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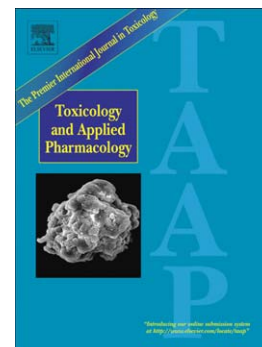
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**Cytochrome P450-mediated activation of the fragrance compound geraniol
forms potent contact allergens**

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Abstract

Contact sensitization is caused by low molecular weight compounds which penetrate the skin and bind to protein. In many cases, these compounds are activated to reactive species, either by autooxidation on exposure to air or by metabolic activation in the skin. Geraniol, a widely used fragrance chemical, is considered to be a weak allergen, although its chemical structure does not indicate it to be a contact sensitizer. We have shown that geraniol autoxidizes and forms allergenic oxidation products. In the literature, it is suggested but not shown that geraniol could be metabolically activated to geranial. Previously, a skin-like CYP cocktail consisting of cutaneous CYP isoenzymes, was developed as a model system to study cutaneous metabolism. In the present study, we used this system to investigate CYP-mediated activation of geraniol. In incubations with the skin-like CYP cocktail, geranial, neral, 2,3-epoxygeraniol, 6,7-epoxygeraniol and 6,7-epoxygeranial were identified. Geranial was the main metabolite formed followed by 6,7-epoxygeraniol. The allergenic activities of the identified metabolites were determined in the murine local lymph node assay (LLNA). Geranial, neral and 6,7-epoxygeraniol were shown to be moderate sensitizers, and 6,7-epoxygeranial a strong sensitizer. Of the isoenzymes studied, CYP2B6, CYP1A1 and CYP3A5 showed high activities. It is likely that CYP1A1 and CYP3A5 are mainly responsible for the metabolic activation of geraniol in the skin, as they are expressed constitutively at significantly higher levels than CYP2B6. Thus, geraniol is activated through both autooxidation and metabolism. The allergens geranial and neral are formed via both oxidation mechanisms, thereby playing a large role in the sensitization to geraniol.

Abbreviations

CYP, cytochrome P450; EC3, estimated concentration required to produce an SI of 3; FM, fragrance mix; GC, gas chromatography; LLNA, local lymph node assay; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; rhCYP, recombinant human cytochrome P450; SI, stimulation index; TRIS, tris(hydroxymethyl)aminomethane.

Introduction

Contact sensitization and its clinical manifestation, allergic contact dermatitis, are caused by low molecular weight chemicals which are able to penetrate the skin. It is generally accepted that they must bind to high molecular weight compounds, such as proteins or peptides, in order to become immunogenic. In many cases, a prerequisite for this binding is the chemical activation of the compounds to more reactive species either by autoxidation on exposure to air or by metabolic activation, e.g. by CYP-mediated metabolism in the skin. As the main interface between the body and the environment, the skin possesses not only immunocompetent cells such as the Langerhans cells but also multiple CYP-isoenzymes (Baron *et al.*, 2001; Oesch *et al.*, 2007).

The CYP enzymes have been identified as the most important metabolic enzymes in the skin (Ahmad and Mukhtar, 2004). The expression of CYP genes in human skin keratinocytes has previously been reported, showing constitutive expression of CYP1A1, CYP1B1, CYP2B6, CYP2E1 and CYP3A5 (Baron *et al.*, 2001). These results were confirmed on the translational level by immunohistochemistry, immunoblot and catalytic assays. The expression of additional CYP genes has been shown in other studies (Ahmad and Mukhtar, 2004; Oesch *et al.*, 2007).

Fragrance compounds are the second most common cause of contact allergy after nickel (de Groot and Frosch, 1997). Many fragrance compounds are themselves non-sensitizers, but are activated to form strong allergens. For example, limonene and linalool (the most commonly used fragrance compounds), form skin sensitizers by autoxidation processes (Karlberg *et al.*, 2008), while eugenol is activated by CYP-mediated metabolic oxidation (Bertrand *et al.*, 1997).

Geraniol (*E*-3,7-dimethyl-2,6-octadien-1-ol, Figure 1) is one of the compounds in the fragrance mix (FM) that is used for clinical screening of contact allergy among consecutive dermatitis patients. The FM consists of seven chemicals and one natural mixture. Geraniol is responsible for only 5% of positive patch test reactions to the individual components of FM (Schnuch *et al.*, 2004) and is thus considered to be a weak allergen. Geraniol occurs naturally in large amounts in, among others, the essential oils of rose, palmarosa and citronella, and is

therefore a widely used fragrance chemical. It has been shown to be present in 76% of investigated deodorants on the European market (Rastogi et al., 1998) and in 41% of domestic and household products (Rastogi et al., 2001). Studies also show that it is present in 33% of cosmetics based on natural ingredients (Rastogi et al., 1996).

The formation of immunogenic complexes normally takes place between electrophilic haptens and nucleophilic functional groups on proteins. Geraniol is not itself electrophilic, and is therefore not expected to be a contact sensitizer according to common structure activity relationship (SAR) rules (Karlberg et al., 2008 and refs therein). We have previously shown that geraniol is readily autoxidized upon air exposure, forming several compounds with allergenic activity (Hagvall *et al.*, 2007). The major allergenic autoxidation products identified were geranial and neral (Figure 1). However, we could also demonstrate that chemically pure geraniol itself had a certain sensitizing capacity (Hagvall *et al.*, 2007), which indicates that geraniol may be metabolically activated in the skin to become protein reactive. To the best of our knowledge, the metabolic activation of geraniol has been suggested in the literature, but never shown. Thus, we wanted to investigate whether CYP-mediated activation of geraniol in the skin occurs in addition to the formation of reactive species by autoxidation.

Previous work within our group led to the development of a skin-like CYP cocktail as a model system of cutaneous metabolism (Bergstrom et al., 2007). In the present study, this system was used to investigate the possible metabolic activation of geraniol by cutaneous CYP isoenzymes. The effect of cutaneous metabolism on the allergenic activity was evaluated by determining the sensitizing capacity of the identified metabolites. Our results show that geraniol is an example of a contact sensitizer that can be activated by two parallel pathways – autoxidation and CYP-mediated metabolic activation.

Materials and methods

Chemicals

Geraniol, geranial (*E*-3,7-dimethyl-2,6-octadienal) and neral (*Z*-3,7-dimethyl-2,6-octadienal) (Figure 1) were obtained from Bedoukian Inc. (USA) and distilled under reduced pressure before use. Acetone p.a. was purchased from Merck (Germany) and olive oil was purchased from Apoteket AB (Sweden). Magnesium chloride (1 M) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Aldrich (Germany). Recombinantly expressed CYP bacosomes (coexpressed with P450-reductase in *E. Coli*) were purchased from Tebu-bio (Germany).

Instrumentation and mode of analysis

Column chromatography was performed using Geduran Si 60 (0.063-0.200 mm) silica gel. TLC was performed using silica gel plates and developed with anisaldehyde dip as previously described (Bergstrom et al., 2006). ^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, respectively. Electron-ionization mass spectral data analysis (70 eV) was performed on a Hewlett-Packard model 5973 mass spectrometer connected to a Hewlett-Packard 6890 gas chromatograph, equipped with a split/splitless inlet and an HP-5MSI fused silica capillary column (30 m x 0.25 mm, 0.25 μm film thickness).

Synthesis of reference compounds

2,3-Epoxy-3,7-dimethyl-oct-6-en-1-ol (2,3-epoxygeraniol) (Fig. 1). The synthesis was performed as previously described (Bezard et al., 1997) starting from geraniol.

(*E*)-6,7-Epoxy-3,7-dimethyl-oct-2-en-1-ol (6,7-epoxygeraniol) (Fig. 1). The synthesis was performed as previously described (Rebelo et al., 2005). ^1H and ^{13}C NMR data and mass spectral data agree with literature values (Martins et al., 2001).

(*E*)-6,7-Epoxy-3,7-dimethylpent-2-enal (6,7-epoxygeranial) (Fig. 1). 70% *m*-CPBA (1.9 g, 7.7 mmol) was added to a solution of geranial (1.2 g, 7.7 mmol) in CH_2Cl_2 (50 mL) at 0 °C. The reaction mixture was stirred for 2 h, after which time NaOH (10 mL, 2 M aqueous solution) was added. The organic phase was dried over MgSO_4 and concentrated. The crude

product was purified by column chromatography on silica gel eluting with ethyl acetate/*n*-hexane (1:9), which gave 6,7-epoxygeraniol in 90% yield. ^1H NMR and mass spectral data agree with those in the literature (Martins et al., 2001). ^{13}C NMR δ 191.2, 162.7, 127.6, 63.4, 58.6, 37.4, 26.8, 24.8, 18.8, 17.7.

Stability experiments of studied epoxides

Prior to performing the metabolism experiments, the chemical stabilities of the epoxides to the incubation conditions (tris(hydroxymethyl)aminomethane (TRIS) buffer (300 mM, pH 7.4), 60 min, 37 °C) were investigated. Stock solutions of each epoxide (1.0 mM) in acetonitrile were prepared. The stability experiments were initiated by addition of the epoxide stock solution (2.5 μL) to prewarmed TRIS buffer (500 μL , 300 mM, pH 7.4). The solutions were either incubated at 37 °C for 60 min or extracted immediately without incubation. The extracts were analyzed using GC/MS and synthesized reference compounds. After 60 min of incubation, 71% of the 2,3-epoxygeraniol, 59% of the 6,7-epoxygeraniol and 79% of the 6,7-epoxygeraniol remained. Although these epoxides are only moderately stable, they can be readily detected and quantified.

Incubations with the skin-like CYP cocktail

The skin-like CYP cocktail was prepared by mixing the recombinantly expressed CYP batosomes (CYP1A1, CYP1B1, CYP2B6, CYP2E1, CYP3A5) as previously described (Bergstrom et al., 2007). As geraniol has the potential to form several metabolites, a second experiment was performed where the rhCYP enzymes were present at 5 times the original concentrations (total CYP content 110 pmol), in order to enable the detection of metabolites formed in low amounts. The CYP content of the more concentrated cocktail corresponds to that of the previously reported liver-like CYP cocktail (Bergstrom et al., 2007).

Sample incubations contained substrate (50 μM), TRIS buffer (300 mM, pH 7.4), MgCl_2 (30 mM) and NADPH (1 mM) in a total volume of 500 μL . The incubations were performed in duplicate and controls were run in the absence of NADPH or CYP. The incubations were initialized by the addition of NADPH after 3 min of pre-incubation at 37 °C and terminated after 60 min by extraction with *n*-hexane/dichloromethane (1:1, 1000 μL) containing 1,2,3,5-tetramethylbenzene as internal standard. The extracts were collected after centrifugation at 3000 rpm for 10 min and analyzed using gas chromatography/mass spectrometry (GC/MS).

Incubations with single recombinant human (rh) CYP enzymes

The incubations were performed using individual recombinantly expressed human CYP bacosomes (20 pmol; CYP 1A1, 1B1, 2B6, 2E1, 3A5, all coexpressed with CYP-reductase in *E. coli*, Tebu-bio). Otherwise, the incubations were performed as described above.

GC/MS analysis of geraniol and metabolites

Analysis was performed using the following method settings: the temperature program started at 35 °C for 1 min, increased by 5 °C/min ending at 160 °C for 1 min, followed by an increase of 15 °C/min ending at 250 °C for 5 min. The injection volume was 3 µL. The mass spectrometer was used in selected ion monitoring (SIM) mode and the *m/z* values were monitored as follows: at t_R = 13.5 – 17.0 min 134, 119 (internal standard); at t_R = 17.0 – 17.5 min 134, 79, 69 (nerol); at t_R = 17.5 – 17.8 min 136, 134, 69 (geraniol and geranial); at t_R = 17.8 – 20.0 min 140, 82, 67 (2,3-epoxygeraniol); at t_R = 20.0 – 20.75 min 152, 109, 81 (6,7-epoxygeraniol); at 20.75 – 39.0 min 150, 135, 79 (6,7-epoxygeranial).

Sensitization experiments in mice

The sensitizing potencies of the chemicals were investigated using the murine local lymph node assay (LLNA) (Kimber et al., 1995, OECD Guideline no. 429, 2002) as previously described (Hagvall et al., 2007). The animals were housed in cages with hepa-filtered airflow, under conventional conditions in light-, humidity- and temperature-controlled rooms. The animals were allowed to acclimate for one week prior to use. All animal procedures were approved by the local ethics committee. Each compound was tested at five different concentrations, using mice in groups of three. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), i.e. the test group/control group ratio. Test materials that caused an SI of 3 or higher at one or more concentrations 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 (Basketter et al., 1999). 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 (Kimber et al., 2003).

Results

Incubation of geraniol using the skin-like CYP cocktail. The following metabolites were detected in the incubations using the lower concentration of the skin-like CYP cocktail (22 pmol/500 μ L): geranial, neral, 2,3-epoxygeraniol and 6,7-epoxygeraniol. Among these, geranial was the major metabolite formed (Figure 2a), followed by 6,7-epoxygeraniol. The same metabolites, together with 6,7-epoxygeranial, were detected in the incubations using the higher concentration of the skin-like CYP cocktail (110 pmol/500 μ L). 6,7-Epoxygeranial was formed in low amounts (Figure 2b), but otherwise the pattern of formation observed was the same as in the incubations using the lower concentration of the skin-like CYP cocktail.

Incubation of geraniol using single rhCYP enzymes. Single rhCYP enzyme incubations were carried out to investigate the role of each enzyme in the metabolic activation of geraniol in the skin-like rhCYP cocktail (Figure 3). Of the enzymes present in the cocktail, CYP1A1, CYP2B6 and CYP3A5 showed high activities, whereas CYP1B1 and CYP2E1 showed low activities. CYP2B6 showed the highest activity of the five isoenzymes studied. However, in the incubations using CYP2B6 alone, only oxidation of the alcohol moiety was observed, i.e. the only products formed were the aldehydes geranial and neral. In the incubations using CYP1A1 and CYP3A5, epoxidation of the double bonds was also observed, with 2,3-epoxygeraniol and 6,7-epoxygeraniol being identified in addition to geranial and neral. In the incubations using CYP1A1, the major product was 6,7-epoxygeraniol, followed by geranial. In the incubations using CYP3A5, the opposite result was observed, as geranial was the major product followed by 6,7-epoxygeraniol. 2,3-Epoxygeraniol was the only product detected in the incubations using CYP1B1 and CYP2E1. The minor metabolite 6,7-epoxygeranial, which was observed in the incubations using the higher concentration of the skin-like CYP cocktail (110 pmol/500 μ L), was not detected in any of the incubations using single enzymes.

Sensitization experiments in mice. The sensitizing potency of 6,7-epoxygeraniol and 6,7-epoxygeranial was determined using the LLNA (Table 1). 6,7-Epoxygeraniol was shown to be a moderate sensitizer with an EC₃ value of 0.42 M (7.2%), whereas 6,7-epoxygeranial was shown to be a strong sensitizer with an EC₃ value of 0.082 M (1.4%).

Discussion

We have for the first time described the CYP-mediated metabolism of geraniol, where five metabolites (geranial, neral, 2,3-epoxygeraniol, 6,7-epoxygeraniol and 6,7-epoxygeranial) were identified (Figure 1). The metabolic activation of geraniol was studied using a recently developed skin-like CYP cocktail, which provides a convenient method for performing investigations of cutaneous metabolism using the CYP isoforms present in the skin (Bergstrom et al., 2007). Geranial was the major metabolite formed, followed by 6,7-epoxygeraniol. The minor metabolite 6,7-epoxygeranial was shown to be the strongest allergen of all those identified, while geranial, neral and 6,7-epoxygeraniol were moderate sensitizers and 2,3-epoxygeraniol a non-sensitizer.

With regard to the experiments using single rhCYP enzymes, CYP2B6 showed a very high activity when using geraniol as the substrate (Figure 3). However, CYP1A1 and CYP3A5 are more likely to be responsible for the majority of cutaneous metabolism of geraniol, since they are constitutively expressed at significantly higher levels in human skin compared to CYP2B6 (Baron et al., 2001, Bergstrom et al., 2007). This is reflected in the skin-like CYP cocktail where CYP1A1 and CYP3A5 constitute 16% and 25% respectively of the cocktail's total CYP content, compared to 0.16% for CYP2B6 (Bergstrom et al., 2007). The remaining isoenzymes studied, CYP1B1 and CYP2E1, showed low activities. Although the levels of expression of these in the skin are similar to those of CYP1A1 and CYP3A5, metabolism of geraniol by CYP1B1 and CYP2E1 is of minor importance with respect to contact allergy, as the only metabolite formed is 2,3-epoxygeraniol, a non-sensitizer.

Both geranial and neral were detected in the metabolic experiments conducted in this study; however, geranial is the only aldehyde that would be expected to be formed by the CYP-mediated oxidation of geraniol. It has been reported that the presence of proteins can catalyze the isomerisation of geranial to neral, even at pH 7 (Wolken et al., 2000), which explains the detection of neral in the incubation experiments. Even though neral could undergo further CYP-mediated metabolic oxidation to give 6,7-epoxyneral, this compound was not identified in the incubations. The most likely reason for this is that it was formed in amounts too low to be detected.

The results from the LLNA experiments identified 6,7-epoxygeranial as a strong allergen and 6,7-epoxygeraniol as a moderate allergen. Even though 6,7-epoxygeranial has two electrophilic sites that are able to react with proteins, it is believed that only the most reactive site in the molecule will participate in the formation of the hapten-protein complex due to kinetic competition (Nazih et al., 1993). However, this does not explain the stronger allergenic activity of 6,7-epoxygeranial compared to 6,7-epoxygeraniol, as both of these epoxides are similar in structure and geranial, which contains the same electrophilic α,β -unsaturated aldehyde moiety as 6,7-epoxygeranial, only is a moderate allergen. The difference in allergenic activity of these two epoxides might be due to the stronger irritant effect of geranial compared to geraniol. Citral, the isomeric mixture of geranial and neral, is a well-known irritant in clinical studies (Heydorn et al., 2003), whereas irritation reactions to geraniol are less frequent. It is well known that concurrent application of allergens and irritants increases the skin response (Pedersen et al., 2004). For citral, its irritating effect has been shown to have an important impact on its sensitizing capacity (Heydorn et al., 2003). The same relationship may apply for the corresponding 6,7-epoxides of geranial and geraniol.

Previous studies in our group have shown that the sensitizing capacities of epoxides vary over a wide range, from non-sensitizing, sterically hindered epoxides to more reactive allylic epoxides (Bergstrom et al., 2006; Skold et al., 2008). We have previously identified 2,3-epoxygeraniol as a non-sensitizer (Figure 4), (Hagvall et al., 2007), which is thus less allergenic than geraniol itself. In 2,3-epoxygeraniol, the formation of a reactive conjugated system is blocked, which most probably explains the decrease in sensitizing capacity compared to geraniol.

As with other unsaturated monoterpenes, e.g. limonene and linalool, geraniol can also be activated by autoxidation upon exposure to air (Karlberg et al., 1994; Skold et al., 2004). The primary autoxidation products, hydroperoxides, are the most potent sensitizers in the autoxidation mixtures. The fragrance terpenes limonene and linalool thereby go from being non-allergenic/weak allergens to commonly-encountered sensitizing oxidation mixtures, which show positive patch test reactions in 2-3% of patch tested consecutive dermatitis patients (Matura et al., 2002; Matura et al., 2005). It has been shown that only non-sensitizing or weak sensitizers are metabolically formed from limonene (Bergstrom et al., 2006) and linalool (Meesters et al., 2007). In contrast to these results, we have demonstrated in this study that geraniol is metabolically activated to give strong and moderate sensitizers. Among

the metabolites formed, geranial and neral have also been shown to form by air oxidation. The relative clinical importance of these two activation pathways is thus not as easily differentiated for geraniol as for limonene and linalool and warrants further investigation.

Geranial is commonly encountered by the general population in the form of the isomeric mixture citral, which is frequently used both as a fragrance and a flavour in food and beverages (Peacock and Kuneman, 1985; Rastogi *et al.*, 2001). In a recent multicenter study, a large number of concomitant reactions (83%) to geraniol and citral were discovered in patients diagnosed with contact allergy to fragrances (Schnuch *et al.*, 2007). The frequency of concomitant reactions was higher than those to cinnamic alcohol and cinnamic aldehyde, a well-known prohaptens-haptens pair. We have previously identified geranial and neral as moderate allergens (Hagvall *et al.*, 2007) and their sensitizing capacities are included in Figure 4 for comparison with the sensitizing capacities of the newly identified metabolites described in this study. The results from the multicenter study by Schnuch *et al.* indicate that formation of geranial by cutaneous metabolism could be a major cause of contact allergy to pure, i.e. not oxidized, geraniol. Patients with negative patch test reactions to citral and positive patch test reactions to geraniol might be sensitized to one or both of the other sensitizing metabolites, 6,7-epoxygeraniol or 6,7-epoxygeranial. However, as the purity of geraniol used for patch testing is not stated in clinical articles, positive reactions to geraniol could also be caused by geraniol hydroperoxide, a primary autoxidation product which is not formed metabolically (Hagvall *et al.*, 2007).

We have for the first time investigated the CYP-mediated metabolic activation of geraniol and demonstrated that strong and moderate sensitizers are formed. Two epoxide metabolites of geraniol, 6,7-epoxygeraniol and 6,7-epoxygeranial were identified as new allergens. This work demonstrates that the recently-developed skin-like CYP cocktail functions well and could serve as an important tool to investigate cutaneous metabolism of various compounds *in vitro*. The main conclusion from this work is that the allergenic activity of pure geraniol can be explained by cutaneous metabolic activation to sensitizing metabolites. Thus, geraniol is activated through both autoxidation and metabolism. The allergens geranial and neral are formed via both oxidation mechanisms, thereby playing a large role in the sensitization to geraniol.

Conflict of interest

The authors state no conflict of interest

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Figure captions

Figure 1. Structures of geraniol and identified metabolites.

Figure 2. Total amounts detected in the incubations with skin-like CYP cocktail (**a**) 22 pmol and (**b**) 110 pmol. Geranial is the main metabolite formed in both cases. In the more concentrated cocktail, the secondary metabolite 6,7-epoxygeranial can be detected. ND = not detected.

Figure 3. Total amounts detected in the incubations with single rhCYP enzymes. 20 pmol of enzyme was used in all incubations. Neral (□), geranial (□), 2,3-epoxygeraniol (■), 6,7-epoxygeraniol (■). Only 2,3-epoxygeraniol was detected in the incubations with CYP 1B1 and CYP 2E1. 6,7-Epoxygeranial was not detected in any of the incubations with single rhCYP enzymes.

Figure 4. Metabolism of geraniol creates metabolites with stronger allergenic activity compared to geraniol. Dose-response curves for compounds tested in the local lymph node assay (LLNA). The concentrations are given in M. Geraniol^a (◇), geranial^a (●), neral^a (○), 2,3-epoxygeraniol^a (□), 6,7-epoxygeraniol (■), 6,7-epoxygeranial (▲).

^a Previously published data (Hagvall *et al.*, 2007).

Table 1. LLNA^a Responses for Metabolites of Geraniol

test material and concentration (% w/v)	[³ H]thymidine incorporation (dpm/lymph node)	SI	EC3 value		classification
			(%)	(M)	
6,7-epoxygeraniol			1.38	0.082	strong
control	1171				
0.01	1047	0.89			
0.1	1229	1.05			
1	2977	2.54			
5	8605	7.35			
10	8614	7.36			
6,7-epoxygeraniol			7.12	0.42	moderate
control	1001				
0.1	765	0.76			
1	1132	1.13			
10	3890	3.88			
20	4736	4.73			
60	5279	5.27			

^a The sensitization experiments were performed as described in Materials and Methods. The SIs correspond to the increase in 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 (Kimber et al., 2003).

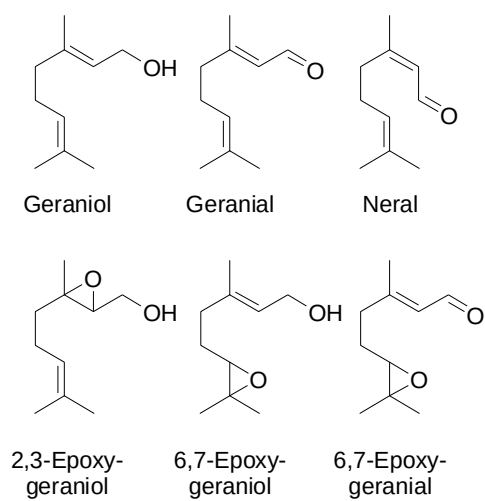
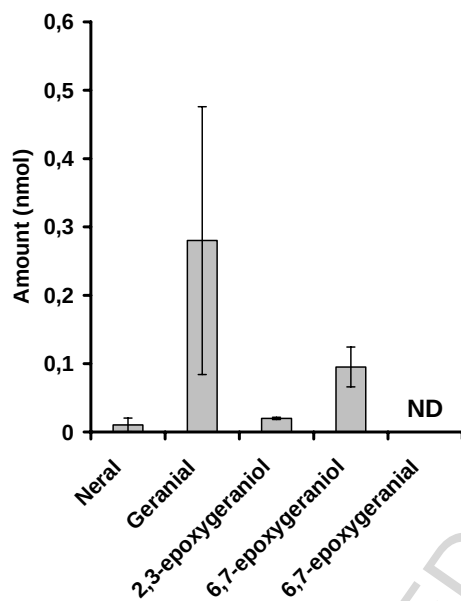
Figure 1

Figure 2

a



b

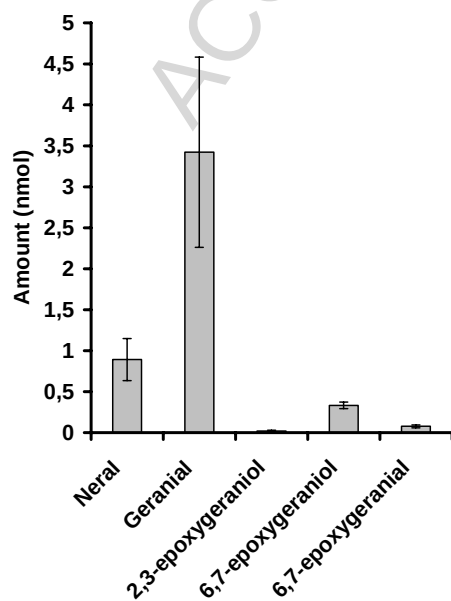


Figure 3

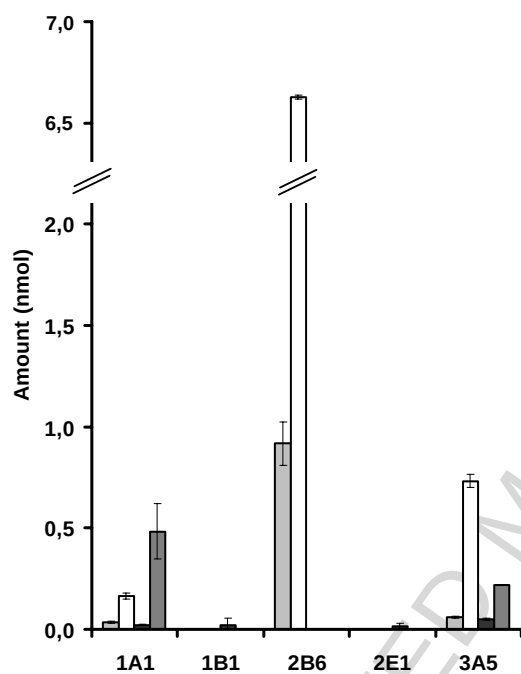


Figure 4

