

Mechanism of the antigen formation of carvone and related α , β -unsaturated ketones

ANNA-MALIN NILSSON¹, ELISABETH GÄFVERT¹, LOURDES SALVADOR², KRISTINA LUTHMAN², MAGNUS BRUZE³, BIRGITTA GRUVBERGER³, J. LARS G. NILSSON¹ AND ANN-THERESE KARLBERG^{1,4}

¹Occupational Dermatology, National Institute for Working Life, Stockholm, Sweden

²Department of Organic Pharmaceutical Chemistry, Uppsala Biomedical Centre, Uppsala University, Uppsala, Sweden

³Department of Occupational and Environmental Dermatology, University Hospital MAS, Malmö, Sweden

⁴Department of Medicine, Karolinska Institutet, Stockholm, Sweden

In the present study, the mechanism for the antigen formation of α , β -unsaturated ketones was investigated. A series of analogues of carvone ((5*R*)-5-isopropenyl-2-methyl-2-cyclohexenone) with altered chemical reactivity and with retained overall structure or with retained reactivity and altered three-dimensional structure were synthesized. These analogues were tested for cross-reactivity in carvone-sensitized animals. Cross-reactivity was observed for analogue **3** ((5*R*)-5-isopropyl-2-methyl-2-cyclohexen-1-one). No cross-reactions were observed for analogues **1** ((2*R*,5*R*)-5-isopropenyl-2-methyl cyclohexanone) and **4** ((5*R*)-2,3-dimethyl-5-isopropenyl-2-cyclohexene-1-one). Both those compounds also failed to induce sensitization. These findings demonstrate that α , β -unsaturated ketones form antigens after a nucleophilic attack at the β -carbon with soft nucleophiles such as thiol in cysteine and not with the formation of a Schiff's base after a nucleophilic attack at the carbonyl carbon with nitrogen nucleophiles. Furthermore, no cross-reactivity was observed between *R*- and *S*-carvone indicating the importance of the 3-dimensional structure of haptens (and antigens) in T-cell recognition. The analogues were also tested for cross-reactivity on patients allergic to carvone. The results from the animal study were confirmed.

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In allergic contact dermatitis, environmental exposure to chemicals (haptens) that penetrate the skin barrier is responsible for sensitization and the development of dermatitis. To induce contact allergy the allergenic compound, the hapten, must have certain chemical characteristics, e.g., skin penetration is dependent on the lipophilicity and the size of the hapten molecule. The hapten structure must have at least 1 chemically reactive site to enable an association with skin macromolecules (proteins) to produce the full antigen. Except in the case of allergenic metals, this association is generally accomplished by the formation of covalent bonds. This bond formation with proteins could result either from an electrophilic-nucleophilic reaction or from a radical-mediated reaction (1, 2). Some compounds, prohaptens, must first be activated by metabolic changes or autooxidation (1, 3) before this binding reaction can occur.

There is a considerable need for robust *in vitro*

tests for prediction of the allergenic properties of a chemical before it is introduced on the market, but no such tests exist today. The work on predictive models based on immunological assays has so far failed (4). For primary prevention we still rely on animal testing, but this raises ethical issues that are now of great concern within the European Union. Structure-activity relationship (SAR) studies have gained increased interest as a possible complement to animal studies for prediction of the sensitizing capacity of various chemicals (5). DEREK (Deductive Estimation of Risk from Existing Knowledge) is an expert system that is currently marketed for detection of xenobiotic effects, e.g., the risk that a new molecule might act as a skin sensitizer (6). The system consists of a control program that analyzes the structure of the molecules, and a database of substructures known to be associated with allergenic activity.

For α , β -unsaturated carbonyl compounds, covalent

lent binding to a macromolecule may occur via a Michael addition of a nucleophilic group in amino acids in proteins. This has been demonstrated for α -methylene- γ -butyrolactones (7 and refs. therein). The other possibility for binding of α,β -unsaturated aldehydes and ketones would be via a Schiff's base formation between the carbonyl group and a primary amino group of, e.g., lysine in a protein molecule. This mechanism has been argued to be predominant in the allergen formation from cinnamal (8) although a β -carbon reaction is a possibility for this molecule (9). The corresponding α,β -unsaturated ketones have, to the best of our knowledge, not been investigated in this respect.

The aim of this study was to investigate the mechanism for the antigen formation of α,β -unsaturated ketones using *R*-carvone and related structures as model compounds. *R*-Carvone may react either via a nucleophilic attack at the β -carbon or via a Schiff's base formation. *R*-Carvone was chosen because it is a small stable molecule with known allergenic activity and a structure that allows the synthetic structural changes necessary for SAR studies. A series of analogues of *R*-carvone with altered chemical reactivity in electrophilic-nucleophilic reactions, but with overall structure retained, was synthesized. Cross-reactivity and sensitizing capacity were measured in guinea pig studies. Patch testing was performed in subjects with known contact allergy to *R*-carvone. Also, the enantiospecificity of the antigen was studied by testing the cross-reactivity of *R*- and *S*-carvone.

Materials and Methods

Chemicals

(*R*)-(-)-carvone ((5*R*)-5-isopropenyl-2-methyl-2-cyclohexenone) (98%) [6485-40-1], (*S*)-(+)-carvone ((5*S*)-5-isopropenyl-2-methyl-2-cyclohexenone) (96%) [2244-16-8] and cyclohexenone (930-68-7) were obtained from Sigma-Aldrich, Sweden. Diethyl ether and tetrahydrofuran (THF) were dried by distillation over sodium benzophenone ketyl. Dry toluene was obtained by distillation over CaH_2 . Methanol, *N,N*-dimethylformamide (DMF), and dichloromethane were dried over molecular sieves. Freund's complete adjuvant (FCA), consisting of paraffin oil, mannide monooleate and heat-killed dried *Mycobacterium tuberculosis* and Freund's incomplete adjuvant (FIA), consisting of paraffin oil and mannide monooleate, were purchased from Difco (Detroit, Mich., USA).

Spectroscopic and chromatographic methods

^1H and ^{13}C NMR spectra were obtained using a JEOL JNM-EX270/EX400 spectrometer with

solutions in CDCl_3 (residual CHCl_3 δ 7.26 and CDCl_3 δ 77.0 as internal standards). Chemical shifts and coupling constants were obtained from 1-dimensional spectra and resonances were assigned from ^1H - ^1H and ^1H - ^{13}C correlation experiments. Optical rotations were measured in CHCl_3 at ambient temperature using a Perkin-Elmer 241 polarimeter. Low-resolution electron-impact mass spectral data (70 eV) were obtained on a Hewlett Packard MSD HP5971A connected to a GC HP 5890 series 2, equipped with an HP-1 (25 m $\times$ 0.2 mm i.d.) column. TLC was performed on aluminum sheets precoated with silica gel 60 F254 with detection by UV light and 5% phosphomolybdic acid in ethanol. Column chromatography was performed on silica gel using Kieselgel 60 (230-400 mesh).

Synthesis

(2*R*, 5*R*)-5-isopropenyl-2-methyl cyclohexanone **1** and (2*S*, 5*S*)-5-isopropenyl-2-methyl cyclohexanone **2**. The synthesis was performed as described in literature (10, 11). ^1H NMR (270 MHz) δ 1.02 (d, 3H, J =6.6 Hz, H7), 1.35 (qd, 1H, J =13.0, 3.5 Hz, H6'), 1.54-1.70 (m, 1H, H4'), 1.72 (br s, 3H, H10), 1.87-1.95 (m, 1H, H5), 2.11 (m, 1H, H6''), 2.20-2.46 (m, 4H, H2, H3, and H4''), 4.71 and 4.74 (br s, and t, respectively, 2H, J =1.5 Hz, H9); ^{13}C NMR (67.5 MHz) δ 14.3 (C7), 20.4 (C10), 30.7 (C5), 34.9 (C6), 44.7, 46.8 and 47.0 (C2, C3, and C4), 109.5 (C9), 147.6 (C8), 212.6 (C1); MS 152 (M^+ 23%), 137 (18%), 109 (40%), 95 (62%), 82 (39%), 81 (40%), 67 (100%); **1** $[\alpha]_D^{25} = +15.2^\circ$ (*c* 1.1); **2** $[\alpha]_D^{25} = -17.1^\circ$ (*c* 1.0) $[\alpha]_D^{25} = -16.5^\circ$ (neat).

(5*R*)-5-isopropenyl-2-methyl-2-cyclohexen-1-one **3**.

Batch I: The synthesis was performed as described in the literature (12). The product of batch I contained 6% carvone according to ^1H NMR: ^1H NMR (270 MHz) δ 0.89 (dd, 6H, J =6.8, 1.0 Hz, H9 and H10), 1.54 (sext, 1H, J =6.7 Hz, H8), 1.74 (pent, 3H, J =14.7 Hz, H6'), 2.28-2.40 (m, 1H, H4''), 2.50 (ddd, 1H, J =16.0, 3.6, 1.6 Hz, H6''), 6.72 (ddd, 1H, J =6.1, 2.6, 1.4 Hz, H3); ^{13}C NMR (67.5 MHz) δ 15.6 (C7), 19.4 and 19.5 (C9 and C10), 29.8 (C4), 32.0 (C8), 41.9 (C5), 42.0 (C6), 135.2 (C2), 145.3 (C3), 200.6 (C1); $[\alpha]_D^{25} = -55^\circ$ (*c* 1). The method was modified as follows, giving batch II, which was used in the patient study: *R*-carvone (0.3 ml, 1.9 mmol) and tris(triphenylphosphine)rhodium chloride (50 mg, 0.054 mmol) in dry toluene (5 ml) were hydrogenated for 24 h. Purification gave a colourless oil (0.04 g, 14%) with spectral data consistent with batch I which was not contaminated with carvone according to GC/MS.

(5*R*)-2,3-dimethyl-5-isopropenyl-2-cyclohexene-1-one **4**. The synthesis is based on methods de-

scribed in the literature (13, 14). (*S*)-(+)-Carvone (0.97 g, 6.4 mmol) in dry diethyl ether (4.5 ml) was added to a freshly-prepared ice-cold suspension of methyl magnesium iodide (prepared from Mg (0.372 g, 15.3 mmol) and CH_3I (830 μl , 13.3 mmol) in dry diethyl ether (6.5 ml). After stirring at room temperature for 2.5 h, the mixture was cooled in an ice bath and quenched with phosphate buffer (pH 7, 30 ml, 0.2 M $\text{NaH}_2\text{PO}_4/0.2$ M Na_2HPO_4). After separation of the layers, the milky aqueous layer was acidified with concentrated HCl until clear and extracted with diethyl ether (3 $\times$ 30 ml). The combined ether extracts were washed with brine (20 ml), dried (Na_2SO_4), and concentrated to furnish the alcohol **5** as a slightly yellow oil (1.02 g, ^1H NMR δ 5.40 (m, 1H, H3), 4.74 (s, 2H, H9), 1.74 and 1.33 (2s, 6H and 3H, respectively, 3 CH_3 ,s) which was used as such in the oxidation step. The crude alcohol **5** (0.70 g) dissolved in dry dichloromethane (6 ml) was added to a stirred suspension of pyridinium chlorochromate (1.366 g, 6.34 mmol) and silica gel (1.4 g, 230–400 mesh) in dichloromethane (4 ml) at room temperature under nitrogen. After 2.5 h, the reaction mixture was applied to a silica gel column and eluted with dichloromethane. Concentration of the eluent gave a yellow oil that was subjected to flash column chromatography (pentane:ethyl acetate 20:1) which rendered the product as a colorless oil (0.43 g, 60%): ^1H NMR (270 MHz) δ 1.72 (s, 3H, H10), 1.73 (m, 3H, H7), 1.93 (d, 3H, $J=0.8$ Hz, C3- CH_3), 2.20–2.36 and 2.48–2.67 (m, 3H and 2H, respectively, H4, H5, and H6), 4.72 and 4.77 (s and t, respectively, 2H, $J=1.3$ Hz, H9); ^{13}C NMR (67.5 MHz) δ 10.7 (C7), 20.4 (C10), 21.5 (C3- CH_3), 37.8, 41.2, and 42.4 (C4, C5, and C6), 110.2 (C9), 130.6 (C2), 146.7 (C8), 154.2 (C3), 198.9 (C1); MS 164 ($M+18\%$), 149 (5%), 122 (50%), 107 (100%), 96 (78%), 91 (23%), 79 (21%), 77 (20%), 67 (64%); $[\alpha]_D = -119^\circ$ (c 1.2). These spectral data are consistent with structure **4**.

Chemical reactivity of *R*-carvone

Reactivity towards a sulfur nucleophile. The reaction was performed as described in the literature (15) producing **6** in 79% yield.

Reactivity towards a nitrogen nucleophile. *R*-Carvone (0.25 g, 1.66 mmol) was dissolved in methanol (8 ml) in a round-bottom flask kept under nitrogen. Benzylamine (0.36 ml, 3.3 mmol) was added with stirring at room temperature. TLC examination showed that the reaction was slow. After 3 days the solvent was evaporated under vacuum and the excess benzylamine was removed by flash chromatography on silica (95:5 CHCl_3 :MeOH). The product was extracted with 3 M HCl (4 $\times$ 10

ml). The aqueous layer was treated with 5 M NaOH to pH >9 and was thereafter extracted with ether (5 $\times$ 10 ml). The organic phase was washed with water, dried (Na_2SO_4) and concentrated under vacuum to give **7** as a brown oil (0.33 g, 83%, contaminated with 17% carvone according to GC/MS): ^1H NMR (400 MHz) δ 1.7 (s, 3H, H7), 1.88 (s, 3H, H10), 2.0–2.5 (m, 4H, H4, H5 and H6ax), 2.9 (ddd, 1H, $J=16, 4, 2$ Hz, H6eq), 4.65 (app d, 2H, H12), 4.73–4.78 (m, 2H, H9), 6.35 (m, 1H, H3) and 7.15–7.35 (m, 5H, Ph); MS 239 ($M+32\%$), 224 (12%), 148 (17%), 91 (100%), 77 (5%). These spectral data are consistent with structure **7**.

Allergenic activity and cross-reactivity in guinea pigs

The experiments were approved by the ethics committee. Female, outbred, albino Dunkin Hartley guinea pigs weighing 250–300 g were purchased from Sahlins AB Försöksdjursfarm, Malmö, Sweden and Bio Jet service, Uppsala, Sweden. The animals were housed in Macrolon cages, kept on a guinea pig standard diet and water *ad lib*. In all studies, the concentrations of *R*- and *S*-carvone were chosen according to earlier experience (16) and the concentrations of the synthesized analogues were chosen to be equimolar to those of carvone.

Cross-reactivity studies on *R*-carvone, *S*-carvone, **1, **2**, **3** and cyclohexenone.** Guinea pigs were sensitized according to Freund's complete adjuvant test (FCAT) (17). Challenge testing was performed under occlusion according to a procedure described earlier (18). On days 0, 6 and 10 the animals received 1 intradermal injection (0.1 ml) on the upper back for induction. 3 groups of 15 animals in each were used. The 1st group was injected with *R*-carvone (5% w/w, 3.3×10^{-4} mol/g) in Freund's complete adjuvant FCA/ H_2O emulsion (1:1). The 2nd group was injected with *S*-carvone (5% w/w) in FCA/ H_2O emulsion. The 3rd group was a control group sham-treated with an FCA/ H_2O emulsion. Closed challenge testing (*i.e.* under occlusion) was performed on day 21. The test material (0.015 g) was applied on the shaved flanks of the animals for 24 h using aluminum test chambers (Finn Chambers®, *i.e.* d. 8 mm, Epitest Ltd Oy, Tuusula, Finland). The reactions were assessed at 48 h and 72 h after application. The minimum criterion for a positive reaction was a confluent erythema. All animals were challenged with *R*-carvone (1%, 6.7×10^{-5} mol/g), *S*-carvone (1% and 0.2%, 1.3×10^{-5} mol/g), **1** (1% and 0.2%), **2** (1% and 0.2%) in olive oil and a vehicle control. Pretests on FCA-treated guinea pigs showed that the compounds did not act as irritants at the concen-

trations used. *Rechallenge 1*: On day 48 the animals sensitized to *R*-carvone were retested with *R*-carvone (0.2% and 0.1%, 6.7×10^{-6} mol/g) and **3** (Batch I, 1%) in olive oil and a vehicle control. *Rechallenge 2*: On day 64 the animals sensitized to *R*-carvone were boosted with an injection of *R*-carvone (5% w/w) in FCA/ H₂O -emulsion and 12 new control animals received an FCA/ H₂O -emulsion. 2 weeks later the animals were tested with *R*-carvone (0.5%, 3.3×10^{-5} mol/g), cyclohexenone (1.28%) in olive oil and a vehicle control. The 0.5% concentration of *R*-carvone was chosen since 0.2% used in the 1st rechallenge did not induce a significant response at the 72-h reading.

Cross-reactivity study on R-carvone and 4. Guinea pigs were sensitized according to Freund's complete adjuvant test (FCAT) as described above. However, at the 2nd and 3rd induction injections the animals received the test compounds in a Freund's incomplete adjuvant FIA/H₂O emulsion as opposed to the 1st injection with FCA/H₂O emulsion. The modification was performed according to recommendations from the ethics committee. 2 groups of 15 animals each were used. One group was induced with *R*-carvone (5%) and the other group was a sham-treated control group. The animals were challenged with *R*-carvone (5% and 1%) in petrolatum, **4** (5.5% and 1.1%) in petrolatum and a vehicle control. The concentrations had been shown to be non-irritating in pretests on FCA-treated guinea pigs.

Sensitizing capacity of 1 and 4. 2 experiments were performed according to the FCAT/FIAT method as described above. In the 1st experiment ($n=15$ exposed and 15 controls) the exposed group was injected with **1** (5.1%) while in the 2nd experiment ($n=15$ exposed and 15 controls) the exposed group was injected with **4** (5.5%). The animals in the 1st experiment were challenged with **1** (5.1% and 1.0%) in petrolatum, and in the 2nd experiment with **4** (5.5% and 1.1%) in petrolatum. Challenge testing with *R*-carvone (5%) in petrolatum and a vehicle control was performed in both experiments.

Studies on cross-reactivity in humans with known contact allergy to R-carvone. The study was approved by the ethics committee. 6 females (age 57–76 years), who earlier had shown a positive patch test response to *R*-carvone, were patch tested with *R*-carvone, *S*-carvone and analogues **1**, **3** (Batch II) and **4**. *R*-Carvone was applied in concentrations of 1%–5% and the other test compounds were applied in equimolar concentrations. The vehicle used was petrolatum. The test material was applied on the upper back using small Finn Chambers and Scanpor. The patches were removed 48 h after application and a 1st scoring of reac-

tions was performed 3–4 days after the application. A 2nd scoring of reactions was performed 6–7 days after application. The reactions were scored according to the International Contact Dermatitis Research Group (ICDRG) recommendations (19).

Statistics

The results from the animal experiments were evaluated using the Fisher exact test and the McNeman test.

Lipophilicity determinations

The log P values for carvone, **1**, **2**, **3** and **4** were calculated using the computer program ACD Log P DB from Advanced Chemistry Development. The predictive program is based on experimental log P values for over 3600 structures with 500 different structural fragments.

Results

Synthesis of carvone analogues 1–4

4 carvone analogues were synthesized in this study (Fig. 1). The analogues were designed to have altered chemical reactivity in electrophilic-nucleophilic reactions; in **1** and **2** a Michael reaction is prevented by saturation of the conjugated double bond, and in **4** the 3-position is sterically hindered by an extra methyl group. The synthetic pathways are described in Fig. 2. Compounds **1** and **2** were obtained by a selective reduction of the conjugated double bond in *R*- or *S*-carvone, respectively, using tri-sec-butylborohydride at -78°C as reducing agent. Compound **3** was obtained

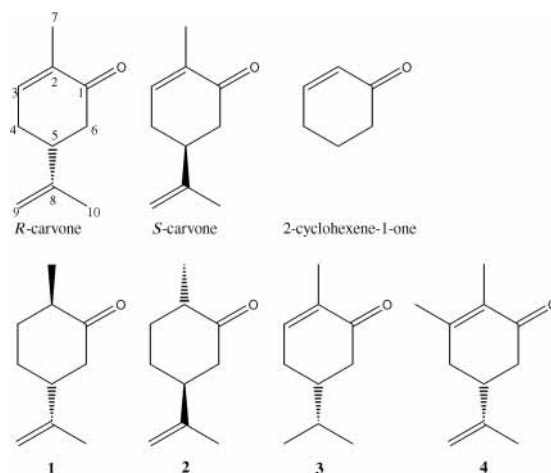


Fig. 1. Chemical structures of the compounds included in the study.

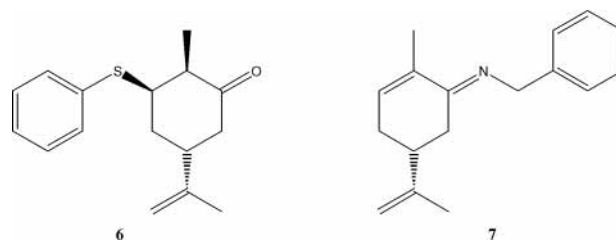
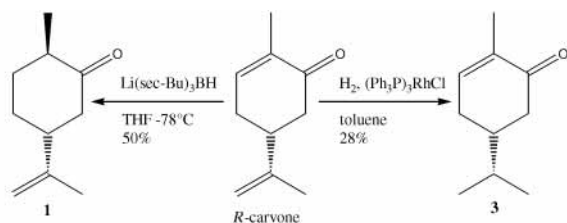
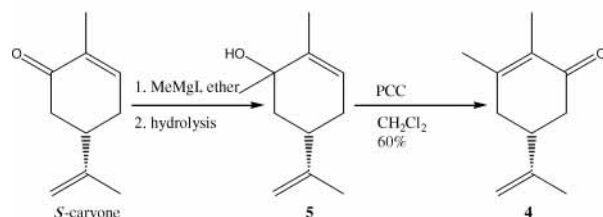
Fig. 3. Chemical structures of product **6** and **7**.

Fig. 2. Synthetic pathways.

by selective hydrogenation of the double bond in the isopropenyl group of carvone by use of a sterically demanding rhodium catalyst. The methyl group on C-3 in **4** was introduced via methylation of *S*-carvone with CH_3MgI . An allylic tertiary alcohol intermediate (**5**) was formed which was subsequently oxidized to **4** using pyridinium chlorochromate.

Chemical reactivity of *R*-carvone towards sulfur and nitrogen nucleophiles

The reactivity of carvone towards sulfur and nitrogen nucleophiles was studied using thiophenol and benzylamine, respectively, as models of amino acids in proteins. The only product obtained in re-

actions with thiophenol was a Michael addition product (Fig. 3) according to ^1H NMR spectroscopy. Sulfur nucleophiles are expected to participate in Michael additions since they are considered as soft nucleophiles. The reactive site of carvone towards this sulfur nucleophile was confirmed to be the β -position. The only product formed in the reaction of carvone with benzylamine was a Schiff's base (Fig. 3) according to ^1H NMR and GC/MS. The harder nitrogen nucleophiles are expected to react at the carbonyl group to a greater extent. The reactive site of carvone towards this nitrogen nucleophile was also found to be the carbonyl group.

Cross-reactivity studies on *R*-carvone, *S*-carvone, **1**, **2**, **3** and cyclohexenone

2 animal groups were sensitized to *R*- and *S*-carvone respectively. No cross-reactivity was observed between the 2 compounds (Table 1). Neither could any cross-reactivity to analogues **1** and **2** be demonstrated.

The results from the 1st rechallenge are shown

Table 1. Study of cross-reactivity; results from challenge testing with *R*- and *S*-carvone, **1** and **2** in carvone-sensitized animals

Challenge material (% w/w)		Group 1 ^a (n=15)	Group 2 ^b (n=15)	Controls (n=15)
<i>R</i> -carvone (1)	48 h	13 (<0.001) ^c	0	0
	72 h	15 (<0.001)	1	0
<i>S</i> -carvone (1)	48 h	0	11 (<0.001)	0
	72 h	1	13 (<0.001)	0
<i>S</i> -carvone (0.2)	48 h	0	6 (<0.01)	0
	72 h	2	7 (<0.005)	0
compound 1 (1)	48 h	0	0	0
	72 h	0	0	0
compound 1 (0.2)	48 h	0	0	0
	72 h	0	0	0
compound 2 (1)	48 h	0	0	0
	72 h	1	0	0
compound 2 (0.2)	48 h	0	0	0
	72 h	1	0	1
olive oil	48 h	1	0	0
(vehicle control)	72 h	1	0	2

^a) Induced with *R*-carvone 5% in FCA/H₂O emulsion intradermally.

^b) Induced with *S*-carvone 5% in FCA/H₂O emulsion intradermally.

^c) *p*-value (exposed/controls).

in Table 2. A significant response was observed to *R*-carvone at the 48 h reading and cross-reactivity was observed to **3**. This test compound contained 6% carvone, *i.e.*, 0.06% in the test preparation. This amount is below the 0.1% test preparation of carvone that gave a non-significant response com-

pared to the controls at the 48 and 72 h readings. Thus, the observed reactions to the test preparation of **3** strongly indicate cross-reactivity.

The results from the 2nd rechallenge are presented in Table 3. A significant response was observed to *R*-carvone. No cross-reactivity was observed for cyclohexenone.

Table 2. Study of cross-reactivity; results from 1st rechallenge in *R*-carvone-sensitized animals, tested with *R*-carvone and **3**

Challenge material (% w/w)		Group 1 ^a (n=15)	Controls (n=15)
<i>R</i> -carvone (0.2)	48 h	6 (<0.01) ^b	0
	72 h	3	0
<i>R</i> -carvone (0.1)	48 h	2 ^c	0
	72 h	1	0
compound 3 (1)	48 h	6 (<0.01)	0
	72 h	5 (<0.05)	0
olive oil (vehicle control)	48 h	0	0
	72 h	0	0

^a) Induced with *R*-carvone 5% in FCA/H₂O emulsion intradermally.

^b) *P*-value (exposed/controls) (Fisher exact test)

^c) statistical calculations of the response obtained when testing with *R*-carvone compared to the response to **3**: 48 h 2 versus 6 *P*>0.20; 72h 1 versus 5 *P*>0.10 (McNeman test).

Table 3. Study of cross-reactivity; results from 2nd rechallenge in *R*-carvone-sensitized animals tested with *R*-carvone and cyclohexenone

Challenge material (% w/w)		Group 1 ^a (n=15)	Controls ^b (n=12)
<i>R</i> -carvone (0.5)	48 h	8 (<0.01) ^c	0
	72 h	10 (<0.001)	0
cyclohexenone (1.28)	48 h	0	0
	72 h	0	0
olive oil (vehicle control)	48 h	0	0
	72 h	0	0

^a) Induced with *R*-carvone 5% in FCA/H₂O emulsion intradermally.

^b) New controls

^c) *P*-value (exposed/controls).

Table 4. Study of cross-reactivity; results from challenge testing with *R*-carvone and **4** in *R*-carvone-sensitized animals

Challenge material (% w/w)		Exposed ^a (n=15)	Controls (n=15)
<i>R</i> -carvone (5)	48 h	11 (<0.001) ^b	0
	72 h	9 (<0.001)	0
<i>R</i> -carvone (1)	48 h	7 (<0.01)	0
	72 h	8 (<0.005)	0
compound 4 (5.5)	48 h	0	1
	72 h	0	1
compound 4 (1.1)	48 h	0	0
	72 h	0	0
petrolatum (vehicle control)	48 h	0	0
	72 h	0	2

^a) Induced with *R*-carvone 5% in FCA(FIA)/H₂O emulsion intradermally.

^b) *P*-value (exposed/controls).

Cross-reactivity study on *R*-carvone and **4**

The cross-reactivity of **4** was studied in animals induced with *R*-carvone. A significant response was observed to *R*-carvone, but no cross-reactivity to **4** could be observed (Table 4).

Sensitizing capacity of analogues **1** and **4**

Compounds **1** and **4** were found to be devoid of sensitizing capacity. Only 1 of the 15 exposed animals in each respective experiment showed a positive response to the highest test concentration of **1** and **4**. The lower concentrations of **1** and **4**, carvone and the vehicle gave no reactions.

Studies on cross-reactivity in humans with known contact allergy to *R*-carvone

All subjects reacted to *R*-carvone (Table 5). Cross-reactions were found to analogue **3** (Batch II) in 4 subjects, whereas no reactions were found to analogues **1** and **4** (Table 5). This is in accordance with the results from the animal studies. 3 subjects reacted to *S*-carvone.

Lipophilicity determinations

The log *P* values were calculated giving the following results: carvone (2.23), **1** and **2** (2.43), **3** (2.41) and **4** (2.82).

Discussion

The present study demonstrates that α,β -unsaturated ketones form antigens after a nucleophilic attack at the β -carbon with soft nucleophiles such as thiol in cysteine. This mechanism corresponds to the reaction of allergenic α,β -methylene lactones that form allergens after an attack at the β -carbon by a sulfur nucleophile from a cystein residue of a protein molecule (7 and refs therein). Hydrogenation of the α,β -double bond as well as introduction of a β -methyl group as a steric hindrance resulted in inactive compounds. Since both the saturated ketone **1** and the methyl-substituted compound **4** were inactive in sensitization studies, we conclude that Schiff's base formation is not a mechanism in the allergen formation. This was

Table 5. Study of cross-reactivity in humans allergic to *R*-carvone; results from challenge testing with *R*- and *S*-carvone, **1**, **3**, and **4**^a

Challenge material		Subjects					
		a	b	c	d	e ^c	f ^c
<i>R</i> -carvone	5.0% in petrolatum	++ ^b	++	++	+++	++	++
	3.0	+	++	+	+++	++	++
	2.0	++	++	+	+++	++	+
	1.0	+	++	—	++	++	—
<i>S</i> -carvone	5.0	—	+	—	++	++	—
	3.0	—	+	—	++	+	—
	2.0	—	+	—	++	—	—
	1.0	—	+	—	+	—	—
compound 1	5.1	—	—	—	—	—	—
	1.0	—	—	—	—	—	—
compound 3	5.1	—	++	—	++	—	+
	1.0	—	++	—	+	—	—
compound 4	5.5	—	—	—	—	—	—
	1.1	—	—	—	—	—	—

^a) A 2nd reading confirmed the results.

^b) Scores according to the ICDRG standard (19), —=no visible reaction, +=palpable erythema (edema), ++=erythema and infiltration, papules and +++=vesicular, bullous reaction.

^c) Tested 2×. On the 1st test occasion only *R*-carvone and *S*-carvone were tested, while **1**, **3** and **4** were tested on a 2nd test occasion.

further substantiated using benzylamine as a model for the nitrogen nucleophile. As model compounds, we have used a series of analogues of *R*-carvone. *R*-Carvone is a potent contact allergen with a simple structure (16, 20, 21) that can be modified to obtain compounds with various desired properties. Studying the sensitizing capacity of carvone in particular, we also investigated the influence of the isopropenyl group and the enantiomeric form on the allergenic activity.

In the development of predictive models or systems such as the DEREK data base (6), it is important to use carefully designed structure-activity experiments with pure chemicals and appropriate animal models. The compounds have to be chosen in such a way that they can give the possibility of answering the questions posed. The allergenic potential and chemical reactivity of structural analogues can be observed in guinea pig studies and correlated to differences in chemical structure and to physicochemical properties (22, 23). Other structure-activity investigations use the local lymph node assay (LLNA) in mice (24–26). This method provides an objective endpoint, thus permitting quantitative comparison of the sensitizing capacity of different molecules. Since the LLNA only expresses the induction phase and does not allow any cross-reactivity studies. Therefore, this method was not suitable for our present investigation.

In the present study a guinea pig assay was used. In some of the animal experiments, FIA was used instead of FCA as adjuvant at 2nd and 3rd induction, due to considerations of the ethics committee on animal experiments. FIA seemed to give some-

what lower reactivity than that to the traditional method. However, these experiments are too few to draw general conclusions and this was not the objective of the study.

The difference in chemical reactivity of carvone towards hard and soft nucleophiles was investigated. The Michael addition product from the reaction of carvone with thiophenol (a cysteine mimic) was formed rapidly, whereas the reaction with benzylamine (a lysine mimic) resulted in the formation of a Schiff's base only after a reaction time of several days. These results were useful in the interpretation of the results from the cross-reactivity studies.

Cross-reactivity with carvone was not observed from **1** where the 2,3 double bond is saturated or from **4** where C-3 is substituted with a methyl group. Both these compounds are still ketones but the reactivity towards nucleophilic groups in proteins is greatly changed compared to carvone; in **1** the reactive site at C-3 is absent and in **4** the reactive site is sterically hindered and thus less available for reaction with nucleophiles. Since compounds **1** and **4** did not cross-react with carvone we wanted to investigate their sensitizing capacity, to see if they could bind to proteins and form antigens via some other mechanism. Both compounds failed to induce an allergic reaction in the animals. These findings support the suggestion that C-3 is the chemically reactive site in the antigen formation of carvone. Theoretically, the carbonyl groups in **1** and **4** could bind to a macromolecule, e.g., via an amine group of lysine residues, to form Schiff's bases as shown in our model studies.

However, since **1** and **4** had no sensitizing ca-

capacity and since the chemical reaction between a primary amine and carvone was demonstrated to be considerably slower than the Michael reaction with a sulfhydryl group, it seems that Schiff's base formation is not involved in the antigen formation with these compounds. Furthermore, there is a possibility that **4** can form an antigen via the β -carbon in spite of the methyl group. Such an antigen may not cross-react with the antigen from *R*-carvone itself. Since we found no sensitization in the guinea pigs, we conclude that a methyl group results in a steric hindrance of the β -carbon that is large enough to prevent a nucleophilic attack from proteins in the skin. Thus, the β -carbon of α,β -unsaturated ketones must be unsubstituted if the ketone should function as a hapten.

Cross-reactivity between *R*-carvone and **3** was expected since both molecules contain the same reactive site (the α,β -unsaturated ketone) that is likely to be involved in the nucleophilic reaction with macromolecules forming the antigens. In addition, both molecules have the *R* configuration at C-5. The only difference is the substituent at C-5, which is an isopropyl group in **3** but an isopropenyl group in carvone. Thus, the double bond in the C-5 substituent is of minor importance for T-cell recognition.

We wanted to investigate further the importance of the isopropenyl group on the allergenic activity, to obtain an indication of how much the hapten structure can be changed and still be recognized by the T-cell. Therefore, we challenged the *R*-carvone-sensitized animals with cyclohexenone, which contains the same α,β -unsaturated system as carvone but lacks the C-5 isopropenyl substituent and the C-2 methyl group. No cross-reactions to cyclohexenone were observed, which indicates that the isopropenyl group (or less likely the C-2 methyl group) is necessary for hapten recognition, although it is not directly involved in the antigen formation. The steric bulk seems to be of importance in antigen-T-cell interactions.

Carvone also contains a chiral center at C-5. Our experiments showed both *R*- and *S*-carvone to be strong allergens, but no cross-reactions were observed. This demonstrates that antigen formation is not only dependent on the reactive site but also on the 3-dimensional structure of the molecule. Enantiospecificity in contact allergy has previously been demonstrated, e.g., for (+)- and (-)-frullanolides and also for some β -substituted α -methylene- γ -butyrolactones (27–29). Different hypotheses have been put forward to explain why enantiospecificity is observed for some haptens and not for others. One hypothesis is that enantiospecificity is seen only if the molecule contains more than 1 chiral center (30). Our findings do not

support this theory, since *R*- and *S*-carvone do not cross-react (Table 1), even though there is only 1 chiral center in the molecules. Another suggestion is that enantiospecificity occurs when a polar group (e.g. a hydroxyl group) is attached to the asymmetric center (31). Such a polar group could interact with the protein by hydrogen bonding, thus resulting in different antigenic determinants in the 2 enantiomers. In carvone the substituent at the asymmetric carbon atom is an isopropenyl group, and any interaction between this group and the carrier protein would be via weak van der Waals forces. The most likely explanation is that the enantiospecificity of carvone is due to a space-filling phenomenon. Carvone is a small molecule and the distance between the reactive site and the asymmetric carbon is short. These circumstances are probably also of importance in the recognition of the 2 enantiomers.

Not only the chemical reactivity but also the skin permeability of a molecule is of importance in contact allergy. Since the skin permeability is related to the lipophilicity of a molecule, the log *P*-values (octanol/water partition coefficient) of carvone and the synthesized analogues were calculated. The molecules showed only small differences in their partition coefficients, indicating that the skin penetration capacity is similar for these compounds and the small differences do not explain the differences in allergenic activity. We therefore conclude that the different potentials of skin sensitization are due to differences in chemical reactivity of the compounds towards skin proteins.

6 human subjects with known contact allergy to *R*-carvone were recruited among those tested with a dental screening series due to oral mucosal reactions or due to periorbital dermatitis. Carvone is present in the majority of toothpastes as a constituent of spearmint oil, which is the most commonly used flavouring agent in these products. 4 of the subjects also reacted to spearmint oil in the dental test. The subjects were retested with *R*-carvone and the analogues and also with *S*-carvone. The same pattern of cross-reactivity to the analogues as we observed in the animal studies was also seen in humans. It should be emphasized that batch II of **3** did not contain any residual carvone. 3 of the subjects reacted to both *R*- and *S*-carvone. These could be considered as simultaneous reactions, since the subjects could be sensitized to both *R*- and *S*-carvone. The latter is present in caraway oil used as a flavouring in toothpaste. *S*-carvone is also present in dill seed oil and ginger grass oil that are used in fragrances.

For primary prevention, it is important to investigate all possible routes of antigen formation from a molecule. Carvone and its analogues could the-

oretically form covalent bonds to the macromolecules via the β - carbon or via a Schiff's base. We found that only the former mechanism was of importance. A dual mechanism of antigen formation has, however, been observed for 15-hydroperoxyabietic acid, a hapten reacting via the radical mechanism (2).

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Address:

*Ann-Therese Karlberg
Occupational Dermatology
National Institute for Working Life
SE-112 79 Stockholm
Sweden*