it is not reliable on its own, without also analyzing samples for the presence of hydroperoxides with a chromatographic technique.

Identification and Quantification of Autoxidation Products of Geraniol. Air-exposed geraniol was subjected to flash chromatography and preparative HPLC in order to isolate the major oxidation products. To facilitate the identification of oxidation products, several potential oxidation products were synthesized or obtained from other sources, and their chromatographic and spectral properties were compared with those of the isolated oxidation products. The following compounds were identified: geranial **3**, neral **4**, geranyl formate **5**, 7-hydroperoxy-3,7-dimethyl-octa-2,5-dien-1-ol (**6**), epoxygeraniol **7**, and 3,7-dimethyl-octa-2,5-diene-1,7-diol (**8**) (Figure 1). Neither hydroperoxide **11** nor its analogous secondary oxidation product **12** could be detected in the autoxidation mixture.

After 10 weeks of air exposure, the oxidation mixture of geraniol contained 2.9% (w/w) of **3**, 0.66% of **4**, 0.73% of formate **5**, 1.2% of hydroperoxide **6**, 3.2% of epoxide **7**, and 0.21% of diol **8**. After 45 weeks of air exposure, **3**, **4**, and **5** had accumulated to 3.8%, 1.3%, and 3.3%, respectively, whereas the concentration of **6** had decreased to 0.47%. The concentrations of the secondary oxidation products **7** and **8** were almost unchanged: 3.5% and 0.57%, respectively.

In the autoxidation study of linalool, the concentration of hydroperoxide **9** mounted to 16% (*3*), whereas in the present study, the concentration of hydroperoxide **6**, analogous to that of **9**, only reaches a tenth of that. The formation of aldehyde via a hydroxyhydroperoxide is a competing pathway, dominating over the formation of **6**, which means that the amount of hydroperoxide in air-exposed geraniol never reaches the levels in air-exposed linalool. Because the aldehydes are known to be unstable (*24*), undergoing further oxidation and possibly polymerization, the total content of primary and secondary oxidation products is less in air-exposed geraniol compared to that in linalool.

The presence of different compounds with such diverse structures in the oxidation mixture of geraniol posed a difficult analytical problem. Terpenes in general are easily analyzed with GC-FID, and oxidation products other than the hydroperoxide were quantified using GC-FID. Thermolabile compounds such as hydroperoxides are decomposed under these conditions, and we therefore used a previously developed HPLC-UV method (*3*) for the quantification of the hydroperoxide in the oxidation mixture. However, the sensitivity of the HPLC-UV method is low because of low UV-absorbance with the exception of the α,β-unsaturated aldehydes **3** and **4.** Therefore, the quantification of hydroperoxides in oxidation mixtures is difficult. To ensure the accuracy of the quantifications and the comparability between the GC-FID and the HPLC-UV methods, the quantifications of geraniol, **3**, and **4** in the air-exposed samples were compared between the two methods with the same result (data not shown).

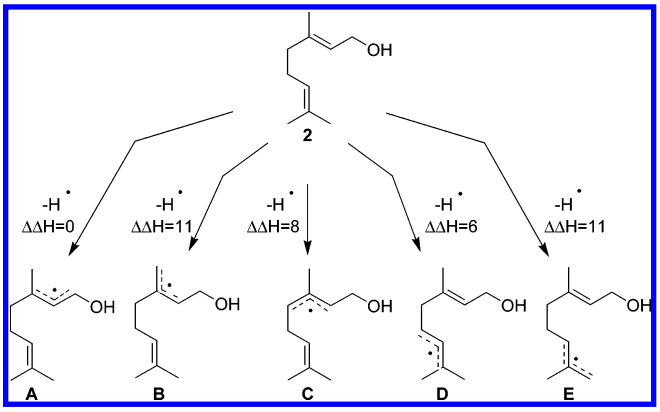

Figure 4. Illustration of five radicals that can be formed by hydrogen abstraction from geraniol. Enthalpy changes for forming the various radicals are given in relation to the enthalpy change calculated for forming radical A.

Mechanisms for the Major Reaction Pathways at the Autoxidation of Geraniol. Computational chemistry was used to give supplemental information on possible mechanisms at the autoxidation of geraniol. The relative stabilities of the five different types of radicals formed from geraniol by abstracting the different allylic hydrogen atoms were investigated (Figure 4). Hydrogen abstraction reactions are generally slow and selective for the most easily abstracted hydrogen atoms, which typically are the most weakly bonded ones. This suggests a correlation between activation energy and bond strength, the BEP relationship (*25*), thereby partly eliminating the need to calculate the saddle point characteristics. Thus, from the relative enthalpies, we conclude that radical A is most likely to be formed, and therefore, the oxidation of geraniol should be dominated by the oxidation products of this radical (Figure 4). Radicals D and E (Figure 4) are analogous to two radicals formed in the autoxidation of linalool, which proceed to give the main linalool hydroperoxides **9** and **10** (*3*). In the case of geraniol we found only small amounts of hydroperoxide **6**, whereas **11** was not detected, which can be explained by the lower stabilities of D and E relative to that of A (Figure 4), although D is sufficiently stable to be formed. The mechanisms for the formation of **6** and **11** are analogous to what is described earlier for **9** and **10** and will not be commented on further (*3*).

In Figure 5, a radical chain mechanism for the formation of hydroperoxides from A and subsequent decomposition to aldehyde and hydrogen peroxide is presented. An oxygen molecule may add to radical A at two sites, either to the carbon atom adjacent to the hydroxy group, forming *13a*, or to the trisubstituted carbon atom adjacent to the methyl group, forming *13b*. The subsequent abstraction of a hydrogen atom from a neighboring geraniol molecule is favorable, and the radical chain reaction proceeds. Of the resulting hydroperoxide products, *14a* is most likely formed because it is more stable than *14b* by 6 kcal/mol. However, these hydroperoxides are not stable and decompose readily to aldehydes and hydrogen peroxide. Hydroperoxides *14a* and *14b* can decompose to geranial **3** and hydrogen peroxide through a general acid-catalyzed fragmentation reaction. Although the decomposition is endothermic by 8 kcal/mol, the Gibbs free energy is favorable because of the release of hydrogen peroxide. Neral **4** can be formed from *14b* in a manner analogous to that of geranial because there is free rotation of the single bond adjacent to the double bond. This is in accordance with the identification of the aldehydes in the oxidation mixture. Because the Z-configuration is less favored

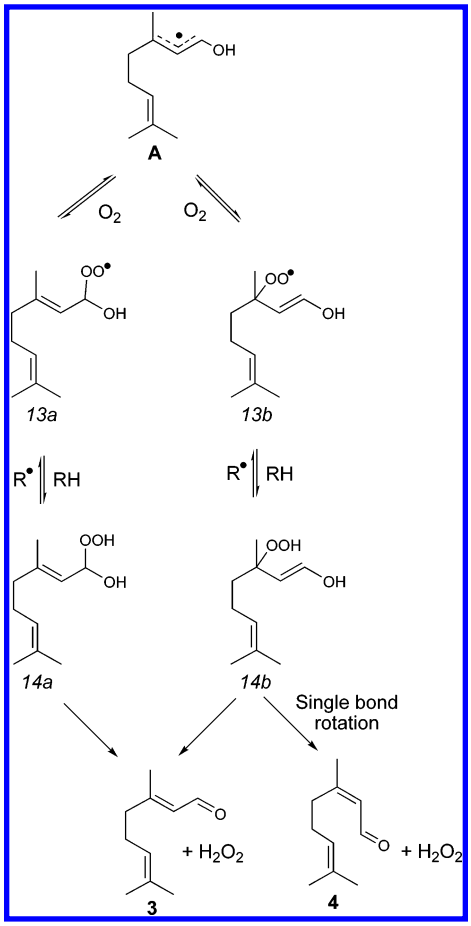

Figure 5. Suggested mechanism for the formation of hydrogen peroxide, **3**, and **4** from radical A. The reversible reaction of A with triplet state oxygen will give peroxyl radicals *13a* and *13b*. Subsequent hydrogen atom abstraction will give hydroperoxides *14a* and *14b*, which readily decompose to hydrogen peroxide and aldehyde.

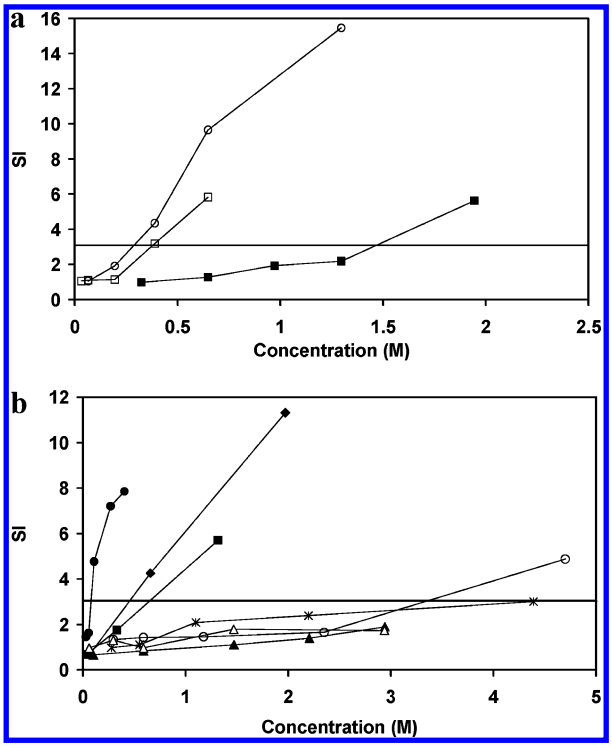

Figure 6. Dose−response curves for the compounds tested in the local lymph node assay (LLNA). The concentrations are given in M. The molar concentrations of oxidized geraniol are calculated using the molecular weight of geraniol. (a) Pure geraniol (■), 10 week air-exposed geraniol (○), and 45 week air-exposed geraniol (□). (b) Hydroperoxides **6** and **11** (●), geranial (♦), neral (■), geranyl formate (*), epoxygeraniol (○), hydrogen peroxide (▲), and alcohol **8** (△).

than the E-configuration, geranial is formed to a greater extent than neral (Figure 5).

It is well-known that olefins react with singlet oxygen to form peroxides and hydroperoxides (*26*) at elevated temperatures and that secondary alcohols oxidize to ketone and hydrogen peroxide under the same conditions (*27*). Allylic and benzylic alcohols have an elevated reactivity compared to that of saturated compounds. A mechanism has been proposed in which a hydroxyhydroperoxide is formed and immediately breaks down into ketone and hydrogen peroxide (*28*). These reactivity experiments were generally performed with different kinds of catalysts to start the reaction. We have shown that the very same process takes place with the allylic alcohol geraniol under conditions similar to that of the normal storage and handling of this terpene. The process is slower because the temperature is lower, and no catalyst is added.

Skin Sensitizing Potency. The LLNA can be used to determine the relative skin sensitizing potency of chemicals via interpolation of the quantitative dose−response data generated. The sensitizing potency of pure geraniol and two different samples of air-exposed geraniol was determined in order to investigate the effect of autoxidation on allergenic activity (Table 2, Figure 6a). The EC3 value of pure geraniol was 1.45 M. The oxidized samples induced greater proliferation and gave EC3 values of 4.4% (w/v) (0.28 M) and 5.8% (w/v) (0.37 M) for geraniol air-exposed 10 weeks and 45 weeks, respectively. Molarity was calculated using the molecular weight of geraniol.

The sensitizing capacity of the identified oxidation products from oxidized geraniol was investigated (Table 3). Hydrogen peroxide was shown to be a non-sensitizer, with no clear dose response (Figure 6b). Hydrogen peroxide is a well-known irritant, and the dose−response curve reflects its toxicity. The mixture of hydroperoxides **6** and **11** (5:3) was found to be strongly allergenic with an EC3 value of 0.077 M. The potency of these hydroperoxides is in the same range as that shown for the corresponding mixture of linalool hydroperoxides (*3*, *15*, *29*). According to our experience so far, all hydroperoxides tested show an EC3 value of 1% (*15*, *29*). We thus conclude that there is no difference in the sensitizing potency of **6** and **11**. Aldehydes **3** and **4** were moderate allergens and gave EC3 values of 0.45 and 0.64 M, respectively. In the oxidation mixtures investigated, they were formed in a ratio of 4:1, which indicates that **3** is more important for the allergenic activity of air-exposed geraniol. According to the literature (*30*−*32*), aldehydes show a wide range of EC3 values (<1 up to 50% (w/v)) depending on the structure. Aldehydes **3** and **4** fit into the group of α,*β*-unsaturated aldehydes, which are known to form antigens via Michael addition and to be moderate sensitizers (*33*).

Epoxide **7** was identified as a very weak allergen with an EC3 value of 3.3 M. Also, epoxides show a wide range of sensitizing capacities. In previous autoxidation studies, limonene oxide and linalylacetate epoxide (unpublished results) were found to be non-sensitizers in the LLNA. These epoxides are sterically hindered and, therefore, less reactive in contrast to less substituted allylic epoxides, which we recently showed to be highly reactive and sensitizing (*34*). Formate **5** was a very weak sensitizer, and gave an EC3 value of 4.4 M. This result is consistent with previous results from the study of autoxidation of ethoxylated surfactants, where a non-sensitizing ethoxylated

Table 3. LLNA Responses for Oxidation Products of Air-Exposed Geraniol

test material and concn (% w/v)	[^{3}H]thymidine incorporation (DPM/lymph node)	SI	EC3 value (%)	EC3 value (M)	classification
hydrogen peroxide					non-sensitizing
control	631				
0.34%	416	0.66			
2%	532	0.84			
5%	699	1.1			
7.5%	882	1.4			
10%	1201	1.9			
geraniol			1.4	0.077	strong
hydroperoxides **6**, **11**					
control	916				
0.5%	1341	1.5			
1%	1494	1.6			
2%	4365	4.8			
5%	6599	7.2			
7.5%	7195	7.9			
geranial[a] **3**			6.8	0.45	moderate
control	604				
1%	426	0.71			
10%	2568	4.3			
30%	6831	11.3			
neral[a] **4**			9.7	0.64	moderate
control	886				
0.5%	600	0.68			
5%	1550	1.8			
20%	5060	5.7			
geranyl formate[b] **5**			79.4	4.4	very weak
control	846				
5%	830	0.98			
10%	936	1.1			
20%	1768	2.1			
40%	2022	2.4			
80%	2548	3.0			
epoxygeraniol **7**			56.7	3.3	very weak
control	801				
5%	1072	1.3			
10%	1143	1.4			
20%	1172	1.5			
40%	1332	1.7			
80%	3910	4.9			
geraniol alcohol **8**					non-sensitizing
control	668				
1%	648	0.97			
5%	872	1.3			
10%	645	0.97			
25%	1196	1.8			
50%	1165	1.7			

[a] Purified by distillation prior to sensitization experiments. [b] Purified by preparative chromatography prior to sensitization experiments.

formate was identified in the oxidation mixture of air-exposed poly(oxyethylene) alcohols (*35*). Diol **8** did not give an EC3 value within the concentrations tested and is, therefore, considered a non-sensitizer. This is consistent with the sensitization data of linalool alcohol, which was shown to be a non-sensitizer (*3*).

Our experiments showed that the autoxidation of geraniol greatly influenced the sensitizing effect of geraniol by the formation of allergenic oxidation products. The oxidized samples had moderate sensitizing capacity, quite different from that of pure geraniol (Figure 6a). The dose−response curves for the oxidized samples of geraniol are steep and resemble the steep curve of geraniol hydroperoxide, although they are shifted to the right (Figure 6b). The sensitizing capacity of air-exposed geraniol is similar to that of air-exposed linalool, although the concentration of hydroperoxide in air-exposed geraniol is about 10 times less than that in air-exposed linalool (*3*), which shows that the total composition is of importance for the sensitizing capacity of the oxidation mixture. Augmentation of skin response by a combination of allergens or allergens and irritants is described in the literature (*36*).

Clinical studies with oxidized linalool and limonene show a high frequency of positive reactions among eczema patients (*5*, *6*, *37*), whereas the pure substances give very few reactions (*38*, *39*). Geraniol gives a higher frequency of positive reactions compared to limonene and linalool (*8*), and pure geraniol shows a higher sensitizing potency in the LLNA compared to pure linalool (*3*) and pure limonene (unpublished results), which means that allergic reactions to geraniol could occur. The metabolic oxidation of alcohols to aldehydes is known (*40*), and another possible route for hapten formation from geraniol is via metabolism in the skin to geranial.

The present study shows that the sensitizing potency of the fragrance compound geraniol increased by air exposure because of the formation of contact allergenic oxidation products. The hydroperoxide formed is believed to be the major contributor to allergenic activity, together with the aldehydes geranial and neral. Geraniol is included in FM I, used for screening consecutive dermatitis patients. Cases of allergy to the oxidation products of geraniol will not be diagnosed unless patients are tested with the air-exposed material. Thus, our observations once more emphasize the need for testing with the right material for screening contact allergy. On the basis of the present study and previous experience, we recommend that the possibility of autoxidation and the subsequent formation of contact allergenic oxidation products are considered in risk assessments performed on fragrance terpenes.

Acknowledgment. The skilful technical assistance of Petri Karhunen is gratefully acknowledged. Dr. Per-Ola Norrby and Dr. J. Lars G. Nilsson are acknowledged for valuable comments and discussions. This work was financially supported by the Research Institute for Fragrance Materials, Inc. (RIFM). The work is part of the platform of Göteborg Science Center for Skin Research at the Faculty of Science, Göteborg University.

References

(1) Karlberg, A. T., Magnusson, K., and Nilsson, U. (1992) Air oxidation of d-limonene (the citrus solvent) creates potent allergens. *Contact Dermatitis 26*, 332−340.
(2) Karlberg, A. T., Shao, L. P., Nilsson, U., Gafvert, E., and Nilsson, J. L. (1994) Hydroperoxides in oxidized d-limonene identified as potent contact allergens. *Arch. Dermatol. Res. 286*, 97−103.
(3) Skold, M., Borje, A., Harambasic, E., and Karlberg, A. T. (2004) Contact allergens formed on air exposure of linalool. Identification and quantification of primary and secondary oxidation products and the effect on skin sensitization. *Chem. Res. Toxicol 17*, 1697−1705.
(4) Skold, M., Borje, A., Matura, M., and Karlberg, A. T. (2002) Studies on the autoxidation and sensitizing capacity of the fragrance chemical linalool, identifying a linalool hydroperoxide. *Contact Dermatitis 46*, 267−272.
(5) Matura, M., Goossens, A., Bordalo, O., Garcia-Bravo, B., Magnusson, K., Wrangsjo, K., and Karlberg, A. T. (2002) Oxidized citrus oil (R-limonene): a frequent skin sensitizer in Europe. *J. Am. Acad. Dermatol. 47*, 709−714.
(6) Matura, M., Skold, M., Borje, A., Andersen, K. E., Bruze, M., Frosch, P., Goossens, A., Johansen, J. D., Svedman, C., White, I. R., and Karlberg, A. T. (2005) Selected oxidized fragrance terpenes are common contact allergens. *Contact Dermatitis 52*, 320−328.
(7) de Groot, A. C., and Frosch, P. J. (1997) Adverse reactions to fragrances. A clinical review. *Contact Dermatitis 36*, 57−86.
(8) Schnuch, A., Lessmann, H., Geier, J., Frosch, P. J., and Uter, W. (2004) Contact allergy to fragrances: frequencies of sensitization from 1996 to 2002. Results of the IVDK*. *Contact Dermatitis 50*, 65−76.
(9) Rastogi, S. C., Johansen, J. D., Frosch, P., Menne, T., Bruze, M., Lepoittevin, J. P., Dreier, B., Andersen, K. E., and White, I. R. (1998) Deodorants on the European market: quantitative chemical analysis of 21 fragrances. *Contact Dermatitis 38*, 29−35.
(10) Rastogi, S. C., Heydorn, S., Johansen, J. D., and Basketter, D. A. (2001) Fragrance chemicals in domestic and occupational products. *Contact Dermatitis 45*, 221−225.

(11) Rastogi, S. C., Johansen, J. D., and Menne, T. (1996) Natural ingredients based cosmetics. Content of selected fragrance sensitizers. *Contact Dermatitis 34*, 423−426.
(12) Heinmöller, P., Kurth, H.-H., Rabong, R., Turner, W. V., Kettrup, A., and Gäb, S. (1998) Selective HPLC analysis of n-alkyl hydroperoxides up to C18H38O2. *Anal. Chem. 70*, 1437−1439.
(13) Svensson, S., Olin, A. C., Larstad, M., Ljungkvist, G., and Toren, K. (2004) Determination of hydrogen peroxide in exhaled breath condensate by flow injection analysis with fluorescence detection. *J. Chromatogr., B 809*, 199−203.
(14) Knapp, H., Straubinger, M., Fornari, S., Oka, N., Watanabe, N., and Winterhalter, P. (1998) (S)-3,7-Dimethyl-5-octene-1,7-diol and related oxygenated monoterpenoids from petals of *Rosa damascena* Mill. *J. Agric. Food Chem. 46*, 1966−1970.
(15) Bezard, M., Karlberg, A. T., Montelius, J., and Lepoittevin, J. P. (1997) Skin sensitization to linalyl hydroperoxide: support for radical intermediates. *Chem. Res. Toxicol. 10*, 987−993.
(16) Kimber, I., Hilton, J., Dearman, R. J., Gerberick, G. F., Ryan, C. A., Basketter, D. A., Scholes, E. W., Ladics, G. S., Loveless, S. E., House, R. V., and Guy, A. (1995) An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology 103*, 63−73.
(17) Basketter, D. A., Lea, L. J., Dickens, A., Briggs, D., Pate, I., Dearman, R. J., and Kimber, I. (1999) A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. *J. Appl. Toxicol. 19*, 261−266.
(18) Kimber, I., Basketter, D. A., Butler, M., Gamer, A., Garrigue, J. L., Gerberick, G. F., Newsome, C., Steiling, W., and Vohr, H. W. (2003) Classification of contact allergens according to potency: proposals. *Food Chem. Toxicol. 41*, 1799−1809.
(19) Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Montgomery, J. A., Jr., Vreven, T., Kudin, K. N., Burant, J. C., Millam, J. M., Iyengar, S. S., Tomasi, J., Barone, V., Mennucci, B., Cossi, M., Scalmani, G., Rega, N., Petersson, G. A., Nakatsuji, H., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Klene, M., Li, X., Knox, J. E., Hratchian, H. P., Cross, J. B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R. E., Yazyev, O., Austin, A. J., Cammi, R., Pomelli, C., Ochterski, J. W., Ayala, P. Y., Morokuma, K., Voth, G. A., Salvador, P., Dannenberg, J. J., Zakrzewski, V. G., Dapprich, S., Daniels, A. D., Strain, M. C., Farkas, O., Malick, D. K., Rabuck, A. D., Raghavachari, K., Foresman, J. B., Ortiz, J. V., Cui, Q., Baboul, A. G., Clifford, S., Cioslowski, J., Stefanov, B. B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Martin, R. L., Fox, D. J., Keith, T., Al-Laham, M. A., Peng, C. Y., Nanayakkara, A., Challacombe, M., Gill, P. M. W., Johnson, B., Chen, W., Wong, M. W., Gonzalez, C., and Pople, J. A. (2004) *Gaussian 03*, revision B.05, Gaussian, Inc., Wallingford, CT.
(20) Lee, C. T., Yang, W. T., and Parr, R. G. (1988) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron-density. *Phys. Rev. B 37*, 785−789.
(21) Becke, A. D. (1993) Density-functional thermochemistry 3. The role of exact exchange. *J. Chem. Phys. 98*, 5648−5652.
(22) Mair, R. D., and Graupner, A. J. (1964) Determination of organic peroxides by iodine liberation procedures. *Anal. Chem. 36*, 194−204.
(23) Parejo, I., Petrakis, C., and Kefalas, P. (2000) A transition metal enhanced luminol chemiluminescence in the presence of a chelator. *J. Pharmacol. Toxicol. Methods 43*, 183−190.
(24) Liang, C. P., Wang, M. F., Simon, J. E., and Ho, C. T. (2004) Antioxidant activity of plant extracts on the inhibition of citral off-odor formation. *Mol. Nutr. Food Res. 48*, 308−317.
(25) Jensen, F. (1999) *Introduction to Computational Chemistry*, Wiley, Chichester, U.K.
(26) Bolland, J. L. (1949) Kinetics of Olefin Oxidation. *Q. Rev., Chem. Soc. 3*, 1−21.
(27) Parlant, C., Sérée de Roch, I., and Balaceanu, J.-C. (1964) Cinétique de l'oxydation des alcools secondaires en phase liquide. *Bull. Soc. Chim. Fr.* 3161−3169.
(28) Sérée de Roch, I. (1968) Oxydations en phase liquide par l'oxygène moléculaire. *Belg. Chem. Ind. 33*, 994−1006.
(29) Mutterer, V., Arnau, E. G., Karlberg, A. T., and Lepoittevin, J. P. (2000) Synthesis and allergenic potential of a 15-hydroperoxyabietic acid-like model: trapping of radical intermediates. *Chem. Res. Toxicol. 13*, 1028−1036.
(30) Basketter, D. A., Wright, Z. M., Warbrick, E. V., Dearman, R. J., Kimber, I., Ryan, C. A., Gerberick, G. F., and White, I. R. (2001) Human potency predictions for aldehydes using the local lymph node assay. *Contact Dermatitis 45*, 89−94.
(31) Patlewicz, G., Basketter, D. A., Smith, C. K., Hotchkiss, S. A. M., and Roberts, D. W. (2001) Skin-sensitization structure-activity relationships for aldehydes. *Contact Dermatitis 44*, 331−336.
(32) Patlewicz, G. Y., Wright, Z. M., Basketter, D. A., Pease, C. K., Lepoittevin, J. P., and Arnau, E. G. (2002) Structure-activity relationships for selected fragrance allergens. *Contact Dermatitis 47*, 219−226.
(33) Patlewicz, G. Y., Basketter, D. A., Pease, C. K., Wilson, K., Wright, Z. M., Roberts, D. W., Bernard, G., Arnau, E. G., and Lepoittevin, J. P. (2004) Further evaluation of quantitative structure−activity relationship models for the prediction of the skin sensitization potency of selected fragrance allergens. *Contact Dermatitis 50*, 91−97.
(34) Bergstrom, M. A., Luthman, K., Nilsson, J. L. G., and Karlberg, A. T. (2006) Conjugated dienes as prohaptens in contact allergy: In vivo and in vitro studies of structure−activity relationships, sensitizing capacity, and metabolic activation. *Chem. Res. Toxicol. 19*, 760−769.
(35) Bergh, M., Shao, L. P., Magnusson, K., Gafvert, E., Nilsson, J. L., and Karlberg, A. T. (1999) Atmospheric oxidation of poly(oxyethylene) alcohols. Identification of ethoxylated formates as oxidation products and study of their contact allergenic activity. *J. Pharm. Sci. 88*, 483−488.
(36) Pedersen, L. K., Johansen, J. D., Held, E., and Agner, T. (2004) Augmentation of skin response by exposure to a combination of allergens and irritants - a review. *Contact Dermatitis 50*, 265−273.
(37) Karlberg, A. T., and Dooms-Goossens, A. (1997) Contact allergy to oxidized d-limonene among dermatitis patients. *Contact Dermatitis 36*, 201−206.
(38) Frosch, P. J., Pilz, B., Andersen, K. E., Burrows, D., Camarasa, J. G., Dooms-Goossens, A., Ducombs, G., Fuchs, T., Hannuksela, M., Lachapelle, J. M., et al. (1995) Patch testing with fragrances: results of a multicenter study of the European Environmental and Contact Dermatitis Research Group with 48 frequently used constituents of perfumes. *Contact Dermatitis 33*, 333−342.
(39) Santucci, B., Cristaudo, A., Cannistraci, C., and Picardo, M. (1987) Contact dermatitis to fragrances. *Contact Dermatitis 16*, 93−95.
(40) Guengerich, F. P. (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol. 14*, 611−650.

TX700017V