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Skin Sensitization of Epoxyaldehydes: Importance of Conjugation

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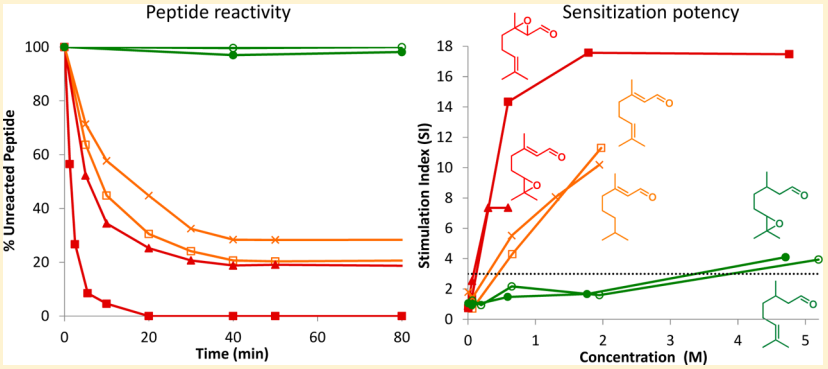
Supporting Information



ABSTRACT: Structure−activity relationship (SAR) models are important tools for predicting the skin sensitization potential of new compounds without animal testing. In compounds possessing a structural alert (aldehyde) and an activation alert (double bond), it is important to consider bioactivation/autoxidation (e.g., epoxidation). In the present study, we have explored a series of aldehydes with regard to contact allergy. The chemical reactivity of these 6 aldehydes toward a model hexapeptide was investigated, and their skin sensitization potencies were evaluated using the local lymph node assay (LLNA). Overall, we observed a similar trend for the *in vitro* reactivity and the *in vivo* sensitization potency for the structural analogues in this study. The highly reactive conjugated aldehydes (α,β-unsaturated aldehydes and 2,3-epoxyaldehydes) are sensitizing moieties, while nonconjugated aldehydes and nonterminal aliphatic epoxides show low reactivity and low sensitization potency. Our data show the importance of not only double bond conjugation to aldehyde but also epoxide−aldehyde conjugation. The observations indicate that the formation of nonconjugated epoxides by bioactivation or autoxidation is not sufficient to significantly increase the sensitization potency of weakly sensitizing parent compounds.

INTRODUCTION

Allergic contact dermatitis (ACD) is a common occupational and environmental health problem caused by chemicals (haptens) in contact with the skin. Once an individual has become sensitized, the contact allergy remains throughout life since no curative therapy is known. To prevent from ACD, exposure to the allergenic compound must be avoided. Thus, it is important to identify skin sensitizing chemicals and evaluate their sensitization potential in order make proper risk assessments before a chemical is introduced on the market. Currently, the *in vivo* murine local lymph node assay (LLNA) is the method of choice to determine the skin sensitization potential.[1] Because of ethical considerations, a number of alternative assays have been developed in recent years including *in vitro* methods, chemical reactivity, and *in silico* computational (quantitative) structure−activity relationship [(Q)SAR] methods.[2−6]

The first SAR study correlating chemical reactivity and skin sensitization was reported by Landsteiner and Jacobs in 1936.[7] They postulated that small organic molecules can become sensitizing entities only after binding to a skin protein. As haptens themselves are too small to induce an immune response, they must form an immunogenic complex by reacting with macromolecules (proteins) in the skin. The details of this process are still unknown, even though extensive research has been carried out. A plausible mechanism for organic compounds acting as haptens is the formation of covalent bonds between electrophilic haptens and nucleophilic amino acid side chain moieties in skin proteins.[8] Reactive amino acid residues suggested to play a role in skin sensitization include cysteine (thiol), lysine (amino), and to a lesser extent arginine, histidine, methionine, and tyrosine.[9] Following this, it has been shown that the skin sensitization potential of a chemical in many cases can be predicted by its ability to react with peptides.[10,11] The relationship between molecular structure and

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reactivity that forms the basis for structural alerts is based on well-established principles of mechanistic organic chemistry. However, some compounds need to be activated via different mechanisms, e.g., outside the skin via autoxidation (prehaptens) or in the skin via bioactivation (prohaptens), to be able to react with skin proteins forming immunogenic complexes.[2] Some chemicals could be activated via both routes, e.g., compounds containing double bonds that are susceptible to oxidation by autoxidation or by bioactivation.[12−18]

No truly predictive QSAR model for skin sensitization potential can be established without access to carefully evaluated experimental data from well-designed structure−activity experiments. Only a limited number of SAR/QSAR studies of structurally closely related compounds have been carried out, for example, with halogenated aromatics,[19] benzaldehydes,[20] dienes,[12] oximes,[21] aldehydes,[22] and epoxides.[23−26] To get a complete picture, it is necessary to consider different mechanisms of activation of pro- and prehaptens and possibilities for cross-reactivity. Epoxidation is described as an important activation route, which can occur either metabolically in the skin or by autoxidation outside the skin. A double bond is the activation alert moiety in both cases, but the mechanism is quite different. The metabolic oxidation occurs via an enzymatically catalyzed oxidation of the double bond.[27,28] In autoxidation, the epoxide is secondary to the formation of the primary oxidation products, the hydroperoxides, which are formed on allylic carbons due to a radical mechanism with hydrogen atom abstraction.[2]

The sensitization potency of epoxides can vary significantly due to minor changes in chemical structure.[12,23−25] It is therefore important to investigate whether a possible epoxidation will increase the sensitization potency of the compound in question. In previous studies,[12,23] conjugated dienes in or in conjunction with a six-membered ring have been shown to be prohaptens which can be metabolically activated to epoxides. Related alkenes containing isolated double bounds or an acyclic conjugated diene show weak sensitizing effects. The difference in sensitization potencies was found to be due to the high reactivity and sensitization potency of the allylic epoxides. A possible activation due to autoxidation was investigated for one of the conjugated dienes, α-terpinene.[15] It was found that the same allylic epoxides seen after bioactivation were formed also in this process. Other conjugated epoxides formed after air exposure are those of abietic acid; they were shown to be strong skin sensitizers in guinea pigs.[15,29] The diglycidyl ether of bisphenol A and the three isomers of the diglycidiyl ether of bisphenol F have also been identified as strong allergens.[30] These compounds are terminal aliphatic diepoxides connected in the β-positions to phenoxy groups. Studies on simplified model analogues have shown the importance of the presence of a heteroatom, the length of the chain between the heteroatom and the epoxide moiety, and the presence of an aromatic ring system for the reactivity and sensitization potency of the epoxides.[24,25] Aldehydes have been classified into different groups according to the reactivity at the carbonyl group and the presence of related functionalities,[22,31] e.g., α,β-unsaturated aldehydes are known to act as electrophilic haptens in Michael additions with proteins.[22] Recently, Natsch et al. investigated benzaldehydes,[20] the general SAR identified 2-hydroxybenzaldehydes, as the most reactive derivatives forming stable Schiff bases.

The aim of the present study was to investigate whether the positioning of the epoxide relative to the aldehyde affects the chemical reactivity and the sensitization potency. One model compound is an α,β-unsaturated aldehyde with a moderate sensitization potency (**1**). Another model is an aliphatic aldehyde with a nonconjugated double bond (**5**). We have also investigated the corresponding aldehydes with epoxides in the positions of the double bonds (**2**, **3**, and **6**). By doing this, we were able to predict if epoxidation of a specific double bond affected the sensitization potency of the studied aldehyde. The reactivity was assessed in a model using the series of aldehydes **1**−**6** (Figure 1) and a previously used peptide.[32] Data from

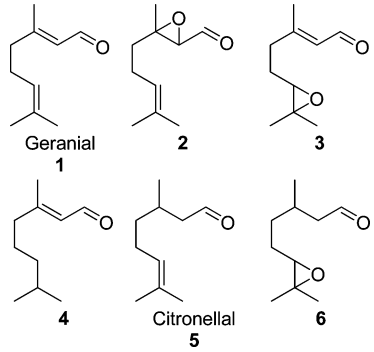

Figure 1. Structures of compounds **1** to **6**. The epoxidation and reduction of double bonds in **1** were studied. Compounds **2** and **3** contain epoxide moieties in the 2,3- and 6,7-positions, respectively. In **4** and **5**, the double bonds are reduced in the 6,7- and 2,3-positions, respectively. In compound **6**, a combination of reduction of the double bond in the 2,3-position and an epoxidation in the 6,7-position is introduced.

both the depletion of the unreacted peptide and the formed adducts were analyzed. The confirmation of the adduct structures was obtained via the reaction with *N*-acetyl-L-cysteine methyl ester (*N*-ACME) and ethanethiol. The sensitization potencies of compounds **1**−**6** was screened using the murine LLNA.[1]

EXPERIMENTAL PROCEDURES

Caution: *This study involves skin sensitizing compounds which must be handled with care.*

Instrumentation and Mode of Analysis. ^{1}H and ^{13}C NMR spectroscopy was performed on a Jeol Eclipse 400 spectrometer at 400 and 100 MHz, respectively, using $CDCl_3$ or DMSO-d_6 solutions, (residual undeuterated solvent: chloroform ^{1}H δ 7.25, ^{13}C δ 77.2 and dimethyl sulfoxide ^{1}H δ 2.50, ^{13}C δ 39.5 as internal standards). ^{1}H and ^{13}C NMR spectra were assigned using ^{13}C distortionless enhancement by polarization transfer, ^{1}H−^{1}H correlation spectroscopy, ^{1}H−^{13}C heteronuclear multiple quantum coherence, and ^{1}H−^{13}C heteronuclear multiple bond correlation experiments. The following abbreviations, or a combination thereof, were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad.

Electron-ionization mass spectral analysis (70 eV) was performed on a Hewlett-Packard 5973 mass spectrometer connected to a gas chromatograph (Hewlett-Packard 6890). The GC was equipped with a cool on-column capillary inlet and an HP-5MSi fused silica capillary column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies, Palo Alto, CA, USA). Helium was used as carrier gas, and the flow rate was 1.2 mL/min. The temperature program started at 35 °C for 1 min, increased by 10 °C/min, and ended at 250 °C for 5 min. For mass spectral analysis, the mass spectrometer was used in the scan mode detecting ions with m/z values ranging from 50 to 1500.

LC/MS analyses were performed using electrospray ionization (ESI) on a Hewlett-Packard 1100 HPLC/MS. The system included a

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vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector, and a single quadrupole mass spectrometer. The HPLC was equipped with a HyPURITY C18 column (150 × 3 mm i.d., particle size 3 μm, Thermo Hypersil-Keystone, Thermo Electron Corp., Bellafonte, PA, USA). The mobile phase consisted of 0.005% pentafluoropropanoic acid, 0.1% acetic acid, and 5% acetonitrile in water (solvent A) and 0.005% pentafluoropropanoic acid, 0.1% acetic acid, and 50% water in acetonitrile (solvent B). A linear gradient from 0% to 100% B in 20 min, followed by 10 min of isocratic elution at 100% B was used. The column was equilibrated with 0% B for 10 min between each run. Aliquots of 2 μL were injected onto the column and eluted with a flow rate of 0.40 mL/min, and the column temperature was set to 40 °C. The ESI interface was used in positive ionization mode with the following spray chamber settings: nebulizer pressure, 40 psig; capillary voltage, 3500 V; drying gas temperature, 350 °C; and drying gas flow rate, 10 L/min. The fragmentor voltage was set to 120 V. The mass spectrometer was used in scan mode detecting molecular ions with m/z values ranging from 50 to 2000.

Chemistry. Geraniol was purchased from Aldrich Chemicals (Stockholm, Sweden). Geranial (*E*-3,7-dimethyl-2,6-octadienal) and 6,7-dihydrogeraniol (*E*-3,7-dimethyl-oct-2-en-1-ol) were obtained from Bedoukian Research, Inc. (USA). (±)-Citronellal ((±)-3,7-dimethyl-oct-6-enal) was purchased from Alfa Aesar GmbH (Karlsruhe, Germany). Ac-Pro-His-Cys-Lys-Arg-Met-OH (98%) was purchased from Peptide 2.0 Inc. (Chantilly, Virginia, USA). Acetone p.a. was purchased from Merck (Darmstadt, Germany) and olive oil from Apoteket AB (Göteborg, Sweden). Water was purified with a Purelab ultra (Genetic) system from Elga Labwater (High Wycombe, UK). Unless otherwise indicated, reagents were obtained from commercial suppliers and used without further purification. Column chromatography was performed using Merck silica gel Geduran Si 60 (0.063−0.200 mm). TLC was performed using silica gel coated aluminum plates (Merck, 60 F254) and developed with anisaldehyde dip (2.1 mL of acetic acid, 5.1 mL of anisaldehyde, and 7 mL of H_2SO_4 in 186 mL of ethanol) followed by heating. Short path distillation was performed using a Buchi GKR-50 Kugelrohr distiller. The purity of both synthesized and purchased test compounds was >98% (GC/MS) before the testing of sensitization potential. Compounds **2**,[33] **3**,[14,34] and **12**[35] were synthesized as described in the literature.

(*E*)-3,7-Dimethyl-oct-2-enal (6,7-Dihydrogeranial, 4). A solution of 6,7-dihydrogeraniol (2.0 g, 13 mmol) in dichloromethane (10 mL) was added dropwise to a stirred solution of Dess-Martin periodinane (6.5 g, 15 mmol) in dichloromethane (40 mL). The reaction was followed by TLC. After 2 h, the reaction mixture was diluted with diethyl ether (100 mL), poured into saturated aqueous $NaHCO_3$ (100 mL) containing $Na_2S_2O_3$ (20 g), and stirred for 20 min. Diethyl ether (100 mL) was added to the reaction mixture, and the layers were separated. The organic layer was washed with a saturated aqueous $NaHCO_3$ (100 mL) and water (50 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure to a yellow oil. The crude product was purified by column chromatography (pentane/ethyl acetate 99:1) to give **4** as a colorless oil (1.5 g, 75%). ^{1}H and ^{13}C NMR data were in agreement with those in the literature.[36,37] ESI-MS (70 eV), m/z (%) 154 (1) (M^+), 139 (9), 111 (12), 97 (35), 84 (100), 69 (27).

6,7-Epoxy-3,7-dimethyl-octanal (Epoxycitronellal, 6). The synthesis was performed as described in the literature, starting from citronellal (**5**) (1.2 g, 7.7 mmol).[38] Compound **6** was obtained as a diastereomeric mixture (0.88 g, 67%) in a 1:1 ratio. ^{1}H NMR ($CDCl_3$) (mixture of diastereomers) δ 0.97 (d, 3H, J = 6.6 Hz), 1.24/1.25 (s, 3H), 1.29 (s, 3H), 1.40−1.47 (m, 2H), 1.48−1.58 (m, 2H), 2.07−2.15 (m, 1H), 2.25−2.29 (m, 1H), 2.37−2.45 (m, 1H), 2.68 (t, 1H, J = 6.2 Hz), 9.75 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 18.8/18.9, 19.9/20.0, 25.0, 26.5/26.6, 28.1, 33.7, 51.0/51.1, 58.4/58.5, 64.3/64.4, 202.6/202.7. ESI-MS (70 eV), m/z (%) 170 (3) (M^+), 152 (39), 137 (23), 123 (64), 109 (100), 95 (31), 81 (87), 69 (60).

General Procedure for the Preparation of *N*-ACME Conjugates. A mixture of hapten (0.5 mmol) and *N*-ACME (0.5 mmol) in ammonium acetate buffer (2.5 mL, 100 mM, pH 7.4) and DMSO (2.5 mL) was stirred at 37 °C for 4 h. Diethyl ether (10 mL) was added to the reaction mixture, and the layers were separated. The organic layer was washed with water (3 × 5 mL) and brine (5 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by column chromatography (hexane/ethyl acetate 1:1 to 0:1).

***N*-ACME Conjugate of 1 (7).** Geranial (**1**) was reacted with *N*-ACME according to the general procedure. The reaction yielded **7** as a diastereomeric mixture (96 mg, 58%) in a 1:1 ratio. ^{1}H NMR (DMSO-d_6) (mixture of diastereomers) δ 1.34/1.35 (s, 3H, H10), 1.56 (t, 2H, J = 8.4 Hz, H4), 1.57 (s, 3H, H8 or H9), 1.64 (s, 3H, H8 or H9), 1.84 (s, 3H, $COCH_3$), 1.99−2.07 (m, 2H, H5), 2.56−2.57 (m, 2H, H2), 2.75−2.88 (m, 2H, SCH_2), 3.64 (s, 3H, $COOCH_3$), 4.38−4.41 (m, 1H, SCH_2CH), 5.06−5.13 (m, 1H, H6), 8.39 (d, 1H, J = 8.1 Hz, N*H*), 9.70−9.72 (m, 1H, H1). ^{13}C NMR (DMSO-d_6) δ 18.0 (C8 or C9), 22.8 ($COCH_3$), 23.0 (C5), 26.0 (C8 or C9), 26.2/26.3 (C10), 29.1 (SCH_2), 40.6 (C4), 47.0 (C3), 52.2/52.3 (C2), 52.7 ($COOCH_3$), 52.8 (SCH_2CH), 124.3 (C6), 131.8 (C7), 169.9 ($COCH_3$), 171.6 ($COOCH_3$), 202.7 (C1). LC-MS (API-ES, 120 V), m/z (%) [M+Na] 352 (30), [M+H] 330 (100).

***N*-ACME Conjugate of 4 (8).** Dihydrogeranial **4** was reacted with *N*-ACME according to the general procedure. The reaction yielded **8** as a diastereomeric mixture (112 mg, 68%) in a ratio of 1:1. ^{1}H NMR (DMSO-d_6) (mixture of diastereomers) δ 0.85 (d, 6H, J = 6.7 Hz, H8 and H9), 1.10−1.18 (m, 2H, H5), 1.29−1.38 (m, 2H, H6), 1.33/1.34 (s, 3H, H10), 1.49−1.57 (m, 3H, H4 and H7), 1.84 (s, 3H, $COCH_3$), 2.54 (d, 2H, J = 2.8 Hz, H2), 2.73−2.83 (m, 2H, SCH_2), 3.68 (s, 3H, OCH_3), 4.39 (q, 1H, J = 7.5 Hz, SCH_2CH), 8.39 (d, 1H, J = 7.5 Hz, N*H*), 9.70−9.72 (m, 1H, H1). ^{13}C NMR (DMSO-d_6) δ 21.8 (C6), 22.8 ($COCH_3$), 23.0 (C8 or C9), 23.1 (C8 or C9), 26.3/26.4 (C10), 27.9 (C7) 29.1 (SCH_2), 39.2 (C5), 40.8 (C4), 47.2 (C3), 52.3 (C2), 52.7 ($COOCH_3$), 52.8 (SCH_2CH), 169.9 ($COCH_3$), 171.6 ($COOCH_3$), 202.8 (C1). LC-MS (API-ES, 120 V), m/z (%) [M+Na] 354 (26), [M+H] 332 (100).

General Procedure for the Preparation of Ethanethiol Conjugates. A mixture of hapten (0.25 mmol) and ethanethiol (0.5 mmol) in ammonium acetate buffer (2.5 mL, 100 mM, pH 7.4) was stirred at 37 °C for 24 h. Diethyl ether (5 mL) was added to the reaction mixture, and the aqueous layer was extracted with diethyl ether (3 × 5 mL). The combined organic layer was washed with water (3 × 5 mL) and brine (5 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by column chromatography (hexane/ethyl acetate 95:5 to 80:20).

Ethanethiol Conjugate of 2 (9). 2,3-Epoxygeranial **2** was reacted with ethanethiol according to the general procedure. The reaction yielded a mixture of diastereomers **9a** and **9b** (38.2 mg, 66%) in a ratio of 3:1.

9a: ^{1}H NMR ($CDCl_3$) δ 1.23 (t, 3H, J = 7.3 Hz, SCH_2CH_3), 1.34 (s, 3H, H10), 1.56−1.68 (m, 2H, H4), 1.60 (s, 3H, H8 or H9), 1.66 (s, 3H, H8 or H9), 2.04−2.12 (m, 2H, H5), 2.29 (s br, 1H, OH), 2.42−2.53 (m, 2H, SCH_2), 3.05 (d, 1H, J = 5.5 Hz, H2), 5.05−5.10 (m, 1H, H6), 9.43 (d, 1H, J = 5.5 Hz, H1). ^{13}C NMR ($CDCl_3$) δ 14.7 (SCH_2CH_3), 17.9 (C8 or C9), 22.2 (C5), 24.8 (C10), 25.8 (C8 or C9), 25.9 (SCH_2), 40.7 (C4), 63.5 (C2), 73.7 (C3), 123.6 (C6), 132.8 (C7), 194.0 (C1). ESI-MS (70 eV), m/z (%) 230 (1) (M^+), 212 (52), 183 (12), 169 (6), 155 (10), 144 (56), 115 (75), 82 (53), 69 (100).

9b: ^{1}H NMR ($CDCl_3$) δ 1.23 (t, 3H, J = 7.3 Hz, SCH_2CH_3), 1.28 (s, 3H, H10), 1.56−1.68 (m, 2H, H4), 1.60 (s, 3H, H8 or H9), 1.66 (s, 3H, H8 or H9), 2.04−2.12 (m, 2H,H5), 2.33 (s br, 1H, OH), 2.42−2.53 (m, 2H, SCH_2), 3.09 (d, 1H, J = 5.5 Hz, H2), 5.05−5.10 (m, 1H, H6), 9.39 (d, 1H, J = 5.5 Hz, H1). ^{13}C NMR ($CDCl_3$) δ 14.7 (SCH_2CH_3), 17.9 (C8 or C9), 22.3 (C5), 24.7 (C10), 25.5 (C8 or C9), 25.8 (SCH_2), 40.5 (C4), 64.0 (C2), 72.7 (C3), 123.7 (C6), 132.6 (C7), 193.7 (C1). ESI-MS (70 eV), m/z (%) 230 (1) (M^+), 212 (52), 183 (12), 169 (6), 155 (10), 144 (56), 115 (75), 82 (53), 69 (100).

Ethanethiol Conjugates of 3 (10 and 11). Epoxygeranial **3** was reacted with ethanethiol according to the general procedure. The reaction yielded monoconjugate **10** (35.5 mg, 62%) and biconjugate **11** (20.0 mg, 27%). Monoconjugate **10** was obtained as a mixture of two diastereomers **10a** and **10b** in a 2:1 ratio.

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10a: ^{1}H NMR ($CDCl_3$) δ 1.16 (s, 3H, H8 or H9), 1.24 (t, 3H, J = 7.3 Hz, SCH_2CH_3), 1.28 (s, 3H, H8 or H9), 1.87−1.92 (m, 2H, H5), 2.18 (s, 3H, H10), 2.34−2.39 (m, 1H, H6), 2.52−2.75 (m, 4H, H4 and SCH_2), 5.89 (d, 1H, J = 7.7 Hz, H2), 9.98 (d, 1H, J = 7.7 Hz, H1). ^{13}C NMR ($CDCl_3$) δ 15.5 (SCH_2CH_3), 17.9 (C10), 25.9 (C8 or C9), 27.1 (C8 or C9), 29.2 (C5), 29.9 (SCH_2), 39.3 (C4), 60.8 (C6), 73.0 (C7), 127.6 (C2), 163.6 (C3), 191.3 (C1). ESI-MS (70 eV), m/z (%) 230 (1) (M^+), 215 (4), 187 (1), 172 (6), 154 (2), 125 (3), 88 (100).

10b: ^{1}H NMR ($CDCl_3$) δ 1.16 (s, 3H, H8 or H9), 1.24 (t, 3H, J = 7.3 Hz, SCH_2CH_3), 1.28 (s, 3H, H8 or H9), 1.36−1.46 (m, 2H, H5), 1.98 (s, 3H, H10), 2.30−2.33 (m, 1H, H6), 2.52−2.75 (m, 4H, H4 and SCH_2), 5.87 (d, 1H, J = 7.7 Hz, H2), 10.01 (d, 1H, J = 7.7 Hz, H1). ^{13}C NMR ($CDCl_3$) δ 15.4 (SCH_2CH_3), 25.1 (C10), 26.1 (C8 or C9), 27.1 (C8 or C9), 29.3 (C5), 31.4 (SCH_2), 31.6 (C4), 60.9 (C6), 73.1 (C7), 128.9 (C2), 163.9 (C3), 191.1 (C1). ESI-MS (70 eV), m/z (%) 230 (1) (M^+), 215 (4), 187 (1), 172 (6), 154 (2), 125 (3), 88 (100).

Biconjugate **11** was obtained as a mixture of two diastereomers in a 1:1 ratio. ^{1}H NMR ($CDCl_3$) (mixture of diastereomers) δ 1.16 (s, 3H, H8 or H9), 1.21 (t, 3H, J = 7.5 Hz, SCH_2CH_{3b}), 1.24 (t, 3H, J = 7.5 Hz, SCH_2CH_{3a}), 1.28 (s, 3H, H8 or H9), 1.35−1.42 (m, 2H, H5α), 1.39 (s, 3H, H10), 1.53−1.65 (m, 1H, H4α), 1.82−1.95 (m, 1H, H5β), 2.10−2.20 (m, 4H, H4β), 2.31 (dd, 1H, J = 2.1 and 11.2 Hz, H6), 2.45−2.64 (m, 6H, H2, SCH_{2a}, SCH_{2b}), 9.49/9.86 (t, 1H, J = 2.9 Hz, H1). ^{13}C NMR ($CDCl_3$) δ 14.1/14.2 ($SCH_2C_bH_3$), 15.4 ($SCH_2C_aH_3$), 21.6 (SC_bH_2), 25.7/25.8 (C10), 26.5 (C8 or C9), 26.9/27.0 (C5), 27.0/27.1 (C8 or C9), 29.0 (SC_aH_2), 39.7/39.8 (C4), 46.0 (C3), 52.9/53.0 (C2), 61.5/61.6 (C6), 72.9/73.0 (C7), 201.5/201.6 (C1). ESI-MS (70 eV), m/z (%) 292 (0.8) (M^+), 277 (0.7), 263 (1), 234 (16), 205 (57), 173 (27), 115 (35), 88 (100).

Reactions of Derivatives 1−6 with the Model Peptide Ac-Pro-His-Cys-Lys-Arg-Met-OH (AcPHCKRM). All solvents were degassed with argon prior to use. Solutions of **1** in dimethyl sulfoxide (DMSO) (40 mM, 100 μL) together with potassium phosphate buffer (100 mM, pH 7.4) (200 μL) were added to a vial purged with argon containing AcPHCKRM in DMSO (4 mM, 100 μL). Accordingly, final concentrations of **1** and the model peptide in the reaction mixture were 10 mM and 1 mM, respectively. The reaction was kept under argon at room temperature and was monitored with HPLC/$UV_{210\ nm}$/ESI/MS every 40 min for 24 h. As the HPLC run time was 40 min, to be able to calculate rate constants, samples were collected from five different sets of reactions (the first set was analyzed at 0 and 40 min, the second at 5 min, the third at 10 and 50 min, the fourth at 20 min, and the fifth at 30 min). Derivatives **2**−**6** were treated in a manner identical to that of **1**, and their reactions were investigated using the method described above. Because of the fast reaction rate, **2** was monitored at 0, 1.25, 2.5, 5.5, and10 min (with five different sets of reactions).

Experimental Animals. Female CBA/Ca mice, 8 or 9 weeks of age, were purchased from B&K Sollentuna. The mice were housed in "hepa" filtered air flow cages and kept on standard laboratory diet and water ad libitum. The local ethics committee in Gothenburg approved the study.

Sensitization Potential of 2 and 4−6 in Mice. The LLNA[1] was used to assess the sensitization potential. Mice in six groups of three animals each were treated by topical application on the dorsum of both ears with the test compound (25 μL) dissolved in acetone/olive oil (AOO) (4:1 v/v) or with the vehicle control. All solutions were freshly prepared for every application. Each compound was tested in five different concentrations. Treatments were performed daily for three consecutive days (day 0, 1, and 2). Sham treated control animals received the vehicle alone. On day 5, all mice were injected intravenously via the tail vein with [methyl-^{3}H]thymidine (2.0 Ci/mmol, Amersham Biosciences, UK) (20 μCi) in PBS (containing 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4) (250 μL). After 5 h, the mice were sacrificed, the draining lymph nodes were excised and pooled for each group, and single cell suspensions of lymph-node cells in PBS were prepared using cell strainers (Falcon, BD labware, 70 μm pore size). Cell suspensions were washed twice with PBS, precipitated with TCA (5%), and left in the refrigerator overnight. The samples were then centrifuged, resuspended in TCA (5%) (1 mL), and transferred to scintillation cocktail (10 mL) (EcoLume, INC Radiochemicals, USA). The [methyl-^{3}H]thymidine incorporation into DNA was measured by β-scintillation counting on Beckman LS 6000TA Instruments. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), i.e., test group/control group ratio. Test materials that at one or more concentrations caused an SI greater than 3 were considered to be positive in the LLNA. EC3 values (the estimated concentration required to induce an SI of 3) were calculated by linear interpolation.

RESULTS AND DISCUSSION

Design of Studied Derivatives. To explore whether the positioning of the epoxides relative to the aldehyde affects the chemical reactivity and the sensitization potency, we designed a series of six aldehydes (**1**−**6**, Figure 1). Similarity within the designed series regarding chemical structure, molecular weight, and hydrophobicity was considered important in order to, as accurately as possible, allow comparison of allergenic potency in relation to structure and avoid variations in skin penetration capacities. The moderate skin sensitizer geranial **1** was used as a model compound, and the epoxidation of each of the double bonds in **1** was studied to mimic possible activation, while the reduction of the double bonds was investigated to explore what happens when such activation is no longer possible. Compounds **1**, **3**, and **4** are α,β-unsaturated aldehydes; in the 6,7-position, **1** has a double bond, **3** has an epoxide, and **4** a reduced double bond. Derivative **2** is a 2,3-epoxyaldehyde with an unsaturation in the 6,7-position. For the nonconjugated aldehydes (**5** and **6**), **5** has a 6,7-double bond, while **6** has an epoxide moiety in this position.

Synthesis. Different oxidation pathways were employed to produce compounds **2**−**4** and **6** (for an illustration of the synthetic strategy, see Scheme S1 in Supporting Information). A two-step procedure was used to obtain 2,3-epoxygeranial **2** from geraniol. In the first step, a Sharpless epoxidation of the allylic alcohol was performed to afford 2,3-epoxygeraniol **12** in a yield of 87%.[35] The corresponding aldehyde **2** could thereafter be obtained in good yield (89%) by oxidation of the alcohol using an optimized Doering procedure.[33] Aldehyde **4** was prepared in 74% yield according to a literature procedure using 6,7-dihydrogeraniol as starting material and Dess-Martin periodinane as oxidative agent.[39] The epoxyaldehydes **3** and **6** were synthesized via a classical *meta*-chloroperbenzoic acid (*m*CPBA) epoxidation in 90% and 67% yield, respectively.

Reactivity of 1−6 toward the Model Peptide Ac-Pro-His-Cys-Lys-Arg-Met-OH (AcPHCKRM). To investigate the chemical reactivity of compounds **1**−**6**, their reactions with the nucleophilic hexapeptide AcPHCKRM were analyzed at pH 7.4 with a 10-fold excess of test chemical in a mixture of phosphate buffer/DMSO. A similar peptide, with no acetyl protection on the *N*-terminal position, has previously been used to assess the reactivity of potential contact allergens.[23−25,40−42] The N-acetylation was performed to exclude reactions with proline as previously described.[24,25,42]

The stability of the peptide AcPHCKRM, under the experimental conditions used, was verified before the start of the reactivity experiments. DMSO is well known to be able to activate the dimerization of peptides containing cysteine; however, in the performed experiments no dimerization or oxidation of the peptide was detectable after 24 h. The stability of **1**−**6** was explored to exclude any risk of hydrolysis of the epoxides, but no degradation was observed for any of the

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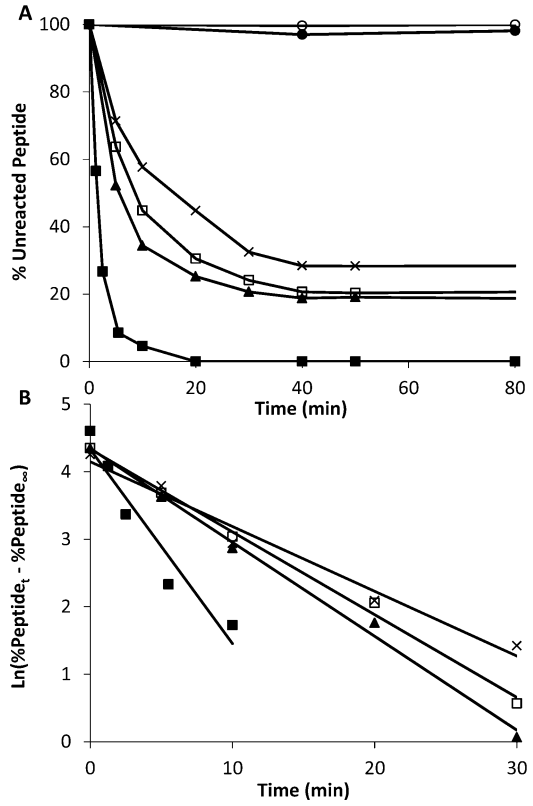

Figure 2. (A) Depletion (consumption rate) of the model peptide AcPHCKRM obtained with **1** (□), **2** (■), **3** (▲), **4** (×), **5** (○), and **6** (●), normalized to 100%. (B) Determination of rate constants for the depletion of the unreacted peptide AcPHCKRM. Plot of ln(% unreacted peptide$_t$ − % unreacted peptide$_\infty$) vs time obtained with **1** (□), **2** (■), **3** (▲), **4** (×), and linear regressions. Reactivity experiments were performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1) with a 10-fold excess of the hapten. The reactions were kept under argon at room temperature and monitored with HPLC/UV/ESI/MS.

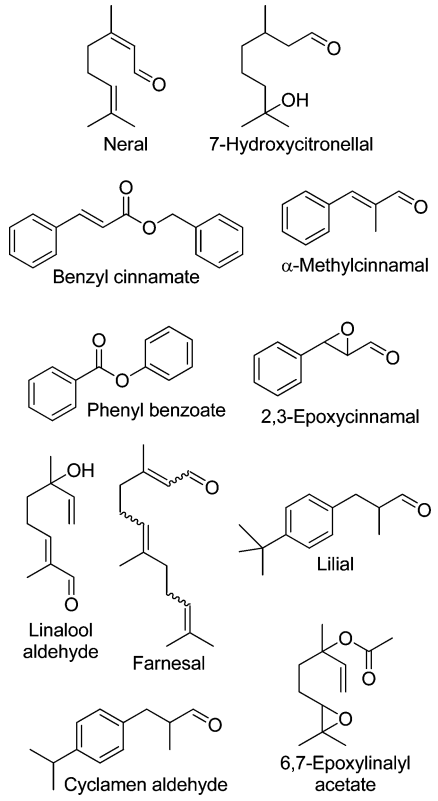

Figure 3. Compounds with related structure elements from the literature.

derivatives within 24 h under the conditions of the reactivity experiment.

The percentage peptide depletion was calculated at different time points over a period of 24 h for **1**−**6** (Figure 2A). Of the six compounds, only **2** gave complete peptide depletion, which occurred already after 20 min. In the reactions with **1**, **3**, and **4**, the unreacted peptide was depleted to 21%, 19%, and 28%, respectively, after 40 min. Thereafter, only minimal depletion was seen in the reaction mixtures with these compounds. The peptide depletion for the α,β-unsaturated aldehydes **1**, **3**, and **4** is similar to data found in the literature for citral (isomeric mixture of geranial **1** and neral (Figure 3)) with a cysteine peptide.[10] We observed no depletion of the unreacted peptide with **5** or **6** after 80 min, but later some depletion mainly due to dimerization was observed (see below). Thus, in our experimental conditions, the nonconjugated aldehyde moiety in **5** and **6** was not reactive enough to cause observable peptide depletion. The low reactivity could be partially explained by the presence of the hydrated form of the aldehyde as described by Merckel et al.[43] It has been described in the literature that the structural analogue 7-hydroxycitronellal (Figure 3) can cause depletion of peptides containing cysteine or lysine.[10] In that experimental setup, cysteine depletion was caused by peptide dimerization, whereas in the assay with the lysine peptide, the test conditions were selected to favor Schiff base formation by applying a 100-fold excess of the test chemical and keeping a high pH in the solution (pH 10).[10] Recently, Natsch et al. have shown that 7-hydroxycitronellal reacted quantitatively with a lysine peptide under *in vivo* conditions to form a stable amide adduct. Also observed was a deaminated lysine peptide, as an aldehyde function had been introduced via hydrolysis of an isomerized imine intermediate.[20] No such products were observed under our experimental conditions. The presence of the 6,7-epoxide moiety in **6** did not cause a significant depletion of free peptide compared to that in **5**.

Interestingly, in all reactions except that of **2**, a peptide dimer was observed in the reactivity experiments as indicated with the appearance of ions *m*/*z* 1624.5, 812.3, and 542.2. These ions correspond to $[M_{\text{peptide dimer}}+H]^+$, $[M_{\text{peptide dimer}}+2H]^{2+}$, and $[M_{\text{peptide dimer}}+3H]^{3+}$, respectively. In reactions with **1**, **3**, and **4**, the dimer appeared after 80 min, with **6** after 120 min, but only after 280 min with **5**. The ability of aldehydes to catalyze dimer formation of an unreacted peptide containing cysteine has previously been described.[44,45] Using our analytical settings, the retention times of the dimer and the unreacted peptide were similar; therefore, no ratio could be calculated, but the unreacted peptide remained after 24 h in experiments with **5** and **6**. Dimer formation could not explain the lack of reactivity of the nonconjugated aldehydes **5** and **6**. However, it could be an explanation for the noncompletion observed for the reaction with the α,β-unsaturated aldehydes **1**, **3**, and **4**.

Linear regression analysis was performed for the conjugated aldehydes **1**−**4** from data plotted as shown in Figure 2B. The rate constants of the pseudo-first-order reaction, which are proportional to the slopes, are given in Table 1. A difference in rate for the depletion was observed between the faster 2,3-epoxyaldehyde **2** (k_{obs} 0.288·min^{-1}) and the slower reacting

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Table 1. Reactivity of 1−4 toward the Model Peptide AcPHCKRM Expressed as Rate Contants[a]

	results from pseudo-first-order plot of ln (% $Pept_t$ − % $Pept_\infty$) vs time[b]	
compd	k_{obs} (min^{-1})	R^2
1	0.123	0.995
2	0.288[c]	0.930[c]
3	0.139	0.995
4	0.096	0.976

[a]The reactivity experiments were all performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1) with a 10-fold excess of the hapten. The reactions were kept under argon at room temperature and monitored with HPLC/UV/ESI/MS. [b]Aliquots were analyzed at 5, 10, 20, 30, and 40 min. [c]Aliquots were analyzed at 1.25, 2.5, 5.5, and 10 min.

α,β-unsaturated aldehydes **1**, **3**, and **4** (k_{obs} 0.096−0.139 min^{-1}). It can be concluded that the 2,3-epoxyaldehyde moiety in **2** is more reactive toward the peptide than the α,β-unsaturated aldehyde moiety in **1**, **3**, and **4**.

Cysteine adducts (Figure 4) were rapidly formed in reactivity experiments of the peptide and **1−4** as indicated by the appearance of $[M^* + H]^+$ and $[M^* + 2H]^{2+}$ ions (* denotes one haptenation) in the mass spectra (Table 2). In addition, signals corresponding to y^*_5, y^*_4, y_3, y_2, b_2, and b_1 fragments were observed (see Figure S1 in the Supporting Information for fragment assignments), showing that the cysteine residue of the peptide had been modified. The results are in good agreement with the well known high reactivity of cysteine.[46] A cysteine adduct was also detected after 200 min in the reactions between epoxycitronellal **6** and the peptide. The presence of small amounts of adduct in the absence of significant depletion of unreacted peptide might be due to a reversibility in adduct formation. This phenomenon has been reported in the literature.[44,45] For example, in the case of benzyl cinnamate and α-methylcinnamal (Figure 3), no significant peptide depletion was observed, while low levels of adduct were identified, or in the case of phenyl benzoate (Figure 3), a tyrosine-containing peptide was not depleted, although an acylated adduct was detected.[44,45]

As no adduct was observed in the reactivity experiment with citronellal (**5**), the conjugate formed from **6** and the peptide was possibly due to a ring-opening of the epoxide on C6 by the cysteine (Figure 4E). In the reactivity experiments with **1** and **4**, only one cysteine adduct was detected, probably corresponding to the Michael addition of peptide to the α,β-unsaturated aldehyde (Figures 4A,D and 5A,D). For the two epoxyaldehydes **2** and **3**, more complicated patterns were observed (Figures 4B,C and 5B,C); four and two adducts were identified, respectively.

In the reactivity experiment with **2**, we observed two cysteine adducts ($CA1_2$ and $CA2_2$) after only 1.25 min as revealed by the presence of y^*_5, y^*_4, y_3, y_2, b_2, and b_1 fragments in the mass spectra (Figure 5B and Table 2). $CA1_2$ contained the base ions m/z 981.3 and 491.3 corresponding to $[(M_{peptide} + M_2) + H]^+$ and $[(M_{peptide} + M_2) + 2H]^{2+}$, respectively. $CA1_2$ could be the result of the epoxide ring-opening via a nucleophilic substitution at C2 with the cysteine residue (Figure 4B). The ions observed for $CA2_2$ were m/z 963.3 and 482.1 with the interpretation of $[(M_{peptide} + M_2 - 18) + H]^+$ and $[(M_{peptide} + M_2 - 18) + 2H]^{2+}$. We hypothesize that $CA1_2$ loses water to form the α,β-unsaturated aldehyde $CA2_2$ (Figure 4B). After 2.5 min, a third cysteine adduct ($CA3_2$) was present in the reaction mixture (identified by the presence of y^*_5, y^*_4, y_3, y_2, b_2, and b_1 fragments (Table 2)). $CA3_2$ gives the ions m/z 1005.5 and 503.1 for $[(M_{peptide} + M_2 + 24) + H]^+$ and $[(M_{peptide} + M_2 + 24) + 2H]^{2+}$ or $[(M(CA2_2) + 42) + H]^+$ and $[(M(CA2_2) + 42) + 2H]^{2+}$. The polarity of the cysteine adducts decreased from $CA1_2$ > $CA2_2$ > $CA3_2$ according to the retention time on the HPLC column.

After 80 min, $CA1_2$, $CA2_2$, $CA3_2$, and a diadduct were present in the reaction mixture as indicated by the appearance of the ions m/z 1135.5 and 566.3, $[M^{**} + H]^+$ and $[M^{**} + 2H]^{2+}$ ions (** denotes two haptenations), in the mass spectrum (Table 3). On the basis of y^{**}_5, y_2, b^*_3, b_2, and b_1 fragments, the new product was assigned to be a cysteine and lysine diadduct. This diadduct could result from a Schiff base formation between the lysine residue of $CA2_2$ and a second equivalent of 2,3-epoxyaldehyde **2** followed by hydrolysis of the

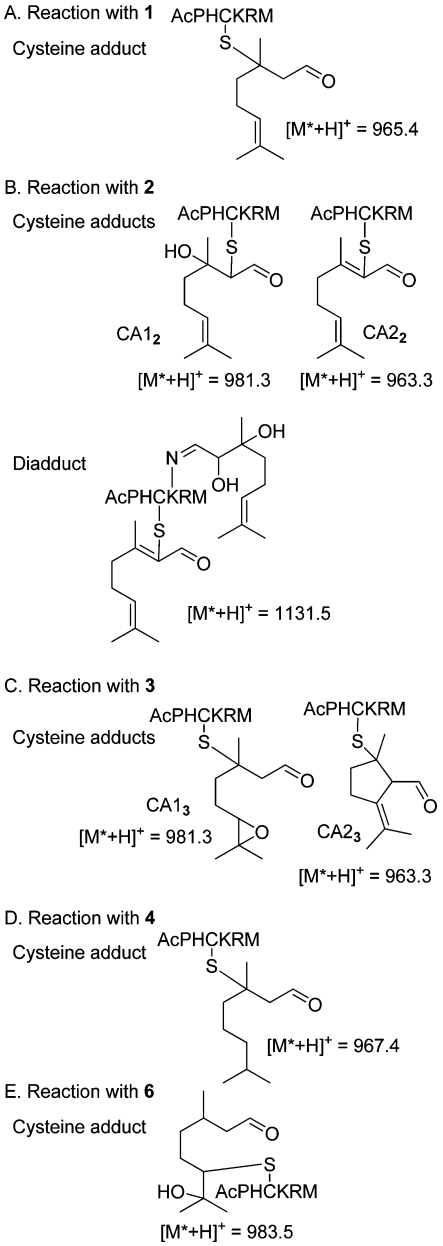

Figure 4. Suggested structures for adducts formed during the reactivity experiments with the model peptide AcPHCKRM and **1−4** and **6**.

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Table 2. Ions and Fragments Observed for Monoadducts Formed in the Reactivity Experiment with AcPHCKRM[a]

compd (MW)	adduct	$[M^* + H]^+$ *m/z*	$[M^* + 2H]^{2+}$ *m/z*	y_5^* *m/z*	y_4^* *m/z*	y_3 *m/z*	y_2 *m/z*	b_2 *m/z*	b_1 *m/z*
1 (152.2)		965.4	483.2	826.3	689.3	434.2	306.2	277.1	140.1
2 (168.2)	($CA1_2$)	981.3	491.3	842.2	705.4	434.2	306.2	277.2	140.1
	($CA2_2$)	963.3	482.1	824.4	687.4	434.2	306.2	277.2	140.1
	($CA3_2$)	1005.5	503.1	866.4	nd[b]	434.2	306.2	277.1	140.1
3 (168.2)	($CA1_3$)	981.3	491.1	842.2	705.2	434.0	306.0	277.1	140.1
	($CA2_3$)	963.3	482.1	824.2	687.2	434.0	306.0	277.0	140.1
4 (154.2)		967.4	484.2	828.4	691.3	434.2	306.1	227.1	104.1
6 (170.2)		983.5	492.2	844.4	707.3	434.2	306.1	277.1	140.1

[a]The reactivity experiments were all performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1) with a 10-fold excess of the hapten. The reactions were kept under argon at room temperature and monitored with HPLC/UV/ESI/MS. M* or y* indicates haptenated peptide or haptenated fragments, respectively. The mass of the unhaptenated peptide is 813.2. The masses of the unhaptenated fragments are as follows: b_1, 140.1; b_2, 277.1; y_2, 306.2; y_3, 434.1; y_4, 537.3; y_5, 674.3. [b]nd: not detected.

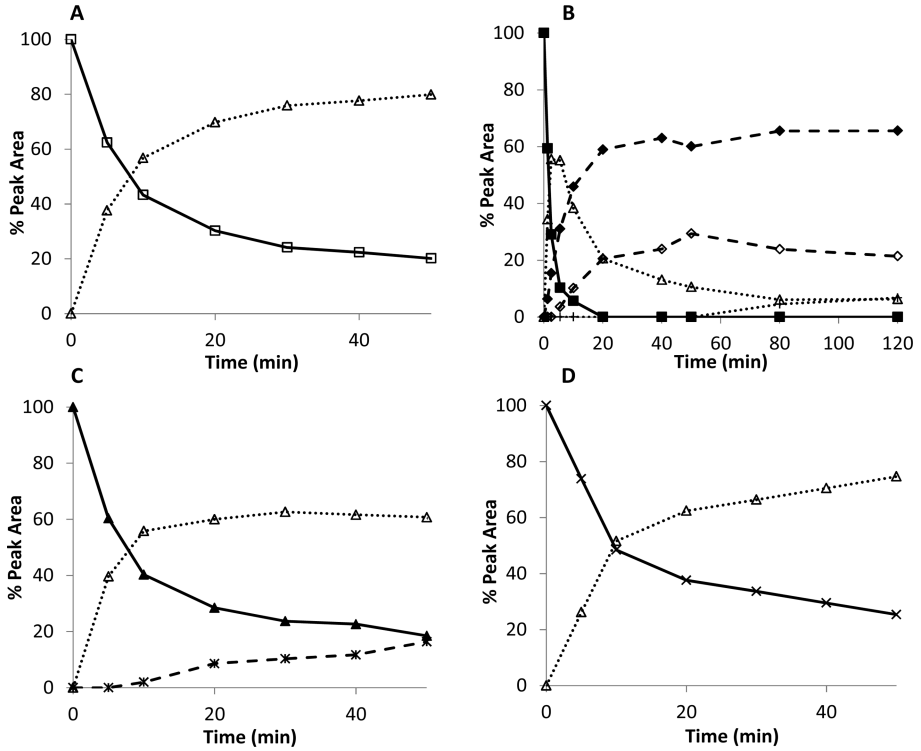

Figure 5. Unreacted peptide AcPHCKRM depletion and adduct formation normalized to 100%. Obtained with (A) **1**: (□) unreacted peptide and (Δ) cysteine adduct. (B) 2: (■) unreacted peptide, (Δ) cysteine adduct $CA1_2$, (♦) cysteine adduct $CA2_2$, (◇) cysteine adduct $CA3_2$, and (■) diadduct. (C) **3**: (▲) unreacted peptide, (Δ) cysteine adduct $CA1_3$, and (∗) cysteine adduct $CA2_3$. (D) **4**: (×) unreacted peptide and (Δ) cysteine adduct. Reactivity experiments were performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1) with a 10-fold excess of the hapten. The reactions were kept under argon at room temperature and monitored with HPLC/UV/ESI/MS.

epoxide to give the diol (Figure 4B). After 1000 min, no unreacted peptide nor $CA1_2$ was left in the reaction mixture, while $CA2_2$, $CA3_2$, and the diadduct were present in relative amounts of 6%, 16%, and 78%, respectively. The 2,3-epoxyaldehyde **2** was clearly more reactive than the *α,β*-unsaturated aldehydes **1**, **3**, and **4** used in this study. Interestingly, a larger number of adducts was also formed with **2**.

In the reactivity experiment with **3**, two cysteine adducts ($CA1_3$ and $CA2_3$) were present already after 10 min. The presence of the fragments y^*_5, y^*_4, y_3, y_2, b_2, and b_1 confirmed the modification of the cysteine residue (Table 2). $CA1_3$ contained the base ions *m/z* 981.3 and 491.3 corresponding to $[(M_{peptide} + M_2) + H]^+$ and $[(M_{peptide} + M_2) + 2H]^{2+}$, respectively. $CA1_3$ could be formed via the Michael addition of **3** by the cysteine residue of the peptide (Figure 4C). It is unlikely that this rapidly formed adduct ($CA1_3$) could be due to an epoxide ring-opening as we saw very small amounts of cysteine adduct in the experiment with **6**. The ions observed for the less polar $CA2_2$ adduct were *m/z* 963.3 and 482.1 defined as $[(M_{peptide} + M_2 - 18) + H]^+$ and $[(M_{peptide} + M_2 - 18) + 2H]^{2+}$. $CA2_3$ could be formed via an intramolecular cyclization with loss of water (Figure 4C). After 1000 min, $CA1_3$, $CA2_3$, and the unreacted peptide were present in relative amounts of 67%, 28%, and 10%, respectively. The epoxide in the 6,7-position did not affect the rate of the peptide depletion but increased the number of adducts that was formed.

Interestingly, the 6,7-epoxyaldehyde **3** was observed to only form monoadducts and did not form any cross-linked peptides (Michael addition and S_N2 epoxide ring-opening). A potential reason for the observed lack of cross-linking is the low reactivity

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Table 3. Ions and Fragments Observed for the Diadduct Formed in the Reactivity Experiment with AcPHCKRM[a]

compd (MW)	$[M^{**} + H]^+$ *m/z*	$[M^{**} + 2H]^{2+}$ *m/z*	y_5^{**} *m/z*	y_4^{**} *m/z*	y_3^{*} *m/z*	y_2 *m/z*	b_3^{*} *m/z*	b_2 *m/z*	b_1 *m/z*
2 (168.2)	1131.5	566.3	992.5	nd[b]	nd[b]	306.2	548.2	277.1	140.1

[a]The reactivity experiments were all performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1) with a 10-fold excess of the hapten. The reactions were kept under argon at room temperature and monitored with HPLC/UV/ESI/MS. M*, y*, or b* indicates haptenated peptide or haptenated fragments, respectively. The mass of the unhaptenated peptide is 813.2. The masses of the unhaptenated fragments are as follows: b_1, 140.1; b_2, 277.1; y_2, 306.2; y_3, 434.1; y_4, 537.3; y_5, 674.3. [b]nd: not detected.

of the aliphatic epoxide in the 6,7-position as described above for **6**.

Reactivity of 1−4 toward *N*-ACME. To confirm the structures of the adducts that were formed when reacting the conjugated aldehydes **1**−**4** with the peptide, an amino acid derivative (*N*-acetyl-cysteine methyl ester, *N*-ACME) was reacted with **1**−**4** to allow purification and structure elucidation using NMR spectroscopy. *N*-ACME formed conjugates with **1**−**4**. As expected, adducts from **1** and **4** were formed via a Michael addition of the cysteine on the *α,β*-unsaturated aldehydes providing the conjugates **7** and **8** (Figure 6) in

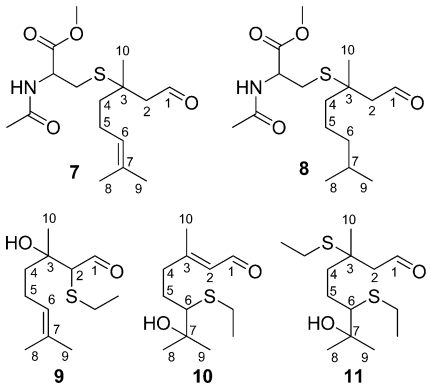

Figure 6. Structures of *N*-ACME conjugates **7** and **8** and ethanethiol conjugates **9**−**11**.

moderate yields. Since epoxyaldehydes **2** and **3** were obtained as a mixture of diastereomers (ratio 1:1), their reactions with *N*-ACME gave complicated mixtures of isomers whose NMR spectra were too difficult to interpret.

Reactivity of 2 and 3 toward Ethanethiol. To circumvent the problem with the formation of complex mixtures of isomers, ethanethiol was used instead of *N*-ACME. The reaction of **2** with ethanethiol gave **9** (Figure 6) and showed, as anticipated, that the opening of the epoxide ring occurred by a nucleophilic substitution at C2. More surprisingly, two different conjugates **10** and **11** (Figure 6) were obtained from the reaction with **3** and ethanethiol. Unlike the reactivity experiment with the model peptide, the epoxide in the 6,7-position of **3** reacted with the thiol group via an S_N2 reaction at C6 to afford **10**. This reaction is possible with a small nucleophile like ethanethiol, but it is unlikely that the rapidly formed peptide adduct $CA1_3$ (Figure 4) resulted from an S_N2 reaction at C6. Indeed, the reactivity of the 6,7-epoxy moiety toward the model peptide was shown to be very low as observed in the experiment with the peptide and **6**. The second conjugate that was characterized was the diadduct **11** resulting from an epoxide ring-opening reaction at C6 and a Michael addition of a second equivalent of ethanethiol. This confirms the importance Michael addition reaction for **3**.

Skin Sensitization Potency. The LLNA was used to assess the skin sensitization potencies of **2** and **4**−**6**. The LLNA results for **1** and **3** have previously been reported.[13,14] LLNA results are expressed as EC3 values; an EC3 value is the estimated concentration of a compound required to induce a 3-fold increase in sensitization compared to that in a control. A higher EC3 value indicates a less sensitizing compound compared to a compound with a low EC3 value.

The EC3 values obtained for **2**, **4**, **5**, and **6** were 0.12 M (2.1% w/v), 0.29 M (4.5% w/v), 3.5 M (57% w/v), and 3.4 M (60% w/v), respectively (Figure 7 and detailed information in

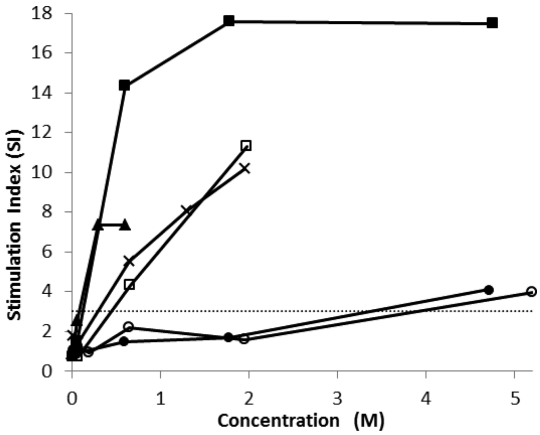

Figure 7. Dose−response curves for **1** (□), **2** (■), **3** (▲), **4** (×), **5** (○), and **6** (●), tested in the LLNA. The horizontal dotted line marks an SI of 3, the cutoff limit for a compound to be considered a sensitizer. EC3 values are as follows: **1**, 0.45 M (6.8% w/v);[13] **2**, 0.12 M (2.1% w/v); **3**, 0.082 M (1.4% w/v);[14] **4**, 0.29 M (4.5% w/v); **5**, 3.5 M (60% w/v); **6**, 3.4 M (57% w/v).

Table S1, the Supporting Information). Compound **2** was expected to be a sensitizer since it was shown to be the most reactive in the reactivity experiment. Interestingly, another 2,3-epoxyaldehyde (2,3-epoxycinnamal) was recently reported as a strong sensitizer (EC3 = 0.015 M).[18] Compound **4** was also expected to be a sensitizer analogous to the results from the previously published sensitization studies of **1** (0.45 M)[13] and **3** (0.082 M)[14] and taking into account the reactivity results in which **1**, **3**, and **4** had similar rate constants. The *α,β*-unsaturated aldehydes (**1** and **4**) show sensitization potencies similar to what is observed for the structurally related linalool aldehyde (EC3 = 0.52 M) and farnesal (EC3 = 0.54 M) (Figure 3).[47,48] The importance of the conjugation for the sensitization potency was confirmed when the nonconjugated aldehydes **5** and **6** gave very high EC3 values of 3.5 and 3.4 M, respectively. This result is in good agreement with the low reactivity of the aliphatic aldehyde and nonterminal aliphatic epoxide.

The two epoxyaldehydes **2** and **3** showed similar sensitization potencies independent of whether the epoxide is conjugated. Compound **2** contains a 2,3-epoxyaldehyde moiety instead of an *α,β*-unsaturated aldehyde, while **3** contains an aliphatic epoxide in addition to the *α,β*-unsaturated aldehyde.

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The ability to cross-link proteins could be a potential reason for the enhanced response of **3** in the LLNA compared to compounds **1** and **4**. The Schiff base alert from the nonconjugated aldehydes **5** and **6** was insufficient to induce sensitization. In literature, aliphatic nonconjugated aldehydes have been reported as weak sensitizers with EC3 values of approximately 1.0 M as demonstrated for 7-hydroxycitronellal, lilial, and cyclamen aldehyde (Figure 3).[48] The presence of an aliphatic epoxide in the 6,7-position (as in **6**) was not enough to increase the sensitization potency. This observation is in good agreement with previous studies showing that 6,7-epoxylinalyl acetate was a nonsensitizer when tested up to 2.8 M and that nonallylic epoxides in terpene derivatives were weak sensitizers.[12,38] Thus, the classification based on the LLNA experiments was congruent with the results from the reactivity experiments.

Investigations to predict the sensitization potential of a compound must include an evaluation of its ability to form sensitizers due to activation outside the skin via autoxidation or in the skin via bioactivation. In addition to a structural alert (α,β-unsaturated aldehyde), some compounds possess an activation alert such as double bonds. It has previously been shown that epoxides can be formed from double bonds via both activation routes, although the mechanisms are completely different.[12−15,18] Autoxidation is a free radical chain reaction that results in the formation of several oxidation products.[2] The reaction depends on the ease of abstraction of a hydrogen atom followed by a possibility of stabilization of the carbon radical. This is obtained in compounds with abstractable allylic hydrogen atoms, which allows for the reaction with oxygen radicals forming primary oxidation products (hydroperoxides). As the hydroperoxides are unstable, they will form secondary oxidation products, e.g., aldehydes, alcohols, and epoxides.[2] The moderate skin sensitizer geranial (**1**) has been shown to autoxidize, forming the stronger contact allergen 6,7-epoxygeranial (**3**) as a main oxidation product.[49]

Compounds containing double bonds are susceptible to epoxidation via P450 enzymes.[27,28] P450 enzymes are also present in the skin.[50] A recent study[51] has established the capacity of Langerhans cells to metabolize 7,12-dimethylbenz-[α]anthracene (DMBA) into DMBA-*trans*-3,4-diol via P450 1B1, which is subsequently delivered to neighboring keratinocytes. DMBA-*trans*-3,4-diol is further metabolized by P450 1A1 to the mutagenic DMBA-1,2-epoxy-*trans*-3,4-diol.[51]

For compounds with structures like that of **1**, formation of the same epoxides both outside the skin and in the skin should be considered. When the epoxidation occurs in the structures with α,β-unsaturation, an increase in the sensitization potency was seen in the already moderately sensitizing compounds. However, epoxidation did not increase the sensitization potency of the nonconjugated aldehyde **5**. In this case, both the aldehyde **5** and the epoxyaldehyde **6** are weakly sensitizing compounds. It could be concluded that activation by nonallylic epoxidation does not per se transform a weak sensitizing compound into a potent sensitizer.

CONCLUSIONS

In the present study, we have investigated the reactivity and sensitization potency of a series of aldehydes. The classification based on the LLNA experiments was congruent with the results from the reactivity experiments. The compounds range from strong to weak sensitizers confirming that even small changes in the chemical structure result in differences in sensitization potency. Our data showed that the highly reactive conjugated aldehydes, both α,β-unsaturated aldehydes and 2,3-epoxyaldehydes, are sensitizing moieties and should be considered as structural alerts. Moreover, nonconjugated aldehydes and nonterminal nonconjugated epoxide moieties have low reactivity and low sensitization potency. Our results show the importance not only of double bond conjugation to aldehyde but also of epoxide-aldehyde conjugation. The observations indicate that the formation of nonconjugated epoxides by bioactivation or autoxidation is not sufficient to significantly increase the sensitization potency of a weakly sensitizing parent compound.

ASSOCIATED CONTENT

Supporting Information

Synthetic scheme, complete LLNA information and structure and fragments of peptide AcPHCKRM are available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

ACD, allergic contact dermatitis; AcPHCKRM, Ac-Pro-His-Cys-Lys-Arg-Met-OH; DMSO, dimethyl sulfoxide; EC3, estimated concentration required to produce a stimulation index of 3; ESI, electrospray ionization; LLNA, local lymph node assay; *N*-ACME, *N*-acetyl-L-cysteine methyl ester; (Q)SAR, (quantitative) structure−activity relationship; SI, stimulation index

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Supporting Information

Skin Sensitization of Epoxyaldehydes – Importance of Conjugation

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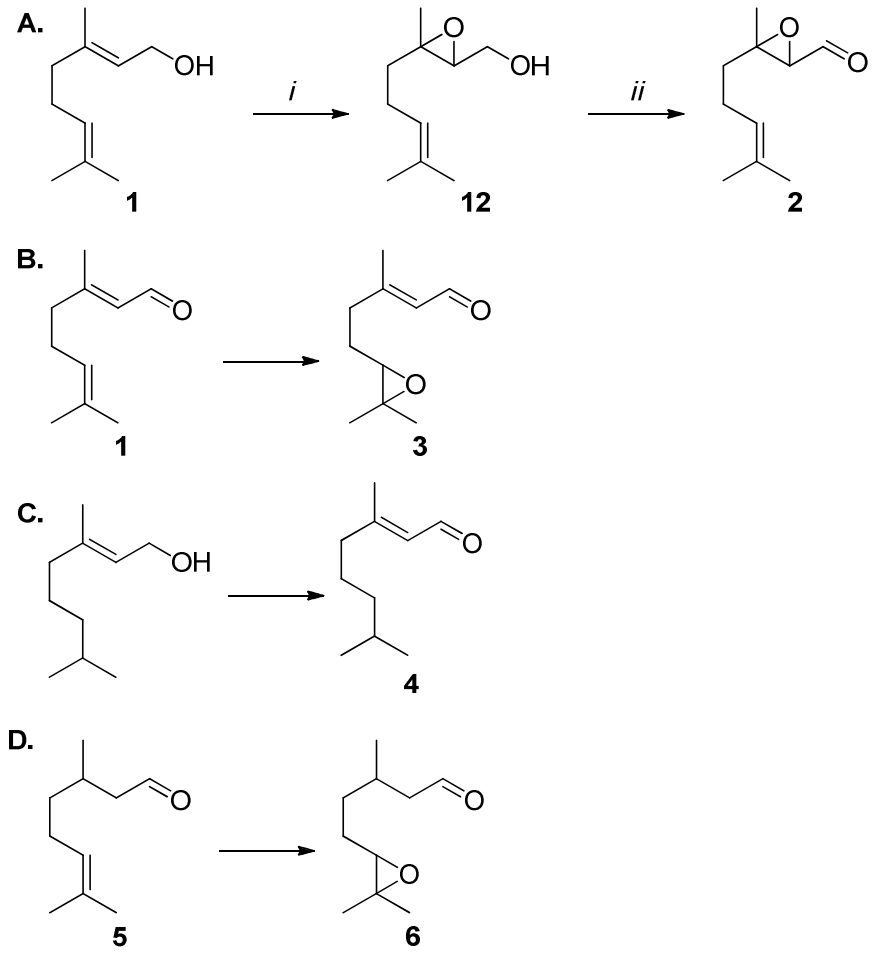
Scheme S1. Synthesis of compounds 2, 3, 4 and 6.[a]



[a]Reagents and conditions: A. (*i*) *t*BuOOH, Ti(O*i*Pr)$_4$, CH_2Cl_2, RT, 3h; (*ii*) DMSO, triethylamine, SO_3·pyridine complex, CH_2Cl_2, RT, 1h. B. and D. *m*CPBA, CH_2Cl_2, 0 °C, 2h. C. Dess Martin periodinane, CH_2Cl_2, RT, 2h.

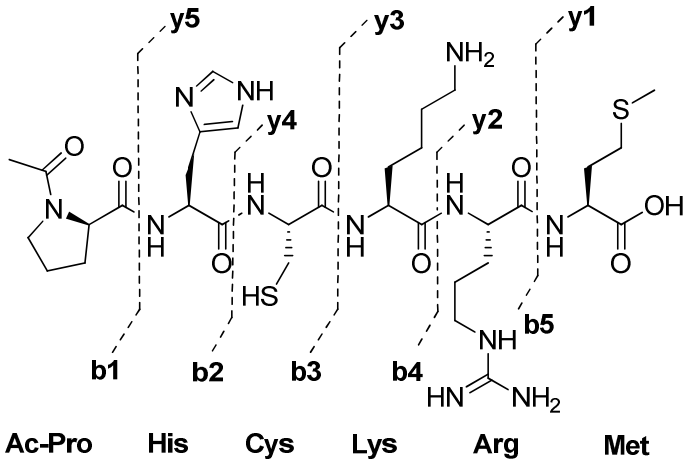
Table S1. Detailed results from the LLNA[a]

Compound and test concn (% w/v)	Test concn (M)	[^{3}H]thymidine incorporation (dpm/lymph node)	SI	EC3 value (% w/v)	EC3 value (M)
2					
Control		336			
0.1	$6.0*10^{-3}$	249	0.74		
1	$60*10^{-3}$	492	1.5	2.1	0.12
10	0.60	4810	14		
30	1.8	5896	18		
80	4.8	5863	17		
4					
Control		263			
0.1	$6.5*10^{-3}$	472	1.8		
1	$65*10^{-3}$	370	1.4	4.5	0.29
10	0.65	1451	5.5		
20	1.3	2118	8.1		
30	1.9	2676	10		
5					
Control		308			
1	$65*10^{-3}$	384	1.2		
3	0.19	291	0.94	60	3.5
10	0.65	669	2.2		
30	1.9	492	1.6		
80	5.2	1216	3.9		
6					
Control		282			
0.1	$5.9*10^{-3}$	297	1.1		
1	$59*10^{-3}$	262	0.93	57	3.4
10	0.59	418	1.5		
30	1.8	472	1.7		
80	4.7	1156	4.1		

[a]Groups of mice were treated with the test substance in five different concentrations, on the dorsum of both ears for three consecutive days. Sham treated control animals received the vehicle alone. On day five, all mice were injected intravenously with PBS (250 μL) containing 20 μCi of [methyl-^{3}H]thymidine. After 5 h the mice were sacrificed, the draining lymph nodes were excised and pooled for each group, single cell suspensions of lymph-node cells were prepared, and the thymidine incorporation into DNA was measured by β-scintillation counting. The increase in thymidine incorporation relative to vehicle-treated controls was derived for each experimental group and recorded as stimulation index (SI). The EC3 values (the estimated concentration required to induce an SI of 3) were calculated using linear interpolation.

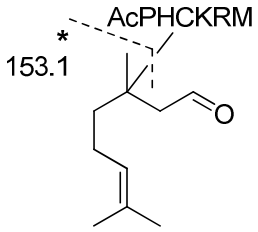
Figure S1. Fragment assignments for Ac-Pro-His-Cys-Lys-Arg-Met (AcPHCKRM)

The haptenated positions on the peptide were determined by analysis of the fragmentation pattern of the mass spectra of conjugates formed between compound and peptide. Peptides are known to form specific fragments by collision-induced decomposition (CID).[1] The most common fragments observed in the mass spectra of AcPHCKRM conjugates were b- and y-fragments.[2]



Molecular ion mass:
MH^+: 813.2
b- and y- fragment masses:

b1: 140.1	y1: 150.1
b2: 277.1	y2: 306.2
b3: 380.1	y3: 434.2
b4: 508.2	y4: 537.3
b5: 664.3	y5: 674.3



Possible stucture for covalently bound **1**

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