

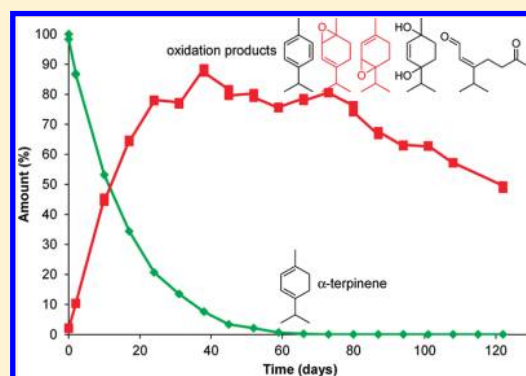
α -Terpinene, an Antioxidant in Tea Tree Oil, Autoxidizes Rapidly to Skin Allergens on Air Exposure

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ABSTRACT: The monoterpene α -terpinene is used as a fragrance compound and is present in different essential oils. It is one of the components responsible for the antioxidant activity of tea tree oil. α -Terpinene is structurally similar to other monoterpenes, e.g., limonene, known to autoxidize on air exposure and form allergenic compounds. The aim of the present study was to investigate the possible autoxidation of α -terpinene at room temperature. To investigate the sensitization potency of air-exposed α -terpinene and the oxidation products formed, the murine local lymph node assay was used. Chemical analysis showed that α -terpinene degrades rapidly, forming allylic epoxides and *p*-cymene as the major oxidation products and also hydrogen peroxide. Thus, the oxidation pathway differs compared to that of, e.g., limonene, which forms highly allergenic hydroperoxides as the primary oxidation products on autoxidation. The sensitization potency of α -terpinene was increased after air-exposure. The allylic epoxides and a fraction, in which only an α,β -unsaturated aldehyde could be identified, were shown to be strong sensitizers in the local lymph node assay. Thus, we consider them to be the major contributors to the increased sensitization potency of the autoxidized mixture. We also investigated the presence of α -terpinene and its oxidation products in four different tea tree oil samples of various ages. α -Terpinene and its oxidation products were identified in all of the tea tree oil samples. Thus, from a technical perspective, α -terpinene is a true antioxidant since it autoxidizes rapidly compared with many other compounds, preventing these from degradation. However, as it easily autoxidizes to form allergens, its suitability can be questioned when used in products for topical applications, e.g., in tea tree oil but also in cosmetics and skin care products.



INTRODUCTION

α -Terpinene (1-isopropyl-4-methylcyclohexa-1,3-diene, **1**, Figure 1A) is a naturally occurring cyclic monoterpene produced in the secondary metabolism of plants. It has been identified in numerous plant extracts and is present in several commonly used essential oils, especially in tea tree oil (TTO).¹ α -Terpinene (**1**) is sometimes present in fragrances at low levels as part of natural oils (e.g., peppermint oil²) that are used in the actual fragrances. According to the ISO standard 4730: 2004, TTO must contain α -terpinene (**1**) at a concentration of 5–13%.³ Studies have shown that α -terpinene (**1**) has antioxidant activity^{4,5} and that it is one of the most important constituents responsible for the antioxidant activity of TTO.⁵ TTO is isolated mainly by steam-distillation of the leaves and branchlets from the Australian plant *Melaleuca alternifolia*.^{3,6,7} The oil is used as a topical antimicrobial and anti-inflammatory product^{6–9} mainly for the treatment of cutaneous infections such as acne and tinea pedis.^{10,11} Terpinene-4-ol, γ -terpinene, 1,8-cineole, and *p*-cymene (**2**) are other compounds that according to the ISO standard must be present in TTO.³

Contact allergy to TTO is reported in the literature.^{12–14} Both freshly distilled and air exposed TTO have been shown to

be sensitizing in guinea pigs, with a 3-fold increase in the sensitization potency for the air exposed TTO.¹⁵ α -Terpinene (**1**) is considered to be one of the components responsible for contact allergy to TTO.¹³

A contact allergen is generally an electrophile able to react with nucleophilic amino acid residues on proteins in the skin, thereby forming immunogenic hapten–protein complexes.¹⁶ However, prehapten and prohaptens are sensitizing compounds despite low chemical reactivity. They require prior activation to become reactive¹⁷ and able to induce contact allergy. Prehapten are activated outside the skin, often by air oxidation, whereas prohaptens are bioactivated in the skin.^{16,17} We have previously shown that α -terpinene (**1**) is a prohaptens and a moderate sensitizer in the murine local lymph node assay (LLNA).¹⁸ α -Terpinene (**1**) has structural features in common with other monoterpene prehapten (e.g., limonene). Accordingly, it is expected to autoxidize on air exposure to form allergenic compounds.^{16,19}

The aim of the present study was to investigate if α -terpinene (**1**) can act as a prehapten. We therefore exposed α -terpinene (**1**)

Received: November 9, 2011

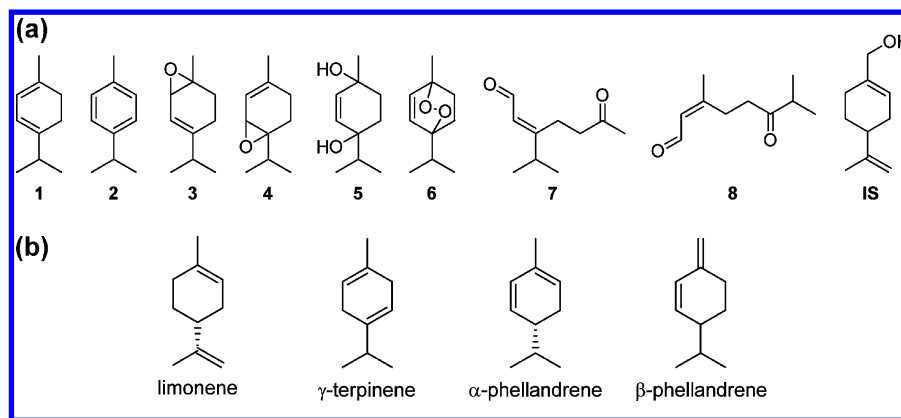


Figure 1. (A) Structures of compounds studied. α -Terpinene (1), *p*-cymene (2), 1,2 epoxide (3), 3,4-epoxide (4), diol (5), ascaridole (6), (*E*)-3-isopropyl-6-oxohept-2-enal (7), (*Z*)-3,7-dimethyl-6-oxooct-2-enal (8), and perillyl alcohol (IS). Compounds 1–5 and 7 were identified in the autoxidation mixture. (B) Structures of compounds discussed.

to air and studied its sensitization potential before and after air exposure using the LLNA. Autoxidation products were identified, quantified, and assessed for sensitization potency. The use of α -terpinene (1) as an antioxidant as stipulated in the literature is also discussed.

EXPERIMENTAL PROCEDURES

Caution: α -Terpinene and its oxidized products are skin-sensitizing, and should be handled with care.

Chemicals. Unless otherwise stated, reagents and solvents were obtained from commercial suppliers and used without further purification. α -Terpinene (1) and *p*-cymene (2) were purchased from Sigma Aldrich (Steinheim, Germany). α -Terpinene (1) was purified by column chromatography on silica gel (eluent: *n*-pentane 100%) to >95% purity prior to the air exposure experiment. The [methyl-³H]-thymidine was obtained from Perkin-Elmer (Waltham, MA, USA), the scintillation fluid was purchased from EcoLume INC Radiochemicals (Solon, OH, USA), acetone p.a. was obtained from Merck (Darmstadt, Germany), and olive oil was purchased from Apoteket AB (Göteborg, Sweden) and used in the sensitization experiments. Water was purified with a Purelab ultra (Genetic) system from Elga Labwater (High Wycombe, UK). The purity of synthesized and purchased compounds was >98% (GC/MS) before testing of sensitization potency, except for the allylic epoxides (3 and 4) which had a purity of 93% (NMR) with traces of *p*-cymene (2).

Instrumentation and Analysis. Column chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM). The reactions and column chromatographic separations were monitored using TLC on silica plated aluminum sheets (Silica gel 60 F₂₅₄). UV or anisaldehyde dip (a solution of 2.1 mL of acetic acid, 5.1 mL of anisaldehyde, and 7 mL of sulphuric acid in 186 mL of ethanol) followed by heating was used for detection.

NMR spectroscopy was performed on a JEOL Eclipse+ 400 MHz spectrometer, using CDCl₃ as solvent. Chemical shifts (δ) are reported in ppm relative to CHCl₃ at δ 7.26 and δ 77.1 for ¹H and ¹³C, respectively.

Photo-oxidation was performed using a Rayonet reactor equipped with 16 UV lamps (350 nm).

GC/MS analyses were carried out using a Hewlett-Packard model 6890 gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a cool on-column injector, with a fused silica column (HP5-MSi, 30 m x 0.25 mm, 0.25 μ m film thickness), connected to a Hewlett-Packard model 5973 mass selective detector (MSD, Agilent Technologies, Waldbronn, Germany), used in scan mode monitoring ions at *m/z* range of 50–500. The samples were electron ionized at 70 eV. The GC temperature program started at 55 °C for 1.5 min and increased with 10 °C/min or 5 °C/min to 250 °C.

Preparative HPLC was performed on a Gilson HPLC pump (model 305) and a Gilson UV/vis detector (model 119) using a ZORBAX

RX-SIL column (21.2 x 250 mm, 7 μ m, Agilent Technologies). 70% *tert*-Butyl methyl ether in hexane was used as eluent, and the flow rate was 21 mL/min. The UV/vis detector was operated at a wavelength of 254 nm.

LC/MS/MS analyses were performed using a Hewlett-Packard 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) connected to a 6410 triple-quadrupole MS detector (G6410B, Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source (G1948B, Agilent Technologies, Santa Clara, CA, USA). The LC system included a vacuum degasser, a binary pump, an autosampler, a column oven (the latter two thermostatted), a UV-detector set at a wavelength of 220 nm (detecting *p*-cymene (2)) or 254 nm (detecting α -terpinene (1) and fraction X).

The ESI interface was used in positive ionization mode with the following spray chamber settings: fragmentor voltage, 90 V; collision energy, 0 eV; nebulizer pressure, 40 psig; capillary voltage, 4500 V; drying gas temperature, 200 °C at a flow rate of 12 L/min; dwell time, 200 ms; and Δ EMV 0. The fragmentor voltage and collision energy were optimized based on reference compounds of the allylic epoxides (3 and 4) and ascaridole (6). The analyses were performed in multiple-reaction monitoring (MRM) mode using the *m/z* 153.1 to 135.1 transitions for the allylic epoxides (3 and 4), the diol (5), and perillyl alcohol (internal standard (IS)) and the *m/z* 169.1 to 151.1 transitions for fraction X and ascaridole (6). The autoxidation samples were also analyzed using a scan method. However, no additional major peaks were observed in scan mode compared to the MRM method.

The mobile phases used were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). Separations were performed on a ZORBAX Eclipse Plus C18 Rapid Resolution HT column (2.1 x 50 mm, 1.8 μ m, Agilent Technologies) using a flow rate of 0.2 mL/min and a column oven temperature of 25 °C. The LC gradient method started at 10% B and increased to 40% B over 10 min. This composition was held isocratically for 10 min, whereafter it was increased to 100% B, and then held for 5 min.

Peroxide test strips (0–100 ppm) were used for the detection of hydrogen peroxide in an undiluted sample of 24 days autoxidized α -terpinene. β -Scintillation counting was carried out using a Beckman LS 6000 TA Instruments.

Air Exposure Procedure. A sample of pure α -terpinene (1, ca. 50 mL) was air-exposed in an Erlenmeyer flask (100 mL) at room temperature and under a daylight lamp (Philips Master TL-D 90 De Luxe, 18W/950) lit for 12 h a day. The neck of the flask was covered with aluminum foil to prevent contamination. The oxidation mixture was stirred four times daily for 1 h, as previously described.^{19,20} Samples were taken on a regular basis and were stored at –72 °C under argon prior to analysis using LC/UV or LC/MS/MS for quantification of α -terpinene (1) and its major oxidation products.

Quantification of α -Terpinene and Oxidation Products Using LC/UV and LC/MS/MS. The degradation of α -terpinene (1) and formation of oxidation products were determined for α -terpinene

air-exposed for 0 to 17 weeks using LC/UV and LC/MS/MS. The amounts of α -terpinene (1), *p*-cymene (2), and fraction X, respectively, were quantified using UV, while MS was used for the allylic epoxides (3 and 4) and the diol (5). Quantification was performed using external standard curves from pure reference compounds. Perillyl alcohol was used as an IS to determine the relative response factors. An equal amount of IS was added to air-exposed α -terpinene after sampling.

Synthesis of the Reference Compounds. *1,2-Epoxy-4-isopropyl-1-methylcyclohex-3-ene* (3) and *1,2-Epoxy-1-isopropyl-4-methylcyclohex-3-ene* (4) (*Allylic Epoxides*, *1,2-Epoxy* (3) and *3,4-Epoxy* (4)). Epoxidation was performed in manner similar to that described in the literature.²¹ α -Terpinene (compound 1, 3.44 g, 25 mmol) was added to a mixture of dichloromethane (75 mL) and potassium carbonate (15.03 g, 109 mmol), whereafter the mixture was cooled to 0 °C on an ice bath. Peracetic acid (40%, 9.62 g, 50 mmol) was pretreated with anhydrous sodium acetate (0.107 g, 1.3 mmol), and the solution was added dropwise to the α -terpinene mixture under vigorous stirring at 0 °C. After 30 min, the mixture was filtered by suction, and the filtrate was concentrated under reduced pressure. The crude product was distilled at 74–76 °C under reduced pressure using a water aspirator to give a colorless oil consisting of a 2:1 mixture of 3 and 4 (1.13 g, 29%). The product contained traces of *p*-cymene (2) according to NMR analysis. ¹H NMR data are in agreement with published data.²¹ **Compound 3:** ¹³C NMR δ 20.7, 21.1, 22.0, 23.6, 27.6, 34.9, 55.6, 60.4, 114.3, 151.1. **Compound 4:** ¹³C NMR δ 18.1, 18.6, 23.5, 24.2, 26.7, 34.0, 54.3, 66.1, 117.5, 142.1.

1-Isopropyl-4-methylcyclohex-2-ene-1,4-diol (5). The hydration was performed based on a literature procedure.²¹ The allylic epoxides (compounds 3–4, 0.507 g, 3.33 mmol) were added to water (5 mL) in a round bottomed flask, and the mixture was shaken for 10 min and left overnight. The water was coevaporated with toluene under reduced pressure to give a white solid. The product was recrystallized using 70% diethyl ether in hexane; the mixture was refluxed at 40 °C under vigorous stirring, and the solution was left to crystallize on ice. The product was filtered by suction and washed with hexane, affording white needle shaped crystals (271 mg, 48%). ¹H NMR: δ 0.90 (d, 3H, *J* = 6.96 Hz), 0.96 (d, 3H, *J* = 6.59 Hz), 1.32 (s, 3H), 1.55 (m, 1H), 1.71–1.95 (m, 4H), 5.61 (d, 1H, *J* = 9.89 Hz), 5.71 (d, 1H, *J* = 9.89 Hz). ¹³C NMR: δ 16.5, 17.7, 27.1, 29.7, 33.6, 37.5, 67.3, 71.7, 133.6, 135.5.

1-Isopropyl-4-methyl-2,3-dioxabicyclo[2.2.2]oct-5-ene (*Ascaridole*, 6). Ascaridole (6) was prepared from α -terpinene (compound 1, 0.735 g, 5.39 mmol) using a photo-oxidation reaction based on a literature procedure.²² After a reaction time of 3 h, the crude product was purified by column chromatography. 10% Ethyl acetate in hexane was initially used as the eluent. The concentration of ethyl acetate was increased to 20% in steps of 5% to finally elute the product as a pale yellow oil (0.477 g, 52%). ¹H and ¹³C NMR data agree with published data.²³

Isolation and Identification of (*E*)-3-Isopropyl-6-oxohept-2-enal (7). Air exposed α -terpinene was fractionated using flash chromatography to isolate fraction X, which was further subjected to preparative HPLC. The most polar compound in the fraction was isolated and identified as compound 7. No other components in the fraction could be identified. ¹H NMR: δ 1.10 (d, 6H, *J* = 6.96 Hz), 2.17 (s, 3H), 2.38 (m, 1H), 2.65 (t, 2H, *J* = 7.87 Hz), 2.81 (t, 2H, *J* = 7.87 Hz), 5.89 (d, 1H, *J* = 7.69 Hz), 9.98 (d, 1H, *J* = 7.32 Hz). ¹³C NMR: δ 21.5, 24.5, 30.0, 35.8, 43.7, 125.3, 172.2, 191.4, 206.5.

Sensitization Experiments Using the LLNA.²⁴ All animal procedures were approved by the local ethics committee. Female CBA/Ca mice, around 8 weeks of age, were purchased from Scanbur or NOVA SCB/Charles River (Sollentuna, Sweden or Germany, respectively). The assay was performed using controls and 5 test concentrations (range: 0.01–30% w/v) of oxidation mixtures or oxidation products of α -terpinene as shown in Table 1. Briefly, groups of female CBA/Ca mice (*n* = 3) received 25 μ L of the test compound dissolved in vehicle (acetone/olive oil 4:1, v/v), on the dorsum of the ears daily for 3 consecutive days. Control animals were treated with vehicle alone. All mice were injected intravenously 5 days after the first treatment, with 250 μ L of phosphate-buffered saline (PBS) containing 20 μ Ci

of [³H]thymidine. After 5 h, the draining auricular lymph nodes were excised and pooled for each group, and single cell suspensions of lymph node cells were prepared. The relative thymidine incorporation was measured by β -scintillation counting. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), i.e., 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.²⁵ The sensitization potency of the test compounds was classified according to the following: <0.1 extreme; ≥ 0.1 – <1 strong; ≥ 1 – <10 moderate; ≥ 10 – <100 weak.²⁶

TTO Analysis. Four bottles of TTO (A–D) with different storage times (A, more than 10 years; B, ca. 10 years; C, newly bought; D, newly bought) were obtained. The four samples of TTO were analyzed using the method described above for identification of α -terpinene (1) and its possible oxidation products according to the results obtained in the investigation of autooxidation of α -terpinene. Solutions (1 mg/mL w/v) of TTO in methanol were diluted with the mobile phases used to a concentration of approximately 0.05 mg/mL (v/v) prior to analysis.

RESULTS AND DISCUSSION

This study shows that α -terpinene (1) undergoes rapid autooxidation on air exposure and forms oxidation products with structural features known to induce contact allergy, i.e., allylic epoxides and α,β -unsaturated aldehydes. These oxidation products were also shown to be strong sensitizers in the murine LLNA. The sensitization potency of autooxidized α -terpinene was approximately 9 times higher compared to that of pure α -terpinene (1). Thus, α -terpinene (1) can be considered as a prehapten as well as a prohaptent since we have previously shown a metabolic activation of this compound.¹⁸

Quantification of α -Terpinene and Oxidation Products after Air Exposure. The concentration of α -terpinene (1) decreased rapidly when exposed to air at room temperature. Using our standard experimental setup for autooxidation studies (see Experimental Procedures), the concentration of α -terpinene (1) had decreased to 53% after 10 days and 21% after 24 days, and after 66 days, α -terpinene (1) was not detected in the oxidation mixture (method limit of detection 0.2%) (Figure 2). The autooxidation rate of α -terpinene (1) was shown to be faster than that of limonene, another monocyclic terpene prehapten (Figure 1B). When using the same experimental set up for the autooxidation of limonene, 60% remained after 56 days.

An LC/UV/MS method was developed to detect the formed oxidation products. Chromatograms from analysis of the α -terpinene sample taken after 24 days are shown in Figure 3. Quantification using synthesized or commercially available reference compounds was performed on samples taken from the oxidation mixture during a period of 122 days (17 weeks) every seventh day, starting on the second day (Figure 2). It was noted that the oxidation products were present in the oxidation mixture at approximately the same concentrations after 20 days of air exposure until the end of the experiment (122 days). After 24 days of air exposure, the oxidation mixture contained 21% α -terpinene (1), 26% 1,2-epoxide (3), 12% 3,4-epoxide (4), 12% diol (5), and 20% *p*-cymene (2). In addition, the mixture contained 7% of a fraction X consisting of (*E*)-3-isopropyl-6-oxohept-2-enal (7) and unidentified compounds (Figures 1A and 2).

A difference in elution behavior was observed for the allylic epoxides (3 and 4), probably due to the shape selectivity of the C₁₈ stationary phase, leading to different retention times. The 1,2-epoxide (3) was shown to be formed at a higher concentration than the 3,4-epoxide (4), which could be due to less

Table 1. LLNA^a Responses for Air-Exposed α -Terpinene (1), *p*-Cymene (2), the Allylic Epoxides (3 and 4), Fraction X, and Ascaridole (6)

compound and test concentration (% w/v)	^{[3]H} thymidine incorporation (dmp/lymph node)	EC3			classification
		SI	(% w/v)	(M)	
air-exposed α -terpinene 1, 3 w			0.9		strong
control	1075				
0.1	857	0.8			
1	3380	3.2			
5	14168	13			
10	18399	17			
25	13365	12			
air-exposed α -terpinene 1, 7 w			1.0		strong
control	1290				
1	3900	3.0			
5	13074	10			
10	16781	13			
15	10593	8.2			
30	12074	9.4			
<i>p</i> -cymene 2			>30	>2.2	weak
control	655				
0.1	565	0.9			
1	787	1.2			
5	905	1.4			
15	1823	2.8			
30	1497	2.3			
allylic epoxides 3 and 4 (2:1 ratio)			0.2	0.02	strong
control	238				
0.1	283	1.2			
1	3094	13			
10	4062	17			
20	4824	20			
30	7618	32			
fraction X			1.1		moderate/strong
control	184				
0.01	204	1.1			
0.1	345	1.9			
1	518	2.8			
10	2735	15			
30	4339	24			
ascaridole 6			1.8	0.11	moderate/strong
control	855				
0.01	892	1.0			
0.1	1178	1.4			
1	1689	2.0			
5	7919	9.3			
15	8705	10			

^aThe LLNA was performed as described in the Experimental Procedures. The stimulation indices (SI) correspond 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. The sensitizing potency of the test compounds was classified according to the following: EC3 <0.1% extreme, EC3 ≥0.1% – <1% strong, EC3 ≥1% – <10% moderate, and EC3 ≥10% – <100% weak.²⁶

steric hindrance. A certain degree of degradation of the epoxides to the diol (5) was observed in the standards, which was compensated for in the quantification.

Fraction X, eluting between the diol (5) and the 1,2-epoxide (3), contained several compounds, but two major peaks were seen in the LC/MS/MS and LC/UV (absorbing UV light at 254 nm) analyses (Figure 3). The amount of fraction X in the oxidation mixture was estimated and is shown in Figure 2. After isolation of the fraction, one of the major compounds

was identified as (*E*)-3-isopropyl-6-oxohept-2-enal (7) using LC/UV, LC/MS/MS, and NMR. No other compounds in fraction X could be identified due to their instability.

No hydroperoxides were detected in the autoxidation mixture by the developed LC/UV/MS method. The presence of hydrogen peroxide (approximately 30 ppm) was indicated in the 24 days air exposed sample of autoxidized α -terpinene using a hydrogen peroxide strip test. The endoperoxide ascaridole (6) that has been proposed to be present in TTO¹⁵ was not detected

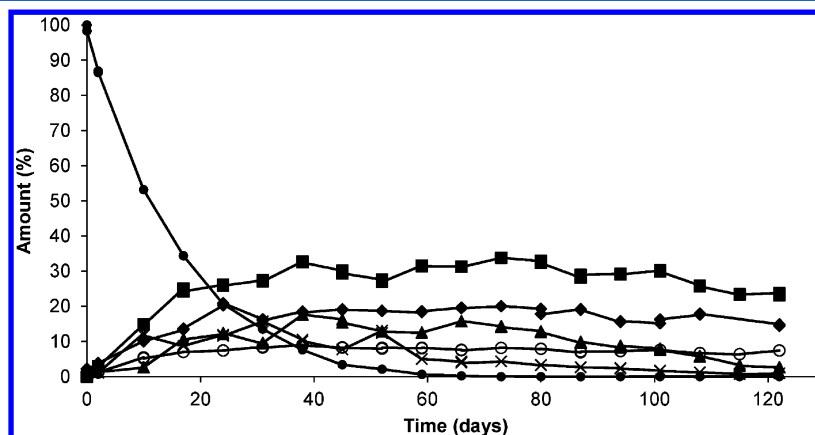


Figure 2. Degradation of α -terpinene and the formation of oxidation products in air-exposed α -terpinene over time. The concentrations of α -terpinene (1, ●), 1,2-epoxide (3, ■), *p*-cymene (2, ◆), 3,4-epoxide (4, ▲), fraction X (○), and diol (5, ×) are shown. Quantification was performed by LC/UV and LC/MS/MS analysis.

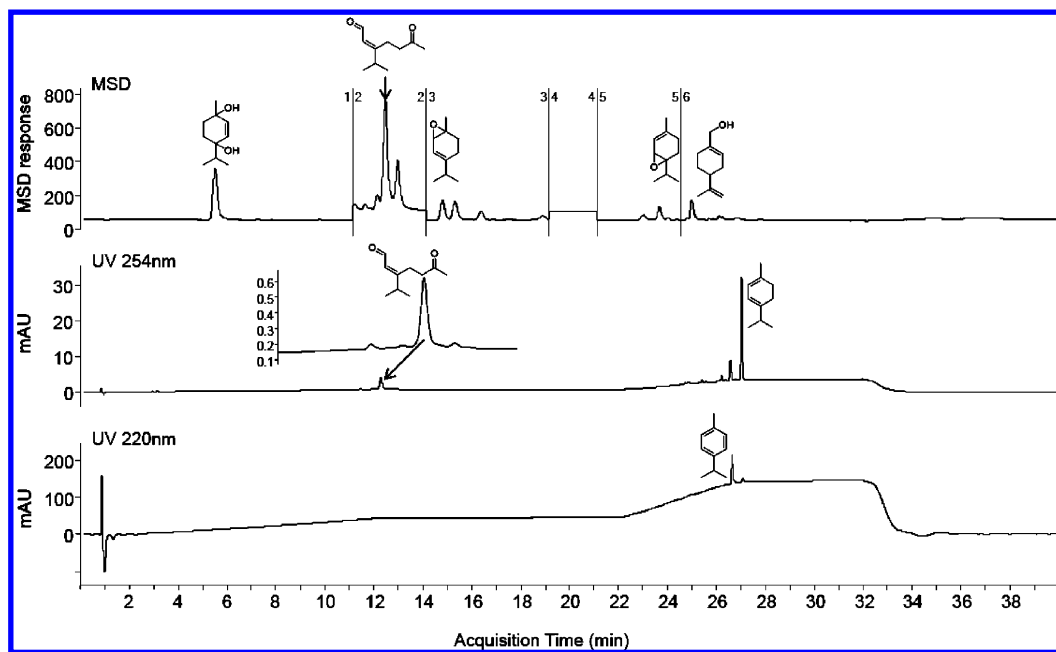


Figure 3. LC/UV and LC/MS/MS chromatograms of an autoxidized sample of α -terpinene after 24 days. Upper: MRM chromatogram showing diol (5) at 5.4 min, the α,β -unsaturated aldehyde (7) at 12.1 min, 1,2-epoxide (3) at 14.4–16.6 min, 3,4-epoxide (4) at 22.6–23.7 min, and the IS at 24.9 min. Middle: UV chromatogram at 254 nm showing the α,β -unsaturated aldehyde (7) at 12.1 min and α -terpinene (1) at 26.9 min. Lower: UV chromatogram at 220 nm showing *p*-cymene (2) at 26.5 min. Note: The allylic epoxides (3 and 4) are analyzed as mixture of isomers; therefore, several peaks are detected.

in the autoxidation mixture of α -terpinene during the time period studied. Using a reference compound, we could demonstrate the absence of a signal at the retention time of ascaridole (6, 19.9 min) at all time points during the autoxidation period (Figure 3).

Polymerization occurred over time, indicated by the increase of the viscosity of the oxidation mixture, but no attempts to identify polymerization products were made. Because of their high molecular weight, polymers are considered to be of low interest from a contact allergenic point of view due to poor skin penetration potential.

Mechanistic Considerations. In the present study, we show that *p*-cymene (2), allylic epoxides (3 and 4), and hydrogen peroxide are formed from air-exposed α -terpinene. No hydroperoxides were identified, which differs from the results obtained when other common fragrance terpenes, i. e., limonene, linalool, and linalyl acetate, were investigated under the same experimental conditions.^{20,27,28} In those studies, a significant amount of allergenic hydroperoxides was detected, which in turn formed secondary oxidation products with various allergenic potentials, e.g., epoxides, alcohols, and carbonyl compounds.^{20,27,28} Despite their differences in structure and allergenic potency, they all have a common mechanistic starting point, the homolytic cleavage of their parent hydroperoxide group. When investigating the autoxidation pattern of another fragrance terpene, geraniol, only minor amounts of hydroperoxides were detected, while hydrogen peroxide together with geraniol, nerol, and epoxygeraniol were the major oxidation products.²⁹ The epoxygeraniol could not be attributed to any of the detected hydroperoxides. Instead, a computational study of the geraniol autoxidation mechanism showed that the formation of epoxygeraniol could be attributed to the addition of a hydroperoxyl radical to the double bond of a new molecule of geraniol thereby yielding epoxygeraniol and a hydroxyl radical.³⁰

It has previously been shown that the peroxidation reaction of γ -terpinene (Figure 1B) results in *p*-cymene (2) and a hydroperoxyl radical, which can be converted to hydrogen peroxide during the propagation step of the radical chain reaction.³¹ This result is in accordance with the observations in the present study, where *p*-cymene (2) and hydrogen peroxide are formed from air exposed α -terpinene (Figure 4A). It is therefore possible that the chain-carrying reaction in the autoxidation of α -terpinene is the same as that shown for γ -terpinene, as we detected no hydroperoxides in this study. Similar to what has previously been shown for epoxygeraniol,³⁰ the α -terpinene epoxides (3 and 4) may be formed via the addition of a hydroperoxyl radical to one of the α -terpinene double bonds, resulting in ring-closure (Figure 4B). In such a mechanism, the concentration of *p*-cymene (2) and epoxides (3 and 4) should run in parallel. The observation that *p*-cymene (2) reaches a steady state concentration during the oxidation is clearly in agreement with published data on the autoxidation of *p*-cymene (2).^{32,33}

The structure of the α,β -unsaturated aldehyde (7) suggests an oxidative cleavage of a double bond in α -terpinene (1). This can be achieved via an intramolecular 4-exotrig cyclization, according to Baldwin's rule, of the peroxyradical to its double bond forming a 1,2-dioxetane (Figure 4C). 1,2-Dioxetanes are notoriously unstable and known to cleave to their corresponding carbonyl compounds. The other α,β -unsaturated aldehyde (8, Figure 4C) was not detected, probably due to steric hindrance.

Sensitization Potency of Air Exposed α -Terpinene and Its Oxidation Products. The murine LLNA²⁴ was used to determine the sensitization potency of air exposed α -terpinene and its identified oxidation products *p*-cymene (2), the allylic epoxides (3 and 4), and fraction X (Table 1 and Figure 5). In addition, ascaridole (6) was tested in the LLNA (Table 1 and Figure 5). The following EC₃ values were afforded for air exposed α -terpinene: three weeks oxidized α -terpinene, 0.9% w/v

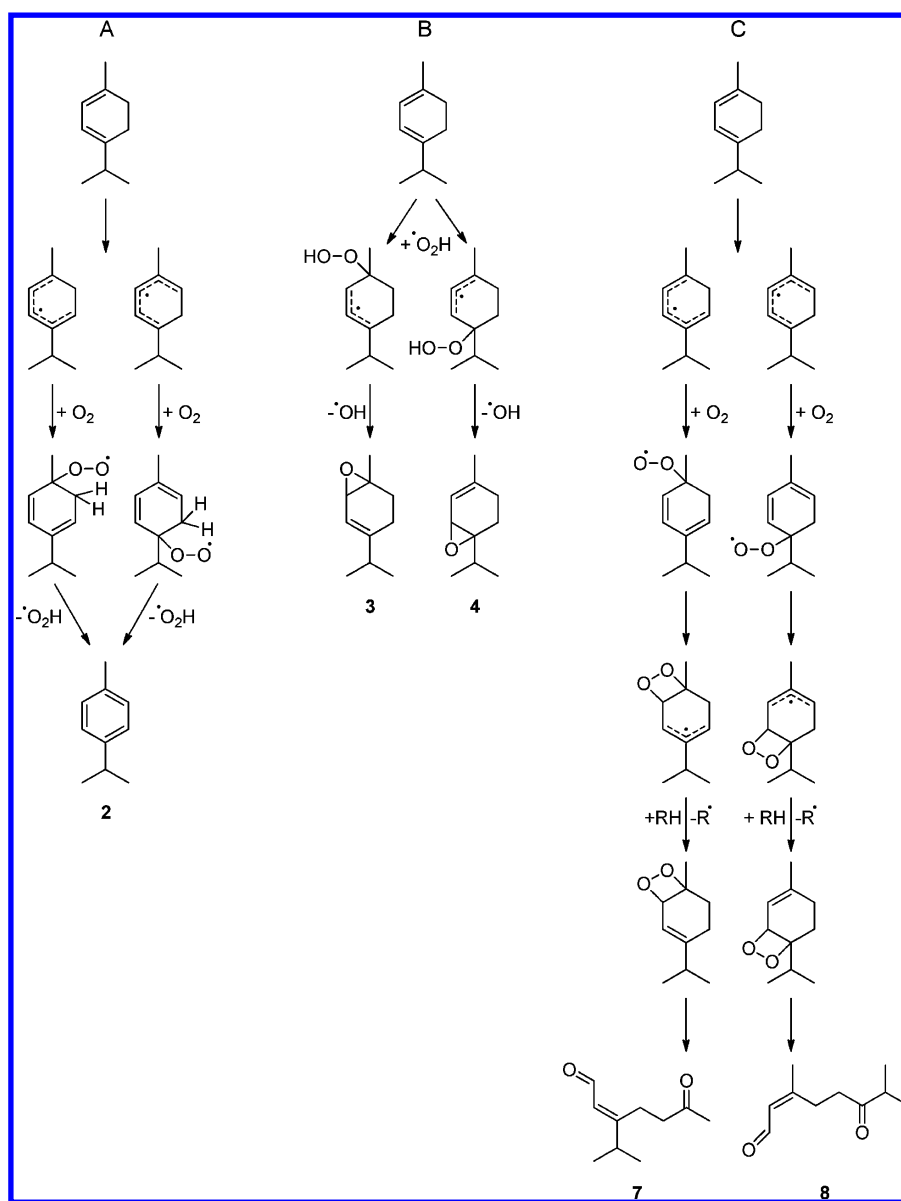


Figure 4. Mechanistic proposals from air-exposed α -terpinene. (A) Formation of *p*-cymene (2) and hydrogen peroxide. (B) Formation of the allylic epoxides (3 and 4). (C) Formation of (*E*)-3-isopropyl-6-oxohept-2-enal (7) and (*Z*)-3,7-dimethyl-6-oxooct-2-enal (8).

(content of pure α -terpinene: ca. 20%); seven weeks oxidized α -terpinene, 1.0% w/v (content of pure α -terpinene: ca. 2%). The allylic epoxides (3 and 4) were tested in a 2:1 mixture, which was found to be strongly sensitizing (EC₃: 0.2% w/v, 0.02 M). No EC₃ value was obtained for *p*-cymene (2) at the concentrations tested (0.1–30% w/v, 0.0075–2.2 M) indicating that *p*-cymene (2) is a weak sensitizer. Fraction X and ascaridole (6) were identified as potent sensitizers (EC₃: 1.1% w/v and EC₃: 1.8% w/v, 0.11M, respectively). The diol (5) was not tested in the LLNA since alcohols are assumed to be weak or nonsensitizing.³⁴

Both the allylic epoxides (3 and 4) and fraction X contributed considerably to the increased sensitization potency of air exposed α -terpinene. For the potency of oxidized α -terpinene, there is no significant difference between three and seven weeks of air exposure (EC₃: 0.9 vs 1.0% w/v, respectively). This correlates well with the analytical data, which showed that the same concentrations of the oxidation products were present in the oxidation mixture in average from 20 days of air exposure onward.

Previous studies have shown pure α -terpinene (1) to be a moderate sensitizer with an EC₃ value of 8.9% w/v (0.65 M).¹⁸ Thus, autoxidized α -terpinene has an increased sensitization potency (approximately 9-fold) compared to that of the pure compound. Notably, the dose–response relationship of pure α -terpinene (1) increased rapidly at a concentration of 10% and above (Figure 5). This could be a result of the metabolic activation as in general a higher dose of a prohapten is needed to achieve sensitization from metabolites compared to that with application of the direct haptens.

α -Terpinene (1) has previously been demonstrated to be a prohapten as it is sensitizing despite not being inherently protein reactive.¹⁸ In studies of metabolic activation using liver microsomes supplemented with the reactive metabolite trapper glutathione, glutathione conjugates with a mass corresponding to the reaction with allylic epoxides were also detected.¹⁸ This is in accordance with geraniol, which forms identical major allergens by both autoxidation and metabolic activation.^{29,35} Also for geraniol, it was demonstrated that a higher dose is required

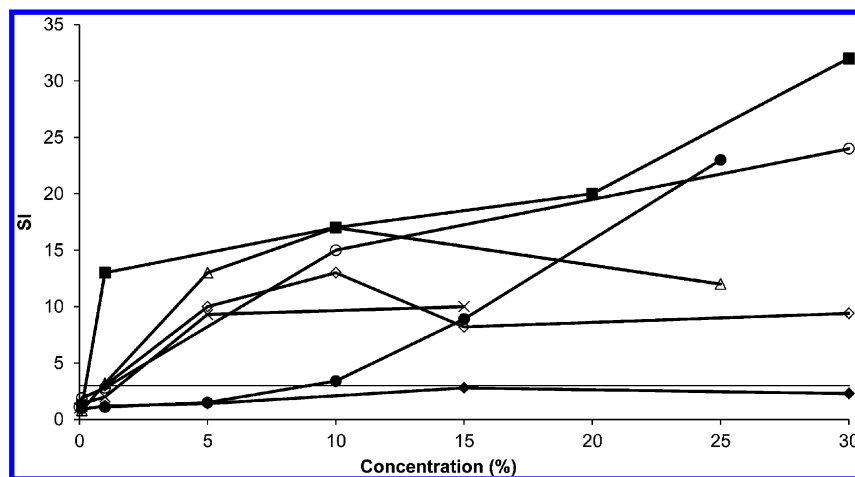


Figure 5. Dose–response curves in the murine LLNA for pure and air-exposed α -terpinene and the oxidations products of α -terpinene detected. Pure nonoxidized α -terpinene (1, ●), air-exposed α -terpinene, three (Δ) and seven weeks (◊), allylic epoxides (3 and 4, ■), fraction X (○), ascaridole (6, ×), and *p*-cymene (2, ◆). The results of pure α -terpinene (1) originate from an earlier published study¹⁸ inserted here to demonstrate the difference in sensitization potency between pure and air-exposed α -terpinene. The horizontal line marks a stimulation index (SI) of 3, the cutoff limit for a compound to be considered a sensitizer according to the used method.

for sensitization based on metabolic activation compared to that from the air exposed samples. Pure geraniol has an EC3 value of 22% w/v, while geraniol air exposed for 10 weeks has an EC3 value of 4.4% w/v.²⁹ Activation of prehapten can be prevented to a certain extent by optimizing handling and storage strategies and by the addition of suitable antioxidants. In contrast, this is not possible for prohaptens, for which the allergenic activity is inherent in the molecule.

The likelihood of monoterpenes acting as both prehapten and prohaptens due to activation outside the body and in the skin must be considered from a safety perspective. In our previous investigation of α -terpinene (1), we also studied the bioactivation of two other naturally occurring monoterpenes α -phellandrene and β -phellandrene (Figure 1B), which, like α -terpinene (1), are dienes with conjugated double bonds.¹⁸ The two phellandrenes were shown to induce contact allergy with sensitization potencies similar to that of α -terpinene (EC3 values: 5.4% w/v for α -phellandrene and 5.6% w/v for β -phellandrene).¹⁸ As they formed glutathione-trapped epoxides in microsomal incubations, it was postulated that these compounds act as prohaptens.¹⁸ Because of their similarity in structure, α - and β -phellandrene will probably autoxidize via pathways similar to those of α -terpinene (1), i.e., forming reactive compounds with allergenic effects.

Fraction X was found to be strongly sensitizing in the LLNA. However, as it was tested only as a crude mixture it is difficult to assess the individual sensitization potency of its constituents. The identified compound (7) might be a moderate sensitizer, due to its structural similarity with geraniol, another α,β -unsaturated aldehyde, which is a moderate sensitizer (EC3: 6.8% w/v, 0.45M).²⁹ Therefore, the other unidentified compounds in fraction X most likely contribute to the sensitization potency of the fraction.

TTO Analysis. It is known that the composition of TTO changes over time in the presence of air, light, moisture, and warmth.¹⁵ Therefore, chemical analysis of four different TTO products was performed to identify α -terpinene (1) and if possible its oxidation products. According to GC/MS analysis, 8%, 5%, 11%, and 10% of α -terpinene (1) were identified in the TTO bottles A (more than 10 years), B (ca. 10 years), C (newly bought), and D (newly bought), respectively. On the basis of

the developed LC/UV and LC/MS/MS methods, we could identify the following oxidation products from α -terpinene (1) in all samples of TTO: *p*-cymene (2), 1,2-epoxide (3), diol (5), and (*E*)-3-isopropyl-6-oxohept-2-enal (7). The 3,4-epoxide (4) was only identified in bottle B. No quantification of the oxidation products was performed due to the complexity of the mixtures. We cannot exclude that the oxidation products identified originate from any of the other compounds present in TTO, e.g., γ -terpinene. As other terpenes in TTO, e.g., γ -terpinene, also may autoxidize to form similar degradation products as α -terpinene (1), the risk of contact allergy is increased. Moreover, when TTO is applied to already damaged skin for treatment of cutaneous infections, it is not surprising that it is a well-known cause of contact allergy and allergic contact dermatitis.

CONCLUSIONS

α -Terpinene (1) can be considered as a prehapten as well as a prohaptens as it can be activated to strong contact allergens both by autoxidation and bioactivation. It is likely a cause of contact allergy to TTO as both α -terpinene (1) and its oxidation products were found in commercial TTO bottles.

α -Terpinene (1) has been suggested as an agent for maintaining the oxidative stability of different matrices, such as food, cosmetics, and medicaments.^{4,5,31} From a technical perspective, it is a true antioxidant, thus preventing these from degradation until it is consumed itself. However, as it easily forms allergens via autoxidation, its suitability can be questioned when used in products for topical applications (e.g., in TTO, but also in cosmetics and skin care products). In general, it is important to consider the fate of antioxidants and their potential to form other products after air exposure. A good antioxidant would be one that is able to inhibit the chain propagation step of autoxidation and then quickly degrade to a nontoxic compound.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Martina Swing and Carolina Glamheden for laboratory assistance. The skillful technical assistance of Dr. Niamh O'Boyle, Petri Karhunen, Susanne Exing, Anders Eliasson, and Gabriella Wendt are acknowledged. Dr. Mate Erdelyi is gratefully acknowledged for discussion and valuable help with NMR experiments and Professor Per-Ola Norrby for valuable discussions. This work is part of the Centre for Skin Research (SkinResQU) at the University of Gothenburg, Sweden.

■ ABBREVIATIONS

DAD, diode array detector; EC3, estimated concentration required to produce a stimulation index of 3; ESI, electrospray ionization; IS, internal standard; LLNA, local lymph node assay; MRM, multiple reaction monitoring; MSD, mass selective detector; PBS, phosphate-buffered saline; SI, stimulation index; TTO, tea tree oil.

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