

Studies on the autoxidation and sensitizing capacity of the fragrance chemical linalool, identifying a linalool hydroperoxide

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Fragrances are among the most common causes of allergic contact dermatitis. The two monoterpenes linalool and *d*-limonene are the most frequently incorporated fragrance chemicals in scented products. Previous studies on *d*-limonene show that this monoterpene oxidizes on air exposure (autoxidation) and that allergenic oxidation products are formed. Due to structural similarities, linalool might also form allergenic oxidation products on air exposure. The aim of the present study was to study the autoxidation of linalool and to investigate the sensitizing potential of linalool before and after air exposure. Linalool was oxidized for 10 weeks and gas chromatographic analyses showed that the content of linalool decreased to about 80%. The chromatograms revealed the formation of other compounds during oxidation. One of the major oxidation products was isolated and identified as 7-hydroperoxy-3,7-dimethyl-octa-1,5-diene-3-ol. This substance is, to the best of our knowledge, described for the first time. In sensitization studies in guinea pigs, linalool of high purity gave no reactions, while linalool that had been oxidized for 10 weeks sensitized the animals. It is concluded that autoxidation of linalool is essential for its sensitizing potential.

Key words: autoxidation; fragrance allergy; FCAT; gas chromatographic analysis; guinea pigs; hydroperoxide; linalool; NMR; sensitization studies; terpene. © Blackwell Munksgaard, 2002.

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Linalool **1** (Fig. 1) occurs naturally in more than 200 oils from herbs, leaves, flowers and wood. It is present in the oils of rosewood, petitgrain, linaloe seed, bergamot, rose, jasmine, coriander and lavender. There are 2 forms of linalool: R(–)-linalool (licareol) and S(+)-linalool (coriandrol), and the racemate of these 2 forms is referred to as linalool. The distribution of the two enantiomers varies in different plants (1). Linalool is produced either by steam distillation and solvent extraction from plant raw materials, or by chemical synthesis, and is widely used because of its fresh flowery odour.

Fragrances are the most common cause of allergic contact dermatitis next to nickel. People come into daily contact with fragrance materials in cosmetics, scented hygiene and skin care products and also in domestic and occupational products. Studies where the frequencies of occurrence of the most common fragrance ingredients in cosmetics and other scented products have been examined show that linalool is the most frequently

incorporated fragrance (2, 3). In one study (4), 73 deodorants were analysed and 97% of these were shown to contain linalool. Furthermore linalool is, together with *d*-limonene **2** (Fig. 1), the most common fragrance in domestic and occupational products (5). In spite of this, reports on contact allergy to linalool are rare (6–8). It might be the case that linalool itself is non-allergenic and that allergens are formed on handling and storage. Linalool has a structure similar to that of *d*-limonene. We have in previous studies (9, 10) found that *d*-limonene itself lacks allergenic activity, but forms strong contact allergens on air exposure. The diterpene abietic acid, which is the major component of colophonium, is also activated on air exposure. It is not allergenic in itself but oxidation products with various sensitizing capacities are readily formed due to autoxidation (11).

The aim of the present study was to investigate the potential autoxidation of linalool and to identify compounds formed on air exposure. We also

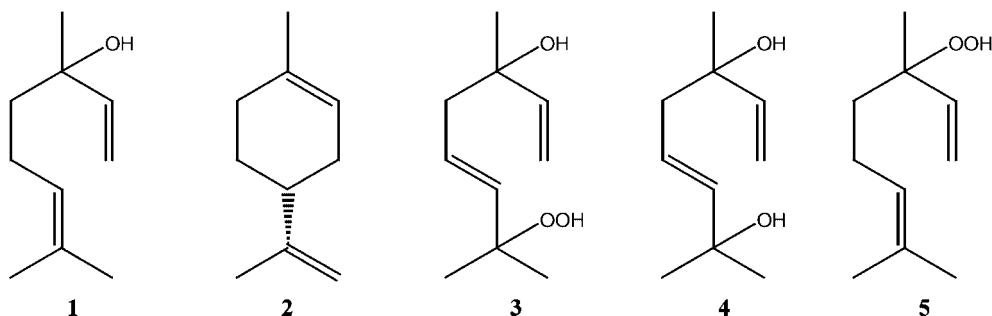


Fig. 1. Linalool (1), *d*-limonene (2), 7-hydroperoxy-3,7-dimethyl-octa-1,5-diene-3-ol (3), 2,6-dimethyl-octa-3,7-diene-2,6-diol (4), 3,7-dimethyl-octa-1,6-diene-3-yl-hydroperoxide (5).

wanted to investigate the sensitizing capacity before and after air exposure in animal experiments.

Materials and Methods

Chemicals

Linalool (3,7-dimethyl-1,6-octadien-3-ol) with a purity stated to be 97% was purchased from Lancaster synthesis Ltd (Eastgate, Lancashire, England). One sample was purified prior to sensitization experiment 1. No contaminants were detected with gas chromatographic (GC) analyses. Another linalool sample was stirred in a flask (the top was covered with para film to prevent contamination and to diminish evaporation) at room temperature for 1 h, 4 $\times$ a day, as previously described (12). Linalool air-exposed for 10 weeks was used in sensitization experiment 2. The rest of the product was kept in the refrigerator during the study. Triphenylphosphine (PPh_3) was purchased from Acros Organics (Morris Plains, NJ, USA). Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were purchased from Difco (Detroit, MI, USA). Non-stabilized white pet. was purchased from Kebo Laboratory AB (Stockholm, Sweden).

Instrumentation and mode of analysis

GC analyses were carried out using an HP 6890 gas chromatograph equipped with an on-column injector and an FID detector, using a fused silica column (HP-5; 0.25 μm , 30 m, 0.25 mm i.d.) and nitrogen as carrier gas. The column temperature was 35°C at injection and 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 internal standard. ^1H and ^{13}C NMR spectra were recorded on a JEOL eclipse + 400 MHz spectrometer using CDCl_3 as solvent. Chemical shifts (δ) are reported in ppm relative to CHCl_3 at δ 7.25 and δ 77.0 for ^1H and ^{13}C spectra, respec-

tively. Multiplicities are indicated by s (singlet), d (doublet), dd (double doublet) and ddd (double, double doublet).

Isolation of the hydroperoxide

Samples of oxidized linalool were subjected to flash chromatography on silica gel columns (Merck silica gel 60, 0.063–0.200 mm) in order to isolate hydroperoxides from the oxidation mixture. Ethyl acetate/hexane was used as eluent (gradient elution). The separation was monitored by thin layer chromatography (TLC; alumina sheets, silica gel 60 F_{254} , Merck,) and a solution of anisaldehyde, sulphuric acid and acetic acid in ethanol was used as visualizing reagent. The purity of the isolated fractions was controlled with GC. Structure elucidation was performed using ^1H and ^{13}C NMR spectroscopy.

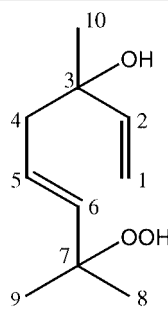
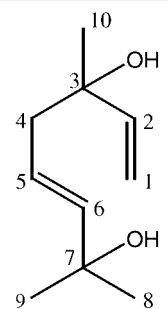
Reduction of the hydroperoxide to the corresponding alcohol

The isolated hydroperoxide was reduced to the corresponding alcohol using PPh_3 . The pure hydroperoxide was dissolved in CDCl_3 in an NMR tube and 1.1 equivalents of PPh_3 were added. The reaction was followed with ^{13}C NMR. The spectrum of the product was compared to the spectrum of the starting material.

Experimental sensitization studies

The sensitization experiments were performed in female Dunkin-Hartley albino guinea pigs (average weight 300–400 g) obtained from HB Lidsköpings Kaninfarm (Lidsköping, Sweden) and Bio Jet service (Uppsala, Sweden). The animals were housed in Macrolon cages, kept on a guinea pig standard diet and water *ad libitum*. Two experiments were carried out using Freund's complete adjuvant test (FCAT) method (13) in its modified version with closed challenge testing (14). The con-

Table 1. ^1H and ^{13}C NMR data for the linalool hydroperoxide and the corresponding alcohol

 				
Position	^1H [JHz]	^{13}C	^1H [JHz]	^{13}C
1	5.06 (dd), 10.7, 1.2	112.22	5.04 (dd), 10.8, 1.3	112.01
1	5.19 (dd), 17.3, 1.2		5.19 (dd), 17.4, 1.3	
2	5.91 (dd), 17.3, 10.7	144.72	5.91 (dd), 17.4, 10.8	144.72
3		72.77		72.64
4	2.31 (dd), -13.4, 6.3	45.20	2.28 (dd), -13.4, 6.5	45.05
4	2.26 (dd), -13.4, 7.0		2.22 (dd), -13.4, 7.6	
5	5.68 (ddd), 15.9, 6.3, 7.0	126.55	5.60 (ddd), 15.6, 7.6, 6.5	121.67
6	5.61 (d), 15.9	137.99	5.70 (d), 15.6	142.66
7		82.05		70.72
8	1.32 (s)	24.39	1.30 (s)	29.90
9	1.32 (s)	24.28	1.30 (s)	29.84
10	1.28 (s)	27.70	1.26 (s)	27.43

centrations used at challenge were chosen on the basis of pretests on FCA-treated animals, where all the concentrations were shown to be non-irritating. The experiments were approved by the local ethics committee.

Experiment 1. For induction, the test animals ($n = 14$) received intradermal injections (0.1 mL) of linalool (5.1% w/w, 3.3×10^{-4} mol/g) on the upper back, on days 0, 6 and 9. The test substance was dissolved in an FCA/water (1/1) emulsion for the 1st injection and in an FIA/water (1/1) emulsion for the 2nd and 3rd injections. The animals in the control group ($n = 14$) were injected with only the FCA/water and the FIA/water emulsions, respectively. Challenge testing was performed on D19 using Finn Chambers[®] (Epitest Ltd Oy, Tuusula, Finland). The test material, linalool 5.1% in pet., and a vehicle control with pet. were applied on shaved flanks for 24 h and reactions were assessed at 48 h and 72 h after the start of exposure. The minimum criterion for a positive reaction was a confluent erythema.

Experiment 2. The experiment was performed according to the procedure described above. Two groups of 15 animals each were used. Induction was performed on days 0, 5 and 9. The exposed group was induced with oxidized linalool (5.1% w/w) and the control group was treated with the FCA(FIA)/water emulsions. Challenge testing was performed on D20. Oxidized linalool was tested at concentrations of 1.0% (6.7×10^{-5} mol/g) and 5.1%. On D47 the animals were rechallenged with

oxidized linalool at concentrations 2.6% (1.7×10^{-4} mol/g) and 10.3% (6.7×10^{-4} mol/g). They were also challenged with a sample of non-purified linalool (5.1%) that had been stored in the refrigerator during the study. At both challenge and rechallenge, a vehicle control with pet. was applied on all animals.

Statistical analysis

The results from the sensitization experiments were statistically evaluated using the Fisher exact test. A p -value of < 0.05 was considered statistically significant.

Results

Oxidation and chemical analysis

During 10 weeks of air exposure, the amount of linalool decreased to about 80%. The GC analyses of the oxidized linalool revealed a complex mixture of compounds which was shown to contain alcohols and hydroperoxides. One of the major oxidation products was isolated from the oxidation mixture by flash chromatography and identified, using ^1H and ^{13}C NMR, as 7-hydroperoxy-3,7-dimethyl-octa-1,5-diene-3-ol **3** (Fig. 1).

To further establish the structure of the hydroperoxide a reduction with PPh_3 to the corresponding alcohol (**15**) was performed in an NMR tube. The NMR spectra of the resulting alcohol were

Table 2. Results from the sensitization experiments on oxidized linalool in guinea pigs using the FCAT method with closed challenge testing

Test material	No. of animals with positive reactions ^a							
	1st challenge				Rechalleng			
	Exposed ^b (n = 15)		Controls (n = 15)		Exposed ^b (n = 15)		Controls (n = 15)	
	48 h	72 h	48 h ¹	72 h ²	48 h ³	72 h ⁴	48 h ⁵	72 h ⁶
Oxidized linalool								
10.3%	nt	nt	nt	nt	13 ^d	13 ^d	4	5
5.1%	8 ^c	7 ^d	0	0	nt	nt	nt	nt
2.6%	nt	nt	nt	nt	4 ^e	5 ^e	0	0
1.0%	1	0	0	0	nt	nt	nt	nt
Non-oxidized linalool ^f								
5.1%	nt	nt	nt	nt	3	3	0	0
pet.	0	0	0	0	0	0	0	0

^aNo. of animals with a confluent erythema 48 and 72 h after application of the test material.

^bInduction: 5.1% of oxidized linalool in FCA(FIA)/H₂O emulsion intradermally.

^cSignificantly different from controls, $p < 0.001$.

^dSignificantly different from controls, $p < 0.01$.

^eSignificantly different from controls, $p < 0.05$.

^f97% as stated by the producer. Not further purified.

nt = not tested.

consistent with the data published for 2,6-dimethyl-3,7-octadiene-2,6-diol **4** (Fig. 1) (16). ¹H and ¹³C NMR spectra (Table 1) for the hydroperoxide **3** are almost identical to the spectra of its corresponding alcohol, except for one important difference. The tertiary carbon (C7) bearing the hydroperoxide or hydroxy group gives rise to a signal at 82.1 ppm for the hydroperoxide and at 70.7 ppm for the alcohol. The same 10 ppm downfield shift has been observed for similar hydroperoxides in comparison to their corresponding alcohols (17, 18).

Sensitization studies

Experiment 1. Pure linalool did not sensitize the animals. No reactions to linalool were found in the exposed animals or in the controls.

Experiment 2. The animals induced with oxidized linalool became sensitized (Table 2). A significant response was found to the concentrations 2.6, 5.1 and 10.3%, but not to 1.0%. Three reactions were seen to the non-oxidized, unpurified linalool but the response was not significant. At rechalleng, 5 of the control animals gave positive reactions when tested with 10.3% oxidized linalool, which suggests that these animals became sensitized at the first challenge testing.

Discussion

In the present study, the autoxidation of linalool **1** was investigated. It was found that linalool oxidizes on air exposure and that the content of linalool decreases over time. In the complex mixture

of oxidation products, one of the major products formed was isolated and identified as the hydroperoxide **3**. The sensitizing capacity of linalool, before and after oxidation, was investigated. Linalool itself had no sensitizing capacity, while the oxidation mixture, obtained after air exposure for 10 weeks, sensitized guinea pigs in an FCAT experiment.

The autoxidation of terpenes on air exposure is a well-known phenomenon and we have previously described the oxidation process for *d*-limonene **2** (10). The oxidation of linalool is somewhat slower. After 10 weeks of air exposure, about 20% of the linalool is consumed, compared to 60% for *d*-limonene. The primary oxidation products seem more stable in the case of linalool and fewer polymers are formed. Autoxidation of terpenes initially produces hydroperoxides, unstable compounds that degrade to other, secondary oxidation products, finally leading to the formation of polymeric material. The linalool hydroperoxide identified is described for the first time, to the best of our knowledge. This allylic hydroperoxide was found to be relatively stable, which could be explained by the tertiary structure of the hydroperoxide. A previously published linalyl hydroperoxide **5** (Fig. 1) (17) was synthesized and searched for in the oxidation mixture of linalool, but it could not be detected. This hydroperoxide was originally synthesized for investigation of the sensitization mechanism of allylic hydroperoxides, and not as a candidate for being a sensitizing compound in oxidized linalool.

To act as a skin sensitizer, a compound must possess certain chemical properties that make it re-

active with skin proteins. Thus, an understanding of the mechanism of antigen formation is important for the prediction of allergenic activity of different chemicals. With regard to structure–activity relationships, linalool would not be considered a contact allergen since the molecular structure of linalool does not contain any protein-reactive groups. Hydroperoxides, though, are known contact allergens and in the cases of *d*-limonene (19) and colophonium (20), hydroperoxides were identified as oxidation products and were also shown to be strong contact allergens. The exact mechanism of the binding of hydroperoxides to skin proteins is still unknown, but several studies show that the antigen formation could be due to radical reactions (17, 19, 21, 22).

Linalool and oxidized linalool were tested for sensitizing capacity in guinea pigs. The results show that linalool is not a sensitizer but that substances with allergenic properties are formed on air exposure. An insignificant response was seen to linalool of commercial quality, not further purified. The content of oxidation products might be enough for some elicitation in sensitized animals. The purity of linalool used in products or test preparations is rarely considered. This might explain why reports in the literature on the sensitizing capacity of linalool show divergent results. A weak sensitizing capacity was observed in the local lymph node assay (23). Testing with undiluted linalool resulted in a stimulation index of 8.3. A chemical is considered to be a sensitizer if a stimulation index of 3 or greater is achieved at any concentration. However, after purification of linalool, the stimulation index decreased to 4.9 (personal communication). This could be explained by a decrease in the concentration of oxidation products, although the difference is within biologic variations. In human maximization tests, linalool caused no sensitization in 25 volunteers, when tested at 8% and 20% concentrations (24, 25).

Very few clinical studies have reported cases of contact allergy to linalool (6–8). Storage and handling conditions naturally influence the degree of oxidation of products and therefore also the risk of adverse effects. Because of the extensive usage of linalool, undiagnosed contact allergy to oxidized linalool might be frequent among patients with allergic contact dermatitis, since screening with oxidized linalool and linalool hydroperoxide is not performed. In previous clinical studies (26, 27) on oxidized limonene and its hydroperoxides, 1–6% of patients who underwent patch testing showed positive reactions. This frequency is equal to the prevalence of contact allergy to the most common contact allergens in the standard series. The present study is part of

a research project on fragrance allergy in Europe. In a following study, the clinical relevance of our findings will be investigated by patch testing consecutive dermatitis patients with oxidized linalool.

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