

## Hydroperoxides form specific antigens in contact allergy

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Concomitant positive reactions to colophonium, oxidized limonene, and/or oxidized linalool are recorded in patch test studies. The main allergens in these patch test mixtures are hydroperoxides, which form antigens by a radical pathway. Theoretically, concomitant reactions can be explained not only by concomitant sensitization or by true cross-reactions but also by the hydroperoxides acting as oxidizing agents on skin proteins to form non-specific antigens without hapten–protein binding. The aim of this study was to explore concomitant reactions and cross-reactivity patterns among hydroperoxide haptens. We investigated whether individuals allergic to the main allergen in colophonium, 15-hydroperoxyabietic acid, would also react to limonene hydroperoxide or linalool hydroperoxide. Only 1 of 29 individuals reacted to more than 1 hydroperoxide. The cross-reactivity pattern among cumene hydroperoxide, limonene hydroperoxide, 1-(1-hydroperoxy-1-methylethyl) cyclohexene (cyclohexene hydroperoxide), and 15-hydroperoxydehydroabietic acid was investigated in guinea-pigs. No general cross-reactivity was observed. Cross-reactions between cumene hydroperoxide and cyclohexene hydroperoxide show that similarity in the overall structure and the way of antigen formation are needed. Quantum calculations were used to determine the formation energies of the intermediary radicals. We concluded that hydroperoxides form specific antigens and that formation of non-specific antigens is unlikely. The concomitant patch test reactions described in the literature are best explained as a result of multiple sensitizations.

**Key words:** allergic contact dermatitis, colophonium, cross-reactivity, Freund's Complete Adjuvant Test, guinea-pig, 15-hydroperoxyabietic acid, limonene, linalool, oxidation products, patch testing, quantum calculations. © Blackwell Munksgaard, 2006.

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In recent clinical studies, significant concomitant positive patch test reactions have been observed involving the colophonium and the oxidized fragrance terpenes, limonene and linalool (1–3). The main allergens in these materials are hydroperoxides. Theoretically, the concomitant reactions can be explained not only by concomitant sensitization or by true cross-reactions but also by the hydroperoxides acting as oxidizing agents on skin proteins to form non-specific antigens without hapten–protein binding.

Colophonium is an easily oxidized natural material with a complex chemical composition, rendering a 2%–5% frequency of positive patch test reactions at consecutive testing in dermatology clinics (4, 5). It is used in a variety of products, mainly, adhesives, emulgators, and soldering fluxes, and also in the sizing of paper (6). The major

component in colophonium is abietic acid (Fig. 1, **1**), which easily autoxidizes on exposure to air, forming allergenic oxidation products. The hydroperoxide of abietic acid, 15-hydroperoxyabietic acid (15-HPA) **2**, is identified as the major allergen in colophonium (7). In clinical patch test screening of individuals allergic to colophonium using chromatographically isolated 15-HPA methyl ester, a positive patch test reaction to the hydroperoxide was seen in 57% of the individuals (8).

Limonene **3** is a common fragrance terpene, which is widely used not only in cosmetics but also in a variety of domestic and industrial cleansers, often in concentrations of several per cent (9). Consecutive testing showed a 2%–3% frequency of positive patch test reactions in patients with dermatitis when testing with oxidized limonene

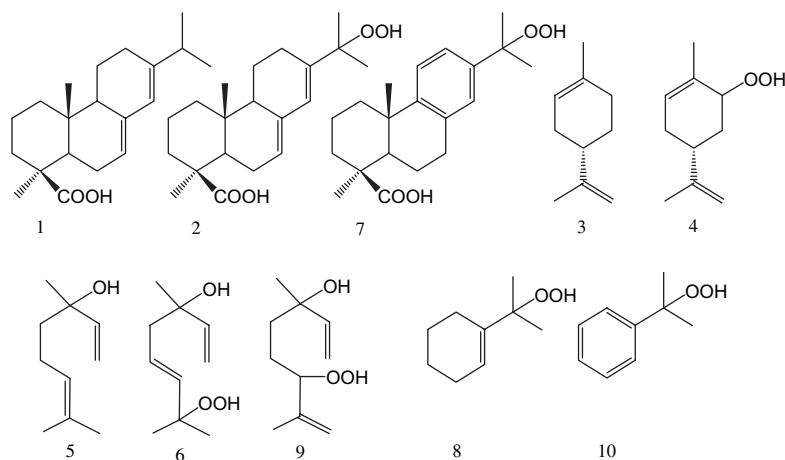


Fig. 1. Abietic acid **1**; 15-hydroperoxyabietic acid (15-HPA) **2**; 15-hydroperoxydehydroabietic acid (15-HPDA) **7**; R-limonene **3**; R-limonene-2-hydroperoxide, 2-hydroperoxy-*p*-menta-6,8-diene (limonene hydroperoxide) **4**; linalool **5**; 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol (linalool hydroperoxide) **6**<sup>a</sup>; 6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol (linalool hydroperoxide) **9**<sup>a</sup>; 1-(1-hydroperoxy-1-methylethyl) cyclohexene (cyclohexene hydroperoxide) **8**; and cumene hydroperoxide **10**. <sup>a</sup>The linalool hydroperoxide used in the clinical study was a 5:3 mixture of compounds **6** and **9**.

(1, 2). Clinical and experimental studies have shown that non-oxidized limonene is not allergenic in itself (1, 2, 10, 11) but autoxidizes on contact with air, thereby forming allergenic oxidation products. Several oxidation products have been identified, and both experimental and clinical studies point out limonene-2-hydroperoxide **4** as the main allergenic component (1, 2, 10–12).

Linalool **5** is another common fragrance terpene found in cosmetics, hygiene products, and household chemicals (9). Consecutive testing showed a 1.3% frequency of positive patch test reactions to oxidized linalool in patients with dermatitis (3). As described above for abietic acid and limonene, linalool readily autoxidizes on exposure to air and a hydroperoxide **6** has been found to be the major contact allergen (3, 13, 14).

The classical model for hapten–protein interaction involves nucleophilic groups of the protein which react with the electrophilic hapten. The electrophilic hapten is a molecule with at least 1 polarized chemical bond. The resulting reaction is a nucleophilic substitution or a nucleophilic addition, whereby a covalent bond between the hapten and the protein is created, forming a complete antigen (15). Abietic acid, limonene, and linalool are considered to be prohaptens and all contain unsaturated double bonds, which react with oxygen in air to form hydroperoxides. The hydroperoxides are not electrophiles, and if they act as haptens, they represent an alternative mode of reaction involving radical coupling between the hapten and the protein or, alternatively, by intramolecular radical rearrangements, forming compounds that may function as electrophilic haptens (16).

In experiments on guinea-pigs, epidermal pretreatment with the antioxidants ascorbic acid and

$\alpha$ -tocopherol, reduced sensitization as well as the response at elicitation of the contact allergic reaction to limonene-2-hydroperoxide **4** (17). Using the same experimental set-up, the antioxidants did not influence the allergenic activity of the potent electrophilic hapten chlorothalonil or of the metallic allergen cobalt, which is believed to form an allergenic coordination complex (17). The mechanism behind the observed protective effect of 1 or both of the antioxidants on the reaction to limonene hydroperoxide is likely to be either a reduction of the hydroperoxide or a trapping of the free radical formed by the hydroperoxide. In both cases, limonene-2-hydroperoxide should be converted to the corresponding alcohol that is non-allergenic, as described in earlier studies (10).

Besides concomitant sensitization, 2 mechanisms can be considered to explain the findings of simultaneous positive patch test reactions to the oxidized fragrance terpenes and colophonium, in recent clinical studies (1–3). In 1 proposed mode of action leading to specific antigens, the hapten binds to a host protein by radical coupling. For this to happen, the hydroperoxides undergo homolytic cleavage of the peroxy group to form a free radical that may either directly couple and form a covalent bond to proteins or form epoxides through intramolecular rearrangements. In this case, only structurally closely related hydroperoxides would cross-react.

A second mode of action would be that the hydroperoxides form non-specific antigens. As hydroperoxides and peroxides are oxidizing agents, they may oxidize functional groups in the proteins, e.g. the phenolic groups in tyrosine or the sulfur moieties of cysteine and methionine. Theoretically, structurally different hydroperoxides

and peroxides could form the same oxidized and immunogenic proteins. Hydroperoxides reacting in this manner would be non-specific agents and cross-reactions between hydroperoxides with significantly different structures would be observed in animal experiments or at patch testing.

The aim of this study was to explore the 2 above-proposed mechanisms for antigen formation from allergenic hydroperoxides using 3 different experimental approaches. We investigated if individuals allergic to the allergenic hydroperoxide in colophonium, 15-HPA, would also react to other terpene hydroperoxides, e.g. limonene hydroperoxide and linalool hydroperoxide. The potential cross-reactivity between hydroperoxides with different structures was further studied in guinea-pigs. Quantum calculations were used to determine the thermodynamic stability of the intermediary radicals.

## Materials and Methods

### Chemicals

Limonene-2-hydroperoxide (2-hydroperoxy-*p*-mentha-6,8-diene) **4** (12), 15-hydroperoxydehydroabietic acid (15-HPDA) **7** (18), and 1-(1-hydroperoxy-1-methylethyl) cyclohexene (cyclohexene hydroperoxide) **8** (19) were synthesized as described earlier. Linalool hydroperoxide was prepared according to the literature (14) and obtained as a 5:3 mixture of 7-hydroperoxy-3,7-dimetylocta-1,5-diene-3-ol **6** and 6-hydroperoxy-3,7-dimetylocta-1,7-diene-3-ol **9**. 15-HPA **2** was synthesized from methyl abietate according to the procedure by Haberkorn et al. (20). Cumene hydroperoxide **10** [in mixture with cumene alcohol (4:1)] and olive oil were purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden.

### Patch testing

Patch test material for 15-HPA was stored at –70 centigrade, while patch test materials for limonene hydroperoxide and linalool hydroperoxide were stored at –20 centigrade until used. Based on previous experience (21), we used non-stabilized petrolatum for the hydroperoxide patch test preparations. 15-HPA was tested at 1% pet., limonene hydroperoxide at 0.5% pet., and linalool hydroperoxide at 0.5% pet., as these concentrations were equimolar and in accordance with previous experience (2, 8). Standard patch test material for colophonium 20% pet. was obtained from Chemotechnique Diagnostics AB, Malmö, Sweden. Small Finn Chambers<sup>®</sup> (8 mm; Epitest Ltd Oy, Tuusula, Finland) on Scanpor<sup>®</sup> tape (Norgesplaster A/S, Vennessla, Norway) were used

for the study. Patch test materials were left under occlusion for 48 hr. Readings were performed according to the International Contact Dermatitis Research Group recommendation (22).

Individuals with known positive patch test reactions of 2+ or 3+ to colophonium were selected from 1600 patients with dermatitis, patch tested between 2001 and 2003 at the Department of Dermatology, Sahlgrenska University Hospital, Göteborg. A letter was sent to 44 individuals who met the above-mentioned criteria. The response rate was 40 of 44 (91%) individuals, of whom 31 agreed to contact further. 2 individuals withdrew, and 29 were included in the study. Exclusion criteria were ultraviolet therapy or sunlight to the back within 4 weeks of testing and ongoing medication with cytostatic drugs or oral cortisone. The mean age was 48.6 years. The group consisted of 5 males and 24 females.

In earlier patch testing, 7 of the 29 (24%) individuals had reacted solely to colophonium. In 8 of the 29 (28%) individuals, previous testing had showed 1 other contact allergen, 6 of the 8 cases reacted to nickel. In 4 of the 29 (14%) individuals, 2–3 other contact allergens were known and 8 of 29 (28%) individuals had earlier reacted to 4 or more contact allergens. 7 of the 29 (24%) had reacted to fragrance mix or *Myroxylon perierae* resin (balsam of Peru). Patch testing was performed in 2004. The study was approved by the local ethics committee.

### Sensitization and cross-reactivity studies in guinea-pigs

Female, outbred, albino Dunkin–Hartley guinea-pigs weighing 250–300 g were purchased from AB Sahlins Försöksdjursfarm, Malmö, Sweden. The animals were housed in Macrocolon cages, kept on a guinea-pig standard diet, and fed water ad libitum. The local ethics committee approved the study, which was performed according to the Freund's complete adjuvant test method, with closed challenge testing (23, 24). For induction, the animals received an intradermal injection (0.1 ml) of the test material in Freund's complete adjuvant (FCA)/H<sub>2</sub>O emulsions on the upper back on days 0, 6, and 10. The concentrations used for induction were chosen according to previous experience (12). 3 groups of 15 animals in each were used. The first group (group A) was injected with cumene hydroperoxide 1% (in FCA/H<sub>2</sub>O). The second group (group B) was injected with limonene-2 hydroperoxide 1.5% (in FCA/H<sub>2</sub>O). The third group was a control group, sham-treated with FCA/H<sub>2</sub>O emulsion only. Challenge testing was performed on day 21. All

test materials were applied using olive oil (0.015 g) on the shaved flanks of the animals for 24 hr using Finn Chambers<sup>®</sup>. All groups were challenged with cumene hydroperoxide 1.0%, 0.3%, 0.1%, with limonene-2-hydroperoxide 1.0%, 0.3%, with cyclohexene hydroperoxide 3.0%, and with olive oil as a vehicle control. Group A and the controls were also tested with 15-HPDA 5.0%, while group B was additionally tested with limonene-2-hydroperoxide 0.1%. Visual evaluation of the skin reactions was conducted 48 and 72 hr after application. The minimum criterion for a positive reaction was a confluent erythema. The test concentrations were shown to be non-irritating in pretest on FCA-treated animals.

The results were evaluated statistically using Fisher's exact test to compare the number of reactions in the exposed groups with the number of reactions in the control group. A *P* value of <0.05 was considered statistically significant.

### Quantum calculations

In this study, all calculations were done using the GAUSSIAN 03 (Gaussian, Inc., Pittsburgh, PA, USA) program package (25). The geometry optimization for all stationary points were performed using the 6-31G (d, p) basis set (26) and the density functional theory method, together with the hybrid functional B3LYP (27). 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  $\Delta H$  (298 K) was calculated.

## Results

### Concomitant reactions and cross-reactivity in the clinical study

In the clinical study, 28 of the 29 (96.5%) individuals previously diagnosed with contact allergy to colophonium showed positive patch test reactions

to colophonium at retesting. 13 (46%) of these 28 individuals also showed a positive reaction to 15-HPA. Limonene hydroperoxide and linalool hydroperoxide were tested simultaneously with 15-HPA. 1 individual (1/29) reacted to linalool hydroperoxide and colophonium but not to 15-HPA or to limonene hydroperoxide (Table 1). 2 other individuals (2/29) showed positive reactions to limonene hydroperoxide and colophonium but not to linalool hydroperoxide. 1 of these individuals was also allergic to 15-HPA (Table 1).

### Sensitizing capacity and cross-reactivity pattern in guinea-pigs

The results from the 72-hr reading are shown in Table 2. Cumene hydroperoxide **10** and limonene hydroperoxide **4** resulted in significant sensitization in their respective groups, but no cross-reactivity was observed between the 2 groups (Table 2). Cross-reactions to cyclohexene hydroperoxide **8** were seen in the animals sensitized to cumene hydroperoxide but not in those sensitized to limonene hydroperoxide. No cross-reactions were observed to 15-HPDA **7** (Table 2).

At 48 hr, the only significant response observed was to limonene hydroperoxide 1.0% in the group induced with limonene hydroperoxide as irritation was seen in the controls to all tested compounds. This might be due to the fact that all compounds were tested at the same time to decrease the risk of obtaining sensitization by repeated challenge testing.

### Quantum calculations

The thermodynamic stability of 3 potential radicals **8a–8c** formed from cyclohexene hydroperoxide **8**, where a hydroxy radical (HO<sup>•</sup>) is abstracted after breaking the oxygen–oxygen bond (Fig. 2A), were found to be very similar at approximately 40 kcal/mol. Also, the thermodynamic stability of the oxygen radical **10a** from cumene hydroperoxide **10** requires 40 kcal/mol, but the intramolecular

Table 1. Reaction patterns in 3 individuals who reacted to limonene hydroperoxide or linalool hydroperoxide in the clinical patch test study

| Patient no. | Limonene hydroperoxide |                 | Linalool hydroperoxide |    | 15-HPA |    | Colophonium |    |
|-------------|------------------------|-----------------|------------------------|----|--------|----|-------------|----|
|             | D3 <sup>a</sup>        | D6 <sup>a</sup> | D3                     | D6 | D3     | D6 | D3          | D6 |
| 1           | +                      | –               | –                      | –  | ++     | ++ | ++          | ++ |
| 2           | –                      | –               | +                      | –  | –      | –  | +           | ++ |
| 3           | +                      | +               | –                      | –  | –      | –  | +           | ++ |

15-HPA, 15-hydroperoxyabietic acid.

<sup>a</sup>Day of reading the patch test reaction.

<sup>b</sup>Readings according to the International Contact Dermatitis Research Group (ICDRG) guidelines (22).

Table 2. Sensitizing capacity and cross-reactivity pattern in guinea-pigs according to the Freund's complete adjuvant test method<sup>a</sup>

|                        | Challenge materials  |     |     |                        |     |                 |                           |         |           |
|------------------------|----------------------|-----|-----|------------------------|-----|-----------------|---------------------------|---------|-----------|
|                        | Cumene hydroperoxide |     |     | Limonene hydroperoxide |     |                 | Cyclohexene hydroperoxide | 15-HPDA | Olive oil |
| Test concentration (%) | 1.0                  | 0.3 | 0.1 | 1.0                    | 0.3 | 0.1             | 3.0                       | 5.0     |           |
| Group A <sup>b</sup>   | 13 <sup>c</sup>      | 2   | 1   | 2                      | 1   | NT              | 10 <sup>d</sup>           | 0       | 0         |
| Group B <sup>e</sup>   | 6                    | 1   | 1   | 15 <sup>c</sup>        | 5   | 2               | 7                         | NT      | 0         |
| Controls <sup>f</sup>  | 4                    | 0   | 0   | 4                      | 1   | NT <sup>g</sup> | 3                         | 1       | 0         |

15-HPDA, 15-hydroperoxydehydroabietic acid.

NT, not tested.

<sup>a</sup>The number of positive animals at 72 hr after challenge is given.

<sup>b</sup>In group A, 15 animals were induced with 1% cumene hydroperoxide.

<sup>c</sup> $P < 0.001$  (exposed/controls).

<sup>d</sup> $P < 0.01$  (exposed/controls).

<sup>e</sup>In group B, 15 animals were induced with 1.5% limonene-2-hydroperoxide.

<sup>f</sup>Control group (15 animals) was sham-treated.

<sup>g</sup> $P$  value (exposed/controls) was calculated using the number of reactions to 0.3% limonene hydroperoxide in the controls.

rearrangements of the radical to form **10b** and **10c** require additional energy (Fig. 2B).

### Discussion

No unspecific responses in the allergenic activity were observed for the hydroperoxides investigated. Neither the human data nor the animal study support the hypothesis that hydroperoxides act as oxidizing agents to form non-specific antigens. Specific allergenic activity caused by specific antigens, where the hapten binds to a host protein, was consistently observed and supported by theoretical chemical calculations.

In the clinical study on individuals with known positive patch test reactions to colophonium, no increase of concomitant contact allergy to hydroperoxide allergens was found as only 1 individual reacted to more than 1 hydroperoxide (limonene hydroperoxide and 15-HPA), despite 15 individuals reacting to hydroperoxide haptens in total (Table 1).

In the animal study, no general cross-reactivity could be observed between the hydroperoxides investigated (Table 2). Both cumene hydroperoxide **10** and limonene hydroperoxide **4** were sensitizers in guinea-pigs, but no cross-reactivity was seen between the 2 compounds. However, the animals sensitized to cumene hydroperoxide also reacted to the monocyclic model compound cyclohexene hydroperoxide **8**.

A direct antigen formation due to coupling between the oxygen radical and the proteins in the skin seems to be a major pathway for antigen formation for hydroperoxides. However, hydroperoxides may also undergo intramolecular rearrangements, leading to epoxides that function as electrophilic haptens. Our quantum calculations indicate that the intermediates **8a–c** are thermody-

namically equally stable for allylic tertiary hydroperoxides as seen for the cyclohexene hydroperoxide **8** (Fig. 2A). This indicates that these 3 radicals may appear together and that the 2 epoxides **8d** and **8e** both are potential products that could function as electrophilic haptens. For aromatic tertiary hydroperoxides, e.g. cumene hydroperoxide **10**, the formation energy for the oxygen radical is equal to that of the allylic tertiary hydroperoxide cyclohexene hydroperoxide **8**. However, as the intramolecular rearrangement of **10a** to **10b** and **10c** involves breakage of the aromaticity in **10a**, which requires additional energy, a direct coupling between the oxygen radical **10a** and a protein would be the preferred reaction pathway for antigen formation (Fig. 2B). Thus, we can conclude that the way of antigen formation giving cross-reactions between **8** and **10** has to take place as a direct coupling between the oxygen radical and the skin proteins. This result agrees with the cross-reactivity previously seen between the allylic tertiary hydroperoxide 15-HPA **2** and the aromatic tertiary hydroperoxide 15-HPDA **7** (16). Cross-reactivity was observed to 15-HPA in animals sensitized to 15-HPDA, with no possibilities to form epoxides (16).

The cyclohexene hydroperoxide **8** was found to be a sensitizer in a previous study that investigated the reaction pathways (20). The possibility of this allylic tertiary hydroperoxide to react by way of epoxides due to intramolecular radical rearrangements was investigated by cross-reactivity studies. No reactions to the synthesized epoxides tested in the animals sensitized to **8** were observed (20). However, there is a possibility that also other epoxides, except those tested, are formed. Previous investigations on 15-HPA and epoxides of abietic acid showed cross-reactivity between the tested compounds (16). This indicates that antigen formation by intramolecular radical rearrangements is also possible for allylic tertiary hydroperoxides.

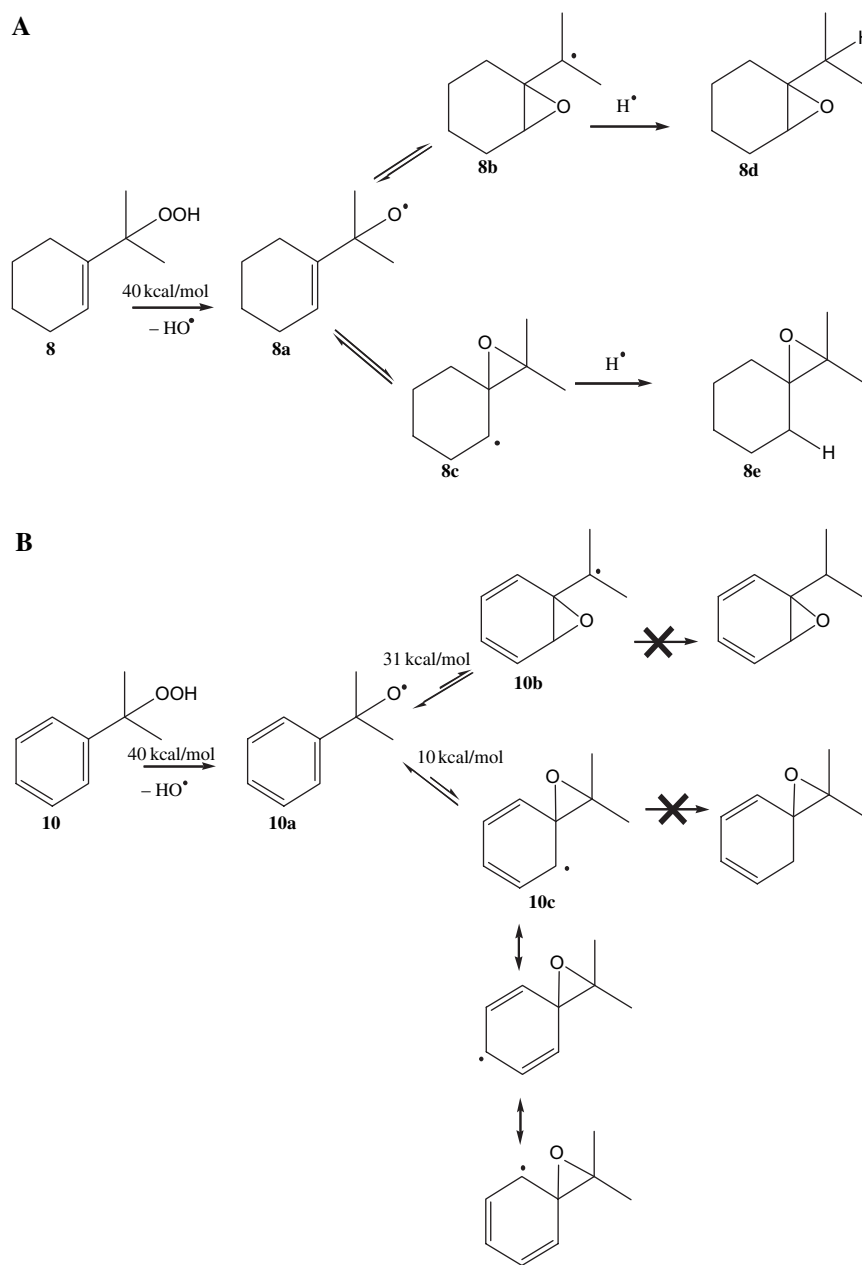


Fig. 2. Reaction pathways for oxygen-centred radicals formed from cyclohexene hydroperoxide **8** and cumene hydroperoxide **10**. **A** Calculated formation energies of the radicals **8a–c**, formed from the homolytic cleavage of the oxygen–oxygen bond in cyclohexene hydroperoxide **8**. **B** Calculated formation energies of the radicals **10a–c**, formed from the homolytic cleavage of the oxygen–oxygen bond in cumene hydroperoxide **10**.

The present results indicate that cumene hydroperoxide and cyclohexene hydroperoxide in analogy with 15-HPA and 15-HPDA are able to form antigens that activate the same T-cell clones due to their similarity in structure. However, the importance of the overall structure must be pointed out. 15-HPDA has 3 fused 6-membered rings, among which the C-ring is aromatic, resembling the structure of cumene hydroperoxide. No cross-reactivity was observed between these 2 molecules, which is interpreted to be due to the difference in the over-

all structure of cumene hydroperoxide and 15-HPDA. Likewise, although similar in size, limonene hydroperoxide and cumene hydroperoxide will not cross-react due to the large difference in overall structure.

The obtained results rule out the possibility of formation of non-specific or somewhat specific antigens. This type of antigen formation could be caused by structural rearrangement of the protein without the hapten binding to the protein, e.g. by oxidizing the macromolecule. In such a case,

a more random cross-reactivity would have been observed and not the 1 that is a result of hapten–protein binding.

We found no evidence for the hypothesis that hydroperoxides reacting by a radical mechanism form non-specific antigens. The designed patch test study gave the clinical evidence; the cross-reactivity study in animals confirmed and broadened the evidence; and the chemical calculations gave theoretical support for the hypothesis that hydroperoxides form specific antigens where the hapten binds to a host protein. The results indicate that T-cell clones specific for the direct hydroperoxide route are present. Kinetics may determine which way dominates for molecules where both this route and the route by intramolecular rearrangement of the radicals are possible. Thus, the concomitant patch test reactions described in the literature are best explained as the result of concomitant sensitization caused by simultaneous exposure to a wide variety of fragrance chemicals and colophonium components in products in everyday life. However, the multiple sensitizations may be based on an individual susceptibility to react to hydroperoxide antigens, e.g. as a result of antioxidant deficiency, which is the subject of ongoing studies.

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